Characterization of Auxin Receptors and Auxin-Ethylene Interaction During Pea Fruit Development

by

Charitha Pramod Anuruddha Jayasinghege

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in
Plant Science

Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract

Normal development of a pea (*Pisum sativum* L.) fruit requires the presence of seeds within the fruit. Removal of seeds from young pea fruits results in slowing of pericarp (fruit) growth and subsequent abscission. Indole-3-acetic acid (IAA) and 4-chloroindole-3-acetic acid (4-Cl-IAA) are two types of naturally occurring auxins in pea. 4-Cl-IAA can mimic the presence of seeds by stimulating deseeded pericarp growth, but IAA does not. Differential interactions of the two auxins with gibberellin and ethylene are at least partially responsible for their differential action on pericarp growth, but the underlying mechanisms are not completely understood. In this thesis, experiments were designed to further understand seed and auxin regulation of pericarp ethylene biosynthesis and signaling pathways and to examine the relationship between auxin (IAA and 4-Cl-IAA) effects on pericarp growth and the auxin receptors within this tissue. Effects of seeds and the two auxins on pericarp ethylene biosynthesis were evaluated by analyzing the 1-aminocyclopropane-1-carboxylic acid synthase (*PsACS1*, *PsACS2* and *PsACS4*; enzyme converts *S*-adenosyl-Met to ACC) and ACC oxidase (*PsACO1*, *PsACO2* and *PsACO3*; enzyme converts ACC to ethylene) gene transcript abundances, the ethylene precursor ACC level, and the ACO enzyme activity in deseeded pericarps and deseeded pericarps treated with IAA or 4-Cl-IAA. The results showed that the two auxins regulate pericarp ethylene biosynthesis in distinctly different manners. 4-Cl-IAA strongly stimulated pericarp *PsACS1* and *PsACO1* transcript levels and mimicked the role of seeds in maintaining low levels of pericarp *PsACS4*, *PsACO2* and *PsACO3* transcripts. IAA had low or no effect on modulating pericarp *PsACS* or *PsACO* transcript levels compared to 4-Cl-IAA. Associated with these *PsACS* and *PsACO* transcript abundance patterns, IAA and 4-Cl-IAA differentially modulated pericarp ACC level and ACO enzyme activity. Transcript abundance of the genes that code for the ethylene receptors

PsERS1 and PsETR2, and signaling-related proteins PsEBF1 and PsEBF2, which act as negative regulators of ethylene signaling, increased in response to ethylene. Application of 4-Cl-IAA, but not IAA, increased the transcript abundance of those genes indicating that the suppression of ethylene signaling through the stimulation of ethylene receptor or EBF gene transcript abundance could be associated with the ethylene action inhibitory effect of 4-Cl-IAA.

The relationship between the auxin (IAA and 4-Cl-IAA) effects on pericarp growth and the auxin receptors within the pericarp tissue was examined by functionally characterizing selected pea auxin receptors in *Arabidopsis* and relating the transcript abundance patterns of pea TIR1/AFB family members to pollination status, seed removal and to auxin and ethylene treatments. Functional characterization of *PsTIR1a* and *PsTIR1b* in *Arabidopsis tir1-10* and *Attir1-10 afb2-3* auxin receptor mutants indicates that they code for functional auxin receptors and that they mediate the comparatively stronger auxin effect of 4-Cl-IAA compared to that of IAA. Expression of *PsAFB2* in *Attir1-10 afb2-3* double mutants indicates that PsAFB2 is also a functional auxin receptor. The transcript profiles of five pea TIR1/AFB family gene members (*PsTIR1a*, *PSTIR1b*, *PsAFB2*, *PsAFB4* and *PsAFB6*) in fruit tissues during early development suggested that the expression of these auxin receptors is developmentally regulated. Expression of *PsTIR1b* and *PsAFB6* was hormone regulated, where developing seeds and 4-Cl-IAA suppressed *PsTIR1b* and *PsAFB6* transcript accumulation, and ethylene stimulated *PsAFB6* transcript accumulation in the pericarps. Expression of the auxin-responsive *DR5::GUS* reporter construct and auxin content in the pericarps was related to auxin receptor transcript abundance, which suggested that the modulation of pericarp auxin level and the auxin sensitivity could be important in regulating the auxin action in developing pea fruit. Overall, the ability of 4-Cl-IAA but not IAA to mimic the presence of seeds in stimulating pericarp development is associated

with their differential effects on ethylene biosynthesis, ethylene signaling, and the auxin receptor abundance. Common receptors likely perceive the two auxins, but the modulation of auxin receptor abundance and the possible differences in IAA and 4-Cl-IAA affinities to those receptors may initiate differential auxin effects.

Preface

All the experiments in this thesis were originally designed by Drs. Jocelyn Ozga and Dennis Reinecke, Plant BioSystems Group, Department of Agricultural, Food and Nutritional Science, University of Alberta, and I modified the designs for implementation. All the experiments and data analyses were completed by me with the contributions made by the following people as described: Dr. Dennis Reinecke contributed to the experiments by creating the *DR5::GUS* plants used in Chapter 3, identifying the pea *PsTIR1a* and *PsAFB4* homologs and preparing most of the auxin solutions. Kosala Waduthanthri contributed to Chapter 2 by isolating and sequencing the *PsACO2* and *PsACO3* genes and analyzing the qRT-PCR efficiencies of the primers and probes designed for those genes. Dr. Harleen Kaur contributed to Chapter 3 and 4 of this thesis by performing qRT-PCR to verify the expression of pea auxin receptors in transgenic *Arabidopsis* plants which generated the Appendix Figs. B4, C1 and D4. Dilini Adihetti contributed to Chapter 3 by analyzing *DR5::GUS* expression in wild-type pea pericarps, which helped in generating Appendix Fig. B5.

Dedication

To Mr. Bernard Withanaarachchi

For his generosity

Acknowledgements

There are many people who have helped me in various ways throughout my studies. My first and foremost thanks go to Dr. Jocelyn Ozga for giving me the opportunity to do my Ph.D. with her, and for all the guidance and advice she has given me for the successful completion of my studies. Pursuing graduate studies in Canada was once a dream and as a supervisor, Dr. Ozga helped me in every way to make it a reality. I am also thankful to my committee members Dr. Janice Cooke and Dr. Enrico Scarpella for all their help and showing me the correct direction in the situations I was not sure about the next step.

Members of the Dr. Ozga/Reinecke lab were always willing to help me during my research work. I thank Dr. Dennis Reinecke for his help in analyzing the gene sequences, preparation of auxin solutions and giving me advice and support on various other things throughout this study. Dr. Reinecke also created the *DR5::GUS* plants that I used in this study. I thank our lab technician Dakshina Pahala Vithanage for her help throughout this research including the assistance with sample collection, preparation of growth media and other solutions, and taking care of my plants whenever needed. I thank Dr. Harleen Kaur for her help with gene expression analysis in transgenic plants, and fellow graduate students Dinuka Waduthanthri and Dilini Adhihetti for their help including solution preparation, gene cloning, and sequencing. I am also grateful to other current and past members of our lab including Aruna Wickramarathna, Prasad Savada, Na Gao, Anais Brown, Harleen Kaur, Gabor and Szidonia Botar, Alena Jin, Han Yang and Dhanuja Abeysingha for their help or in sharing some time with me for a coffee or chat. I was very fortunate to work in an always friendly environment with their smiling faces. I would also thank Dr. Tyler Donner from Dr. Scarpella's lab for his guidance in working with *Arabidopsis* and Amanda Gregoris from Dr. Cooke's lab for her help in phylogenetic tree creation.

There are many people in the Department of Agricultural, Food and Nutritional Science (AFNS) and the Molecular Biology Service Unit (MBSU) who helped me in various ways in my studies. I thank all of them for their helpfulness and kindness. In particular, I should mention the names of Jody Forslund, Nikki-Karyssa Scott and Kelvin Lien at AFNS, whom I felt were always willing to take an extra step in helping me.

Life as a student in a foreign country could have been difficult without all the Sri Lankan-Canadian families and fellow Sri Lankan students at the University of Alberta who helped me making Canada my second home. I especially thank Dr. Laki Goonewardene who helped me adjusting to my new life in Canada when I first arrived in Edmonton and his continued caring for us as a family. I am also grateful to the families of Gayan Perera and Gayan Amarasuriya for being with us like members of our own family.

I also take this as an opportunity to thank those who helped me during my undergraduate studies, including my teachers, friends, relatives and complete outsiders. Without their assistance, a Ph.D. would have been an unachievable dream.

Finally, I thank my family members for all they have done for me throughout this journey. I am very grateful to my mother and my late father who made enormous sacrifices to make me who I am today. I thank my father- and mother-in-law for all their help including taking care of kids during the final stage of my studies. I should re-mention the name of Dakshina Pahala Vithanage not as the technician of our lab but as my wife. I believe it is well worth mentioning her name more than once given all the sacrifices she has made. Dakshina took care of all the family burdens during past several years as a wife, as a mom and as the primary breadwinner of the family. I also thank with a smile on my face to my older son Akain who barely understood that his father has lots of work to do on campus, and the little one Nethush who recently learned to wave his hand when I leave to campus, for reminding me that I have a beautiful world beyond my life as a graduate student.

Table of contents

CHAPTER 3

TIR1 AUXIN-RECEPTOR MEDIATED REGULATION OF AUXIN ACTION IN DEVELOPING PEA FRUIT 70

List of Tables

List of Figures

List of Abbreviations

Abbriviation	Definition
1-MCP	1-methylcyclopropene
2, 4-D	2,4-dichlorophenoxyacetic acid
2OG Fe(II)	2-oxoglutarate-dependent-Fe (II)
4-Cl-IAA	4-chloroindole-3-acetic acid
4-MU	4-methylumbelliferone
AA	Amino acid
ABCB	ATP BINDING CASSETTE TYPE B
ABP1	AUXIN BINDING PROTEIN 1
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	1-aminocyclopropane-1-carboxylic acid oxidase
ACS	1-aminocyclopropane-1-carboxylic acid synthase
AIBA	α-aminoisobutyric acid
ALAAT	alanine aminotransferase
AOA	Aminooxyacetic acid
ARF	AUXIN RESPONSE FACTOR
ASK1	ARABIDOPSIS SKP1-LIKE 1
Aux/IAA	AUXIN/INDOLE-3-ACETIC ACID
AUX1	AUXIN RESISTANT 1
AuxRE	Auxin response element
AVG	1-aminoethoxyvinyl-glycine
CaMV	Cauliflower mosaic virus
Cas9	CRISPR-associated 9
cDNA	Complementary deoxyribonucleic acid
CDPK	Calcium-dependent protein kinase
CDS	Coding sequence
Co^{2+}	Cobalt ions
Col-0	Columbia
CRISPR	Clustered regularly interspaced palindromic repeats

CTAB	Cetyltrimethylammonium bromide
CTR1	CONSTITUTIVE TRIPLE-RESPONSE 1
CUL1	CULLIN 1
DAA	Days after anthesis
DAI	Days after imbibition
DAO	DIOXYGENASE FOR AUXIN OXIDATION (DAO)
DPB	DIMERIZATION PARTNER B
EBF1	EIN3 BINDING F-BOX 1
EIL1	EIN3/ ETHYLENE INSENSITIVE 3-LIKE 1
EIN4	ETHYLENE INSENSITIVE 4
EOL	ETO1-like
ERS1	ETHYLENE RESPONSE SENSOR 1
ETO1	ETHYLENE OVER-PRODUCER 1
ETR1	ETHYLENE RESPONSE 1
GA	Gibberellin
GACC	1-(γ-L-glutamylamino) ACC
GH3	GRETCHEN HAGEN 3
GUS	ß-glucuronidase
HPLC-MS/MS	High-performance liquid chromatography-tandem mass spectrometry
IAA	Indole-3-acetic acid
IAM	indole-3-acetamide
IBA	Indole-3-butyric acid
IBFQ	Iowa Black Fluorescent Quencher
INS	INDOLE SYNTHASE
$InsP_6$	inositol hexakisphosphate
IPyA	indole-3-pyruvic acid
IAOx	indole-3-acetaldoxime
LAX	LIKE AUX 1
LDOX	leucoanthocyanidin dioxygenase
MACC	malonyl-ACC
MP	Monopteros

MAPK	mitogen-activated protein kinase
MUG	4-methylumbelliferyl glucuronide
NAA	1-naphthaleneacetic acid
NBD	2, 5-norbornadiene
$nLgfwt^{-1}h^{-1}$	Nano-liters per gram fresh weight per hour
nmol $gfwt^{-1}$	Nanomoles per gram fresh weight
ng $gdwt^{-1}$	Nanograms per gram dry weight
NPA	N-1-naphthylphthalamic acid
NR	NEVER-RIPE
NRCC SK	National Research Council, Saskatoon, Canada
PAA	Phenylacetic acid
PCR	Polymerase Chain Reaction
PIN 1	PIN-FORMED 1
PVP	Polyvinylpyrrolidone
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RBX1	RING-BOX 1
RNA	Ribonucleic acid
RNAi	RNA interference
rRNA	Ribosomal RNA
rSAP	Shrimp alkaline phosphatase
SAM	s-adenosylmethionine
SHY2	SHORT HYPOCOTYL 2
SKP	S PHASE KINASE-ASSOCIATED PROTEIN
SP	Split pericarp with seeds
SPNS	Split pod no seeds
STS	Silver thiosulfate
TAA	TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS
TAM	Tryptamine
TAR	TRYPTOPHAN AMINOTRANSFERASE RELATED
TIBA	2,3,5-triiodobenzoic acid
TIR1	TRANSPORT INHIBITOR RESPONSE 1

TOE	Target of ETO1
TPL	TOPLESS
TPR	TPL-RELATED
TSA	Transcriptome Shotgun Assembly
WT	Wild-type
YUC	YUCCA

CHAPTER 1

GENERAL INTRODUCTION

1.1 AUXINS

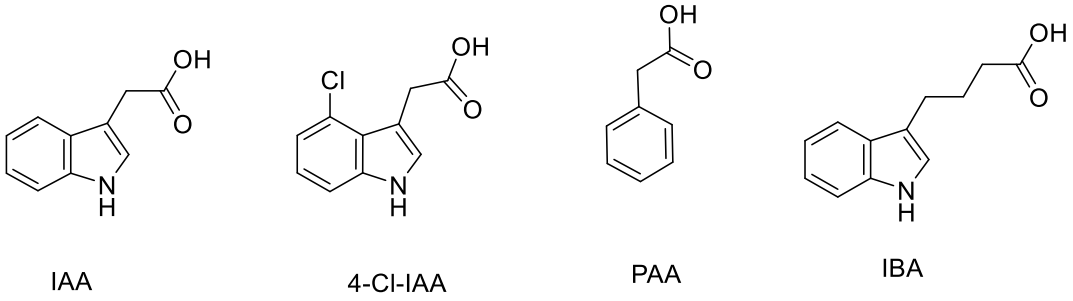
In the late 19th century, Charles Darwin predicted the existence of an "influence" that causes plants to bend toward light and roots to grow down (Darwin and Darwin, 1881). The accuracy of his prediction became evident a few decades later as the compound mediating this "influence" was isolated into agar blocks and was shown to regulate the bending of *Avena sativa* (oat) coleoptiles depending on its localization (Went, 1926). In this experiment, Went (1926) isolated a growth stimulatory substance from *A. sativa* coleoptile tips by keeping them on an agar block. When a small piece of this agar was placed on one side of a decapitated coleoptile, the coleoptile bent towards the opposite side indicating that the growth regulator in the agar block simulates the growth of the underlying half of the coleoptile (Went, 1926). Subsequently, the chemical structure of this growth regulator was recognized as indole-3-acetic acid (IAA) and was categorized under a group of phytohormones termed 'auxins' (Went and Thimann, 1937). The term 'auxin', which means 'to grow' in Greek (Abel and Theologis, 2010), is used commonly for the class of compounds which have effects similar to that of IAA, such as the ability to stimulate the curvature of *Avena* coleoptiles (Went and Thimann, 1937).

In addition to the most ubiquitous auxin, IAA, three other naturally occurring compounds, indole-3-butyric acid (IBA), phenylacetic acid (PAA) and 4-chloroindole-3-acetic acid (4-Cl-IAA) have been classified as plant auxins (Fig. 1; Simon and Petrášek, 2011). Of these, IBA and PAA are also found commonly in plants (Wightman and Lighty, 1982; Epstein and Ludwig-Müller, 1993; Sugawara et al., 2015). 4-Cl-IAA appears to be mainly limited to few members of Fabaceae family within the Fabeae and Trifoleae tribes including *Vicia faba* (broad bean), *Pisum sativum* (pea), *Lens culinaris* (lentil), *Trifolium repens* (white clover), *Medicago truncatula* (barrelclover), and *Melilotus indicus* (Reinecke et al., 1999; Lam et al., 2015). 4-Cl-IAA was not detected in *Lotus japonicus, Glycine max* (soybean) or *Cicer arietinum* (Chickpea; Reinecke, 1999; Lam et al., 2015). Based on these reports of 4-Cl-IAA occurrence in the

Fabaceae family, Lam et al. (2015) hypothesized that the Fabeae and Trifoleae tribes started producing 4-Cl-IAA after the divergence of the genus *Cicer* from their common ancestor roughly about 25 million years ago. The only plant species which was reported to contain 4-Cl-IAA outside the Fabaceae family is *Pinus sylvestris* (Scots pine; Ernstsen and Sandberg, 1986), which has recently been questioned (Lam et al., 2015).

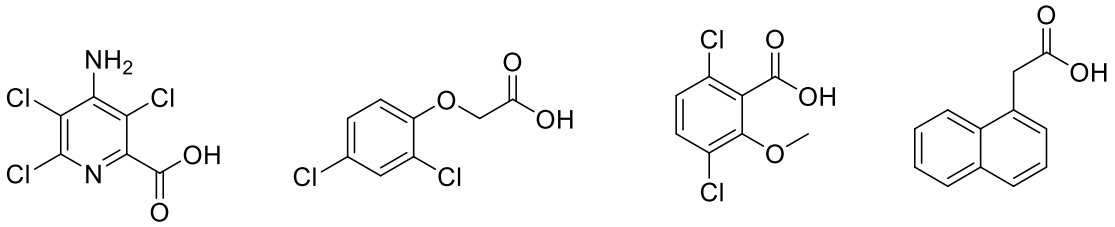
With its foremost importance in plant development, the role of auxin in plants has been extensively characterized since its discovery. Auxins regulate many developmental processes within plants due to their ability to modulate cell division and orient cell expansion (Perrot-Rechenmann, 2010). The transport and localization of auxins with tissues and cells is also a key element in auxin-regulated growth and development (Vanneste and Friml, 2009). Auxin-regulated developmental processes include phototropism and gravitropism (Muday, 2001), plant root development (Overvoorde et al., 2010), initiation of lateral organ development and their arrangement within plants (Reinhardt et al., 2000) and reproductive development (Ozga and Reinecke, 2003; Sundberg and Ostergaard, 2009). The ubiquitously occurring auxin IAA is known to mediate almost all of these processes while the specific roles of other naturally occurring auxins in plant development are less known. In agriculture, IBA has been commonly used as a rooting agent (Gianfagna, 1995). However, the current knowledge indicates that naturally occurring IBA does not have intrinsic auxin activity, but the conversion of IBA to IAA stimulates the auxin response (Strader et al., 2011; Schlicht et al., 2013). Phenylacetic acid is considered a weak auxin and is generally present in plants at high concentrations compared to IAA (Sauer et al., 2013; Sugawara et al., 2015). Microarray analysis data from *Arabidopsis* seedlings show that PAA can induce the same set of early auxin-responsive genes as IAA, but a higher concentration is required (Sugawara et al., 2015). Gravitropic stimulations with maize (*Zea mays L.*) coleoptiles show that the distribution mechanism of PAA is different from that of IAA. In those gravitropic stimulation experiments, only IAA but not PAA formed concentration gradients, suggesting that PAA is not actively or directionally transported (Sugawara et al., 2015).

Natural Auxins

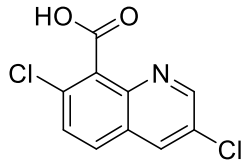


IAA 4-Cl-IAA PAA IBA

Synthetic auxins



picloram 2,4-D Dicamba NAA



quinclorac

Figure 1.1. Chemical diversity of naturally occurring plant auxins and selected synthetic auxins. A chemically diverse group of compounds has been classified as auxins based on their effects on plants. The presence of a free carboxyl group appears to be an important feature for their action. In addition, the majority of the synthetic auxins are chlorinated and this is important in their activity and plant selectivity. The figure was developed based on Cobb and Reade (2010) and Simon and Petrášek (2011).

1.1.1 Synthetic auxins

A number of synthetic compounds with auxin activity have been identified and are commonly used in the agricultural industry (Gianfagna, 1995). These synthetic auxins include chemically diverse compounds such as 2,4-dichlorophenoxyacetic acid (2, 4-D), 3,6-dichloro-2-methoxybenzoic acid (Dicamba), 1-naphthaleneacetic acid (NAA), 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (Picloram) and 3,7-dichloro-8-quinolinecarboxylic acid (Quinclorac) (Fig. 1.1; Cobb and Reade, 2010). The presence of a free carboxyl group appears to be a common characteristic of all auxins (Cobb and Reade, 2010). In addition, almost all of the commercially available synthetic auxins (Cobb and Reade, 2010; Alberta Agriculture and Forestry, 2016) are halogenated primarily with chlorine, reminiscent of the natural plant auxin 4-Cl-IAA (Fig. 1.1). The presence of halogens and their positioning in the auxin molecule are known to be important in auxin activity as well as in plant selectivity (Reinecke et al., 1995; Reinecke et al., 1999; Cobb and Reade, 2010).

Synthetic auxins are widely used as herbicides given their selective action and higher stability resulting in a stronger effect within the plant compared to natural auxins (Cobb and Reade, 2010; Grossmann, 2010). When higher concentrations of auxins are used as herbicides, it causes an auxin imbalance in plants, dramatically changing the plant metabolism including stimulation of ethylene and abscisic acid that can act to inhibit growth. These changes eventually disrupt normal plant functions leading to death (Grossmann, 2010). Auxin is also used for many other agricultural applications including stimulation of root initiation in stem cuttings, stimulation of fruit set in the absence of pollination or under unfavorable environmental conditions, stimulation of fruit abscission to remove excess fruit early in development, and to delay fruit drop at the time of harvest in tree fruits crops (Gianfagna, 1995).

1.1.2 Auxin signaling pathway in plants

Even though auxin was discovered as a plant hormone in the 1930's (cited from Went and Thimann, 1937), a clear picture of how plants perceive auxin and initiate the cellular auxin response was not available until recently (Abel and Theologis, 2010). Identification of the *Arabidopsis* TRANSPORT INHIBITOR RESPONSE 1 (TIR1) protein as an auxin receptor was a breakthrough in clarifying this pathway (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005).

Subsequent studies showed that the remaining five AUXIN SIGNALING F-BOX (AFB) homologs in *Arabidopsis*, AFB1 through AFB5 can also bind auxin in a manner similar to TIR1 (Dharmasiri et al., 2005b; Prigge et al., 2016). Binding of auxin with TIR1/AFB proteins requires a second co-receptor protein AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA; Fig. 1.2). Neither TIR1/AFB nor Aux/IAA proteins bind with auxin at appreciable levels alone (Calderón Villalobos et al., 2012); however, auxin, which acts like a molecular glue, brings the two proteins together making a TIR1/AFB-Aux/IAA co-receptor complex for auxin perception (Tan et al., 2007).

The TIR1/AFB family of F-box proteins form a ubiquitination ligase (E3) complex with S PHASE KINASE-ASSOCIATED PROTEIN 1 [SKP1; or ARABIDOPSIS SKP1-LIKE1 (ASK1)], CULLIN 1 (CUL1), and RING-BOX 1 (RBX1) known as SCF $^{TIR1/AFB}$ (Wang and Estelle, 2014). Auxin-aided binding of the Aux/IAA co-receptors to TIR1/AFB proteins of the SCF $^{TIR1/AFB}$ complex results in ubiquitination of Aux/IAAs and subsequent degradation by 26S proteasome (Wang and Estelle, 2014). AUXIN RESPONSE FACTOR (ARF) transcription factors are activated with the degradation of the Aux/IAAs leading to auxin-mediated gene expression and auxin response. In *Arabidopsis,* there are 29 different Aux/IAA proteins and 23 different ARFs (Salehin et al., 2015). Both Aux/IAAs and ARF proteins have been shown to interact with other proteins within the same families as well as between Aux/IAAs and ARFs. It is believed that multimerization of Aux/IAAs and ARFs can occur and instead of an Aux/IAA-ARF dimer, multiple Aux/IAAs may be required for efficient repression of an ARF in the absence of auxin (Wang and Estelle, 2014). In the repression process, Aux/IAAs recruit the corepressor proteins TOPLESS (TPL) and TPL-RELATED (TPR). This is believed to affect the chromatin conformation preventing the transcription of auxin-responsive genes by ARF (Wang and Estelle, 2014; Salehin et al., 2015). It is not well understood how ARFs regulate auxin-mediated gene expression; but it is known that some ARFs act as transcription activators and some behave as repressors (Wang and Estelle, 2014).

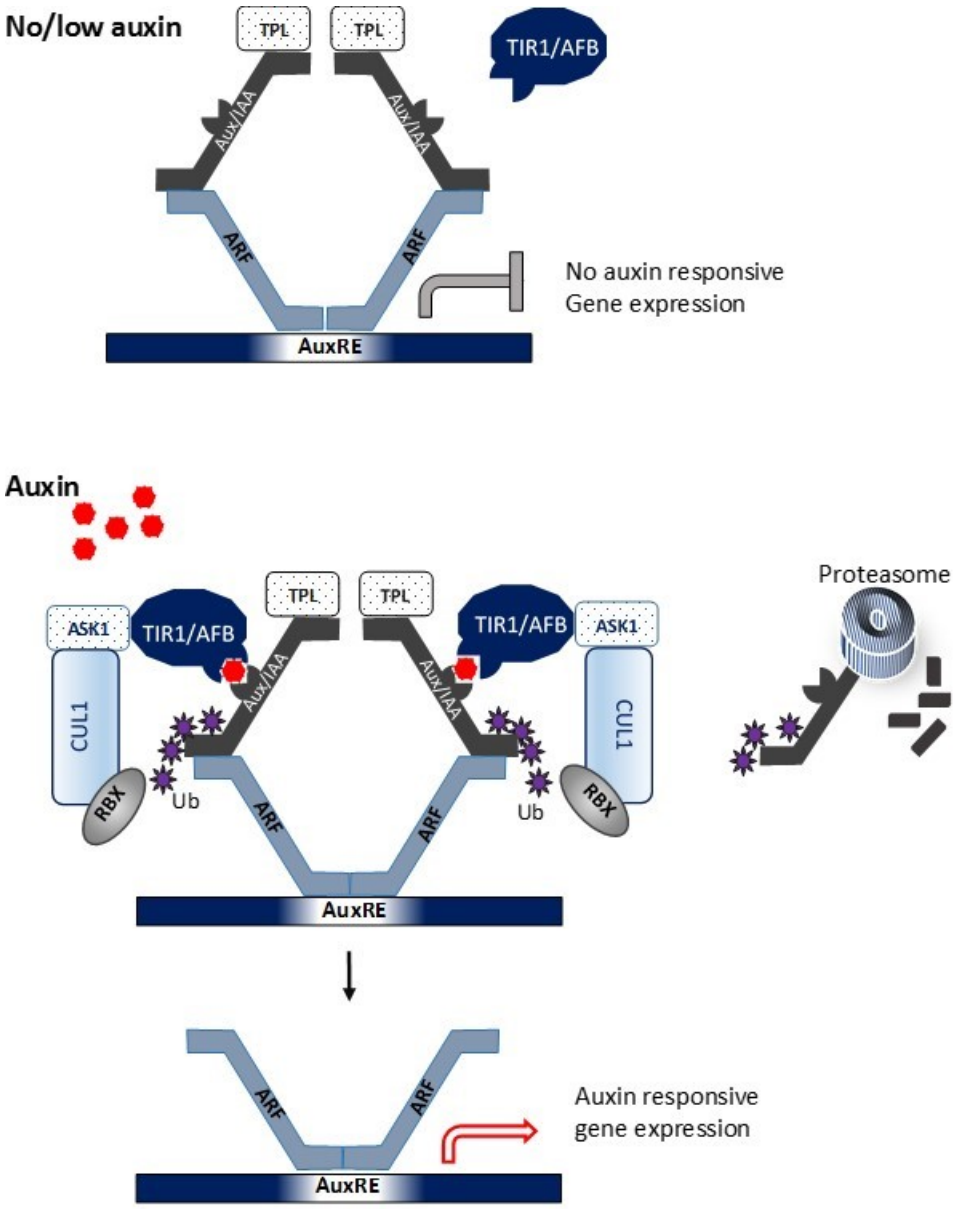


Figure 1.2. Mechanism of auxin signaling through the TIR1/AFB-mediated pathway. In the absence of auxin, Aux/IAA repressors bind with ARF transcription factors and repress the auxin-responsive gene expression. Aux/IAAs recruit TPL co-repressors in this repression process. When auxin is present, TIR1/AFB F-box proteins and Aux/IAA repressors act as co-receptors in auxin perception. Auxin acting like a molecular glue brings the TIR1/AFB and Aux/IAA proteins together. This association causes $SCF^{TIR1/AFB}$ mediated ubiquitination of Aux/IAAs. Degradation of ubiquitinated Aux/IAAs by 26S proteasome releases ARF transcription factors initiating auxin-responsive gene expression. The figure was developed based on Wang and Estelle (2014) and Salehin et al. (2015). Ub: Ubiquitin.

Auxin binding with TIR1/AFB appears to be the primary mechanism of auxin perception; however, two other types of proteins, AUXIN BINDING PROTEIN 1 (ABP1) and S-PHASE KINASE-ASSOCIATED PROTEIN 2A (SKP2A), have been reported to bind with auxin (cited from Grones and Friml, 2015). In contrast to earlier reports suggesting an important role of ABP1 in plant development, a recent study with two new *Arabidopsis* ABP1 mutants (*abp1-c1* and *abp1-TD1*) found no developmental defect in the mutants, and their auxin-responsive gene expression was similar to that of wild-type (Gao et al., 2015). These observations indicate that the ABP1 protein is not necessary and does not play an essential role in auxin signal perception during normal plant development (Gao et al., 2015). SKP2A is an F-box protein important in the degradation of cell cycle transcription factors including DIMERIZATION PARTNER B (DPB; Jurado et al., 2008). Relatively limited information is available about the importance of auxin binding with SKP2A. It is likely that auxin binding with the F-box protein SKP2A enhances its degradation as well as its interaction with DPB (Jurado et al., 2010).

1.1.3 Auxin biosynthesis and homeostasis

Plants synthesize IAA through two distinct mechanisms which have been categorized depending on whether the amino acid Tryptophan (Trp) is a precursor of the biosynthetic pathway, the Trp-dependent and Trp-independent pathways (Gao and Zhao, 2014). The Trp-dependent pathway is comparatively well characterized and different genes associated with this pathway have been recognized (Gao and Zhao, 2014). The IAA synthesis rates in plants, which are not likely achievable only through the Trp-dependent pathway, and the IAA synthesis ability of Trp biosynthesis mutants support the existence of Trp-independent pathway, but the associated molecular mechanism or genes are not known (Gao and Zhao, 2014; Tivendale et al., 2014). In a recent study, cytosol-localized INDOLE SYNTHASE (INS) has been reported as an important enzyme which may play a key role in Trp-independent IAA biosynthesis (Wang et al., 2015). However, numerous unanswered questions and the incompleteness of the available data question the existence and the importance of Trp-independent pathway as a major root of auxin biosynthesis (Nonhebel, 2015).

The Trp-dependent IAA biosynthesis has been postulated to occur through several different pathways, which have been named based on the intermediate immediately downstream of the Trp; indole-3-acetaldoxime (IAOx) pathway, tryptamine (TAM) pathway, indole-3-acetamide

(IAM) pathway and the indole-3-pyruvic acid (IPyA) pathway (Tivendale et al., 2014). Of these, the IPyA pathway has been recognized as a major mechanism of auxin biosynthesis in plants (Zhao, 2012; Gao and Zhao, 2014). The IPyA pathway is a simple two-step pathway which is regulated by enzymes encoded by two families of genes, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 and its close homologs TRYPTOPHAN AMINOTRANSFERASE RELATED (TAA/TARs), and the YUCCA (YUC) genes. In the first step Trp is converted into IPyA by TAA family of aminotransferases. IAA is synthesized from IPyA in the second step by YUC family of flavin monooxygenases (cited from Zhao, 2012). In addition to IAA biosynthesis, the IPyA pathway also likely regulates the 4-Cl-IAA biosynthesis in pea. Expression of pea *PsTAR1* and *PsTAR2* in *Escherichia coli* converted 4-Cl-Trp to 4-Cl-IPyA, and a *PsTAR2* knockout mutant produced low levels of 4-Cl-IAA in pea seeds at later developmental stages. Furthermore, when deuterium labeled Trp was injected to pea seeds, deuterated 4-Cl-Trp and 4-Cl-IAA were detected, suggesting that 4-Cl-IAA is synthesized from chlorinated Trp possibly through IPyA pathway (Tivendale et al., 2012). Recent studies also provide evidence that plants may synthesize the other naturally occurring auxin PAA in a similar manner from the amino acid phenylalanine through the intermediate phenylpyruvate (Dai et al., 2013; Cook et al., 2016).

In plant tissues, IAA levels can be modified through biosynthesis, transportation, degradation and conversion into amino acid, peptide or sugar conjugates (Ludwig-Muller, 2011). Amino acid conjugates of IAA include several different forms including indole-3-acetyl-alanine (IAA-Ala), indole-3-acetyl-leucine (IAA-Leu), indole-3-acetyl-aspartate (IAA-Asp) and indole-3-acetyl-glutamate (IAA-Glu). Of these, IAA-Ala and IAA-Leu are considered to be reversible conjugates and thus act as storage forms of IAA, whereas IAA-Asp and IAA-Glu are believed to be destined for degradation (Ludwig-Muller, 2011). In pea, IAA-Asp is the most predominant auxin conjugate (Sudi, 1964; Nordström and Eliasson, 1991). Oxidation of IAA to 2-oxindole-3-acetic acid (oxIAA) has been recognized as an important route of IAA metabolism in several plant species including maize (Reinecke and Bandurski, 1981; Reinecke and Bandurski, 1983) rice (*Oryza sativa* L. ; Zhao et al., 2013) and *Arabidopsis* (Pěnčík et al., 2013). The molecular mechanism associated with IAA oxidation remained elusive until the *DIOXYGENASE FOR AUXIN OXIDATION* (*DAO*) encoded 2-oxoglutarate-dependent-Fe (II) (2OG Fe(II)) dioxygenase in rice was shown to catalyze the oxidation of IAA to oxIAA (Zhao et al., 2013).

The rice *dao* mutants showed defects in reproductive development including flowers with indehiscent anthers and parthenocarpic seeds which could not germinate, indicating that the conversion of IAA to oxIAA is an important mechanism of IAA catabolism during reproductive development (Zhao et al., 2013). Recently, the *Arabidopsis* DAO1 was also shown to play a major role in IAA catabolism (Mellor et al., 2016; Porco et al., 2016; Zhang et al., 2016). The *Atdao1* loss of function mutants (*Atado-1* and *Atdao-3*) contained significantly reduced oxIAA levels and showed slight developmental defects including elongated pistills and reduced fertility. The free IAA levels in the *Atdao1* mutants were not strongly affected, but IAA-Glu and IAA-Asp levels were increased, indicating that IAA oxidation and conjugation collaboratively maintain the optimal IAA levels in plans (Mellor et al., 2016; Porco et al., 2016; Zhang et al., 2016). OxIAA has also been reported in pea (Stolárik et al., 2015), but minimal information is available on this IAA metabolism pathway in pea.

1.2 ETHYLENE

Long before the discovery of ethylene as a plant hormone, ethylene from smoke was used to induce fruit ripening and to stimulate the formation of female flowers in cucumber (cited from Abeles et al., 1992). In the late 19th century, Dimitry Nikolayevich Neljubov observed that etiolated pea seedlings exposed to an unknown compound in the laboratory air grew horizontally (diageotropism) with thicker and shorter epicotyls, a phenotype which was subsequently termed triple response (Knight and Crocker, 1913; Abeles et al., 1992). Neljubov's studies eventually led to the identification of ethylene as the compound responsible for the triple response in the early 1900's (cited from Abeles et al., 1992; Bakshi et al., 2015). However, ethylene was not recognized as a plant hormone until its natural existence in plants and its role in regulating natural fruit ripening was established in 1935 (cited from Bakshi et al., 2015).

In plants, the enzyme *S*-adenosylmethionine synthetase converts the amino acid methionine into *S*-adenosylmethionine (SAM) which is the precursor for the subsequent two-step process of ethylene biosynthesis (cited from Roje, 2006; Argueso et al., 2007; Fig. 1.3). In the first step, SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). In the second step, ACC is oxidized to ethylene by the enzyme ACC oxidase (ACO; cited from Bürstenbinder and Sauter, 2012). In general, the step catalyzed by ACS is considered to be

the major regulatory step in ethylene biosynthesis; however, the oxidation step catalyzed by ACO may regulate the rate of ethylene biosynthesis in certain physiological processes (cited from Dorling and McManus, 2012).

Both ACS and ACO are encoded by a small families of genes. In *Arabidopsis,* there are 10 ACS genes and five ACO genes (Dorling and McManus, 2012; Harpaz-Saad et al., 2012). Of the 10 *ACS* genes, only eight genes are considered fully functional as *ACS3* is a pseudogene, and ACS1 protein is functional only as a heterodimer (cited from Harpaz-Saad et al., 2012). Transcriptional regulation of *ACS* and *ACO* genes is considered to be a major regulatory mechanism of ethylene biosynthesis (Xu and Zhang, 2015). Different ACS and ACO gene family members can become transcriptionally active depending on conditions such as tissue type, developmental stage, or environmental stimuli (cited from Dorling and McManus, 2012; Harpaz-Saad et al., 2012).

In addition to regulation at the transcriptional level, ACS protein turnover is also considered to be an important regulatory step in ethylene biosynthesis (Xu and Zhang, 2015). Furthermore, the ethylene precursor ACC can undergo conjugation, limiting its availability for ethylene biosynthesis. Generally ACC is converted into malonyl-ACC (MACC) (Dorling and McManus, 2012), but a second form of ACC conjugate known as 1-(γ-L-glutamylamino) ACC (GACC) has been reported in tomato (*Solanum lycopersicum*; Martin et al., 1995). In addition to conjugation, it is possible for ACC to be transported from its site of synthesis through the xylem to other tissues, serving as a substrate for ethylene production (Bradford and Yang, 1980; Tudela and Primo-Millo, 1992; English et al., 1995).

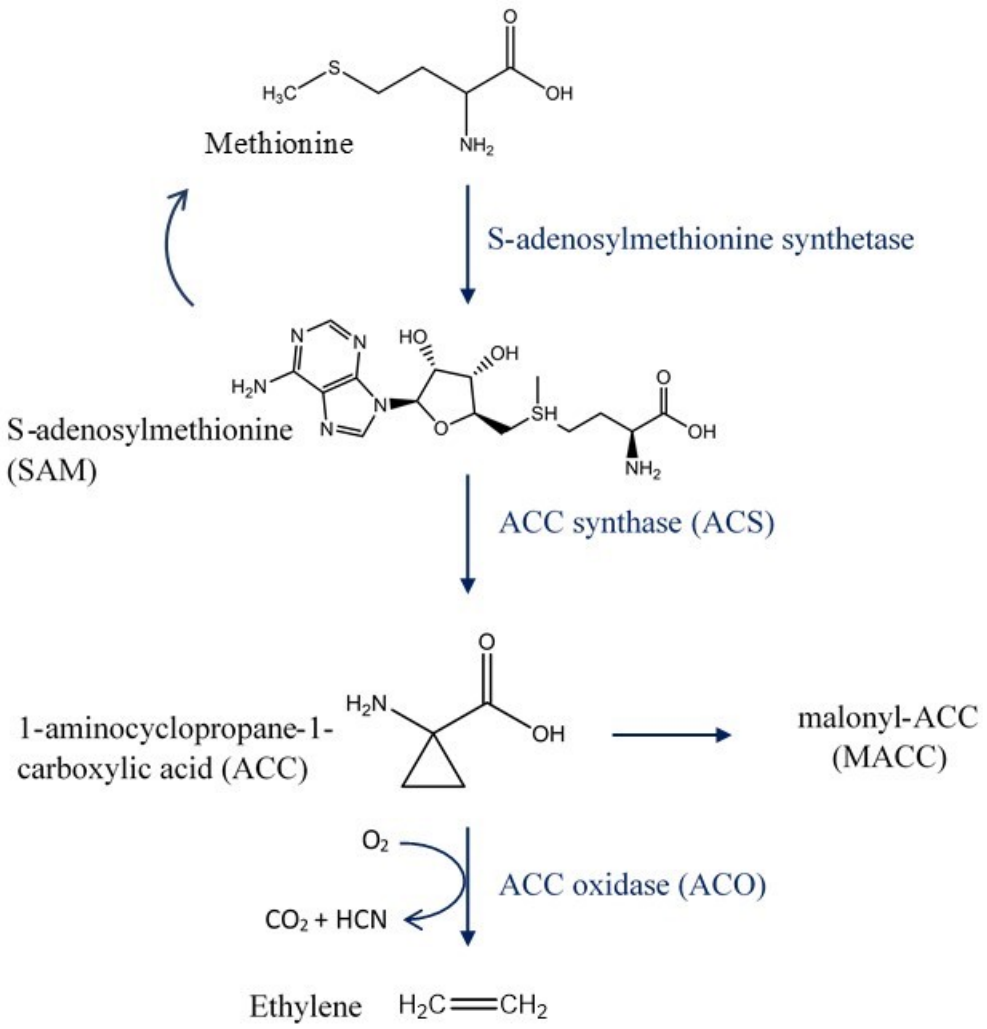


Figure 1.3. Ethylene biosynthetic pathway. The ethylene precursor s-adenosylmethionine (SAM) is synthesized from the amino acid methionine. SAM is converted to ethylene in a two-step pathway. In the first step, SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). In the next step, ACC is oxidized into ethylene by the enzyme ACC oxidase. ACC can also be removed from the biosynthesis pathway by conjugation. The most common form of ACC conjugate is malonyl-ACC (MACC). The figure was developed based on Argueso et al. (2007) and Pech et al. (2010).

1.2.1 Ethylene signaling

The triple response phenotype which led to the discovery of ethylene as a plant hormone also became an important developmental response in understanding the ethylene signaling pathway. Certain ethylene signaling mutants show triple response phenotypes even in the absence of ethylene, while other mutants do not show this phenotype even in the presence of ethylene (Guzmán and Ecker, 1990; Hua, 2015). In *Arabidopsis*, there are five ethylene receptors, ETHYLENE RESPONSE 1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR 1 (ERS1), ERS2 and ETHYLENE INSENSITIVE 4 (EIN4; Hua et al., 1998). In the absence of ethylene, ethylene receptors repress ethylene signaling through the activation of the negative regulator CONSTITUTIVE TRIPLE-RESPONSE 1 (CTR1; Hua and Meyerowitz, 1998; Fig. 1.4). CTR1 is a serine/threonine protein kinase which phosphorylates the downstream signaling component EIN2 (Ju et al., 2012; Qiao et al., 2012). This likely leads to the destabilization of EIN3/ ETHYLENE INSENSITIVE 3-LIKE 1 (EIL1) transcription factors preventing the expression of ethylene responsive genes. When ethylene binds to its receptors, the receptors no longer activate CTR1, which prevents phosphorylation of EIN2 by CTR1 (Ju et al., 2012; Qiao et al., 2012). This appears to cause the cleavage of the C-terminal domain of EIN2, which is subsequently translocated to the nucleus, where it stabilizes EIN3, allowing the expression of ethylene responsive genes (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). The levels of EIN3/EIL1 transcription factors are regulated by two F-box proteins EIN3 BINDING F-BOX 1 (EBF1) and EBF2, which mediate their degradation through 26S proteasome-mediated pathway. Recent studies show that the C-terminal end of EIN2 is also capable of suppressing the translation of EBF1 and EBF2 proteins (Li et al., 2015; Merchante et al., 2015). In *Arabidopsis*, transcript abundance of *EBF2* increases in response to ethylene, likely providing a negative feedback loop for ethylene signaling (Potuschak et al., 2003; Gagne et al., 2004). Ethylene also increases the transcript abundance of its receptors *ERS1, ERS2* and *ETR2* (Hua et al., 1998), which also acts as a negative feedback mechanism, as ethylene-unbound receptors negatively regulate the ethylene signaling pathway (Merchante et al., 2013).

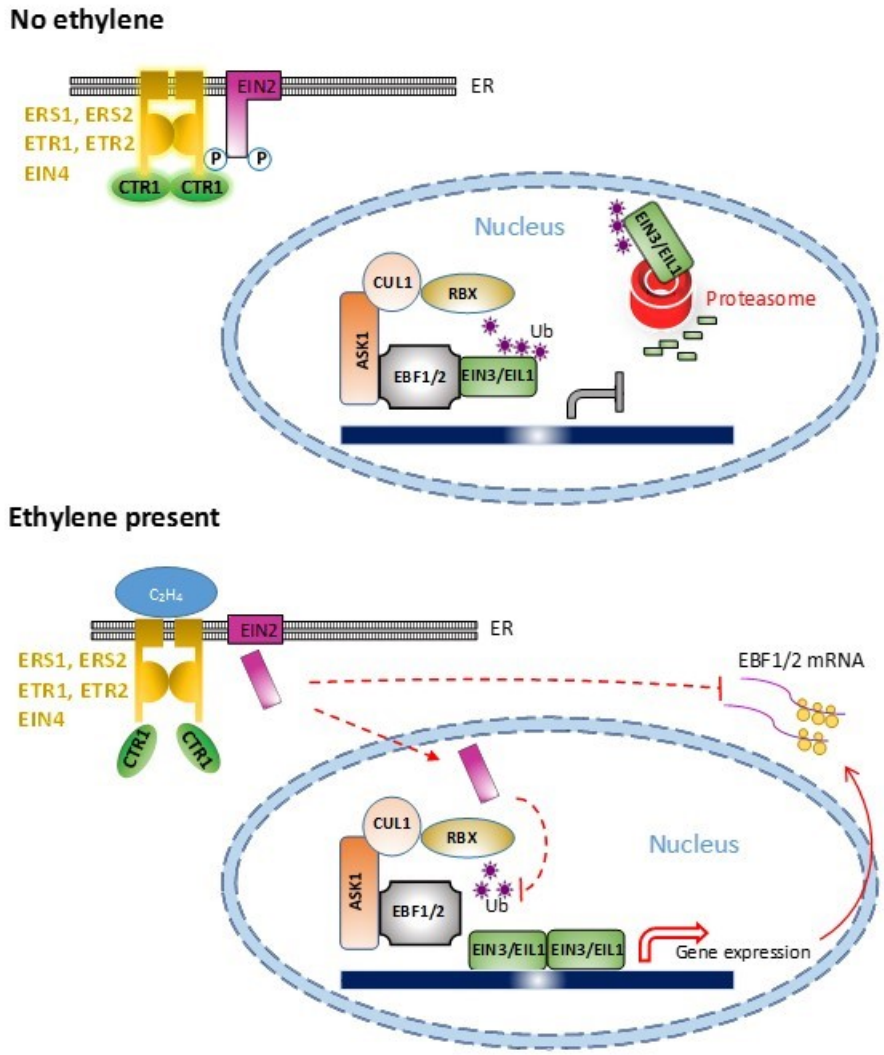


Figure 1.4. Ethylene signaling pathway. In the absence of ethylene, ethylene receptors activate CTR1 proteins. Active CTR1 phosphorylates EIN2 proteins preventing the cleavage of EIN2 C-terminal end. Downstream transcription factors EIN3 and EINL1 are degraded by F-box proteins EBF1 and EBF2 ($SCF^{EBF1/EBF2}$) through 26S proteasome-mediated pathway. Degradation of EIN3/EINL1 transcription factors stops ethylene responsive gene expression. When ethylene is present, ethylene bound receptors no longer activate CTR1, which prevents CTR1 mediated phosphorylation of EIN2. Dephosphorylation cleaves the C-terminal end of EIN2, which moves into the nucleus and stabilizes EIN3 and EIL1 apparently by preventing the $SCF^{EBF1/2}$ mediated degradation. Furthermore, EIN2 C-terminal end suppresses the translation of EBF1/2 mRNA. Stabilization of EIN3/EIL1 transcription factors initiates the expression of ethylene responsive genes including EBF2. The figure was developed based on Merchante et al. (2013) and Zheng and Zhu (2016). ER: endoplasmic reticulum, P: phosphate, Ub: ubiquitin.

1.3 REGULATION OF REPRODUCTIVE DEVELOPMENT BY AUXIN AND ETHYLENE

1.3.1 Roles of auxin during reproductive development

Auxin is important in almost all the aspects of reproductive development from the formation of floral meristem to floral morphogenesis, and from pollination to fruit development (Sundberg and Ostergaard, 2009; Dresselhaus and Schneitz, 2014). The formation of floral primordium requires site-specific accumulation and depletion of auxin (Heisler et al., 2005). Abnormalities in auxin distribution due to mutations in auxin transporter proteins such as *pin-formed1* (*pin1*), or due to the application of auxin transport inhibitors such as N-1-naphthylphthalamic acid (NPA), disrupt the formation of flower buds in *Arabidopsis* (Przemeck et al., 1996; Okada et al., 1991). The auxin transcription factor *ARF5* mutant (*monopteros*; *mp*) also forms inflorescence without flowers (Cheng and Zhao, 2007) indicating both auxin distribution patterns as well as an intact auxin-signaling pathway are important in floral initiation. *ARF3* (*ETTIN*; *ETT*) mutants such as *ett-1* show the importance of auxin in floral morphogenesis as *ett* flowers contain a number of abnormalities including increased number of petals and sepals and reduced number of stamens (Sessions et al., 1997). Furthermore, *ett* flowers have smaller ovaries and an abnormal distribution of stigma and style tissues (Sessions and Zambryski, 1995). Floral development is also affected in *Arabidopsis ARF6* and *ARF8* mutants such as *arf6-2* and *arf8-3*. The mutant flowers showed delayed stamen development and reduced fecundity. The *arf6-2* and *arf8-3* double mutants show severe defects where the flower development terminated as infertile closed buds and therefore produce no seeds (Nagpal et al., 2005).

Auxin also appears to be important in pollination and fertilization events. Increased auxin levels and an associated activation of auxin-responsive promoter *DR5* has been detected in the stigma and style tissue during pollen germination and pollen tube growth in *Nicotiana tabacum* (cited from Sundberg and Ostergaard, 2009). The importance of auxin in subsequent fruit set and development was first noted with its ability to induce parthenocarpic fruit development (development of fruits without ovule fertilization; Gustafson, 1936; Gustafson, 1939; Gustafson, 1942). Furthermore, alteration of normal auxin function can result in abnormalities in fruit development. For example, in tomato, antisense-mediated downregulation of *SlAux/IAA9* co-

receptors caused fruit set prior to pollination and fertilization causing fruits to develop simultaneously with flowers. Precocious fruit enlargement positioned the stigma out of reach of the stamens preventing self-pollination which contributed to parthenocarpic fruit development in those plants (Wang et al., 2005). Transcripts of *SlARF7* accumulate in the ovaries of developing tomato flowers, and they are depleted after pollination. Downregulation of *SlARF7* by RNA interference induced the formation heart-shaped parthenocarpic fruits (de Jong et al., 2009). Parthenocarpic fruit development has also been reported in tomato plants in response to the overexpression of plum auxin receptor *PslTIR1* (El-Sharkawy et al., 2016). Pea has also been used as a model system for studying auxin in fruit development (van Huizen et al., 1995; Ozga and Reinecke, 1999; Ozga et al., 2002; Ozga et al., 2009). Studies with developing pea fruit have led to the now established mechanism that auxins stimulate gibberellin biosynthesis, which is at least partially responsible for auxin-induced fruit growth (Ozga et al., 2009).

1.3.2 Roles of ethylene during reproductive development

As an inducer of petal senescence and fruit ripening, the vast majority of research on ethylene in reproductive development is focused on flower senescence and the later stages of fruit development that are associated with ripening. In horticultural plants like morning glory (*Ipomoea spp.*) and carnation (*Dianthus spp.*), flower (petal) senescence is stimulated by ethylene, and delayed by ethylene signaling or biosynthesis inhibitors (cited from Reid, 1995). Application of ethylene action inhibitors such as silver thiosulfate (STS) and 1-methylcyclopropene (1-MCP) has provided the basis for the commercial use of floral life extenders (Reid et al., 1980; Serek et al., 1995). The role of ethylene in fruit ripening is extensively researched. Climacteric fruits (like tomato and banana), are defined as fruits that show a sharp increase in ethylene evolution and respiration with the initiation of ripening. Coordinated regulation of ACC and ACO gene expression appears to be responsible for the induction of ethylene biosynthesis in the ripening process of climacteric fruits (cited from Lelièvre et al., 1997).

Other roles of ethylene in reproductive development include the regulation of flowering time. In pineapple (*Ananas comosus*), as well as mango (*Mangifera indica*) and wax apple (*Syzygium samarangense*), application of ethylene stimulates flower initiation (Reid, 1995). However in *Arabidopsis*, flowering was delayed in the constitutive ethylene response mutant

ctr1-1 and plants continually exposed to ethylene (Achard et al., 2007). Ethylene is also important in floral sex determination in certain species of the Cucurbitaceae family including cucumber (*Cucumis sativus*) and melon (*Cucumis melo*). In these species, ethylene modifies the ratio of male to female flowers as ethylene induces the femaleness (cited from Vandenbussche and Van Der Straeten, 2012).

Research on the role of ethylene in early fruit development has not received an adequate amount of attention (Ruan et al., 2012). However, certain studies indicate that ethylene plays an important role during early fruit development both before and after pollination events. In pistils of pollinated tomato flowers, a peak of ethylene evolution has been detected within hours after pollination, which then decreases to prepollinated levels within 24 hours. Pistil and petal transcript levels of certain *ACS* and *ACO* genes are also modulated in response to pollination (Llop-Tous et al., 2000). In orchid (*Phalaenopsis spp*.), increased *Phal-ACS2* and *Phal-ACS3* transcript levels were correlated with increased ACS enzyme activity in stigma and ovaries in response to pollination (Bui and O'Neill, 1998). Furthermore, in tobacco, transgenic inhibition of *ACO1* expression arrested the ovule maturation (De Martinis and Mariani, 1999). Pollen tubes germinating on the stigma of those plants did not reach the ovules. External application of ethylene restored the ovule development in *ACO1*-silenced tobacco plants and stimulated the pollen tube growth into the micropyle allowing ovule fertilization and subsequent seed development (De Martinis and Mariani, 1999).

1.4 AUXIN-ETHYLENE INTERACTIONS DURING PLANT DEVELOPMENT

A microarray analysis of *Arabidopsis* root tips exposed to IAA or ethylene showed that 27% of the ethylene-regulated genes were also regulated by auxin, while 18% of auxin-regulated genes were also regulated by ethylene. Even among the genes regulated only by auxin, 46% showed an altered expression in *ein2-5* plants which are incapable of transmitting an ethylene signal. Similarly, 28% of the ethylene-specific genes were affected in AUXIN RESISTANT 1 (AUX1) mutant *aux1-7*, where auxin transport is partially affected (Stepanova et al., 2007). These findings indicate the presence of complex interactions between auxin and ethylene.

A well-known auxin-ethylene interaction is the auxin-mediated stimulation of ethylene biosynthesis. Auxin stimulated expression of ACS genes can be considered as a primary

mechanism of auxin-stimulated ethylene biosynthesis (Abel and Theologis, 1996; Argueso et al., 2007; Robles et al., 2013). In *Arabidopsis*, the transcript abundance of all the functional ACS genes has been reported to be increased by IAA in seedling or root tissues (Tian et al., 2002; Yamagami et al., 2003; Tsuchisaka and Theologis, 2004; Lee et al., 2009). The presence of at least one auxin response element (AuxRE) within the 2 kb upstream promoter region has been reported for *AtACS4*, *AtACS6*, *AtACS8, AtACS9* and *AtACS11* genes (Lee et al., 2009). Consistent with auxin-mediated expression of *ACS*, a gain of function IAA3 (SHORT HYPOCOTYL 2; SHY2) mutant *shy2-2*, which acts as a repressor of auxin-responsive gene regulation, reduced the auxin responsive expression of *AtACS4*, *AtACS6* and *AtACS8* (Tian et al., 2002). Furthermore, an *Arabidopsis* mutant expressing a truncated version of *ARF2* (*arf2-6*) showed a reduced expression of *AtACS2*, *AtACS6* and *AtACS8* genes when tested in flowers. Consistent with this, senescence of both flowers and the plants were delayed in the *arf2-6* mutants (Okushima et al., 2005). Examples also exist for auxin stimulated modulation of ethylene biosynthesis in pea. IAA increased the transcript abundance of *PsACS1* and *PsACS2*, as well as the ACS enzyme activity, in the internodes of etiolated pea seedlings (Peck and Kende, 1998b).

Auxin can also induce *ACO* gene expression, which can either be dependent or independent of IAA-induced ethylene (Peck and Kende, 1995; Chae et al., 2000). In the stems of etiolated pea seedlings, *PsACO1* transcript abundance and ACO enzyme activity increased in response to IAA treatment. Application of IAA with ethylene action inhibitor 2, 5-norbornadiene (NBD) prevented the IAA-induced increase in *ACO1* transcript abundance and ACO enzyme activity (Peck and Kende, 1995). These data indicate that *PsACO1* expression is mediated through IAA-induced ethylene in etiolated pea internodes. In etiolated rice seedlings, *OsACO2* transcripts, but not that of *OsACO3,* accumulated in response to auxin. Application of ethylene action inhibitor NBD or ethylene biosynthesis inhibitor 1-aminoethoxyvinyl-glycine (AVG) did not affect the IAA induction of *OsACO2* transcript abundance, suggesting that the IAA-mediated regulation of *OsACO2* expression is independent of IAA-induced ethylene (Chae et al., 2000).

Data also support the presence of auxin responsive elements (AuxREs) in the 5′ promoter regions of *ACO* genes. In *Arabidopsis*, expression of the *ß-glucuronidase* (*GUS*) reporter gene driven by the pine (*Pinus taeda L.*) *ACO2* promoter responded to exogenous IAA (Yuan and Dean, 2010). In a similar study, the *GUS* gene expressed under the control of the tomato *ACO1*

promoter in tobacco seedlings responded to externally applied ethylene as well as auxin (Khodakovskaya et al., 2006). In the stems of those seedlings, *SlACO1* promoter-driven *GUS* gene expression was reduced in response to the removal of shoot apex (an endogenous auxin source) or the application of auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) directly under the intact apex. Furthermore, analysis of *SlACO1* 5′ promoter sequence revealed putative AuxREs indicating that auxin may directly regulate *SlACO1* promoter function (Khodakovskaya et al., 2006).

In addition to auxin stimulation of ethylene biosynthesis, there is evidence for ethylene-stimulated auxin biosynthesis, as well as ethylene-mediated regulation of auxin transport (Robles et al., 2013). For example, the immediate ethylene precursor, ACC, induced IAA biosynthesis in *Arabidopsis* seedlings, while application of the ethylene biosynthesis inhibitor AVG reduced the rate of auxin biosynthesis (Swarup et al., 2007). Expression of the *GUS* reporter gene driven by the auxin-responsive *IAA2* promoter showed reduced *GUS* expression in the roots of the ethylene insensitive mutant *ein2,* while *GUS* expression was increased in the constitutive ethylene signaling mutant *ctr1* indicating that intact ethylene signaling pathway was important in the auxin response (Swarup et al., 2007).

Auxin transport into plant cells is mediated by AUX1 and LIKE AUX 1 (LAX) proteins (cited from Muday et al., 2012). Auxin efflux from cells is mediated by PINFORMED (PIN) and ATP BINDING CASSETTE TYPE B/ P-GLYCOPROTEIN/ MULTIDRUG RESISTANCE (ABCB/PGP/MDR) proteins (cited from Muday et al., 2012). Transcript analysis of genes encoding auxin transport proteins or expression analysis of *GUS* or green fluorescent proteins driven by auxin transport gene promoters indicate that the expression of *PIN1*, *PIN2*, *PIN4* and *AUX1* in *Arabidopsis* is regulated by ethylene (cited from Muday et al., 2012; Robles et al., 2013). Therefore, it is likely that ethylene may also have a role modulating the auxin transport within plants (Muday et al., 2012; Robles et al., 2013). Overall, these data support that auxin and ethylene interact at multiple levels to influence plant growth and development.

1.5 PEA AS A MODEL SYSTEM FOR STUDYING HORMONAL INTERACTIONS IN FRUIT DEVELOPMENT

Pea is a climbing annual plant (Elzebroek and Wind, 2008), with a general life span of 12-18 weeks depending on the cultivar (Food and Agriculture Organization of the United Nations, 2016). Generally, plants start flowering in about 45 days after planting and flowering lasts about 2 to 4 weeks (Elzebroek and Wind, 2008). As a cool-season legume, pea plants require temperatures between 7 °C and 24 °C for normal development (Lim, 2012), while optimal temperature for growth is around 13 to 23 °C (Cutforth et al., 2007). Pea is an economically important crop in Canada. As the world's leading pea producer (Smýkal et al., 2012), Canada accounted for 32% of the world pea production in 2010 (Pulse Canada, 2016).

In pea, flowers normally self-pollinate 24 to 36 h before flower opening and fertilization has occurred by the time of complete flower opening (Cooper, 1938). Pea fruits contain a single carpel with the ovules attached to the ventral suture of the ovary. This simple fruit structure with its moderate size makes pea fruit easy to manipulate at a young developmental stage (Ozga et al., 1992). Pea is also a classical genetic model. The early use of pea as a model plant has been traced back to 1799 when Thomas Andrew Knight used pea to study heredity (cited from Smýkal, 2014). This was followed by the detailed experiments on heredity by Gregor Mendel in 1860's using pea as a model plant (cited from Smýkal et al., 2012). As a long used plant model in scientific research, detailed morphological, anatomical and physiological characterization of pea fruits has been carried out over a long period (Gregory, 1903; Bisson and Jones, 1932; Cooper, 1938; McKee et al., 1955; Eeuwens and Schwabe, 1975; García-Martínez and Carbonell, 1980; Ozga and Reinecke, 2003). The availability of detailed information and the ease of fruit manipulation make pea an ideal model for fruit development studies. Especially the ability of easy seed removal and hormonal applications by split pericarp technique make it a better model compared to other commonly used model plants like *Arabidopsis* and tomato when analyzing the role of seeds and hormones in fruit development (Ozga et al., 1992; Ozga and Reinecke, 2003). However, the larger genome size and repetitive nature of the pea (*P. sativum* L.) genome has delayed its sequencing (Smýkal et al., 2012).

1.6 ROLES OF AUXIN AND ETHYLENE DURING PEA FRUIT DEVELOPMENT

Seeds play a critical role in normal fruit development as removal or destruction of developing seeds arrests ovary development causing ovary senesce and abscission (Eeuwens and Schwabe, 1975; Ozga et al., 1992; Ozga et al., 2003; Ozga et al., 2009). Plant hormones are involved in coordinating pea pericarp growth with developing seeds (Eeuwens and Schwabe, 1975; Ozga et al., 1992; Ozga et al., 2009), but the mechanisms of hormonal regulation are complex.

Gibberellins (GAs) and/or auxins such as 4-Cl-IAA have been implicated as hormonal signals coordinating pericarp growth in pea. 4-Cl-IAA and IAA are naturally occurring auxins found in developing pea fruits with high concentrations in the seeds (Marumo et al., 1968; Magnus et al., 1997; Reinecke, 1999). 4-Cl-IAA can stimulate deseeded pea pericarp growth, mimicking the presence of seeds. These data suggest that 4-Cl-IAA may be a seed-derived signal promoting fruit growth (Ozga and Reinecke, 1999; Ozga et al., 2002; Ozga et al., 2009). In contrast, the ubiquitously occurring auxin IAA is incapable of rescuing deseeded pea pericarp growth (Reinecke et al., 1995). Seeds and 4-Cl-IAA are capable of regulating the transcript abundance of specific GA biosynthesis and metabolism genes in the pericarps. During GA biosynthesis, GA 20-oxidase (encoded by *PsGA20ox1* and *PsGA20ox2*) converts GA_{53} to GA_{20}, and GA 3-oxidase (encoded by *PsGA3ox1* and *PsGA3ox2*) converts GA_{20} to bioactive GA_1. GA 2-oxidases (encoded by *PsGA2ox1* and *PsGA2ox2*) catabolize GA_{20} to GA_{29}, reducing the precursor availability for GA_1 biosynthesis, and GA_1 to its inactive catabolite GA_8 (Ozga et al., 2009).

In the presence of developing seeds or in response to the treatment of deseeded pericarps with 4-Cl-IAA but not with IAA, higher transcript abundance of *PsGA20ox1*, *PsGA3ox1* and a reduced transcript abundance of *PsGA2ox1* was detected in the pericarps (Ozga et al., 2009). Associated with this transcript abundance profile, conversion of $[^{14}C]GA_{12}$ to $[^{14}C]GA_1$ was observed in deseeded pericarps only if treated with 4-Cl-IAA (Ozga et al., 2009). These data support the hypothesis that seed-derived auxin (4-Cl-IAA in pea) is transported to the pericarp, where it stimulates GA biosynthesis by differentially regulating the expression of pericarp GA biosynthesis and catabolism genes for increased production of bioactive GA_1, which in part, stimulates pericarp growth (Ozga et al., 2009).

The ability of 4-Cl-IAA, but not IAA, to stimulate GA biosynthesis in the pericarp is likely a major factor in 4-Cl-IAA-mediated pericarp growth. However, 4-Cl-IAA can also minimize the inhibitory effects of ethylene on pericarp growth, but this is not the case for IAA (Johnstone et al., 2005). Reinecke et al. (1995) found that although 4-Cl-IAA-stimulated deseeded pericarp growth at 1 to 100 μM, IAA at 10 μM or higher concentrations inhibited deseeded pericarp growth. As a pretreatment with silver thiosulfate (STS; an inhibitor of ethylene action) blocked the IAA-induced inhibition of deseeded pericarp growth, IAA-induced ethylene was implicated as the growth inhibitory factor in the pericarp assays (Johnstone et al., 2005). The difference in growth response between the two auxins was not attributed to differences in auxin-induced ethylene evolution, as the 4-Cl-IAA-stimulated ethylene evolution profile from deseeded pea pericarps was similar to that of IAA (Johnstone et al., 2005). Furthermore, the application of ethephon to 4-Cl-IAA-treated deseeded pericarps had minimal to no effect on pericarp growth (Johnstone et al., 2005). Overall, these data suggest that the interactions of IAA and 4-Cl-IAA with ethylene in pea fruit tissue is fundamentally different; however, the specific mechanisms that bring about the different auxin-ethylene interactions are not known.

1.7 HYPOTHESES

There were two main hypotheses in my thesis research. The first hypothesis was that 4-Cl-IAA, the pericarp growth stimulating auxin, affects the ethylene biosynthesis and/or signaling pathway in a fundamentally different manner than IAA, the non-growth stimulating auxin. The second was that the differential effects of IAA and 4-Cl-IAA on pericarp growth are modulated through the auxin receptors involved in their perception. The following specific objectives were addressed in this study to evaluate the above hypotheses:

Objective 1 (Chapter 2):

To determine if developing seeds, IAA, 4-Cl-IAA differentially modulate the ethylene biosynthesis or signaling pathways, the transcript abundance of selected ethylene biosynthesis and signaling related genes were analyzed under different developmental conditions and in response to different hormonal treatments. Effect of transcript level modulations on the ethylene

precursor ACC and the ACO enzyme activity were also analyzed in pericarps treated with IAA and 4-Cl-IAA.

Objective 2 (Chapter 3):

To determine if auxin receptors are involved in bringing about the differential effect of IAA and 4-Cl-IAA on pea fruit development, two TIR1 homologs of pea (*PsTIR1a* and *PsTIR1b*) were isolated and functionally characterized in *Arabidopsis* auxin receptor mutant backgrounds. Auxin regulation of *PsTIR1a* and *PsTIR1b* transcript abundance and the expression of auxin-responsive *DR5* promoter driven *β-D-glucuronidase* (*GUS*) reporter were evaluated to determine the possible mechanism behind the differential action of IAA and 4-Cl-IAA on pericarp development.

Objective 3 (Chapter 4):

To understand the possible involvement of other TIR1/AFB family members in the differential action of IAA and 4-Cl-IAA, the expression profiles of the putative auxin receptors *PsAFB4* and *PsAFB6* were analyzed under different developmental conditions and in response to various treatments including IAA and 4-Cl-IAA. The possibilities of the functional characterization of those receptors in *Arabidopsis* auxin receptor mutant backgrounds were also evaluated.

CHAPTER 2

REGULATION OF ETHYLENE BIOSYNTHESIS AND SIGNALING BY DEVELOPING SEEDS AND TWO NATURALLY OCCURRING AUXINS IN PEA FRUIT

2.1 INTRODUCTION

Fruit development involves a complex interaction of molecular, biochemical and physiological changes regulated in part by an intricate hormonal network to transform an ovary into a mature fruit. The role of the plant hormone ethylene in the stimulation of fruit ripening in climacteric fruit is well known and it has been extensively characterized (Klee and Giovannoni, 2011); however, only limited information is available on the role of ethylene during early fruit development. Although ethylene evolution data (Orzáez et al., 1999) suggest that ethylene plays a role in the senescence of non-pollinated pea fruits, little is known about the regulation of ethylene biosynthesis, receptor and signaling genes during fruit set. Orzáez et al. (1999), using semi-quantitative Northern analysis, found that the mRNA levels of *PsACO1*, and a putative *ERS* ethylene receptor gene, increased with concomitant increases in ethylene production in non-pollinated carpels. However, the significance of the transcript level changes of these two genes within the suite of ethylene biosynthesis, receptor and signaling genes to affect fruit set is not known. Transcriptomic studies, mainly with tomato, indicated that a number of putative ethylene biosynthesis and signaling genes are differentially expressed in response to pollination and during early fruit development, suggesting coordinated regulation of ethylene biosynthesis and signaling in these processes (Vriezen et al., 2008; Wang et al., 2009).

Developing seeds play a critical role in normal fruit development as removal or destruction of developing seeds arrests ovary development and leads to ovary senescence and abscission (Eeuwens and Schwabe, 1975; Ozga et al., 1992). 4-Cl-IAA and indole-3-acetic acid (IAA) are naturally occurring auxins found in developing pea seeds (Marumo et al., 1968; Magnus et al., 1997; Reinecke, 1999). 4-Cl-IAA treatment to deseeded pea pericarps can mimic the presence of seeds with respect to growth, suggesting that this auxin may act as a seed-derived signal promoting fruit growth (Ozga and Reinecke, 1999; Ozga et al., 2002; Ozga et al., 2009).

However, the ubiquitously occurring auxin, IAA, was incapable of rescuing the growth of deseeded pericarps (Reinecke et al., 1995). The ability of 4-Cl-IAA, but not IAA, to stimulate pericarp GA biosynthesis is likely a major factor in 4-Cl-IAA-mediated pericarp growth (Ozga et al., 2009). However, 4-Cl-IAA can also minimize the growth inhibitory effects of ethylene on pericarp tissue, while IAA does not (Johnstone et al., 2005). Reinecke et al. (1995) found that although 4-Cl-IAA stimulated deseeded pericarp growth at 1 to 100 μM, IAA at 10 μM or higher inhibited deseeded pericarp growth. As a pretreatment with the ethylene action inhibitor silver thiosulfate (STS) blocked the IAA-induced inhibition of deseeded pericarp growth, IAA-induced ethylene was implicated as the growth inhibitory factor in the pericarp assays (Johnstone et al., 2005). The difference in growth response between 4-Cl-IAA and IAA was not attributed to differences in auxin-induced ethylene evolution, as both auxins stimulated similar ethylene evolution profiles when applied to deseeded pea pericarps (Johnstone et al., 2005). Furthermore, application of the ethylene releasing agent ethephon to 4-Cl-IAA-treated deseeded pericarps had minimal effect on pericarp growth (Johnstone et al., 2005). Overall, these data suggest that the interaction of these two naturally occurring auxins with ethylene in pea fruit tissue is fundamentally different; however, the specific mechanisms that bring about the different auxin-ethylene interactions are not known.

In this study, the expression of ethylene biosynthesis and signaling-related genes in pea fruit tissue was profiled in rapidly growing pea fruit (pollinated ovaries with developing seeds) and compared to fruit that will senesce (non-pollinated ovaries with unfertilized ovaries) to correlate ethylene gene expression profiles to pericarp growth status. Furthermore, seed, auxin and ethylene regulation of pericarp ethylene biosynthesis gene expression, ACC levels, ACC oxidase enzyme activity, and ethylene receptor and signaling gene expression were studied to investigate the underlying mechanisms involved in seed-, 4-Cl-IAA- and IAA-specific regulation of the ethylene biosynthesis and signaling pathway in pea fruit tissues during fruit set. From these studies, we developed a model of auxin and ethylene regulation of early pea fruit development that supports the working hypothesis that auxin (4-Cl-IAA in pea) produced in the developing seeds stimulates growth and development in the surrounding ovary by regulating a network of hormonal pathways in the ovary including the modulation of ethylene biosynthesis and response.

2.2 MATERIALS AND METHODS

2.2.1 Plant material and experimental procedures

Pea (*Pisum sativum* L. cv. I_3 Alaska-type) mature dry pea seeds were planted in 3-L pots containing a 4:1 (v/v) mixture of potting medium (Sunshine Mix #4/LA4, Sun Gro Horticulture, MA, USA) and sand. In each pot, five seeds were planted at a depth of 2.5 cm. After about two weeks, the seedlings were thinned to three per pot. Plants were grown in a growth chamber under cool white fluorescent lights with an average photon flux density of 235 µmol m^{-2} s^{-1} (measured with a LI-COR LI-188 photometer) and 16 h [light (19 ºC)/8 h dark (17 ºC)] photoperiod. Plants were fertilized with a solution of 28-14-14 (N-P-K) fertilizer when they were about 30-50 cm tall. Throughout plant development, the main shoot apex remained intact, while expanding lateral shoots were removed for all fruit experiments.

For the fruit developmental study, flowers from flowering nodes 1 to 6 were tagged at the date of anthesis (0 DAA). In addition to the date of anthesis, for consistency only pericarps within a specific length range were used as follows: 1 DAA: 8-12 mm, 2 DAA: 15-20 mm, 3 DAA: 26-33 mm. For non-pollinated fruits, floral buds at -2 DAA were emasculated and tissues were collected at -2, 0, 1, 2 and 3 DAA. Fruits were collected onto ice and immediately dissected into seed/ovule, pericarp wall, pericarp dorsal vascular suture and pericarp ventral vascular suture tissues (Fig. 2.1 C), except for those at -2 DAA, where the ovules were removed from the fruit, and the pericarps with the stigma and style attached were harvested with no further dissection. Tissues were frozen in liquid N_2 immediately after harvest and dissection, and they were immediately stored in -80 ºC until further processing.

For the fruit hormonal application studies, one fruit per plant from the 3rd to 6th flowering nodes was used. All the other flower buds and lateral shoots were removed as they emerged. The pericarps remained attached to the plant during the entire experiment. Hormonal treatments were applied to the pericarps using the split-pod technique (Ozga et al., 1992). Fruits at 2 DAA measuring 15 to 20 mm in length were split down the dorsal suture (opposite from the attachment of the seeds) and seeds were either left intact (SP) or removed (SPNS). Surgical manipulation of the pea fruit was completed 12 h prior to hormone application, with one exception. STS was applied to the pericarp immediately after splitting and deseeding, with subsequent hormonal application occurring 12 h after STS application. Deseeded pericarps were

treated with IAA or 4-Cl-IAA (50 μM in 0.1% aqueous Tween 80; 30 μL), ethephon (1000 mg L^{-1} in 0.1% aqueous Tween 80; 30 μL), or STS (1 mM in 0.1% aqueous Tween 80; 30 μL) alone or in combination. Auxin or auxin-ethephon combinations were IAA plus 4-Cl-IAA, IAA plus ethephon, 4-Cl-IAA plus ethephon, in 0.1% aqueous Tween 80 (30 μL total). Auxin or ethephon combinations with STS were IAA plus STS, 4-Cl-IAA plus STS, or ethephon plus STS, with a final concentration of auxins at 50 μM (30 μL), ethephon at 1000 mg L^{-1} (30 μL), and STS at 1 mM in 0.1% aqueous Tween 80 (30 μL). The SP and SPNS controls were treated with 0.1% aqueous Tween 80 (30 μL). When fruits were treated with both auxin and ethephon, ethephon was applied 90 min after the auxin treatment and samples were collected based on the time after auxin treatment. All solutions were applied directly to the inside surface of the pericarp wall (endocarp). All surgically modified pericarps were covered with plastic bags to maintain high humidity. Pericarps were harvested at 2, 8, and 12 h after the hormone treatment (or 14, 20, and 24 h after pericarp splitting or splitting and deseeding). The STS treated samples were also collected simultaneously with other samples (i.e. 14, 20 and 24 h after STS). Fruits were harvested and immediately transferred into liquid N_2, and subsequently stored at -80 ºC until further processing.

For the auxin application seedling studies, mature-dry pea seeds (*P. sativum* L. cv. I_3 Alaska-type) were sterilized with 70% ethanol for 20 to 30 sec, then gently shaken in a 1.2% (v/v) aqueous NaOCl solution with 0.1% Tween 80 (wt/v) for 20 min. Seeds were then rinsed four times with sterile deionized water and placed on two layers of Whatman #1 filter paper moistened with 40 mL of sterilized deionized water in 150 mm X 15 mm Petri plates. An additional filter paper layer was placed on top of the seeds; the plates were sealed with Parafilm and covered with aluminum foil to exclude light, and placed at room temperature (~ 22 ºC) for two days. After two days, germinated seeds were transferred to sterile Magenta GA-7 vessels (60 x 60 x 95 mm; 4 seeds per vessel) containing 1 layer of Whatman #1 filter paper at the bottom wetted with 9 mL of sterile deionized water or filter sterilized aqueous IAA (1 μM) or 4-Cl-IAA (1 μM) solutions. During the transfer, seeds were exposed to low light conditions for a short period of time. The Magenta vessels were covered with loosely fitting sterile Petri dish lids (to allow for some air exchange and limit ethylene accumulation in the headspace in the presence of auxin), and placed in the dark at room temperature (~ 22 ºC) for two days. After two days in the Magenta vessels, the 4 DAI seedlings were dissected into plumule, epicotyl and root-tip (6-7

mm) tissues, the tissue lengths were recorded, and then the tissues were immediately frozen in liquid nitrogen. Subsequently tissues were stored at -80 ºC until further processing.

For the ethephon-treated seedling studies, mature, dried seeds (four per vessel) were directly imbibed in Magenta GA-7 Vessels on a layer of Whatman #1 filter paper wetted with 8 mL of 15 mg L^{-1} ethephon (in 0.1% aqueous Tween 80) or 0.1% aqueous Tween 80 (v/v). The vessels containing the ethephon solution were cover with tight-fitting lids, while the vessels containing the 0.1% aqueous Tween 80 were covered with loose-fitting Petri dish lids. All vessels were incubated at room temperature in the dark for four days. After four days in dark, seedlings with a clearly emerged epicotyl and root were dissected into plumule, epicotyl and root-tip (6-7 mm) tissues, and the tissues were measured (epicotyl width at the base adjacent to the attachment of the cotyledons, and epicotyl and root length), immediately frozen in liquid nitrogen, and then subsequently stored at -80 ºC until further processing.

2.2.2 RNA Isolation

Frozen tissues were ground in liquid N_2 to a fine powder using a pre-chilled mortar and pestle. Approximately 40-60 mg of ground tissue per sample was used for total RNA isolation using a modified TRIzol (Invitrogen, CA, USA) method as described in Ayele et al., (2006). The concentration of extracted total RNA was measured at A_{260} using a NanoDrop (ND-1000 or 2000c; Thermo Scientific, MA, USA) spectrophotometer, and RNA quality and integrity were estimated using OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios and gel electrophoresis, or an Agilent 2100 bioanalyzer (Agilent Technologies, CA, USA). Given high RNA quality, the total RNA was DNase-treated (Ambion DNA-free Kit; Thermo Scientific) as per the manufacture's protocol. Following DNase treatment, the concentration of the total RNA samples was requantified at A_{260} using a NanoDrop (ND-1000 or 2000c) spectrophotometer and then diluted with nuclease free water to obtain to obtain a concentration of 40 ng μL^{-1}. The accuracy of the dilution was reverified at A_{260}; then the total RNA samples were stored at -80 ºC until use in qRT-PCR assays.

2.2.3 qRT-PCR assays

qRT-PCR primers and probes for *PsACS1*, *PsACS2*, *PsACS4*, *PsACO1*, *PsACO2*, *PsACO3*, *PsERS1*, *PsETR2*, *PsEBF1* and *PsEBF2* were designed with Primer Express Software (Version 3.0; Life Technologies, CA, USA) using the non-minor groove binding option. The ethylene biosynthesis and signaling gene probes were double-quenched probes (Integrated DNA Technologies, IA, USA) with an Iowa Black FQ (IBFQ) quencher at the 3′ end, and a ZEN quencher positioned 9 bp from the 6-FAM fluorescent dye containing 5′ end (Appendix Table A1). Primers and probe for the 18S rRNA control were designed by Ozga et al. (2003) with 5′ VIC as probe fluorescent dye and 3′ TAMRA as the quencher (Applied Biosystems, CA, USA).

For all the transcript targets, reverse transcription and quantification were performed as one-step reactions with TaqMan One-Step RT-PCR Master Mix Reagents Kit or TaqMan RNA-to-Ct 1-Step Kit (Applied Biosystems). Each 25 µL reaction contained 200 ng of total RNA (5 µL of 40 ng μL^{-1}), 12.5 µL of 2X master mix, 0.6 µL of 40X MultiScribe/RT Enzyme Mix, 300 nM each of forward and reverse primers (1.5 µL of each 5 µM primer), 100 nM of probe (0.5 µL of 5 µM) and 3.4 µL of nuclease-free water (Ambion). The reactions were performed in MicroAmp Fast Optical 96-well reaction plates covered with MicroAmp Optical Adhesive Film (Applied Biosystems) in a StepOnePlus Real-Time PCR system (Applied Biosystems). The thermal cycler conditions were: 48 ºC for 30 min (reverse transcription), 95 ºC for 10 min (AmpliTaq Gold DNA Polymerase activation), 40 cycles of amplification at 95 ºC for 15 sec (denaturation) and 60 ºC for 1 min (primer annealing and extension). Each sample was run in duplicate, and the average of these two technical replicates was used as the sample value. A common pooled total RNA sample was run on each qRT-PCR plate for use as a control to correct for plate to plate variation (Ayele et al., 2006) as follows:

*Normalized Ct value of sample = (Ct value of the common sample in the standard plate / Ct value of the common sample in the sample plate) * Ct value of sample*

The pea 18S small subunit nuclear ribosomal RNA gene was used as a loading control to estimate variation in input total RNA concentration across all samples. For 18S rRNA quantitation, the total RNA samples were diluted to 20 pg μL^{-1} (100 pg total RNA per 25 µL reaction) and assays were performed as described above. The coefficient of variation of the 18S

rRNA Ct among all the samples was 3.0% or lower; therefore, the target amplicon expression values were not normalized to the 18S signal (Livak and Schmittgen, 2001; Ozga et al., 2009).

The relative transcript abundance was calculated using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001), where ΔCt is the Ct difference of the sample being analyzed and an arbitrary value equal to or greater than the highest assayed Ct value. The arbitrary Ct value was set at 25 for all ethylene signaling genes. For ethylene biosynthesis genes, the arbitrary Ct values were set at 36 for *ACS* genes 28 for *ACO* genes. The efficiency of the qRT-PCR reaction was calculated for each target gene by performing qRT-PCR reactions with a series of total RNA dilutions typically from 150 to 0.015 ng μL^{-1} (or 750 ng to 0.075 ng per reaction) with three technical replicates for each concentration. The Ct values were plotted against the log RNA amount, and linear regression (r^2) was calculated. With an acceptable r^2, reaction efficiency (E) was calculated using the formula: $E = (10^{[-1/slope]}-1) * 100$ (See Appendix Table A1 for reaction efficiencies).

2.2.4 Cloning and sequencing of ethylene biosynthesis and signaling genes

Sequences of the putative ethylene biosynthesis and signaling pathway genes *PsACS1*, *PsACS2*, *PsACS3*, *PsACO1* and *PsERS1* were available in the GenBank database (accession numbers AF016458, AF016459, AB049725, M98357 and AF039746.1, respectively). The partial sequences of *PsACS4*, *PsETR2*, *PsEBF1* and *PsEBF2* genes were identified using a small scale Next Generation Sequencing database derived from 10 DAA seed coats of *P. sativum* L. cvs. I_3 (Alaska-type), Courier, Canstar, Solido and LAN 3017, using a Roche 454 Titanium pyrosequencer at the National Research Council, Saskatoon, Canada (NRCC SK). This database consisted of approximately 1.1 million reads with an average length of 336 bp. These reads have been *de novo* assembled into coatings using the program Newbler 2.5 under the default parameters. The final database contained about 19,000 unigenes (contigs and single reads which faild to assembled to contigs) and was accessible through the National Research Council Plant Biotechnology Institute, Canada as a non-public database (Ferraro, 2014). The coatings obtained from the database were assembled manually or using CAP3 sequence assembly program (Huang and Madan, 1999) to obtain the full-length coding sequences (CDSs). The putative full-length sequences of *PsACO2* and *PsACO3* were identified using blastn and tblastn search for *P. sativum* entries in the NCBI Transcriptome Shotgun Assembly (TSA) database which contain

computationally assembled sequences from primary data such as from Next Generation Sequencing Technologies.

For confirmation, full-length CDSs of *PsACS4*, *ACO2*, *ACO3*, *PsETR2*, *PsEBF1* and *PsEBF2* genes were amplified from pea fruit or seedling tissue cDNA (Appendix Fig. A1 to A6). SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) or qScript Flex cDNA Kit (Quantabio, MA, USA) were used for the cDNA synthesis with oligo-dT primers as per the manufacturers' protocols. The PCR amplifications of the CDSs were done using Phusion Hot Start II DNA Polymerase (Thermo Scientific), Q5 High-Fidelity DNA Polymerase (New England Biolabs, MA, USA) or the LongRange PCR Kit (Qiagen, Qiagen, Hilden, Germany; Appendix Table A2). The PCR primers, reaction composition, and cycling conditions are given in Appendix Tables A3, A4 and A5, respectively. The PCR amplicons were used directly as the template for sequencing of *PsACS4*, *PsEBF1, PsACO2* and *PsACO3*. For *PsETR2* and *PsEBF2*, the PCR products were cloned into a pCR8/GW/TOPO vector (Invitrogen) and transformed into chemically competent *E. coli* cells using the pCR8/GW/TOPO TA Cloning Kit (Invitrogen) as per the manufacturer's protocol. Plasmids extracted from transformed *E. coli* cultures using GeneJET Plasmid Miniprep Kit (Thermo Scientific) were used for sequencing. All gene sequencing was completed at the Molecular Biology Service Unit, Department of Biological Sciences at the University of Alberta, Edmonton, Alberta, Canada.

The *PsACS1* gene was reported to produce two different mRNAs, *PsACS1a* and *PsACS1b,* due to the presence of an alternate transcription initiation site (Peck and Kende, 1998*a*). As a result, the *PsACS1b* transcript lacks the first 383 nucleotides and encodes a non-functional protein which lacks at least the first 98 amino acids compared to *PsACS1a* (Peck and Kende, 1998*a*). Therefore, to avoid the analysis of non-functional *PsACS1b*, qRT-PCR primers and probes were designed to the 5′ coding region of the mRNA which is unique to *PsACS1a*.

The CDS of *PsACS4* (*P. sativum* L. cv. I_3 Alaska-type) harbors 98-99% sequence homology to portions of the 5′ and 3′ ends of *PsACS3* (*P. sativum* L. var. Saccharatum; GenBank accession number AB049725). However, an approximately 400 bp region (about 800 to 1200 bp from the start codon) was markedly different in *PsACS4* than *PsACS3,* with only 68% similarity at the nucleotide level (Appendix Fig. A7) and 62% similarity at the amino acid level. PCR reactions with cDNA synthesized from total RNA extracted from 6 DAA pericarp wall or 8 DAA seed tissues of *P. sativum* L. cv. I_3 (Alaska-type) produced no target-specific amplicon

when using a forward primer that binds to an identical 5′ region sequence of *PsACS3* and *PsACS4* (5′-CAAGAAATGGGTTTGGAAAA-3′) and a reverse primer specific to the variable region of *PsACS3* (5′-CCACCGTAACTTTGCGAACA-3′). However, the same forward primer produced a target-specific band in PCR reactions when used with a reverse primer that binds to the 3′ region of *PSACS4* that varies from that of *PsACS3* (5′-TGAACTCTCCGCTAAAAACTTATCC-3′). Furthermore, primers designed to amplify the full-length sequence of *PsACS3/ PsACS4*, which bind to the identical sequences in the 5′ and 3′ regions of *PsACS3* and *PsACS4* (Appendix Table A3), amplified only one PCR product from the pea cv. I_3 Alaska-type cDNA. This amplicon had 100% sequence similarity to the *PsACS4* CDS (Appendix Fig. A1). However, as a precaution to ensure that only *PsACS4* transcripts would be targeted, the primers and the probes for *PsACS4* were designed to target the unique region of the *PsACS4* CDS (Appendix Table A1).

2.2.5 Phylogenetic analysis and conserved domain search

The amino acid sequence alignment for phylogenetic tree creation for ACS and ACO was done using the MUSCLE sequence alignment program (Edgar, 2004) under the default settings in MEGA 7.0 (Edgar, 2004; Kumar et al., 2016). Best model selection and maximum likelihood tree creation with 1000 bootstrap replicates for ACS and ACO were also done in MEGA 7.0. For the ACS tree (Appendix Fig. A9), the LG model was used with a Gamma distribution (+ G). For the ACO tree (Appendix Fig. A10), the LG model with a Gamma distribution and invariant sites (G+I) was used (the number of discrete gamma categories was 4). The analysis involved 40 amino acid sequences for the ACS tree and 39 amino acid sequences for the ACO tree. All positions with less than 95% site coverage were eliminated.

The *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*) ACS sequences used in the ACS phylogenetic tree are from Booker and DeLong (2015), and that of *Hevea brasiliensis* from Zhu et al. (2015; see Appendix E.1 for all the sequences and their GenBank accession numbers). For the ACO phylogenetic tree, sequences of *Arabidopsis*, tomato, *Malus domestica,* and *Petunia x hybrida* were from Ruduś et al. (2013). The *Medicago truncatula* ACO sequences were obtained by a BLAST search of the *M. truncatula* Genome Project v4.0 database (http://jcvi.org/medicago/). Uniport accession numbers for the *M. truncatula* ACO proteins are G7KH99, G7J9K5, G7KJU7, A0A072V1X2, and G7ILQ8. The same Uniport accession

numbers have been used for naming the MtACO proteins in the phylogenetic tree (see Appendix E.2 for all the amino acid sequences and their GenBank accession numbers).

The amino acid sequence alignments for identification of conserved domains and residues in ACS and ACO were done using the PRALINE multiple sequence alignment application accessed through http://www.ibi.vu.nl/programs/pralinewww/ (Simossis and Heringa, 2003; Simossis and Heringa, 2005) under the default settings (gap opening penalty, 12.0; gap extension penalty, 1.0; amino acid substitution matrix, BLOSUM62; alignment strategy, homology-extended alignment). The PRALINE output is presented with color schemes for amino acid conservation as given in the figure legends of Appendix Figs. A10 and A11.

2.2.6 ACC and ACC-conjugate content, and ACO activity analyses

2.2.6.1 Plant material

Pericarp samples of 4-Cl-IAA- and IAA-treated fruits were collected 12 h after hormone treatment, and the control treatments (intact, SP and SPNS) were collected at the equivalent time-point (24 h after pericarp splitting and seed removal) for ACC and ACO analyses. Samples were immediately frozen in liquid N_2, and stored in -80 ^{o}C until further processing. Pericarp samples were ground in liquid N_2 using a pre-chilled mortar and pestle. Each biological replicate was composed of approximately 1.5 g of tissue (composed of a minimal of 6 pericarps), of which approximately 1 g of ground tissue was used for the ACC and ACC-conjugate analyses, and 0.5 g was used for ACO enzyme activity analysis.

2.2.6.2 Analysis of free ACC and total ACC

The ACC and ACC-conjugate contents were determined by quantitating the amount of ethylene liberated from ACC with NaOCl in the presence of Hg^{2+} as described by Bulens et al. (2011), with minor modifications. Approximately 1 g of ground pericarp tissue and 4.5 mL of 5% (wt/v) aqueous sulfosalicylic acid were added into a 15 mL tube and vortexed to mix. The homogenate was gently shaken at 4 ^{o}C for 30 min then centrifuged at 4,284 g for 10 min at 4 ^{o}C. A 500 µL aliquot of supernatant was placed into a separate tube for total ACC analysis. Both the

500 µL aliquot and the remaining plant extract of about 4 mL were frozen in liquid N_2 and stored at -80 ºC until further processing.

For free ACC analysis, the frozen plant extract was thawed on ice and 1.4 mL was transferred to a 7 mL glass vial. While keeping the vial on ice, 400 µL of 10 mM aqueous $HgCl_2$ was added to the plant extract and the vial was immediately closed with a rubber septum. In the presence of Hg^{2+} and basic reaction conditions (pH 10 or higher), NaOCl can efficiently convert ACC into ethylene (Concepcion et al., 1979). Therefore, to induce conversion of ACC into ethylene, 200 µL of NaOCl-NaOH mixture (2:1, v/v), freshly prepared by mixing 6 M NaOH and 5% aqueous NaOCl (v/v), was delivered into the vial by injection through the septum. The sample was vortexed for exactly 5 sec, kept on ice for exactly 4 min for the reaction to occur, and then vortexed exactly for 5 sec prior to removal of a headspace sample for GC analysis.

For total ACC (free ACC plus conjugated ACC) analysis, the vial with 500 µL of plant extract was thawed on ice, and 200 µL of 6 M HCl was added. The sample was vortexed and incubated in a boiling water bath for 3 h to facilitate acidic hydrolysis of ACC conjugates. Subsequently, the vials were kept at room temperature for 15 min to cool down prior to the addition of 200 µL of 6 M NaOH to neutralize the solution. The vials were then centrifuged at 21,000 g for 6 min to separate the supernatant containing total ACC. The conversion of ACC to ethylene was performed as in free ACC analysis, but with half-size reaction volumes. Specifically, 100 µL of acidic hydrolyzed sample was used for total ACC analysis, which was diluted with sterile deionized water to make 700 µL (placed into a 7 mL vial). Into the 700 µL sample, 200 µL of 10 mM aqueous $HgCl_2$ was added. The vial was sealed with a rubber septum, and 100 µL of freshly prepared NaOCl-NaOH mixture (2:1, v/v) was injected into the vial through the rubber septum. After vortexing and incubating the vial for the exact periods of time as described in free ACC analysis, a headspace sample was removed for ethylene analysis.

2.2.6.3 Analysis of ACO enzyme activity

The enzyme activity of ACO was analyzed by incubating the ACO extracts with an excess amount of ACC to release ethylene as described in Bulens et al. (2011). The amount of ethylene formed from ACC depends on the ACO enzyme activity in the extract. Frozen ground tissue (0.5 g) was transferred to a 2.0 mL microfuge tube, and 50 mg of polyvinylpyrrolidone (PVP) was added. PVP binds with polyphenols which can otherwise interfere with the ACO enzyme

activity. For ACO extraction, 1 mL of 400 mM MOPS extraction buffer (pH 7.2; see MOPS buffer preparation in Appendix A) was added to the sample followed by vortexing and incubation of the sample in a 4 ºC water bath for 10 min while gently shaking. The sample was then centrifuged at 21,000 g for 30 min at 4 ºC, and the supernatant was frozen in liquid N_2, then stored at -80 ºC until further processing.

To measure the ACO enzyme activity, the frozen supernatant was thawed on ice, 400 µL of the extract was transferred to a 7 mL glass vial, and 3.6 mL of MOPS reaction buffer (see MOPS buffer preparation in Appendix A) was added. The sample vial was immediately closed with a rubber septum, vortexed for 5 sec and incubated in a 30 ºC water bath for 1 h while gently shaking. The vial was vortexed again for 5 sec to release ethylene and a headspace sample was immediately taken for ethylene measurement. At the beginning and end of each set, a blank reaction was run by replacing the supernatant extract with sterile deionized water. These blank samples were used to estimate and correct for ethylene formed by the reaction solutions through non-enzymatic chemical reactions.

2.2.6.4 Ethylene analysis by gas chromatography

Ethylene from the reaction vial headspace was quantitated using a Varian 3400 gas chromatograph fitted with a 2.9 m x 6.35 mm HP plot-Q column connected to a flame-ionization detector. The column temperature was isothermal at 35 ºC, with the injection and detector ports set at 150 ºC and 250 ºC, respectively. Helium was used as the carrier gas. Injector head space pressure was set at 6.895 kPa giving a relative retention time for ethylene of 1.2 min. Ethylene standard curves were created by injecting different volumes (generally 10-250 µL) of 10 ppm analytical grade ethylene standard (Praxair, Danbury, USA). The injection volume was 50 µL for free ACC determination, except for 4-Cl-IAA-treated pericarp samples where the injection volume was 20 µL. The injection volumes for total ACC and ACO enzyme activity determination were 100 µL and 200 µL, respectively, for all samples. The number of ethylene moles released was calculated using the ideal gas law considering that the headspace air pressure within the vial remained constant at 101.325 KPa during the reaction.

2.3 RESULTS AND DISCUSSION

2.3.1 Regulation of ethylene biosynthesis and signaling gene expression in rapidly growing fruit and fruit targeted for senescence

Pollination and ovule fertilization was followed by rapid development of pea fruits in pericarp length and fresh weight (Fig. 2.1, A and B). In contrast, minimal growth occurred in the pericarps of non-pollinated ovaries (Fig. 2.1, A and B); however, the non-pollinated pericarps were still turgid and green at 3 DAA, and the fruit pedicel was still attached to the plant. Ethylene biosynthesis and signaling gene transcript abundance was profiled in whole pericarps at -2 DAA (before self-pollination occurs), and in the wall, dorsal vascular suture, and ventral vascular suture of the pericarp from 0 to 3 DAA in pollinated and non-pollinated ovaries (Fig. 2.1 C).

For ethylene biosynthesis gene expression, three putative *ACS* genes, *PsACS1* (GenBank accession number: AF016458), *PsACS2* (GenBank accession number: AF016459) and *PsACS4* (GenBank accession number: KX255646; sequenced from pea; Appendix Fig. A1), and three putative *ACO* genes, *PsACO1* (GenBank accession number: M98357), *PsACO2* (GenBank accession number: KX261617; sequenced from pea; Appendix Fig. A2) and *PsACO3* (GenBank accession number: KX261618; sequenced from pea; Appendix Fig. A3) were profiled. Marker genes for the ethylene signaling pathway were selected based on previous observations (mainly from *Arabidopsis* and tomato) that they become transcriptionally active in response to ethylene (Hua et al., 1998; Kevany et al., 2007; Konishi and Yanagisawa, 2008; Yang et al., 2010). Thus, the gene expression patterns of two putative ethylene receptor genes, *PsERS1* (GenBank accession number: AF039746), and *PsETR2* (GenBank accession number: KX261619; sequenced from pea; Appendix Fig. A4), and two putative genes of ethylene signal transduction, *PsEBF1* (GenBank accession number: KX261620; sequenced from pea; Appendix Fig. A5) and *PsEBF2* (GenBank accession number: KX261621; sequenced from pea; Appendix Fig. A6) that code for F-box proteins that are negative regulators of ethylene signaling were profiled.

The *ACS* genes code for enzymes that catalyze the synthesis of the ethylene precursor ACC. Phylogenetic analysis of the pea ACS proteins grouped them with functional ACS isoforms in *Arabidopsis* (AtACS2, AtACS4-AtACS9 and AtACS11; Yamagami et al., 2003; Appendix Fig. A8). Based on the C-terminus length and the presence of phosphorylation sites,

the ACS proteins are divided into three types (Grierson, 2012; Xu and Zhang, 2015). The phylogenetic tree grouped PsACS2 and PsACS4 with Type I ACS and PsACS1 with Type II ACS (Appendix Fig. A8). The Type I ACS isoforms have a longer C-terminus and contain three mitogen-activated protein kinase (MAPK) phosphorylation sites (Liu and Zhang, 2004) and one calcium-dependent protein kinase (CDPK) site (Sebastià et al., 2004). All of these features associated with Type I ACS could be recognized in PsACS2 and PsACS4 (Appendix Fig. A10). Phosphorylation of the MAPK and CDPK motifs of type I ACS proteins increases the stability of the protein, which is otherwise rapidly turned over by 26S proteasome-mediated degradation (cited from Booker and DeLong, 2015). Sequence analysis of PsACS1 showed that it has a shorter C-terminus and contain only a putative CDPK site as characteristic to Type II ACS (Appendix Figs. A8 and A10; Grierson, 2012). Type II ACS proteins are also degraded through a 26S proteasome-mediated pathway. The E3 ligase component ETHYLENE OVER-PRODUCER 1 (ETO1) or ETO1-like (EOL) proteins interact with the type II ACS at the C-terminal domain known as Target of ETO1 (TOE). The TOE domain consists of the consensus sequence WVF, RLSF and R/D/E rich region which is conserved in numerous species (Yoshida et al., 2006; Booker and DeLong, 2015). The PsACS1 sequence contains the conserved WVF sequence and a modified RLSF sequence (where F has been substituted with S, see Appendix Fig. A10, box from AA 484 to 499).

2.3.2 Increased *PsACS1* expression is associated with the presence of developing seeds, whereas *PsACS2* and *PsACS4* expression is associated with programed senescence in the ovary

PsACS1 transcript abundance was higher in the vascular sutures than in the wall of the pericarp (0 to 3 DAA; Fig. 2.2, A and B). Following pollination and fertilization at 0 DAA, *PsACS1* transcript level was higher in the pericarp ventral vascular suture where the developing seeds are attached (Figs. 2.1 C and 2.2 A) compared to the pericarp dorsal suture or wall tissue, and it gradually decreased as the fruit developed from 0 to 3 DAA (Fig. 2.2 A). In the absence of pollination, the variation in *PsACS1* transcript abundance among the pericarp tissues was relatively minor (Fig. 2.2 B). As *PsACS1* expression is auxin responsive (Peck and Kende, 1995; see below), it is possible that the pattern of *PsACS1* expression in the ovary tissues reflects higher auxin levels in the vascular sutures subtending the developing seeds in the pollinated

fruits. *PsACS1* transcript abundance was higher in the developing seeds of pollinated fruits which are enriched in auxins (Magnus et al., 1997) compared to non-pollinated ovules (0-3 DAA; Appendix Table A6). In contrast to *PsACS1*, transcript abundance of *PsACS2* and *PsACS4* increased approximately 2-fold (compare Fig. 2.2, C and D) and 10- to 20-fold (compare Fig. 2.2, E and F), respectively, in all pericarp tissues (both vascular sutures and the wall) of non-pollinated fruits compared to that of the pollinated fruits by 3 DAA. *PsACS2* transcript levels in the ovules of non-pollinated fruits were also about 2-fold higher compared to seeds of pollinated fruits (Appendix Table A6). These data suggest that the expression of *PsACS2* and *PsACS4* may be up-regulated by senescence signals.

2.3.3 *PsACO2* and *PsACO3* expression is associated with programed senescence in the ovary

The ACO proteins that catalyze the oxidation of ACC to ethylene are encoded by a small family of genes (usually 3 to 4 members) that belong to a large superfamily of ferrous-dependent non-heme oxygenases (Ruduś et al., 2013). Within this family, tomato SlACO1-3, apple (*Malus domestica*) MdACO1 and *Arabidopsis* AtACO4 have been shown to exhibit ACO activity (cited from Booker and DeLong, 2015). Phylogenetic analysis clusters PsACO1, PsACO2 and PsACO3 with ACC oxidases which are known to be functional (Booker and DeLong, 2015; Appendix Fig. A9). Furthermore, alignment of pea ACO with apple and petunia (*Petunia hybrida*) ACO1 shows that amino acids known to be important in ACO activity are conserved in PsACO1, PsACO2 and PsACO3 (Appendix Fig. A11). One modification was noted, a histidine (H) occurs at amino acid (AA) position 296 in PsACO3 instead of a glutamine (Q). However, an ACO family protein from *Medicago truncatula* also contained an H at AA position 296, suggesting that this variation is not exclusive to PsACO3 (amino acid positions with respect to the Appendix Fig. A11). The functions of the conserved AA sequences include binding sites for bicarbonate, ascorbic acid and the Fe (II) cofactor, required for the activation of ACO, and the binding sites for the ACC substrate (Shaw et al., 1996; Zhang et al., 2004; Dilley et al., 2013).

Within the pericarp, the transcripts of *PsACO1, PsACO2* and *PsACO3* were more abundant in the vascular sutures than in the wall tissue (Fig. 2.3; Appendix Table A6). In the absence of pollination, *PsACO1* transcript abundance remained elevated in the pericarp ventral suture of non-pollinated ovaries (where the seeds are attached to the ovary by the funiculus), while in the

other tissues transcript level decreased as observed in pollinated pericarps (Fig. 2.3 A and B). *PsACO1* transcript abundance was similar in ovules from non-pollinated ovaries and developing seeds from pollinated ovaries (Appendix Table A6). In contrast to *PsACO1*, the *PsACO2* and *PsACO3* transcript abundance increased 5- to 10-fold in all pericarp tissues of non-pollinated ovaries compared to that of pollinated ovaries (compare Fig. 2.3, C to D and E to F). Transcript levels of *PsACO2* and *PsACO3* in the ovules from non-pollinated fruits were also about 2-fold higher compared to developing seeds from pollinated fruits (Appendix Table A6). The increase in transcript abundance of *PsACO2* and *PsACO3* in the pericarp and ovule tissues from non-pollinated ovaries suggests that up-regulation of these genes is involved in programed senescence in these tissues. The expression pattern of *PsACO1* differs from that of *PsACO2* and *PsACO3* in pollinated and non-pollinated pericarp and seed/ovule tissues, suggesting complex regulation of ACO protein pools at the transcript level in theses tissues.

Overall, in the absence of pollination, the elevated transcript abundance of the ACC synthase genes *PsACS2* and *PsACS4,* and the ACC oxidase genes *PsACO2* and *PsACO3* suggests that the capacity to produce ACC and convert ACC to ethylene increases in non-pollinated ovaries, facilitating ovary senescence. This is consistent with elevated ethylene evolution from non-pollinated pea ovaries by 2 DAA compared to pollinated ovaries (cv. Alaska; Orzáez et al., 1999). Similarly, tomato transcriptomic data suggest that the transcript abundance of putative *ACS* and *ACO* genes are elevated in the ovaries of non-pollinated fruits compared to that in pollinated fruits (Vriezen et al., 2008).

2.3.4 Effect of seeds on pericarp ethylene biosynthesis during fruit set

Two DAA pea pericarps with seeds (either intact pericarps or split pericarp with intact seeds) will continue to grow and the fruit will set; however, if the pericarps are split and the seeds removed, pericarp growth is inhibited, and the pericarps will subsequently abscise (Ozga et al., 1992; Fig. 2.4). Minimal to no changes in the transcript abundance of the pericarp *PsACS* (*PsACS1*, *PsACS2* and *PsACS4*) and *PsACO* (*PsACO1*, *PsACO2* and *PsACO3*) ethylene biosynthesis genes from 2 DAA intact fruits were observed throughout the 12 h experimental period (Fig. 2.5). Associated with this transcript abundance pattern, intact pea pericarps contained trace amounts of free ACC (approximately 0.03 nmol $gfwt^{-1}$; Fig. 2.6 A), a moderate

level of ACC conjugate (19 nmol $gfwt^{-1}$; Fig. 2.6 C), and evolved low levels of ethylene (7-27 nL $gfwt^{-1}h^{-1}$, 2-3 DAA; Johnstone et al., 2005).

In the split-pericarp assays, pericarps are split down the dorsal suture to access the seeds, which stimulates wound-induced ethylene evolution (Johnstone et al., 2005). To avoid wound-induced ethylene evolution (which peaks approximately 4 h after pericarp splitting) and allow time for seed-derived factors to diminish in the pericarp tissue in the treatments where the seeds are removed, the pericarps were assessed 12 h after splitting and deseeding (Ozga et al., 1992; Johnstone et al., 2005). By 24 h after pericarp splitting, the transcript abundances of the *PsACS* and *PsACO* ethylene biosynthesis genes in the pericarps with seeds (SP) were similar to that of the intact pericarps, except for that of *PsACS1,* which had increased by 3-fold (Fig. 2.5). The *PsACS* and *PsACO1* transcript abundance patterns in SP pericarps were associated with increased free ACC (0.9 nmol $gfwt^{-1}$; 25-fold; Fig. 2.6 A), ACC conjugate (32 nmol $gfwt^{-1}$; 1.5-fold; Fig. 2.6 C), and ethylene evolution levels (Johnstone et al., 2005) 24 h after pericarp splitting compared to pericarps of intact fruit.

Seed removal (compare SPNS with SP) increased pericarp *PsACS4*, *PsACO2* and *PsACO3* transcript abundance (Fig. 2.5 C, E and F; 7-, 2.5-, and 5-fold, respectively, by 24 h after deseeding) and eliminated the increase in pericarp *PsACS1* transcript abundance (Fig. 2.5 A). In general, the lack of developing seeds (SPNS, Fig. 2.5 and non-pollinated ovaries, Figs. 2.2 and 2.3) was a primary factor influencing the pericarp *PsACS* and *PsACO* ethylene biosynthesis gene expression patterns. Elevated *PsACS4* transcript levels in deseeded pericarps were correlated with a 6-fold higher free ACC level (5.7 nmol $gfwt^{-1}$) and 2-fold higher ACC conjugate level (60 nmol $gfwt^{-1}$) than pericarps with seeds (SP; Fig. 2.6 A and C). The rate of ethylene evolution was also higher in deseeded pericarps (SPNS; from 8 to 24 h after pericarp splitting and deseeding) compared to pericarps with seeds (SP; Johnstone et al., 2005). As the ACO enzyme activity was similar in pericarp with (SP) and without seeds (SPNS; 24 h after pericarp splitting; Fig. 2.6 D), it is likely that the increase in ACC levels was the primary factor in increasing ethylene evolution in deseeded pericarps which will subsequently senesce. For comparison, the pericarp ACC levels in the intact pollinated pea fruit are similar to ACC levels observed in pre-climacteric fruit of banana (less than 0.05 nmol $gfwt^{-1}$), and the pericarp ACC levels in the deseeded pea pericarp (SPNS) are similar to those in ripening banana fruit during the climacteric rise (5 nmol $gfwt^{-1}$; Hoffman and Yang, 1980).

2.3.5 4-Cl-IAA and IAA increase pericarp *PsACS1* transcript abundance; however, only 4-Cl-IAA reduces the transcript abundance of pericarp *PsACS4*

Hormone treatments were applied to the pericarp 12 h after splitting and deseeding to avoid wound-induced ethylene evolution and allow time for seed-derived factors to diminish in the pericarp tissue (Ozga et al., 1992; Johnstone et al., 2005). 4-Cl-IAA stimulates deseeded pea pericarp growth and IAA does not (Reinecke et al., 1995), and this effect was observed within the time frame of this experiment (Fig. 2.4 A). The naturally occurring auxin 4-Cl-IAA dramatically increased the transcript abundance of *PsACS1* in deseeded pericarps, with peak transcript levels occurring 2 h after application (1800-fold increase), followed by a gradual decline in transcript levels from 2 to 12 h after treatment (compare 4-Cl-IAA to SPNS; Fig. 2.7 A, Appendix Table A7).

In contrast to *PsACS1*, 4-Cl-IAA reduced the transcript abundance of pericarp *PsACS4* (4-fold; Fig. 2.7 C), and did not affect that of *PsACS2* (compare 4-Cl-IAA to SPNS; Fig. 2.7 B). The increase in *PsACS1* transcript abundance by 4-Cl-IAA is not likely due to 4-Cl-IAA-induced ethylene, as the ethylene releasing agent ethephon applied alone or in combination with 4-Cl-IAA had minimal to no effect on pericarp *PsACS1* transcript abundance (Fig. 2.7 A; Appendix Table A7). Additionally, pretreatment of the pericarp with the ethylene action inhibitor STS did not affect the rise in *PsACS1* transcript abundance 2 h after 4-Cl-IAA treatment; however, the auxin response was attenuated at 8 and 12 h after hormone treatment, suggesting that interaction with an activated ethylene signaling pathway prolongs the duration of the 4-Cl-IAA response (Fig. 2.8 A). Consistent with the marked increase in *PsACS1* transcript levels in response to 4-Cl-IAA, application of 4-Cl-IAA to deseeded pericarps markedly increased the level of free ACC (6-fold higher) and conjugated ACC (2-fold higher) compared to that in the deseeded pericarp controls (SPNS; Fig. 2.6 A and C).

Application of the other naturally occurring auxin IAA to deseeded pericarps also increased *PsACS1* transcript level 2 h after application (approximately 40-fold), but to a much lesser extent than that of 4-Cl-IAA. By 8 h after application, *PsACS1* transcript level in IAA-treated deseeded pericarps was similar to the deseeded pericarp control (compare IAA to SPNS; Fig. 2.7 A; Appendix Table A7). IAA had minimal effects on *PsACS2* and *PsACS4* transcript abundance (Fig. 2.7 B and C). In contrast to 4-Cl-IAA, deseeded pericarps treated with IAA contained less free ACC (3-fold) and slightly lower ACC conjugate levels compared to SPNS

controls (Fig. 2.6, A and C). In vegetative tissue, Peck and Kende (1998*b*) reported IAA induction of *PsACS1* and *PsACS2* transcript abundance in the third (highest) internode of 5 to 6-day old etiolated pea seedlings, with *PsACS1* exhibiting a much stronger induction than *PsACS2* 2 h after IAA treatment. By 6 h after IAA treatment, the transcript abundance of *PsACS1* and *PsACS2,* and ACC synthase enzyme activity were markedly lower (Peck and Kende, 1998*b*).

2.3.6 4-Cl-IAA and IAA increase pericarp *PsACO1* transcript abundance; however, only 4-Cl-IAA reduces the transcript abundance of pericarp *PsACO2* and *PsACO3*

Auxin-induced pericarp *PsACS1* transcript abundance peaked 2 h after hormone application, whereas 4-Cl-IAA (11-fold) and IAA (about 2-fold) increased pericarp *PsACO1* transcript abundance 8 to 12 h after application (Fig. 2.9 A). The increase in *PsACO1* transcript abundance by 4-Cl-IAA was similar in magnitude when 4-Cl-IAA was applied alone or in combination with ethephon or IAA at 8 and 12 h after hormone application (Fig. 2.9 A). However, pretreatment of the pericarp with the ethylene action inhibitor STS reduced by half the magnitude of the 4-Cl-IAA-induced increase in *PsACO1* transcript abundance (by 8 h after hormone application; Fig. 2.8 D), while the IAA-induced increase was minimally affected by STS pretreatment (Fig. 2.9 A). As the level of *PsACO1* transcript abundance increased about 2-fold with ethephon treatment by 8 h after application (compared to the SPNS control; Fig. 2.8 D), and this effect was reversed by pretreatment with STS, our data suggest that *PsACO1* expression in young pea fruit is influenced by auxin and ethylene independently, and by auxin-induced ethylene. Peck and Kende (1995) found that the ethylene action inhibitor 2,5-norbornadiene inhibited the IAA-induced increase in *PsACO1* transcript abundance (using Northern analysis) and ACO oxidase activity in pea internode tissue, and they concluded that IAA promoted the accumulation of *PsACO1* transcript and the increase in ACC oxidase activity through IAA-induced ethylene in this tissue.

Similar to that observed for *PsASC4* (Fig. 2.7 C), 4-Cl-IAA suppressed the increase in *PsACO2* and *PsACO3* transcript abundance in deseeded pericarps (compare 4-Cl-IAA with SPNS; Fig. 2.9 B and C), mimicking the presence of the seeds (compare with SP and SPNS treatments). Additionally, ethephon treatment did not increase *PsACO2* and *PsACO3* transcript abundance of 4-Cl-IAA-treated pericarps (Fig. 2.9 B and C). As *PsACO2* and *PsACO3* transcript levels were stimulated in deseeded pericarps by ethylene (between 8 to 12 h after ethephon

application; Figs. 2.8 E and F, 2.9 B and C), and this increase was inhibited by pretreatment of the pericarp with STS (Fig. 2.8 E and F), these data indicate that 4-Cl-IAA may act to reduce ethylene activation of *PsACO2* and *PsACO3* expression. The 4-Cl-IAA-modulated pericarp *PsACO* transcript abundance profile was associated with no change in pericarp ACO enzyme activity (Fig. 2.6 D).

In contrast to 4-Cl-IAA, application of IAA to deseeded pericarps only increased *PsACO1* transcript abundance by 2-fold (8 and 12 h after hormone application; compare IAA and SPNS; Fig. 2.9 A), and had no clear effect on *PsACO2* and *PsACO3* transcript abundance compared to the SPNS control (8 and 12 h after hormone application; Fig. 2.9 B and C). This IAA-modulation of ACO transcript abundance pattern was associated with a slightly higher ACO enzyme activity level (50 to 70%) in IAA-treated pericarps compared to the SPNS control and 4-Cl-IAA-treated pericarps (Fig. 2.6 D). The lower ACC content observed in IAA-treated pericarps is likely due to higher ACO activity in these pericarps compared to the SPNS control (Fig. 2.6).

2.3.7 ACO apparent rate-limiting step in 4-Cl-IAA-treated pea fruit

Generally, the conversion of SAM to ACC by the enzyme ACS is considered as the rate-limiting step of ethylene production in plant tissues; however, ACO activity has been reported as the rate-limiting step in several systems (Dorling and McManus, 2012; Xu and Zhang, 2015) including post-climacteric tomato (Van de Poel et al., 2012). Similarly, in post-climacteric avocado (*Persea americana Mill.*), free ACC level increased to 100 nmol/g fwt, but no induction of ethylene evolution was observed (Hoffman and Yang, 1980) suggesting that ACO could be the rate-limiting step. Although 4-Cl-IAA-treated deseeded pea pericarps contained 20-fold higher ACC levels than those treated with IAA (Fig. 2.6 A), the ethylene evolution profiles from these tissues were similar (Johnstone et al., 2005), suggesting that in 4-Cl-IAA-treated deseeded pericarps, ACO is the rate-limiting step for ethylene biosynthesis.

It has been suggested recently that ACC may function as a signaling molecule, independent from its role as a precursor for ethylene biosynthesis (Yoon and Kieber, 2013), in processes such as root cell expansion (Xu et al., 2008) and root response to impaired cell wall biosynthesis (Tsang et al., 2011). The Tsang et al. (2011) study demonstrated that an active signaling process is present in *Arabidopsis* roots that reduce elongation when cell wall biosynthesis is impaired. This signaling process in cell wall damaged roots could be disrupted resulting in restored root

elongation in the short term by inhibiting ACC biosynthesis, auxin signaling, and superoxide production. In the present study, 4-Cl-IAA's ability to block ethylene response in deseeded pericarp (Johnstone et al., 2005) was associated with a dramatic increase in *PsACS1* transcript abundance, and the accumulation of very high ACC levels in this tissue. If ACC is functioning as a signaling molecule independent from its role as a precursor for ethylene biosynthesis in pea pericarp, our data suggest that ACC may be involved in attenuating ethylene response in this tissue.

The ACO protein has also been proposed to have regulatory roles independent of its ability to convert ACC to ethylene (Dilley et al., 2013). Analysis of the apple MdACO1 protein suggests that it has several protein-protein interactions and phosphokinase motifs (Dilley et al., 2013), and in apple fruits, the MdACO1 protein was shown to bind and phosphorylate proteins in a ripening dependent manner, further supporting the possibility of additional regulatory roles for this protein (Dilley et al., 2013). MdACO1 also contains putative cysteine protease motifs (Appendix Fig. A11) and shows a significant homology to tomato cysteine proteinase (LeCp; Dilley et al., 2013), which has protease enzymatic activity and, upon elicitor signaling, exhibits transcriptional factor activity that induces *LeACS2* expression (renamed *SlACS2*; Matarasso et al., 2005). In pea fruit, the transcript abundance of pericarp *PsACO1* was markedly induced by 4-Cl-IAA (Figs. 2.8 D and 2.9 A), a pericarp growth-stimulating auxin (Fig. 2.4; Reinecke et al., 1995). In contrast to *PsACO1*, the transcript abundance of pericarp *PsACO2* and *PsACO3* was reduced by 4-Cl-IAA, but stimulated by ethylene, and associated with tissues that will be undergoing senescence (Figs. 2.3 D and F, 2.5 E and F, 2.8 E and F, 2.9 B and C). It is possible that the pericarp *PsACO* transcript profile induced by 4-Cl-IAA results in a pool of ACO proteins that maintain the ACO enzyme activity at levels similar to pericarps with seeds (Intact and SP; Fig. 2.6 D). It is also possible that the *PsACO1* transcript was not translated to functional ACC oxidase protein, or if translated to protein, the function of at least part of the protein varied from that of ACC oxidation to ethylene. Further research on ACO proteins and their levels would aid in determining if the PsACO1 protein has additional regulatory roles during early pea fruit growth and development.

2.3.8 Ethylene signaling during fruit set

Ethylene is known to induce the expression of ethylene receptor and *EBF* genes. For example, in *Arabidopsis*, ethylene stimulates the transcript levels of *AtERS1*, *AtERS2* and *AtETR2* receptors and that of the *AtEBF2* (Hua et al., 1998; Gagne et al., 2004; Konishi and Yanagisawa, 2008). In tomato, ethylene stimulates the transcript levels of *NR, SlETR4* and *SlETR6* ethylene receptors and that of the *SlEBF2* (Yang et al., 2010). This ethylene-dependent increase of receptor and *EBF* transcript levels is believed to be an important part of the feedback regulatory mechanisms for damping the ethylene effect (Merchante et al., 2013). Consistently, ethephon increased the transcript abundance of pericarp ethylene receptors *PsERS1* and *PsETR2* (2- to 3-fold, Fig. 2.10 A and D), and that of the *PsEFB* genes, with the increase in *PsEBF2* transcript levels being the most prominent (18-fold by 8 h after treatment; Fig. 2.10 G and J). Pretreatment of pericarps with STS inhibited the ethylene-induced increase in transcript abundance of the ethylene receptor and signaling-related genes (Fig. 2.10 A, D, G, and J), providing further evidence that these responses were ethylene-induced.

The ethylene receptor (*PsERS1* and *PsETR2*) and signaling-related genes (*PsEBF1* and *PsEBF2*) showed minimal to no spatial variation in their transcript abundance among the pericarp tissues (Fig. 2.11; Appendix Table A6). In pollinated pericarps, a gradual reduction in the transcript abundance of *PsERS1*, *PsETR2*, and *PsEBF2* occurred from 0 to 3 DAA (Fig. 2.11 A, C, and G), with *PsEBF1* transcript abundance decreasing from 0 to 1 DAA (Fig. 2.11 E), likely reflecting reduced ethylene signaling, as ethylene evolution from the ovaries decreased after pollination/fertilization of the pea ovary (Orzáez et al., 1999). Similarly, transcript levels of the ethylene receptor *NEVER-RIPE* (*NR*; Lashbrook et al., 1998), *SlEBF1* and *SLEBF2* (Yang et al., 2010) decreased in tomato fruits following pollination. Furthermore, microarray analysis suggests that the transcript abundance of a number of putative ethylene signaling-related genes (*ERF* transcription factor genes and putative receptor genes showing homology to *ETR1* and *EIN4*) change during tomato fruit set indicating that the modulation of ethylene signaling is an important process during early fruit development (Wang et al., 2009).

In the absence of pollination, the pericarp transcript abundance of the ethylene receptor (*PsERS1* and *PsETR2*) and the signaling-related genes (*PsEBF1* and *PsEBF2*) remained elevated compared to pollinated fruits (compare pollinated to non-pollinated fruits; Fig. 2.11). The higher transcript abundances of the pea ethylene receptor and signalling-related genes in the pericarps

of non-pollinated fruits are consistent with increased ethylene action in these fruits that will undergo senescence.

2.3.9 Developing seeds and auxin regulation of pericarp ethylene signaling

Compared to intact pericarps, transcript abundance of the ethylene receptor genes, *PsERS1* and *PsETR2*, and the signaling gene *PsEBF2,* was higher in deseeded pericarps 12 h after pericarp splitting and deseeding (0 h after treatment), and their levels declined over the next 12 h to levels nearing that of the intact pericarps (Fig. 2.12). The peak of wound ethylene was observed 4 h after pea pericarp splitting (SP) or splitting and seed removal (SPNS), followed by lower ethylene evolution levels in both treatments with time (Johnstone et al., 2005). Therefore, the minimal differences of ethylene receptor and signalling gene transcript abundances by 20 to 24 h after pericarp splitting or splitting and seed removal (8 to 12 h after treatment; Fig. 2.12) likely reflects the decline of ethylene levels (that can up-regulate their expression; Ciardi and Klee, 2001; Konishi and Yanagisawa, 2008; see below) in both SP and SPNS over the experimental period.

4-Cl-IAA application to deseeded pericarps increased the transcript abundance of the ethylene receptors *PsERS1* and *PsETR2* (most prominent at 8 h after hormone treatment with a 2.5- and 4-fold increase, respectively; Fig. 2.10 B and E), and the ethylene signaling genes *PsEBF1* and *PsEBF2* (approximately 2- and 6-fold at 8 h; Fig. 2.10, H and K) compared to the SPNS control. IAA had no clear effect on pericarp ethylene receptor and EBF transcript levels (Fig. 2.10 C, F, I and L). Pretreatment of pericarps with STS inhibited the 4-Cl-IAA-induced increases in transcript abundance of the ethylene receptor and signaling-related genes (Fig. 2.10 B, E, H, and K), suggesting a functional ethylene receptor complex is required for the 4-Cl-IAA response. The 4-Cl-IAA-specific increase of pericarp ethylene receptor and *EBF* gene transcript abundance (not observed with IAA application) is likely not attributed to higher ethylene evolution levels in 4-Cl-IAA-treated pericarps, as 4-Cl-IAA and IAA induced similar ethylene evolution levels in deseeded pericarps (Johnstone et al., 2005). Increasing pea pericarp ethylene receptor and/or *EFB* transcript levels may be part of the mechanism that leads to inhibition of ethylene action by 4-Cl-IAA in deseeded pea pericarps, as observed by Johnstone et al. (2005)

2.3.10 Auxin and ethylene regulation of ethylene biosynthesis and signaling-related gene expression varies in fruit and seedling tissues

The regulation of ethylene biosynthesis gene expression by the auxins IAA and 4-Cl-IAA in fruit tissue (where 4-Cl-IAA stimulates fruit growth and IAA does not) was compared to that in 4 days after imbibition (DAI) dark grown pea seedling tissues (where both auxins inhibit root elongation). When etiolated pea seedlings are exposed to ethylene they produce triple-response phenotypes which are characterized by shorter, thicker shoots (epicotyls) and diageotropism (Knight and Crocker, 1913). A triple-response seedling phenotype was observed when seedlings were grown at 10 µM 4-Cl-IAA in our system, apparently due to the auxin-induced ethylene, but not at 1 µM 4-Cl-IAA (see epicotyl length; Appendix Fig. A12 B). Therefore, for the gene expression studies, pea seedlings (2 DAA) were grown for 48 h in the presence of IAA or 4-Cl-IAA at 1 µM, which inhibited root elongation, but did not induce a triple-response seedling phenotype (Appendix Fig. A12 A).

Although the auxin incubation period in the pericarp assays was shorter than that in the seedling assays, trends in auxin regulation of *PsACS* and *PsACO* genes in both assays were evident. Following the 48 h auxin incubation period, 4-Cl-IAA strongly induced *PsACS1* transcript level in the root tip (15-fold) and epicotyl tissues (6-fold), while no clear trend was evident with IAA (Fig. 2.13 A). *PsACS2* transcript abundance was generally not affected by IAA or 4-Cl-IAA in the seedling tissues (Fig. 2.13 B); however, increased transcript abundance of *PsACS2* was reported in the first internode of etiolated pea seedlings 1 to 2 h after IAA application (Peck and Kende, 1995). 4-Cl-IAA and IAA similarly affected the transcript abundance of *PsACS4, PsACO1*, *PsACO2* and *PsACO3* in the seedling root tips, where they increased *PsACO2* and *PsACO3* (Fig. 2.13 E and F), and had minimal to no effects on *PsACS4* and *PsACO1* (Fig. 2.13 C and D) transcript abundance. In the epicotyl tissue, 4-Cl-IAA slightly reduced *PsACO2* and *PsACO3* transcript abundance, and IAA did not (Fig. 2.13 E, and F). Neither auxin affected the transcript abundance of the *PsACS* or *PsACO* genes in the plumule tissue. Overall, these data indicate that effect of auxins on ethylene biosynthesis gene expression is tissue specific, as well as auxin-type specific. Furthermore, the auxin 4-Cl-IAA affects ethylene biosynthesis gene expression in a dramatically different manner in fruit tissue where it stimulates growth, than in root tips, where it inhibits growth.

The effect of ethylene on seedling ethylene biosynthesis and signaling genes was also studied in 4 DAI etiolated seedlings grown continually in the presence of ethephon (15 mg L^{-1} in 0.1% aqueous Tween 80) or 0.1% aqueous Tween 80 (control). Ethylene (via ethephon application) had no clear effect on *PsACS1* transcript abundance in the seedling tissues (Fig. 2.14 A), similar to that observed in the pericarp (Fig. 2.7 A and 2.8 A). Ethylene increased the transcript abundance of its biosynthesis genes, *PsACS2*, *PsACS4, PsACO2* and *PsACO3* in root tip tissues in a feed-forward manner (Fig. 2.14 B, C, E and F), and this was correlated with inhibition of root elongation (Appendix Fig. A12). *PsACO1* transcript abundance was higher in plumule and epicotyl tissues of ethephon-grown seedlings, but not in the root tip tissues (Fig. 2.14 D). Similar to that observed in pericarp tissues (Fig. 2.10 A, D, G and J), ethephon induced the transcript abundance of the ethylene receptor (*PsERS1* and *PsETR2*), and signaling-related genes (*PsEBF1* and *PsEBF2*) in the seedlings tissues (Fig. 2.15), indicating ethylene induction of the feedback regulatory loop to diminish ethylene signalling output.

In summary, increased pericarp *PsACS4*, *PsACO2* and *PsACO3* transcript abundance (observed in non-pollinated ovaries and deseeded fruit) was associated with pericarp senescence, suggesting that expression of these ethylene biosynthesis genes is up-regulated by senescence-related factors/conditions for increasing the capacity to produce ethylene for senescence induction in this tissue. Feed-forward regulation of ethylene biosynthesis by ethylene is likely involved in the senescence process mainly via ethylene induction of *ACO* gene expression (Fig. 2.16 A).

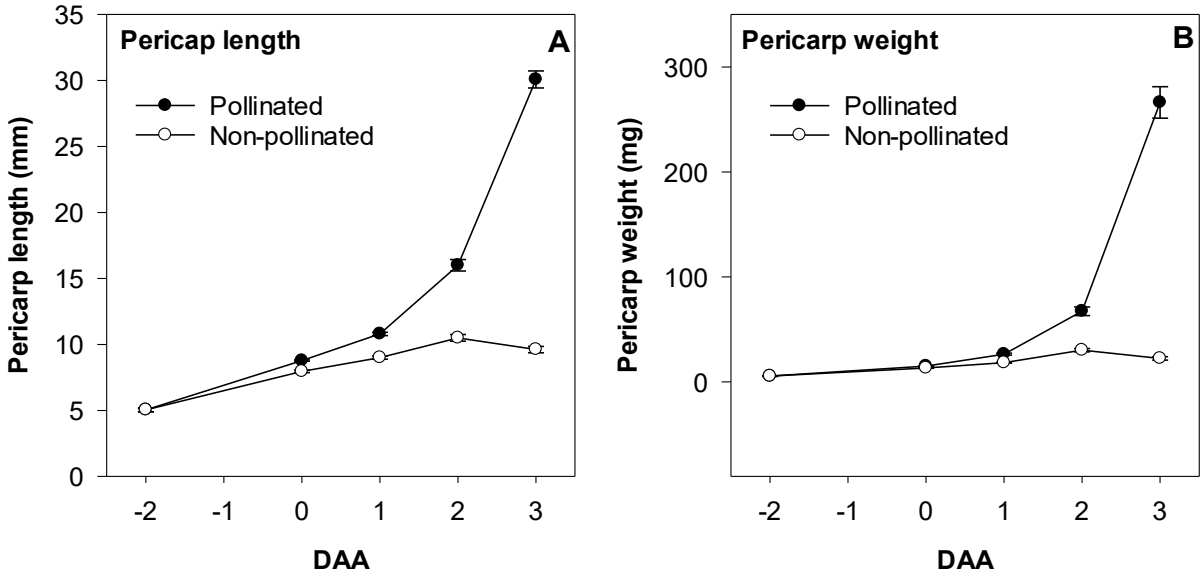
Auxin regulates the transcript abundance of pericarp *ACS* and *ACO* gene family members in a developmental-, tissue-, and auxin-specific manner. Application of 4-Cl-IAA to deseeded pericarps stimulated pericarp growth and inhibited the increase in transcript abundance of the pericarp ethylene biosynthesis genes *PsACS4*, *PsACO2* and *PsACO3* (Fig. 2.16 A), mimicking the presence of seeds. IAA did not rescue growth or inhibit the increase in *PsACS4*, *PsACO2* and *PsACO3* transcript abundance in deseeded pericarps (Fig. 2.16 A).

The marked induction of pericarp *PsACS1* transcript abundance by 4-Cl-IAA (Fig. 2.16 A) was associated with high pericarp ACC and ACC conjugate levels (Fig. 2.16 B). The pericarp *PsACO* transcript abundance profile was substantially modulated by 4-Cl-IAA, and this transcript profile was associated with no change in ACO enzyme activity compared to that in

seeded or deseeded controls (Fig. 2.16 A and B). IAA modulated the transcript abundance of the pericarp *PsACS* and *PsACO* genes to a much lesser extent than that of 4-Cl-IAA (Fig. 2.16 A), and the IAA-induced ethylene biosynthesis gene expression profile was associated with a decrease in pericarp ACC content and a modest increase in ACO enzyme activity compared to that in seeded or deseeded controls (Fig. 2.16 A and B). These data, together with the previously reported data that ethylene evolution levels were similar in 4-Cl-IAA- and IAA-treated deseeded pericarps (Johnstone et al., 2005), suggest that ACC oxidase is the limiting step for ethylene synthesis in 4-Cl-IAA-treated pericarps, but not in IAA-treated pericarps. Recent studies suggest that the ethylene biosynthesis precursor ACC may act as an independent signaling molecule (Yoon and Kieber, 2013). Furthermore, it has been suggested that the ACO protein may have regulatory functions in addition to its role in converting ACC to ethylene (Dilley et al., 2013). It is possible that the effects of 4-Cl-IAA on the expression of the *PsACS* and *PsACO* genes, which modify pericarp ACC levels and the ACO protein pools, leads to a reduction of the pericarp's response to ethylene (as noted by Johnstone et al., 2005).

With respect to ethylene signaling, 4-Cl-IAA increased the transcript abundance of the pericarp ethylene receptor and signaling-related (*EFB*) genes in a manner similar to that of the ethylene releasing agent ethephon, whereas IAA had minimal effects on the transcript abundance of these genes (Fig. 2.16 C). These data suggest that both ethylene and 4-Cl-IAA have the ability to diminish ethylene signalling output by up-regulating the expression of the pericarp ethylene receptor and signaling-related (*EFB*) genes. 4-Cl-IAA-induced increases in pea pericarp ethylene receptor and *EFB* transcript abundance (Fig. 2.16 C) may be part of the mechanism involved in the inhibition of ethylene action by 4-Cl-IAA in deseeded pea pericarps (observed by Johnstone et al., 2005). It also appears that 4-Cl-IAA's effect on ethylene receptor and *EFB* transcript levels is not a direct consequence of 4-Cl-IAA-induced ethylene evolution, as 4-Cl-IAA and IAA induce similar ethylene evolution levels in deseeded pericarps (Johnstone et al., 2005), but they elicit different effects on the transcript abundance of these genes.

Overall, the data in this study support the working hypothesis that auxin (4-Cl-IAA in pea) produced in the developing seeds stimulates growth and development in the surrounding ovary by regulating a network of hormonal pathways in the ovary including stimulation of gibberellin biosynthesis (Ozga et al., 2009) and modulation of ethylene biosynthesis and response (Fig. 2.16 D).



C

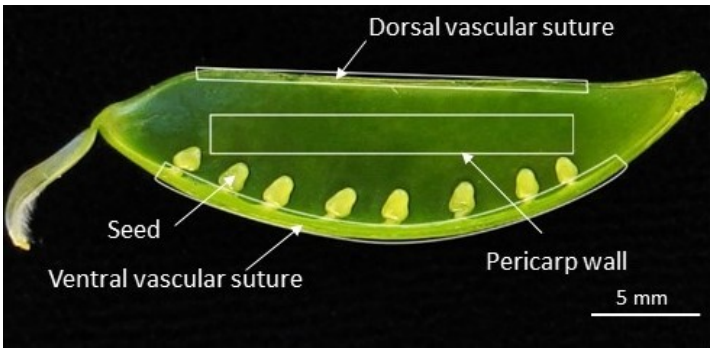


Figure 2.1. Growth of pollinated and non-pollinated pea fruits (*P. sativum L.* cv. I_3 Alaska-type). Pericarp length (**A**) and fresh weight (**B**) of pollinated and non-pollinated pea fruits from -2 to 3 days after anthesis (DAA). A representative pollinated pea fruit (ovary; 3 DAA) depicting the pericarp wall, dorsal vascular suture, and ventral vascular suture sections, and seeds, harvested for gene expression analysis (**C**). For the non-pollinated fruits, flowers were emasculated at -2 DAA to prevent pollination. Data are means ± SE; n=12 to 88.

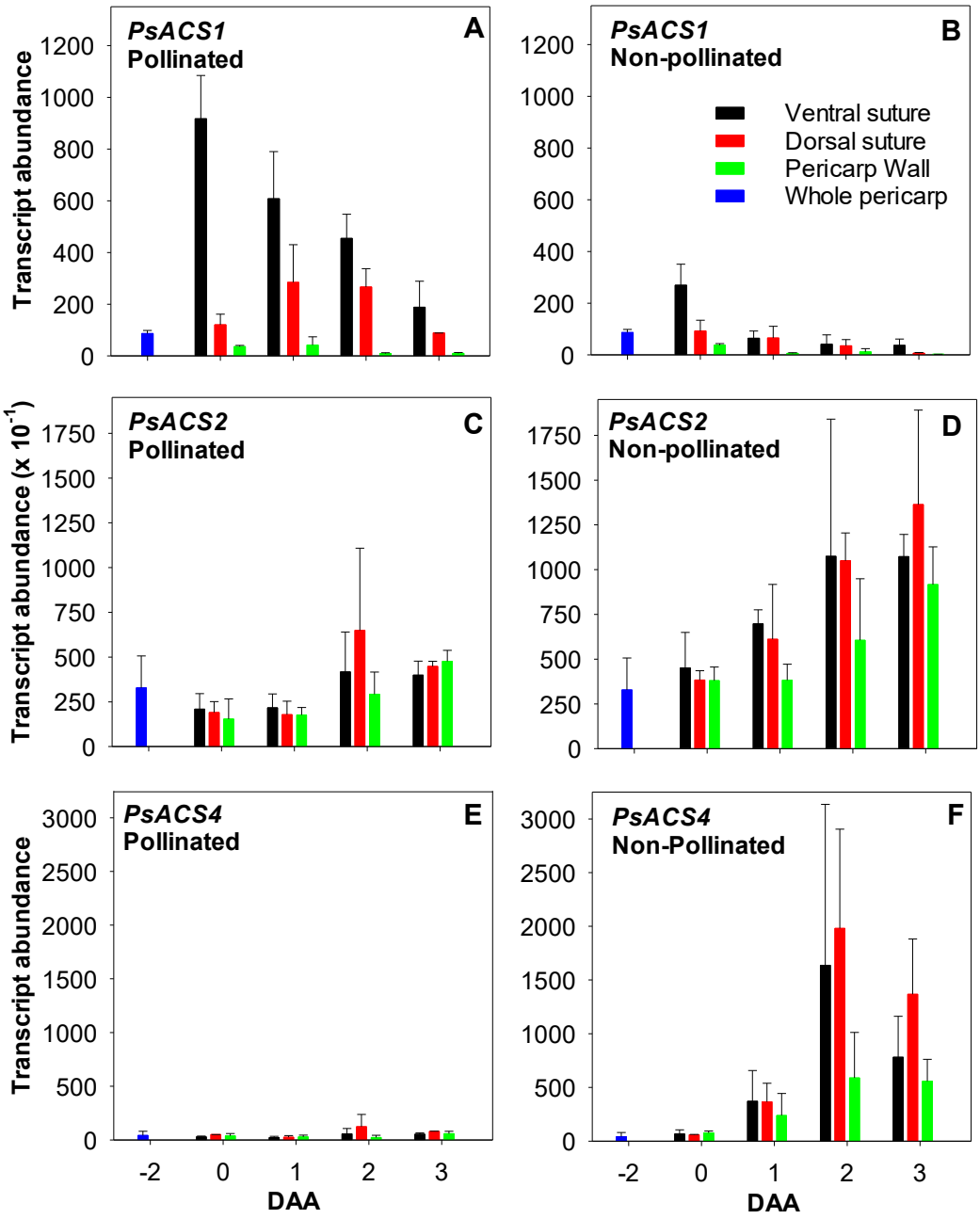


Figure 2.2. Relative transcript abundance of the ethylene biosynthesis genes *PsACS1* (**A** and **B**), *PsACS2* (**C** and **D**) and *PsACS4* (**E** and **F**) in pollinated and non-pollinated pea fruits. Whole pericarps were assessed at -2 days after anthesis (DAA), and pericarp tissues (ventral vascular suture, dorsal vascular suture, and wall) were assessed at 0 to 3 DAA. For non-pollinated fruits, flowers were emasculated at -2 DAA to prevent pollination. Data are means ± SD, n=3, with n=2 for a few samples where the tissues were limited due to small tissue size. Each sample is composed of a minimum of four pericarp tissues.

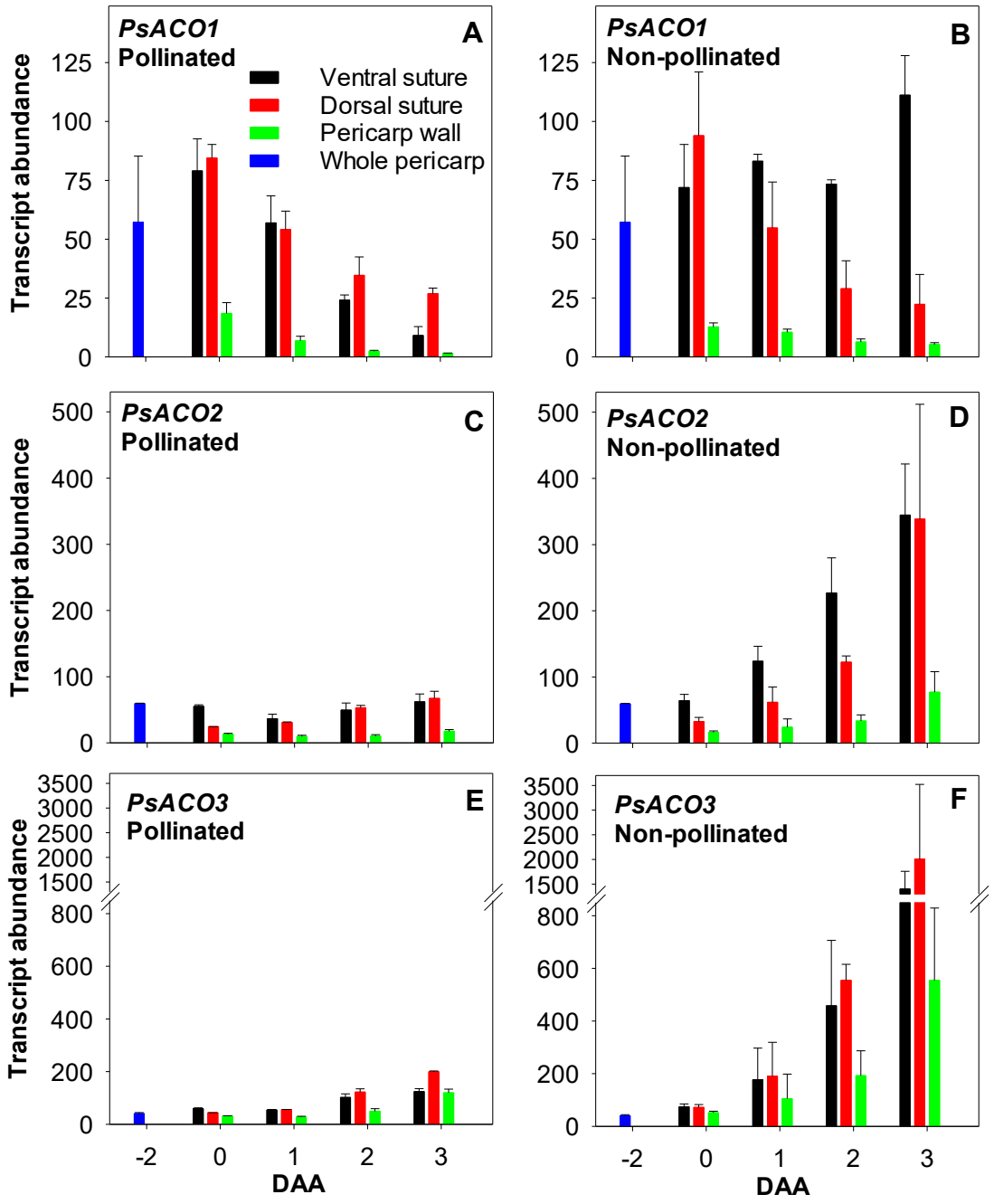


Figure 2.3. Relative transcript abundance of the ethylene biosynthesis genes *PsACO1* (**A** and **B**), *PsACO2* (**C** and **D**) and *PsACO3* (**E** and **F**) in pollinated and non-pollinated pea fruits. Whole pericarps were assessed at -2 days after anthesis (DAA), and pericarp tissues (ventral vascular suture, dorsal vascular suture, and wall) were assessed at 0 to 3 DAA. For non-pollinated fruits, flowers were emasculated at -2 DAA to prevent pollination. Data are means ± SD, n=3, with n=2 for a few samples where the tissues were limited due to small tissue size. Each sample is composed of a minimum of four pericarp tissues.

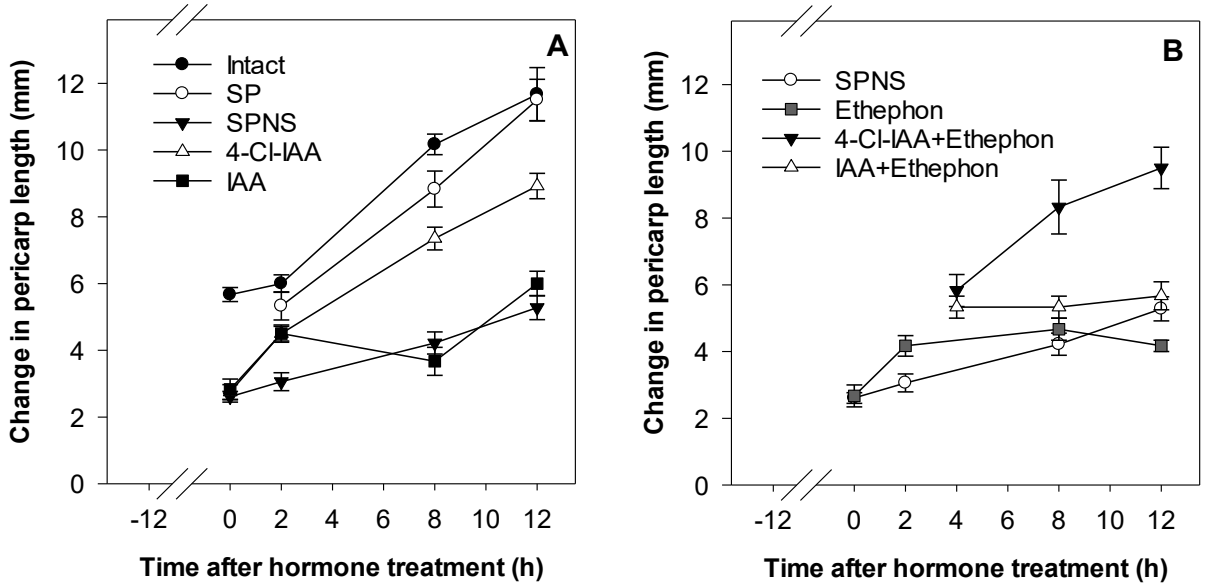


Figure 2.4. Effect of seed removal and hormone treatment on pea pericarp growth (final length minus initial length 12h after pericarp splitting). Two days after anthesis (DAA) pericarps were split (SP), or split and deseeded (SPNS), and deseeded pericarps were treated with 30 µL of 50 µM IAA or 4-Cl-IAA (**A**), ethephon (1000 mg/L) alone or ethephon in combination with 50 µM IAA or 4-Cl-IAA (**B**). All the hormones were in 0.1% aqueous Tween 80 and treatments were applied 12 h after splitting and deseeding. SPNS and SP controls were treated with 30 µL of 0.1% aqueous Tween 80. When treated with both ethephon and auxin, ethephon was applied 90 min after auxin treatment (with length measurements based on the time of auxin application). Data are means ± SE, n=6 to 18.

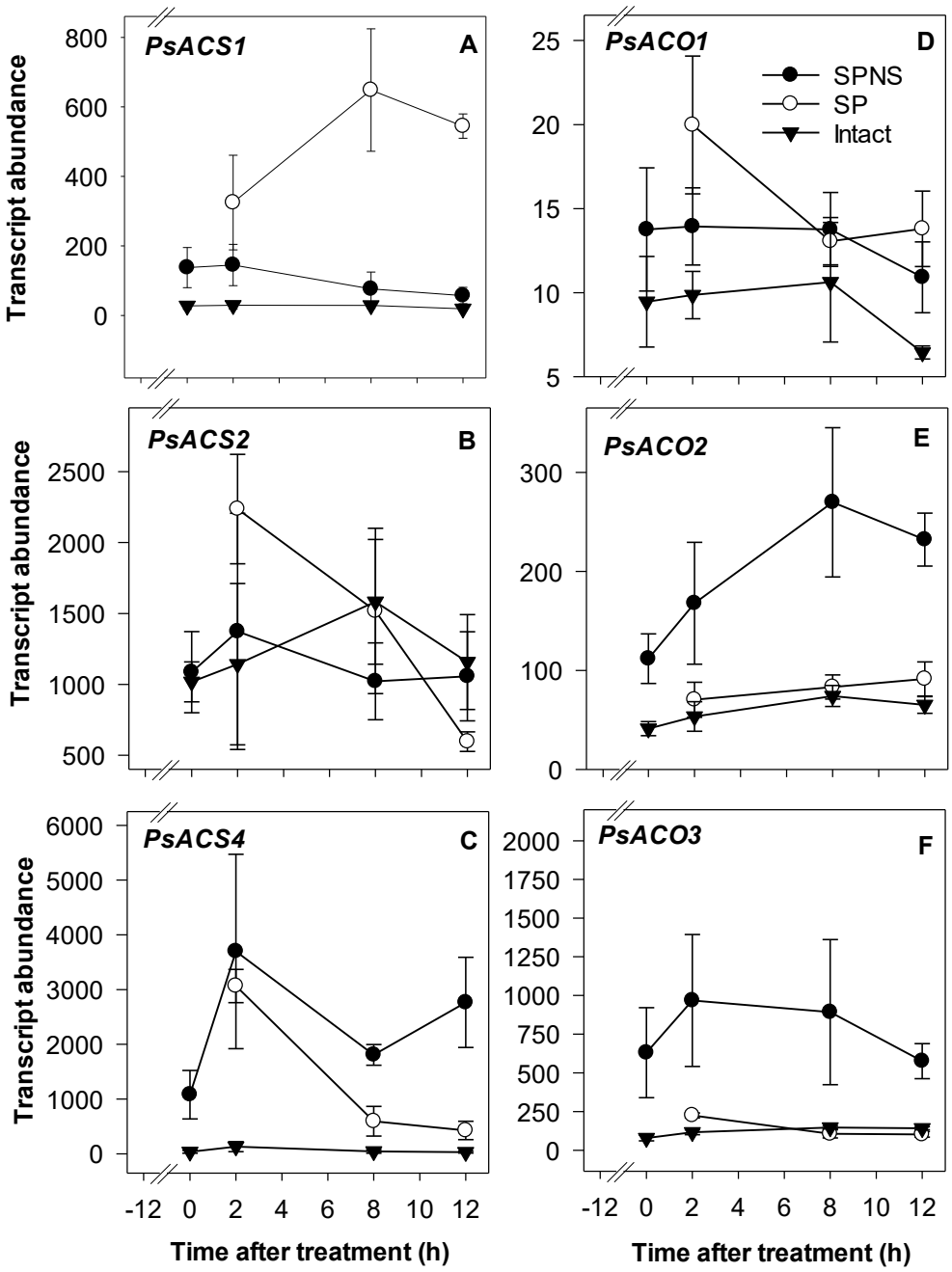


Figure 2.5. Relative transcript abundance of the ethylene biosynthesis genes *PsACS1* (**A**), *PsACS2* (**B**), *PsACS4* (**C**), *PsACO1* (**D**), *PsACO2* (**E**) and *PsACO3* (**F**) in pericarps of intact, split, or split and deseeded pollinated ovaries. Two days after anthesis (DAA) pericarps were either left intact, split (SP), or split and deseeded (SPNS). An aqueous 0.1% Tween 80 solution was added to the SP or SPNS pericarps 12 h after splitting (at 0 treatment time). Data are means ± SD, n=3 to 8.

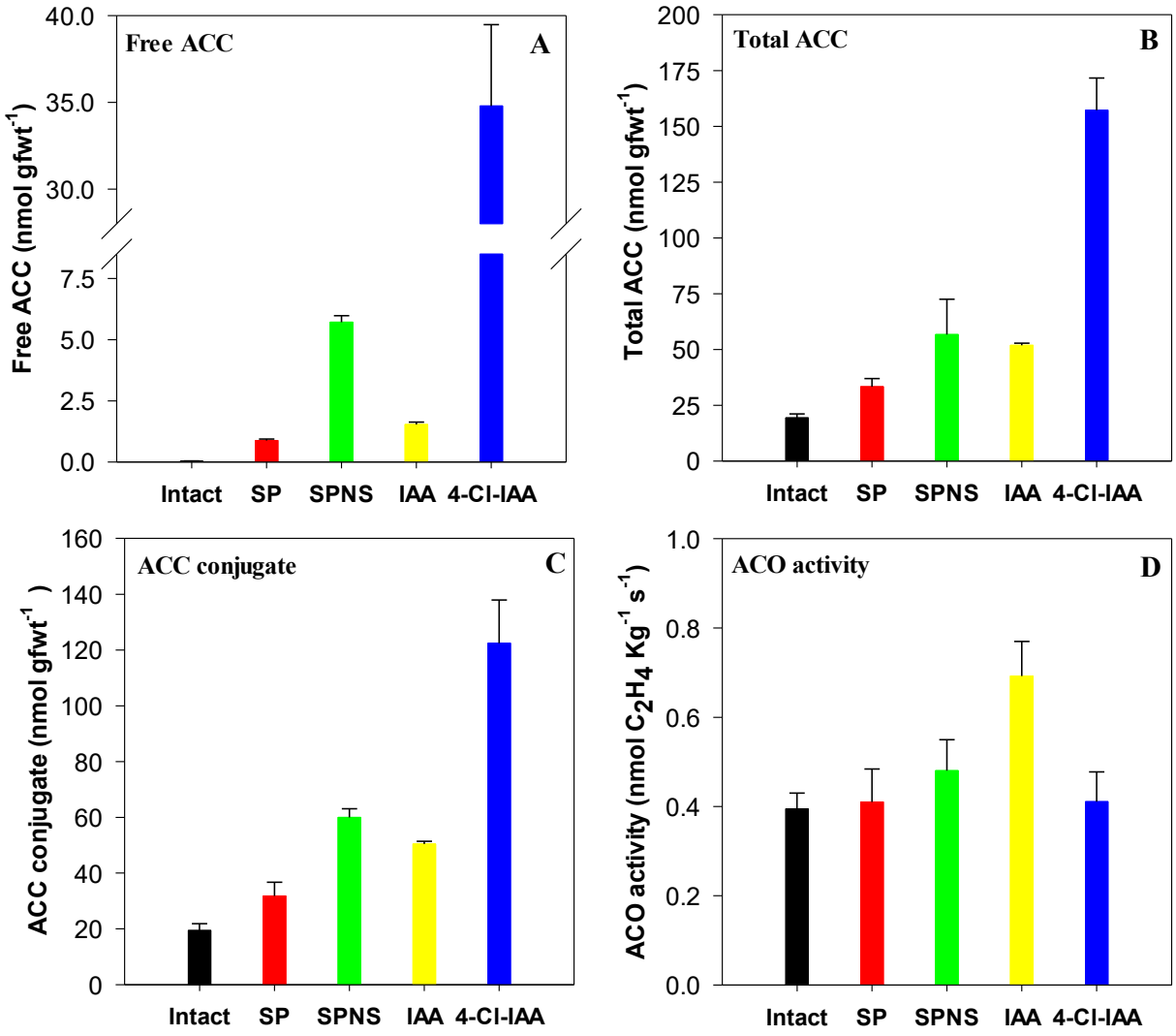


Figure 2.6. Free ACC, ACC conjugate, and total ACC content, and ACO enzyme activity of pea pericarps with seeds and deseeded pericarp treated with IAA or 4-Cl-IAA. Free ACC (**A**), total ACC (**B**), ACC conjugate (**C**) and ACO enzyme activity (**D**) in 2 days after anthesis (DAA) pea pericarps that were intact, split (SP), or split and deseeded (SPNS) and treated with 30 µL of 50 µM IAA or 4-Cl-IAA in aqueous 0.1% Tween 80, or aqueous 0.1% Tween 80 (SPNS control) 12 h after deseeding. Assays were completed on tissues 12 h after the hormone treatment. Data are means ± SD n=3, except for intact, SP and SPNS free ACC and ACC conjugate analysis, where n=2.

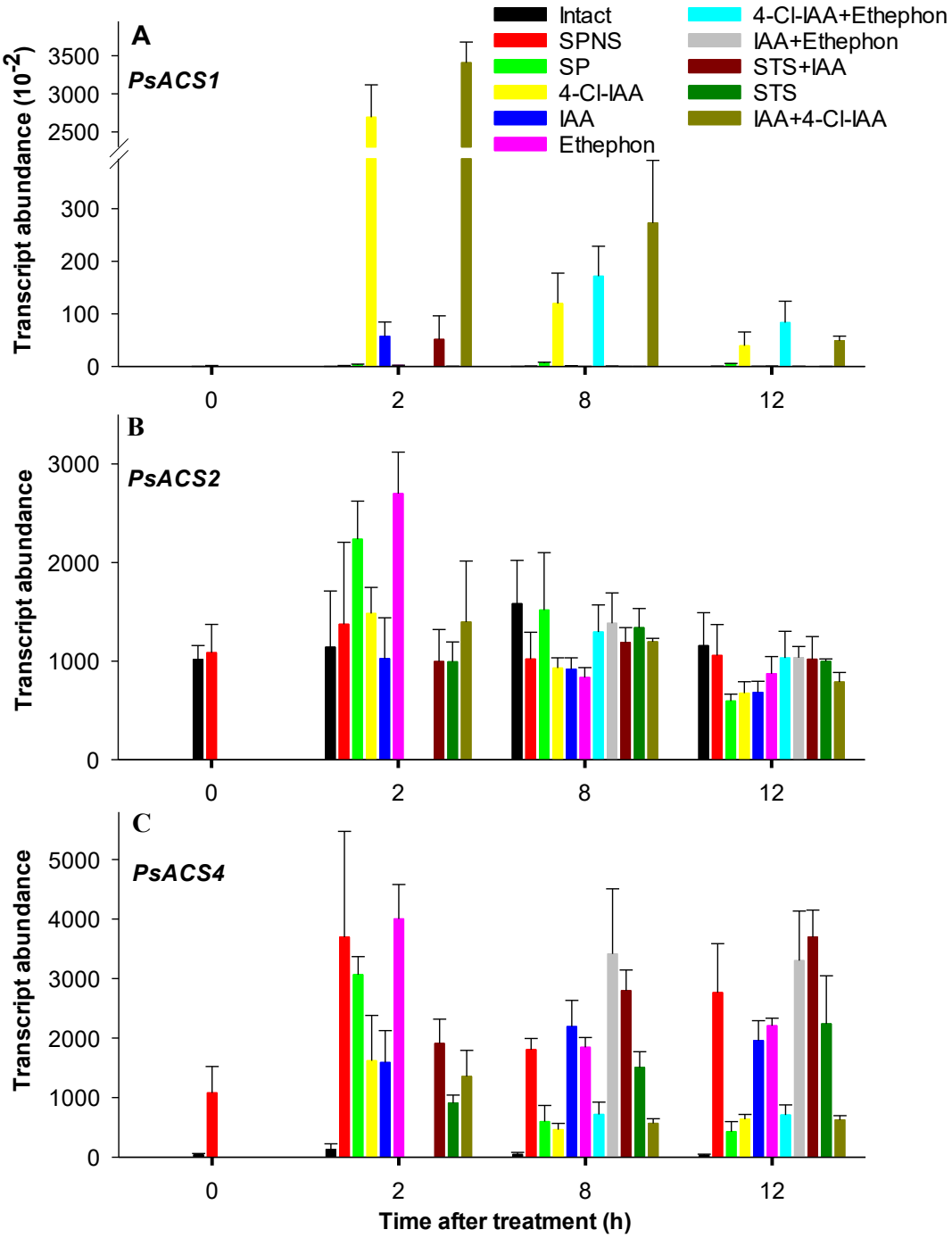


Figure 2.7. Effect of seed removal and hormone treatment on the relative transcript abundance of the ethylene biosynthesis genes *PsACS1* (**A**), *PsACS2* (**B**), and *PsACS4* (**C**) in the pericarps of pollinated pea ovaries. Two days after anthesis (DAA) pericarps were either left intact, split (SP), or split and deseeded (SPNS) and treated 12 h after deseeding with 4-Cl-IAA (50 µM), IAA (50 µM), or ethephon (1000 mg L^{-1}) in 0.1% aqueous Tween 80 (30 µL) alone or in

combination. Auxin or auxin-ethephon combinations were IAA plus 4-Cl-IAA, 4-Cl-IAA plus ethephon, or IAA plus ethephon in 0.1% aqueous Tween 80 (30 μL total). When fruits were treated with both auxin and ethephon, ethephon was applied 90 min after the auxin treatment and samples were collected based on the time after auxin treatment. Because of the delayed ethephon application, the auxin plus ethephon treated pericarps were not studied at the 2 h time-point. Deseeded pericarps were also pretreated with STS (1 mM in 0.1% aqueous Tween 80, 30 μL) at pericarp splitting and deseeding (STS treatment), and IAA (50 μM in 0.1% aqueous Tween 80, 30 μL) was applied to STS pretreated pericarps (IAA plus STS treatment). The SP and SPNS controls were treated with 0.1% aqueous Tween 80 (30 μL). All the samples were collected with respect to the time after hormone treatment. Data are means ± SD, n=3 to 8, with the exception of STS plus IAA 2 h treatment, where n=2.

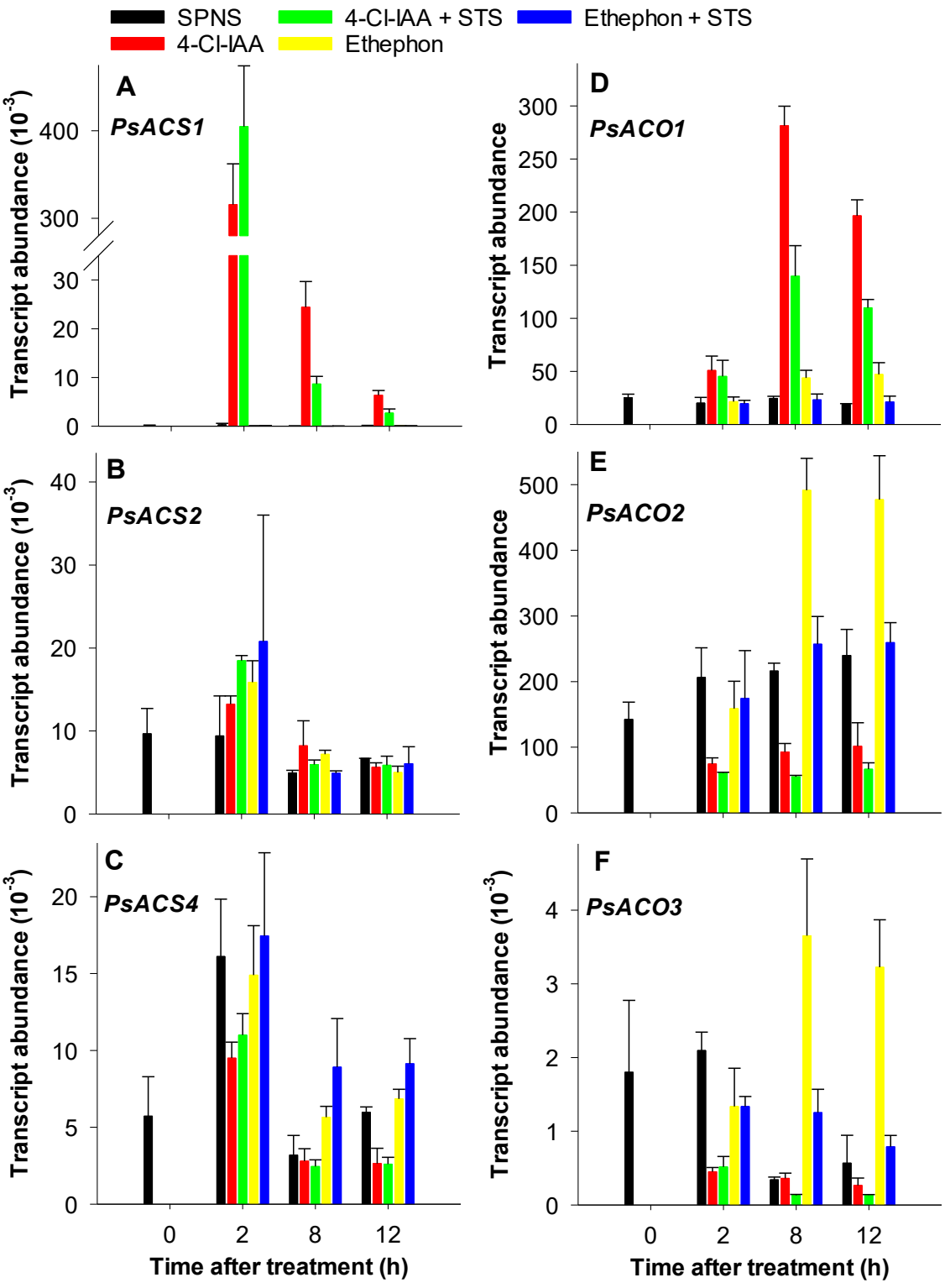


Figure 2.8. Effect of STS pretreatment on 4-Cl-IAA and ethephon regulation of ethylene biosynthesis gene transcript abundance in deseeded pea pericarps [*PsACS1* (**A**), *PsACS2* (**B**),

PsACS4 (**C**), *PsACO1* (**D**), *PsACO2* (**E**) and *PsACO3* (**F**)]. Two days after anthesis (DAA) pericarps were split and deseeded (SPNS) and immediately treated with STS (1 mM; 30 μL) or they were not treated. Twelve h after splitting and deseeding, pericarps were treated with 30 μL of 4-Cl-IAA (50 μM) or ethephon (1000 mg L^{-1}) in 0.1% aqueous Tween 80. Data are means ± SD, n=3 (with the exceptions, STS plus ethephon 12 h, SPNS 12 h, ethephon 8 and 12 h, STS plus 4-Cl-IAA 8 h, where n=2).

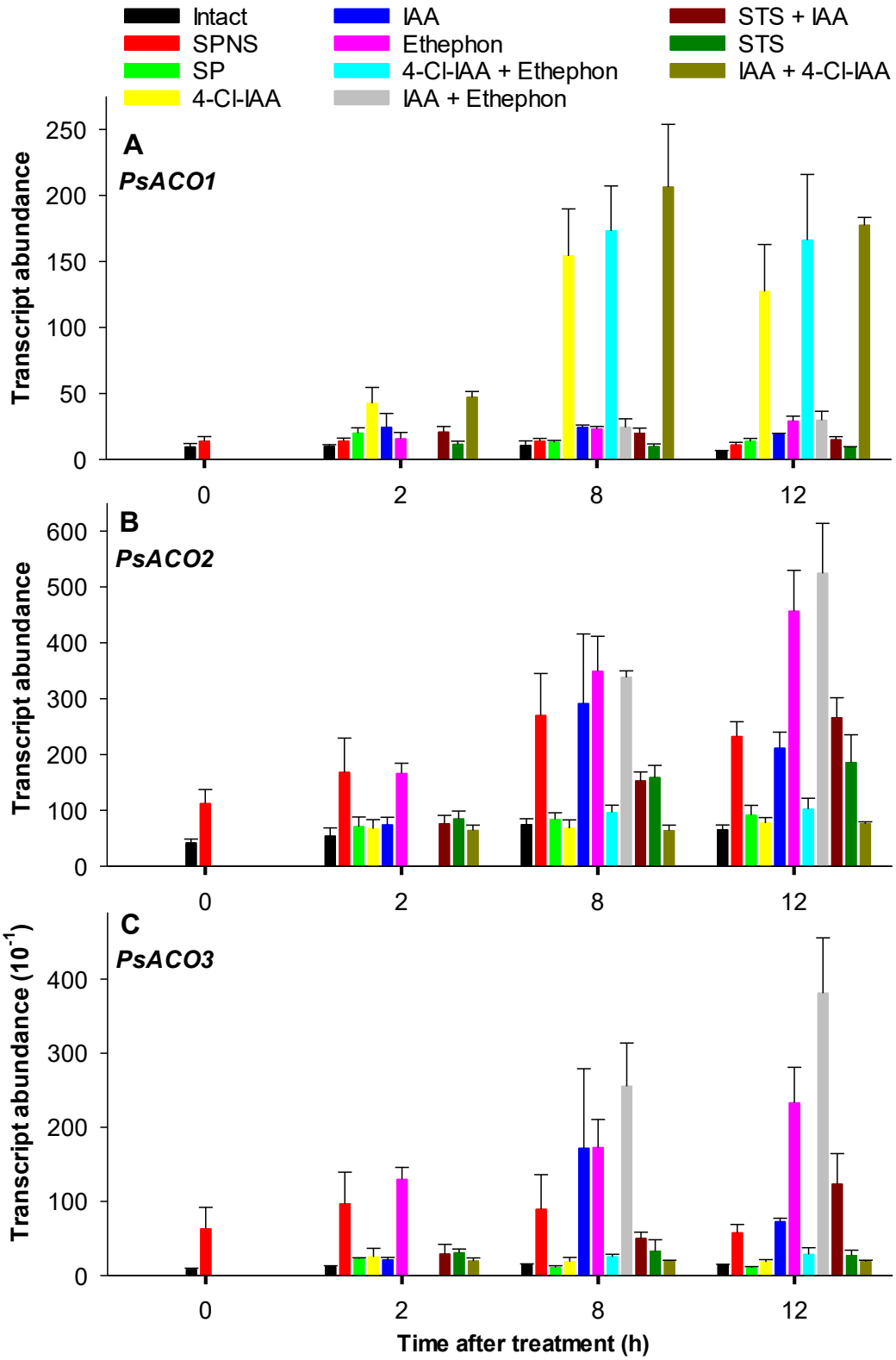


Figure 2.9. Effect of seed removal and hormone treatment on the relative transcript abundance of the ethylene biosynthesis genes *PsACO1* (**A**), *PsACO2* (**B**), and *PsACO3* (**C**) in the pericarps

of pollinated pea ovaries. Two days after anthesis (DAA) pericarps were either left intact, split (SP), or split and deseeded (SPNS) and treated 12 h after deseeding with 4-Cl-IAA (50 μM), IAA (50 μM), or ethephon (1000 mg L^{-1}) in 0.1% aqueous Tween 80 (30 μL) alone or in combination. Auxin or auxin-ethephon combinations were IAA plus 4-Cl-IAA, 4-Cl-IAA plus ethephon, or IAA plus ethephon in 0.1% aqueous Tween 80 (30 μL total). When fruits were treated with both auxin and ethephon, ethephon was applied 90 min after the auxin treatment and samples were collected based on the time after auxin treatment. Because of the delayed ethephon application, the auxin plus ethephon-treated pericarps were not studied at the 2 h time-point. Deseeded pericarps were also pretreated with STS (1 mM in 0.1% aqueous Tween 80, 30 μL) at pericarp splitting and deseeding (STS treatment), and IAA (50 μM in 0.1% aqueous Tween 80, 30 μL) was applied to STS pretreated pericarps (IAA plus STS treatment). The SP and SPNS controls were treated with 0.1% aqueous Tween 80 (30 μL). All the samples were collected with respect to the time after hormone treatment. Data are means ± SD, n=3 to 8, with the exception of STS plus IAA 2 h treatment, where n=2.

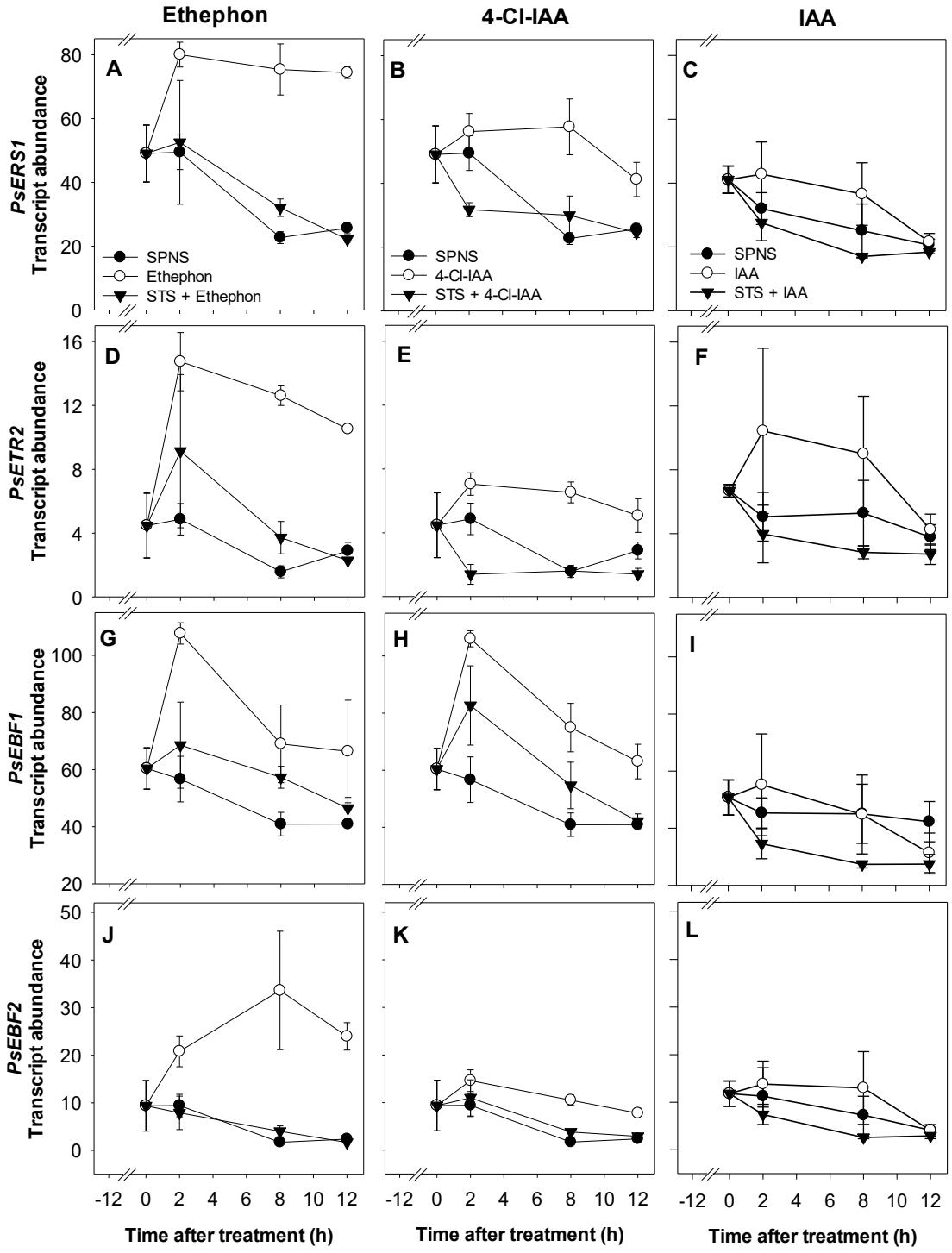


Figure 2.10. Effect of seed removal and hormone treatment on the relative transcript abundance of the ethylene receptor genes *PsERS1* (**A**, **B**, **C**) and *PsETR2* (**D**, **E**, **F**), and the ethylene signalling-related genes *PsEBF1* (**G**, **H**, **I**) and *PsEBF2* (**J**, **K**, **L**). Two days after anthesis (DAA) pericarps were left intact, split (SP), or split and deseeded (SPNS). Deseeded pericarps

were immediately treated with STS (1 mM; 30 μL) or they were not treated. Twelve h after splitting and deseeding, pericarps were treated with IAA (50 μM, 30 μL), 4-Cl-IAA (50 μM, 30 μL) or ethephon (1000 mg L^{-1}, 30 μL) in 0.1% aqueous Tween 80. SPNS and SP controls were treated with 30 μL of 0.1% aqueous Tween 80. Data are means ± SD, n=3 to 8, with the exception of STS + IAA 2 h treatment, where n=2.

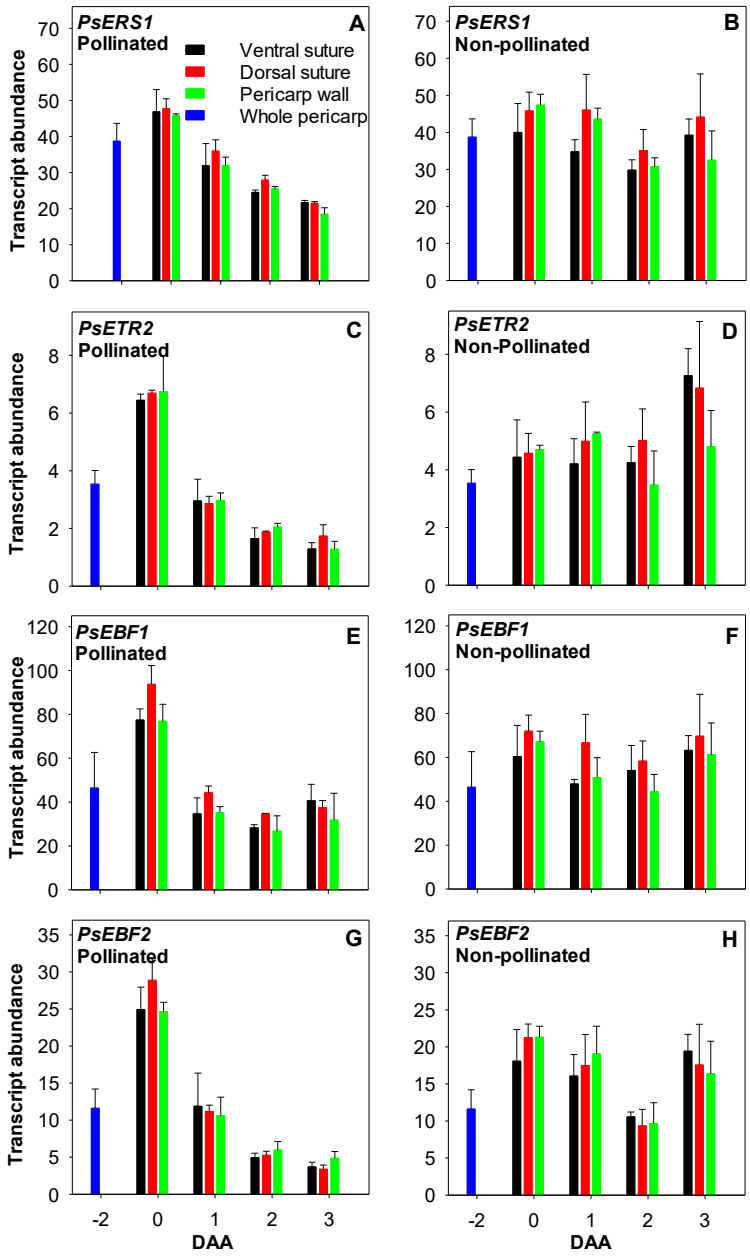


Figure 2.11. Relative transcript abundance of the ethylene receptor genes *PsERS1* (**A** and **B**) and *PsETR2* (**C** and **D**), and the signaling-related genes *PsEBF1* (**E** and **F**) and *PsEBF2* (**G** and **H**) in pollinated and non-pollinated pea fruits. Whole pericarps were assessed at -2 days after anthesis (DAA), and pericarp tissues (ventral vascular suture, dorsal vascular suture, and wall) were assessed at 0 to 3 DAA. For non-pollinated fruits, flowers were emasculated at -2 DAA to prevent pollination. Data are means ± SD; n=3, with n=2 for a few samples where the tissues were limited due to small tissue size. Each sample is composed of a minimum of four pericarp tissues.

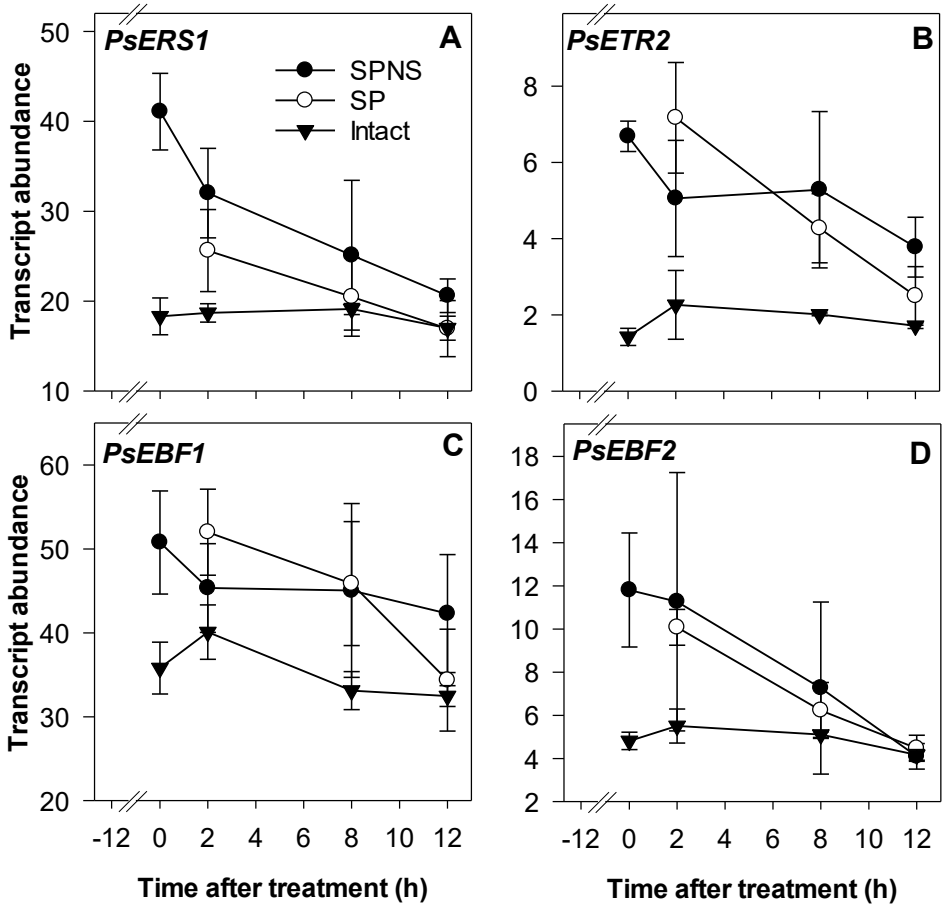


Figure 2.12. Effect of seed removal on the relative transcript abundance of the ethylene receptor genes, *PsERS1* (**A**) and *PsETR2* (**B**), and the ethylene signaling-related *EFB* genes, *PsEBF1* (**C**) and *PsEBF2* (**D**), in pericarps of pollinated pea ovaries. Two days after anthesis (DAA) pericarps were left intact, split (SP), or split and deseeded (SPNS). An aqueous 0.1% Tween 80 solution was added to the SP or SPNS pericarps 12 h after splitting (at 0 treatment time). Data are means ± SD, n=3 to 8.

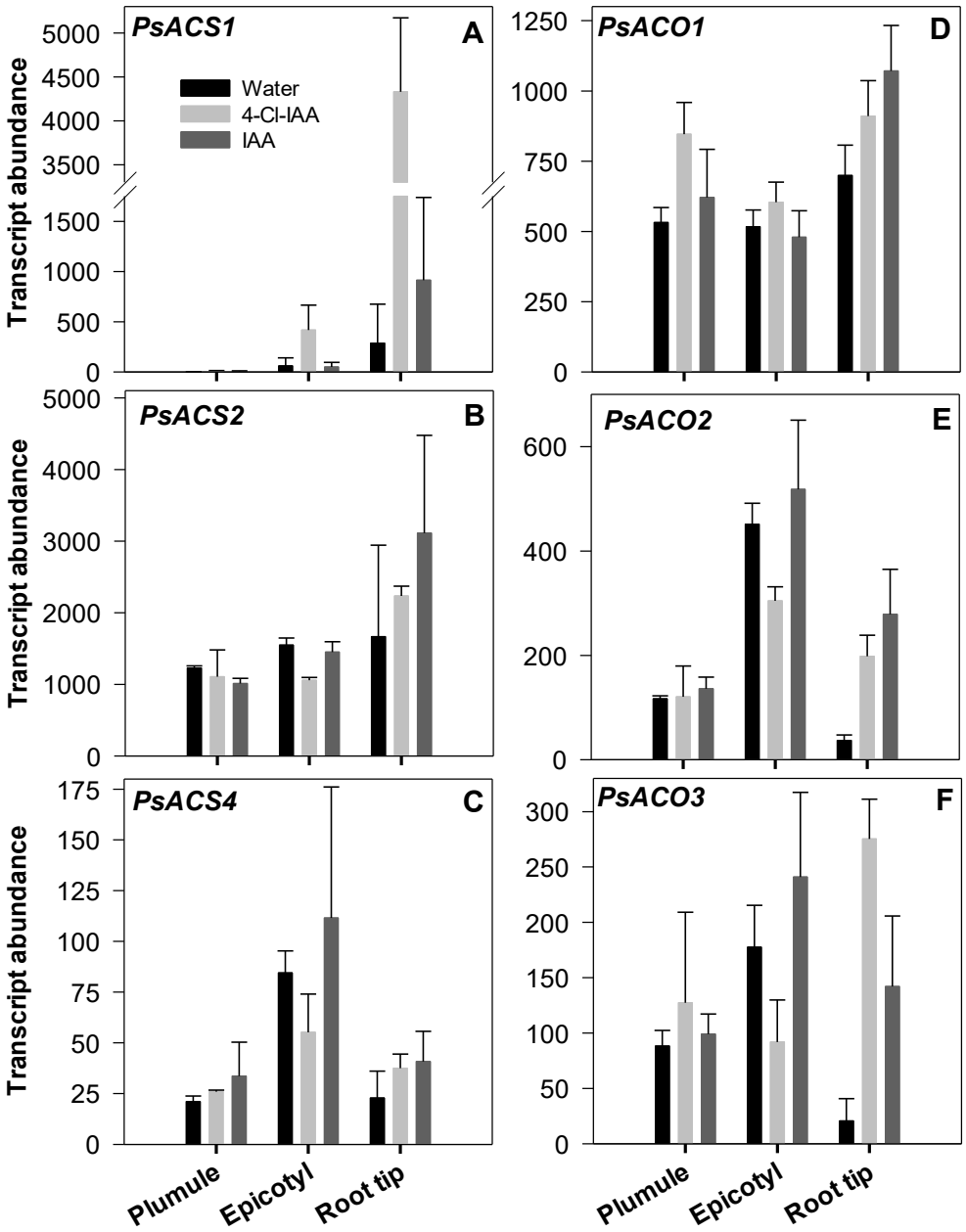


Figure 2.13. Effect of auxin treatment on the relative transcript abundance of the ethylene biosynthesis genes *PsACS1* **(A)**, *PsACS2* **(B)**, *PsACS4* **(C)**, *PsACO1* **(D)**, *PsACO2* **(E)** and *PsACO3* **(F)** in the plumule, epicotyl, and root tip tissues of 4 DAI (days after imbibition) etiolated pea seedlings. Seeds were imbibed in water in the dark for two days. Germinated seeds selected for uniformity were grown in the presence of 0 or 1 µM aqueous 4-Cl-IAA or IAA for an additional two days under dark conditions. Data are means ± SD, n=3, except for the 4-Cl-IAA root tip treatment, where n=2 to 3.

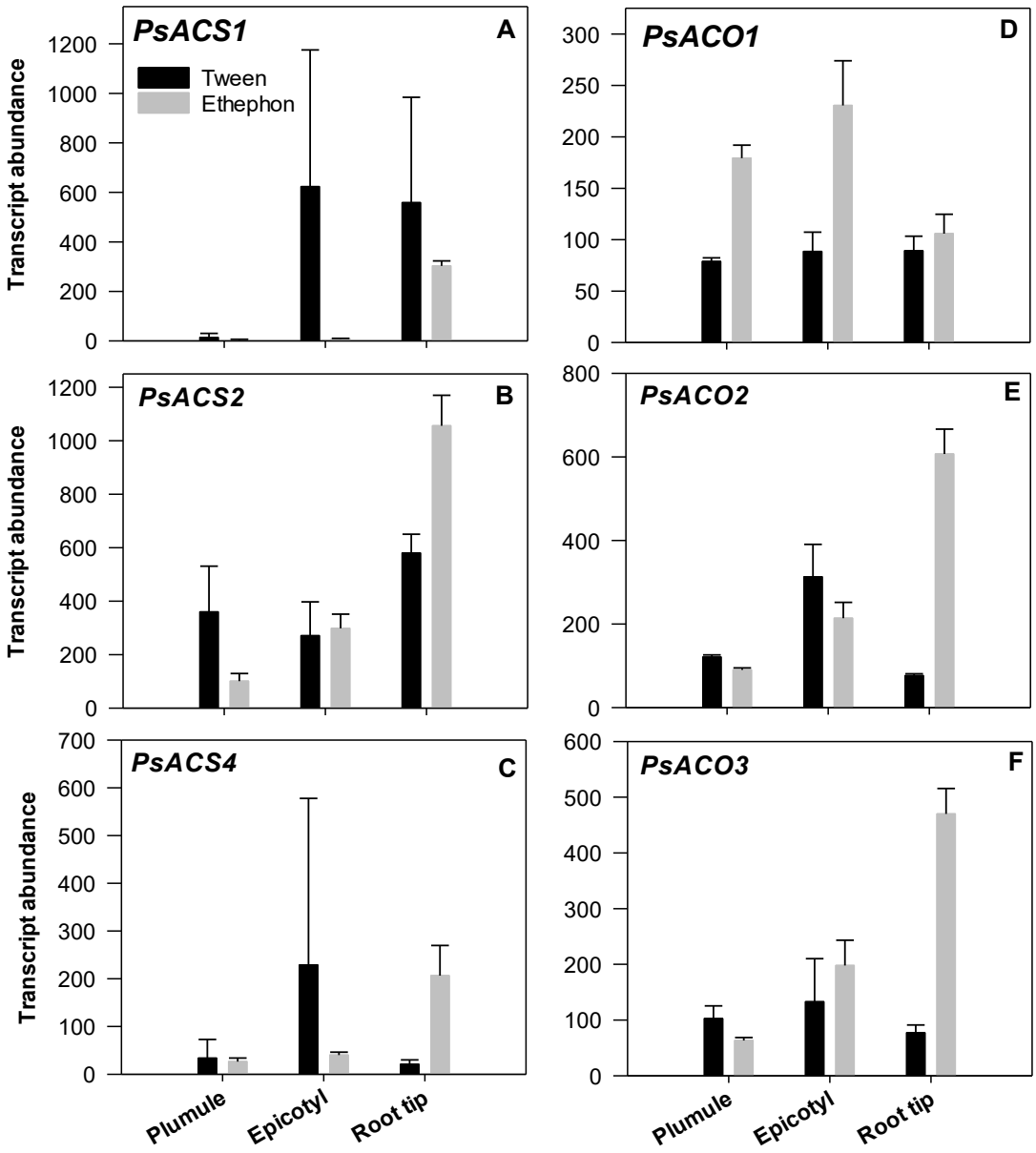


Figure 2.14. Effect of ethephon on the relative transcript abundance of the ethylene biosynthesis genes *PsACS1* **(A)**, *PsACS2* **(B)**, *PsACS4* **(C)**, *PsACO1* **(D)**, *PsACO2* **(E)** and *PsACO3* **(F)** in plumule, epicotyl and root tip tissues of 4 DAI (days after imbibition) etiolated seedlings. Seeds were imbibed and grown in the presence of 15 mg L^{-1} ethephon (in 0.1% aqueous Tween 80) or in 0.1% aqueous Tween 80 in the dark for four days. Data are means ± SD, n=3.

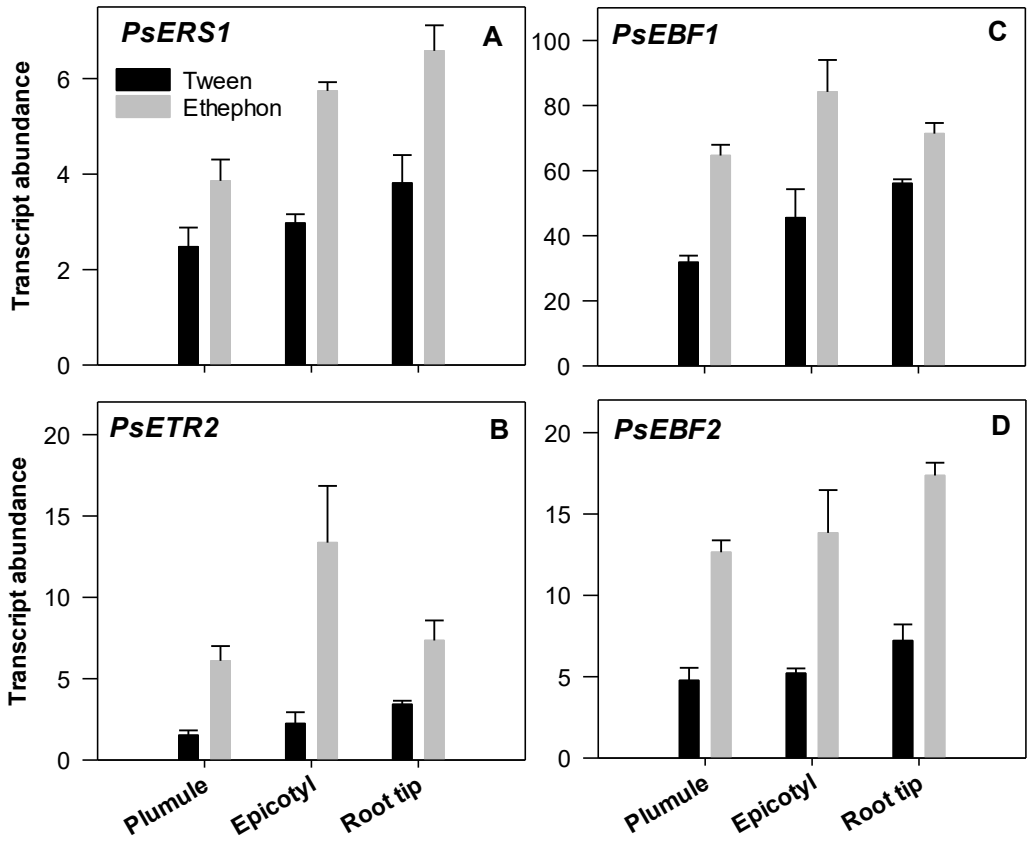


Figure 2.15. Effect of ethephon on the relative transcript abundance of the ethylene receptor genes, *PsERS1* **(A)** and *PsETR2* **(B)**, and the signaling-related genes, *PsEBF1* **(C)** and *PsEBF2* **(D)**, in plumule, epicotyl and root tip tissues of 4 DAI (days after imbibition) etiolated seedlings. Seeds were imbibed and grown in the presence of 15 mg L^{-1} ethephon (in 0.1% aqueous Tween 80) or in 0.1% aqueous Tween 80 in the dark for four days. Data are means ± SD, n=3.

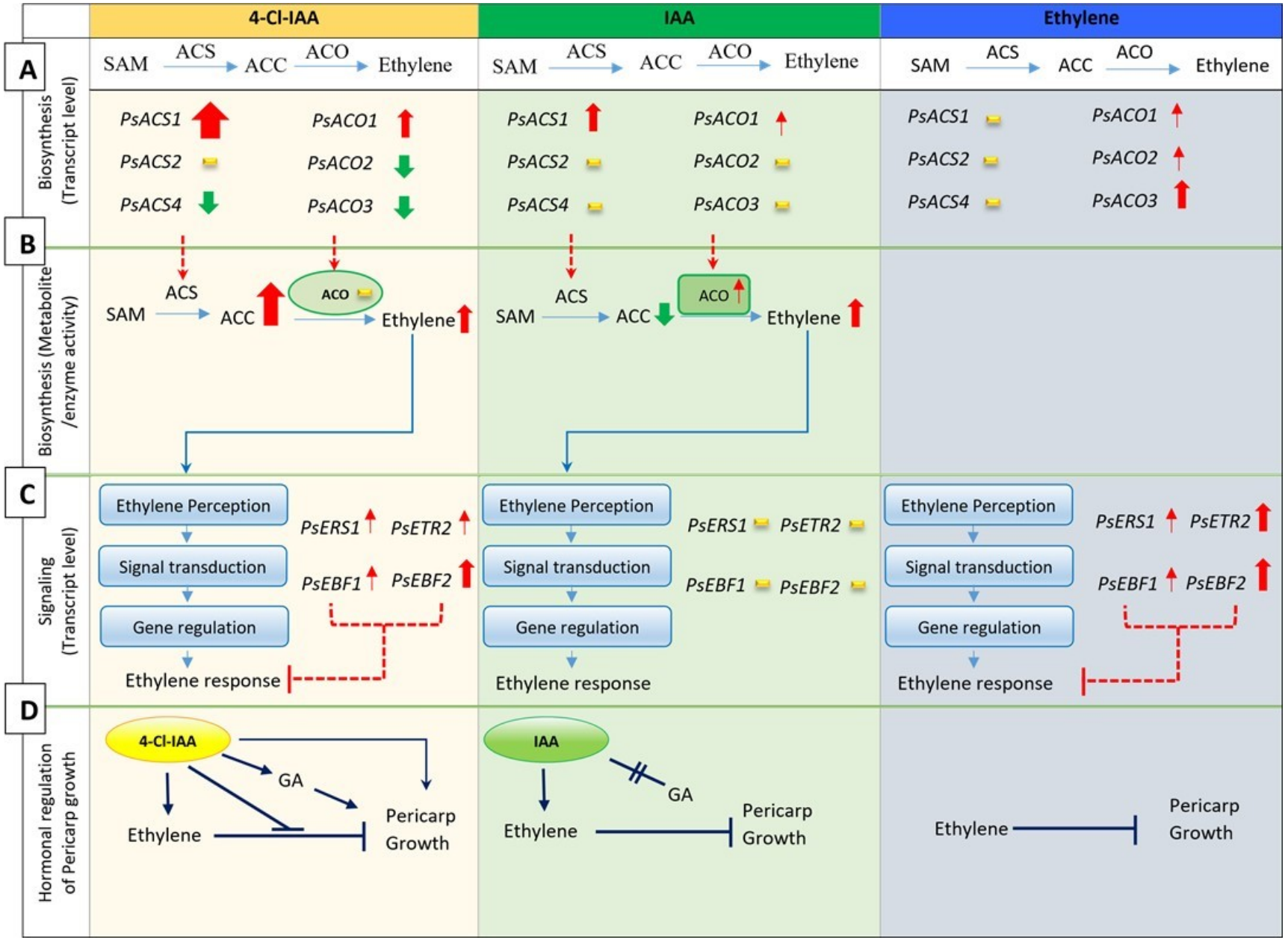

4-Cl-IAA
IAA
Ethylene
A
Biosynthesis (Transcript level)
SAM ACS ACC ACO Ethylene
PsACS1
PsACS2
PsACS4
PsACO1
PsACO2
PsACO3
B
Biosynthesis (Metabolite /enzyme activity)
ACS
ACO
C
Signaling (Transcript level)
Ethylene Perception
Signal transduction
Gene regulation
Ethylene response
PsERS1
PsETR2
PsEBF1
PsEBF2
D
Hormonal regulation of Pericarp growth
4-Cl-IAA
GA
Ethylene
Pericarp Growth
IAA

Figure 2.16. Working model of auxin and ethylene regulation of early pea fruit development. 4-Cl-IAA stimulated deseeded pericarp growth and inhibited the increase in transcript abundance of the pericarp ethylene biosynthesis genes *PsACS4*, *PsACO2* and *PsACO3*, mimicking the presence of seeds, IAA did not **(A)**. The auxin-specific gene expression and ACO metabolite data, together with the previously reported data that ethylene evolution levels were similar in 4-Cl-IAA- and IAA-treated deseeded pericarps (Johnstone et al., 2005), suggest that ACC oxidase activity limited ethylene synthesis in 4-Cl-IAA-treated pericarps, but not in IAA-treated pericarps **(B)**. Both ethylene and 4-Cl-IAA diminish ethylene signalling output by up-regulating the expression of the pericarp ethylene receptor and signaling-related (*EFB*) genes **(C)**. Overall, these data support the working hypothesis that auxin (4-Cl-IAA in pea) produced in the developing seeds stimulates growth and development in the surrounding ovary by regulating a network of hormonal pathways in the ovary including stimulation of gibberellin biosynthesis (Ozga et al., 2009) and modulation of ethylene biosynthesis and response **(D)**. Red and green arrows show an increase and decrease of given target or response, respectively, with arrow thickness representing response magnitude. Minimal to no response is represented by a yellow dash.

CHAPTER 3

TIR1 AUXIN-RECEPTOR MEDIATED REGULATION OF AUXIN ACTION IN DEVELOPING PEA FRUIT

3.1 INTRODUCTION

In pea, fruits do not grow in the absence of developing seeds. The lack of pollination or manual seed removal cause pericarps to senescence and subsequently abscission (Ozga et al., 1992; Ozga and Reinecke, 1999). Application of 4-chloroindole-3-acetic acid (4-Cl-IAA), a naturally occurring auxin in pea, stimulates deseeded pericarp development. The other naturally occurring auxin indole-3-acetic acid (IAA) does not induce pericarp development (Reinecke et al., 1995). Application of IAA can even be inhibitory on pericarp growth at concentrations above 10 µM. In contrast, 4-Cl-IAA stimulates pea pericarp development at concentrations ranging from 1 to 100 µM (Reinecke et al., 1995). 4-Cl-IAA-specific mediation of pericarp gibberellin (GA) biosynthesis is at least partially responsible for this growth stimulatory effect (Ozga et al., 2003; Ozga et al., 2009). 4-Cl-IAA, but not IAA, also inhibits ethylene response in deseeded fruit (pericarps; Johnstone et al., 2005). Data in Chapter 2 outline how IAA and 4-Cl-IAA regulate ethylene biosynthesis as well as pericarp ethylene action in distinctly different manners. These differential interactions of IAA and 4-Cl-IAA with the ethylene and GA biosynthesis and signaling pathways likely play a primary role determining the effects of the two auxins on pericarp development. However, the underlying mechanisms leading to these differential auxin effects in early pea fruit development are not known.

Higher concentrations or continued daily application of IAA were not effective in stimulating pea pericarp development. Therefore, the comparatively lower activity of IAA or its lower chemical stability are not likely the primary reasons for its inability to induce pericarp development (Reinecke et al., 1995; Reinecke, 1999). Auxin analogs with varying substitutions at the fourth position of the indole ring, or analogs with chlorine or fluorine substituents at different positions of the indole ring, have been evaluated for their ability to induce pericarp development (Reinecke et al., 1995; Reinecke et al., 1999). These studies show that the position of the substituent, its size, and lipophilicity are important factors determining auxin activity

(Reinecke et al., 1999). Based on these observations, it has been speculated that the differential effects of IAA and 4-Cl-IAA on pea pericarp development may rest at the receptor level. The possible differences in their transport within the tissues leading to a differential action has also not been excluded (Reinecke, 1999; Reinecke et al., 1999).

All six *Arabidopsis* TRANSPORT INHIBITOR RESPONSE 1/ AUXIN SIGNALING F-BOX (TIR1/AFB) proteins interact with AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) co-receptors in an auxin-dependent manner. These interactions suggest that all of the *Arabidopsis* TIR1/AFB proteins may be involved in the auxin perception (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Prigge et al., 2016; see Fig. 1.2). The interaction between TIR1/AFB and Aux/IAA protein family members appears to depend on the two protein combination, type of auxin present and the concentration of auxin (Calderón Villalobos et al., 2012). For instance, AtTIR1-IAA7 co-receptor complex showed a higher affinity to IAA than picloram. Furthermore, when the AtTIR1-IAA7 complex was compared with the AtAFB5-IAA7 complex, picloram affinity was greater in the co-receptor complex with AtAFB5 (Calderón Villalobos et al., 2012). It is possible that the differential activity of IAA and 4-Cl-IAA is at least partially due to the specific TIR1/AFB family members involved in their perception and signal transduction. Consistent with this possibility, genes coding two putative auxin receptors in pea, *PsAFB2* and *PsAFB6,* showed differential auxin-induced expression patterns in young pea fruit (Nadeau, 2009). Transcript abundance of *PsAFB2* was not modulated by auxin (IAA or 4-Cl-IAA) or the presence of developing seeds in pea pericarps. However, 4-Cl-IAA mimicked the presence of developing seeds in repressing *PsAFB6* transcript abundance in the pericarp, IAA did not (Nadeau, 2009). These transcript profiles suggest that the pool of auxin receptors may be modulated depending on the developmental stage or auxin availability, and these changes may eventually modulate the auxin response.

To test the hypothesis that auxin receptors are involved in the differential activity of IAA and 4-Cl-IAA in pea pericarp growth, auxin receptors in pea need to be identified and characterized. In *Arabidopsis*, characterization of auxin receptor function using root growth assays showed AtTIR1 as the most prominent receptor in auxin perception (Parry et al., 2009). Two pea orthologs of *AtTIR1* named *PsTIR1a* and *PsTIR1b* were identified in this study. The two pea auxin receptors were functionally characterized in *Arabidopsis* auxin receptor mutant backgrounds to evaluate their role in the perception of 2,4-D, IAA and 4-Cl-IAA. Auxin

regulation of *PsTIR1a* and *PsTIR1b* transcript abundance and the expression of auxin-responsive *DR5* promoter driven *β-D-glucuronidase* (*GUS*) reporter were evaluated to determine the possible mechanism behind the differential action of IAA and 4-Cl-IAA on pericarp development.

3.2 MATERIALS AND METHODS

3.2.1 Pea plant materials for gene expression studies

The pea cultivar *Pisum sativum* L. cv. I_3 (Alaska-type) was used for all the studies with pea. Mature-dry pea seeds were planted in 3-L pots containing a 4:1 (v/v) mixture of Sunshine Mix #4/LA4 (SunGro Horticulture, MA, USA) and sand. Seeds (five per pot) were planted at a depth of 2.5 cm. After about two weeks, the seedlings were thinned to three per pot. Plants were grown in a growth chamber under cool white fluorescent lights with an average photon flux density of 235 μmol m^{-2} s^{-1} and 16 h light (19 ºC)/8 h dark (17 ºC) photoperiod. The main shoot apex of the plants remained intact while expanding lateral shoots were removed as they developed.

For the fruit developmental study, fruits from flowering nodes 1 to 6 were tagged at the date of anthesis (0 DAA) and fruits within a specific pericarp length range (1 DAA: 8-12 mm, 2 DAA: 15-20 mm, 3 DAA: 26-33 mm) and/or average seed weight range (5 DAA: 1.2-2.5 mg, 6 DAA: 5-7 mg, 7 DAA: 14-16 mg, 8 DAA: 20-28 mg, 10 DAA: 70-100 mg) were used. For non-pollinated fruits, floral buds at -2 DAA were emasculated, and tissues were collected at -2, 0, 1, 2 and 3 DAA. Fruits were collected onto ice and immediately dissected into seed/ovule, pericarp wall, pericarp dorsal vascular suture and pericarp ventral vascular suture tissues (Appendix Fig. B1), except for those at -2 DAA, where the ovules were removed from the fruit, and the pericarps with the stigma and style attached were harvested. Tissues were frozen in liquid N_2 immediately after harvest and dissection, and stored in -80 ºC until further processing.

For the hormonal treatment studies deseeded pericarps were treated with IAA or 4-Cl-IAA (50 μM in 0.1% aqueous Tween 80; 30 μL), ethephon (1000 mg L^{-1} in 0.1% aqueous Tween 80; 30 μL), or STS (1 mM in 0.1% aqueous Tween 80; 30 μL) alone or in combination. Auxin or auxin-ethephon combinations were IAA plus 4-Cl-IAA, IAA plus ethephon, 4-Cl-IAA plus

ethephon, in 0.1% aqueous Tween 80 (30 µL total). All the treatments and sample collections were done as described in Chapter 2.

For the auxin and ethylene treatment of pea seedlings, they were grown in the presence of auxin and ethylene as described in Chapter 2. Briefly, for the auxin application, 2 DAI water-grown seedlings were transferred to water, IAA (1 µM) or 4-Cl-IAA (1 µM) and grown there for two additional days. For ethylene treatment, sterilized seeds were imbibed in a 0.1% aqueous Tween 80 solution with or without 15 mg L^{-1} ethephon and grown there for four days. All the assays were done in Magenta GA-7 vessels, at room temperature (~ 22 ºC) and under continuous dark conditions. For the analysis of *PsTIR1a, PsTIR1b and PsAFB2* transcript abundance in 12-day old seedling tissues, plants were grown under 16 h light (19 ºC) and 8 h dark (17 ºC) conditions. When the seedlings are 12 days old, they had 6 to 7 nodes below the shoot apex. Leaves attached to the 4th node were collected as mature leaves (average length 31 ± 2 mm, average width 26 ± 2 mm). Leaves attached to the 6th or 7th node were collected as immature leaves (Average length 8 ± 2 mm). Internodes between 3rd and 4th nodes were collected as mature internodes (average length 25 ± 5 mm). Internodes between 6th and 7th nodes (5th and 6th nodes in a few seedlings where the 7th node was not developed) were collected as immature internodes (average length 4 ± 2 mm). Shoot apex was also collected from all the seedlings. All the samples were immediately frozen in liquid nitrogen and stored at -80 ºC until RNA extraction.

3.2.2 Identification of pea *TIR1/AFB* family members and the cloning and sequencing of *PsTIR1a*, *PsTIR1b*, *PsAFB2* and *AtTIR1*

In the Ozga/Reinecke lab, five putative TIR1/AFB family auxin receptor homologs in pea were identified by screening a small scale next generation sequencing database where I was responsible for identifying the *PsTIRb* homolog. The sequencing database was derived from 10 days after anthesis (DAA) seed coats of *P. sativum* L. cvs. I_3 (Alaska-type), Courier, Canstar, Solido and LAN 3017 as described in Chapter 2. The database was screened using blastn and blastx programs with *TIR1/AFB* family sequences of *Arabidopsis* and *M. truncatula* as the queries. Sequences obtained from the database screening were assembled into full-length coding sequences (CDS) manually or using the CAP3 sequence assembly program (Huang and Madan, 1999).

Complete CDSs of *PsTIR1a, PsTIR1b* and *PsAFB2* were PCR amplified from pea tissues of *P. sativum* L. cv. I_3 (Alaska-type) with the PCR primers given in Appendix Table B1. A pooled sample of complementary DNA (cDNA) synthesized from the total RNA of 6 DAA pea pericarp walls and 8 DAA seeds was used to amplify *PsTIR1a*. A pooled sample of cDNA synthesized from the total RNA of 3 and 6 DAA whole pericarps, 7 DAA seeds, and the shoot apex of 12-day old pea seedlings was used for the amplification of *PsTIR1b* and *PsAFB2*. For the PCR amplification of *AtTIR1*, cDNA was synthesized from the total RNA of about a month old *Arabidopsis* leaf and inflorescence tissues. cDNA was synthesized using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, CA, USA) with oligo-dT primers as per the manufacturer provided protocol. All PCR amplification of genes were done with Phusion Hot Start II polymerase (Thermo Scientific, MA, USA; Appendix Table A4), except for *PsTIR1a*, for which LongRange PCR Kit (Qiagen, Hilden Germany; Appendix Table B2) was used.

The pCR8 vector (Invitrogen) used for initial cloning contained single 3′ deoxythymidine (T) overhangs and required deoxyadenosine (A) overhangs in the insert for successful TA cloning. Therefore, the PCR products obtained with Phusion polymerase for *PsTIR1b*, *PsAFB2* and *AtTIR1* were cleaned with a QIAquick PCR Purification Kit (Qiagen). Purified PCR products were incubated with 1x PCR buffer with $MgCl_2$, 300 µM deoxyadenosine triphosphate and 0.05 units/µL Taq DNA polymerase at 37 ºC for 30 min to introduce deoxyadenosine overhangs. Adenylated products were run on an agarose gel, correct size band was gel purified and cloned into the pCR8 vector. Cloned vectors were transformed into chemically competent *E. coli* cells by chemical transformation as per the protocol provided with pCR8/GW/TOPO TA Cloning Kit (Invitrogen). Plasmids were extracted from transformed *E. coli* cultures using GenElute Plasmid Miniprep Kit (Sigma, MO, USA) or with GeneJET Plasmid Miniprep Kit (Thermo Scientific) and used for sequencing of the insert. All the sequencing reactions were done at the Molecular Biology Service Unit, Department of Biological Sciences, University of Alberta.

3.2.3 RNA extraction and qRT-PCR analysis for pea gene expression analysis

RNA extraction and subsequent qRT-PCR analysis were completed as described in Chapter 2 with TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, CA, USA; Part Number: 4309169) or with One-step qRT-PCR Master Mix (RNA-to-Ct 1-Step Kit,

Applied Biosystems, Part Number: 4392938). Reaction setup and qRT-PCR conditions were similar for both kits and are given in Chapter 2. Primers and probes used for the qRT-PCR analysis and their reaction efficiencies are given in Appendix Table B3. All the qRT-PCR probes for the auxin receptor genes were double-quenched, with Iowa Black FQ (IBFQ) quencher at the 3′ end, and ZEN quencher at 9 bp from the 5′ end and 6-FAM fluorescent dye at 5′ end (Integrated DNA Technologies, IA, USA). The CDSs of *PsTIR1a* and *PsTIR1b* share 82% identity. Therefore, to verify the target specificity of qRT-PCR primers designed for *PsTIR1a* and *PsTIR1b*, their PCR products were cloned into pCR8 vectors as discussed below. Five independent clones containing PCR products obtained with each primer pair were sequenced to verify that only the target gene was amplified.

The 18S small subunit nuclear rRNA of pea was quantified as a loading control to estimate the variation of input total RNA for each qRT-PCR. Primers and probe for the 18S rRNA control were designed by Ozga et al. (2003) with 5′ VIC as the fluorescent reporter of the probe and 3′ TAMRA as the quencher (Applied Biosystems). The coefficient of variation of the Ct values of 18S rRNA amplicon across all the samples was less than 3%. Therefore, the target amplicon expression values were not normalized to the 18S signal (Livak and Schmittgen, 2001; Ozga et al., 2009). When analyzing the pea auxin receptor transcript levels in transgenic *Arabidopsis* plants (discussed below), *Arabidopsis* protein phosphatase 2A subunit A3 (AtPP2AA3; *Arabidopsis* Gene ID: AT1G13320; Appendix Table B3) was used as the internal reference for normalization of qRT-PCR data as described below (Czechowski et al., 2005; Wang et al., 2014).

3.2.4 Creation of transgenic *Arabidopsis* plants expressing *PsTIR1a*, *PsTIR1b* and *AtTIR1*

3.2.4.1 Wild-type and mutant lines of Arabidopsis

All the *A. thaliana* lines used in this study were of Columbia (Col-0) ecotype background. Wild-type (WT; Col-0; CS 70000) and T-DNA insertion mutant lines *Attir1-10* (SALK_090445C) and *Atafb2-3* (SALK_137151) were obtained from the Arabidopsis Biological Resource Center (ABRC). Backgrounds of all the mutants were cleaned once by backcrossing them with Col-0 plants. Heterozygous plants obtained by backcrossing were allowed to self-pollinate, and the progenies homozygous for the mutation were verified by PCR analysis as

described in O'Malley and Ecker (2010). Primers used for the verification of homozygous T-DNA insertion (Appendix Table B4) were designed using the T-DNA primer designing tool available online through the Salk Institute Genomic Analysis Laboratory (SIGnAL: http://signal.salk.edu/tdnaprimers.2.html). Background cleaned *Atafb2-3* and *Attir1-10* were crossed with each other to obtain double mutants, and progenies from this cross which are homozygous for the double mutation were identified by PCR as described above.

3.2.4.2 Isolation of AtTIR1 promoter

DNA was extracted from 10-day old *Arabidopsis* Col-0 seedling tissues using a Cetyltrimethylammonium bromide (CTAB)-based method. Briefly, 400 µL of CTAB DNA extraction buffer [100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% CTAB, 1% polyvinylpyrrolidone (PVP)] was added into ground tissue sample (about 100 mg) and incubated for 15 min while mixing every 5 min. Into this mixture, 400 µL of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed well and centrifuged for 5 min to isolate the aqueous layer. An equal amount of isopropanol was added to the separated aqueous phase and centrifuged for 5 min to precipitate the DNA. The DNA pellet was cleaned by washing with 70% ethanol before dissolving in nuclease-free water. This DNA was used to isolate the *AtTIR1* promoter region about 3 kb in length (-1 bp to – 3008 bp region from the start codon) using primers given in Appendix Table B1. The PCR amplified product was used as a template for a second PCR reaction to incorporate restriction endonuclease sites at the ends of the *AtTIR1* promoter region and this amplicon was inserted into the pCR8 vector and transformed into chemically competent *E. coli* cells as described above. The sequence accuracy of the complete promoter sequence in the pCR8 vector was verified by sequencing as described above.

3.2.4.3 Transformation vector construction

A modified version of pCAMBIA1300, hereafter referred to as pCM1300-polyA, was obtained from Dr. E. Scarpella at the University of Alberta. In pCM1300-polyA, the *CaMV 35S* promoter driving the expression of the selectable marker has been replaced with the mannopine synthase promoter to avoid influencing the transgene expression (Yoo et al., 2005; Gudynaite-Savitch et al., 2009). Additionally, the *CaMV 35S* polyadenylation sequence has been

incorporated towards the end of the pUC18 polylinker (flanked by SacI and EcoRI restriction recognition sites) as a transcription terminator.

Expression vectors were created by placing the *PsTIR1a, PsTIR1b* and *AtTIR1* CDSs under the *AtTIR1* 3 kb promoter region (Appendix Fig. B2). Restriction endonuclease sites required for cloning were incorporated into CDSs by PCR with primers containing restriction endonuclease recognition sites (Appendix Table B5). The promoter and transgene were incorporated into the vector in two independent steps. Each time the vector was digested with the two appropriate restriction endonuclease enzymes and treated with shrimp alkaline phosphatase (rSAP; New England Biolabs, MA, USA) as per the manufacturer provided protocol to avoid self-ligation which may result due to incomplete double digestion. Ligation of restriction endonuclease-digested inserts into the vector was done with T4 DNA ligase (Invitrogen) as per the manufacturer recommendations. The complete CDS and the regions spanning each ligation site were sequenced to verify the accuracy of the construct.

The *Agrobacterium tumefaciens* Strain GV3101 used in the transformation was kindly provided by Dr. E. Scarpella at the University of Alberta. Competent GV3101 cells were prepared by growing them in LB medium at 29 °C until reaching an OD260 value of 1 to 1.5. The cells were pelleted by centrifugation (4000g for 30 min) and washed three times with cold 1 mM HEPES buffer (1 mM HEPES in water, pH 7) before resuspending in 10% glycerol. Recombinant plasmids carrying *pAtTIR1*::*cPsTIR1a*, *pAtTIR1*::*cPsTIR1b* or *pAtTIR1*::*cAtTIR1* were transformed into *A. tumefaciens* strain GV3101 by electroporation. The GV3101 strain has rifampicin and gentamicin resistance as chromosomal and helper plasmid markers, respectively. Therefore, 10 µg/mL rifampicin and 25 ug/mL gentamicin were included in all the culture media to maintain selectivity. Additionally, 50 µg/mL kanamycin was used in the growth media for the selection of bacterial transformants carrying pCM1300-polyA.

3.2.4.4 Arabidopsis transformation

Floral-dip transformation of *Arabidopsis* was done as described in Zhang et al. (2006). Briefly, *Agrobacterium* GV3101 containing the transgene construct grown in a 5 mL culture for two days at 28 °C was used to inoculate 1500 mL of LB medium. Newly inoculated culture was grown at 28 °C for 22-24 h, spun down at 5500 rpm at 4 ºC for 10 min, and the pellet was re-

dissolved in 1500 mL of 5% (wt/vol) sucrose containing the surfactant Silwet L-77 (0.05% vol/vol).

Background cleaned *Arabidopsis tir1-10* or *tir1-10 afb2-3* double mutant plants (T_0 generation) were grown in 10 x 10 cm pots with each pot containing about 20 plants. Three pots of plants were used in each transformation (Appendix Fig. B3). Whole aerial parts of flowering plants were dipped in the *Agrobacterium* suspension for about 10 seconds. Plants were then covered with plastic bags and kept horizontally to the ground for about 16- 24 h. After that, plastic covers were removed, and plants were grown normally at 23 ºC and 16 h light until the seed harvest (T_1 seeds).

3.2.4.5 Hygromycin selection of transformed Arabidopsis

The method for hygromycin selection of transformed plants was adapted from Bent (2006). Seeds from floral-dipped plants (T_1 seeds) were sterilized with 70% ethanol for 2 min and then with 2% NaOCl containing 0.1% Tween 80 for 10 min while vortexing every 2-3 min. Finally, the seeds were washed four times with sterile water and suspended in a sterile solution of 0.1% Agarose. The seed suspension was spread on 150 x 15 mm Petri plates containing half-strength Murashige and Skoog (MS) nutrient agar medium with 25 mg/L hygromycin. The plates were kept in the dark at 4 ºC for four days; then they were exposed to light to induce germination at room temperature (approximately 22 ºC) for 6-8 h prior to incubation in continuous dark conditions at room temperature for four days. At the end of the fourth day, the hygromycin-resistant transformants of the T_1 generation could be differentiated from non-transformants by their elongated hypocotyl and closed cotyledons (Bent, 2006).

Selected hygromycin-resistant transformants were moved to a peat-based potting medium (Sunshine Mix #4/ LA4, Sun Gro Horticulture) and grown in a growth chamber (21-22 ºC and 12 h photoperiod with fluorescent light) covered with a transparent dome to maintain high humidity conditions. The dome was gradually opened after a few days allowing the seedlings to adapt the normal growth conditions, and then it was completely removed. The presence of the target transgene construct in plants was verified by a direct PCR with a leaf tissue sample and using the Phire Plant Direct PCR kit (Thermo Scientific) as per the manufacturer recommendations. For the plants transformed with *PsTIR1a* and *PsTIR1b*, the PCR primer pair was chosen as the forward primer binds to the AtTIR1 promoter and the reverse primer binds to the CDS of the

transgene construct. In control plants transformed with *AtTIR1*, the forward primer binds to the CDS and reverse primer binds to the *CaMV 35S* terminator sequence of the transgene construct (Appendix Table B6).

3.2.4.6 Selection of transgenic lines for the phenotypic analysis

About nine independent transformants (T_1 generation) were obtained from floral-dip transformation. Seeds of each independent T_1 transformant line were grown to obtain 12 T_2 plants. A portion of T_3 seeds (about 30 to 60) from each of those individual plants was grown independently on half-strength MS medium with 25 mg/L hygromycin to see if the parent plant was homozygous for the transgene construct (Appendix Fig. B3). The hygromycin selection assay was repeated for putative homozygous lines with about 100 seeds to reverify that those lines are homozygous. Independent transgenic lines showing an approximately 1:2:1 (homozygous: hemizygous: wild-type) segregation ratio for hygromycin resistance at T2 generation were considered to contain single insertions and selected for subsequent studies. Generally, about five of the nine independent transformant lines segregated as single gene insertion lines.

Quantitative PCR for transgene expression analysis was done with total RNA extracted from the leaves of about month-old greenhouse-grown plants. Reaction conditions are as described in Chapter 2. *Arabidopsis AtPP2AA3* was used as the internal reference (Czechowski et al., 2005; Wang et al., 2014). Data were analyzed using $2^{-\Delta\Delta Ct}$ method considering the WT *Arabidopsis* as the reference sample (Livak and Schmittgen, 2001). In final studies, two independent transgenic lines were selected for a particular analysis given that they are homozygous for the insert, appear to have a single insertion, and express the transgene at reliable levels (Appendix Fig. B4). In addition, their root elongation phenotype in preliminary analysis was also taken into consideration. The only exception to the above selection criteria is with *pAtTIR1::cPsTIR1a* in *Attir1-10*, where segregation ratios were not studied and thus may contain lines with more than a single insertion.

3.2.5 *Arabidopsis* growth assays

The root elongation assay was adapted from Parry et al. (2009) and was done with T_4 generation transgenic plants. *Arabidopsis* seeds sterilized as described above were suspended in a sterile solution of 0.1% aqueous agarose (wt/v) and placed on 1% aqueous (wt/v) agar plates (100 x 100 x 15 mm square plates with grid) containing half-strength MS and 1% (wt/v) sucrose. The plates were kept in the dark at 4 ºC for four days for seed stratification and then moved to a growth chamber at 22 °C and continuous fluorescent light. Seedlings were grown vertically for four days and selected uniform size seedlings were transferred to plates (100 x 100 x 15 mm square plates with grid) containing half-strength MS medium with 1% sucrose as above but with or without auxins. For the plates containing auxin, filter-sterilized auxins were added to autoclaved growth media to make the final auxin concentrations 50 or 70 nM for 2,4-D and 400 or 800 nM for IAA and 4-Cl-IAA. Analysis of root elongation with 2,4-D was performed under fluorescent light (average photosynthetic flux density; 155 ± 20 µmol m^{-2} s^{-1}). Root elongation assays with IAA and 4-Cl-IAA were done under yellow plastic film (which filtered out the wavelengths between 350 nm to 500nm with a maximum absorbance at 430 nm) to prevent the photodegradation of auxin in the growth media (Stasinopoulos and Hangarter, 1990). Covering with yellow plastic film reduced the average photosynthetic flux density the plates were exposed to about 100 ± 10 µmol m^{-2} s^{-1}. Each plate contained two seedlings each (considered as technical replicates during analysis) of all the relevant transgenic or mutant lines with appropriate controls used for comparison. The total number of seedlings per root growth assay plate was always 12. Seedling root tip positions were marked on the day of transfer to the new plates and were remarked after three days. Plates were photographed using a Canon-PC1438 camera positioned horizontally to the vertically kept plates. Root elongation was measured using ImageJ software (1.49v; Abràmoff et al., 2004) as described in Doerner, 2008. Root elongation of each genotype in auxin medium is expressed as a percentage of the same genotype in the medium without auxin.

3.2.6 Phylogenetic tree creation and the sequence analysis of pea auxin receptors

For the creation of the phylogenetic tree, predicted amino acid sequences of the pea auxin receptors PsTIR1a, PsTIR1b, PsAFB2, PsAFB4, and PsAFB6 were aligned with the auxin

receptor proteins of *A. thaliana* (Parry et al. 2009), apple (*Malus x domestica*; Devoghalaere et al. 2012) and tomato (*Solanum lycopersicum*; Ben-Gera et al. 2012) using the MUSCLE sequence alignment program (Edgar, 2004) under default settings in MEGA 7.0 (Kumar et al., 2016). A neighbor-joining tree was created using the MEGA 7.0 program with 1000 bootstrap replicates, Poisson correction model and partial deletion treatment with a site coverage cutoff set to 95%. The tree was rooted with EIN3 BINDING F-BOX 1 (EBF1) and EBF2 sequences of *Arabidopsis*, tomato and pea (see Appendix E.3 for the sequences of all the proteins used in the phylogenetic tree).

Conservation of amino acids important in auxin perception was analyzed by aligning the predicted amino acid sequences of the pea (*P. sativum*) auxin receptors with those of *Arabidopsis*. Sequences were aligned using the PRALINE sequence alignment program (Simossis and Heringa, 2003; Simossis and Heringa, 2005) under default settings as described in Chapter 2.

3.2.7 Pea *DR5::GUS* expression experiments

3.2.7.1 DR5::GUS lines in pea

All the plants used in the study were in *P. sativum* L. cv. I_3 (Alaska-type) background. The *DR5::GUS* construct in pRD400 vector (DeMason and Polowick, 2009) was transformed into *P. sativum* L. cv. I_3 (Alaska-type) by Dennis M. Reinecke as described in Reinecke et al. (2013) and T_3 generation plants homozygous for the transgene were used for these studies.

3.2.7.2 Histochemical analysis for GUS activity

DR5-driven expression of the *GUS* marker gene was monitored in pea fruit from prepollination to 10 DAA [*DR5::GUS* line: A DR5P-R24A (7-3) (P-2)]. Thin fresh-tissue fruit cross sections (<1 mm thick) of the mid-pericarp region of prepollinated (-2 DAA) and pollinated ovaries at 0, 3, 5, 8 and 10 DAA were made by hand using a scalpel #10 blade. A second line of *DR5::GUS* plants ([A DR5P-R22A (3-3) (P-19) + (P-3)] was used to analyze the effects of auxin treatments on *DR5::GUS* expression. Two DAA deseeded (SPNS) pericarps were treated with IAA or 4-Cl-IAA in 0.1% aqueous Tween 80, or 0.1% aqueous Tween 80

(treatments described in Chapter 2) and pericarps were collected at 2, 8 and 12 h after treatment. Fresh-tissue fruit cross sections from the mid-pericarp region were made from the auxin-treated pericarps as described above. The pericarp sections were submerged in 12- or 24-well tissue culture plates containing GUS staining solution [1 mM 5-Bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc), 100 mM sodium-phosphate buffer (pH 7), 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% Triton X100 and 10 mM EDTA (Hull and Devic, 1995)]. Samples were placed in a vacuum desiccator for 30 min and then were incubated in the dark at 37 °C. After 12 h, samples were washed with 70% ethanol for about three times before taking micrographs using a Zeiss SteREO Discovery V8 (Carl Zeiss MicroImaging GmbH, Jena, Germany) or Olympus SZ61 (Olympus Corporation, Tokyo, Japan) stereo microscope. Histochemical analysis was repeated with a second independently transformed *DR5::GUS* line for the verification of staining patterns. In order to verify that the staining observed in the transgenic plants is due to the expression of *DR5::GUS*, SPNS pericarps treated with IAA, 4-Cl-IAA or 0.1% aqueous Tween 80 (treatments described in Chapter 2) were also analyzed in WT plants and no GUS staining was detected at 8 h after hormonal treatments (Appendix Fig. B5).

3.2.7.3 Fluorometric quantification of GUS activity

The GUS activity was quantified using a fluorescent assay protocol with the GUS substrate 4-methylumbelliferyl glucuronide (MUG assay; adapted from Jefferson, 1987). Pericarp wall tissues of 3, 5, 8 and 10 DAA fruits were analyzed for the developmental variation of GUS activity. Effect of seed removal and IAA and 4-Cl-IAA treatments on GUS activity was analyzed in 2 DAA deseeded pericarps treated with IAA or 4-Cl-IAA (50 μM in 0.1% aqueous Tween 80; 30 μL). The SPNS controls were treated with 0.1% aqueous Tween 80. All the treatments were done as described in Chapter 2. GUS activity was analyzed at 2, 8 and 12 h after treatments.

For the MUG assay, four pericarp wall discs per fruit (two from each side of the pericarp wall, which are coming from the middle half region of the pericarp) were taken using a cork borer (6 mm diameter). The tissues were immediately frozen in liquid nitrogen, then stored at -80 °C. Tissues from two fruits were pooled as a single sample (biological replicate). Three biological replicates were always used for the assay. Frozen tissue samples were ground by bead-

beating. For each ground tissue sample 150 µL of GUS extraction buffer [50 mM $NaPO_4$ (pH 7.0), 10 mM Na_2EDTA, 0.1% Sodium lauroyl sarcosinate, 0.1% Triton X-100, l0 mM β-Mercaptoethanol] was added, vortexed for 1 min and spun down at 14,800 rpm at 4 °C for 10 min. After centrifugation, the supernatant (protein extract) was separated out, frozen in liquid N_2, and stored at -80 °C. Before performing the MUG assay, tissue extracts of the IAA- and 4-Cl-IAA-treated samples were diluted 3- and 5-fold, respectively, due to very high GUS activity in those samples. Twenty microliters of undiluted or diluted tissue extracts were added to microcentrifuge tubes containing 200 µL of pre-warmed (37 °C) assay buffer (1 mM MUG in extraction buffer), and the tubes were vortexed for a few seconds. The tubes containing the mixture were transferred to a 37 °C shaking water bath, and 60 µL aliquots were taken out at pre-determined time intervals and placed into microcentrifuge tubes containing 540 µL of stop buffer (0.2 M Na_2CO_3). For the developmental assay samples, the 60 µL aliquots were taken out at 15, 30 and 45 min after initiating the reaction. For hormone-treated samples and respective SPNS and intact controls, reactions were stopped at 5, 10 and 15 min after initiation. The amount of the end product, 4-methylumbelliferone (4-MU), produced during the reaction period was analyzed by fluorescence. Each sample (190 µL) was aliquoted in triplicate into microtiter plates (Costar 96 well, black, flat bottom plates, Corning Incorporated, NY, USA) and sample fluorescence was measured using a SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices, USA; excitation, emission, and cut-off bandwidths were set to 365 nm, 455 nm, and 435 nm, respectively). A 9-point dilution series of a 4-MU (Sigma) standard with concentrations ranging from 4000 nM to 15.625 nM was run on each plate. The 5-point range appropriate for the fluorescence intensity of samples was selected for the creation of the calibration curve. Each sample was analyzed twice, and the average was taken for the calculations.

The MUG assay data was normalized to total protein content of the sample (Cervera, 2004). For estimation of the total sample protein content, the Bio-Rad Protein Assay (Bio-Rad Laboratories, CA, USA) kit based on the Bradford (1976) method was used. Reactions were set up as per the manufacturer's standard procedure for microtiter plates. Briefly, 2 µL of the protein extract was diluted 50-fold. Ten µL of the diluted protein extract was mixed with 200 µL of diluted dye reagent and aliquoted into microtiter plates (Costar 96 well clear, flat bottom plates; Corning Incorporated). Four technical replicates were analyzed per sample. After 10 min, sample absorbance was measured at 595 nm in a SpectraMax M3 Multi-Mode Microplate Reader

(Molecular Devices, CA, USA). A 3-point dilution series of bovine serum albumin (ranging from 0.06- 0.24 mg/mL) was used to construct a calibration curve.

3.2.8 Hormonal analysis

Pea fruit samples were collected from *P. sativum* L. cv. I_3 (Alaska-type) plants expressing the *DR5::GUS* transgene [A DR5P-R22A (3-3) (P-19) + (P-3)] for hormonal analysis. The samples included whole pericarps (0, 3, 5 and 8 DAA), pericarp wall (5 and 8 DAA), ventral vascular suture (5, 8 DAA), dorsal vascular suture (5, 8 DAA) and seeds (8 DAA). Also, whole pericarps of non-pollinated fruits, which were obtained by the emasculation of flower buds at -2 DAA, were collected at 3 DAA for hormone analysis. All the samples were stored at -80 ºC, and subsequently lyophilized (Virtis Ultra 35L Freeze Dryer, NY, USA) and ground to a fine powder. Each biological replicate consisted of tissues from a minimum of 10 fruits. Hormonal analysis was done by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) at the National Research Council, Saskatoon, Canada (NRCC SK; see Appendix B, supplementary materials and method for the hormone analysis protocol provided by NRCC SK).

3.2.9 Statistical analysis

The data means in all experiments are the average of biological replicates (independent samples). The number of biological replicates is given for each experiment within the specific data set. In graphs showing developmental data, the minimum number of biological replicates were three or higher. Therefore, standard error (SE) is shown by error bars in all the graphs showing developmental data. In other graphs, occasionally there were samples with only two biological replicates. Due to this occasional limitation of biological replicates, standard deviation (SD) is shown by error bars in all the other graphs, unless stated otherwise in the figure legend.

In *Arabidopsis* root elongation assays, the probability values (P-values) were calculated using a two-tailed T-test assuming unequal variance. Data analysis was done in Microsoft Excel 2013 using the Analysis ToolPak. Statistical significance was declared at $P \leq 0.01$ for comparisons between the means.

3.3 RESULTS AND DISCUSSION

3.3.1 TIR1/AFB family of auxin receptors in pea

The full-length CDS of the five TIR1/AFB family members of *P. sativum* were verified by cloning and sequencing. The five putative auxin receptors included two previously reported genes *PsAFB2* and *PsAFB6* (Nadeau, 2009; Appendix Figs. B6 and D2), and three new genes. A phylogenetic analysis predicted that two of the new sequences are TIR1 homologs; therefore, they were named as *PsTIR1a* and *PsTIR1b* (Fig. 3.1, Appendix Figs. B7 and B8; GenBank accession numbers: KX954124 and KX954125 respectively). The third new sequence grouped with *Arabidopsis* AFB4 clade of auxin receptors which contains *AtAFB4* and *AtAFB5*; therefore, it was named *PsAFB4* (see Chapter 4, Appendix Fig. D1; GenBank accession number: KX954126).

In addition to the transcriptomic sequence database mentioned above, sequence assemblies of several transcriptome sequencing projects of pea have become publically available over the past several years. Those transcriptome sequences were derived from pea tissues including seedling tissues such as cotyledons, epicotyl and hypocotyl, reproductive tissues such as flowers, pods and seeds, and vegetative tissues such as stem, tendril, roots and leaves at different developmental stages (Franssen et al., 2011; Kaur et al., 2012; Duarte et al., 2014; Sudheesh et al., 2015). Screening of sequence assemblies generated by the above sequencing projects in the NCBI Transcriptome Shotgun Assembly (TSA) database revealed no additional putative auxin receptor homologs.

The predicted amino acid sequences of PsTIR1a and PsTIR1b are 83% identical, and they share 79% and 76% identities with the AtTIR1 protein, respectively. As a comparison, PsAFB2 shares 75 % amino acid identity with AtAFB2 (Nadeau, 2009). In the *Arabidopsis* TIR1 protein, the leucine-rich repeat domain contains a pocket for auxin binding. The bottom of this pocket contains an inositol hexakisphosphate ($InsP_6$) molecule which is likely acting as a structural cofactor (Tan et al., 2007). When auxin is bound in the TIR1 pocket, an Aux/IAA co-receptor protein fits into the pocket on top of the auxin molecule (Tan et al., 2007). Comparison of *Arabidopsis* and pea auxin receptor proteins show that all the amino acids important for auxin binding and interaction with $InsP_6$ in AtTIR1 (Tan et al., 2007) are also conserved in the pea PsTIR1a, PsTIR1b and PsAFB2 proteins, except for a single amino acid substitution in PsAFB2,

where the amino acid residue valine (V) 463 of AtTIR1 has been substituted with isoleucine (V463I); a similar substitution was also seen in AtAFB2 (see the amino acid position 516 of the Appendix Fig. B9). The majority of AtTIR1 amino acids involved in the interactions with the AtIAA7 co-receptor are also conserved in pea PsTIR1a, PsTIR1b and PsAFB2 proteins (Appendix Fig. B9).

3.3.2 PsTIR1a and PsTIR1b auxin receptors can respond to IAA and 4-Cl-IAA

According to the current knowledge of auxin receptors in *Arabidopsis*, the AtTIR1 receptor plays the most prominent role in auxin perception (Parry et al., 2009). To evaluate the role of *PsTIR1a* and *PsTIR1b* as auxin receptors and their role in IAA and 4-Cl-IAA perception, they were introduced into *Arabidopsis tir-10* and *Attir1-10 afb2-3* mutants under the regulation of *Arabidopsis TIR1* promoter (*pAtTIR1::cPsTIR1a*, *pAtTIR1::cPsTIR1b*). As a control, the CDS of *AtTIR1* was also introduced into *Attir-10* and *Attir1-10 afb2-3* mutants under the same promoter (*pAtTIR1::cAtTIR1*). The *Attir1-10* plants contain a T-DNA insertion close to the 5′ end region of the CDS and therefore, are likely null mutants (Parry et al., 2009). In *Atafb2-3* mutants, a T-DNA insertion in the promoter region results in reduced gene expression (Parry et al., 2009). As reported previously by Parry et al. (2009), *Arabidopsis tir1-10* plants show reduced auxin sensitivity (greater root elongation) when grown in the presence of 2,4-D (50 and 70 nM) compared to that of the WT (Col-0; Appendix Fig. B10). Compared to the *Attir1-10* single mutants, *Attir1-10 afb2-3* double mutants showed a further reduction in 2,4-D sensitivity in the root growth assays (Appendix Fig. B10). Similar results have been previously reported with the *Attir1-1* allele, where 2,4-D sensitivity was lower in *Attir1-1 afb2-3* double mutant compared to *Attir1-1* single mutant (Parry et al., 2009).

The two auxins IAA and 4-Cl-IAA were also effective in inhibiting the root elongation in *Arabidopsis* seedlings. Inhibition of root elongation in WT seedlings was greater with 4-Cl-IAA than IAA when compared at the same concentration (Fig. 3.2). However, IAA inhibited root elongation to the same extent as 4-Cl-IAA (at the same concentration) in the *Arabidopsis tir1-10 afb2-3* double mutant (Fig. 3.2). It is possible that 4-Cl-IAA, which is not a natural auxin in *Arabidopsis*, is not subjected to conjugation, degradation or transport within *Arabidopsis* in a manner similar to that of IAA. Increased stability or the differential transport efficiencies may be associated with the stronger root elongation inhibitory effect of 4-Cl-IAA in WT seedlings. In

tir1-10 afb2-3 double mutant, the reduced auxin sensitivity may be masking the differential effects of the two auxins. However, it is also possible that the two auxins show differential affinities to *Arabidopsis* auxin receptors and therefore the differential effects of the two auxins are at least partially mediated through the *AtTIR1* and/or *AtAFB2* receptors.

Functional characterization of *PsTIR1a* and *PsTIR1b* was conducted in *Attir1-10* single mutant and *Attir1-10 afb2-3* double mutant backgrounds, using two independent transgenic lines per genotype background. Transgenic plants expressing *PsTIR1a* (*pAtTIR1::cPsTIR1a*) or *PsTIR1b* (*pAtTIR1::cPsTIR1b*) restored 2,4-D inhibition of root growth in *Arabidopsis tir-10* and *tir1-10 afb2-3* mutants to levels similar to that of mutants expressing the *AtTIR1* transgene (*pAtTIR1::cAtTIR1*), and they are consistent with the auxin root inhibiting activity exhibited by the WT seedlings (Figs. 3.3 and 3.4). Restoration of auxin sensitive root growth in *Arabidopsis* auxin-resistant mutants indicates that both *PsTIR1a* and *PsTIR1b* are functional auxin receptors. Expression of *PsTIR1a* or *PsTIR1b* in the *Arabidopsis tir1-10 afb2-3* double mutant also restored IAA and 4-Cl-IAA sensitive root growth inhibition to levels comparable to that of mutants expressing the *AtTIR1* transgene (Fig. 3.5). Similar to *Arabidopsis* WT plants, *tir1-10 afb2-3* double mutant seedlings expressing *PsTIR1a*, *PsTIR1b,* or *AtTIR* genes showed stronger inhibition of root elongation in the presence of 4-Cl-IAA than IAA, especially at 800 nM (Appendix Fig. B11). The absence of a differential auxin response to IAA and 4-Cl-IAA in the *tir1-10 afb2-3* double mutant and the restoration of differential response in the mutants expressing *AtTIR1*, *PsTIR1a* and *PsTIR1b* (Appendix Fig. B11) suggest that the stronger auxin response elicited by 4-Cl-IAA compared to IAA (at the same concentration) is at least partially mediated through TIR1. Overall, these findings suggest that *PsTIR1a* and *PsTIR1b* code for auxin receptors that in the presence of IAA, 4-Cl-IAA or 2,4-D can elicit an auxin response. In addition, expression of pea *PsAFB2* CDS under the regulation of *AtTIR1* promoter also increased the 2,4-D sensitivity of *Attir1-10 afb2-3* mutants in root growth assays, which indicates that PsAFB2 is also a functional auxin receptor (see Appendix C for the functional characterization of PsAFB2).

3.3.3 *PsTIR1a* and *PsTIR1b* auxin receptor expression profiles in pea seedling tissues

In 12 DAI pea seedlings, *PsTIR1a* transcript abundance was similar in the shoot apex, immature leaf, immature internode and mature internode tissues, but was slightly lower in

mature leaves (Fig. 3.6 A). In contrast to *PsTIR1a*, transcript abundance of *PsTIR1b* was higher in the immature and mature leaves, and the shoot apex, than that observed in the immature or mature internode tissue (Fig. 3.6 B). For comparison, transcript levels of *PsAFB2*, originally identified and cloned by Nadeau (2009), were also profiled in the 12-day old pea seedlings. In contrast to *PsTIR1,* transcript abundance of *PsAFB2* was higher in immature seedling tissues than in mature tissues (Fig. 3.6 C). Screening of microarray data shows that the transcript levels of *Arabidopsis TIR1*, *AFB1* and *AFB2* genes are also regulated in tissue type and developmental stage dependent manners (Schmid et al., 2005; Winter et al., 2007; Appendix Fig.B12)

3.3.4 Regulation of *PsTIR1a*, *PsTIR1b* and *PsAFB2* gene expression profiles during early pea fruit development

In pea, flowers are self-pollinated approximately 24 h before flower opening (by -1 DAA), and fertilization takes place by 0 DAA (Cooper, 1938). Developmental regulation of *PsTIR1a, PsTIR1b* and *PsAFB2* transcript levels was analyzed in -2 DAA prepollinated fruit, and in the pericarp wall, dorsal and ventral vascular suture tissues, and the seeds of pollinated pea fruits from 0 to 10 DAA (Fig. 3.7; see Appendix Fig. B1 for a diagram of fruit tissues). In all the pericarp tissues and seeds, *PsTIR1a, PsTIR1b,* and *PsAFB2* transcript levels were highest immediately after fertilization (0 DAA; Fig 3.7 and Appendix Fig. B13). The transcript levels of *PsTIR1a, PsTIR1b,* and *PsAFB2* gradually declined in the pericarp and seeds with fruit development. The trend of *PsAFB2* transcript abundance in different pericarp tissues was similar to the previously reported *PsAFB2* transcript abundance profile in whole pericarps (Nadeau, 2009). Within the pericarp, transcript abundance of *PsTIR1a* was slightly higher in the dorsal vascular suture compared to pericarp wall over this developmental period. A similar trend was observed for *PsTIR1b* from 4 to 10 DAA. Transcript abundance of *PsAFB2* was relatively uniform in all the pericarp tissues (Fig. 3.7).

The presence of higher auxin receptor transcript levels during early ovary growth and development followed by a gradual reduction of their levels with further fruit development appears to be a common trend in a number of species. In cucumber (*Cucumis sativus* L.), peak levels of *CsTIR1* and *CsAFB2* transcripts have been reported in 2 and 4 DAA fruits respectively, which gradually declined afterward (Cui et al., 2014). In developing plum (*Prunus salicina* L.) fruits, peak levels of *PslTIR1*, *PslAFB2* and *PslAFB5* transcripts were reported following

fertilization (at around 7 DAA). Transcript abundance of those genes gradually declined until fruit maturity, when a gradual re-accumulation of *PslTIR1* and *PslAFB2* was observed (El-Sharkawy et al., 2014). In tomato (*Solanum lycopersicum*) ovaries, the transcript abundance of *SlTIR1* was higher at the time of anthesis (0 DAA) but declined by 4 DAA (Ren et al., 2011). The higher abundance of auxin receptor transcripts closely following fertilization events indicates that an increased auxin sensitivity of the ovaries facilitates ovary growth and development. Consistent with this possibility, overexpression of *SlTIR1* in tomato stimulated precocious fruit set and parthenocarpic fruit development (Ren et al., 2011).

In the absence of pollination, pea pericarp growth is inhibited (Fig. 2.1 A and B) and the fruit will subsequently abscise; however, at 3 DAA, non-pollinated ovaries were still green and turgid. In the pericarp wall and dorsal vascular suture tissue, *PsTIR1a* transcript abundance was slightly higher in non-pollinated fruits compared to pollinated fruits, with no change observed in the ventral vascular suture tissue (Fig. 3.8 A; Appendix Table B7). The *PsTIR1b* transcript level increased from 0 to 1 DAA (about 2-fold) and remained elevated through 3 DAA in all pericarp tissues from non-pollinated ovaries compared to that of pollinated ovaries (Fig. 3.8 B; Appendix Table B7). A slight increase in the transcript abundance of *PsAFB2* was observed only at 3 DAA in non-pollinated compared to pollinated pericarp tissues (Fig. 3.8 C, Appendix Table B7). Non-pollinated ovaries of cucumber cultivar 8419s-1 (non-parthenocarpic type), which undergoes ovary senescence, also showed an increase of *CsTIR1* and *CsAFB2* transcript abundance from 0 to 6 DAA, but the parthenocarpic cultivar EC1 (ovary grows in the absence of pollination) showed a decline in those gene transcript levels over the same period (Cui et al., 2014). These observations suggest that a signal associated with ovary development suppresses the auxin receptor transcript abundance.

3.3.5 IAA and IAA-amino acid conjugate content in pericarp and seed tissues during early pea fruit development

Coincident with the decline of pericarp *PsTIR1* and *AFB2* transcript abundance (Fig. 3.7), pericarp IAA level was higher following pollination and fertilization events at 0 DAA and decreased about 3-fold as the fruit developed (5 DAA; Table 3.1; the auxin levels are discussed using ng $gfwt^{-1}$). Magnus et al. (1997) also reported a reduction in pea pericarp IAA levels during early pea fruit development (1.7-fold reduction of free IAA from 3 to 6 DAA). In parallel

to the decline of free IAA level, the level of amino acid conjugates of IAA, indole-3-acetyl-aspartate (IAA-Asp) and indole-3-acetyl-glutamate (IAA-Glu) also declined from 0 to 3 DAA (Table 3.1). IAA-Asp and IAA-Glu are considered as irreversible IAA conjugates destined for degradation (Ludwig-Muller, 2011) and IAA-Asp is likely the most abundant form of IAA conjugate in pea (Sudi, 1964; Nordström and Eliasson, 1991). The decrease in these IAA-amino acid conjugate levels with the reduction of free IAA suggests that the conversion of IAA to IAA-Asp or IAA-Glu is not the reason for reduced pericarp IAA level observed from 0 to 3 DAA. However, the decline of free IAA level from 3 to 5 DAA was concomitant with an increase in IAA-Asp content, suggesting the conjugation of free IAA to IAA-Asp (Table 3.1).

In the absence of pollination and developing seeds within the ovary, free-IAA level was about 4-fold lower, and IAA-Asp was minimally detectable in the pericarp (compare 3 DAA pollinated and non-pollinated pericarps; Table 3.1). These data suggest that developing seeds are a source for pericarp auxins, and/or they stimulate pericarp auxin synthesis. Regardless of the source of pericarp auxin, the higher transcript levels of *PsTIR1b* (and to a lesser extent *PsTIRa* and *PsAFB2*) in non-pollinated pericarps (Fig. 3.8) compared to pollinated pericarps suggest that auxin receptor (particularly *PsTIR1b)* expression is feedback regulated to increase auxin sensitivity under low auxin conditions.

To determine if IAA distribution varies within the different tissues that make up the pea fruit, IAA, IAA-Asp and IAA-Glu were analyzed in the pericarp wall, dorsal vascular suture and ventral vascular suture of 5 and 8 DAA fruits, along with 8 DAA seeds. The seeds contained the highest concentrations of IAA, IAA-Asp and IAA-Glu within the 8 DAA fruit (215-, 2250- and 430-fold higher, respectively, in the seeds compared to the pericarp wall; Table 3.1). The very high level of IAA in seeds is consistent with that previously reported by Magnus et al. (1997), where 12- and 100-fold higher levels of IAA and, 25- and 50-fold higher levels of 4-Cl-IAA were observed in seeds compared to the pericarps of 3 and 6 DAA fruits, respectively. Within the pericarp, the 5 DAA fruits contained approximately 15- and 5-fold higher free IAA, 65- and 15-fold higher IAA-Asp and, 6- and 3-fold higher IAA-Glu levels in the ventral and dorsal vascular sutures compared to the pericarp wall, respectively (Table 3.1). Associated with the high levels of IAA in the vascular sutures, the 5 DAA fruits showed a rapid increase in pericarp length and width (Ozga et al., 2003).

The free IAA and IAA-Asp levels decreased approximately 1.5-fold in the dorsal vascular suture and 2-fold in the ventral vascular suture from 5 to 8 DAA period (Table 3.1). In contrast to vascular sutures, the free IAA level in the pericarp wall increased by about 3-fold from 5 to 8 DAA and the IAA-Asp level increased by about 2-fold. By 8 DAA, the pericarp has obtained most of its maximal length and width, and the pericarp diameter increases rapidly to accommodate the developing seeds (Ozga et al., 2003). The reduction in IAA level in the vascular suture tissues and the increased IAA level in the pericarp wall from 5 to 8 DAA suggest that IAA may be transported from vascular suture to the pericarp wall and the higher IAA level in the pericarp wall could facilitate rapid pericarp diameter expansion. In pea fruits, the seeds are attached to the ventral vascular suture through the funiculus (Appendix Fig. B1). The high levels of IAA in seeds, declining concentration gradient of IAA from seeds through the vascular sutures to the pericarp wall (Table 3.1), and the possible transportation of IAA from vascular sutures to the pericarp wall further support the hypothesis that seeds may act as a source of pericarp auxins.

3.3.6. Modulation of pericarp auxin activity in developing pea fruit

To relate the pericarp *PsTIR1* and *PsAFB2* gene expression profiles with IAA levels and pericarp auxin activity during early pea fruit development, *DR5::GUS* gene expression was analyzed in the fruit from 0 to 10 DAA. The auxin responsive *DR5* promoter is made up of seven tandem repeats of the *DR5* AuxRE (a mutated auxin response element from soybean *GRETCHEN HAGEN 3*; *GH3*) fused upstream of a minimal *CaMV-35S* promoter (-46 truncated *CaMV 35S* promoter; Ulmasov et al., 1995; Ulmasov et al., 1997; DeMason and Polowick, 2009). The endogenous ARF transcription factors recognize the *DR5-AuxRE* and bind to the *DR5* promoter. However, the expression of downstream *GUS* reporter requires the degradation of Aux/IAA repressors, which are bound to the ARF transcription factors. In the presence of auxin, the TIR1/AFB auxin receptors form a co-receptor complex with Aux/IAAs, which initiates the ubiquitination and subsequent degradation of Aux/IAAs through 26S proteasome-mediated pathway (Wang and Estelle, 2014; see Fig 1.2). Due to this auxin-dependent activation of the *DR5* promoter, the localized GUS enzyme activity of *DR5::GUS* plants is commonly used as a marker of auxin maxima (in pea; DeMason and Polowick, 2009). However, *DR5::GUS* expression relies on the transduction of the auxin signal to the *DR5* promoter, which may be affected by changes in the auxin signaling pathway including the type and level of auxin

receptors present in the tissue. Therefore, *DR5::GUS* expression may not necessarily represent the localized auxin concentrations, but it is an indication of auxin response (Vernoux et al., 2011; Brunoud et al., 2012). As both pericarp IAA levels and the auxin receptor transcript abundance were modulated during pea fruit development, plants expressing *DR5::GUS* can be used to obtain a picture of how those changes are related to auxin action in the pericarp.

In prepollinated (-2 DAA) and pollinated ovaries at 0 DAA, intense GUS staining was observed in the pericarp vasculature (Fig. 3.9 A and B). A similar GUS staining pattern could be seen in 3 DAA pericarp, but with a reduced intensity (Fig. 3.9 C). GUS staining intensity gradually decreased from 0 to 5 DAA, with minimal GUS staining observed in the pericarp wall by 8 to 10 DAA (Fig. 3.9 B-F). A relatively distinct GUS staining was detected at the proximal end of the seed adjacent to the attachment to the funiculus (3 and 5 DAA; Fig. 3.9 C and D), and in the funiculus (3 to 10 DAA; Fig. 3.9 C-F).

To quantitate the changes in *DR5::GUS* expression with fruit development, GUS enzyme activity was determined in the pericarp walls of 3 to 10 DAA fruits. The β-glucuronidase enzyme encoded by the *GUS* gene cleaves the substrate MUG into 4-methylumbelliferone (4MU) which can be quantified by fluorescence (Jefferson, 1987). As observed with the GUS-stain intensity, GUS enzyme activity in the pericarp wall was highest in younger fruits (3 DAA), which decreased (approximately 6-fold from 3 to 8 DAA; Fig. 3.10) with the fruit development.

The decline of pericarp GUS activity indicates a reduction of pericarp auxin action during pea fruit development. The decline of pericarp IAA level with pea fruit development suggests that the reduced auxin action over the pea fruit development is at least partially due to the reduced IAA level. Moreover, given that 4-Cl-IAA is a potent inducer of *DR5::GUS* expression (DeMason and Polowick, 2009, discussed below), reduced GUS activity in developing pea fruits suggest that pericarp 4-Cl-IAA level is also reduced or remained at relatively low levels during this developmental period. Consistent with this, 4-Cl-IAA was 8- and 4-fold lower compared to IAA in 3 and 6 DAA pericarps, respectively (Magnus et al., 1997). Furthermore, pericarp 4-Cl-IAA level was similar in 3 and 6 DAA fruits (Magnus et al., 1997).

However, IAA level in the pericarp wall increased from 5 to 8 DAA (Table 3.1), whereas, GUS enzyme activity declined (Fig. 3.10). These data indicate that the IAA level is not the sole regulator of *DR5::GUS* expression in the pericarp wall tissue at this developmental stage. Transcript abundance of the pericarp wall *PsTIR1b* and *PsAFB2* genes was relatively stable

during the 5 to 8 DAA period; however, *PsTIR1a* transcript abundance decreased about 2.5-fold (Fig. 3.7). Also, the transcript abundance of the putative auxin receptors *PsAFB4* and *PsAFB6* decreased approximately 2-fold in the pericarp wall tissue during this period (5 to 8 DAA; Fig. 4.2). It is possible that the reduction in auxin receptor transcript abundance may have played a role in reducing the auxin responsive *DR5::GUS* expression in the pericarp wall tissues from 5-8 DAA. Furthermore, it is also possible that a decline of pericarp 4-Cl-IAA level may also be associated with the reduced *DR5::GUS* expression in the pericarp walls in the 5 to 8 DAA period.

Altogether, these observations indicate that the transcript abundance of pea fruit auxin receptors, pericarp IAA levels, and auxin activity are developmentally and tissue-specifically regulated. The balance of auxin activity during early pea fruit development is likely modulated through the regulation of auxin level as well as the modulation of fruit sensitivity to auxin through the regulation of auxin receptor abundance.

3.3.7 Transcript abundances of pea fruit *PsTIR1* receptors are differentially modulated by IAA and 4-Cl-IAA

When seeds were present (intact and SP), transcript levels of *PsTIR1a* and *PsTIR1b* in the pericarp remained relatively constant (Fig. 3.11 A and B). Seed removal did not affect pericarp *PsTIR1a* transcript abundance (Fig. 3.11 A); however, *PsTIR1b* transcript abundance was approximately 3-fold higher in deseeded pericarps compared to those with seeds (compare SP with SPNS; Fig. 3.11 B). Pericarp *PsAFB2* transcript abundance was also not affected by seed removal (Fig. 3.11 C), which is consistent with the previous observations by Nadeau (2009). The increase of *PsTIR1b* transcript abundance in deseeded pericarps together with higher *PsTIR1b* transcript abundance in non-pollinated pericarps are consistent with the hypothesis that *PsTIR1b* expression is feedback regulated to increase auxin sensitivity under low auxin or other growth-related signals in the pericarp tissue (due to the absence of seeds).

To further determine the signal(s) regulating pericarp *PsTIR1* expression, *PsTIR1a* and *PsTIR1b* transcript abundance profiles were determined in deseeded pericarps treated with the auxins IAA (does not stimulate deseeded pericarp growth), 4-Cl-IAA (stimulates deseeded pericarp growth) or both IAA and 4-Cl-IAA. Transcript abundance of *PsAFB2* was also analyzed as a control, which remains stable in response to externally applied auxins (Nadeau, 2009).

Transcript abundance of *PsTIR1a* was not clearly modulated by exogenous auxins and it remained at relatively constant levels similar to that of *PsAFB2* (Fig. 3.12 A and C). In contrast, *PsTIR1b* transcript abundance was lower in pericarps treated with 4-Cl-IAA or a mix of IAA and 4-Cl-IAA compared to SPNS controls (especially at 2 and 12 h after treatment time-points; Fig. 3.12 B). However, application of IAA alone had no effect on deseeded pericarp *PsTIR1b* transcript abundance. The increased transcript abundance of pericarp *PsTIR1b* under low pericarp growth conditions (the absence of developing seeds), and the suppression of *PsTIR1b* transcript accumulation only in response to the growth active auxin 4-Cl-IAA indicate that *PsTIR1b* expression is likely modulated by growth-related signals including the auxin 4-Cl-IAA to integrate pericarp growth status to auxin sensitivity.

Pericarp ethylene evolution increases in the absence of pollination or due to seed removal from pollinated pericarps (Orzáez et al., 1999; Johnstone et al., 2005). Application of 4-Cl-IAA but not IAA to deseeded pericarps inhibits ethylene action (Johnstone et al., 2005). The increased transcript abundance of *PsTIR1b* in pericarps with no developing seeds, and the suppression of *PsTIR1b* transcript abundance by 4-Cl-IAA but not by IAA, may indicate that pericarp *PsTIR1b* expression is regulated by ethylene. To test if pericarp auxin receptor expresssion is regulated by ethylene, pericarp *PsTIR1a*, *PsTIR1b* and *PsAFB2* transcript abundance was analyzed in deseeded pericarps (SPNS) treated with an ethylene signaling inhibitor silver thiosulfate (STS), an ethylene releasing agent ethephon, or a combination of ethephon and 4-Cl-IAA, or ethephon and IAA. Application of ethephon or STS alone or in the presence of exogenous 4-Cl-IAA or IAA had no clear effect on deseeded pericarp *PsTIR1a* or *PsTIR1b* transcript levels, indicating that their expression is not directly regulated by ethylene in this tissue (Fig. 3.12 A and B). Transcript abundance of *PsAFB2* remained relatively uniform in response to auxin, ethylene, and ethylene action inhibitor treatments, indicating that pericarp *PsAFB2* transcript level is not regulated by the presence of developing seeds or the variations of auxin (Fig, 3.12 C; Nadeau, 2009) or ethylene levels (Fig, 3.12 C). Altogether, these data support that *PsTIR1b* expression is likely modulated by growth-related signals including the auxin 4-Cl-IAA to integrate pericarp growth status to auxin sensitivity.

3.3.8 *DR5::GUS* expression is higher in 4-Cl-IAA-treated pericarps compared to those treated with IAA

In addition to the possible differences of IAA and 4-Cl-IAA perception at the auxin receptor level, differences in IAA and 4-Cl-IAA activity in stimulation of deseeded pericarp growth may also rely on the differential efficiencies of IAA and 4-Cl-IAA uptake by the pericarp or more rapid metabolism of IAA than 4-Cl-IAA. A time course of IAA- and 4-Cl-IAA-induced *DR5::GUS* expression was performed to gain knowledge of the timing of the auxin response in the split pericarp assays. Histochemical analysis showed consistent GUS staining in the vascular tissues of the control pericarps treated with 0.1% aqueous Tween 80 over the 12 h period of study (Fig. 3.13 A-C). In pericarps treated with IAA or 4-Cl-IAA (50 µM), higher GUS staining, particularly in the non-vascular pericarp tissue, was observed by 2 h after auxin treatment compared to the controls. These data indicate that both IAA and 4-Cl-IAA diffused from the endocarp application site into the mesocarp tissue and initiated the auxin response within 2 h after treatment (Fig. 3.13 D and G). GUS staining further increased by 8 to 12 h after treatment in both IAA- and 4-Cl-IAA-treated pericarps, indicating an increased auxin response due to continued auxin uptake from the application site after the 2 h time-point (Fig. 3.13 E, F, H and I). The increase of GUS enzyme activity in pericarp wall tissues of intact fruits and deseeded pericarps treated with IAA, 4-Cl-IAA or aqueous 0.1% Tween 80 was further evaluated by MUG assay. No clear difference of GUS activity could be seen in the intact and SPNS pericarps during the 12 h period of study (Fig. 3.14). In IAA-treated pericarps, GUS enzyme activity was about 4- and 17-fold higher compared to the SPNS control by 2 and 8 h after treatments, respectively. No further increase of GUS activity could be detected between 8 and 12 h after IAA treatment (Fig. 3.14). In 4-Cl-IAA-treated pericarps, GUS activity was about 6-fold higher by 2 h after treatment, and by 8 h the difference was about 150-fold compared to the controls. Again, no further increase of GUS activity could be detected between 8 and 12 h after treatment (Fig. 3.14).

The patterns of GUS enzyme activity suggest that after a 30 µL aqueous solution is applied to the endocarp, both auxins enter the pericarp within 2 h after treatment, and their accumulation maximizes by 8 h after treatment. The GUS enzyme activity was stronger in response to 4-Cl-IAA, which was about 8-fold higher compared to IAA-treated pericarps at 8 and 12 h after treatment. A stronger effect of 4-Cl-IAA on *DR5::GUS* expression compared to IAA has been previously reported in pea root tips, but the difference was less than 2-fold (DeMason and

Polowick, 2009). The differential effect of 4-Cl-IAA and IAA on *DR5::GUS* expression may be due to different efficiencies of IAA and 4-Cl-IAA uptake by the pericarp, more rapid metabolism of IAA than 4-Cl-IAA, or the ability of 4-Cl-IAA to stimulate a stronger auxin response than IAA at the same concentration. However, approximately 8-fold higher levels of free IAA compared to that of 4-Cl-IAA in 3 DAA pericarps, along with auxin conjugate levels equivalent to that of the free forms for both auxins (Magnus et al., 1997), suggest that a faster rate of IAA metabolism in the pericarp is not likely the primary reason for the differential effects of IAA and 4-Cl-IAA on *DR5::GUS* expression.

3.3.9 Auxin and ethylene regulation of *PsTIR1* auxin receptor transcript abundance in seedling tissues

Auxin regulation of gene expression can be tissue dependent. For example, transcript abundance of 1-aminocyclopropane-1-carboxylate oxidase 1 (*PsACO1*) is strongly induced by 4-Cl-IAA in pericarps but not in 4 DAI seedlings (Fig. 2.9 A and 2.13 D). To see if the auxin effects on the *PsTIR1* gene transcript abundances are regulated in a tissue-dependent manner, their transcript levels were evaluated in seedlings exposed to IAA or 4-Cl-IAA. In contrast to pea pericarps where 4-Cl-IAA was growth stimulatory and IAA was not (Reinecke et al., 1995), both auxins inhibit seedling root elongation (Appendix Fig. A12 A). In addition to *PsTIR1* genes, transcript abundance *PsAFB2* was also analyzed as a control which showed a uniform transcript abundance in response to all the treatments in the pericarps. Neither IAA nor 4-Cl-IAA clearly affected *PsTIR1a*, *PsTIR1b* or *PsAFB2* transcript abundance in the plumule, epicotyl or root tip tissues of 4 DAI seedlings (Fig. 3.15). Relatively stable auxin receptor transcript levels in seedlings exposed to auxin is consistent with the *Arabidopsis* auxin receptor expression data, where roots of *Arabidopsis* seedlings were reported to have no change in the *AtTIR1*, *AtAFB1*, *AtAFB2* and *AtAFB3* transcript abundance after growing in IAA medium for 6 h (Parry et al., 2009). Similar to that in pea pericarps, ethylene also had no clear effect on the transcript abundance of *PsTIR1a*, *PsTIR1b* or *PsAFB2* genes in ethephon-grown seedlings (Fig. 3.16).

3.3.10 Modulation of auxin response at the receptor level

The ability of PsTIR1 receptors to elicit an auxin response in the presence of IAA or 4-Cl-IAA in *Arabidopsis* root growth assays suggest that PsTIR1 receptors are involved in perceiving both auxins. Furthermore, the loss of a stronger auxin response elicited by 4-Cl-IAA compared to that of IAA (at the 800 nM concentration) in the absence of TIR1 (in the *Attir1-10 afb2-3* double mutant), and regaining this effect upon reintroduction of TIR1 (*AtTIR1*, *PsTIR1a*, or *PsTIR1b*) into the *Attir1-10 afb2-3* double mutant background, suggests that at least part of this response is mediated through TIR1.

Auxin perception through TIR1/AFB receptors requires the formation of a co-receptor complex with Aux/IAA co-receptors (Calderón Villalobos et al., 2012). There are 29 Aux/IAA proteins in *Arabidopsis,* which have different affinities to different TIR1/AFB proteins. The affinity between TIR1/AFB and Aux/IAA proteins likely depends on the two protein combination, auxin concentration and the auxin type (Calderón Villalobos et al., 2012). Protein binding assays suggest that the auxin affinity of the co-receptor complex is primarily determined by the Aux/IAA protein, but TIR1/AFB protein is also important with certain Aux/IAAs and auxin types (Calderón Villalobos et al., 2012). Therefore, the differential action of IAA and 4-Cl-IAA in the pericarps may be determined by TIR1/AFB and Aux/IAA protein pools and the differential affinities of those protein combinations to IAA and 4-Cl-IAA (Fig. 3.17). Changes in TIR1/AFB and Aux/IAA protein abundances, and IAA and 4-Cl-IAA levels at different developmental stages or under different conditions, may determine the composition of TIR1/AFB-Aux/IAA co-receptor complexes and their stabilities. This may eventually lead to IAA- and 4-Cl-IAA-specific degradation of Aux/IAA proteins initiating an auxin-type specific response in the pericarp. The 4-Cl-IAA-dependent regulation of *PsTIR1b* transcript abundance in pea pericarps but not seedling tissues further supports tissue-dependent modulation of auxin receptor composition. Similar auxin-induced regulation of the transcript abundance of putative auxin receptor *PsAFB6* was observed by Nadeau (2009; also see Chapter 4). The expression profiles of the Aux/IAA genes in different plant species also indicate tissue- and developmental stage-dependent modulation of Aux/IAA protein compositions (Kalluri et al., 2007; Song et al., 2009; Audran-Delalande et al., 2012; Singh and Jain, 2015). In the future, analysis of tissue-specific and auxin-type specific regulation of Aux/IAA gene expression in the pea pericarp system will provide information on potential TIR1/Aux/IAA combinations associated with seed

and auxin regulation of pea fruit development. Once the putative TIR1/AFB-Aux/IAA protein combinations are identified, a detailed protein interaction analysis with IAA and 4-Cl-IAA can be used for the validation of their interactions.

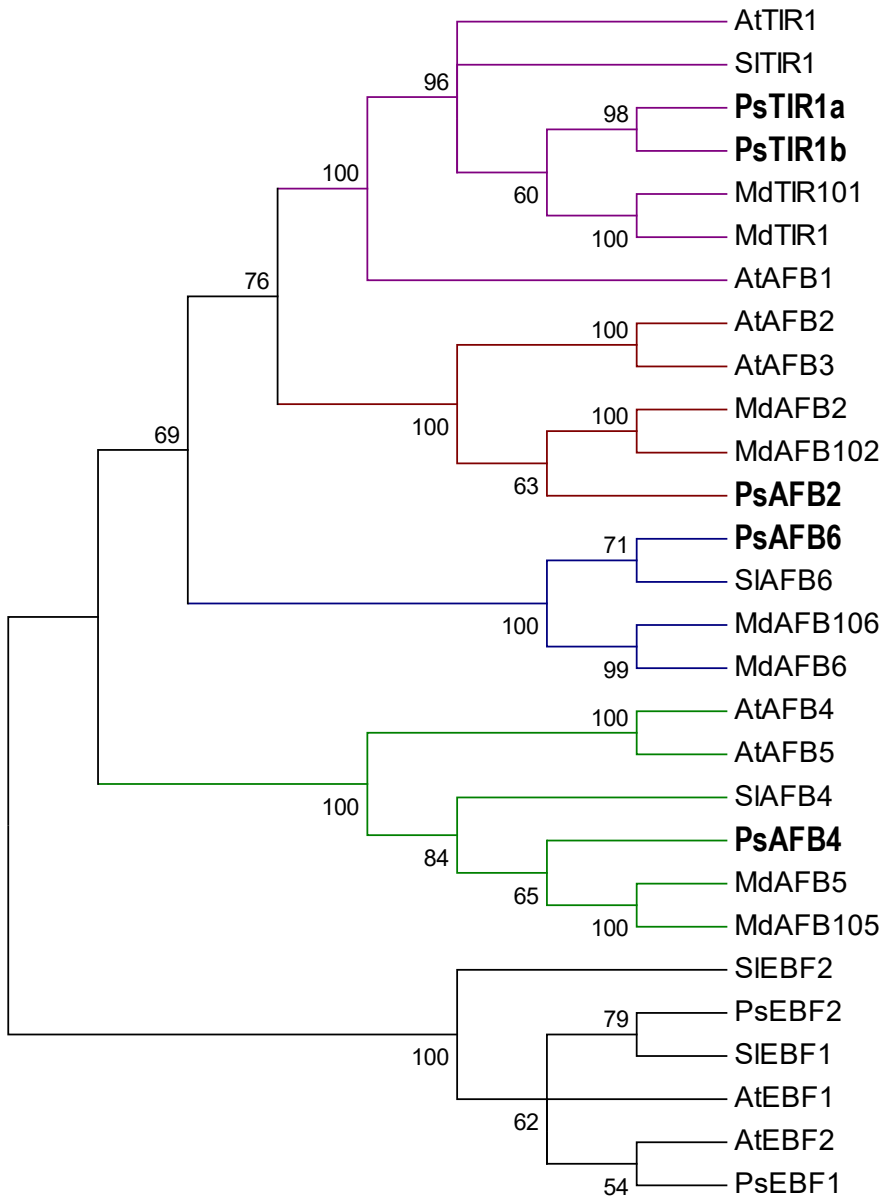


Figure 3.1. A phylogenetic tree showing the association of pea (*P. sativum*) auxin receptor proteins PsTIR1a, PsTIR1b, PsAFB2, PsAFB4, and PsAFB6 with the auxin receptor proteins of *Arabidopsis thaliana* (At), *Malus x domestica* (Md) and *Solanum lycopersicum* (Sl). A neighbor-joining tree was created using the MEGA 7.0 program with 1000 bootstrap replicates and Poisson correction model. All positions with less than 95% site coverage were eliminated. The tree was rooted with EIN3-binding F-box (EBF) protein sequences of selected plant species. Numbers in the branches represent the percentage bootstrap support. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

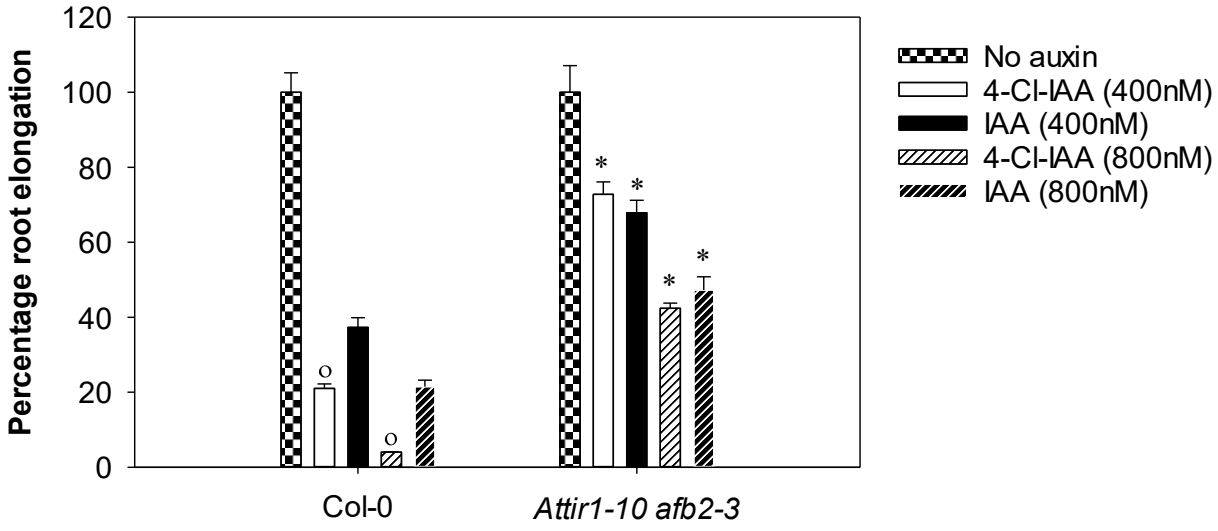


Figure 3.2. The effect of IAA or 4-Cl-IAA on the root elongation of *Arabidopsis* WT (Col-0) and *Attir1-10afb2-3* seedlings. Four-day-old *Arabidopsis* Col-0 and *tir1-10afb2-3* double mutants were transferred to media containing 400 or 800 nM IAA or 4-Cl-IAA and grown for three days prior to measurement of root length. Root elongation of each genotype in auxin is expressed as a percentage compared to the same genotype in medium without auxin. Data are means ± SE (n=8). Comparisons were made using a two-tailed student's T-test.

* Means are significantly different from that of Col-0 $P<0.01$

º 4-Cl-IAA means are significantly different from that of IAA within the same auxin concentration, $P<0.01$

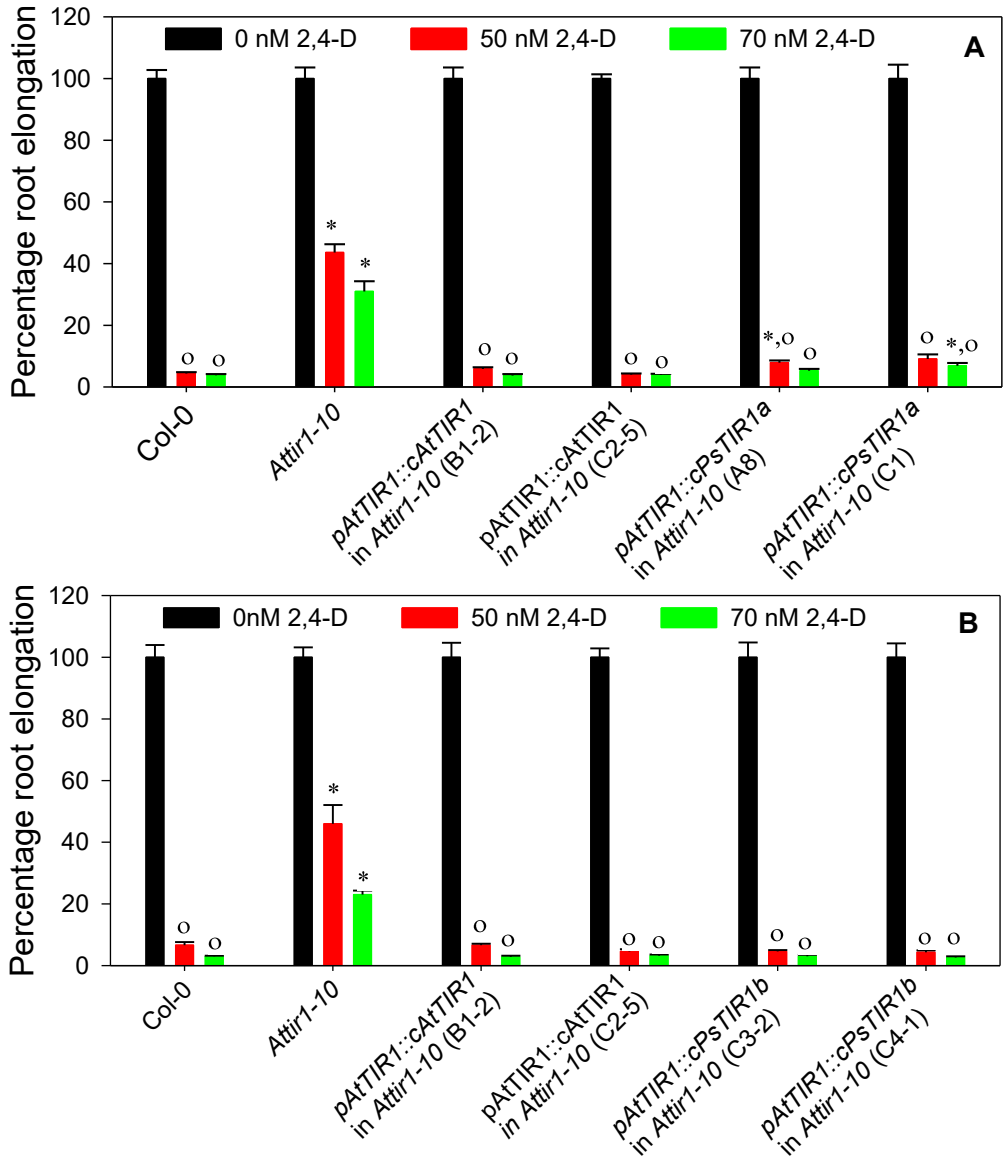


Figure 3.3. Functional characterization of pea PsTIR1a (**A**) and PsTIR1b (**B**) auxin receptors in *Arabidopsis Attir1-10* seedlings. Four-day-old seedlings of *Arabidopsis* WT (Col-0), *tir1-10,* and *tir1-10* expressing *pAtTIR1::cAtTIR1*, *pAtTIR1::cPsTIR1a* (**A**) or *pAtTIR1::cPsTIR1b* (**B**) were transferred to media containing 0, 50 and 70 nM 2, 4-D, and grown for three days prior to measurement of root elongation. Root elongation of each genotype in auxin is expressed as a percentage compared to the same genotype in the media without auxin. Data are means ± SE (n=8). Comparisons were made using a two-tailed student's T-test.

* Means are significantly different from that of WT (Col-0) within respective auxin treatment, P<0.01

º Means are significantly different from that of *Attir1-10* at the same auxin concentration, P<0.01

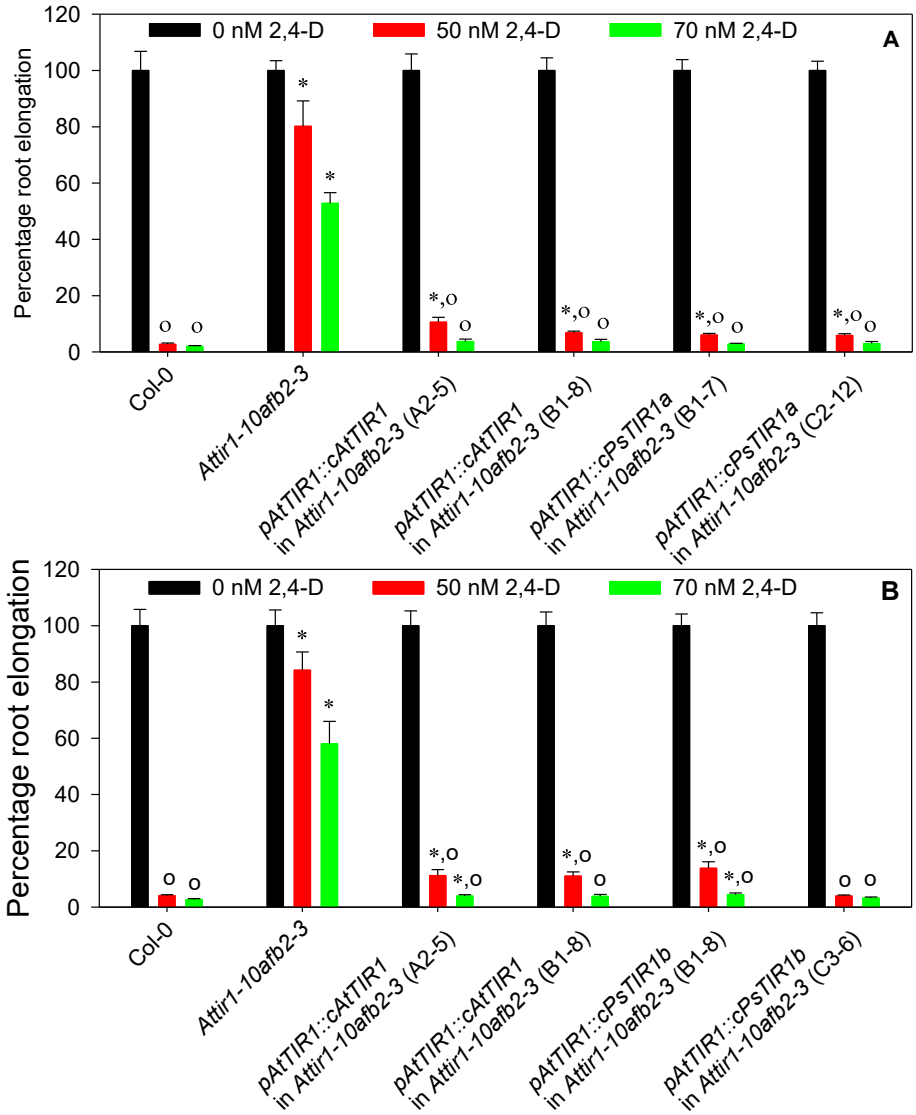


Figure 3.4. Functional characterization of pea PsTIR1a (**A**) and PsTIR1b (**B**) auxin receptors in *Arabidopsis Attir1-10 afb2-3* double mutant seedlings. Four-day-old of *Arabidopsis* WT (Col-0), *tir1-10 afb2-3,* and *tir1-10 afb2-3* expressing *pAtTIR1::cAtTIR1*, *pAtTIR1::cPsTIR1a* (**A**) or *pAtTIR1::cPsTIR1b* (**B**) were transferred to media containing 0, 50 and 70 nM 2, 4-D, and grown for three days prior to measurement of root elongation. Root elongation of each genotype in auxin is expressed as a percentage compared to the same genotype in the media without auxin. Data are means ± SE (n=8). Comparisons were made using a two-tailed student's T-test.

* Means are significantly different from that of WT (Col-0) within respective auxin treatment, P<0.01

º Means are significantly different from that of *Attir1-10 afb2-3* double mutants at same auxin concentration, P<0.01

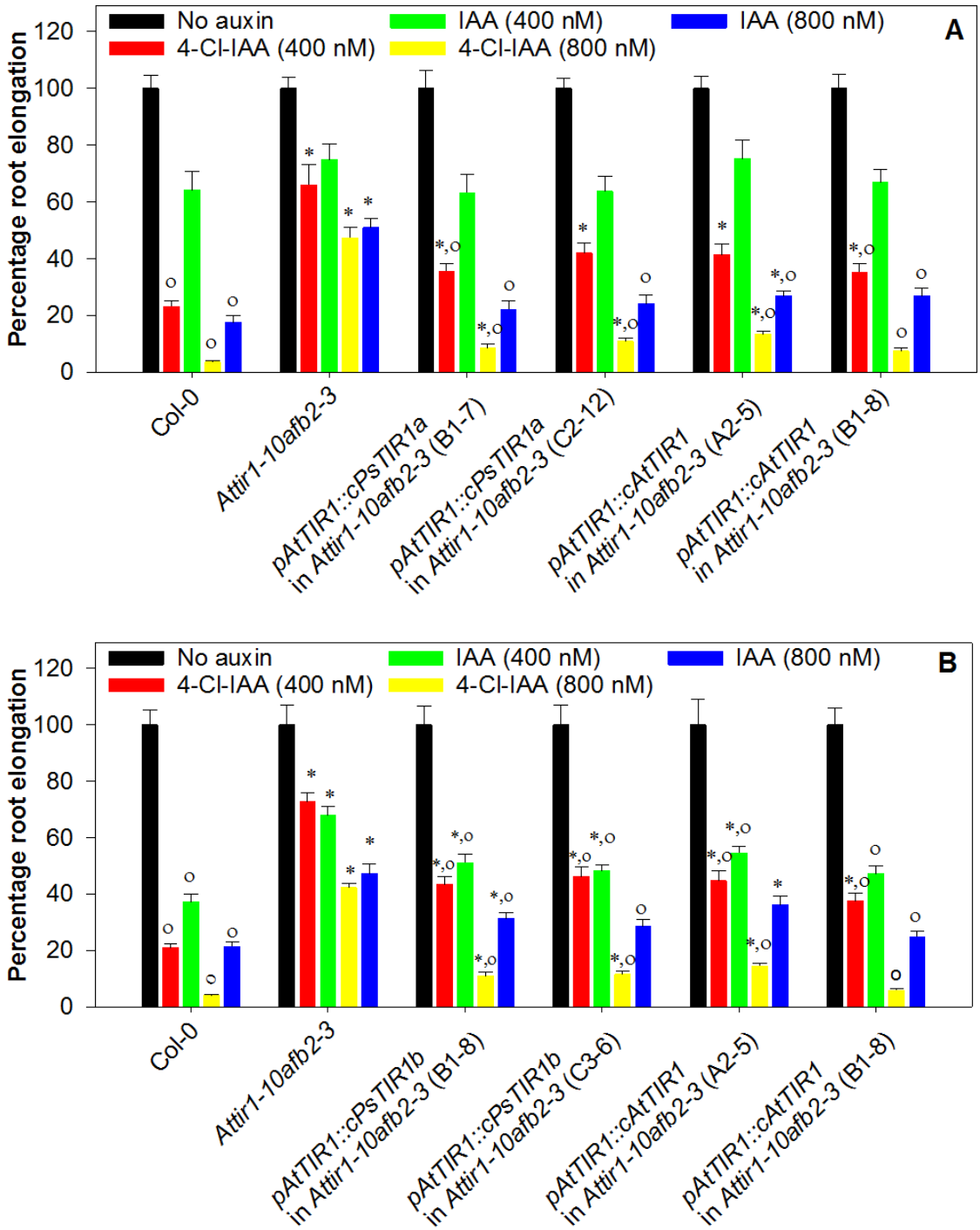


Figure 3.5. IAA and 4-Cl-IAA responsive root elongation of *Arabidopsis Attir1-10 afb2-3* double mutants expressing pea *PsTIR1a* (**A**), *PsTIR1b* (**B**), or *AtTIR1*. Four-day-old Col-0, *tir1-10 afb2-3* and *tir1-10 afb2-3* expressing *pAtTIR1::cAtTIR1*, *pAtTIR1::cPsTIR1a* (**A**) or *pAtTIR1::cPsTIR1b* (**B**) were transferred to media containing 0, 400 and 800 nM IAA or 4-Cl-

IAA and grown for three days prior to measurement of root elongation. Root elongation of each genotype in auxin is expressed as a percentage compared to the same genotype in the media without auxin. Data are means ± SE (n=8). Comparisons were made using a two-tailed student's T-test.

* Means are significantly different from that of WT (Col-0) within respective auxin treatment, $P<0.01$;

º Means are significantly different from that of *Attir1-10 afb2-3* double mutants at same auxin concentration, $P<0.01$

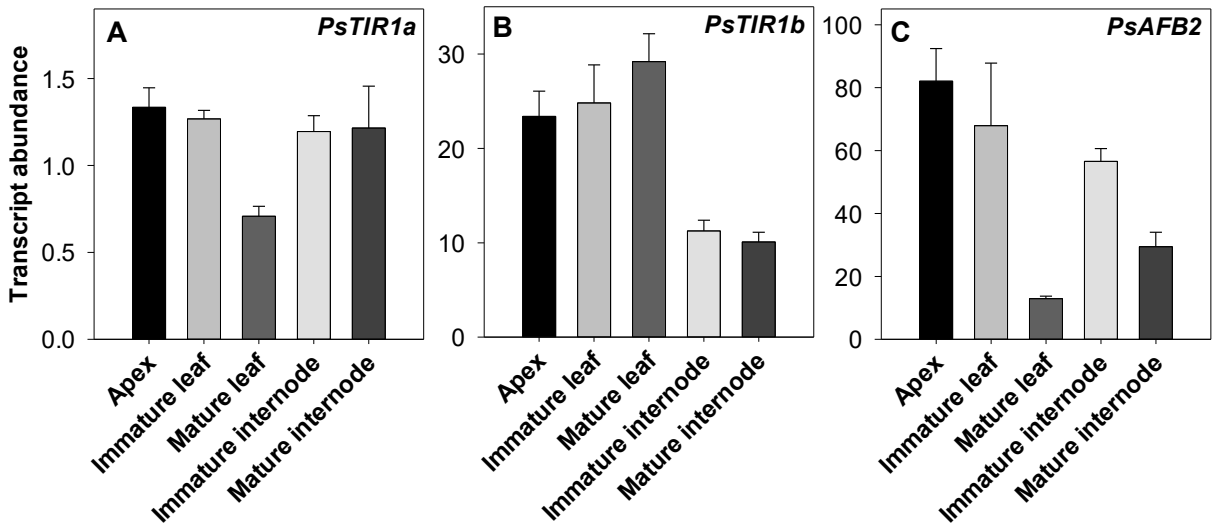


Figure 3.6. Relative transcript abundance of *PsTIR1a* (**A**), *PsTIR1b* (**B**) and *PsAFB2* (**C**) in different tissues of 12-day old pea seedlings. Seedlings were grown under 16 h light (19 ºC) and 8 h dark (17 ºC) photoperiod. Data are means ± SD (n=3).

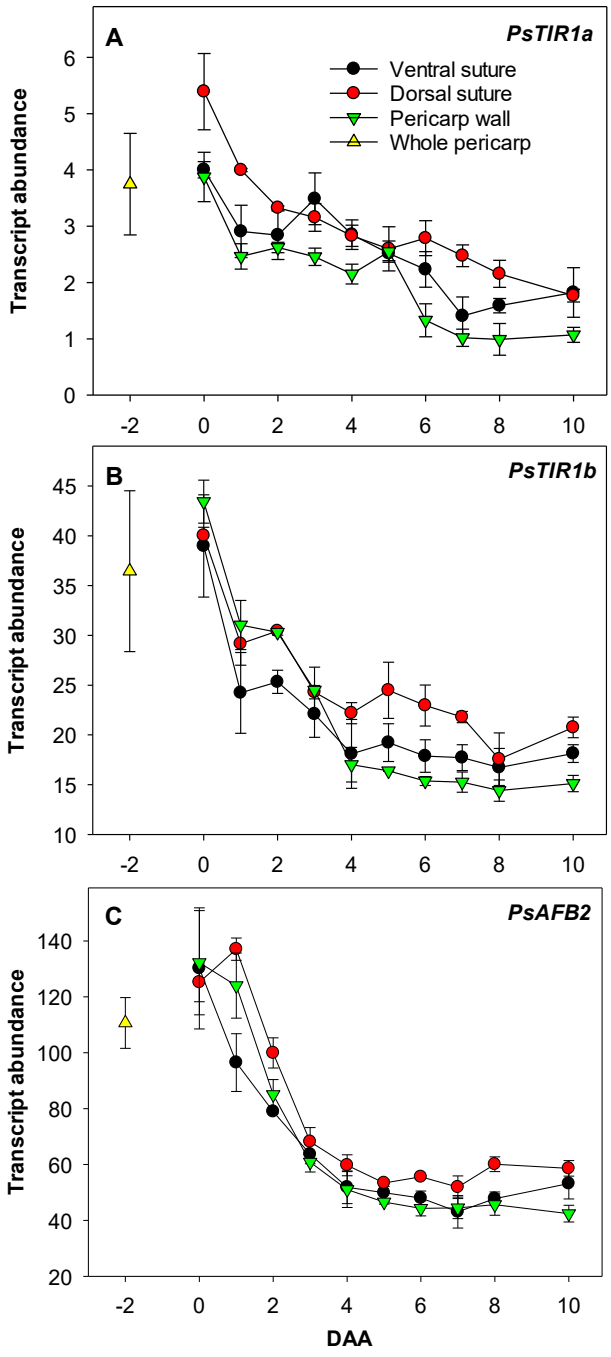


Figure 3.7. Relative transcript abundance of *PsTIR1a* (**A**), *PsTIR1b* (**B**), and *PsAFB2* (**C**) in -2 DAA (days after anthesis) prepollinated whole pericarps, and in the pericarp wall, dorsal vascular suture and ventral vascular suture tissues of pollinated pea fruits from 0 to 10 DAA. Data are means ± SD, n=3 with the exception of 0-3 DAA dorsal suture where n=2. Each sample is a composite of a minimum of three pericarp tissues.

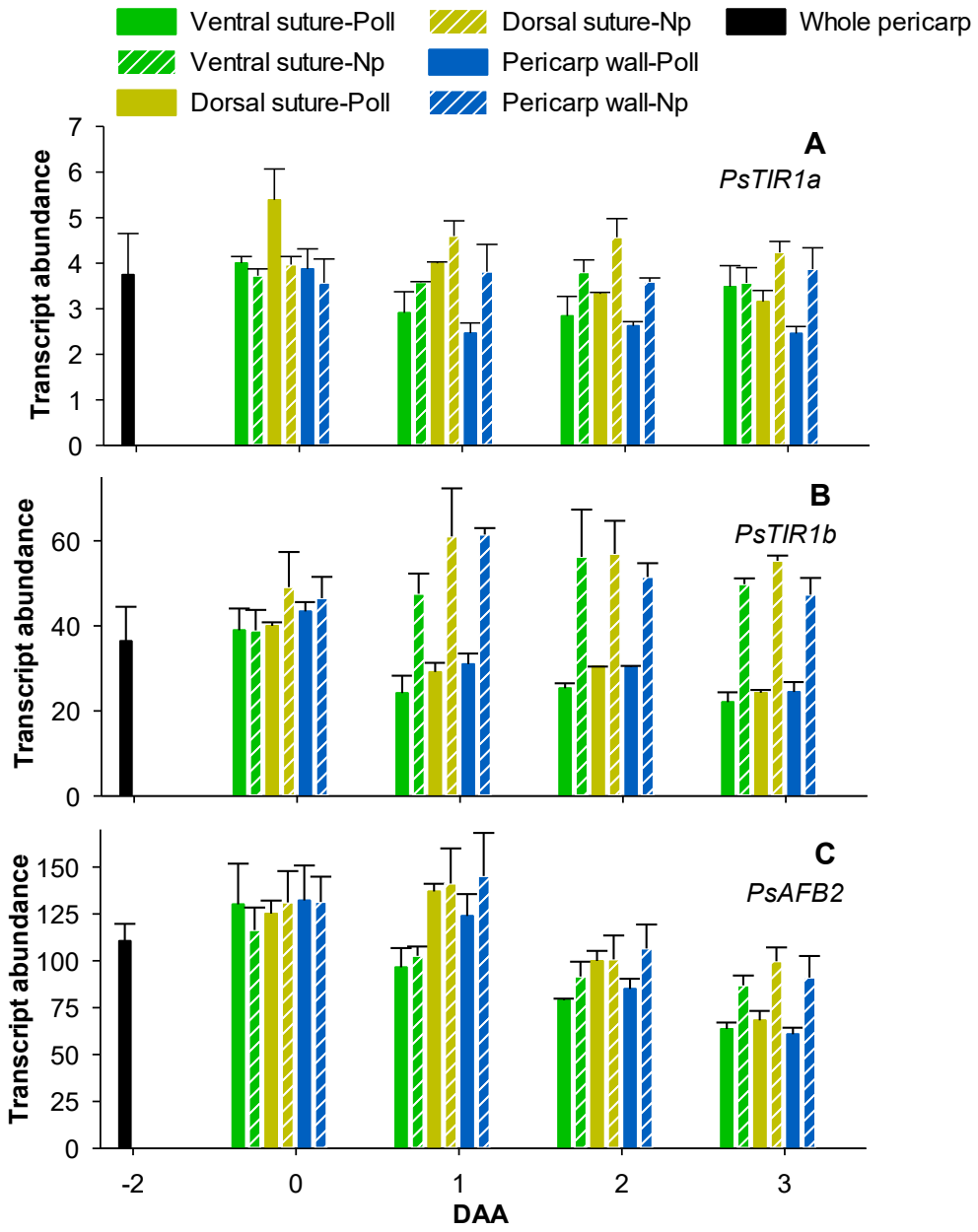


Figure 3.8. Relative transcript abundance of *PsTIR1a* (**A**), *PsTIR1b* (**B**) and *PsAFB2* (**C**) in –2 DAA (days after anthesis) prepollinated whole pericarps and pericarp tissues (ventral vascular suture, dorsal vascular suture, and pericarp wall) of 0 to 3 DAA pollinated (Poll) and non-pollinated (Np) fruits. In non-pollinated fruits, flowers were emasculated at -2 DAA to prevent pollination. Data are means ± SD; n= 3 for whole pericarp and pericarp wall; for ventral and dorsal sutures, the majority of samples were n=3, with n=2 for the remaining samples where the tissues were limited due to small tissue size.

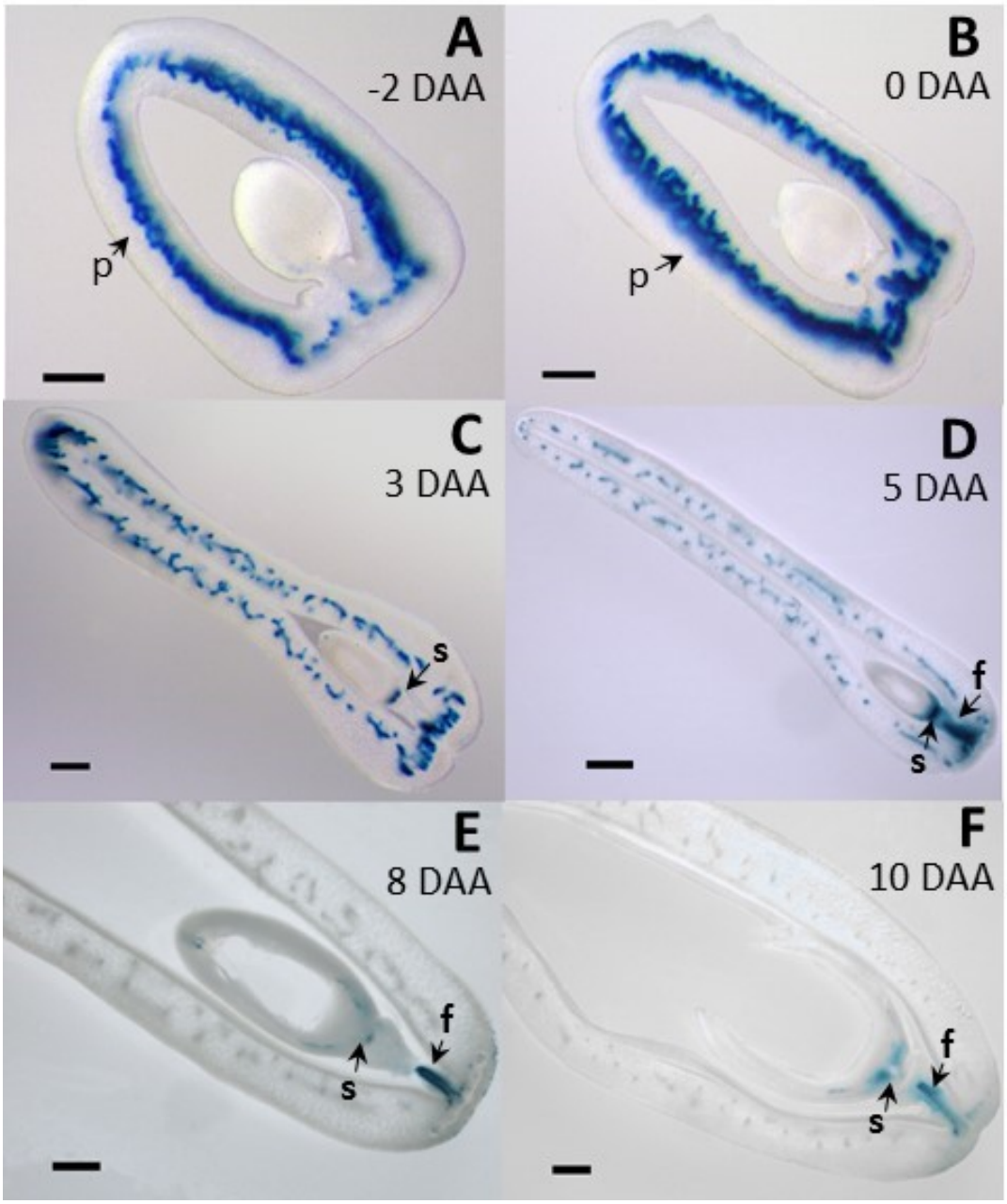


Figure 3.9. Representative micrographs of GUS-stained pea fruit cross-sections from plants expressing *GUS* gene under the regulation of auxin responsive *DR5* promoter (*DR5::GUS*). *DR5::GUS* expression was monitored in -2 DAA (days after anthesis) prepollinated (**A**), 0, 3, 5, 8 and 10 DAA (days after anthesis) pollinated (**B-F**) pea fruits. Arrows indicate the pericarp wall (p), proximal end of the seed (s), and funiculus (f). A, B scale bar = 500 µm; C-F = 1000 µm.

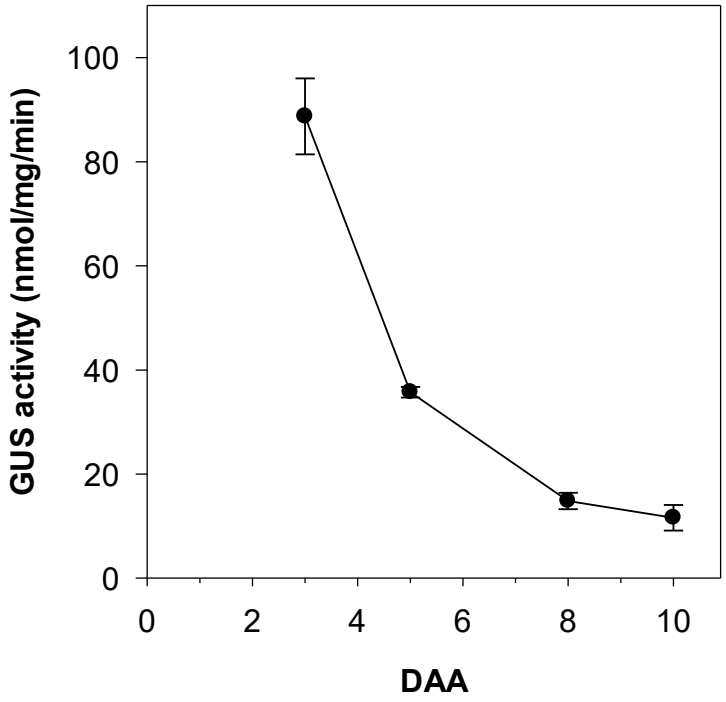


Figure 3.10. GUS (β-glucuronidase) activity detected by MUG (4-methylumbelliferone) assay over the early development of pea fruit in the pericarp walls of plants expressing *GUS* gene under the regulation of *DR5* promoter (*DR5::GUS*). Data are means ± SD, n=3 with each replicate containing two pericarp wall samples. DAA: days after anthesis.

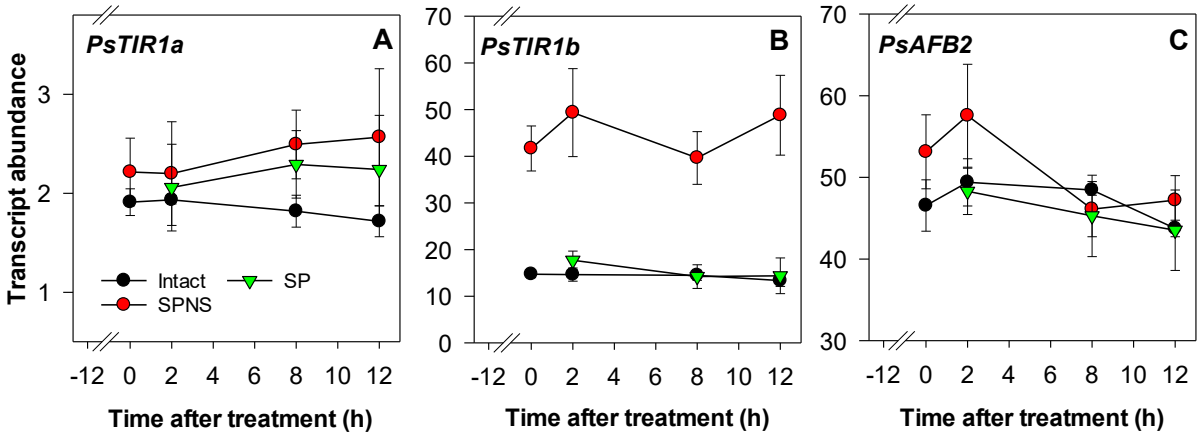


Figure 3.11. Relative transcript abundance of *PsTIR1a* (**A**), *PsTIR1b* (**B**) and *PsAFB2* (**C**) in two days after anthesis pericarps (pods) of pollinated pea ovaries that were intact, split with seeds left intact (split pod; SP) and split with seeds removed (split pod no seeds; SPNS). Splitting of the pods occurred at -12 h, and aqueous Tween 80 (0.1%) as a surfactant control was added at treatment time 0 h. Data are means ± SD, n=3 to 8

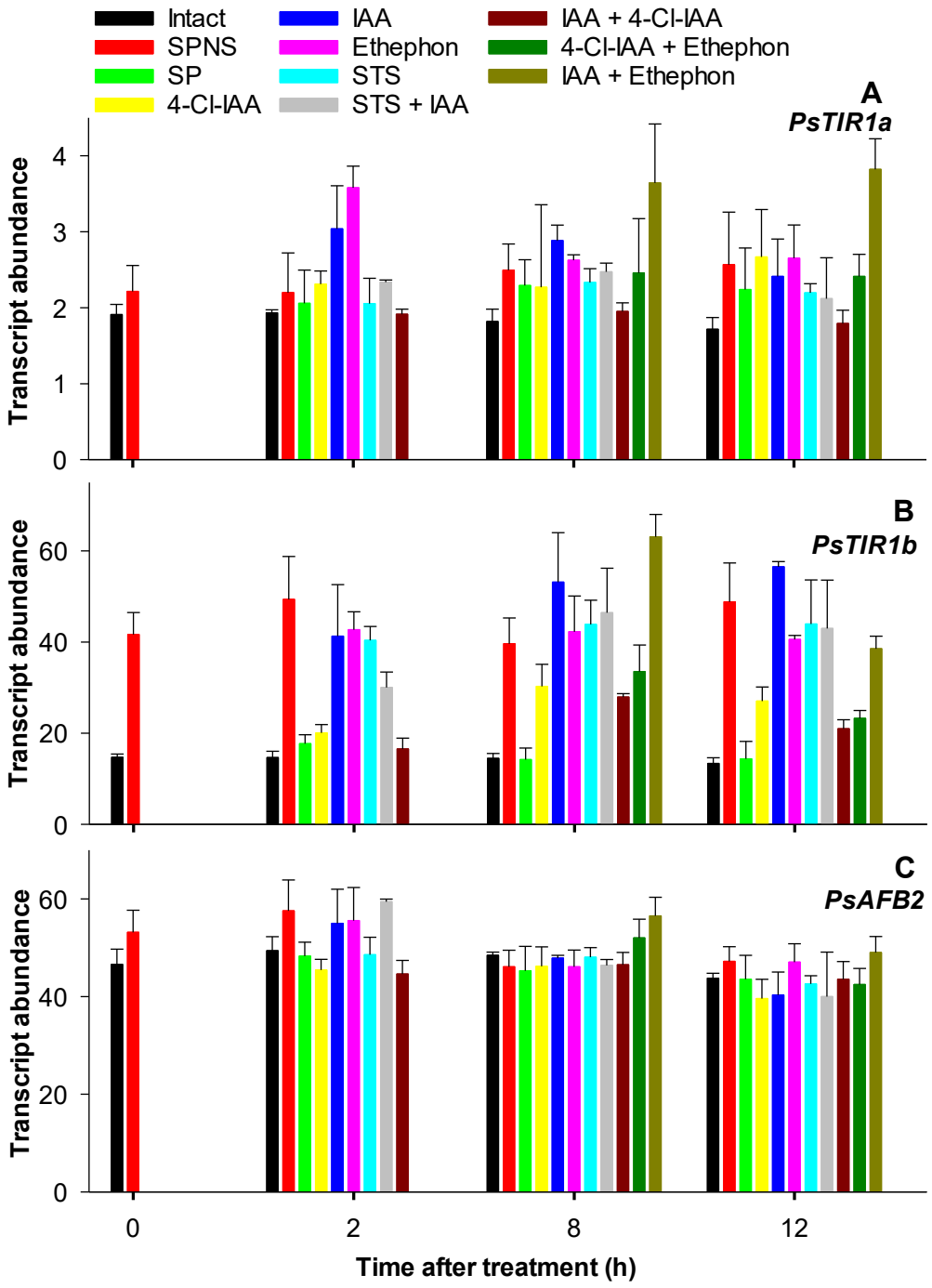


Figure 3.12. Effect of seed removal and hormone treatment on the relative transcript abundance of the ethylene biosynthesis genes *PsTIR1a* (**A**), *PsTIR1b* (**B**), and *PsAFB2* (**C**) in the pericarps of pollinated pea ovaries. Two days after anthesis (DAA) pericarps were either left intact, split (SP), or split and deseeded (SPNS) and treated 12 h after deseeding with 4-Cl-IAA (50 µM),

IAA (50 μM), or ethephon (1000 mg L^{-1}) in 0.1% aqueous Tween 80 (30 μL) alone or in combination. Auxin or auxin-ethephon combinations were IAA plus 4-Cl-IAA, 4-Cl-IAA plus ethephon, or IAA plus ethephon in 0.1% aqueous Tween 80 (30 μL total). When fruits were treated with both auxin and ethephon, ethephon was applied 90 min after the auxin treatment and samples were collected based on the time after auxin treatment. Because of the delayed ethephon application, the auxin plus ethephon-treated pericarps were not studied at the 2 h time-point. Deseeded pericarps were also pretreated with STS (1 mM in 0.1% aqueous Tween 80, 30 μL) at pericarp splitting and deseeding (STS treatment), and IAA (50 μM in 0.1% aqueous Tween 80, 30 μL) was applied to STS pretreated pericarps (IAA plus STS treatment). The SP and SPNS controls were treated with 0.1% aqueous Tween 80 (30 μL). All the samples were collected with respect to the time after hormone treatment. Data are means ± SD, n=3 to 8, with the exception of STS + IAA 2 h treatment, where n=2.

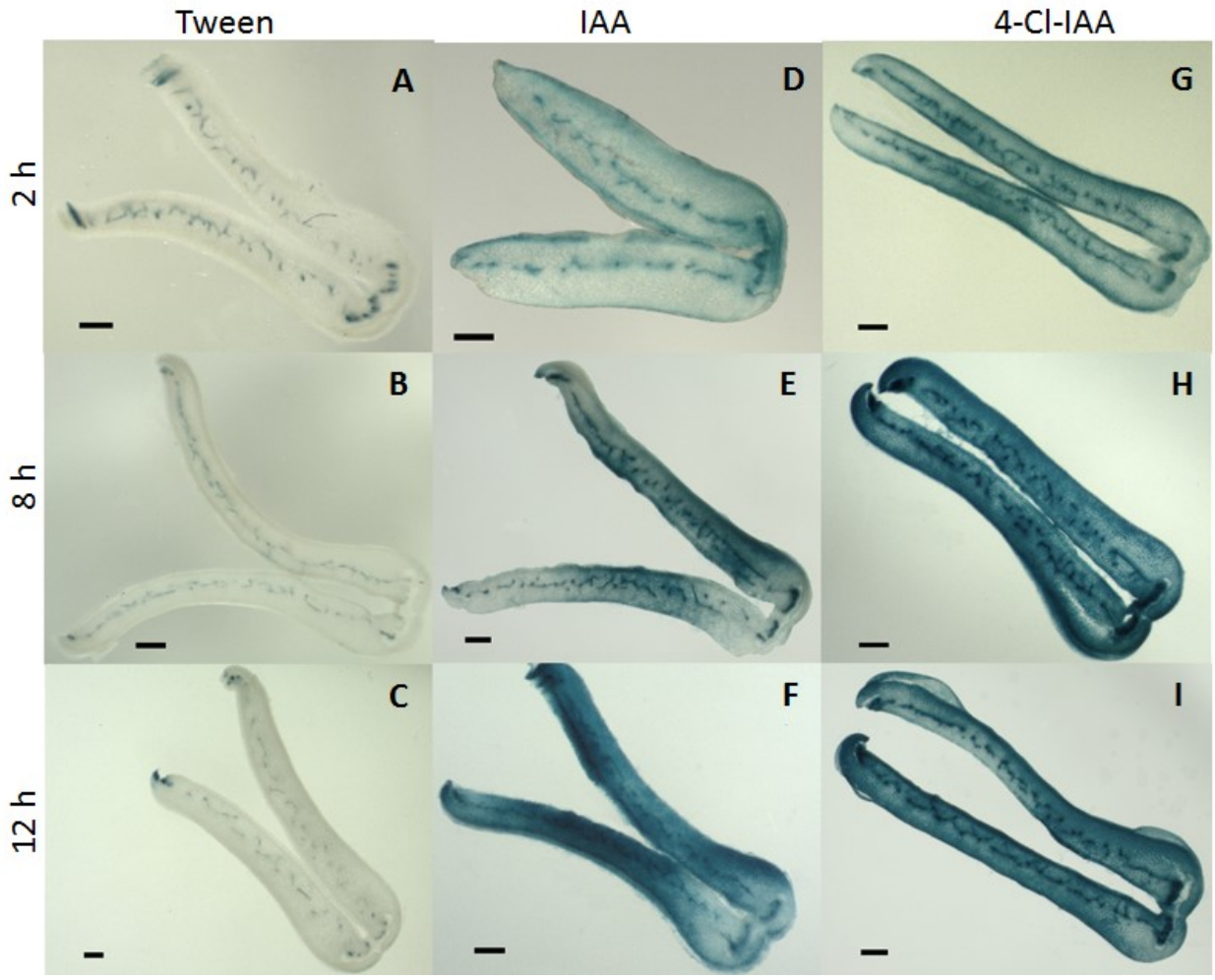


Figure 3.13. GUS (β-glucuronidase) activity in the pericarps of plants expressing *GUS* gene under the regulation of auxin-responsive promoter *DR5* (*DR5::GUS*). Two days after anthesis fruits were split and deseeded (SPNS) and treated with aqueous 0.1% Tween 80 (**A-C**), IAA (50 µM; **D-F**) or 4-Cl-IAA (50 µM; **G-I**). Samples were collected at 2h (**A**, **D** and **G**), 8h (**B**, **E** and **H**) and 12h (**C**, **F** and **I**) after treatment. Scale bars = 500 µm.

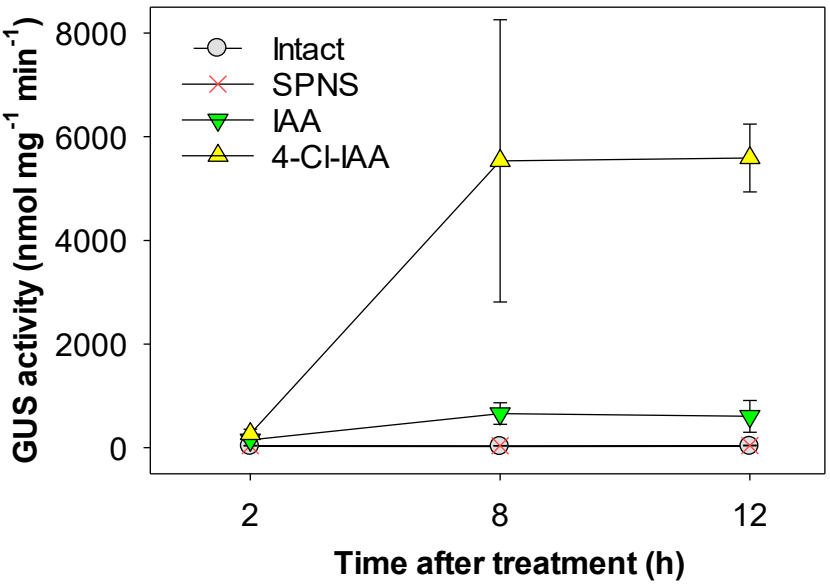


Figure 3.14. Pericarp GUS (β-glucuronidase) activity in response to auxin treatment. In pea plants expressing *GUS* gene under the regulation of auxin-responsive *DR5* promoter (*DR5::GUS*), two days after anthesis pericarps were left intact or split and deseeded (SPNS). SPNS pericarps were treated with 30 µL of IAA or 4-Cl-IAA (50 µM in 0.1% aqueous Tween 80). SPNS controls were treated with 30 µL of 0.1% aqueous Tween 80. Treatments were done 12 h after splitting and deseeding. At 2 h, the GUS activity of intact, SPNS, IAA and 4-Cl-IAA treated fruits were 26.6 ± 3.4, 40.1 ± 6.4, 153.0 ± 21.8 and 267.2 ± 89.6 nmol mg^{-1} min^{-1}, respectively. Data are means ± SD, n=3.

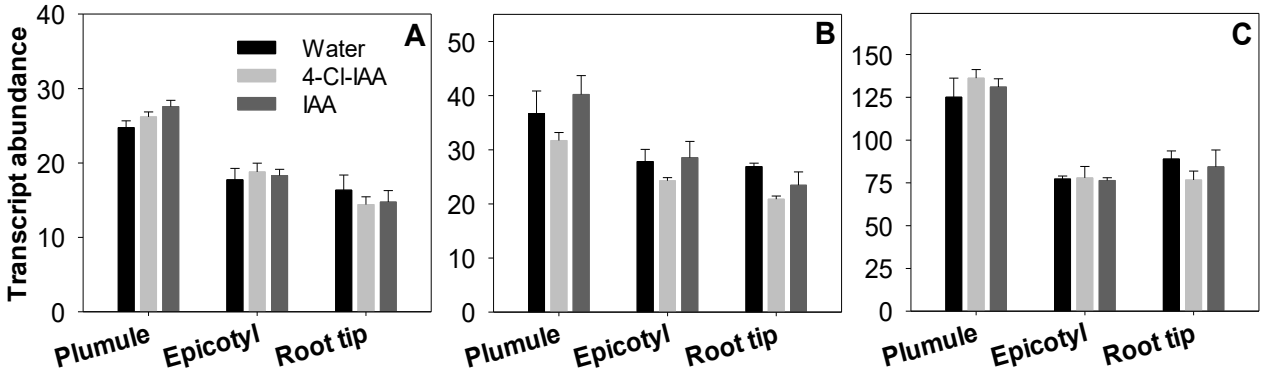


Figure 3.15. Relative transcript abundance of *PsTIR1a* (**A**), *PsTIR1b* (**B**) and *PsAFB2* (**C**) in the plumule, epicotyl and root tip tissues of 4 DAI (days after imbibition) etiolated pea seedlings. Seeds were imbibed in water for two days, then transferred to 4-Cl-IAA (1 µM) or IAA (1 µM) for two more days. Data are means ± SD, n=3

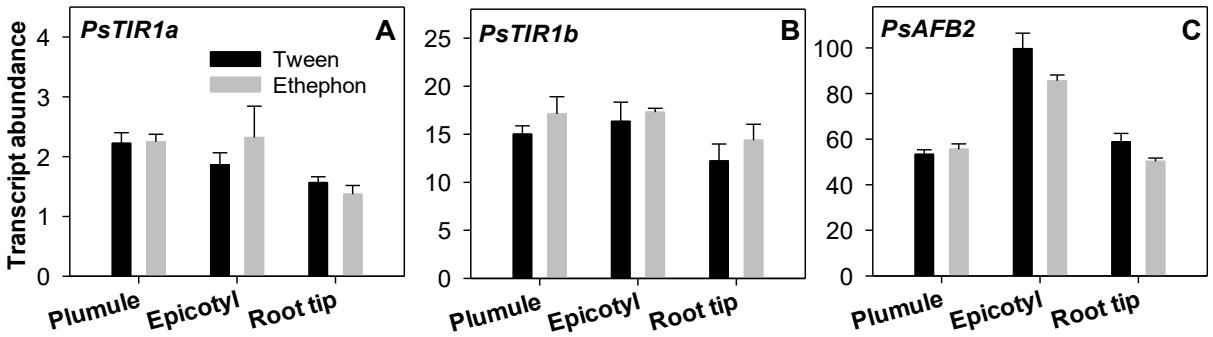


Figure 3.16. Transcript abundance of auxin receptors *PsTIR1a* (**A**), *PsTIR1b* (**B**) and *PsAFB2* (**C**) in plumule, epicotyl and root tip tissues of 4 DAI (days after imbibition) etiolated seedlings grown in the presence of ethephon (15 mg L^{-1} in 0.1% aqueous Tween 80) or aqueous Tween 80. Data are means ± SD, n=3

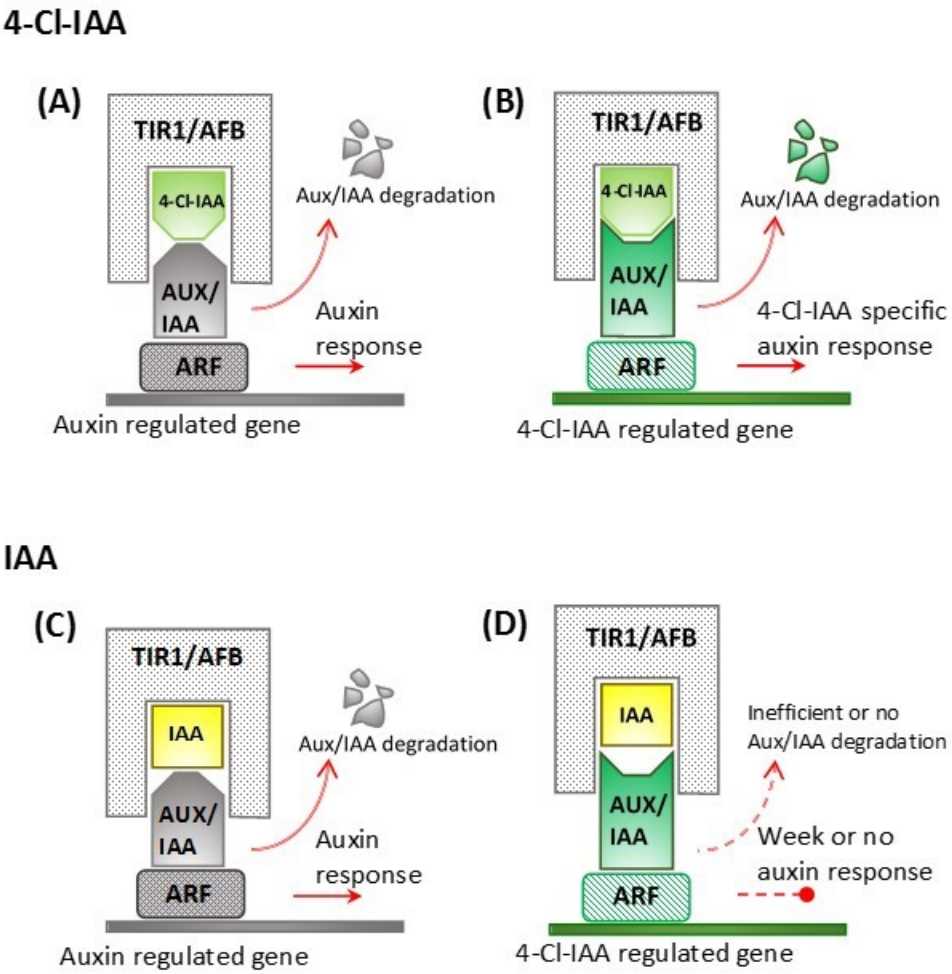


Figure 3.17. A model for the initiation of 4-Cl-IAA-specific response at the level of auxin co-receptors. Auxin act as a 'molecular glue' to bring together TIR1/AFB and Aux/IAA co-receptors (Tan et al., 2007), which initiates the ubiquitination and subsequent degradation of Aux/IAA proteins through 26S proteasome-mediated pathway. Degradation of Aux/IAA proteins releases ARF transcription factors allowing auxin-mediated gene regulation (**A**, **B** and **C**). The formation of a stable co-receptor complex depends on the TIR1/AFB and Aux/IAA protein combinations and the auxin type (Calderón Villalobos et al., 2012). Association of certain TIR1/AFB and Aux/IAA co-receptor combinations only with 4-Cl-IAA may result in 4-Cl-IAA specific gene expression (**C**). Inability to form a similar co-receptor complex or its low stability may cause a weak or no expression of the same gene with IAA (**D**).

Table 3.1. IAA, IAA-asparate (IAA-Asp), and IAA-glutamate (IAA-Glu) levels in pollinated ovaries from 0 to 8 days after anthesis (DAA), 3 DAA non-pollinated ovaries (NP), and 8 DAA seeds of pea (cv I_3, Alaska type; *DR5::GUS* plants) determined using HPLC-MS/MS and heavy-labeled internal standards. [a]

Tissue	(ng gdwt^{-1})			(ng gfwt^{-1})		
	IAA	**IAA-Asp**	**IAA-Glu**	**IAA**	**IAA-Asp**	**IAA-Glu**
Whole pericarp						
0 DAA	292 ± 5[b]	293 ± 14	37 ± 1	61 ± 1	62 ± 3	8 ± 0
3 DAA	168 ± 10	43 ± 16	19 ± 3	25 ± 1	6 ± 2	3 ± 0
3 DAA-NP	38 ± 13	<5[c]	23 ± 6	6 ± 2	<1	4 ± 1
5 DAA	136 ± 1	209 ± 62	13 ± 4	18 ± 0	27 ± 8	2 ± 1
8 DAA	135 ± 11	124 ± 47	8 ± 0	23 ± 2	21 ± 8	1 ± 0
Pericarp wall						
5 DAA	73 ± 12	27 ± 10	5 ± 1	8 ± 1	3 ± 1	1 ± 0
8 DAA	145 ± 24	34 ± 11	4 ± 0	24 ± 4	6 ± 2	1 ± 0
Ventral vascular suture						
5 DAA	774 ± 10	1266 ± 262	40 ± 2	123 ± 2	200 ± 41	6 ± 0
8 DAA	267 ± 30	648 ± 9	22 ± 4	59 ± 7	142 ± 2	5 ± 1
Dorsal vascular suture						
5 DAA	366	359	23	43	43	3
8 DAA	148 ± 7	70 ± 65	20 ± 2	31 ± 1	15 ± 14	4 ± 0
Seed						
8 DAA	32262±700	84497±6280	2704 ± 52	5148±112	13484±1002	431 ± 8

[a] IAA-alanine (IAA-Ala), IAA-leucine (IAA-Leu) and Indole-3-butyric acid (IBA) were also analyzed, but the levels were minute to non-detectable with the exception of IAA-Ala in the seeds of 8 DAA fruits where the average was 47.7 ± 0.4 ng gdwt^{-1}.

[b] Data are means ± SD (n=2 with the exception of 5 DAA Dorsal vascular suture where only a single sample was analyzed). Each replicate contains more than 50 pericarps in early developmental stages to minimum of 10 pericarps at later developmental stages.

CHAPTER 4

DEVELOPMENTAL, SEED AND AUXIN REGULATION OF *PsAFB4* AND *PsAFB6* PUTATIVE AUXIN RECEPTORS IN PEA FRUIT

4.1. INTRODUCTION

The TRANSPORT INHIBITOR RESPONSE 1/ AUXIN SIGNALING F-BOX protein (TIR1/AFB) family of auxin receptors has been extensively studied over the past several years. In *Arabidopsis*, there are six members of TIR1/AFB family receptors; AtTIR1 and AtAFB1 through AtAFB5 (Parry et al., 2009). All of these proteins interact with AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) co-receptors in an auxin-dependent manner indicating their role in auxin perception (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Calderón Villalobos et al., 2012; Prigge et al., 2016). Phylogenetic analysis of auxin receptors divides them into four clades, denoted as TIR1, AFB2, AFB4 and AFB6. Of these groups, AFB6 homologs are proposed to be lost during early evolution in the Brassicaceae and Poaceae families (Parry et al., 2009). Loss of AFB6 homologs in some plant families suggests a non-pivotal role for AFB6 proteins in plant development, possibly due to their functional redundancy with other TIR1/AFB family members. However, the emergence of the AFB6 clade even before the divergence of angiosperm and gymnosperm lineages and their continued existence in some plant families (Parry et al., 2009) suggest that they may be involved in unique or specialized roles.

Studies focused on auxin receptor function has been mainly carried out with the Brassicaceae family member, *Arabidopsis thaliana* as the model species (Parry et al., 2009; Calderón Villalobos et al., 2012; Hu et al., 2012). As *Arabidopsis* does not contain AFB6 homologs, only limited functional characterization of AFB6 genes has been reported. In tomato (*Solanum lycopersicum*), *SlAFB6* has been recognized as a gene of interest during compound leaf development (Ben-Gera et al., 2012). In pull-down assays, which are used to analyze if two proteins interact with each other, the tomato AUX/IAA homolog SlIAA9 (ENTIRE) interacted with SlAFB6 and SlTIR1 in an indole-3-acetic acid (IAA) dependent manner, providing evidence that SlAFB6 acts as a functional auxin receptor (Ben-Gera et al., 2012). The interaction between SlIAA9 and SlAFB6 was stronger than the interaction between SlIAA9 and SlTIR1,

suggesting the possibility of an auxin receptor specificity in SlIAA9 regulation (Ben-Gera et al., 2012).

The tomato SlAFB6 interacting co-receptor SlIAA9 plays a prominent role in tomato fruit development. Antisense-mediated downregulation of *SlIAA9* stimulates fruit set before pollination and produces parthenocarpic fruits (Wang et al., 2005; Wang et al., 2009). To date, it has not been documented that SlAFB6 is also associated with this process. However, a possible involvement of SlAFB6 in tomato fruit development is suggested from transcriptomic data. In the wild tomato species *Solanum pimpinellifolium,* a direct ancestor of *S. lycopersicum,* orthologs of *SlAFB6* and *SlAFB4* were more strongly expressed than the *SlTIR1* ortholog during early fruit development. Furthermore, an ortholog of SlIAA9, which shows strong interactions with SlAFB6, had the highest transcript abundance among AUX/IAA family members in those fruits (Pattison et al., 2015).

The pea (*Pisum sativum* L. cv. I_3 Alaska-type) *PsAFB6* gene was identified in a previous study by Nadeau (2009). In that study, transcript abundance of *PsAFB6* was profiled in pea fruits under several developmental conditions and stages, including -2 to 12 days after anthesis (DAA) whole pericarp and seeds, -1 to 3 DAA emasculated pericarps, and different tissues of 8 to 20 DAA pericarps and seeds. The variation of pericarp *PsAFB6* transcript level in response to seed removal and treatment of deseeded pericarps (split pericarps with no seeds; SPNS) with IAA, 4-chloroindole-3-acetic acid (4-Cl-IAA) or GA_3 has also been studied (Nadeau, 2009). The Nadeau (2009) study showed that the *PsAFB6* transcript level increased in the ovary (pericarp) in the absence of developing seeds (3 DAA emasculated or 2 DAA deseeded pericarps). Application of 4-Cl-IAA inhibited the increase in *PsAFB6* transcripts in the 2 DAA deseeded pericarps. In contrast, IAA-treated deseeded pericarps showed only a transitory reduction at 2 h after treatment while GA_3 treatment had no effect on *PsAFB6* transcript abundance (Nadeau, 2009).

Earlier studies carried out with different *Atafb4* mutant alleles in *Arabidopsis* provided conflicting information regarding its role in plant development. Initially, AFB4 was reported as a negative regulator of auxin action based on the *Atafb4-2* allele, but this report was recently retracted (Greenham et al., 2011; Greenham et al., 2015). In a separate study with the T-DNA insertion mutant *Atafb4-1*, complete loss of AFB4 function was reported to cause a number of developmental defects in *Arabidopsis* (Hu et al., 2012). However, a more recent study carried out with two newer T-DNA insertion mutants of *AtAFB4* (*afb4-8* and *afb4-9*) showed that the lack of

AFB4 function does not cause any visible defects in the plants (Prigge et al., 2016). In *Arabidopsis* root growth assays, the *Atafb4-8* and *Atafb4-9* alleles were slightly resistant to the root elongation inhibitory effect of the synthetic auxin picloram. Furthermore, in pull-down assays, the AtAFB4 protein interacts with AtIAA3 in an IAA dependent manner, indicating that AFB4 can function as an auxin receptor (Prigge et al., 2016). To date, it is not clear if AFB4 plays a role in fruit development, but some studies suggest a possible relationship. A very recent study on *ramosus 2* (*rms2*) mutation in pea suggests that *PsAFB4* is likely the gene associated with this mutation (Ligerot et al., 2016). The *rms2* plants have increased shoot branching, shorter internodes, and reduced shoot and root dry weights (Beveridge et al., 1994). The fruit development of the *rms2* mutants (*rms2-1* and *rms2-2*) was also affected, smaller fruits with a fewer number of seeds were observed in the *rms2* mutants compared with the wild-type (Gélinas-Marion and Ross, 2016). These data on the *rms2* mutant suggest that PsAFB4 may have a role in regulating pea fruit development.

In this study, tissue-specific regulation of *PsAFB4* and *PsAFB6* transcript levels was examined in normally developing and non-pollinated pea ovaries. Seed, IAA, 4-Cl-IAA and ethylene regulation of pericarp *PsAFB4* and *PsAFB6* transcript levels were also examined. Preliminary data on functional characterization of *PsAFB6* in the root elongation assays of *Arabidopsis* auxin receptor mutants are presented, and potential future characterization studies are discussed.

4.2 MATERIALS AND METHODS

4.2.1 Plant material for *PsAFB4* and *PsAFB6* expression analysis in pea

All the studies in pea were done with *P. sativum* L. cv. I_3 (Alaska-type). Plant growth conditions, hormonal treatments, and sample collections have been described in Chapter 3.

4.2.2 RNA extraction and qRT-PCR for gene expression analysis

RNA extraction, qRT-PCR, and subsequent qRT-PCR data analysis were completed as described in Chapter 2. TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied

Biosystems, Part Number: 4309169) was used in all the qRT-PCR analysis. The only exceptions were in the analysis of auxin effect in 4 days after imbibition (DAI) seedling tissues (IAA-, 4-Cl-IAA- and water-grown 4 DAI etiolated seedlings) and in the analysis of *PsAFB6* expression in transgenic *Arabidopsis* plants, where a newer version of One-step qRT-PCR Master Mix (RNA-to-Ct 1-Step Kit, Applied Biosystems, Part Number: 4392938) was used. Reaction setup and qRT-PCR conditions were similar for both kits and are given in Chapter 2. Primers and probes used in qRT-PCR and their reaction efficiencies are given in the Appendix Table D1. All the qRT-PCR probes except that for the 18s rRNA control were designed as double-quenched probes with Iowa Black FQ (IBFQ) quencher at the 3′ end, and ZEN quencher at 9 bp from the 5′ end and 6-FAM fluorescent dye at the 5′ end (Integrated DNA Technologies). The primers and probe for the 18S rRNA control were designed by Ozga et al. (2003) with 5′ VIC as the fluorescent reporter of the probe and 3′ TAMRA as the quencher (Applied Biosystems). The coefficient of variation of the Ct values of the 18S rRNA amplicon across all the samples was less than 3%. Therefore, the target amplicon expression values were not normalized to the 18S signal and data were analyzed using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001; Ozga et al., 2009). The *Arabidopsis* protein phosphatase 2A subunit A3 (*AtPP2AA3*) was used as an internal reference when analyzing the *PsAFB6* expression in transgenic *Arabidopsis* plants and the data analysis was done using the $2^{-\Delta\Delta Ct}$ method as described in Chapter 3.

4.2.3 Cloning and sequencing of *PsAFB4* and *PsAFB6*

The *PsAFB6* coding sequence (CDS) was determined by Nadeau (2009). The putative CDS of *PsAFB4* was identified, and *PsAFB6* was confirmed in a next generation sequencing database of 10 DAA pea seed coats as described in Chapter 2. The full-length CDS of *PsAFB4* and *PsAFB6* (Appendix Figs. D1 and D2) were PCR amplified with RNA extracted from pea tissues of *P. sativum* L. cv. I_3 (Alaska-type). Synthesis of complementary DNA (cDNA) was done using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) with oligo-dT primers as per the manufacturer provided protocol. A pooled cDNA sample of 2 and 6 DAA pericarp wall and 7 DAA seeds were used for the amplification of *PsAFB4*. Complete CDS of *PsAFB6* was amplified with 10 DAA seed coat cDNA. Qiagen LongRange PCR Kit was used for the amplification of *PsAFB6* (Appendix Table B2). Amplification of *PsAFB4* and all the subsequent PCR reactions were done with Phusion Hot Start II polymerase (Thermo Scientific; Appendix

Table A4). The primers used for the amplification of full-length CDS are given in the Appendix Table D2. Cloning of full-length CDS into the pCR8 vector and subsequent sequencing of the insert was done as described in Chapter 3. Primers given in the Appendix Table D3 were used as required for sequencing reactions in addition to those used in the amplification of full-length CDS.

4.2.4 Creation of transgenic *Arabidopsis* plants expressing *PsAFB6*

4.2.4.1 Wild type and mutant lines of Arabidopsis

All the *A. thaliana* lines used in this study were of Columbia (Col-0) ecotype. Wild-type (WT; CS 70000), and *Atafb5-5* (SALK_110643) T-DNA insertion mutant plants were obtained from the Arabidopsis Biological Resource Center (ABRC). Background of the *Atafb5-5* mutant was cleaned once by backcrossing them with Col-0 plants as described in Chapter 3. Mutant homozygosity of background cleaned plants was verified by PCR as described in O'Malley and Ecker (2010) with the primers (LP: GTTGGATCTACCCTCTACCGC, RP: GTGGCAATTGAGTATGATGGG and LBb1.3 T-DNA border primer: ATTTTGCCGATTTCGGAAC) designed using the T-DNA primer designing tool available online through the Salk Institute Genomic Analysis Laboratory (SIGnAL).

4.2.4.2 Isolation of AtAFB5 promoter

DNA was extracted from 10 day-old WT *Arabidopsis* (Col-0) seedlings using the Cetyltrimethylammonium bromide (CTAB)-based method described in Chapter 3. This DNA was used to isolate the *AtAFB5* promoter region about 2 kb in length (-1 bp to -2062 bp region from the start codon) using primers given in Appendix Table D2 (pAtAFB5-sqF1 and pAtAFB5-sqR1).

4.2.4.3 Vector construction and floral dip transformation

Vector construction and floral dip transformation were done as described in Chapter 3. Briefly, the restriction endonuclease sites were incorporated at the ends of *PsAFB6* CDS and the *AtAFB5* promoter by PCR amplifying with primers containing endonuclease restriction

recognition sites (Appendix Table D4). The complete *PsAFB6* CDS was placed downstream of *AtAFB5* promoter (*pAtAFB5::cPsAFB6*) in the modified pCAMBIA1300 (pCM1300-polyA) vector (Appendix Fig. D3). The recombinant plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation, and the construct was subsequently transformed into *Arabidopsis afb5-5* plants by floral-dip transformation. A primer pair which binds to the *AtAFB5* promoter (pAtAFB5-sqF4: TTGATGGAGACAAGAAAGCAGA) and *PsAFB6* CDS (PsAFB6-qRT-RV: TGCTGGCCAGGGTTCATTA) was used for the verification of the presence of the transgene construct in *Arabidopsis*. Homozygous transgenic lines appearing to carry a single insertion were recognized by segregation analysis. Four independent homozygous transgenic lines were selected for final analysis. The relative expression of *PsAFB6* in the selected transgenic lines is given in the Appendix Fig. D4.

4.2.5 *Arabidopsis* root growth assays

The root elongation assays were performed as described in Chapter 3 with the following modifications. Four independent transgenic *Arabidopsis* lines expressing *PsAFB6* (*pAtAFB5::cPsAFB6* in *afb5*-5) were used for the root elongation assay with Col-0 and *Atafb5-5* as controls. Seedlings were grown vertically at 22 ^{o}C under continuous fluorescent light with an average photosynthetic flux density of 70 ±10 µmol m^{-2} s^{-1}. Four-day-old seedlings of about the same size were transferred to growth medium containing specified concentrations of picloram (0, 0.5, 1, 2.5, 5 and 10 µM) and grown under the same growth conditions for three or six more days. Each plate contained two seedlings (considered as technical replicates during analysis) of each transgenic line and the controls. The total number of seedlings per plate was 12. Root tip positions were marked on the day of transfer to new plates and remarked after three or six more days. Plates were photographed as described in Chapter 3 and the root elongation in auxin medium was measured using ImageJ software (Doerner, 2008). Root elongation of each genotype in auxin medium is expressed as a percentage compared to the same genotype in the medium without auxin. Root length differences were compared using two-tailed T-test as described in Chapter 3.

4.3. RESULTS AND DISCUSSION

4.3.1 Transcript abundance of *PsAFB4* and *PsAFB6* is differentially regulated in developing pea fruits.

Full-length coding sequences of *PsAFB4* and *PsAFB6* (Appendix Figs. D1 and D2) were isolated from pea (Chapter 3). The pea AFB4 shares 68% and 70% amino acid identities with *Arabidopsis* auxin receptors AtAFB4 and AtAFB5, respectively. The *Arabidopsis* AtAFB4 and AtAFB5 protein sequences show substitutions in three out of 14 auxin-contacting (H78R, S438A and L439V) and four out of 12 InsP6-contacting (H78R, R401V, M460T and R484Q) amino acids with respect to those positions in AtTIR1 (Tan et al., 2007). The predicted pea AFB4 sequence shared substitutions identical to that of AtAFB4 and AtAFB5 proteins in all those respective positions (Appendix Fig. B9). In the predicted PsAFB6 sequence, the substitutions of auxin-contacting residues were similar to that of AFB4 and AFB5 proteins, but only three substitutions could be seen in the corresponding InsP6-contacting residues (H78R, R401H and M460T; Appendix Fig. B9).

PsAFB4 and *PsAFB6* genes were expressed in the shoot apex, mature internode, immature internode, mature leaf and immature leaf tissues of 12-day old pea seedlings, but at different levels depending on the tissue type or developmental stage. Transcript abundances of *PsAFB4* and *PsAFB6* were highest in the rapidly expanding tissues such as the shoot apex and immature leaves (Fig. 4.1). The lowest transcript abundance was observed in mature leaves, where *PsAFB4* and *PsAFB6* transcript abundances were 5- and 3-fold lower compared to the immature leaves, respectively (Fig. 4.1).

The pericarp transcript abundance pattern of *PsAFB4* was similar to that of *PsTIR1a*, *PsTIR1b* and *PsAFB2* (Fig. 3.7), where levels were higher immediately after pollination and fertilization, then they decreased with fruit development (Fig. 4.2 A). Compared to 0 DAA fruits, *PsAFB4* transcript abundance in the pericarp wall declined by about 3-fold by 8 DAA. In dorsal and ventral vascular suture tissues, *PsAFB4* abundance declined by about 2-fold during the same developmental period (Fig. 4.2 A). A similar developmental pattern was also seen in the seeds where *PsAFB4* transcript abundance declined by about 3-fold from 0 to 8 DAA (Appendix Fig. D5 A).

Pericarp *PsAFB6* transcript abundance differed from that of *PsAFB4*, *PsTIR1a*, *PsTIR1b* and *PsAFB2* during early fruit development (Compare Figs. 3.7 and 4.2 A with 4.2 B). *PsAFB6* transcript abundance was higher in pericarp tissues before pollination (-2 DAA) and lower following ovary pollination and fertilization, similar to that reported by Nadeau (2009). These results suggest that the presence of developing seeds, a presumed source for pericarp auxin, down-regulates *PsAFB6* expression in the pericarp. Another reduction in *PsAFB6* transcript abundance was observed in the pericarp tissues from approximately 5 to 7 DAA (Fig. 4.2 B) when the pericarp initiates expansion in diameter to accommodate the rapidly expanding seeds (Ozga et al., 2003). From 8 to 10 DAA, *PsAFB6* transcript levels increased (Fig. 4.2 B), when the pericarp growth rate in length drops to very low levels (Ozga et al., 2003). The modulation of *PsAFB6* transcript abundance and the parallel developing phase of the pea pericarp suggest an inverse relationship between *PsAFB6* transcript abundance and the growth rate of the pericarp tissues. No change of *PsAFB6* transcript abundance could be seen in the seeds of 0 to 10 DAA fruits (Appendix Fig. D5 B).

4.3.2 Developing seeds modulate *PsAFB4* and *PsAFB6* transcript abundance in the pericarp

In pea fruits, seeds are believed to act as a source of pericarp auxin. Seed derived 4-Cl-IAA is in particular considered to be a major inducer of pericarp development (Ozga and Reinecke, 2003; Ozga et al., 2009). The absence of pollination or removal of developing seeds from young developing fruits slows pericarp growth and stimulates pericarp senescence (Ozga et al., 1992; Ozga and Reinecke, 1999). To understand if the seed regulation of pericarp growth involves the modulation of *PsAFB4* transcript levels and to confirm the previous expression analysis of *PsAFB6* (Nadeau, 2009), their transcript abundances were profiled in non-pollinated ovaries and in 2 DAA deseeded pericarps.

Pollination status had only minor effects on pericarp *PsAFB4* transcript abundance (Fig. 4.3 A). Relatively similar transcript abundance of *PsAFB4* could be detected in the pericarp wall, dorsal vascular suture and the ventral vascular suture tissues from -2 to 3 DAA in pollinated or non-pollinated fruits (Fig. 4.3 A, Appendix Table D5). *PsAFB4* transcript abundance in the seeds/ovules was similar to that of pericarp tissues, and it was slightly elevated in the absence of pollination (Appendix Table D5). In contrast, *PsAFB6* transcript abundance increased in the

pericarp wall, dorsal vascular suture, and ventral vascular suture tissues of the non-pollinated compared to pollinated fruits (Fig. 4.3 B, Appendix Table D5). By 3 DAA, *PsAFB6* transcript level in non-pollinated fruits was about 3-fold higher compared to that of pollinated fruits (Fig. 4.3 B). Fertilization of the ovule did not affect *PsAFB6* transcript abundance (Appendix Table D5). Similar *PsAFB6* transcript abundance patterns were observed by Nadeau (2009) in pea pericarps in the absence of pollination.

The effect of seed removal on pericarp *PsAFB4* and *PsAFB6* transcript levels was analyzed by comparing the transcript abundance in intact, split (SP; where pericarp wall was split along the dorsal vascular suture but seeds remained intact) and SPNS pericarps. Even though the *PsAFB4* transcript abundance remained relatively similar in both pollinated and non-pollinated pericarp tissues (Fig. 4.3 A), seed removal increased *PsAFB4* transcript abundance by about 2-fold (SPNS pericarps; Fig. 4.4 A). In SP pericarps, where seeds remained intact, *PsAFB4* transcript abundance was minimally affected, indicating that seed removal, but not the pericarp splitting increases *PsAFB4* transcript level (Fig. 4.4 A). As reported by Nadeau (2009), pericarp *PsAFB6* transcript abundance also increased in SPNS fruits. During the 12 h period of study (i.e. 12 to 24 h after pericarp splitting and seed removal), *PsAFB6* transcript abundance increased by more than 3-fold in SPNS pericarps compared to that of intact fruits (Fig. 4.4 B). Intact and SP pericarps had relatively similar *PsAFB6* transcript levels (Fig. 4.4 B). These observations suggest that *PsAFB4* and *PsAFB6* transcript levels are regulated in different manners in response to fruit developmental status. Transcript abundance of *PsAFB6* is clearly modulated by both pollination status and manual seed removal from developing fruits and hence similar to regulatory patterns observed with *PsTIR1b* (Chapter 3). Regulation of *PsAFB4* transcript abundance is different from all the other auxin receptors studied (*PsTIR1a*, *PsTIR1b*, *PsAFB2* and *PsAFB6*; Chapter 3) as pericarp *PsAFB4* transcript level increased in response to manual seed removal from pollinated fruits but not in the absence of pollination.

4.3.3 Ethylene and 4-Cl-IAA inversely regulate pericarp *PsAFB6* transcript abundance but neither affect *PsAFB4* transcript abundance

If seeds act as the source of pericarp auxins, the increase of pericarp *PsAFB4* and *PsAFB6* transcript abundance observed in deseeded fruits could be due to the reduced levels of pericarp auxins. Previous observation that 4-Cl-IAA can downregulate *PsTIR1b* (Chapter 3) and *PsAFB6*

(Nadeau, 2009) transcript abundance further support this hypothesis. It is also possible that a senescence or wounding-induced stimulus such as ethylene upregulates the transcript abundance of *PsAFB4* and *PsAFB6*. The ability of 4-Cl-IAA but not IAA to inhibit ethylene response in the pericarp (Johnstone et al., 2005) should result in the suppression of ethylene-induced gene expression changes in 4-Cl-IAA-treated pericarps. The ability of ethylene to regulate pericarp *PsAFB4* and *PsAFB6* transcript was then assessed in deseeded pericarps and deseeded pericarps treated with IAA, 4-Cl-IAA, the ethylene releasing agent ethephon, the ethylene signaling inhibitor silver thiosulfate (STS), or selected treatment combinations.

Application of 4-Cl-IAA, IAA, ethephon, STS or combinations of those treatments had minimal or no effects on the pericarp *PsAFB4* transcript abundance compared to SPNS controls (Fig. 4.5 A). In IAA and ethephon treated pericarps, *PsAFB4* level was relatively similar to SPNS controls (Fig. 4.5 A). This stability of *PsAFB4* transcript abundance in response to IAA and ethylene is consistent with the relatively stable level of *PsAFB4* in non-pollinated pericarps which contain a low level of IAA (Table 3.1) and evolve an increased level of ethylene (Orzáez et al., 1999). In 4-Cl-IAA or STS treated pericarps, *PsAFB4* level was slightly lower compared to the SPNS control, but none of the treatments completely suppressed the increase in *PsAFB4* transcript abundance due to deseeding (Fig. 4.5 A). The relatively minor changes in pericarp *PsAFB4* transcript abundance in response to the applied auxins, ethylene, or STS treatments suggests that the changes in pericarp ethylene or auxin levels caused by seed removal are not the primary factors responsible for higher *PsAFB4* transcript abundance in SPNS pericarps.

Application of 4-Cl-IAA suppressed *PsAFB6* transcript abundance in deseeded pericarps as reported previously by Nadeau (2009). In 4-Cl-IAA-treated deseeded pericarps, *PsAFB6* transcript abundance was similar to that of intact and SP pericarps, indicating that 4-Cl-IAA can completely suppress the accumulation of *PsAFB6* transcripts in deseeded pericarps (Fig. 4.5 B). In contrast to 4-Cl-IAA, application of IAA had only a transitory effect on *PsAFB6* transcript abundance. When analyzed 2 h after the IAA treatment, pericarp *PsAFB6* transcript abundance decreased by about 3-fold compared to the SPNS control. However, IAA-treated pericarps had similar *PsAFB6* transcripts levels to that of the deseeded control when analyzed at 8 and 12 h after the IAA treatment (Fig. 4.5 B). This effect of IAA on deseeded pericarp *PsAFB6* transcript abundance is again similar to that observed by Nadeau (2009). The effect of ethylene on *PsAFB6* transcript abundance was contrary to that of auxin. No clear change in pericarp *PsAFB6*

transcript level could be seen when analyzed at 2 h after the treatment with the ethylene releasing agent ethephon. However, a gradual increase in *PsAFB6* transcript abundance could be detected in ethephon-treated pericarps, which reached about 2-fold higher levels compared to SPNS controls by 12 h after the treatment (Fig. 4.5 B).

Pericarps treated with both IAA and ethephon had increased *PsAFB6* transcript abundance at levels similar to those resulting from ethephon treatment alone (Fig. 4.5 B). In contrast, the pericarps treated with both 4-Cl-IAA and ethephon had reduced levels of *PsAFB6* transcripts at levels similar to those treated with 4-Cl-IAA alone (Fig. 4.5 B). This differential regulation of ethylene-induced *PsAFB6* transcript abundance by IAA and 4-Cl-IAA is consistent with the previous observations that 4-Cl-IAA but not IAA attenuates the pericarp ethylene action (Johnstone et al., 2005).

To further verify ethylene stimulation of pericarp *PsAFB6* transcript abundance, the effect of ethephon on pericarp *PsAFB6* transcript abundance was determined in deseeded pericarps with and without pretreatment with the ethylene action inhibitor STS. As previously observed, treatment with ethephon induced *PsAFB6* transcript abundance compared to the SPNS controls. Pretreatment with STS reduced the ethylene stimulation of *PsAFB6* transcript abundance (Fig. 4.6). These observations further indicate that ethylene has a direct effect in modulating the pericarp *PsAFB6* transcript abundance. Altogether, these data and those of Nadeau (2009) suggest that developing seeds and 4-Cl-IAA suppress, and ethylene induces *PsAFB6* expression in the pericarp of young developing ovaries of pea. 4-Cl-IAA, but not IAA, inhibits the ethylene-induced accumulation of pericarp *PsAFB6* transcript (Fig. 4.7).

4.3.4 Auxin and ethylene regulation of auxin receptor transcript abundance in seedling tissues

In pea, 4-Cl-IAA induces deseeded pea pericarp development while IAA does not (Reinecke et al., 1995; Reinecke et al., 1999). This dramatic differential effect of IAA and 4-Cl-IAA on growth appears to be specific to the ovary tissue. In vegetative tissues, IAA and 4-Cl-IAA act similarly with respect to tissues growth, primarily showing differences in the strength of their action, with 4-Cl-IAA being stronger compared to IAA at the same concentration (cited from Reinecke et al., 1995; Chapter 3). Transcript abundances of *PsAFB4* and *PsAFB6* were studied in different tissues at four days after imbibition (DAI) etiolated seedlings grown in the

presence of IAA, 4-Cl-IAA (1 μM) or in water for two days to see if the two auxins differentially modulate auxin receptor transcript abundance. The effect of ethylene was also studied in 4 DAI etiolated seedlings grown continuously in the presence of ethephon (15 mg L^{-1}).

Relatively similar levels of *PsAFB4* and *PsAFB6* transcripts could be detected in plumule, epicotyl and root tip tissues of the 4 DAI water-grown seedlings (Fig. 4.8). Growing the seedlings in IAA or 4-Cl-IAA inhibited root elongation. The inhibitory effect of 4-Cl-IAA on root elongation was slightly stronger compared to that of IAA ($P<0.01$, students t-test; Appendix Fig. A12 A). The transcript levels of *PsAFB4* or *PsAFB6* were minimally or not affected by exogenous IAA or 4-Cl-IAA in any of the seedling tissues (Fig. 4.8).

In seedlings grown in the presence of ethephon, no clear change in *PsAFB4* transcript abundance could be seen in the root tip and plumule tissues compared to control. However, *PsAFB4* transcript abundance increased about 2-fold in the epicotyl, which indicates that ethylene may regulate *PsAFB4* transcript level in a tissue-dependent manner (Fig. 4.9 A). In contrast to *PsAFB4*, a slight decrease of *PsAFB6* transcripts was observed in all the tissues of ethephon-grown seedlings compared to the controls (Fig. 4.9 B), indicating an ethylene-mediated inhibition of *PsAFB6* transcript in seedling tissues, as opposed to an enhancing effect.

In the pericarp where 4-Cl-IAA stimulates growth, it suppresses *PsAFB6* transcript abundance. In seedling roots where 4-Cl-IAA inhibits growth, it has a minimal effect on *PsAFB6* transcript abundance. These data suggest that the effect of 4-Cl-IAA on *PsAFB6* transcript abundance is tissue specific. The other naturally occurring auxin in pea, IAA, does not modulate *PsAFB6* transcript abundance in the pericarps or the seedlings. The effect of ethylene on auxin receptor transcript abundance also appears to be tissue-dependent. In deseeded pericarps, ethylene stimulates *PsAFB6* transcript abundance and it has no effect on *PsAFB4* transcript abundance. In seedlings, ethylene stimulates epicotyl *PsAFB4* and slightly suppresses *PsAFB6* transcript levels.

4.3.5 Pea AFB6 does not restore picloram sensitivity in *Arabidopsis afb5-5* plants

Pea auxin receptors can be functionally characterized within pea plants by creating *TIR1/AFB* gene mutants using a technique such as clustered regularly interspaced palindromic repeats/CRISPR-associated 9 (CRISPER/Cas9). Functional characterization of the genes can also be done by overexpressing the genes of interest or creating RNA interference (RNAi) lines to

reduce their expression in pea. However, creation of mutants or obtaining overexpression or RNAi lines in pea is a lengthy process. As an alternative approach, characterization of these genes can be attempted in *Arabidopsis* auxin receptor mutants. However, the absence of AFB6 clade of auxin receptors in *Arabidopsis* requires the transformation of *PsAFB6* into a different auxin receptor mutant. There is no obvious choice of a mutant which is ideal for the functional characterization of PsAFB6 in *Arabidopsis*. Compared to other *TIR1/AFB* mutants in *Arabidopsis,* the *Attir1-10* and *Atafb5-5* mutants show discrete root elongation phenotypes in the presence of auxins 2,4-D (2,4-Dichlorophenoxyacetic acid) and picloram (4-amino-3,5,6-trichloropicolinicacid), respectively (Parry et al., 2009; Prigge et al., 2016). Therefore, it is possible to study whether *PsAFB6* restores the auxin sensitivities of the *Attir1-10* and *Atafb5-5* mutants or not. In addition, the higher order mutants such as *Attir1-10 afb2-3* or *Atafb4-8 afb5-5*, which show stronger auxin resistant root growth phenotypes compared to that of the single mutants can also be used (Parry et al., 2009; Prigge et al., 2016).

In *Arabidopsis*, AtAFB4 belongs to the AFB4 clade of auxin receptors which includes one additional member, AtAFB5 (Parry et al., 2009). Members of the AFB4 clade are important in the perception of synthetic auxin picloram (Walsh et al., 2006; Greenham et al., 2011; Greenham et al., 2015). As discussed above, the pea PsAFB6 and *Arabidopsis* AtAFB4 and AtAFB5 contain identical amino acid substitutions in auxin interacting residues when compared with AtTIR1 (Appendix Fig. B9). Therefore, it is possible that PsAFB6 may function in a similar manner to that of *Arabidopsis* AFB4 clade auxin receptors. The ability of PsAFB6 to function as an auxin receptor in the presence of picloram was evaluated by expressing *PsAFB6* in *Arabidopsis Atafb5-5* plants under the regulation of *Arabidopsis AtAFB5* promoter (*pAtAFB5::cPsAFB6*; Appendix Fig. D3). The *Arabidopsis afb5* mutants and the *AtAFB5* promoter is preferable over that of AtAFB4 in a complementation study due to the stronger promoter function of *AtAFB5* and the stronger picloram resistant phenotype of *afb5-5* plants compared to *AtAFB4* promoter and *afb4* mutants, respectively (Prigge et al., 2016). The *Atafb5-5* allele, selected as the mutant background for transformation, contains a T-DNA insertion in the first intron and as a result does not produce full-length *AtAFB5* mRNA (Prigge et al., 2016).

Four independent transgenic lines containing *pAtAFB5::cPsAFB6* in *Atafb5-5* background were selected for phenotypic analysis. Picloram sensitivity of the transgenic plants was analyzed by growing them in 0.5, 1, 2.5, 5 and 10 μM picloram. After four days in picloram, root

elongation of Col-0 seedlings was clearly inhibited by 2.5 µM and higher concentrations of picloram compared to that of *Atafb5-5* seedlings. However, transgenic seedlings expressing *PsAFB6* had elongated roots similar to that of *Atafb5-5* seedlings, indicating that *PsAFB6* does not restore picloram sensitivity in *Arabidopsis* root elongation assays (Fig. 4.10 A). A repeated root elongation assay at 5 µM picloram with increased number of replicates and an extended seedling exposure to picloram (six consecutive days) produced similar results (Fig. 4.10 B). Even though these results show that PsAFB6 is not likely acting as a picloram receptor in *Arabidopsis*, only WT and *Atafb5-5* seedlings were included in the comparison. Transgenic expression of *AtAFB5* in *Atafb5-5* mutant background and simultaneous analysis of their root growth response with the mutants expressing *PsAFB6* could have further verified this results.

4.4 SUMMARY

Developmental-, pollination- and seed-dependent modulations of *PsAFB6* transcript abundance suggest that it might play an important role during pea fruit development. Transcript abundance of pericarp *PsAFB6* increased in the absence of pollination, in response to seed removal, and in response to ethylene treatment. Exogenous 4-Cl-IAA, which rescues the growth of deseeded pericarps, mimics the presence of seeds in maintaining low levels of *PsAFB6* transcripts in deseeded and ethephon-treated deseeded pericarps. Exogenous IAA, which is ineffective in stimulating deseeded pericarp growth, did not maintain low levels of *PsAFB6* transcripts in deseeded pericarps and did not suppress the ethylene stimulation of *PsAFB6* transcript abundance. The ability of 4-Cl-IAA, but not IAA to maintain low levels of *PsAFB6* transcripts in a manner similar to the seeds is consistent with the hypothesis that 4-Cl-IAA may act as a seed signal in stimulating pericarp development (Ozga et al., 2009). Furthermore, the ability of 4-Cl-IAA (but not IAA) to inhibit ethylene stimulation of *PsAFB6* transcript abundance is consistent with the observations that only 4-Cl-IAA (but not IAA) suppresses pericarp ethylene response (Johnstone et al., 2005). The expression of *PsAFB6* in *Atafb5-5* mutants did not restore the picloram sensitivity of the mutants in root growth assays. It is possible that PsAFB6 may not act as a picloram receptor, at least in *Arabidopsis* roots. Further analysis is required to determine the role of PsAFB6 in auxin perception.

Whether or not PsAFB4 has a role in fruit development remains to be determined; however, it appears that the transcript abundance of *PsAFB4* is not regulated in the developing ovary by hormones involved in fruit development such as IAA, 4-Cl-IAA or ethylene.

4.5 FUTURE PERSPECTIVES

Functional characterization of PsAFB4 and PsAFB6 putative auxin receptors is an important primary step in understanding the role of PsAFB4 and PsAFB6 in pea fruit development. Verification of auxin receptor function will help to evaluate if the modulation of *PsAFB4* and *PsAFB6* transcript abundance by developing seeds is important in regulating the auxin action during pea fruit development. As discussed above, PsAFB4 is 68% and 70% identical to the *Arabidopsis* AtAFB4 and AtAFB5 proteins, respectively. In *Arabidopsis*, the *Atafb4* mutants are only weakly resistant to picloram, but *Atafb5-5* mutants and *Atafb4-8 afb5-5* double mutants are strongly picloram resistant (Prigge et al., 2016). Therefore, it may be possible to functionally characterize the role of PsAFB4 in auxin perception by expressing *PsAFB4* in *Atafb5-5* or *Atafb4-8 afb5-5* mutants and analyzing the root elongation in picloram medium.

It is not known if or how effectively the AFB6 clade of auxin receptors will complement any of the auxin receptor mutant phenotypes in *Arabidopsis*. The absence of a picloram sensitive phenotype in *Atafb5-5* seedlings expressing *PsAFB6* further supports the previous observation that picloram is primarily perceived through the AFB4 clade of auxin receptors in *Arabidopsis* (Prigge et al., 2016). The IAA dependent interactions of tomato auxin receptor SlAFB6 with SlIAA9 and AtIAA7 in pull-down assays suggest that PsAFB6 may act as an IAA receptor (Ben-Gera et al., 2012). The natural auxin IAA and the synthetic auxin 2,4-D are perceived primarily through the TIR1 clade of auxin receptors (Parry et al., 2009, Chapter 3). A complementation assay in the *Attir1-10* background or a higher order mutant background such as *Attir1-10 afb2-3* double mutants (Chapter 3) may help in determining the role of PsAFB6 in IAA and 2,4-D perception.

Given the fact that not all the TIR1/AFB family members are equally strong in the auxin perception (Parry et al., 2009; Prigge et al., 2016), characterization of *PsAFB4* and *PsAFB6* functions may be challenging even in a higher order mutant background of *Arabidopsis* showing

a discrete phenotype. As an *in vitro* approach, auxin receptors have been commonly characterized using pull-down assays, where the formation of a co-receptor complex between a selected TIR1/AFB protein and an Aux/IAA co-receptor is shown to be auxin-dependent (Parry et al., 2009; Ben-Gera et al., 2012). In *Arabidopsis,* there are 29 different Aux/IAA proteins which can be divided into 10 different clades based on their sequence similarities (Overvoorde et al., 2005). Not all the Aux/IAA proteins interacts with a particular TIR1/AFB family protein (Calderón Villalobos et al., 2012). Therefore, a careful selection of a suitable Aux/IAA protein is important in pull-down experiments. In this context, a better choice will be to isolate pea orthologs of *Arabidopsis* Aux/IAAs such as *AtIAA3*, *AtIAA7* and *AtIAA9,* which are known to interact with AFB4 or AFB6 clades of auxin receptors (Ben-Gera et al., 2012; Prigge et al., 2016). A homolog of *AtIAA3* could be recognized in pea by screening the GenBank entries (PsIAA4/5; Accession X68215). A putative pea homolog of AtIAA7 could also be identified by screening the NCBI Transcriptome Shotgun Assembly database (Appendix Fig. D6). As the *PsAFB4* and *PsAFB6* complete CDSs have already been cloned, isolation of above Aux/IAA genes from pea will make the characterization of two receptors by pull-down assays feasible. As an alternative approach to pull-down assays, auxin-dependent co-receptor complex formation can also be studied using yeast two-hybrid assays (Calderón Villalobos et al., 2012).

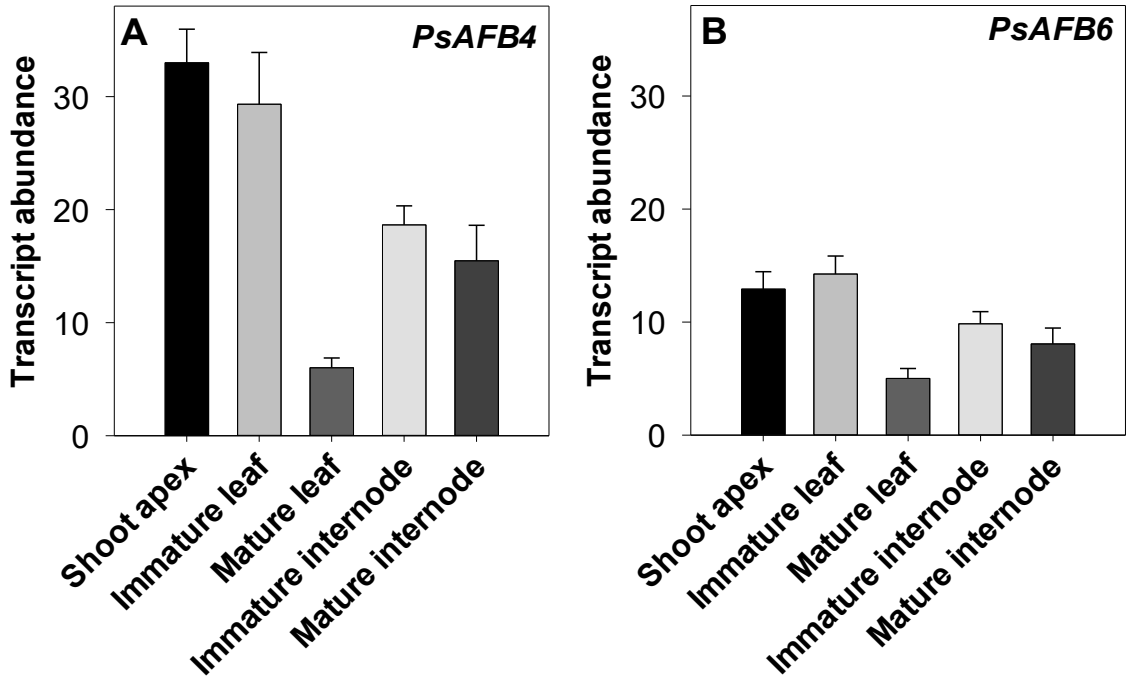


Figure 4.1. Relative transcript abundance of *PsAFB4* (**A**) and *PsAFB6* (**B**) in the shoot apex, and mature and immature leaf and internode tissues of 12-day-old *P. sativum* L. cv. I_3 Alaska-type seedlings. Data are means ± SD (n=3).

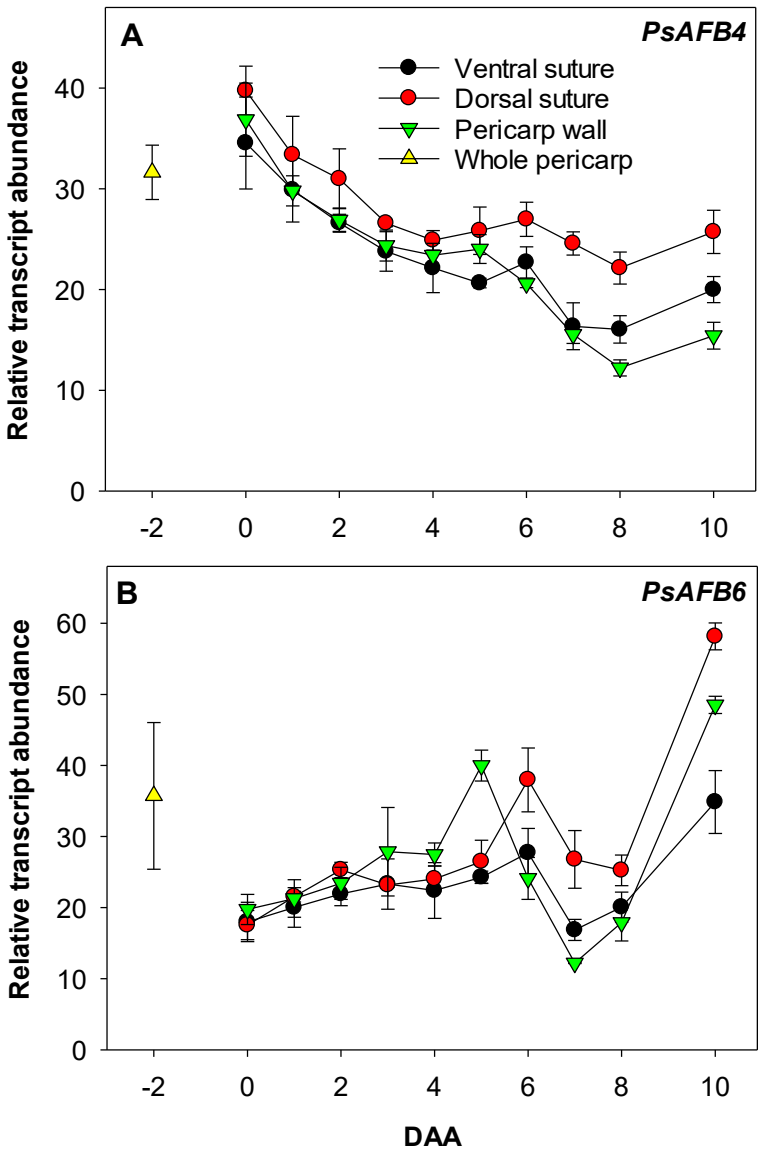


Figure 4.2. Relative transcript abundance of *PsAFB4* (**A**) and *PsAFB6* (**B**) in -2 DAA (days after anthesis) prepollinated whole pericarps, and in the pericarp wall, dorsal vascular suture and ventral vascular suture tissues of pollinated pea fruits from 0 to 10 DAA. Data are means ± SD, n=3 with the exception of 0-3 DAA dorsal suture where n=2. Each sample is a composite of a minimum of three pericarp tissues.

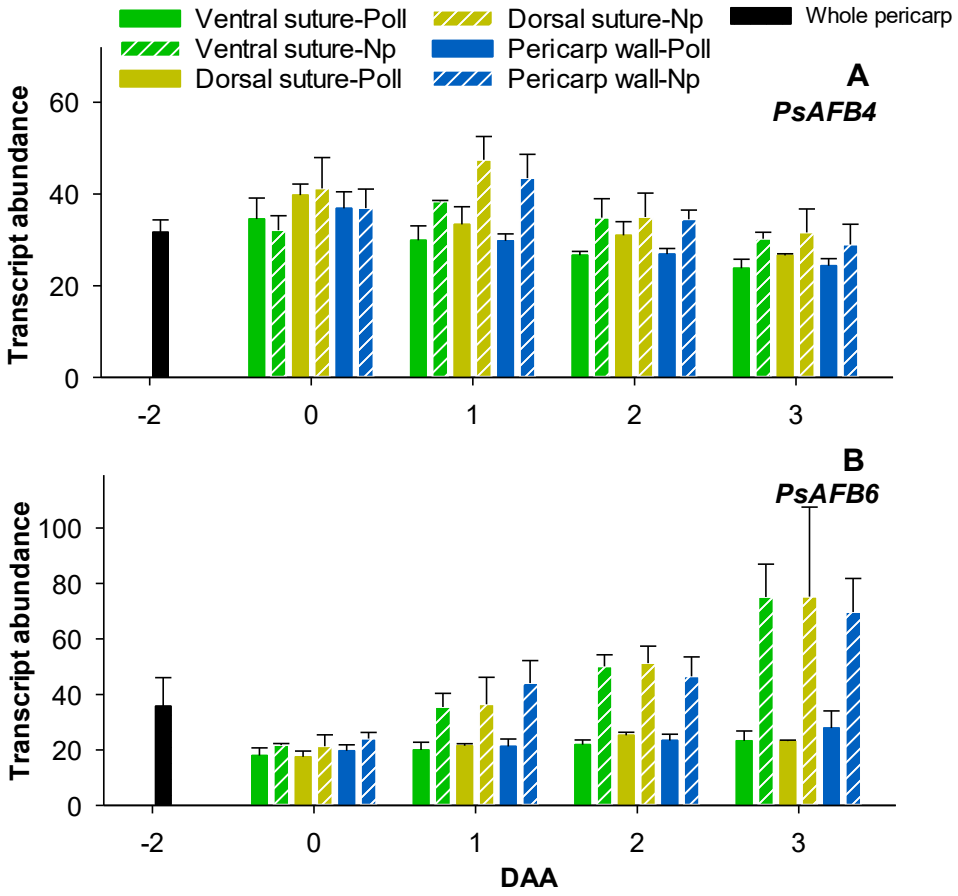


Figure 4.3. Relative transcript abundance of *PsAFB4* (**A**), *PsAFB6* (**B**) in –2 DAA (days after anthesis) prepollinated whole pericarps and pericarp tissues (ventral vascular suture, dorsal vascular suture, and pericarp wall) of 0 to 3 DAA of pollinated (Poll) and non-pollinated (Np) fruits. In non-pollinated fruits, flowers were emasculated at -2 DAA to prevent pollination. Data are means ± SD; n=3 for whole pericarp and pericarp wall; for ventral and dorsal sutures, the majority of samples were n=3, with n=2 for the remaining samples where the tissues were limited due to small tissue size.

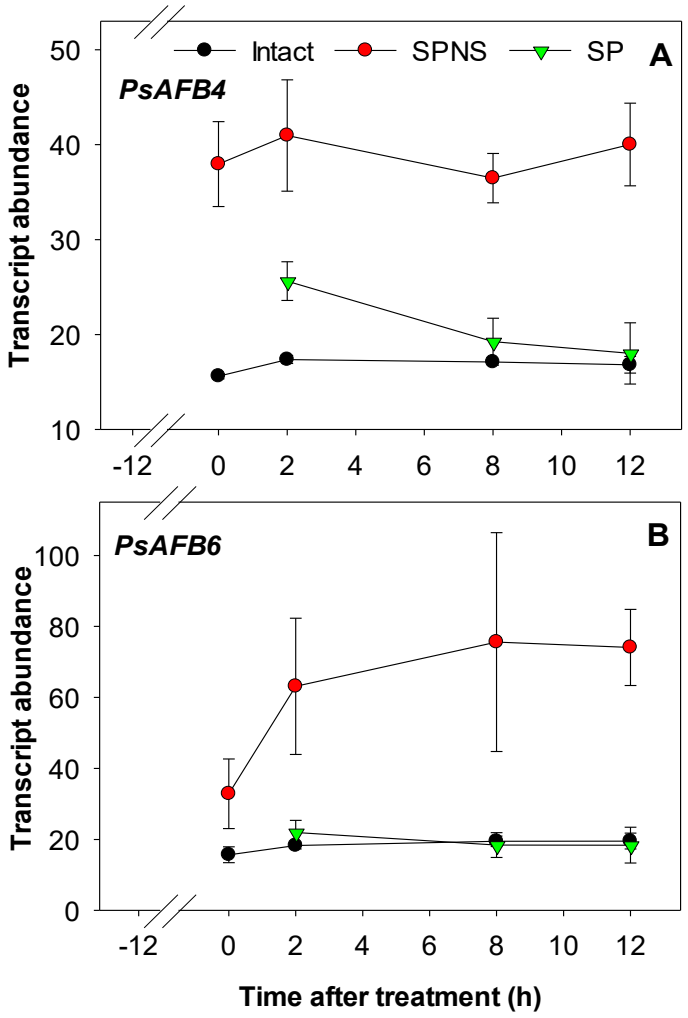


Figure 4.4. Relative transcript abundance of *PsAFB4* (**A**) and *PsAFB6* (**B**) in two days after anthesis pericarps (pods) of pollinated pea ovaries that were intact, split with seeds left intact (split pod; SP) and split with seeds removed (split pod no seeds; SPNS). Splitting of the pods occurred at -12 h, and aqueous Tween 80 (0.1%) as a surfactant control was added at treatment time 0 h. Data are means ± SD, n=3 to 8.

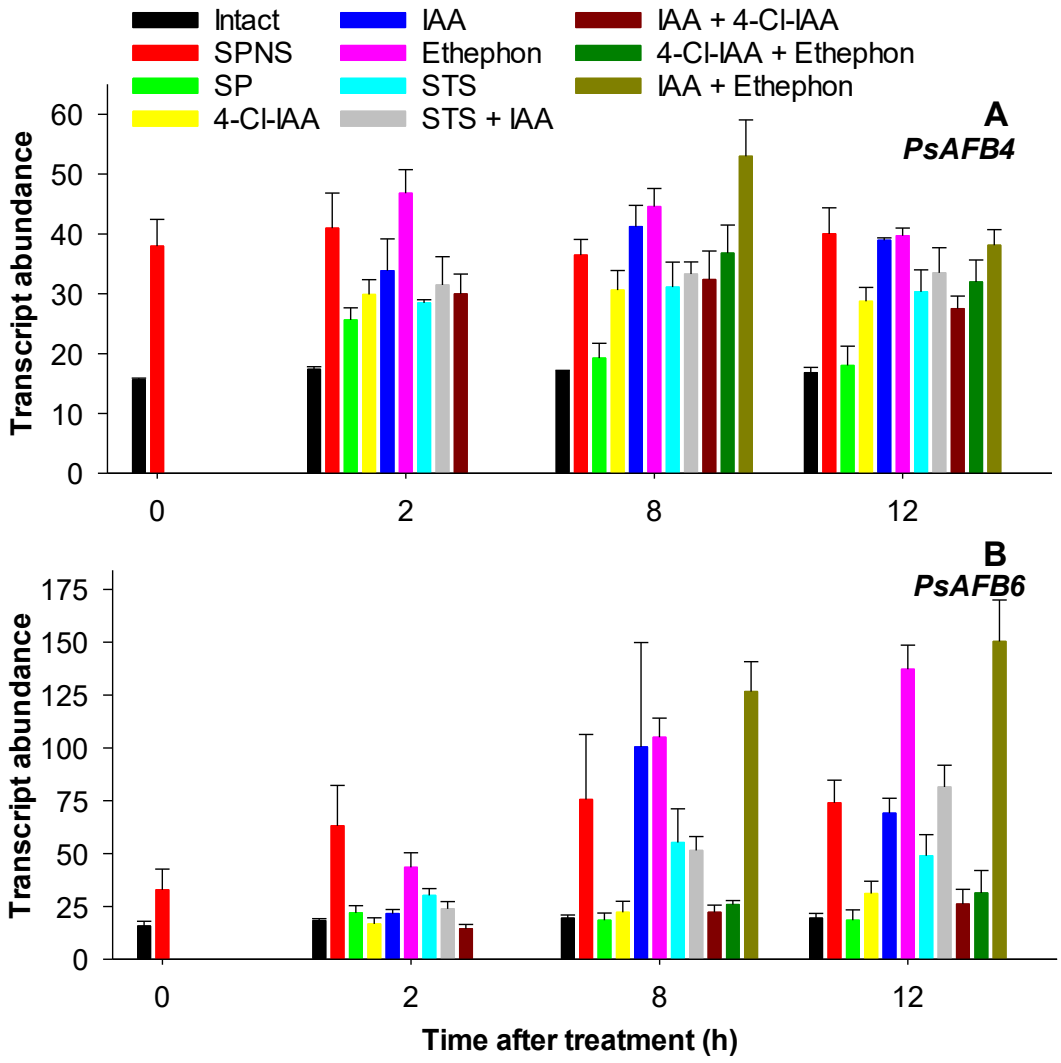


Figure 4.5. Effect of seed removal and hormone treatment on the relative transcript abundance of the ethylene biosynthesis genes *PsAFB4* (**A**) and *PsAFB6* (**B**) in the pericarps of pollinated pea ovaries. Two days after anthesis pericarps were either left intact, split (SP), or split and deseeded (SPNS) and treated 12 h after deseeding with 4-Cl-IAA (50 µM), IAA (50 µM), or ethephon (1000 mg L^{-1}) in 0.1% aqueous Tween 80 (30 µL) alone or in combination. Auxin or auxin-ethephon combinations were IAA plus 4-Cl-IAA, 4-Cl-IAA plus ethephon, or IAA plus ethephon in 0.1% aqueous Tween 80 (30 µL total). When fruits were treated with both auxin and ethephon, ethephon was applied 90 min after the auxin treatment and samples were collected based on the time after auxin treatment. Because of the delayed ethephon application, the auxin plus ethephon-treated pericarps were not studied at the 2 h time-point. Deseeded pericarps were

also pretreated with STS (1 mM in 0.1% aqueous Tween 80, 30 μL) at pericarp splitting and deseeding (STS treatment), and IAA (50 μM in 0.1% aqueous Tween 80, 30 μL) was applied to STS pretreated pericarps (IAA plus STS treatment). The SP and SPNS controls were treated with 0.1% aqueous Tween 80 (30 μL). All the samples were collected with respect to the time after hormone treatment. Data are means ± SD, n=3 to 8, with the exception of STS + IAA 2 h treatment, where n=2.

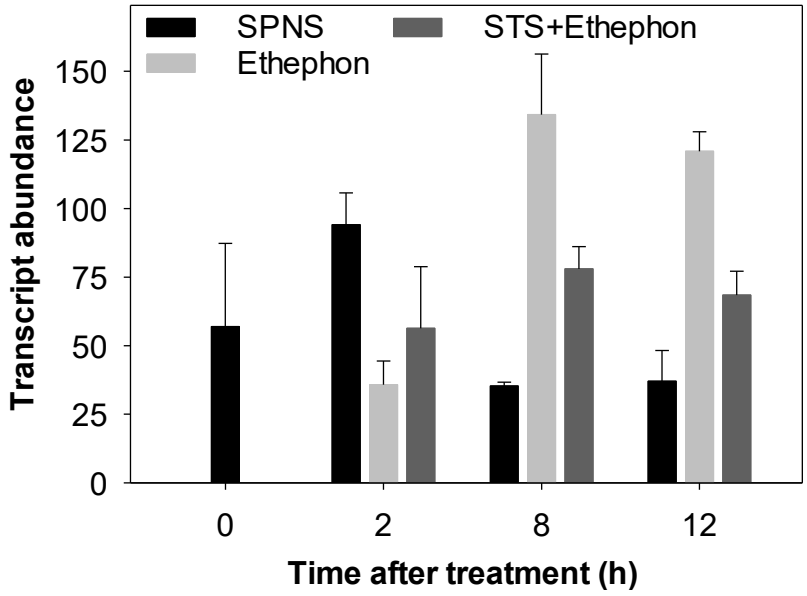


Figure 4.6. Effect of ethylene on pericarp *PsAFB6* transcript abundance. Two days after anthesis pericarps were split and deseeded (SPNS), and immediately treated with ethylene signaling inhibitor silver thiosulfate (STS; 1 mM; 30 µL) or they were left untreated. Twelve h after splitting and deseeding, pericarps were treated with ethephon (1000 mg L^{-1}, 30 µL) in 0.1% aqueous Tween 80. SPNS controls were treated with 30 µL of 0.1% aqueous Tween 80. Data is presented with respect to the time after ethephon/Tween 80 treatment. Data are means ± SD, n=2 to 3.

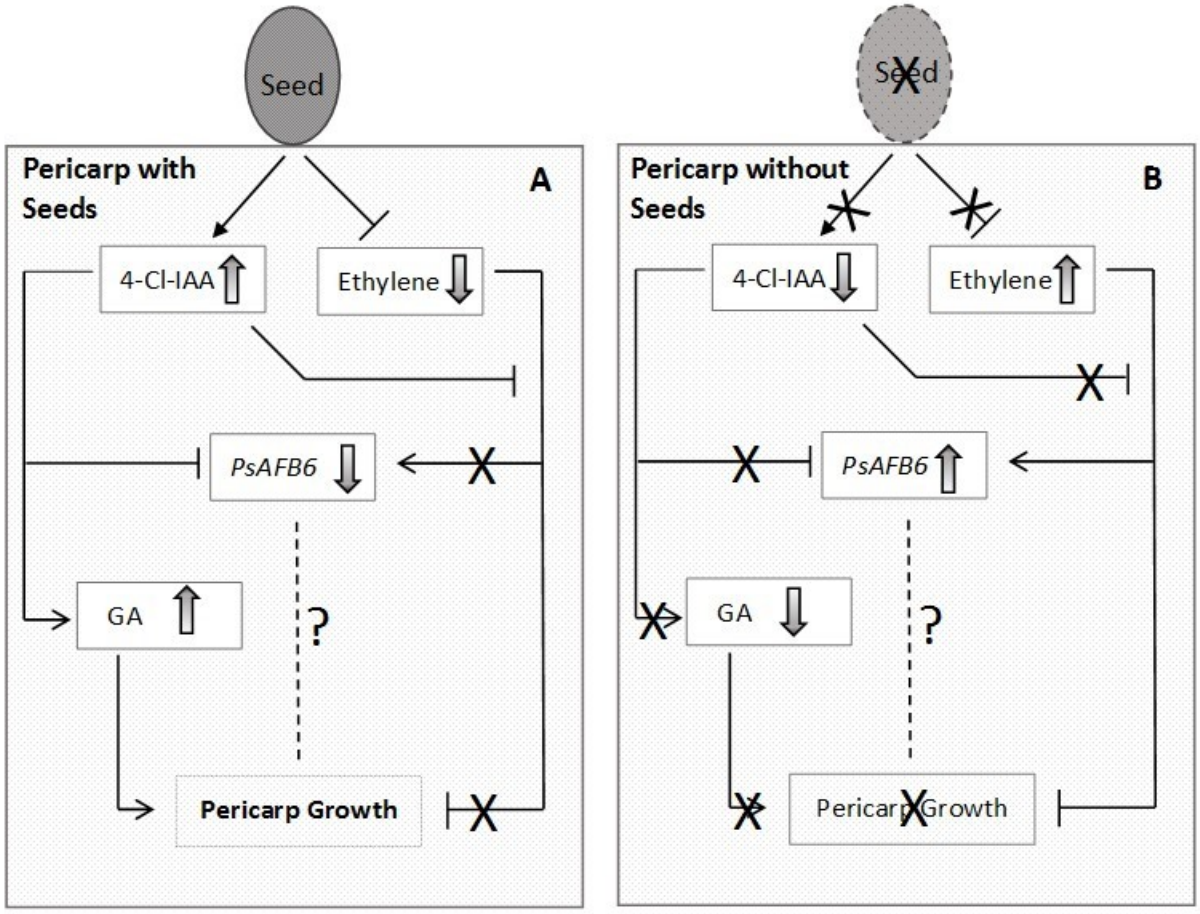


Figure 4.7. Working model of 4-Cl-IAA and ethylene regulation of *PsAFB6* transcript abundance and pericarp development. (**A**) Developing seeds act as a source of pericarp 4-Cl-IAA and inhibit pericarp ethylene biosynthesis. High levels of 4-Cl-IAA in the pericarp stimulates pericarp development through the stimulation of GA biosynthesis and the inhibition of ethylene action. High levels of 4-Cl-IAA and the suppression of ethylene action also inhibit the accumulation of *PsAFB6* transcripts. (**B**) In the absence of developing seeds, pericarp 4-Cl-IAA levels decline and ethylene biosynthesis increases, leading to reduced GA biosynthesis and increased ethylene action which initiates pericarp senescence. Low levels of 4-Cl-IAA and increased ethylene action also stimulate pericarp *PsAFB6* transcript abundance, however, exact role of the putative auxin receptor PsAFB6 in pericarp development or senescence is currently not known.

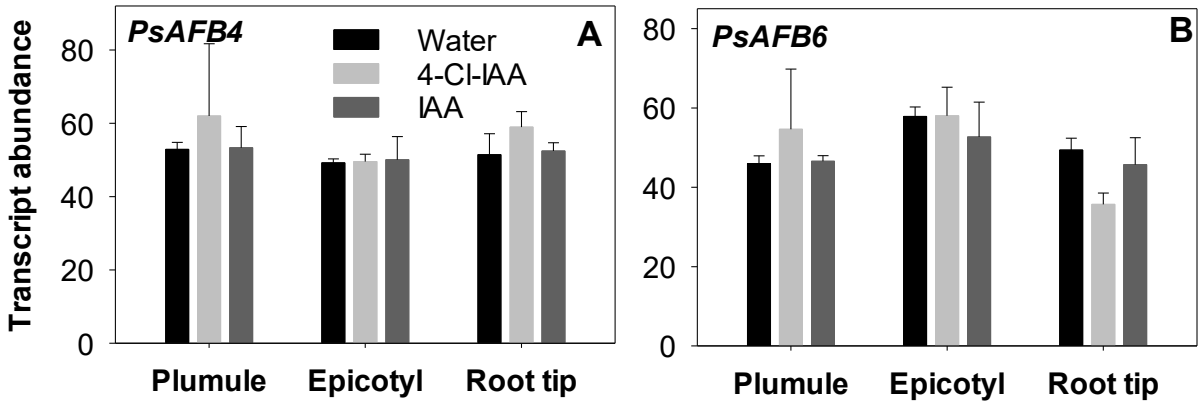


Figure 4.8. Relative transcript abundance of *PsAFB4* (**A**) and *PsAFB6* (**B**) in the plumule, epicotyl and root tip tissues of 4 days after imbibition etiolated pea seedlings. Seeds were imbibed in water for two days, then transferred to 4-Cl-IAA (1 µM) or IAA (1 µM) for two more days. Data are means ± SD, n=3

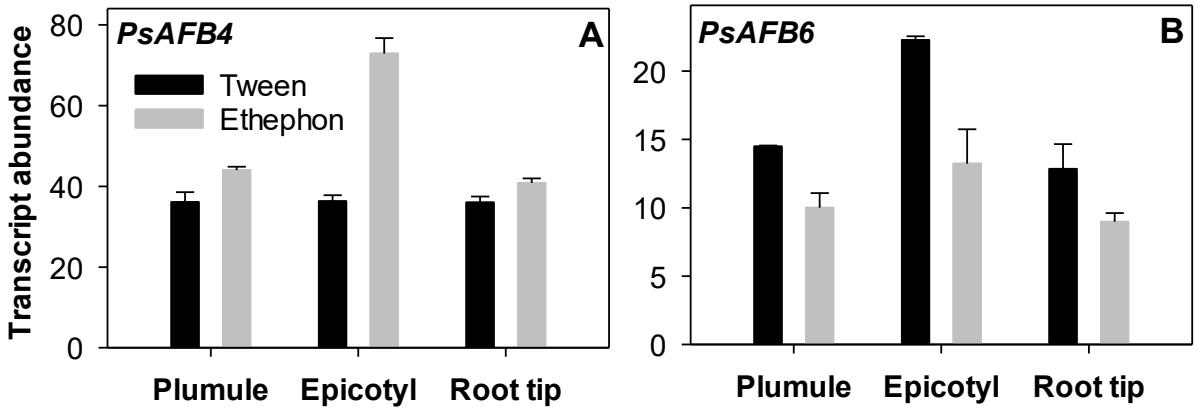


Figure 4.9. Transcript abundance of auxin receptors *PsAFB4* (**A**) and *PsAFB6* (**B**) in plumule, epicotyl and root tip tissues of 4 days after imbibition etiolated seedlings grown in the presence of ethephon (15 mg L^{-1} in 0.1% aqueous Tween 80) or aqueous Tween 80. Data are means ± SD, n=3

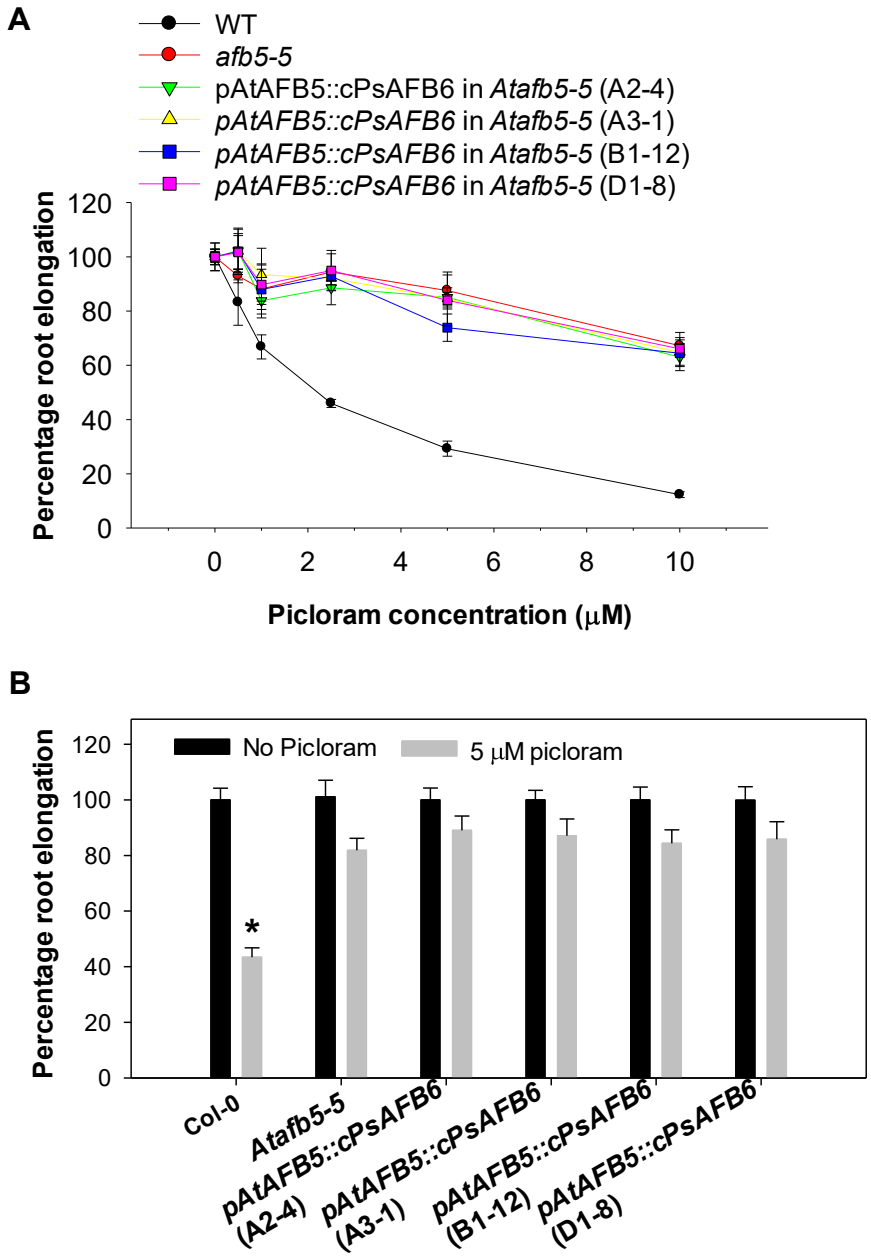


Figure 4.10. Root elongation of *Arabidopsis afb5-5* seedlings and *afb5-5* seedlings expressing *PsAFB6* in 0, 0.5, 1, 2.5, 5, 10 μM (**A**) or 0, 5 μM (**B**) picloram. Four-day-old seedlings were transferred to medium containing picloram and grown for four days (**A**) or six days (**B**) days before root length measurement. Root elongation of each genotype in picloram is expressed as a percentage compared to the same genotype in the media without auxin. Data are means ± SE (A) n= 3-4, (B) n=10. Comparisons were made using a two-tailed student's T-test.

* Means are significantly different from that of *afb5-5* within the same picloram concentration $P<0.01$

CHAPTER 5

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

In young pea fruits, the presence of seeds is a primary requirement for their continued development (Ozga et al., 1992). The naturally occurring auxin 4-chloroindole-3-acetic acid (4-Cl-IAA) mimics the role of seeds and stimulates deseeded pericarp (fruit) development in pea. The ubiquitous plant auxin indole-3-acetic acid (IAA), which is also present naturally in pea, does not induce pericarp development (Reinecke et al., 1995). Stimulation of pea pericarp gibberellin (GA) biosynthesis and the suppression of ethylene action by 4-Cl-IAA but not by IAA are believed to be at least partially responsible for the growth stimulatory effect of 4-Cl-IAA (Table 5.1; Johnstone et al., 2005; Ozga et al., 2009). This research focused on further understanding the mechanism behind differential effects of IAA and 4-Cl-IAA on pea pericarp development. In the first chapter of this thesis, effects of the two auxins on ethylene biosynthesis and signaling pathways were analyzed. The second and third chapters focused on understanding the underlying mechanisms that lead to the differential effects of IAA and 4-Cl-IAA, with a primary emphasis on the functional characterization and expression analysis of TIR1/AFB auxin receptors in pea.

5.1. DIFFERENTIAL REGULATION OF ETHYLENE BIOSYNTHESIS AND SIGNALING BY IAA AND 4-CL-IAA IN DEVELOPING PEA FRUITS

The first study evaluated whether IAA and 4-Cl-IAA differentially modulate ethylene biosynthesis and signaling pathways in developing pea fruits. Transcriptional regulation of *ACS* and *ACO* genes is a major regulatory mechanism of ethylene biosynthesis (Xu and Zhang, 2015). Transcript profiles of pea 1-aminocyclopropane-1-carboxylic acid synthases (*PsACS1*, *PsACS2* and *PsACS4*; enzyme converts *S*-adenosyl-Met to ACC) and ACC oxidases (*PsACO1*, *PsACO2* and *PsACO3*; enzyme converts ACC to ethylene) were analyzed in the pea pericarp to determine

the effect of different developmental conditions and the auxins IAA and 4-Cl-IAA on pericarp ethylene biosynthesis. Analysis of pericarp ACS and ACO gene transcript profiles revealed that their abundances are differentially regulated by developing seeds and auxins. Compared to normally developing fruits, the transcript abundances of *PsACS4*, *PsACO2*, and *PsACO3* were higher in non-pollinated or deseeded (SPNS) pericarps, indicating a correlation of transcript abundance with developing seeds (Figs. 2.2 E and F, 2.3 C, D, E, and F, 2.5. C, E, and F). Exogenous 4-Cl-IAA but not IAA mimicked the role of seeds by decreasing *PsACS4*, *PsACO2*, and *PsACO3* transcript levels and stimulating pericarp development (Figs. 2.4, 2.7 C, 2.9 B and C).

The transcript abundances of *PsACS1* and *PsACO1* genes were modulated differently from those of other *ACS* and *ACO* genes. Pericarps of both pollinated and non-pollinated ovaries showed a decline in *PsACS1* and *PsACO1* transcript levels from 0 to 3 DAA (except in the ventral vascular sutures of the non-pollinated pericarps where *PsACO1* transcript abundance remained high; Figs. 2.2 A and B, 2.3 A and B). Removal of seeds from normally developing fruits also had no clear effect on *PsACS1* and *PsACO1* transcript levels (Fig. 2.5 A and D). These observations suggested that PsACS1 and PsACO1 may not be directly associated with the increased ethylene evolution in non-pollinated or deseeded pericarps (Orzáez et al., 1999; Johnstone et al., 2005). However, the decline of *PsACS1* and *PsACO1* transcript abundance during early fruit development suggested that modulation of their transcript levels is important in fruit development. Exogenous 4-Cl-IAA strongly stimulated *PsACS1* and *PsACO1* transcript abundance. Application of IAA also increased *PsACS1* and *PsACO1* transcript abundance, but the effect was minor compared to that of 4-Cl-IAA (Figs. 2.7 A, 2.9 A). Treatments with ethylene releasing agent ethephon and the ethylene signaling inhibitor silver thiosulfate (STS) indicated that auxins can directly regulate the levels of *PsACS1* and *PsACO1* transcripts (Figs. 2.7 A, 2.8 A and D, 2.9 A).

Associated with changes in *ACS* and *ACO* transcript abundance, the level of ethylene precursor ACC and the activity of the ethylene producing enzyme ACO (which converts ACC to ethylene) were also changed in SPNS and auxin-treated pericarps. The increased ethylene evolution in SPNS pericarps (Johnstone et al., 2005) appeared to be regulated at the level of ACC synthesis, as the pericarp ACC level but not the ACO enzyme activity increased in SPNS pericarps (Fig. 2.6). Application of IAA or 4-Cl-IAA to SPNS pericarps further increased

ethylene evolution at similar levels (Johnstone et al., 2005), but their effects on the pericarp ACC level and ACO enzyme activity were clearly different. Compared to SPNS controls, the IAA-treated pericarps contained a slightly lower level of ACC and showed a slight increase in ACO enzyme activity. Application of 4-Cl-IAA strongly increased pericarp ACC level, but the ACO enzyme activity remained unaffected (Fig. 2.6). Regulation of ACC synthesis is the commonly observed rate-limiting mechanism of ethylene biosynthesis (Harpaz-Saad et al., 2012). The low level of ACC and increased ACO enzyme activity in IAA-treated pericarps indicated that ACC synthesis is also the rate-limiting step of ethylene biosynthesis in the IAA-treated pericarps. However, in 4-Cl-IAA-treated pericarps, ethylene biosynthesis is likely regulated at the ACO enzyme level.

It is unclear if the rate-limiting step of ethylene biosynthesis has any effect on pericarp ethylene action. However, if ACC itself acts as a signaling molecule as suggested by recent studies (Yoon and Kieber, 2013), the very high level of ACC in 4-Cl-IAA-treated fruits may play a role modulating 4-Cl-IAA-specific effects in the pericarps. The ACO enzyme activity of 4-Cl-IAA-treated pericarps remained similar to that of SPNS controls. However, suppression of *PsACO2* and *PsACO3* transcript abundance and the increase of *PsACO1* transcript abundance by 4-Cl-IAA indicated that in response to 4-Cl-IAA, different *ACO* genes code pericarp ACO protein pool. The composition of the ACO protein pool may also have an influence on the differential activity of auxins as ACO proteins are predicted to have regulatory roles in addition to their function in ethylene biosynthesis (Dilley et al., 2013).

The putative ethylene receptors *PsERS1* and *PsETR2* and ethylene signaling-related genes *PsEBF1* and *PsEBF2* were selected as ethylene-signaling markers based on the observations that ethylene induced the transcript levels of their orthologs in *Arabidopsis* and tomato (Hua et al., 1998; Kevany et al., 2007; Konishi and Yanagisawa, 2008; Yang et al., 2010). Ethylene-induced accumulation of the *PsERS1*, *PsETR2*, *PsEBF1* and *PsEBF2* genes in pea was verified by treating the pericarps with ethephon and STS (Fig. 2.10 A, D, G and J). Treatment of deseeded pericarps with 4-Cl-IAA increased the transcript abundance of all the four genes while no clear increase could be seen with IAA (Fig. 2.10). The higher transcript levels with 4-Cl-IAA may not necessarily represent an increased ethylene action as both IAA and 4-Cl-IAA stimulated pericarp ethylene evolution at similar levels, and 4-Cl-IAA was inhibitory on pericarp ethylene action (Johnstone et al., 2005). The ethylene receptors and *EBF* genes act as negative regulators of

ethylene signaling, and the increased levels of their transcript abundance may play a role suppressing the ethylene action in 4-Cl-IAA-treated pericarps (Table 5.1).

In conclusion, the pea fruit ethylene biosynthesis is modulated by developing seeds, and by the auxins IAA and 4-Cl-IAA. Auxin and seed regulation of pericarp ethylene biosynthesis involves the modulation of *PsACS* and *PsACO* transcript levels. Increased ethylene evolution in the absence of developing seeds is associated with an increased transcript abundance of *PsACS4*, *PsACO2* and *PsACO3* genes. 4-Cl-IAA but not IAA mimics the presence of seeds in reducing *PsACS4*, *PsACO2* and *PsACO3* transcript abundance. Transcript levels of *PsACS1* and *PsACO1* are IAA and 4-Cl-IAA inducible, but the stimulatory effect of 4-Cl-IAA is stronger compared to that of IAA. Increased ethylene evolution in 4-Cl-IAA-treated fruits is likely regulated at the ACO enzyme level as 4-Cl-IAA strongly stimulates the ethylene precursor ACC level but does not induce the activity of the ACO enzyme. In IAA-treated fruits, the rate of ethylene biosynthesis is likely regulated at the level of ACC synthesis but not at the level of ACO enzyme activity. The very high level of ACC, possible changes in the ACO protein pool and the increased transcript levels of ethylene receptor and *PsEBF* genes in the pericarps treated with 4-Cl-IAA may have a correlation with the suppression of ethylene action and the stimulation of pericarp growth. Overall, these data show that the two naturally occurring auxins IAA and 4-Cl-IAA have distinctly different effects on ethylene biosynthesis in the pea fruits, which may be associated with the growth stimulatory effects limited to 4-Cl-IAA.

5.1.1. Future perspectives in understanding auxin-ethylene interactions during pea fruit development

In the future, it will be interesting to determine if the different ACC levels and ACO protein pools in IAA- and 4-Cl-IAA-treated pericarps have any effect on pericarp development. Low- or high-expression transgenic plants or mutants of *PsACS* and *PsACO* genes such as *PsACS1*, *PsACO1*, *PsACO2* and *PsACO3*, which show auxin-dependent regulations will be useful in understanding whether the modulations in ethylene biosynthesis are important in auxin action. However, compared to a model plant like *Arabidopsis*, mutants are only available for a limited number of genes in pea, and obtaining transgenic pea plants is also a lengthy process (Sinjushin, 2013; Smýkal, 2014). Chemical inhibitors of ethylene biosynthesis such as aminoethoxyvinylglycine (AVG), aminooxyacetic acid (AOA), α-aminoisobutyric acid (AIBA)

and cobalt ions ($Co^{2+)}$ can be used as an alternative approach to modulate the pericarp ACC levels and ACO enzyme activity (Abeles et al., 1992). The chemicals AVG and AOA, which act as inhibitors of ACC synthase have been used to analyze the roles of ACC in *Arabidopsis* (Tsang et al., 2011). In pea, pretreatment of deseeded pericarps with AVG or AOA will inhibit the accumulation of ACC in 4-Cl-IAA-treated pericarps. The absence of 4-Cl-IAA-induced ACC in the pericarps would reveal whether or not the high levels of ACC in 4-Cl-IAA-treated fruits have any function independent of ethylene. The compound AIBA is a structural analog of ACC which inhibits the ACC transport and the ACO enzyme activity (Abeles et al., 1992). Cobalt ions also act as an inhibitor of ACO enzyme activity (Abeles et al., 1992). Comparison of IAA and 4-Cl-IAA treated pericarps with and without AIBA and Co^{2+} pretreatments may also provide clues if ACC or ACO has additional regulatory roles in the IAA- and 4-Cl-IAA-dependent pericarp growth regulatory responses.

Even though the chemical inhibitors can be easily used to regulate ethylene biosynthesis or action, those compounds may have additional physiological effects (Abeles et al., 1992). For example, AVG and AOA disrupt all the pyridoxal phosphate requiring enzymes including tryptophan aminotransferase, causing a reduction in auxin biosynthesis (Soeno et al., 2010; Tsang et al., 2011). Also, silver ions used as an ethylene signaling inhibitor act as an inducer of auxin efflux (Strader et al., 2009). Because of undesired effects of chemical inhibitors, careful data interpretation is required. In the present study, silver thiosulfate (STS) was used to verify if the transcript abundance modulation of a particular gene with auxin is independent of auxin-induced ethylene. Although the effects of silver ions on auxin transport may have an effect on gene regulation, additional treatments with ethephon, ethephon and IAA, or ethephon and 4-Cl-IAA minimized the chances of data misinterpretation.

In future studies, a recently identified ethylene insensitive *ein2* mutant in pea (AF145; Weller et al., 2015) could be used to examine the effects of auxins on ethylene action. The EIN2 protein plays a central role in ethylene signaling and causes ethylene insensitivity in the loss of function *ein2* mutants (cited from Zheng and Zhu, 2016; Fig. 1.4). Using *ein2* plants together with STS-treated wild-type (WT) plants would be helpful in differentiating any undesired effects caused by STS. Also, it will be interesting to see if higher concentrations of IAA have any inhibitory effect on pericarp growth in *ein2* plants. If IAA-induced ethylene inhibits the pericarp development as suggested by Johnstone et al. (2005), an inhibitory effect may not be visible in

ein2 plants even at high IAA concentrations. Furthermore, *ein2* plants will be helpful in studying whether ACC acts as an independent signaling molecule during pericarp development. In *ein2* plants, there will be no increase of ethylene action in response to the application of ACC, and therefore can be used to recognize ethylene-independent roles of ACC without stimulating any interfering ethylene response.

In this study, the analysis of auxin effect on pericarp ACC levels and the ACO enzyme activity was limited to 12 h after treatment time-point. The 12 h time-point was selected based on the observation that auxin-induced ethylene evolution peak at 12 h after hormone application (Johnstone et al., 2005). However, gene expression data show that the highest transcript abundance variation of some genes occurs at earlier time-points. For example, *PsACS1* transcript abundance peaks around 2 h after auxin treatment and *PsACO1* levels peak around 8 and 12 h after the auxin treatments. Therefore, a detailed evaluation of IAA and 4-Cl-IAA effects on the ACC level and ACO enzyme activity requires the analysis of samples collected at a range of different time-points, particularly between the 2 to 12 h after the auxin treatments.

5.2 FUNCTIONAL CHARACTERIZATION OF PEA AUXIN RECEPTORS AND ANALYSIS OF THEIR EXPRESSION DURING EARLY PEA FRUIT DEVELOPMENT

The underlying mechanisms that lead to the differential effects of IAA and 4-Cl-IAA in pea fruit are not completely understood. It is possible that the differences in auxin uptake and transport, metabolism, or signaling mechanisms are associated with the functional differences of the two auxins (Reinecke, 1999; Reinecke et al., 1999). The third and fourth chapters of this thesis focused on evaluating whether the differential actions of IAA and 4-Cl-IAA initiate at the level of auxin perception.

When the pea *PsTIR1a* and *PsTIR1b* genes were functionally characterized in *Arabidopsis Attir1-10* and *Attir1-10 afb2-3* auxin receptor mutants, both genes increased the 2,4-D sensitivity of the mutants in root growth assays, indicating that *PsTIR1a* and *PSTIR1b* code for functional auxin receptors (Figs. 3.3 and 3.4). The inhibitory effect of 4-Cl-IAA on root elongation was stronger than that of IAA in WT (Col-0) *Arabidopsis* seedlings (Fig. 3.2). Compared to WT plants, *Attir1-10 afb2-3* double mutants were less sensitive to auxins and showed no differential response to IAA and 4-Cl-IAA (Fig3.2 and Appendix Fig. B11). Expression of *Arabidopsis*

AtTIR1, pea *PsTIR1a* or *PsTIR1b* genes restored the IAA and 4-Cl-IAA sensitivity of the *Attir1-10 afb2-3* double mutants, and their root growth inhibition was higher with 4-Cl-IAA compared to that of IAA (Appendix Fig. B11). This root growth assay data indicated that PsTIR1a and PsTIR1b act as IAA and 4-Cl-IAA receptors and the stronger auxin effect of 4-Cl-IAA is at least partially mediated through the TIR1 auxin receptors.

Transcript abundance of pea *PsTIR1/AFB* family members showed spatiotemporal variations. In pea seedlings, transcript abundance of *PsTIR1a*, *PsAFB2*, *PsAFB4* and *PsAFB6* was comparatively higher in the internode, shoot apex and immature leaf tissues compared to that of mature leaves (Figs. 3.6 and 4.1). In contrast, the transcript abundance of *PsTIR1b* was higher in all the leaves compared to that of internodes (Fig. 3.6 B). In pea pericarps, *PsTIR1a*, *PsTIR1b*, *PsAFB2* and *PsAFB4* transcript levels but not the *PsAFB6* transcript level gradually declined with fruit development (Figs. 3.7 and 4.2). Transcript abundance of *PsTIR1b*, *PsAFB4* and *PsAFB6* in the pericarp was regulated by pollination status and/or the developing seeds. Seed removal from normally developing fruits or the absence of pollination increased the *PsTIR1b*, and *PsAFB6* transcript abundance (Figs. 3.8 B, 3.11 B, 4.3 B and 4.4 B). Application of 4-Cl-IAA to SPNS pericarps mimics the role of seeds in suppressing *PsTIR1b* and *PsAFB6* transcript abundance, but IAA had no or only a transitory effect (Figs. 3.12 B and 4.5 B). In young pea fruits, seeds are considered as a source of pericarp auxins (Ozga et al., 2009). The high levels of *PsTIR1b* and *PsAFB6* transcripts in the absence of developing seeds and the reduced levels of those transcripts in 4-Cl-IAA-treated pericarps suggested that high levels of pericarp 4-Cl-IAA in the presence of seeds maintains low levels of *PsTIR1b* and *PsAFB6* transcripts in the pericarp. In contrast to 4-Cl-IAA, ethylene stimulated pericarp *PsAFB6* transcript abundance, which indicated that high transcript abundance of *PsAFB6* in SPNS and non-pollinated pericarps could also be induced by increased ethylene evolution in those pericarps (Orzáez et al., 1999; Johnstone et al., 2005). Seed removal also increased the *PsAFB4* transcript abundance, but the treatment of deseeded pericarps with IAA, 4-Cl-IAA or ethylene had no clear effect. These observations indicated that other seed-derived signals may also be associated with the modulation of auxin receptor transcript abundance in the pericarp (Figs. 4.4 A and 4.5 A). The presence of developing seeds or application of auxins or ethylene had no clear effect on *PsTIR1a* and *PsAFB2* transcript levels. Altogether, these results suggest that the modulation of the TIR1/AFB auxin receptor protein pool in the pea fruit may plays an important role regulating

the auxin action under different developmental conditions and in responses to IAA and 4-Cl-IAA. However, it is not possible to exclude the possibility that the modulation of auxin receptor abundance is only a result of the pericarp development or senescence process and not a direct determinant of the fate of the pericarp. Future studies with auxin receptor overexpression lines or mutants are required to conclude the importance of auxin receptor abundance and the receptor composition in modulating the pericarp growth.

The auxin-responsive *DR5* promoter-driven *GUS* reporter expression in the pea pericarp indicated a correlation between the auxin activity, auxin receptor transcript abundance, and the free IAA levels. In parallel to the gradual decline of auxin receptor transcript abundance, IAA levels and *DR5::GUS* expression also decreased in developing pea pericarps (Table 3.1 and Fig. 3.10). The decline of auxin receptor transcript abundance with the reduction of pericarp *GUS* reporter expression suggested that, in addition to the changes in auxin levels, modulation of pericarp auxin sensitivity also plays a role regulating the pericarp auxin action. In the pericarp wall, *DR5::GUS* expression declined from 5 to 8 DAA fruits despite the concomitant increase of IAA levels, which further supported the possibility that regulation of pericarp auxin sensitivity through the modulation of auxin receptor abundance is important in regulating the pericarp auxin action.

The *DR5::GUS* expression of pericarps treated with IAA and 4-Cl-IAA showed that both IAA and 4-Cl-IAA enter the pericarp tissues and stimulated auxin response within 2 h after the treatment. The *GUS* expression maximized by 8 h after auxin application and persisted at relatively stable levels through 12 h after treatment (Fig. 3.14). However, the GUS activity was stronger in 4-Cl-IAA-treated pericarps compared to those treated with IAA (Fig. 3.14). It is possible that the different efficiencies of IAA and 4-Cl-IAA uptake by the pericarp, comparatively rapid IAA metabolism in the pericarp tissues or the ability of 4-Cl-IAA to stimulate a stronger auxin response than IAA causes this stronger auxin response with 4-Cl-IAA.

In conclusion, the pea *PsTIR1a* and *PsTIR1b* genes code for functional auxin receptors. In *Arabidopsis*, the AtTIR1 auxin receptor plays a pivotal role in the perception of IAA and 4-Cl-IAA and mediating the comparatively stronger root growth inhibitory effect of 4-Cl-IAA. The pea PsTIR1a and PsTIR1b auxin receptors restore the IAA and 4-Cl-IAA sensitivity of *Arabidopsis* auxin receptor mutants in a manner similar to that of *Arabidopsis* TIR1. Therefore in pea, PsTIR1a and PsTIR1b receptors likely play a major role in the perception of both IAA

and 4-Cl-IAA, and the differential effects of IAA and 4-Cl-IAA may at least partially be mediated through those receptors. The modulation of auxin receptor transcript abundance in the pericarp suggests that pericarp auxin receptor levels and the composition is developmentally and auxin specifically regulated, and this could be important in mediating the differential effects of IAA and 4-Cl-IAA.

5.2.1. Future perspectives in understanding mechanism of differential IAA and 4-Cl-IAA action

If a particular TIR1/AFB auxin receptor is more prevalent under certain physiological conditions, the downstream auxin signaling pathway will depend on the Aux/IAA proteins which associate with that receptor in the presence of IAA and 4-Cl-IAA. However, the changes observed in the *TIR1/AFB* transcript abundances may not necessarily represent the changes in their protein levels. In *Arabidopsis*, plants expressing *GUS* reporter as a translational fusion with *AtTIR1*, *AtAFB2* and *AtAFB3* coding sequences showed restricted GUS staining compared to equivalent *promoter::GUS* plants with similar transcript abundances, which revealed that the TIR1/AFB genes undergo post-transcriptional regulation (Parry et al., 2009). Therefore, it is important to determine the changes of TIR1/AFB protein levels using a technique such as Western blotting to verify if the modulations of *TIR1/AFB* transcript levels directly correlate with the auxin receptor abundance in the pea fruit.

Functional characterization of other putative auxin receptors is an important future step in understanding the importance of auxin receptors in the differential action of IAA and 4-Cl-IAA. Especially, it is not clear if PsAFB4 and PsAFB6 act as functional auxin receptors. The *Arabidopsis afb5-5* plants, which show a strong picloram resistant root elongation phenotype, can be used for the functional characterization of PsAFB4 (Prigge et al., 2016, Fig. 4.10). The Arabidopsis *afb4-8* or *afb4-9* mutants may not be suitable for a similar study as those mutants did not show a strong phenotype in root growth assays (Prigge et al., 2016). Expression of pea *PsAFB6* did not restore the picloram sensitivity of the *Arabidopsis Atafb5-5* mutants in root growth assays, which suggested that PsAFB6 is not a picloram receptor in *Arabidopsis* roots (Fig. 4.10). It is possible that PsAFB6 acts as a receptor of other auxins such as 2,4-D, IAA and 4-Cl-IAA, which can be tested by doing a similar complementation study in *Arabidopsis Attir1-10* and *Attir1-10 afb2-3* auxin receptor mutant backgrounds.

Even though the functional characterization of pea auxin receptors in *Arabidopsis* is a possibility, those receptors may not be functional in *Arabidopsis* as the two species are not closely related. Therefore as a next step, the pea auxin receptor genes can be functionally characterized within pea plants itself. A technique such as clustered regularly interspaced palindromic repeats/CRISPR-associated 9 (CRISPER/Cas9) can be used to generate TIR1/AFB mutants in pea. Also, reduced expression of selected genes using a technique such as RNAi or overexpression of selected genes with a stronger promoter such as *CaMV-35S* may provide clues about their importance in the auxin perception and plant development. However, gene mutations or modulations of their transcript abundance may not necessarily provide discrete phenotypes due to the functional redundancies or the possible tissue specificities of the auxin receptors. As an alternative approach, auxin receptors have been commonly characterized *in vitro*. Several different types of protein binding assays have been adapted for the functional characterization of auxin receptors, and for the comparison of their auxin binding efficiencies. Those methods include pull-down assay (Dharmasiri et al., 2005; Kepinski and Leyser, 2005), yeast-two-hybrid assay (Calderón Villalobos et al., 2012), auxin-based degron system (Havens et al., 2012; Shimizu-Mitao and Kakimoto, 2014) and surface plasmon resonance (SPR) assay (Lee et al., 2014). All these assays determine if TIR1/AFB and Aux/IAA co-receptor proteins interact in an auxin-dependent manner and therefore provide *in vitro* evidence if a protein of interest acts as an auxin receptor. When analyzing possible differences in the receptor binding affinities of IAA and 4-Cl-IAA, SPR can be considered as the best method of choice. The SPR assays provide information on the auxin-dependent interactions between TIR1/AFB and Aux/IAA family members and their auxin binding affinities (Lee et al., 2014). A comparison of IAA and 4-Cl-IAA dependent binding of TIR1/AFB and Aux/IAA proteins would provide information if a particular auxin has a strong affinity to a particular TIR1/AFB-Aux/IAA co-receptor complex.

With respect to auxin perception, this study only focused on the identification, expression analysis and functional characterization of TIR1/AFB auxin receptors in pea. Nevertheless, TIR1/AFB and Aux/IAA proteins act as co-receptors in auxin perception (Calderón Villalobos et al., 2012; Figs. 1.2 and 3.17). None of the two protein types successfully bind with auxin in the absence of the other (Calderón Villalobos et al., 2012). Therefore, identification, expression analysis and functional characterization of Aux/IAA proteins have an equal importance when studying the possible differences in IAA and 4-Cl-IAA perception. A detailed study of Aux/IAA

co-receptors would be far more challenging compared to that of TIR1/AFB co-receptors given that there are a number of genes in the Aux/IAAs family. In *Arabidopsis* for example, there are 29 Aux/IAA members (Overvoorde et al., 2005), and it is not clear how many of them are present in pea. The other drawback is the lack of visible defects in single and some higher order Aux/IAA mutants, likely due to their functional redundancies (Overvoorde et al., 2005).

As an initial step, Aux/IAA family members in pea can be identified, and their transcript abundance can be analyzed in the pericarps treated with IAA and 4-Cl-IAA. Auxin modulates the expression of Aux/IAA genes (Goda et al., 2004; Wu et al., 2012) and if IAA and 4-Cl-IAA differentially regulate Aux/IAA transcript levels, it further supports the hypothesis that the differential effects of IAA and 4-Cl-IAA are modulated at the level of auxin receptors. The next step will be the evaluation of IAA and 4-Cl-IAA affinities to different Aux/IAA and TIR1/AFB protein combinations using a method such as SPR.

In this study, the uptake of exogenous IAA and 4-Cl-IAA into the pericarps was analyzed using *DR5::GUS* plants. Even though the GUS activity was stronger in 4-Cl-IAA-treated fruits, both IAA- and 4-Cl-IAA-treated pericarps showed an increase in *GUS* expression by 2 h after treatment, which maximized by 8 h. This same system can be adapted to evaluate the pericarp IAA and 4-Cl-IAA uptake efficiencies. Analysis of *GUS* expression in more frequent time-points from immediately after auxin treatment to until around 8 h after treatment will show if 4-Cl-IAA enter the pericarps more rapidly than IAA. Such an analysis would be helpful in understanding whether the differences in IAA and 4-Cl-IAA uptake efficiencies are associated with the differential effects of IAA and 4-Cl-IAA on pea pericarp development.

5.3 WHY DOES 4-CL-IAA BUT NOT IAA STIMULATE PEA PERICARP DEVELOPMENT?

The ability of 4-Cl-IAA to mimic the presence of the seeds in pericarp development (Reinecke et al., 1995; Ozga et al., 2009), high auxin concentration (IAA and 4-Cl-IAA) in young developing pea seeds (Magnus et al., 1997; Table 3.1) localized by GUS staining to the proximal end of the seed and the funiculus (Fig. 3.9 C to F), and an auxin (IAA) concentration gradient where seeds > ventral vascular suture > pericarp wall (8 DAA; Table 3.1) are

observations consistent with the hypothesis that developing seeds act as a source of pericarp auxins.

The naturally occurring pea auxin 4-Cl-IAA plays a pivotal role in pea fruit development. In the pea pericarps, auxin effect of 4-Cl-IAA is stronger compared that of IAA (Fig. 3.14). Exogenous 4-Cl-IAA stimulates deseeded pericarp growth, but IAA does not stimulate pericarp development even in response to increased concentrations or repeated daily applications (Reinecke et al., 1995), indicating that 4-Cl-IAA is irreplaceable by IAA during pericarp development. Transcript abundance analysis of genes associated with ethylene biosynthesis and signaling (Chapter 2), auxin perception (Chapter 3 and 4) and GA biosynthesis and metabolism (Ozga et al., 2009) indicates that 4-Cl-IAA has strong effects modulating their transcript levels (Table 5.1). IAA has no or relatively minor effects modulating those gene transcript levels.

Suppression of *PsTIR1b* and *PsAFB6* transcript levels, together with the possible modulations of Aux/IAA co-receptor abundance by 4-Cl-IAA may regulate the 4-Cl-IAA-specific gene expression in the pea pericarp. The 4-Cl-IAA mediated stimulation of *PsACS1* and *PsACO1* transcript abundance and the suppression of *PsACS4*, *PsACO2* and *PsACO3* transcript abundance (Chapter 2) suggest that 4-Cl-IAA is important in maintaining the pericarp ethylene biosynthesis at optimum levels during normal pea fruit development (Table 5.1). Also, if the ethylene precursor ACC acts as a signaling molecule (Yoon and Kieber, 2013) and/or if ACO proteins have additional regulatory roles (Dilley et al., 2013), the modulation of pericarp ACC level and ACO protein pools by 4-Cl-IAA may have effects associated with pericarp development. Furthermore, 4-Cl-IAA-mediated stimulation of pericarp *PsERS1*, *PsETR2*, *PsEBF1* and *PsEBF2* transcript abundance (Chapter 2), together with the ethylene action inhibitory effect of 4-Cl-IAA (Johnstone et al., 2005) indicate that 4-Cl-IAA but not IAA could also be important in regulating the ethylene signaling or ethylene action during pericarp development (Table 5.1). The 4-Cl-IAA stimulation of GA biosynthesis and the modulation of GA levels in the pericarp, which was also not seen with IAA, is also believed to play a pivotal role in 4-Cl-IAA-mediated pericarp development (Ozga et al., 2009).

Table 5.1. Factors associated with the 4-Cl-IAA stimulation of pericarp development

	Level of Action	*Action*	*Known or putative effect*
Effects supported by evidence	Auxin perception	4-Cl-IAA suppresses transcript abundance of auxin receptor *PsTIR1b* (Chapter 3) and putative receptor *PsAFB6* (Chapter 4 and Nadeau, 2009)	Modulation of receptor composition may initiate 4-Cl-IAA-specific gene expression. Suppression of *PsAFB6* may also have additional effects preventing pericarp senescence
	Ethylene biosynthesis	Suppresses *PsACS4*, *PsACO2* and *PsACO3* transcript abundance. Stimulates *PsACS1* and *PsACO1* transcript abundance and pericarp ACC level (Chapter 2).	Regulation of ethylene biosynthesis gene transcript levels and ACC level may maintain ethylene biosynthesis at optimum levels.
	Ethylene Signaling	Stimulates ethylene receptor and signaling-related genes *PsERS1*, *PsETR2*, *PsEBF1*, and *PsEBF2* transcript abundance (Chapter 2)	Suppresses ethylene signaling (Chapter 2) and the inhibitory action of ethylene on pericarp development (Johnstone et al., 2005)
	GA biosynthesis	Increases GA20-oxidase 1 and GA 3-oxidase 1 gene expression. Increases the production of bioactive GA_1 and modulates its half-life (Ozga et al., 2009)	Increased pericarp GA levels stimulate pericarp development (Ozga et al., 1992)
Hypothetical effects	Auxin perception	Modulates pericarp Aux/IAA co-receptor abundance	Initiates 4-Cl-IAA-specific gene expression
	Unknown	Modulates pericarp ACO protein pool and ACC level	ACO proteins and ACC may have additional regulatory roles associated with pericarp development

References

Abel S, Theologis A (1996) Early genes and auxin action. Plant Physiol **111**: 9–17

Abel S, Theologis A (2010) Odyssey of auxin. Cold Spring Harb Perspect Biol. doi: 10.1101/cshperspect.a004572

Abeles FB, Morgan PW, Jr. MES (1992) The biosynthesis of ethylene. *In* FB Abeles, PW Morgan, ME Saltveit, eds, Ethyl. Plant Biol., Second Edi. Academic Press, New York, pp 26–55

Abeles FB, Morgan PW, Saltveit Jr. ME (1992) Chapter 1 - Introduction and historical perspectives. *In* FB Abeles, PW Morgan, ME Saltveit, eds, Ethyl. Plant Biol., Second Edi. Academic Press, New York, pp 1–13

Abràmoff MD, Magalhães PJ, Ram SJ (2004) Image processing with ImageJ. Biophotonics Int **11**: 36–42

Achard P, Baghour M, Chapple A, Hedden P, Van Der Straeten D, Genschik P, Moritz T, Harberd NP (2007) The plant stress hormone ethylene controls floral transition via DELLA-dependent regulation of floral meristem-identity genes. Proc Natl Acad Sci USA **104**: 6484–6489

Alberta Agriculture and Forestry (2016) Herbicide group classification by mode of action. http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/prm6487

Argueso CT, Hansen M, Kieber JJ (2007) Regulation of ethylene biosynthesis. J Plant Growth Regul **26**: 92–105

Audran-Delalande C, Bassa C, Mila I, Regad F, Zouine M, Bouzayen M (2012) Genome-wide identification, functional analysis and expression profiling of the Aux/IAA gene family in tomato. Plant Cell Physiol **53**: 659–672

Ayele BT, Ozga JA, Kurepin L V, Reinecke DM (2006) Developmental and embryo axis regulation of gibberellin biosynthesis during germination and young seedling growth of pea. Plant Physiol **142**: 1267–1281

Bakshi A, Shemansky J, Chang C, Binder B (2015) History of research on the plant hormone ethylene. J Plant Growth Regul 1–19

Ben-Gera H, Shwartz I, Shao M-R, Shani E, Estelle M, Ori N (2012) ENTIRE and GOBLET promote leaflet development in tomato by modulating auxin response. Plant J **70**: 903–915

Bent A (2006) *Arabidopsis thaliana* Floral dip transformation method. *In* K Wang, ed, Agrobacterium Protoc. SE - 7. Humana Press, pp 87–104

Beveridge CA, Ross JJ, Murfet IC (1994) Branching mutant rms-2 in *Pisum sativum* (Grafting Studies and Endogenous Indole-3-Acetic Acid Levels). Plant Physiol **104**: 953–959

Bisson CS, Jones HA (1932) Changes accompanying fruit development in the garden pea. Plant Physiol **7**: 91

Booker MA, DeLong A (2015) Producing the ethylene signal: regulation and diversification of ethylene biosynthetic enzymes. Plant Physiol **169**: 42–50

Bradford KJ, Yang SF (1980) Xylem transport of 1-aminocyclopropane-1-carboxylic acid, an ethylene precursor, in waterlogged tomato plants. Plant Physiol **65**: 322–326

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72**: 248–254

Brunoud G, Wells DM, Oliva M, Larrieu A, Mirabet V, Burrow AH, Beeckman T, Kepinski S, Traas J, Bennett MJ, et al (2012) A novel sensor to map auxin response and distribution at high spatio-temporal resolution. Nature **482**: 103–106

Bui AQ, O'Neill SD (1998) Three 1-aminocyclopropane-1-carboxylate synthase genes regulated by primary and secondary pollination signals in orchid flowers. Plant Physiol **116**: 419–428

Bulens I, Van de Poel B, Hertog ML, De Proft MP, Geeraerd AH, Nicolaï BM (2011) Protocol: an updated integrated methodology for analysis of metabolites and enzyme activities of ethylene biosynthesis. Plant Methods **7**: 17

Bürstenbinder K, Sauter M (2012) Early events in the ethylene biosynthetic pathway - regulation of the pools of methionine and S-adenosylmethionine. *In* MT McManus, ed, Annu. Plant Rev. Plant Horm. Ethyl. Wiley-Blackwell, pp 19–52

Calderón Villalobos LIA, Lee S, De Oliveira C, Ivetac A, Brandt W, Armitage L, Sheard LB, Tan X, Parry G, Mao H, et al (2012) A combinatorial TIR1/AFB–Aux/IAA co-receptor system for differential sensing of auxin. Nat Chem Biol **8**: 477–485

Cervera M (2004) Histochemical and fluorometric assays for *uidA* (*GUS*) gene detection. Transgenic plants Methods Protoc. Springer, pp 203–213

Chae HS, Cho YG, Park MY, Lee MC, Eun MY, Kang BG, Kim WT (2000) Hormonal cross-talk between auxin and ethylene differentially regulates the expression of two members of the 1-aminocyclopropane-1-carboxylate oxidase gene family in rice (*Oryza sativa* L.). Plant Cell Physiol **41**: 354–362

Cheng Y, Zhao Y (2007) A role for auxin in flower development. J Integr Plant Biol **49**: 99–104

Ciardi J, Klee H (2001) Regulation of ethylene-mediated responses at the level of the receptor. Ann Bot **88**: 813–822

Cobb A, Reade J (2010) Auxin-type herbicides. Herbic. Plant Physiol., Second edi. Wiley-Blackwell, pp 133–156

Concepcion M, Lizada C, Yang SF (1979) A simple and sensitive assay for 1-aminocyclopropane-1-carboxylic acid. Anal Biochem **100**: 140–145

Cook SD, Nichols DS, Smith J, Chourey PS, McAdam EL, Quittenden LJ, Ross JJ (2016) Auxin biosynthesis: are the indole-3-acetic acid and phenylacetic acid biosynthesis pathways mirror images? Plant Physiol.

Cooper DC (1938) Embryology of *Pisum sativum*. Bot Gaz **100**: 123–132

Cui L, Zhang T, Li J, Lou Q, Chen J (2014) Cloning and expression analysis of Cs-TIR1/AFB2: the fruit development-related genes of cucumber (*Cucumis sativus* L.). Acta Physiol Plant **36**: 139–149

Cutforth HW, McGinn SM, McPhee KE, Miller PR (2007) Adaptation of pulse crops to the changing climate of the northern Great Plains. Agron J **99**: 1684–1699

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible W-R (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in arabidopsis. Plant Physiol **139**: 5–17

Dai X, Mashiguchi K, Chen Q, Kasahara H, Kamiya Y, Ojha S, DuBois J, Ballou D, Zhao Y (2013) The biochemical mechanism of auxin biosynthesis by an arabidopsis YUCCA flavin-containing monooxygenase. J Biol Chem **288**: 1448–1457

Darwin C, Darwin F (1881) The power of movement in plants. John Murray, London

de Jong M, Wolters-Arts M, Feron R, Mariani C, Vriezen WH (2009) The *Solanum lycopersicum* auxin response factor 7 (SlARF7) regulates auxin signaling during tomato fruit set and development. Plant J. doi: 10.1111/j.1365-313X.2008.03671.x

De Martinis D, Mariani C (1999) Silencing gene expression of the ethylene-forming enzyme results in a reversible inhibition of ovule development in transgenic tobacco plants. Plant Cell **11**: 1061–1071

DeMason DA, Polowick PL (2009) Patterns of *DR5::GUS* expression in organs of pea (*Pisum sativum*). Int J Plant Sci **170**: 1–11

Devoghalaere F, Doucen T, Guitton B, Keeling J, Payne W, Ling TJ, Ross JJ, Hallett IC, Gunaseelan K, Dayatilake GA, et al (2012) A genomics approach to understanding the role of auxin in apple (*Malus x domestica*) fruit size control. BMC Plant Biol **12**: 1–15

Dharmasiri N, Dharmasiri S, Estelle M (2005) The F-box protein TIR1 is an auxin receptor. Nature **435**: 441–445

Dharmasiri N, Dharmasiri S, Weijers D, Lechner E, Yamada M, Hobbie L, Ehrismann JS, Jürgens G, Estelle M (2005) Plant development is regulated by a family of auxin receptor F box proteins. Dev Cell **9**: 109–119

Dilley DR, Wang Z, Kadirjan-Kalbach DK, Ververidis F, Beaudry R, Padmanabhan K (2013) 1-Aminocyclopropane-1-carboxylic acid oxidase reaction mechanism and putative post-translational activities of the ACCO protein. AoB Plants. doi: 10.1093/aobpla/plt031

Doerner P (2008) Phenotypic analysis of arabidopsis mutants: Quantitative analysis of root

growth. Cold Spring Harb Protoc **2008**: pdb.prot4960

Dorling SJ, McManus MT (2012) The fate of ACC in higher plants. *In* MT McManus, ed, Annu. Plant Rev. Plant Horm. Ethyl. Wiley-Blackwell, pp 83–115

Dresselhaus T, Schneitz K (2014) The role of auxin for reproductive organ patterning and development. Auxin Its Role Plant Dev. Springer, pp 213–243

Duarte J, Rivière N, Baranger A, Aubert G, Burstin J, Cornet L, Lavaud C, Lejeune-Hénaut I, Martinant JP, Pichon JP, et al (2014) Transcriptome sequencing for high throughput SNP development and genetic mapping in pea. BMC Genomics. doi: 10.1186/1471-2164-15-126

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res **32**: 1792–1797

Eeuwens CJ, Schwabe WW (1975) Seed and pod wall development in *Pisum sativum,* L. in relation to extracted and applied hormones. J Exp Bot **26**: 1–14

El-Sharkawy I, Sherif S, El Kayal W, Jones B, Li Z, Sullivan AJ, Jayasankar S (2016) Overexpression of plum auxin receptor PslTIR1 in tomato alters plant growth, fruit development and fruit shelf-life characteristics. BMC Plant Biol **16**: 56

El-Sharkawy I, Sherif SM, Jones B, Mila I, Kumar PP, Bouzayen M, Jayasankar S (2014) TIR1-like auxin-receptors are involved in the regulation of plum fruit development. J Exp Bot. doi: 10.1093/jxb/eru279

Elzebroek ATG, Wind K (2008) Protein crops. Guid. to Cultiv. plants. CABI, pp 226–262

English PJ, Lycett GW, Roberts JA, Jackson MB (1995) Increased 1-aminocyclopropane-1-carboxylic acid oxidase activity in shoots of flooded tomato plants raises ethylene production to physiologically active levels. Plant Physiol **109**: 1435–1440

Epstein E, Ludwig-Müller J (1993) Indole-3-butyric acid in plants: occurrence, synthesis, metabolism and transport. Physiol Plant **88**: 382–389

Ernstsen A, Sandberg G (1986) Identification of 4-chloroindole-3-acetic acid and indole-3-aldehyde in seeds of *Pinus sylvestris*. Physiol Plant **68**: 511–518

Ferraro KS (2014) Comparative transcriptomics and proanthocyanidin metabolism in pea (*Pisum sativum*) seed coat. PhD thesis, University of Calgary, Calgary, Alberta

Food and Agriculture Organization of the United Nations (2016) *Pisum sativum* L. Grassl. species profiles, http://www.fao.org/ag/AGp/agpc/doc/Gbase/data/Pf000493.HTM

Franssen SU, Shrestha RP, Bräutigam A, Bornberg-Bauer E, Weber APM (2011) Comprehensive transcriptome analysis of the highly complex *Pisum sativum* genome using next generation sequencing. BMC Genomics. doi: 10.1186/1471-2164-12-227

Gagne JM, Smalle J, Gingerich DJ, Walker JM, Yoo S-D, Yanagisawa S, Vierstra RD

(2004) Arabidopsis EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. Proc Natl Acad Sci USA **101**: 6803–6808

Gao Y, Zhang Y, Zhang D, Dai X, Estelle M, Zhao Y (2015) Auxin binding protein 1 (ABP1) is not required for either auxin signaling or arabidopsis development. Proc Natl Acad Sci U S A **112**: 2275–80

Gao Y, Zhao Y (2014) Auxin biosynthesis and catabolism. *In* E Zažímalová, J Petrášek, E Benková, eds, Auxin Its Role Plant Dev. Springer Vienna, Vienna, pp 21–38

García-Martínez JL, Carbonell J (1980) Fruit-set of unpollinated ovaries of *Pisum sativum* L. Planta **147**: 451–456

Gélinas-Marion A, Ross J (2016) Auxin receptors and seed development in pea. 22nd Int. Conf. Plant Growth Subst.

Gianfagna T (1995) Natural and synthetic growth regulators and their use in horticultural and agronomic crops. Plant Horm. Springer, pp 751–773

Goda H, Sawa S, Asami T, Fujioka S, Shimada Y, Yoshida S (2004) Comprehensive comparison of auxin-regulated and brassinosteroid-regulated genes in Arabidopsis. Plant Physiol **134**: 1555–1573

Greenham K, Santner A, Castillejo C, Mooney S, Sairanen I, Ljung K, Estelle M (2011) RETRACTED: The AFB4 auxin receptor is a negative regulator of auxin signaling in seedlings. Curr Biol **21**: 520–525

Greenham K, Santner A, Castillejo C, Mooney S, Sairanen I, Ljung K, Estelle M (2015) Retraction Notice to: The AFB4 auxin receptor is a negative regulator of auxin signaling in seedlings. Curr Biol **25**: 819

Gregory RP (1903) The seed characters of *Pisum sativum*. New Phytol **2**: 226–228

Grierson D (2012) 100 years of ethylene—a personal view. *In* MT McManus, ed, Annu. Plant Rev. Plant Horm. Ethyl. John Wiley & Sons, pp 1–17

Grones P, Friml J (2015) Auxin transporters and binding proteins at a glance. J Cell Sci **128**: 1–7

Grossmann K (2010) Auxin herbicides: current status of mechanism and mode of action. Pest Manag Sci **66**: 113–120

Gudynaite-Savitch L, Johnson DA, Miki BLA (2009) Strategies to mitigate transgene–promoter interactions. Plant Biotechnol J **7**: 472–485

Gustafson FG (1936) Inducement of Fruit Development by Growth-Promoting Chemicals. Proc Natl Acad Sci U S A **22**: 628–636

Gustafson FG (1939) Auxin distribution in fruits and its significance in fruit development. Am J

Bot **26**: 189–194

Gustafson FG (1942) Parthenocarpy: Natural and Artificial. Bot Rev **8**: 599–654

Guzmán P, Ecker JR (1990) Exploiting the triple response of arabidopsis to identify ethylene-related mutants. Plant Cell **2**: 513–523

Harpaz-Saad S, Yoon GM, Mattoo AK, Kieber JJ (2012) The Formation of ACC and competition between polyamines and ethylene for SAM. *In* MT McManus, ed, Annu. Plant Rev. Plant Horm. Ethyl. Wiley-Blackwell, pp 53–81

Havens KA, Guseman JM, Jang SS, Pierre-Jerome E, Bolten N, Klavins E, Nemhauser JL (2012) A synthetic approach reveals extensive tunability of auxin signaling. Plant Physiol **160**: 135–142

Heisler MG, Ohno C, Das P, Sieber P, Reddy G V, Long JA, Meyerowitz EM (2005) Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the arabidopsis inflorescence meristem. Curr Biol **15**: 1899–1911

Hoffman NE, Yang SF (1980) Changes of 1-aminocyclopropane-1-carboxylic acid content in ripening fruits in relation to their ethylene production. J Amer Soc Hort Sci **105**: 492–495

Hu Z, Keçeli MA, Piisilä M, Li J, Survila M, Heino P, Brader G, Palva ET, Li J (2012) F-box protein AFB4 plays a crucial role in plant growth, development and innate immunity. Cell Res **22**: 777–781

Hua J (2015) Isolation of components involved in ethylene signaling. Ethyl. Plants. Springer, pp 27–44

Hua J, Meyerowitz EM (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell **94**: 261–271

Hua J, Sakai H, Nourizadeh S, Chen QG, Bleecker AB, Ecker JR, Meyerowitz EM (1998) EIN4 and ERS2 are members of the putative ethylene receptor gene family in arabidopsis. Plant Cell **10**: 1321–1332

Huang, Madan A (1999) CAP3: A DNA sequence assembly program. Genome Res **9**: 868–877

Hull GA, Devic M (1995) The *β-glucuronidase* (*gus*) reporter gene system gene fusions; spectrophotometric, fluorometric, and histochemical Ddetection. *In* H Jones, ed, Plant Gene Transf. Expr. Protoc. Springer New York, Totowa, NJ, pp 125–141

Jefferson RA (1987) Assaying chimeric genes in plants: The *GUS* gene fusion system. Plant Mol Biol Report **5**: 387–405

Johnstone MG, Reinecke D, Ozga J (2005) The Auxins IAA and 4-Cl-IAA differentially modify gibberellin action via ethylene response in developing pea fruit. J Plant Growth Regul **24**: 214–225

Ju C, Yoon GM, Shemansky JM, Lin DY, Ying ZI, Chang J, Garrett WM, Kessenbrock M,

Groth G, Tucker ML, et al (2012) CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in Arabidopsis. Proc Natl Acad Sci USA **109**: 19486–19491

Jurado S, Abraham Z, Manzano C, López-Torrejón G, Pacios LF, Del Pozo JC (2010) The arabidopsis cell cycle F-box protein SKP2A binds to auxin. Plant Cell **22**: 3891–904

Jurado S, Triviño SD, Abraham Z, Manzano C, Gutierrez C, del Pozo C (2008) SKP2A protein, an F-box that regulates cell division, is degraded via the ubiquitin pathway. Plant Signal Behav **3**: 810–812

Kalluri UC, Difazio SP, Brunner AM, Tuskan GA (2007) Genome-wide analysis of Aux/IAA and ARF gene families in *Populus trichocarpa*. BMC Plant Biol. doi: 10.1186/1471-2229-7-59

Kaur S, Pembleton L, Cogan NOI, Savin KW, Leonforte T, Paull J, Materne M, Forster JW (2012) Transcriptome sequencing of field pea and faba bean for discovery and validation of SSR genetic markers. BMC Genomics. doi: 10.1186/1471-2164-13-104

Kepinski S, Leyser O (2005) The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature **435**: 446–451

Kevany BM, Tieman DM, Taylor MG, Cin VD, Klee HJ (2007) Ethylene receptor degradation controls the timing of ripening in tomato fruit. Plant J **51**: 458–467

Khodakovskaya M, Zhao D, Smith W, Li Y, McAvoy R (2006) Expression of ipt gene controlled by an ethylene and auxin responsive fragment of the LEACO1 promoter increases flower number in transgenic *Nicotiana tabacum*. Plant Cell Rep **25**: 1181–1192

Klee HJ, Giovannoni JJ (2011) Genetics and control of tomato fruit ripening and quality attributes. Annu Rev Genet **45**: 41–59

Knight LI, Crocker W (1913) Toxicity of smoke. Bot Gaz **55**: 337–371

Konishi M, Yanagisawa S (2008) Ethylene signaling in Arabidopsis involves feedback regulation via the elaborate control of EBF2 expression by EIN3. Plant J **55**: 821–831

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol **33**: 1870–1874

Lam HK, McAdam, Scott AM, McAdam EL, Ross JJ (2015) Evidence that chlorinated auxin is restricted to the Fabaceae but not to the Fabeae. Plant Physiol **168**: pp.00410.2015

Lashbrook CC, Tieman DM, Klee HJ (1998) Differential regulation of the tomato ETR gene family throughout plant development. Plant J **15**: 243–252

Lee DJ, Park JW, Lee HW, Kim J (2009) Genome-wide analysis of the auxin-responsive transcriptome downstream of iaa1 and its expression analysis reveal the diversity and complexity of auxin-regulated gene expression. J Exp Bot **60**: 3935–3957

Lee S, Sundaram S, Armitage L, Evans JP, Hawkes T, Kepinski S, Ferro N, Napier RM (2014) Defining binding efficiency and specificity of auxins for SCFTIR1/AFB-Aux/IAA co-receptor complex formation. ACS Chem Biol **9**: 673–682

Lelièvre J-M, Latchè A, Jones B, Bouzayen M, Pech J-C (1997) Ethylene and fruit ripening. Physiol Plant **101**: 727–739

Li W, Ma M, Feng Y, Li H, Wang Y, Ma Y, Li M, An F, Guo H (2015) EIN2-directed translational regulation of ethylene signaling in arabidopsis. Cell **163**: 670–683

Ligerot Y, Germain AD Saint, Citerne S, Kadakia N, Novaretti R, Pillot J-P, Troadec C, Waldie T, Aubert G, Debellé F, et al (2016) The pea branching RMS2 gene encodes the PsAFB4/5 auxin receptor and is involved in an auxin- strigolactone regulation loop. 22nd Int. Conf. Plant Growth Subst.

Lim TK (2012) *Pisum sativum*. Edible Med. Non-Medicinal Plants. Springer, pp 849–866

Liu Y, Zhang S (2004) Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in Arabidopsis. Plant Cell **16**: 3386–3399

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25**: 402–8

Llop-Tous I, Barry CS, Grierson D (2000) Regulation of ethylene biosynthesis in response to pollination in tomato flowers. Plant Physiol **123**: 971–978

Ludwig-Muller J (2011) Auxin conjugates: their role for plant development and in the evolution of land plants. J Exp Bot **62**: 1757–1773

Lulsdorf MM, Yuan HY, Slater SMH, Vandenberg A, Han X, Zaharia LI, Abrams SR (2013) Endogenous hormone profiles during early seed development of C. arietinum and C. anatolicum. Plant Growth Regul **71**: 191–198

Magnus V, Ozga JA, Reinecke DM, Pierson GL, Larue TA, Cohen JD, Brenner ML (1997) 4-chloroindole-3-acetic and indole-3-acetic acids in *Pisum sativum*. Phytochemistry **46**: 675–681

Martin MN, Cohen JD, Saftner RA (1995) A new 1-aminocyclopropane-1-carboxylic acid-conjugating activity in tomato fruit. Plant Physiol **109**: 917–926

Marumo S, Hattori H, Abe H, Munakata K (1968) Isolation of 4-chloroindolyl-3-acetic acid from immature seeds of *Pisum sativum*. Nature **219**: 959–960

Matarasso N, Schuster S, Avni A (2005) A novel plant cysteine protease has a dual function as a regulator of 1-aminocyclopropane-1-carboxylic acid synthase gene expression. Plant Cell **17**: 1205–1216

McKee HS, Robertson RN, Lee JB (1955) Physiology of pea fruits I. The developing fruit. Aust J Biol Sci **8**: 137–163

Mellor N, Band LR, Pencik A, Novak O, Rashed A, Holman T, Wilson MH, Voss U, Bishopp A, King JR, et al (2016) Dynamic regulation of auxin oxidase and conjugating enzymes AtDAO1 and GH3 modulates auxin homeostasis. Proc Natl Acad Sci U S A **113**: 11022–11027

Merchante C, Alonso JM, Stepanova AN (2013) Ethylene signaling: simple ligand, complex regulation. Curr Opin Plant Biol **16**: 554–60

Merchante C, Brumos J, Yun J, Hu Q, Spencer KR, Enríquez P, Binder BM, Heber S, Stepanova AN, Alonso JM (2015) Gene-specific translation regulation mediated by the hormone-signaling molecule EIN2. Cell **163**: 684–697

Muday GK, Rahman A, Binder BM (2012) Auxin and ethylene: collaborators or competitors? Trends Plant Sci **17**: 181–195

Muday KG (2001) Auxins and tropisms. J Plant Growth Regul **20**: 226–243

Nadeau CD (2009) Hormone metabolism and action in developing pea fruit. University of Alberta

Nagpal P, Ellis CM, Weber H, Ploense SE, Barkawi LS, Guilfoyle TJ, Hagen G, Alonso JM, Cohen JD, Farmer EE, et al (2005) Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. Development **132**: 4107–4118

Nonhebel HM (2015) Tryptophan-Independent Indole-3-Acetic Acid Synthesis: Critical Evaluation of the Evidence. Plant Physiol **169**: 1001–1005

Nordström A-C, Eliasson L (1991) Levels of endogenous indole-3-acetic acid and indole-3-acetylaspartic acid during adventitious root formation in pea cuttings. Physiol Plant **82**: 599–605

O'Malley RC, Ecker JR (2010) Linking genotype to phenotype using the Arabidopsis unimutant collection. Plant J **61**: 928–940

Okada K, Ueda J, Komaki MK, Bell CJ, Shimura Y (1991) Requirement of the auxin polar transport system in early stages of Arabidopsis floral bud formation. Plant Cell **3**: 677–684

Okushima Y, Mitina I, Quach HL, Theologis A (2005) AUXIN RESPONSE FACTOR 2 (ARF2): a pleiotropic developmental regulator. Plant J **43**: 29–46

Orzáez D, Blay R, Granell A (1999) Programme of senescence in petals and carpels of *Pisum sativum* L. flowers and its control by ethylene. Planta **208**: 220–226

Overvoorde P, Fukaki H, Beeckman T (2010) Auxin control of root development. Cold Spring Harb Perspect Biol **2**: a001537

Overvoorde PJ, Okushima Y, Alonso JM, Chan A, Chang C, Ecker JR, Hughes B, Liu A, Onodera C, Quach H, et al (2005) Functional genomic analysis of the AUXIN/INDOLE-3-ACETIC ACID Gene Family Members in *Arabidopsis thaliana*. Plant Cell **17**: 3282–3300

Ozga J, Reinecke D (1999) Interaction of 4-chloroindole-3-acetic acid and gibberellins in early pea fruit development. Plant Growth Regul **27**: 33–38

Ozga J, Reinecke D (2003) Hormonal interactions in fruit development. J Plant Growth Regul **22**: 73–81

Ozga JA, Brenner ML, Reinecke DM (1992) Seed effects on gibberellin metabolism in pea pericarp . Plant Physiol **100**: 88–94

Ozga JA, Reinecke DM, Ayele BT, Ngo P, Nadeau C, Wickramarathna AD (2009) Developmental and hormonal regulation of gibberellin biosynthesis and catabolism in pea fruit. Plant Physiol **150**: 448–462

Ozga JA, van Huizen R, Reinecke DM (2002) Hormone and seed-specific regulation of pea fruit growth. Plant Physiol **128**: 1379–1389

Ozga JA, Yu J, Reinecke DM (2003) Pollination-, development-, and auxin-specific regulation of gibberellin 3β-hydroxylase gene expression in pea fruit and seeds. Plant Physiol **131**: 1137–1146

Parry G, Calderon-Villalobos LI, Prigge M, Peret B, Dharmasiri S, Itoh H, Lechner E, Gray WM, Bennett M, Estelle M (2009) Complex regulation of the TIR1/AFB family of auxin receptors. Proc Natl Acad Sci **106**: 22540–22545

Pattison RJ, Csukasi F, Zheng Y, Fei Z, van der Knaap E, Catala C (2015) Comprehensive tissue-specific transcriptome analysis reveals distinct regulatory programs during early tomato fruit development. Plant Physiol **168**: 1684–1701

Pech J-C, Latché A, Bouzayen M (2010) Ethylene biosynthesis. *In* PJ Davies, ed, Plant Horm. Biosynthesis, Signal Transduction, Action, Third edit. Springer Netherlands, pp 115–136

Peck S, Kende H (1995) Sequential induction of the ethylene biosynthetic enzymes by indole-3-acetic acid in etiolated peas. Plant Mol Biol **28**: 293–301

Peck SC, Kende H (1998*a*) A gene encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase produces two transcripts: elucidation of a conserved response. Plant J **14**: 573–581

Peck SC, Kende H (1998*b*) Differential regulation of genes encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase in etiolated pea seedlings: effects of indole-3-acetic acid, wounding, and ethylene. Plant Mol Biol **38**: 977–982

Pěnčík A, Simonovik B, Petersson S V, Henyková E, Simon S, Greenham K, Zhang Y, Kowalczyk M, Estelle M, Zažímalová E, et al (2013) Regulation of auxin homeostasis and gradients in Arabidopsis roots through the formation of the indole-3-acetic acid catabolite 2-oxindole-3-acetic acid. Plant Cell **25**: 3858–3870

Perrot-Rechenmann C (2010) Cellular responses to auxin: division versus expansion. Cold Spring Harb Perspect Biol. doi: 10.1101/cshperspect.a001446

Porco S, Pěnčík A, Rashed A, Voß U, Casanova-Sáez R, Bishopp A, Golebiowska A,

Bhosale R, Swarup R, Swarup K, et al (2016) Dioxygenase-encoding AtDAO1 gene controls IAA oxidation and homeostasis in Arabidopsis. Proc Natl Acad Sci U S A **113**: 11016–11021

Potuschak T, Lechner E, Parmentier Y, Yanagisawa S, Grava S, Koncz C, Genschik P (2003) EIN3-dependent regulation of plant Eethylene hormone signaling by two Arabidopsis F box proteins. Cell **115**: 679–689

Przemeck GKH, Mattsson J, Hardtke CS, Sung ZR, Berleth T (1996) Studies on the role of the Arabidopsis gene MONOPTEROS in vascular development and plant cell axialization. Planta **200**: 229–237

Prigge M, Greenham K, Zhang Y, Santner A, Castillejo C, Mutka AM, O'Malley RC, Ecker JR, Kunkel BN, Estelle M (2016) The Arabidopsis auxin receptor F-box proteins AFB4 and AFB5 are required for response to the synthetic auxin picloram. G3 (Bethesda). doi: 10.1534/g3.115.025585

Pulse Canada (2016) Pulse industry. http://www.pulsecanada.com/pulse-industry

Qiao H, Shen Z, Huang SC, Schmitz RJ, Urich MA, Briggs SP, Ecker JR (2012) Processing and subcellular trafficking of ER-Tethered EIN2 control response to ethylene gas. Sci **338**: 390–393

Reid MS (1995) Ethylene in plant growth, development, and senescence. Plant Horm. Springer, pp 486–508

Reid MS, Paul JL, Farhoomand MB, Kofranek AM, Staby GL (1980) Pulse treatments with the silver thiosulfate complex extend the vase life of cut carnations [*Dianthus caryophyllus*, Senescence]. J. Am. Soc. Hortic. Sci.

Reinecke D (1999) 4-Chloroindole-3-acetic acid and plant growth. Plant Growth Regul **27**: 3–13

Reinecke DM, Bandurski RS (1981) Metabolic conversion of 14C-indole-3-acetic acid to 14C-oxindole-3-acetic acid. Biochem Biophys Res Commun **103**: 429–433

Reinecke DM, Bandurski RS (1983) Oxindole-3-acetic Acid, an Indole-3-acetic Acid Catabolite in *Zea mays*. Plant Physiol **71**: 211–213

Reinecke DM, Ozga JA, Ilić N, Magnus V, Kojić-Prodić B (1999) Molecular properties of 4-substituted indole-3-acetic acids affecting pea pericarp elongation. Plant Growth Regul **27**: 39–48

Reinecke DM, Ozga JA, Magnus V (1995) Effect of halogen substitution of indole-3-acetic acid on biological activity in pea fruit. Phytochemistry **40**: 1361–1366

Reinecke DM, Wickramarathna AD, Ozga JA, Kurepin L V, Jin AL, Good AG, Pharis RP (2013) Gibberellin 3-oxidase gene expression patterns influence gibberellin biosynthesis, growth, and development in pea. Plant Physiol **163**: 929–945

Reinhardt D, Mandel T, Kuhlemeier C (2000) Auxin regulates the initiation and radial

position of plant lateral organs. Plant Cell **12**: 507–518

Ren Z, Li Z, Miao Q, Yang Y, Deng W, Hao Y (2011) The auxin receptor homologue in *Solanum lycopersicum* stimulates tomato fruit set and leaf morphogenesis. J Exp Bot. doi: 10.1093/jxb/erq455

Robles L, Stepanova A, Alonso J (2013) Molecular mechanisms of ethylene–auxin interaction. Mol Plant **6**: 1734–1737

Roje S (2006) S-Adenosyl-l-methionine: beyond the universal methyl group donor. Phytochemistry **67**: 1686–1698

Ross ARS, Ambrose SJ, Cutler AJ, Feurtado JA, Kermode AR, Nelson K, Zhou R, Abrams SR (2004) Determination of endogenous and supplied deuterated abscisic acid in plant tissues by high-performance liquid chromatography-electrospray ionization tandem mass spectrometry with multiple reaction monitoring. Anal Biochem **329**: 324–333

Ruan YL, Patrick JW, Bouzayen M, Osorio S, Fernie AR (2012) Molecular regulation of seed and fruit set. Trends Plant Sci **17**: 656–665

Ruduś I, Sasiak M, Kępczyński J (2013) Regulation of ethylene biosynthesis at the level of 1-aminocyclopropane-1-carboxylate oxidase (ACO) gene. Acta Physiol Plant **35**: 295–307

Salehin M, Bagchi R, Estelle M (2015) SCFTIR1/AFB-based auxin perception: mechanism and role in plant growth and development. Plant Cell **27**: 9–19

Sauer M, Robert S, Kleine-Vehn J (2013) Auxin: simply complicated. J. Exp. Bot.

Schlicht M, Ludwig-Müller J, Burbach C, Volkmann D, Baluska F (2013) Indole-3-butyric acid induces lateral root formation via peroxisome-derived indole-3-acetic acid and nitric oxide. New Phytol **200**: 473–482

Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, Schölkopf B, Weigel D, Lohmann JU (2005) A gene expression map of *Arabidopsis thaliana* development. Nat Genet. doi: 10.1038/ng1543

Sebastià CH, Hardin SC, Clouse SD, Kieber JJ, Huber SC (2004) Identification of a new motif for CDPK phosphorylation in vitro that suggests ACC synthase may be a CDPK substrate. Arch Biochem Biophys **428**: 81–91

Serek M, Sisler EC, Reid MS (1995) Effects of 1-MCP on the vase life and ethylene response of cut flowers. Plant Growth Regul **16**: 93–97

Sessions A, Nemhauser JL, McColl A, Roe JL, Feldmann KA, Zambryski PC (1997) ETTIN patterns the Arabidopsis floral meristem and reproductive organs. Development **124**: 4481–4491

Sessions RA, Zambryski PC (1995) Arabidopsis gynoecium structure in the wild and in ettin mutants. Development **121**: 1519–1532

Shaw J-F, Chou Y-S, Chang R-C, Yang SF (1996) Characterization of the ferrous ion binding sites of apple 1-aminocyclopropane-1-carboxylate oxidase by site-directed mutagenesis. Biochem Biophys Res Commun **225**: 697–700

Shimizu-Mitao Y, Kakimoto T (2014) Auxin sensitivities of all Arabidopsis Aux/IAAs for degradation in the presence of every TIR1/AFB. Plant Cell Physiol **55**: 1450–1459

Simon S, Petrášek J (2011) Why plants need more than one type of auxin. Plant Sci **180**: 454–460

Simossis VA, Heringa J (2003) The PRALINE online server: optimising progressive multiple alignment on the web. Comput Biol Chem **27**: 511–519

Simossis VA, Heringa J (2005) PRALINE: a multiple sequence alignment toolbox that integrates homology-extended and secondary structure information. Nucleic Acids Res **33**: W289–W294

Singh VK, Jain M (2015) Genome-wide survey and comprehensive expression profiling of Aux/IAA gene family in chickpea and soybean. Front Plant Sci **6**: 918

Sinjushin A (2013) Mutation genetics of pea (*Pisum sativum* L.): What is done and what is left to do. Ratar i Povrt **50**: 36–43

Smýkal P (2014) Pea (*Pisum sativum* L.) in biology prior and after Mendel's discovery. Czech J Genet Plant Breed **50**: 52–64

Smýkal P, Aubert G, Burstin J, Coyne CJ, Ellis NTH, Flavell AJ, Ford R, Hýbl M, Macas J, Neumann P (2012) Pea (*Pisum sativum* L.) in the genomic era. Agronomy **2**: 74–115

Soeno K, Goda H, Ishii T, Ogura T, Tachikawa T, Sasaki E, Yoshida S, Fujioka S, Asami T, Shimada Y (2010) Auxin biosynthesis inhibitors, identified by a genomics-based approach, provide insights into auxin biosynthesis. Plant Cell Physiol **51**: 524–536

Song Y, Wang L, Xiong L (2009) Comprehensive expression profiling analysis of OsIAA gene family in developmental processes and in response to phytohormone and stress treatments. Planta **229**: 577–591

Stasinopoulos TC, Hangarter RP (1990) Preventing photochemistry in culture media by long-pass light filters alters growth of cultured tissues. Plant Physiol **93**: 1365–1369

Stepanova AN, Yun J, Likhacheva A V, Alonso JM (2007) Multilevel interactions between ethylene and auxin in Arabidopsis roots. Plant Cell **19**: 2169–2185

Stolárik T, Henselová M, Martinka M, Novák O, Zahoranová A, Černák M (2015) Effect of low-temperature plasma on the structure of seeds, growth and metabolism of endogenous phytohormones in pea (*Pisum sativum* L.). Plasma Chem Plasma Process **35**: 659–676

Strader LC, Beisner ER, Bartel B (2009) Silver ions increase auxin efflux independently of effects on ethylene response. Plant Cell **21**: 3585–3590

Strader LC, Wheeler DL, Christensen SE, Berens JC, Cohen JD, Rampey RA, Bartel B (2011) Multiple facets of Arabidopsis seedling development require indole-3-butyric acid-derived auxin. Plant Cell **23**: 984–999

Sudheesh S, Sawbridge TI, Cogan NOI, Kennedy P, Forster JW, Kaur S (2015) De novo assembly and characterisation of the field pea transcriptome using RNA-Seq. BMC Genomics **16**: 1–15

Sudi J (1964) Induction of the formation of complexes between aspartic acid and indolyl-3-acetic acid or I-naphthalene acetic acid by other carboxylic acids. Nature **201**: 1009–1010

Sugawara S, Mashiguchi K, Tanaka K, Hishiyama S, Sakai T, Hanada K, Kinoshita-Tsujimura K, Yu H, Dai X, Takebayashi Y, et al (2015) Distinct characteristics of indole-3-acetic acid and phenylacetic acid, two common auxins in plants. Plant Cell Physiol **56**: 1–14

Sundberg E, Ostergaard L (2009) Distinct and dynamic auxin activities during reproductive development. Cold Spring Harb Perspect Biol **1**: a001628

Swarup R, Perry P, Hagenbeek D, Van Der Straeten D, Beemster GTS, Sandberg G, Bhalerao R, Ljung K, Bennett MJ (2007) Ethylene upregulates auxin biosynthesis in Arabidopsis seedlings to enhance inhibition of root cell elongation. Plant Cell **19**: 2186–96

Tan X, Calderon-Villalobos LIA, Sharon M, Zheng C, Robinson C V, Estelle M, Zheng N (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature **446**: 640–645

Tian Q, Uhlir NJ, Reed JW (2002) Arabidopsis SHY2/IAA3 inhibits auxin-regulated gene expression. Plant Cell **14**: 301–319

Tivendale ND, Davidson SE, Davies NW, Smith JA, Dalmais M, Bendahmane AI, Quittenden LJ, Sutton L, Bala RK, Le Signor C, et al (2012) Biosynthesis of the halogenated auxin, 4-chloroindole-3-acetic acid. Plant Physiol **159**: 1055–1063

Tivendale ND, Ross JJ, Cohen JD (2014) The shifting paradigms of auxin biosynthesis. Trends Plant Sci **19**: 44–51

Tsang DL, Edmond C, Harrington JL, Nuhse TS (2011) Cell wall integrity controls root elongation via a general 1-aminocyclopropane-1-carboxylic acid-dependent, ethylene-independent pathway. Plant Physiol **156**: 596–604

Tsuchisaka A, Theologis A (2004) Unique and overlapping expression patterns among the Arabidopsis 1-amino-cyclopropane-1-carboxylate synthase gene family members. Plant Physiol **136** : 2982–3000

Tudela D, Primo-Millo E (1992) 1-Aminocyclopropane-1-carboxylic acid transported from roots to shoots promotes leaf abscission in Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) seedlings rehydrated after water stress. Plant Physiol **100**: 131–137

Ulmasov T, Liu ZB, Hagen G, Guilfoyle TJ (1995) Composite structure of auxin response

elements. Plant Cell **7**: 1611–1623

Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell **9**: 1963–1971

Van de Poel B, Bulens I, Markoula A, Hertog MLATM, Dreesen R, Wirtz M, Vandoninck S, Oppermann Y, Keulemans J, Hell R, et al (2012) Targeted systems biology profiling of tomato fruit reveals coordination of the Yang cycle and a distinct regulation of ethylene biosynthesis during postclimacteric ripening. Plant Physiol **160**: 1498–1514

van Huizen R, Ozga JA, Reinecke DM, Twitchin B, Mander LN (1995) Seed and 4-chloroindole-3-acetic acid regulation of gibberellin metabolism in pea pericarp. Plant Physiol **109**: 1213–1217

Vandenbussche F, Van Der Straeten D (2012) The role of ethylene in plant growth and development. *In* MT McManus, ed, Annu. Plant Rev. Plant Horm. Ethyl. Wiley-Blackwell, pp 219–241

Vanneste S, Friml J (2009) Auxin: A trigger for change in plant development. Cell **136**: 1005–1016

Vernoux T, Brunoud G, Farcot E, Morin V, Van den Daele H, Legrand J, Oliva M, Das P, Larrieu A, Wells D, et al (2011) The auxin signalling network translates dynamic input into robust patterning at the shoot apex. Mol Syst Biol **7**: 508

Vriezen WH, Feron R, Maretto F, Keijman J, Mariani C (2008) Changes in tomato ovary transcriptome demonstrate complex hormonal regulation of fruit set. New Phytol **177**: 60–76

Walsh TA, Neal R, Merlo AO, Honma M, Hicks GR, Wolff K, Matsumura W, Davies JP (2006) Mutations in an auxin receptor homolog AFB5 and in SGT1b confer resistance to synthetic picolinate auxins and not to 2,4-dichlorophenoxyacetic acid or indole-3-acetic acid in Arabidopsis. Plant Physiol **142**: 542–552

Wang H, Jones B, Li Z, Frasse P, Delalande C, Regad F, Chaabouni S, Latché A, Pech J-C, Bouzayen M (2005) The Tomato Aux/IAA Transcription Factor IAA9 Is Involved in Fruit Development and Leaf Morphogenesis. Plant Cell **17**: 2676–2692

Wang H, Schauer N, Usadel B, Frasse P, Zouine M, Hernould M, Latché A, Pech J-C, Fernie AR, Bouzayen M (2009) Regulatory features underlying pollination-dependent and -independent tomato fruit set revealed by transcript and primary metabolite profiling. Plant Cell **21**: 1428–1452

Wang H, Wang J, Jiang J, Chen S, Guan Z, Liao Y, Chen F (2014) Reference genes for normalizing transcription in diploid and tetraploid Arabidopsis. Sci Rep **4**: 6781

Wang R, Estelle M (2014) Diversity and specificity: auxin perception and signaling through the TIR1/AFB pathway. Curr Opin Plant Biol **21**: 51–58

Weller JL, Foo EM, Hecht V, Ridge S, Vander Schoor JK, Reid JB (2015) Ethylene Signaling Influences Light-Regulated Development in Pea. Plant Physiol **169**: 115–124

Wen X, Zhang C, Ji Y, Zhao Q, He W, An F, Jiang L, Guo H (2012) Activation of ethylene signaling is mediated by nuclear translocation of the cleaved EIN2 carboxyl terminus. Cell Res **22**: 1613–1616

Went F (1926) On growth-accelerating substances in the coleoptile of *Avena sativa*. Proc K Ned Akad van Wet **30**: 10–19

Went FW, Thimann K V. (1937) Phytohormones. Macmillan, New York, New York, USA

Wightman F, Lighty D (1982) Identification of phenylacetic acid as a natural auxin in the shoots of higher plants. Physiol Plant **55**: 17–24

Winter D, Vinegar B, Nahal H, Ammar R, Wilson G V, Provart NJ (2007) An "Electronic Fluorescent Pictograph" Browser for Exploring and Analyzing Large-Scale Biological Data Sets. PLoS One **2**: e718

Wu J, Peng Z, Liu S, He Y, Cheng L, Kong F, Wang J, Lu G (2012) Genome-wide analysis of Aux/IAA gene family in Solanaceae species using tomato as a model. Mol Genet Genomics **287**: 211–295

Xu J, Zhang S (2015) Ethylene Biosynthesis and Regulation in Plants. *In* C-K Wen, ed, Ethyl. Plants. Springer Netherlands, pp 1–25

Xu S-L, Rahman A, Baskin TI, Kieber JJ (2008) Two leucine-rich repeat receptor kinases mediate signaling, linking cell wall biosynthesis and ACC synthase in Arabidopsis. Plant Cell **20**: 3065–3079

Yamagami T, Tsuchisaka A, Yamada K, Haddon WF, Harden LA, Theologis A (2003) Biochemical diversity among the 1-amino-cyclopropane-1-carboxylate synthase isozymes encoded by the Arabidopsis gene family. J Biol Chem **278**: 49102–49112

Yang Y, Wu Y, Pirrello J, Regad F, Bouzayen M, Deng W, Li Z (2010) Silencing Sl-EBF1 and Sl-EBF2 expression causes constitutive ethylene response phenotype, accelerated plant senescence, and fruit ripening in tomato. J Exp Bot **61**: 697–708

Yoo S, Bomblies K, Yoo S, Yang J, Choi M, Lee J, Weigel D, Ahn J (2005) The 35S promoter used in a selectable marker gene of a plant transformation vector affects the expression of the transgene. Planta **221**: 523–530

Yoon GM, Kieber JJ (2013) 1-Aminocyclopropane-1-carboxylic acid as a signalling molecule in plants. AoB Plants. doi: 10.1093/aobpla/plt017

Yoshida H, Wang KL-C, Chang C-M, Mori K, Uchida E, Ecker JR (2006) The ACC synthase TOE sequence is required for interaction with ETO1 family proteins and destabilization of target proteins. Plant Mol Biol **62**: 427–437

Yuan S, Dean JD (2010) Differential responses of the promoters from nearly identical paralogs

of loblolly pine (*Pinus taeda* L.*)* ACC oxidase to biotic and abiotic stresses in transgenic *Arabidopsis thaliana*. Planta **232**: 873–886

Zhang J, Lin JE, Harris C, Campos Mastrotti Pereira F, Wu F, Blakeslee JJ, Peer WA (2016) DAO1 catalyzes temporal and tissue-specific oxidative inactivation of auxin in Arabidopsis thaliana. Proc Natl Acad Sci U S A **113**: 11010–11015

Zhang X, Henriques R, Lin S-S, Niu Q-W, Chua N-H (2006) Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method. Nat Protoc **1**: 641–646

Zhang Z, Ren J-S, Clifton IJ, Schofield CJ (2004) Crystal structure and mechanistic implications of 1-aminocyclopropane-1-carboxylic acid oxidase—the ethylene-forming enzyme. Chem Biol **11**: 1383–1394

Zhao Y (2012) Auxin biosynthesis: a simple two-step pathway converts tryptophan to indole-3-acetic acid in plants. Mol Plant **5**: 334–338

Zhao Z, Zhang Y, Liu X, Zhang X, Liu S, Yu X, Ren Y, Zheng X, Zhou K, Jiang L, et al (2013) A role for a dioxygenase in auxin metabolism and reproductive development in rice. Dev Cell **27**: 113–22

Zheng Y, Zhu Z (2016) Relaying the Ethylene Signal: New Roles for EIN2. Trends Plant Sci **21**: 2–4

Zhu J-H, Xu J, Chang W-J, Zhang Z-L (2015) Isolation and molecular characterization of 1-aminocyclopropane-1-carboxylic acid synthase genes in *Hevea brasiliensis*. Int J Mol Sci **16**: 4136–4149

Appendix A

Supplementary information for Chapter 2

A

```
   1 ATGGGTTTGG AAAATAATAG CCAGAAGCTT TTATCCAAGA TTGCTACCAA
  51 CAACAAACAT GGTGAAAATT CTCCTTACTT TGATGGATGG AAAGCTTATG
 101 AATCAAACCC ATTTCACCCC ACAAAAAATC CTCAAGGTGT TATCCAAATG
 151 GGTCTTGCTG AAAATCAGCT CTGTTTTGAT TTGATTGAAG AATGGATAAA
 201 GAATAATCCA AAAGCTTCCA TTTGTACTCC AGAAGGAGTC AATCAATTCA
 251 GACATATAGC AAACTTTCAA GACTATCATG GATTGCCAGA ATTCAAAAAT
 301 GCTGTGGCAA ATTTGATGTC AAAAGTGAGA GGTGGTAGGG TGAGATTTGA
 351 TCCTGACCGT TTATTGATGA GTGGAGGAGC AACTGGTGCA AATGAGTTAA
 401 TAATGTTCTG TTTGGCTGAT CCTGGTGATG CTTTTTTGGT ACCTAGCCCT
 451 TATTATCCAG CATTTGTCCG TGACTTGTGT TGGAGAACCG GTTTGCAACT
 501 AATTCCGGTC CAATGTCATA GTTCAAACAA TTTCATGATA ACAAGAGAAG
 551 CACTTGAAGA AGCTCATAAA AAGGCACAAG ACGAAAACAT CAATGTGAAA
 601 GGGTTAATCA TAACAAATCC ATCAAACCCT TTAGGAACAA CAATTGAAAA
 651 AGAAACACTC AAGAGCATAG TGAGTTTCAT CAATGAAAAC AACATTCATC
 701 TAGTGTGTGA CGAAATCTAT TCGGGTACCG TTTACGACAC CCCAAAATTC
 751 GTAAGTGTGT CCGAAATTAT ACAAGAAATG GAAGACATAA AAAAGGATCT
 801 CATTCATATC ATCTATAGTT TATCAAAAGA TATGGGGCTA CCGGGTTTTA
 851 GAGTTGGTTT AGTTTATTCG TACAACGACG AAGTTGTGAG CTGTGGTCGG
 901 AAAATGTCAA GTTTTGGGTT AGTCTCGTCG CAGACACAAT ATTTTCTCGC
 951 TACAATGCTA TCCGATGACA AATTCATGGA TAAGTTTTTA GCGGAGAGTT
1001 CAAGAAGGTT AAGGGCTCGG CGTGAGTTTT TCACAAAGGG ACTCGAAAAG
1051 GTTAACATAA CTTGCTTACC AAGTAATGCA GGACTATTCT TTTGGATGAA
1101 TTTGAGGAGT TTGTTAAAAG AGAAAACGTT CGAAGGCGAA ATGAAGCTTT
1151 GGCGATTGAT TATCAATGAG GTGAAGCTTA ATGTTTCGCC GGGTTCGGCT
1201 TTTGAATGCT CCGAACCGGG TTGGTACCGA GTTTGTTTTG CTAACATGGA
1251 TGAAGAAACT GTTGAGATTG CATTGATGAG AATTAGAGCA TTTGTTAATG
1301 GAAGAGAGAA AGGGGTGAAA AAAGTTGAAA TGAAACGTTG GAAGAGTAAT
1351 CTAAGACTTA GTTTTTCATC AAGAAGGTTT GAAGAGAATG TTATGTCTCC
1401 TCATTCACCA ATTCCTCATT CACCACTTGT TCGAGCAACT TAA
```

B

```
   1 MGLENNSQKL LSKIATNNKH GENSPYFDGW KAYESNPFHP TKNPQGVIQM
  51 GLAENQLCFD LIEEWIKNNP KASICTPEGV NQFRHIANFQ DYHGLPEFKN
 101 AVANLMSKVR GGRVRFDPDR LLMSGGATGA NELIMFCLAD PGDAFLVPSP
 151 YYPAFVRDLC WRTGLQLIPV QCHSSNNFMI TREALEEAHK KAQDENINVK
 201 GLIITNPSNP LGTTIEKETL KSIVSFINEN NIHLVCDEIY SGTVYDTPKF
 251 VSVSEIIQEM EDIKKDLIHI IYSLSKDMGL PGFRVGLVYS YNDEVVSCGR
 301 KMSSFGLVSS QTQYFLATML SDDKFMDKFL AESSRRLRAR REFFTKGLEK
 351 VNITCLPSNA GLFFWMNLRS LLKEKTFEGE MKLWRLIINE VKLNVSPGSA
 401 FECSEPGWYR VCFANMDEET VEIALMRIRA FVNGREKGVK KVEMKRWKSN
 451 LRLSFSSRRF EENVMSPHSP IPHSPLVRAT
```

Figure A1. **A)** The complete coding sequence of the putative ethylene biosynthesis gene *PsACS4* from *P. sativum* L. cv. I_3 (Alaska-type). Forward and reverse qRT-PCR primers and the probe binding sites are in green, blue and red, respectively. **B)** The predicted amino acid sequence of PsACS4 from *P. sativum* L. cv. I_3 (Alaska-type).

A

```
   1 ATGGAGAATT TCCCAATTAT CAACTTAGAG AACATCAATG GTGAGAAGAG
  51 AGAAGCTACT ATGGAGAAAA TCAAAGATGC TTGTGAAAAC TGGGGATTCT
 101 TTGAGCTGGT GAATCATGGC ATACCTCATG ACTTGATGGA CACCGTGGAG
 151 AGATTGACCA AAGAACACTA CAGGAAATGC ATGGAACAAA GATTCAAGGA
 201 ATTAGTAGCT GAGAAAGGGC TTGAAGCTGT TCAAACTGAG GTCAAAGACA
 251 TGGATTGGGA GAGTACCTTC CACTTGCGTC ACCTACCTGA GTCAAACATT
 301 TCAGAGATCC CTGATCTTAG TGATGAATAC AGGAAATCAA TGAAGGAATT
 351 TGCTTTGAGA CTAGAGAAAC TAGCAGAGGA GCTACTAGAC TTGTTATGTG
 401 AAAATCTTGG ACTAGAAAAA GGGTACCTCA AAAAGGCCTT CTATGGATCA
 451 AAAGGACCAA CTTTTGGCAC CAAGATAGCA AACTACCCTC CATGTCCAAA
 501 ACCCGATCTA GTGAAGGGTC TCCGCGCACA CACCGATGCC GGAGGAATCA
 551 TCCTCCTTTT CCAAGACGAC AAAGTCAGTG GCCTCCAACT TCTCAAAGAT
 601 GGCATCTGGG TAGATGTTCC ACCCATGCAT CATTCAATTG TCATCAACCT
 651 CGGTGACCAA CTAGAGGTAA TTACCAATGG TAAATACAAA AGTATAGAGC
 701 ACCGTGTGAT AGCACAAAGT GATGGAACAA GAATGTCCAT AGCCTCATTC
 751 TACAACCCTG GTAGTGACGC TGTTATCTAC CCAGCACCAA CATTGATAGA
 801 AGAAAACAAT GAAATTTACC CAAAATTTGT GTTTGAGGAT TACATGAAAC
 851 TCTACGCTGG GTTAAAGTTT CAGGCCAAAG AACCAAGATT TGAAGCATTT
 901 AAGGAATCAA ATGTTGTTAA TTTGGGACCA ATTGCAACAG TTTAA
```

B

```
   1 MENFPIINLE NINGEKREAT MEKIKDACEN WGFFELVNHG IPHDLMDTVE
  51 RLTKEHYRKC MEQRFKELVA EKGLEAVQTE VKDMDWESTF HLRHLPESNI
 101 SEIPDLSDEY RKSMKEFALR LEKLAEELLD LLCENLGLEK GYLKKAFYGS
 151 KGPTFGTKIA NYPPCPKPDL VKGLRAHTDA GGIILLFQDD KVSGLQLLKD
 201 GIWVDVPPMH HSIVINLGDQ LEVITNGKYK SIEHRVIAQS DGTRMSIASF
 251 YNPGSDAVIY PAPTLIEENN EIYPKFVFED YMKLYAGLKF QAKEPRFEAF
 301 KESNVVNLGP IATV
```

Figure A2. **A)** The complete coding sequence of the putative ethylene biosynthesis gene *PsACO2* from *P. sativum* L. cv. I_3 (Alaska-type). Forward and reverse qRT-PCR primers and the probe binding sites are in green, blue and red, respectively. **B)** The predicted amino acid sequence of PsACO2 from *P. sativum* L. cv. I_3 (Alaska-type).

A

```
   1 ATGATGAACT TCCCAATTAT CAGCTTGGAG AGAATCAATG GTGAGGAGAG
  51 AAAACACACC ATGGAAAAAA TAAAAGATGC TTGTGAGAAT TGGGGATTCT
 101 TTGAGTTGGT GAATCATGGC ATACCTCATG ACCTTATGGA CACACTGGAG
 151 AGGTTGACCA AAGAGCACTA TAGGAAATGC ATGGAGCAGA GGTTCAGGGA
 201 ATTTGTATCA AACAGAGGTC TAGATGCTGT CCAAACTGAG GTCAAAGATA
 251 TGGATTGGGA GAGCACCTTC CATGTTCGCC ACCTCCCGGA ATCAAACATT
 301 TCAGAGGTCC CTGATCTCAG TGATGAATAC AGGAAGGTGA TGAAGGAATT
 351 TGCTTTTAGG TTAGAGAAAC TAGCAGAAGA GCTTTTGGAC TTGTTATGTG
 401 AAAATCTAGG ACTAGAGAAA GGGTACCTCA AAAAGGCTTT CTATGGATCA
 451 AGAGGACCAA CTTTTGGAAC CAAGGTAGCC AACTACCCTC AGTGCCCCAA
 501 TCCAGAGTTG GTGAAGGGTC TTCGCGCTCA CACCGATGCT GGCGGGATCA
 551 TCCTTCTCTT CCAAGACGAC AAGGTCAGTG GCCTACAGCT TCTCAAAGAC
 601 GGCGAGTGGG TTGATGTTCC CCCAATGCGC CACTCCATTG TAGTCAACCT
 651 CGGTGATCAG CTCGAGGTTA TAACAAATGG TAAATACAAG AGTGTGGAGC
 701 ACCGTGTGAT AGCACAAACT AATGGAACAA GAATGTCCAT AGCCTCATTC
 751 TACAACCCTG GTTCTGATGC TGTAATCTAC CCTGCTCCAA AATTGTTGGA
 801 AAAAGAAACA GTGGAAAAAA ACAATGTGTA CCCAAAATTT GTGTTTGAAG
 851 AGTACATGAA GATCTATGCT GGTTTGAAAT TCCATGCTAA GGAACCAAGA
 901 TTTGAAGCCT TGAAAGGATC AAATGAAAAT TTGGGTCCAA TTGCAATTAT
 951 TTAA
```

B

```
   1 MMNFPIISLE RINGEERKHT MEKIKDACEN WGFFELVNHG IPHDLMDTLE
  51 RLTKEHYRKC MEQRFREFVS NRGLDAVQTE VKDMDWESTF HVRHLPESNI
 101 SEVPDLSDEY RKVMKEFAFR LEKLAEELLD LLCENLGLEK GYLKKAFYGS
 151 RGPTFGTKVA NYPQCPNPEL VKGLRAHTDA GGIILLFQDD KVSGLQLLKD
 201 GEWVDVPPMR HSIVVNLGDQ LEVITNGKYK SVEHRVIAQT NGTRMSIASF
 251 YNPGSDAVIY PAPKLLEKET VEKNNVYPKF VFEEYMKIYA GLKFHAKEPR
 301 FEALKGSNEN LGPIAII
```

Figure A3. **A)** The complete coding sequence of the putative ethylene biosynthesis gene *PsACO3* from *P. sativum* L. cv. I_3 (Alaska-type). Binding sites of the forward and reverse qRT-PCR primers and the probe (which were designed for the complementary strand) are in green, blue and red, respectively. **B)** The predicted amino acid sequence of PsACO3 from *P. sativum* L. cv. I_3 (Alaska-type).

A

```
   1 ATGGTAAAAG CAATAGTGTC TGTGCTGTTG ATTACCTCTA TCGTCTTGTG
  51 TGTATCTGCA TCGGATAATG GGTTTCCGAG ATGTAATTGC GACGATGAAG
 101 CGAGTTTGTG GACTATTGAG AGCATTTTGG AGTGTCAAAG AGTTGGTGAT
 151 TTCTTGATTG CTGTGGCCTA CTTCTCAATC CCTATTGAAC TGCTTTATTT
 201 TGTCAGTTGC TCCAACGTCC CTTTCAAATG GGTCCTTGTT CAGTTCATTG
 251 CCTTCATCGT ACTATGTGGA TTGACACATT TGTTGAATGG TTGGACTTAT
 301 GGTCCTCACA CTTTTCAGCT TATGGTGGCA CTTACTATCT TTAAGATTCT
 351 CACTGCGTTG GTGTCTTGTG CTACTGCGAT AACGCTTGTT ACTTTGATTC
 401 CTTTGCTTCT TAAAGTGAAG GTTAGAGAAT TCATGCTCAA GAAAAAGACG
 451 TGGGATCTTG GACGGGAGGT TGGTCTTATT ATGAAGCAAA AGGAGGCGGC
 501 GGTGCATGTA AGAATGCTTA CTCAAGAGAT TCGTAAGTCG CTTGATAGAC
 551 ATACGATTCT GTATACCACT TTGGTGGAGC TTTCTAAAAC GCTAGGCTTG
 601 CAAAATTGTG CTGTGTGGAT GCCTAATGAA GAAAAGACAG TGATGAATCT
 651 CACTCATGAA TTGAACGGAA GGAATTTAAA TATTTCTATA CCGATTACTG
 701 ATGTAGATGT TGTGAGAATT AAGGGAAGCA GTGTAGTGAA AATACTTGGC
 751 TCTGACTCGG CGCTTGCTGT TTCCAGTTGC GTGGTTTCTG GTGATGCAGG
 801 ACCGGTTGCT GCAATCCGAA TGCCAATGCT ACGGGTTTGT AATTTCAAAG
 851 GAGGAACACC TGAGCTAACT CAGGCGTGTT ATGCAATATT GGTCTTGATT
 901 CTTCCCGCCG GAGAGCCTAG ATCTTGGAGC AACCAGGAGC TGGAGATAAT
 951 TAAGGTGGTT GCTGATCAAG TTGCTGTAGC TCTATCCCAT GCTGCAATTC
1001 TTGAAGAGTC TCAACTTATG AGAGAGAAGT TGGAGGAACG CAATCGAGCT
1051 TTGCAACAGG CGAGAAGGAA CGCTATGATG GCAAGCCAGG CAAGAAACTC
1101 GTTTCAGAAA GTCATGAGCG ATGGCATGAG GAGGCCTATG CACTCGATTT
1151 TGGGATTGCT TTCAATGGTA CAAGACGATA ATTTAAAGAA CGAACAGAAA
1201 CTTATTGTGG ATGCAATGCT GAGGACGAGC AATGTGCTAT CAAACTTGAT
1251 AAACGATGCC ATGGACAGCT CAGCAAAAGA TGATGGAAGG TTCCCTTTGG
1301 AGATAAGGTC TTTCGGGTTA CATTCCATGA TAAAAGAAGC AGCTTGCCTT
1351 GCCAAATGCA TGTGTGTTTA TAAGGGCTTG GGTTTTATCG TTGAGGTTGA
1401 TAAGTCTTTA CCGAACAATG TTATGGGTGA TGAGAGGAGA GTTTTTCAGG
1451 TGATTTTGCA TATGGTTGGG AATCTACTTG ATTGCAACCA TGGAGAAGGA
1501 GGGATCCTTG TATTCCGGGT TTCGGCAGAT GCTGGAAGTC AAGGGAGGAA
1551 TGAGAAAGGA TGGACTACCT GGAGACCAAG CTCATCTAGT GGTGATGTAA
1601 ATATTAGATT TGAGATAGGG ATCACCGGTA GCGATTCTGA AGTTGGGAGC
1651 TCAGTTTCCT CGGGACCCGG TGGTAGGACA TATACCAGTG ATAGGTATGA
1701 GGGCAGATTG AGCTTTAGTA TTTGCAAGAG GATAGTCCAG TTGATGCAAG
1751 GGAACTTATG GTTGGTACCA TACACTCATG GCATTCCTCA AAGCATGACA
1801 CTTCTTCTCC GCTTTCAACT ACGACCTTCC ATTGCAATAG CCATCTCTGA
1851 ACCCGGAGAA TCATCAGAAC GCACGTATTC CAATTCAATG CTAAGAGGTC
1901 TGCAAGTTCT GTTAGTTGAC CATGACGATG TAAATAGAGC AGTCACACAA
1951 AAACTGCTTC AGAAACTCGG CTGCTCCGTC ACTTCCGCCT CTTCAGGATT
2001 CGAATGCCTC ACTCTCATCG GACCTGTCGG ATCTTCCATC CAAGTCATTC
2051 TCTTGGATCT CCAAACACCT GACATAGACG GCTTCGAAGT TGCCGCGAGG
2101 ATCCGGAAGT TCAAAAGTGG AAACAGGCCT ATGATTATCG CGTTAACAGC
2151 AAGCGCAGAA GAAGATTTGT GGGAAAGGTG TATGCAGATT GGCGTCAATG
2201 GAGTAATCCG AAAACCGGTT TTGTTGCAAG GAATCGCCAG TGAACTCAGA
2251 AGAATTCTGA TGCAGGGAAA TATATAA
```

B

```
  1 MVKAIVSVLL ITSIVLCVSA SDNGFPRCNC DDEASLWTIE SILECQRVGD
 51 FLIAVAYFSI PIELLYFVSC SNVPFKWVLV QFIAFIVLCG LTHLLNGWTY
101 GPHTFQLMVA LTIFKILTAL VSCATAITLV TLIPLLLKVK VREFMLKKKT
151 WDLGREVGLI MKQKEAAVHV RMLTQEIRKS LDRHTILYTT LVELSKTLGL
201 QNCAVWMPNE EKTVMNLTHE LNGRNLNISI PITDVDVVRI KGSSVVKILG
251 SDSALAVSSC VVSGDAGPVA AIRMPMLRVC NFKGGTPELT QACYAILVLI
301 LPAGEPRSWS NQELEIIKVV ADQVAVALSH AAILEESQLM REKLEERNRA
351 LQQARRNAMM ASQARNSFQK VMSDGMRRPM HSILGLLSMV QDDNLKNEQK
401 LIVDAMLRTS NVLSNLINDA MDSSAKDDGR FPLEIRSFGL HSMIKEAACL
451 AKCMCVYKGL GFIVEVDKSL PNNVMGDERR VFQVILHMVG NLLDCNHGEG
501 GILVFRVSAD AGSQGRNEKG WTTWRPSSSS GDVNIRFEIG ITGSDSEVGS
551 SVSSGPGGRT YTSDRYEGRL SFSICKRIVQ LMQGNLWLVP YTHGIPQSMT
601 LLLRFQLRPS IAIAISEPGE SSERTYSNSM LRGLQVLLVD HDDVNRAVTQ
651 KLLQKLGCSV TSASSGFECL TLIGPVGSSI QVILLDLQTP DIDGFEVAAR
701 IRKFKSGNRP MIIALTASAE EDLWERCMQI GVNGVIRKPV LLQGIASELR
751 RILMQGNI
```

Figure A4. **A)** The complete coding sequence of the putative ethylene receptor gene *PsETR2* from *P. sativum* L. cv. I_3 (Alaska-type). Binding sites of the forward and reverse qRT-PCR primers and the probe (which were designed for the complementary strand) are in green, blue and red, respectively. **B)** The predicted amino acid sequence of PsETR2 from *P. sativum* L. cv. I_3 (Alaska-type).

A

```
   1 ATGTCTCAAG TCTTCGGCTT TTCCGGTGAT AACTTTTGCC ATGGGGGTTC
  51 AATATACACA AACCCCAAGG AAGCAAGCTT CTTCCTGTCT CTTGGCCCTC
 101 AGGTTGATGT CTACTATCCT CCTCAGAAGA GATCACGTGT CAGCGTTCCA
 151 TTTGTTTTCG ATGGAGAATG GTTCGAGCAG AAGCAGAAAA CAACAATTGA
 201 TTCCTTGCCA GATGAGTGTC TCTTTGAGAT CTTTAGAAGG TTGCCTGTGG
 251 GTGAAGAAAG GAGTGCTTGC GCTTGTGTTT CCAAACGCTG GCTTATGCTT
 301 CTAAGCAGTA TTTGCAAGAG CGAAATCTGT AGCAACAAAA ATACCTCAGA
 351 TGGCGAGAAT AAAATGGAAG GTGACAGTCA GGAGTTTGGA GGCGAGGGAT
 401 ACCTTTCCCG GAGCTTGGAA GGAAAGAAGG CAACTGATGT TAGACTTGCC
 451 GCTATTGCAG TTGGAACCGC ATCACGTGGA GGATTGGGAA AGCTTTCTAT
 501 CCGTGGAAAC AACTCAAATC GCGGGGTGAC TACTCTAGGT CTCAAAGCAG
 551 TTGCTAGCGG GTGCCCTTCT CTAAAGGTTC TTTCTCTATG GAATGTTTCC
 601 TCTGTTGGTG ATGAAGGCCT AATTGAGATT GCTAATGGAT GTCAACAGCT
 651 AGAGAAGCTT GACCTTTGCA GATGCCCTTC AATTTCCGAC AAGGCTTTGA
 701 TTGCAGTTGC AAAAAACTGT CCAAATCTAA CAGAGTTATC ATTAGAGTCT
 751 TGTCCTAACA TTCACAATGA AGGTCTACAA GCCATTGGAA AACTCTGCTC
 801 CAATCTGAAG TCTATATCTA TCAAGGATTG TGCTGGTGTT GGTGATCAGG
 851 GAATCGCTGG CCTCTTTTCT TCAACTTCTT TGCTTTGAC AAAGGTGAAG
 901 CTCCAGGCAT TGACTGTTTC TGATCTCTCT CTAGCCGTTA TTGGACACTA
 951 TGGAAAAGCA GTTACCGATC TTGTCCTTAA TTTCCTCCCA AATGTTAGTG
1001 AGAGGGGCTT CTGGGTTATG GGAAATGCTA ATGGATTGCA TAAACTGAAG
1051 TCACTCACAA TTGCATCCTG CAGAGGAGTA ACCGATGTTG GGCTTGAAGC
1101 TGTGGGAAAG GGCTGCCCTA ATCTGAAAAG TGTACACCTT CAAAAGTGTG
1151 CGTTTCTCTC AGACAATGGG TTGATTTCGT TCACCAAGGC TGCTATATCA
1201 CTTGAAAGCC TAAAATTGGA AGAGTGCCAC AGAATTACCC AGTTTGGGTT
1251 TTTCGGTGTC CTTTTTAACT GTGGTGCAAA ATTGAAGGCT CTTTCTCTTG
1301 TAAGCTGCTA TGGAATCAAA GATCTGGACT TGGAATTGTC ACCCTCATCT
1351 CCTTGTGAAT CACTTCGTTC ATTAACAATC TGTAACTGCC CTGGATTTGG
1401 AAATGCTACC CTATCCGTAC TCGGAAAGTT GTGCCCTCAG CTTCAACAGG
1451 TTGAATTGAC TGGACTTAAG GGAGTGACAG ATGCTGGTTT GCTTCCACTG
1501 CTTGAGAGCT CAGAGGCTGG TCTGGTTAAA GTCAATCTTA GTGGTTGTGT
1551 AAATCTTACA GACAAAGTAG TTTCATCCCT GGTCAATCTG CATGGTTGGA
1601 CTCTCGAGAT TCTAAACCTT GAAGGTTGTA AAAACATTAG CAATGCTAGT
1651 TTGATAGCAA TGGCTGAGCA TTGCCAGTTG CTTTGTGATC TTGATGTTTC
1701 CATGTGTGCG ATCTCTGATG CTGGAATAGC AGCCCTGGCA CATGCAAAAC
1751 AGATAAATCT ACAGATTCTT TCTCTGTCAG GCTGCACTTT GGTTACAGAC
1801 AGGAGTTTGC CCGCATTGAG AAAATTGGGC CGCACCCTTT TGGGACTAAA
1851 CGTCCAGCAT TGCAATTCAA TCAGCAGCAG TGCAGTTGAA ATGCTTGTTG
1901 AACTTCTTTG GAGGTGTGAC ATCCTCTCCT GA
```

B

```
  1 MSQVFGFSGD NFCHGGSIYT NPKEASFFLS LGPQVDVYYP PQKRSRVSVP
 51 FVFDGEWFEQ KQKTTIDSLP DECLFEIFRR LPVGEERSAC ACVSKRWLML
101 LSSICKSEIC SNKNTSDGEN KMEGDSQEFG GEGYLSRSLE GKKATDVRLA
151 AIAVGTASRG GLGKLSIRGN NSNRGVTTLG LKAVASGCPS LKVLSLWNVS
201 SVGDEGLIEI ANGCQQLEKL DLCRCPSISD KALIAVAKNC PNLTELSLES
251 CPNIHNEGLQ AIGKLCSNLK SISIKDCAGV GDQGIAGLFS STSLLLTKVK
301 LQALTVSDLS LAVIGHYGKA VTDLVLNFLP NVSERGFWVM GNANGLHKLK
351 SLTIASCRGV TDVGLEAVGK GCPNLKSVHL QKCAFLSDNG LISFTKAAIS
401 LESLKLEECH RITQFGFFGV LFNCGAKLKA LSLVSCYGIK DLDLELSPSS
451 PCESLRSLTI CNCPGFGNAT LSVLGKLCPQ LQQVELTGLK GVTDAGLLPL
501 LESSEAGLVK VNLSGCVNLT DKVVSSLVNL HGWTLEILNL EGCKNISNAS
551 LIAMAEHCQL LCDLDVSMCA ISDAGIAALA HAKQINLQIL SLSGCTLVTD
601 RSLPALRKLG RTLLGLNVQH CNSISSSAVE MLVELLWRCD ILS
```

Figure A5. **A**) The complete coding sequence of the putative ethylene signaling gene *PsEBF1* from *P. sativum* L. cv. I_3 (Alaska-type). Forward and reverse qRT-PCR primers and the probe binding sites are in green, blue and red, respectively. **B**) The predicted amino acid sequence of PsEBF1 from *P. sativum* L. cv. I_3 (Alaska-type).

A

```
   1 ATGCCTGCCC TTGTCAATCA TGGTGGTGAT GATGAACTGT ATCCTGGGGG
  51 CTTGGTGGAT TTGGGGGGTA GGTTGCGTAC TGCTAGTTCC AATGTTGATG
 101 TGTACTCCTC TCCTACGAAG CGAGCTCGAA TCAGTGTCCC GTTTAGTTTT
 151 GGAGATCTTG AGGCTGAGAG TGAGGTGGAT GTGGATTATA AGTCTAGTGT
 201 TGAGATCCTT CCTGACGAAT GTCTGTTTGA GATATTCAGA AGGCTTTCGA
 251 GTGGCAAAGA GAGAAGCTCG TGTGCTTGTG TATCGAGAAA GTGGCTTATG
 301 CTTGTGAGTG GTATATGCAA AGATGAGATT GAAACTGTTG TGAAAACGAT
 351 TTCTTCAGAT GAGAGTCATG AAGATGTTGA AGGTGATGGG TACCTTACGA
 401 GGCGTTTGGA AGGGAAGAAA GCGACTGATG TAAGACTTGC TGCTATTGCT
 451 GTTGGGACTA GTTGGCGCGG TGGGCTTGGG AAGCTGTCGA TTAGAGGAAA
 501 CAACTCTGTT CGAGGTGTTA CAAACCGCGG TCTCTCGGCC GTTGCTAATG
 551 GTTGTCCTTC TCTCAGATCA CTTTCATTGT GGAATGTGTC TTCTGTGGGT
 601 GACAAGGGTC TGTGTGAGAT TGGAAAAAGG TGTCATATGT TGGAGAAGCT
 651 TGACTTGTGC CATTCTCGGT GGATCACGAA CAAGGGTTTG ATTGCGATAG
 701 CTGAAGGTTG CCCTAACTTG ACCACGTTAA ATATTGAATC GTGCTCTAAG
 751 ATTGGAAATG AGGGTTTGCA AGCTGTTGCA AAGTTGTGTC CTAATTTACA
 801 TTCAATCTCT ATCAAGGATT GTCCTCTTGT TGGTGATCAT GGAGTGTCTA
 851 GTTTGTTATC ATCGGCTTCT GAGTTGTCAA GGGTAAAGCT TCAGATTTTG
 901 AATATTACTG ATTTTTCTCT GGCTGTTATT GGCCATTATG GGAAGTCGAT
 951 AACGAATCTG GTTCTTAGTG GACTTCGAAA TGTAAACGAA AGAGGGTTTT
1001 GGGTCATGGG TGTTGCTCAG GGTCTACAGA AACTGGTTTC ACTTACTATA
1051 ACTTCATGCC ATGGGGTAAC TGATGCGAGC ATCGAAGCCA TCGGTAAGGG
1101 TTGCCCAAAT CTGAAGCAGA TGTGCCTTCG CAGGTGTTGC TTTGTATCCG
1151 ACAGTGGATT GGTTGCATTT GCCAAATCCG CGGGATCTCT TGAGAACTTG
1201 CATTTGGAAG AGTGTAACAG GTTCACTCAA TCTGGGATTA TCGGCGCCAT
1251 TTCGAGCATC AAAGCAAAGT TGAAATCACT CACACTTGTG AAGTGTATGG
1301 GAATTAAAGA CATTGAGGTG GAAGTATCTA TGCTTTCACC TTGCGAGTCT
1351 CTTCGATCTG TAACCATTAA AAACTGTCAC GGTATCGGAA GTGCTAGTCT
1401 GGCTGTGATC GGGAAGTTGT GTCCCCAACT TCAGTATGTT GATCTGACCG
1451 GACTTTACGG CATAACAGAC GCAGGCCTTC TCCCACTTTT GGAGAATTGT
1501 GAGGCTGGAC TCGTCAAGGT GAACCTTACG GGTTGCTGGA ACTTGACAGA
1551 TAACATAGTT TCCGCCATGA CCAGACTACA CGGCGGAACT CTCGAAGTAC
1601 TAAATCTCGA CGGATGCTGG AATATTACTG ATGCAAGCTT GGTTGCGATT
1651 GCAGACAACT GCCTACTGCT CAACGATCTA GATGTGTCGA GGTGTGCAAT
1701 CACCGATGCC GGTATATCTG TTCTCTCCGA TGCCATTCAG CTTAGTTTGC
1751 AAGTTCTCTC TATGTCTGGC TGTTCTGAAA TATCAAACAA CTGTATGCCT
1801 TTCCTGAAGA AATTGAGCCA GAACTTGTTG GGATTGAATC TTCAAAACTG
1851 CATTGGAATT GGCAGCAACA CGATTGAGTT GCTCGTGGAG AGCCTGTGGA
1901 GATGTGATAT TCTGGCCTAA
```

B

```
  1 MPALVNHGGD DELYPGGLVD LGGRLRTASS NVDVYSSPTK RARISVPFSF
 51 GDLEAESEVD VDYKSSVEIL PDECLFEIFR RLSSGKERSS CACVSRKWLM
101 LVSGICKDEI ETVVKTISSD ESHEDVEGDG YLTRRLEGKK ATDVRLAAIA
151 VGTSWRGGLG KLSIRGNNSV RGVTNRGLSA VANGCPSLRS LSLWNVSSVG
201 DKGLCEIGKR CHMLEKLDLC HSRWITNKGL IAIAEGCPNL TTLNIESCSK
251 IGNEGLQAVA KLCPNLHSIS IKDCPLVGDH GVSSLLSSAS ELSRVKLQIL
301 NITDFSLAVI GHYGKSITNL VLSGLRNVNE RGFWVMGVAQ GLQKLVSLTI
351 TSCHGVTDAS IEAIGKGCPN LKQMCLRRCC FVSDSGLVAF AKSAGSLENL
401 HLEECNRFTQ SGIIGAISSI KAKLKSLTLV KCMGIKDIEV EVSMLSPCES
451 LRSVTIKNCH GIGSASLAVI GKLCPQLQYV DLTGLYGITD AGLLPLLENC
501 EAGLVKVNLT GCWNLTDNIV SAMTRLHGGT LEVLNLDGCW NITDASLVAI
551 ADNCLLLNDL DVSRCAITDA GISVLSDAIQ LSLQVLSMSG CSEISNNCMP
601 FLKKLSQNLL GLNLQNCIGI GSNTIELLVE SLWRCDILA
```

Figure A6. **A**) The complete coding sequence of the putative ethylene signaling gene *PsEBF2* from *P. sativum* L. cv. I_3 (Alaska-type). Forward and reverse qRT-PCR primers and the probe binding sites are in green, blue and red, respectively. **B**) The predicted amino acid sequence of PsEBF2 from *P. sativum* L. cv. I_3 (Alaska-type).

```
ACS4       1 ATGGGTTTGGAAAATAATAGCCAGAAGCTTTTATCCAAGATTGCTACCAA     50
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3       1 ATGGGTTTGGAAAATAATAGCCAGAAGCTTTTATCCAAGATTGCTACCAA     50

ACS4      51 CAACAAACATGGTGAAAATTCTCCTTACTTTGATGGATGGAAAGCTTATG    100
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3      51 CAACAAACATGGTGAAAATTCTCCTTACTTTGATGGATGGAAAGCTTATG    100

ACS4     101 AATCAAACCCATTTCACCCCACAAAAAATCCTCAAGGTGTTATCCAAATG    150
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3     101 AATCAAACCCATTTCACCCCACAAAAAATCCTCAAGGTGTTATCCAAATG    150

ACS4     151 GGTCTTGCTGAAAATCAGCTCTGTTTTGATTTGATTGAAGAATGGATAAA    200
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3     151 GGTCTTGCTGAAAATCAGCTCTGTTTTGATTTGATTGAAGAATGGATAAA    200

ACS4     201 GAATAATCCAAAAGCTTCCATTTGTACTCCAGAAGGAGTCAATCAATTCA    250
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3     201 GAATAATCCAAAAGCTTCCATTTGTACTCCAGAAGGAGTCAATCAATTCA    250

ACS4     251 GACATATAGCAAACTTTCAAGACTATCATGGATTGCCAGAATTCAAAAAT    300
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3     251 GACATATAGCAAACTTTCAAGACTATCATGGATTGCCAGAATTCAAAAAT    300

ACS4     301 GCTGTGGCAAATTTGATGTCAAAAGTGAGAGGTGGTAGGGTGAGATTTGA    350
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3     301 GCTGTGGCAAATTTGATGTCAAAAGTGAGAGGTGGTAGGGTGAGATTTGA    350

ACS4     351 TCCTGACCGTTTATTGATGAGTGGAGGAGCAACTGGTGCAAATGAGTTAA    400
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3     351 TCCTGACCGTTTATTGATGAGTGGAGGAGCAACTGGTGCAAATGAGTTAA    400

ACS4     401 TAATGTTCTGTTTGGCTGATCCTGGTGATGCTTTTTTGGTACCTAGCCCT    450
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3     401 TAATGTTCTGTTTGGCTGATCCTGGTGATGCTTTTTTGGTACCTAGCCCT    450

ACS4     451 TATTATCCAGCATTTGTCCGTGACTTGTGTTGGAGAACCGGTTTGCAACT    500
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3     451 TATTATCCAGCATTTGTCCGTGACTTGTGTTGGAGAACCGGTTTGCAACT    500

ACS4     501 AATTCCGGTCCAATGTCATAGTTCAAACAATTTCATGATAACAAGAGAAG    550
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3     501 AATTCCGGTCCAATGTCATAGTTCAAACAATTTCATGATAACAAGAGAAG    550

ACS4     551 CACTTGAAGAAGCTCATAAAAAGGCACAAGACGAAAACATCAATGTGAAA    600
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3     551 CACTTGAAGAAGCTCATAAAAAGGCACAAGACGAAAACATCAATGTGAAA    600

ACS4     601 GGGTTAATCATAACAAATCCATCAAACCCTTTAGGAACAACAATTGAAAA    650
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3     601 GGGTTAATCATAACAAATCCATCAAACCCTTTAGGAACAACAATTGAAAA    650

ACS4     651 AGAAACACTCAAGAGCATAGTGAGTTTCATCAATGAAAACAACATTCATC    700
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3     651 AGAAACACTCAAGAGCATAGTGAGTTTCATCAATGAAAACAACATTCATC    700
```

```
ACS4     701 TAGTGTGTGACGAAATCTATTCGGGTACCGTTTACGACACCCCAAAATTC    750
             ||||||||||||||||||||||||||..||||||||||||||||||||
ACS3     701 TAGTGTGTGACGAAATCTATTCGGGTACGTTTTACGACACCCCAAAATTC    750

ACS4     751 GTAAGTGTGTCCGAAATTATACAAGAAATGGAAGACATAAAAAAGGATCT    800
             |||||||||||||||||||||||||||||||||||||||||.|||| ||
ACS3     751 GTAAGTGTGTCCGAAATTATACAAGAAATGGAAGACATAAAAGAGGA-CT    799

ACS4     801 -CATTCATATCATCTATAGTTTATCAAAAGATATGGGGCTACCGGGTTTT    849
              ||||||||.||.||||||||||.|||||||..|.||.||||||||||||
ACS3     800 CCATTCATACCACCTATAGTTTACCAAAAGACTTAGGCCTACCGGGTTTT    849

ACS4     850 AGAGTTGGTTTAGTTTATTCGTACAA-CGACGAAGTTGT-GAGCTGTGGT    897
             |||||.|||||.|||||||||| ||| ||||||||..|| ||    |.|.
ACS3     850 AGAGTGGGTTTTGTTTATTCGT-CAACCGACGAAGACGTAGA----TTGC    894

ACS4     898 CG----GAAAATGTCAAGTTTTGGGTTAGTCTCGTC-GCAGACACAATAT    942
             ||    .||||| ||.||||||||||..|| ||.| |.|.|||||| ||
ACS3     895 CGCACAAAAAAT-TCCAGTTTTGGGTTGCTC-CGGCGGGAAACACAA-AT    941

ACS4     943 -TTTCTCGCTAC-AATGCTATCCGATGACAAATTCATGGATAAGTTTTTA    990
              |||||| ||.| |||..|||.|||||.|||||..|..|||.|.|||||||
ACS3     942 ATTTCTC-CTCCAAATCTTATACGATGGCAAAACCCAGGAAAGGTTTTTA    990

ACS4     991 GCGGAGAGTTCAAGAAGGTTAAGG-----GCT-CGGCGTGAGTTTTTCAC   1034
              |||...||.|||.|     ||||     ||| |||||.|||.|||||||
ACS3     991 -CGGGCGGTCCAAAA-----AAGGCTCGCGCTGCGGCGGGAGGTTTTCAC   1034

ACS4    1035 -AAAGGGACTCGAAAAG-GTTAACATAACTTGCTTACCAAGTAATGCAGG   1082
              ||||||||.|.|||||  |..|  ||.|..||.|..|.||||.|.|..||
ACS3    1035 AAAAGGGACGCCAAAAGTGGGA--ATCAAGTGTTCGCAAAGTTACGGTGG   1082

ACS4    1083 ACTATTCTTTTGGATGAATTTGAGGAGTTTGTTAAAAGAGAAAACGTTCG   1132
             |||.||..|.||.|||.||||      .|.|||||.|||||||..|||
ACS3    1083 ACTCTTGGTGTGCATGCATTT------ATCGTTAAAGGAGAAAACCGTCG   1126

ACS4    1133 AAGGCG-AAATGAAGCTTTGGCGATTGATTATCAATGAGGTGAAGCTTAA   1181
             |||||| ||||||    |||..||.|||||.||.||||.||.|.|.|.|.
ACS3    1127 AAGGCGAAAATGA----TTGCAGAGTGATTGTCCATGAAGTCACGATCAT   1172

ACS4    1182 TGTTTCGCCGGGTTCGGCTTTTGAATGCTCCGAACCGGGTTGGTACCGAG   1231
             ||||...||.|||....|.|||.|.||||||||||.||.||||||.|||
ACS3    1173 TGTTCTACCTGGTGTTTCATTTCATTGCTCCGAACCTGGCTGGTACAGAG   1222

ACS4    1232 TTTGTTTTGCTAACATGGATGAAGAAACTGTTGAGATTGCATTGATGAGA   1281
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3    1223 TTTGTTTTGCTAACATGGATGAAGAAACTGTTGAGATTGCATTGATGAGA   1272

ACS4    1282 ATTAGAGCATTTGTTAATGGAAGAGAGAAAGGGGTGAAAAAAGTTGAAAT   1331
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3    1273 ATTAGAGCATTTGTTAATGGAAGAGAGAAAGGGGTGAAAAAAGTTGAAAT   1322

ACS4    1332 GAAACGTTGGAAGAGTAATCTAAGACTTAGTTTTTCATCAAGAAGGTTTG   1381
             ||||||||||||||||||||||||||||||||||||||||||||||||||
ACS3    1323 GAAACGTTGGAAGAGTAATCTAAGACTTAGTTTTTCATCAAGAAGGTTTG   1372
```

```
ACS4    1382 AAGAGAATGTTATGTCTCCTCATTCACCAATTCCTCATTCACCACTTGTT   1431
             |||||||||||||||.||||||||||||||||||||||||||||||||
ACS3    1373 AAGAGAATGTTATGTCCCCTCATTCACCAATTCCTCATTCACCACTTGTT   1422

ACS4    1432 CGAGCAACTTAA   1443
             ||||||||||||
ACS3    1423 CGAGCAACTTAA   1434
```

Figure A7. Nucleotide sequence alignment of *PsACS4* from *P. sativum* L. cv. I_3 Alaska-type (GenBank accession#: KX255646) and *PsACS3* from *P. sativum* L. var. Saccharatum (GenBank accession#: AB049725) performed using the EMBOSS Needle pairwise sequence alignment program under the default settings (Matrix: DNAfull, Gap penalty, 10.0; Extend penalty, 0.5). The vertical bars (|) represent residues that are identical between the two sequences. Note: the ~ 400 bp internal region of *PsACS3* from *P. sativum* L. var. Saccharatum does not match with that of *PsACS4* from *P. sativum* L. cv. I_3 (Alaska-type) or closely related *Medicago* genes (data not shown), suggesting that the *PsACS3* is a variant only in *P. sativum* var. Saccharatum, or it may be the result of cloning or sequencing errors (See Supplementary Materials and Methods section for further detail).

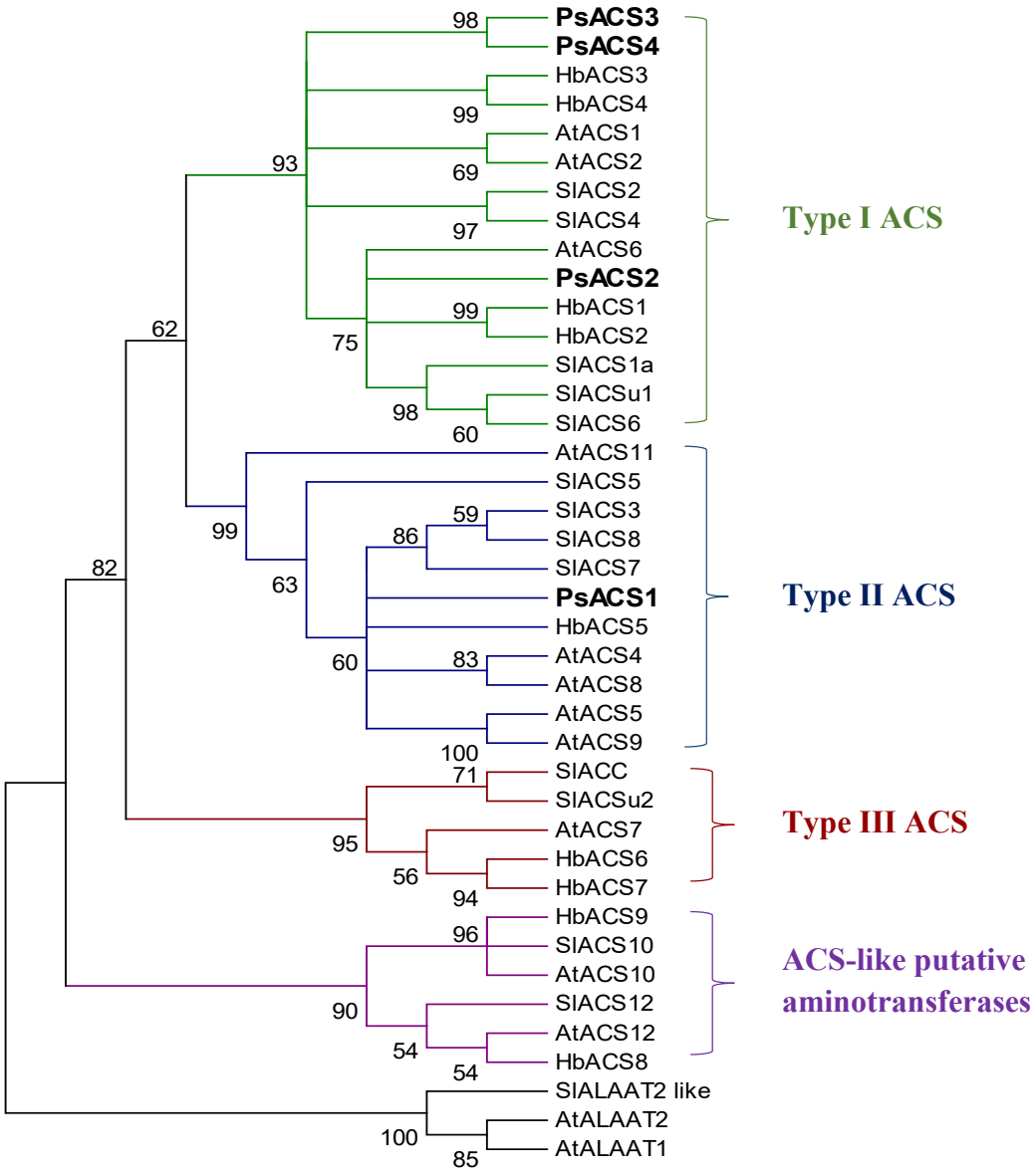


Figure A8. A phylogenetic tree showing the association of pea (*Pisum sativum*) 1-aminocyclopropane-1-carboxylate synthases (PsACSs) within the three ACS protein types of *Arabidopsis thaliana* (At), *Solanum lycopersicum* (Sl) and *Hevea brasiliensis* (Hb) species. The amino acid sequence alignment for the phylogenetic tree creation was done using the MUSCLE sequence alignment program under the default settings in MEGA 7.0. A maximum likelihood tree was created with 1000 bootstrap replicates using the LG model with a Gamma distribution (+ G). The tree was rooted with alanine aminotransferase (ALAAT) sequences of *Arabidopsis* and tomato. Numbers in the branches represent the percentage bootstrap support. Branches with greater than 50% bootstrap support are shown.

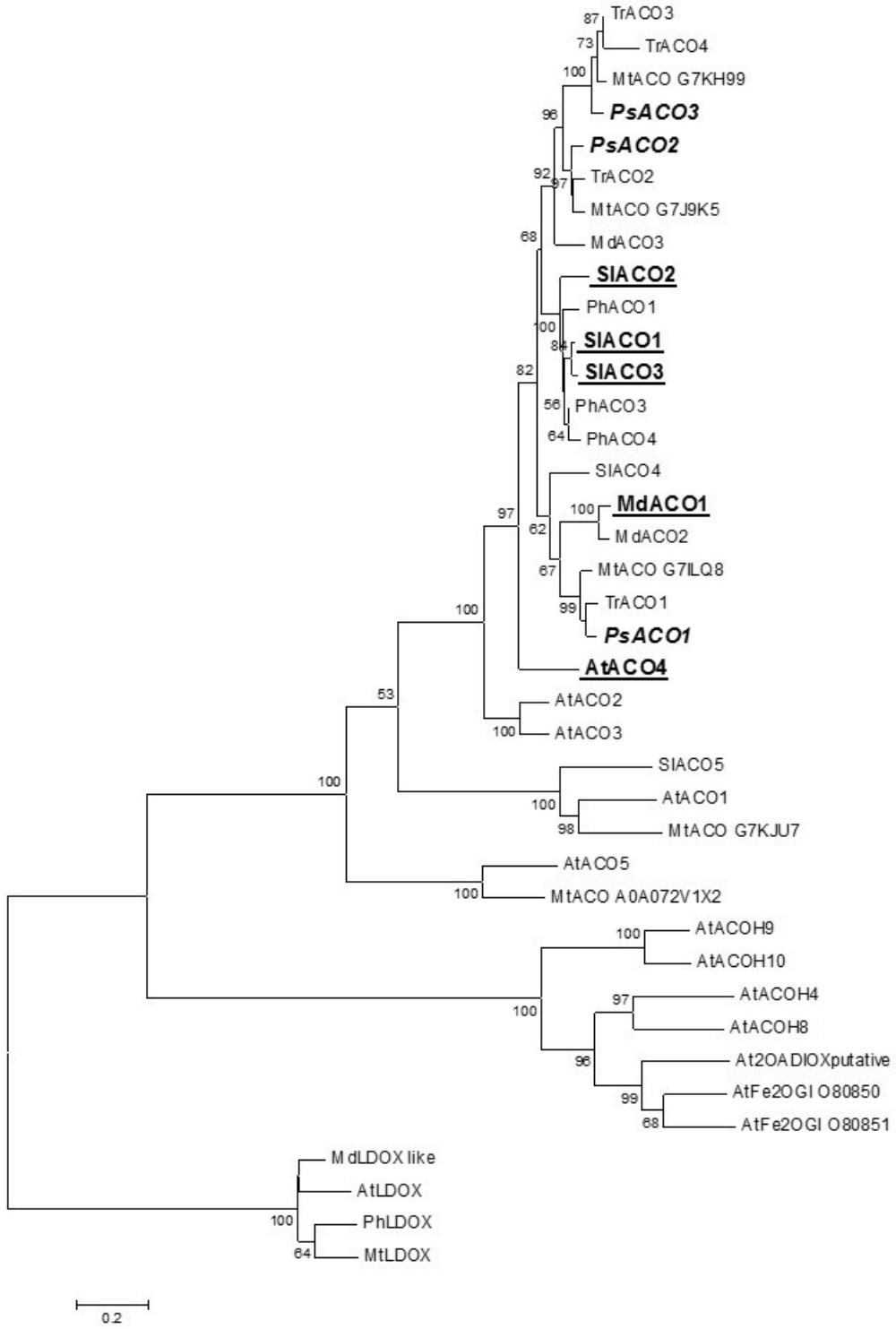
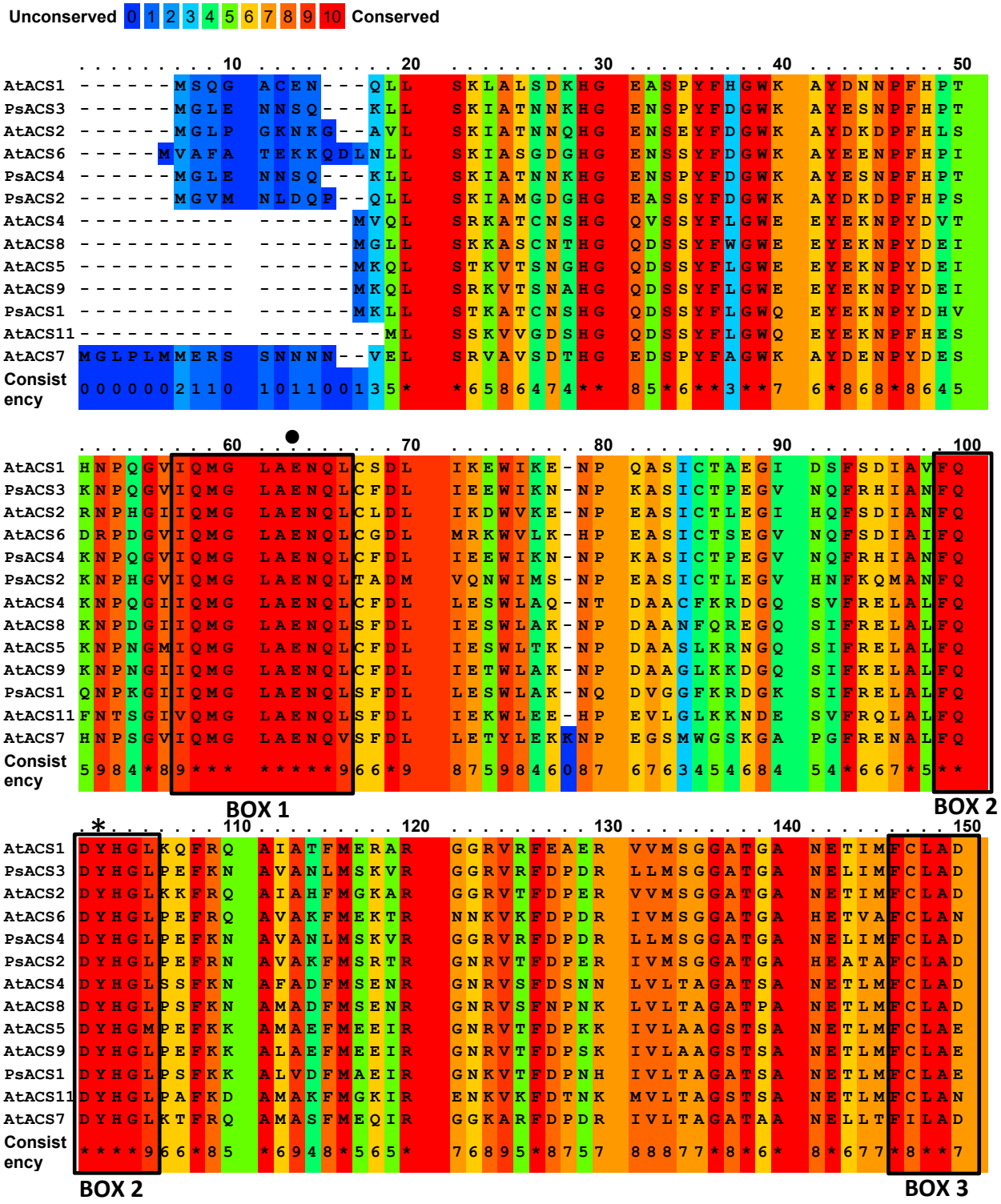
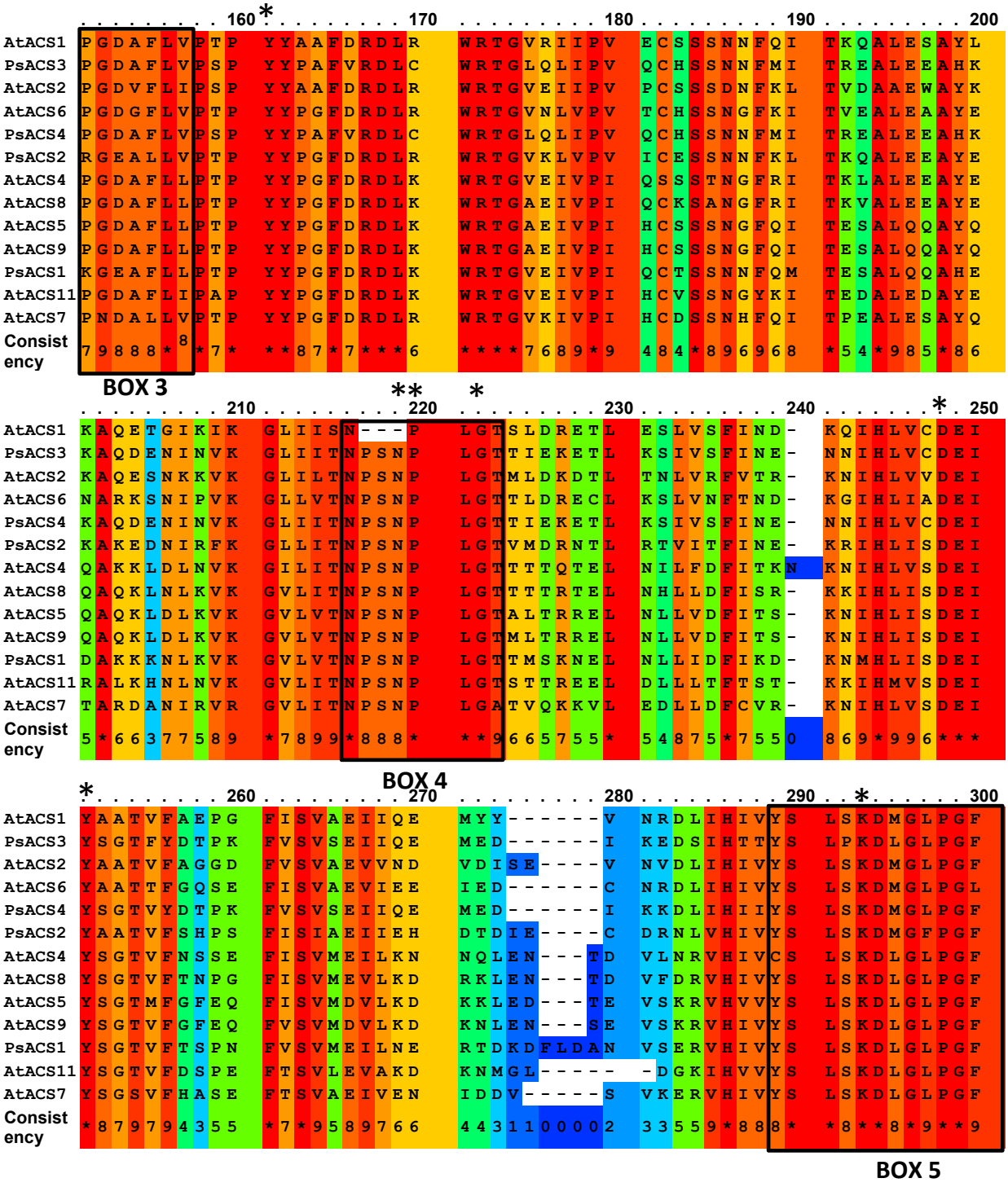
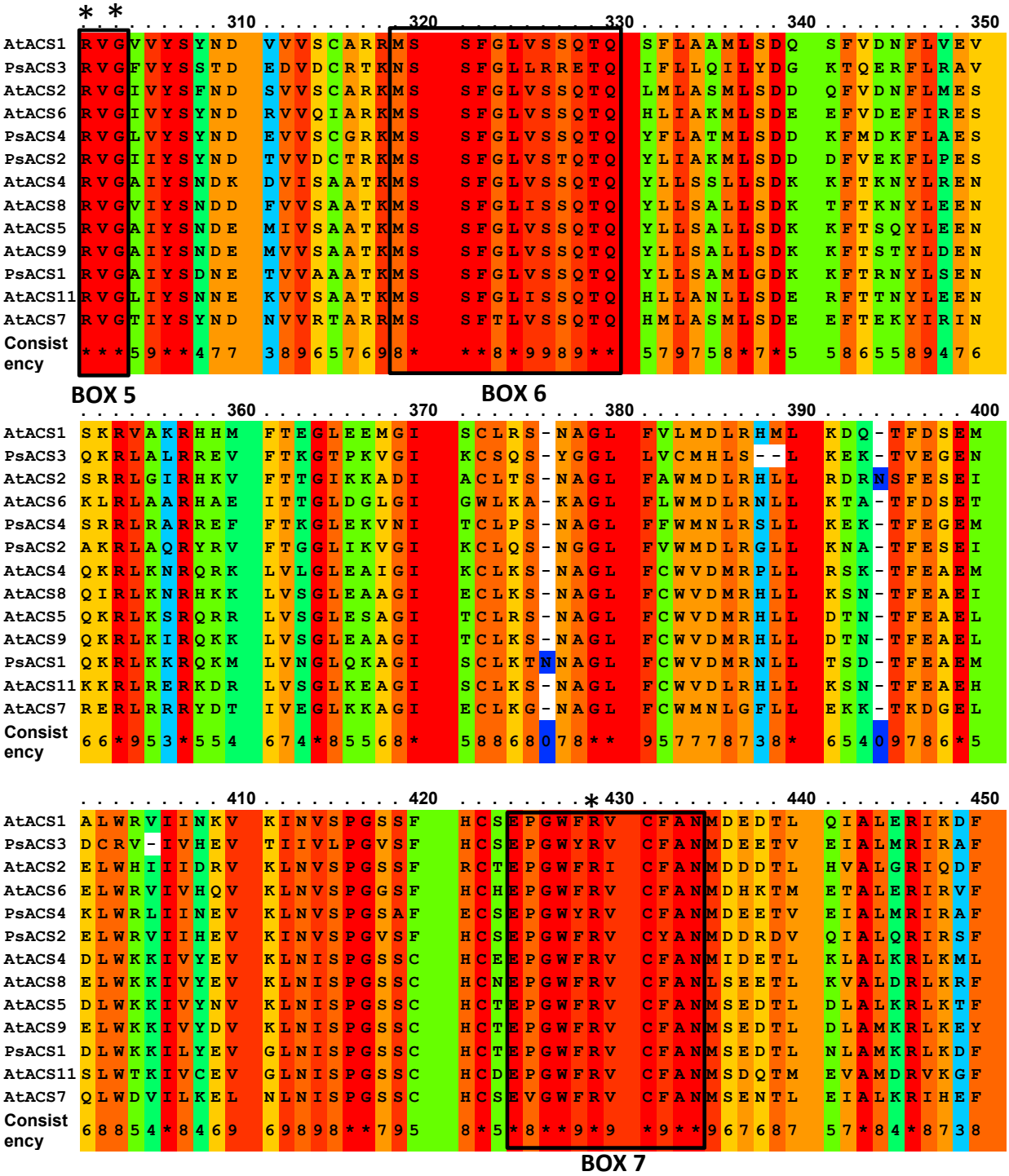


Figure A9. A phylogenetic tree showing the association of pea (*P. sativum*) 1-aminocyclopropane-1-carboxylate oxidases (PsACOs) with the ACO proteins of *Arabidopsis thaliana* (At), *Malus domestica* (Md)*, Medicago truncatula* (Mt), *Petunia x hybrid* (Ph), *Solanum*

lycopersicum (Sl), and *Trifolium repens* (Tr) species. The amino acid sequence alignment for the phylogenetic tree creation was done using the MUSCLE sequence alignment program under the default settings in MEGA 7.0. The maximum likelihood tree was created with 1000 bootstrap replicates using the LG model with a Gamma distribution and invariant sites (G+I); the number of discrete gamma categories was 4. The tree was rooted with leucoanthocyanidin dioxygenase (LDOX) of selected plant species. Bootstrap values with greater than 50 % branch support are shown. Pea ACO sequences are in bold and italicized. The ACO proteins of which activity has been verified *in vitro* (Booker and DeLong, 2015) are in bold and underlined.


Unconserved 0 1 2 3 4 5 6 7 8 9 10 Conserved
AtACS1
PsACS3
AtACS2
AtACS6
PsACS4
PsACS2
AtACS4
AtACS8
AtACS5
AtACS9
PsACS1
AtACS11
AtACS7
Consistency
BOX 1
BOX 2
BOX 2
BOX 3


BOX 3
BOX 4
BOX 5


BOX 5
BOX 6
BOX 7
AtACS1
PsACS3
AtACS2
AtACS6
PsACS4
PsACS2
AtACS4
AtACS8
AtACS5
AtACS9
PsACS1
AtACS11
AtACS7
Consistency

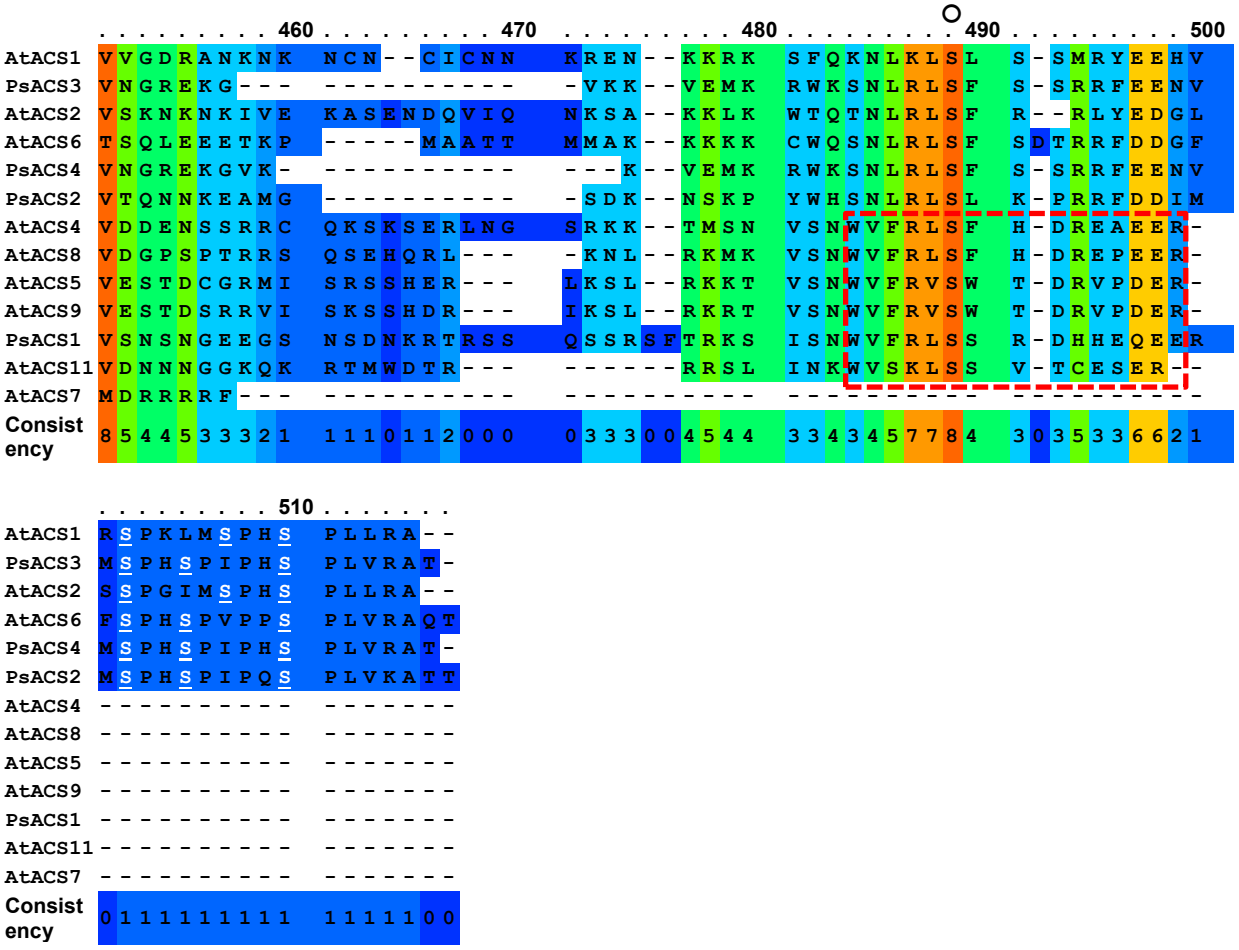
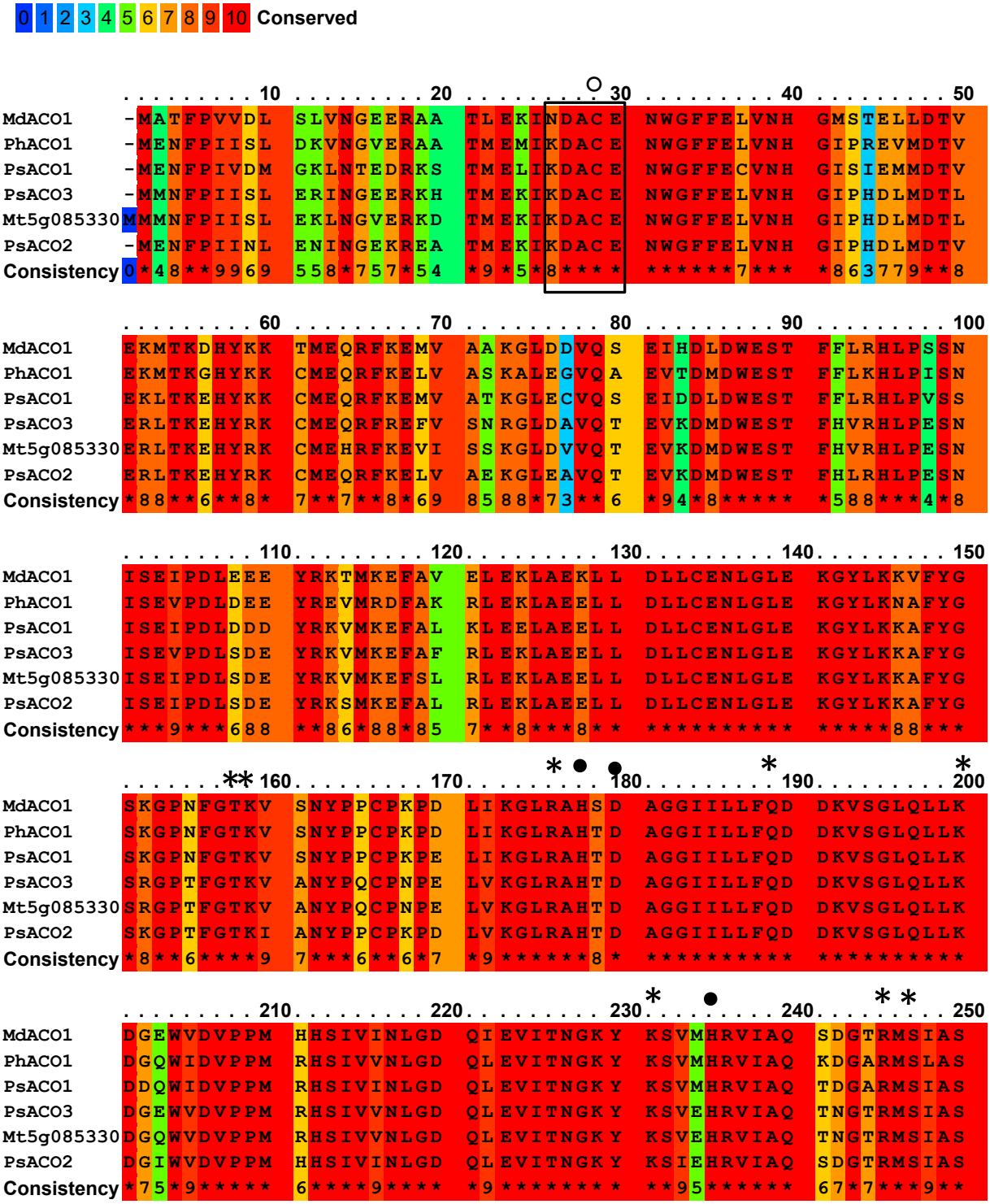


Figure A10. Alignment of predicted pea ACS sequences with enzymatically active *Arabidopsis* ACS isozymes including the AtACS1, which is only active as a heterodimer. Sequences were aligned using the PRALINE multiple sequence alignment tool under default parameters (www.ibi.vu.nl/programs/pralinewww). The seven conserved domains found in the ACS isozymes are marked as boxes 1-7 (Yamagami et al., 2003). The conserved glutamate (E) residue in box 1, which is responsible for substrate specificity, is marked with a solid circle. The amino acids marked with asterisks are conserved among ACS isozymes and also found in various aminotransferases (Yamagami et al., 2003). The *Arabidopsis* AtACS1, AtACS2, AtACS6 are type I ACS. AtACS4, AtACS5, AtACS8, AtACS9 and AtACS11 are type II ACS and AtACS7 is a type III ACS (Xu and Zhang, 2015). The CDPK site found in Type I and Type II ACS is marked with an open circle (Sebastià et al., 2004). The three C-terminal MAPK sites characteristic of type I ACS are marked in white font and underlined (Liu and Zhang, 2004). The C-terminal region with TOE (Target of ETO1) domain which is the target site for ETO1

(Ethylene overproducer 1) interaction during Type II protein turnover is marked in a red dashed box (Yoshida et al., 2006).


0 1 2 3 4 5 6 7 8 9 10 Conserved
MdACO1
PhACO1
PsACO1
PsACO3
Mt5g085330
PsACO2
Consistency

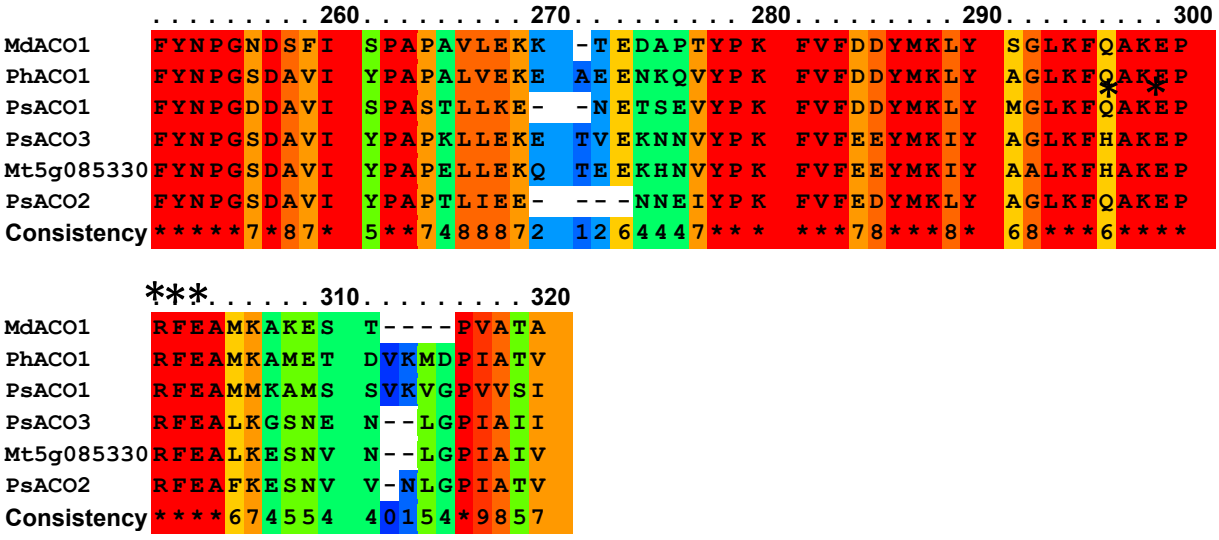


Figure A11. Amino acid sequence alignment of PsACO1, PsACO2 and PsACO3 with *Malus domestica* MdACO1 (UniProt ID Q00985), *Petunia hybrida* PhACO1 (UniProt ID Q08506) and *Medicago truncatula* Mt5g085330 (UniProt ID G7KH99). Sequences were aligned using the PSI-PRALINE multiple sequence alignment tool under default parameters (www.ibi.vu.nl/programs/pralinewww). Amino acids shown to be important in ACO enzyme activity include residues involved in the binding of ACC, bicarbonate, and ascorbic acid; these residues are marked with asterisks (Dilley et al., 2013). The solid black circles mark the amino acid residues involved in Fe(II) binding (Shaw et al., 1996; Zhang et al., 2004). The box with the cysteine residue marked with an open circle represents the putative divalent metal binding site required for cysteine protease activity (Dilley et al., 2013).

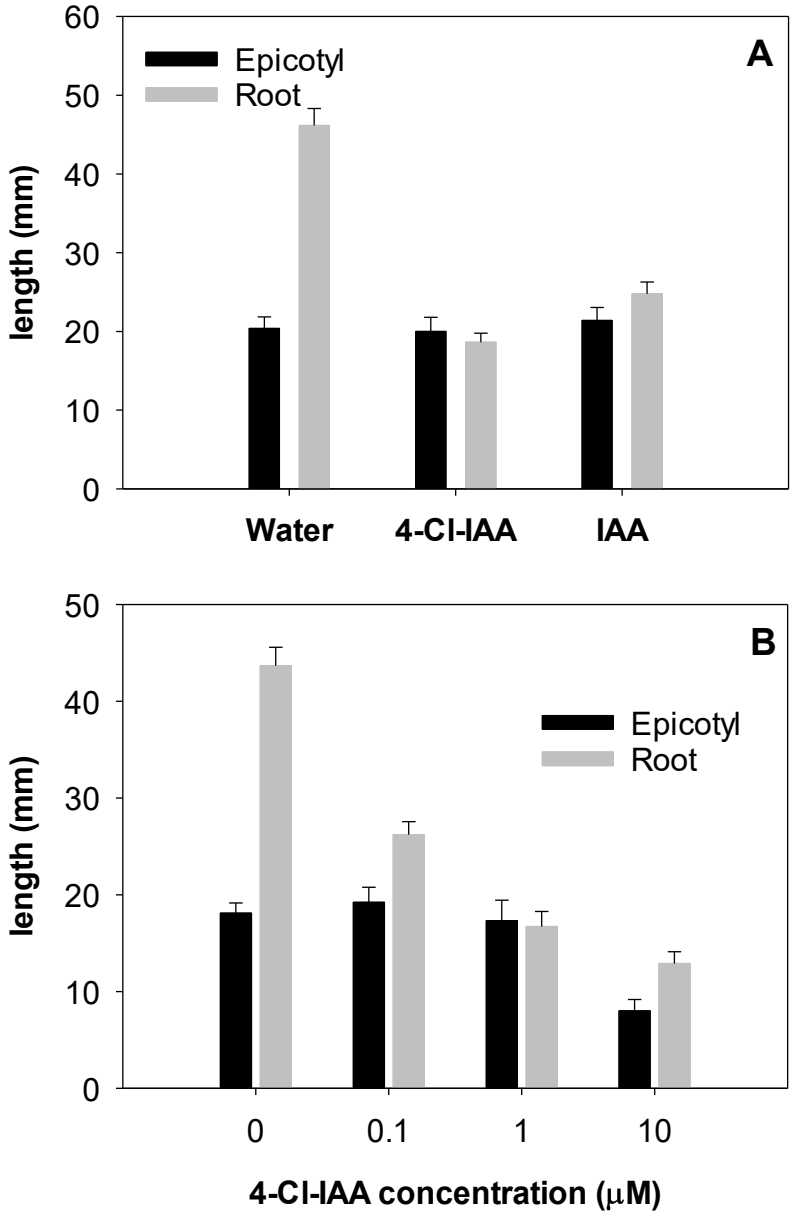


Figure A12. Effect of 4-Cl-IAA and IAA on epicotyl and root length of 4 days after imbibition dark-grown seedlings of *P. sativum* L. cv. I_3 (Alaska-type). Pea seedlings were grown in the presence of **A)** 0 μM or 1 μM aqueous 4-Cl-IAA or IAA; or in the presence of **B)** 0, 0.1, 1 or 10 μM aqueous 4-Cl-IAA. Seeds were initially imbibed in water in the dark for two days. Germinated seeds were then selected for uniformity and transferred to plastic vessels (60 x 60 x 95 mm) containing the treatment solutions and placed under dark conditions for an additional two days (as describe in the Materials and Methods section). Data are means ± SE (A, n= 18-20; B, n=25-30).

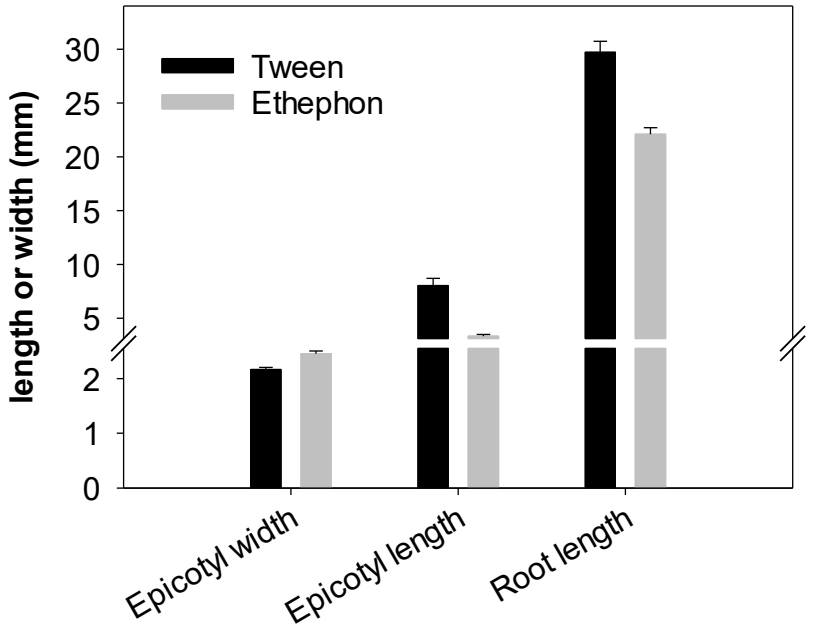


Figure A13. Effect of ethephon on epicotyl width (at the base adjacent to the attachment of the cotyledons), and epicotyl and root length of 4 days after imbibition seedlings of *P. sativum* L. cv. I_3 (Alaska-type). Seeds were imbibed and grown in the presence of 15 mg L^{-1} ethephon (in 0.1% aqueous Tween 80) or 0.1% aqueous Tween 80 in the dark for four days (as described in the Materials and Methods section). Data are means ± SE, n=30.

Table A1. Primers and probes used for transcript abundance quantitation of pea ethylene biosynthesis, receptor, and signaling-related genes by qRT-PCR, and their PCR efficiencies. [a]

Gene	qRT-PCR Efficiency (%)	Primers and Probe	Sequence
PsACS1	93	Forward	CGTTTGAATCCTCCTACATCTTGA
		Reverse	GCAAGAATATGAAAAGAACCCTTATGA
		Probe	TCGGCGAGACCCATCTGAATAATTCCT
PsACS2	94.1	Forward	GCCCGTGAAAACTCTGTACCTT
		Reverse	CGATGATGACTTCGTCGAGAAA
		Probe	CTAACCTCTTCGCACTCTCGGCAAGAAA
PsACS4	100.5	Forward	AGTCTCGTCGCAGACACAATATTT
		Reverse	TGAACTCTCCGCTAAAAACTTATCC
		Probe	TCGCTACAATGCTATCCGATGACAAATTCA
PsACO1	97	Forward	GTGGACTTCAGCTTCTCAAAGATG
		Reverse	CGAGTTGATCACCAAGATTGATG
		Probe	TTGATGTCCCTCCAATGCGTCACTCTATTG
PsACO2	97.2	Forward	CATGTCCAAAACCCGATCTAGTG
		Reverse	TGATGCATGGGTGGAACATCT
		Probe	ACACACCGATGCCGGAGGAATCATC
PsACO3	97	Forward	AATGGAGTGGCGCATTGG
		Reverse	TGGCGGGATCATCCTTCTC
		Probe	AACATCAACCCACTCGCCGTCTTTGA
PsERS1	92.7	Forward	TTGTGGCAATCCAGATTCATCT
		Reverse	GCAAGGCCTCCACTTCCA
		Probe	ATCATCAAGCTACAACCAGAGGCCAAGCA
PsETR2	96.1	Forward	TCTCCAAAGGGAACCTTCCAT
		Reverse	CGAGCAATGTGCTATCAAACTTG
		Probe	TCTTTTGCTGAGCTGTCCATGGCATC
PsEBF1	95.9	Forward	TCAAGGATTGTGCTGGTGTTG
		Reverse	CTGGAGCTTCACCTTTGTCAAA
		Probe	ATCGCTGGCCTCTTTTCTTCAACTTCTTTG
PsEBF2	91.5	Forward	AGACATTGAGGTGGAAGTATCTATGCT
		Reverse	CTTCCGATACCGTGACAGTTTTT
		Probe	TCACCTTGCGAGTCTCTTCGATCTGTAACC

[a] Probe-based qRT-PCR assays were used for the quantification of transcript abundance of the pea ethylene biosynthesis (*PsACS1, PsACS2, PsACS4, PsACO1, PsACO2* and *PsACO3*), ethylene receptor (*PsERS1*and *PsETR2*), and ethylene signaling-related (*PsEBF1* and *PsEBF2*) genes. The probes were labeled with the 6-FAM fluorescent dye at the 5′ end, and double-quenched with Iowa Black FQ (IBFQ) quencher at the 3′ end and the ZEN quencher in the middle.

Table A2. DNA polymerase and the source of total RNA used for PCR amplification of the full-length CDS of the pea ethylene biosynthesis, receptor, and signaling-related genes. [a]

Gene	DNA Polymerase	Source of total RNA used for cDNA synthesis
PsETR2	Phusion Hot Start II DNA Polymerase	3 DAA pericarps, 7 DAA seeds, and 12 DAI seedling tissues (apex, immature and mature leaves, immature internode)
PsEBF1	Phusion Hot Start II DNA Polymerase	3 DAA pericarps, pericarps from the SPNS 12 h treatment, and 12 DAI seedling tissues (immature internode and mature leaf)
PsEBF2	Phusion Hot Start II DNA Polymerase	3 DAA pericarps, pericarps from the SPNS 12 h treatment, and 12 DAI seedling tissues (immature internode and mature leaf)
PsACS4	Qiagen LongRange PCR Enzyme Mix	6 DAA pericarp wall and 8 DAA seeds
PsACO2	Q5 polymerase	2-week-old seedling tissues (mature and young leaves)
PsACO3	Q5 polymerase	2-week-old seedling tissues (mature and young leaves)

[a] The full-length CDS of the pea ethylene biosynthesis (*PsACS4, PsACO2,* and *PsACO3*), receptor (*PsETR2*), and signaling-related (*PsEBF1* and *PsEBF2*) genes were amplified with the listed DNA polymerase and the source of total RNA (from young pea fruit or seedling tissues of cv. I_3 Alaska-type) used for cDNA synthesis.

Table A3. Primers used for the amplification of the full-length CDS of the pea ethylene biosynthesis, receptor, and signaling-related genes. [a]

Gene	Primer	Sequence
PsACS3/ACS4	Forward	CAAGAAATGGGTTTGGAAAA
	Reverse	TGGAAATCCAATTCATCTAGC
PsACO2	Forward	ACCACCTTGTATAGAACTCTAATATGC
	Reverse	AAGGAATAACATCACATCACTCAC
PsACO3	Forward	CAAAAGAGCCAAAAGAAGATAGAA
	Reverse	TGTGTTACCTATTTTCCAAGTACAA
PsETR2	Forward	AGTTGACAGGGCAAGACTGG
	Reverse	TAACAAGAACAAGAATGTGACATGAA
PsEBF1	Forward	CGTCTTCGTTTTCTCTTCGCT
	Reverse	ATTATTGCAGCCATCTAAAAGGC
PsEBF2	Forward	ATGCTCTTCATCCTTTTCTCTCT
	Reverse	ATAAACGATGAACCAAACATGCT

[a] The full-length CDS of the pea ethylene biosynthesis (*PsACS4, PsACO2,* and *PsACO3*), receptor (*PsETR2*), and signaling-related (*PsEBF1* and *PsEBF2*) genes were amplified by PCR using the given primers. The forward primer was designed to bind to the 5′ UTR and the reverse primer to the 3’ UTR of the mRNA, except in the case of *PSACS4*, where the forward primer was designed to bind to a region spanning the 5’ UTR and CDS.

Table A4. PCR reaction components used for PCR amplification of the full-length CDS of the pea ethylene biosynthesis, receptor, and signaling-related genes. [a]

Polymerase	Component	Volume (µL)	Final concentration
Qiagen LongRange PCR kit	10X LongRange PCR buffer with Mg^{2+}	5	1X
	dNTP mix (10mM each)	2.5	500 µM of each
	Forward Primer (5 µM)	4	0.4 µM
	Reverse Primer (5 µM)	4	0.4 µM
	LongRange PCR enzyme mix	0.4	2 units
	Template cDNA	1	variable
	Nuclease-free water	33.1	
	Total reaction volume	50	
Thermo Scientific Phusion Hot Start II DNA Polymerase or NEB Q5 High-Fidelity DNA Polymerase	5X Phusion HF Buffer or 5X Q5 Reaction Buffer	4	1X
	dNTP mix (10 mM each)	0.4	200 µM of each
	Forward Primer (5 µM)	2	0.5 µM
	Reverse Primer (5 µM)	2	0.5 µM
	Template cDNA	1-2	variable
	Phusion Hot Start II DNA Polymerase or Q5 High-Fidelity DNA Polymerase	0.2	0.02 U/µL
	Nuclease-free water	variable	
	Total reaction volume	20	

[a] PCR reactions were set up as per the manufacture's recommendation for PCR amplification of the pea ethylene biosynthesis, receptor, and signaling-related genes. *PsACS4* was PCR amplified using the Qiagen LongRange PCR kit. *PsETR2, PsEBF1* and *PsEBF2* were PCR amplified using the Thermo Scientific Phusion Hot Start II DNA Polymerase. *PsACO2* and *PsACO3* were PCR amplified using the NEB Q5 High-Fidelity DNA Polymerase.

Table A5. PCR cycling conditions used for PCR amplification of the full-length CDS of the pea ethylene biosynthesis, receptor, and signaling-related genes. [a]

Reaction step	Qiagen LongRange PCR kit [b]		Phusion Hot Start II or NEB Q5 High-Fidelity DNA Polymerase [b]	
	Temperature	**Duration**	**Temperature**	**Duration**
Initial Denaturation	93 ºC	3 min	98 ºC	30 s
Denaturation	93 ºC	15 s	98 ºC	10 s
Primer annealing	Variable	30 s	Variable	20 s
Extension	68 ºC	1 min/kb	72 ºC	30 s/kb
Final extension	68 ºC	10 min	72 ºC	10 min

[a] PCR amplification of the full-length pea ethylene biosynthesis, receptor, and signaling-related genes was completed using the listed PCR cycling conditions. *PsACS4* was PCR amplified using the Qiagen LongRange PCR kit. *PsETR2, PsEBF1* and *PsEBF2* were PCR amplified using the Thermo Scientific Phusion Hot Start II DNA Polymerase. *PsACO2* and *PsACO3* were PCR amplified using the NEB Q5 High-Fidelity DNA Polymerase.

[b] The number of amplification cycles was 35.

Table A6. Relative transcript abundance of ethylene biosynthesis, receptor and signaling-related genes in pericarp wall, pericarp dorsal and ventral vascular sutures, and ovules or seeds of pollinated and non-pollinated fruits (1 to 3 DAA).

Gene	Pollinated				Non-pollinated [a]			
	Seeds	Pericarp wall	Pericarp Dorsal suture	Pericarp Ventral suture	Ovule	Pericarp wall	Pericarp Dorsal suture	Pericarp Ventral suture
PsACS1	696±433 [b]	20.5±7.6	213.3±49.3	417±72.4	134.4± 55.3	6.7±2.5	39.3±13.1	45.3±10.1
PsACS2	725.6±60.3	3152±499	4253±1211	3444±524	1916±537	6351±1035	9639±1480	9794±1595
PsACS4	1080±152	36.4±7.9	76.4±27.5	43.5±10.4	808.2±180.2	461.4±102.2	1221±327	999±354
PsACO1	174.8±22.4	3.6±0.9	38.5±5.5	30.1±7.4	139.5±23.8	7.4±0.9	37±7.0	90±7.1
PsACO2	3.2±1.0	12.6±1.4	50.4±7.0	49.4±4.7	9.8±2.1	44.9±10.0	153.6±47.8	245.3±37.3
PsACO3	45.3±13.2	65.4±14.1	125.9±26.3	93.4±10.7	134±52.9	283.9±85.6	780.3±341.6	741±214.4
PsERS1	50.5±3.4	25.3±2	28.5±2.7	26±1.8	57.1±5.1	35.6±2.5	41.5±3.2	34.6±1.9
PsETR2	7.0±1.2	2.1±0.3	2.2±0.2	2.0±0.3	8.7±1.1	4.5±0.4	5.5±0.5	5.4±0.6
PsEBF1	40.4±1.7	31.2±2.7	38.7±2.0	34.5±2.5	62.7±7.1	52.2±4.0	64.3±4.3	55.9±3.4
PsEBF2	14.7±2.3	7.1±1	6.6±1.5	6.8±1.5	17.6±2.7	15±1.8	14.4±1.9	15.3±1.6

[a] In non-pollinated fruits, flowers were emasculated at -2 DAA to prevent pollination.

[b] Data are means within tissues (harvested 1 to 3 DAA) ± SE, n= 3-4 in ovules/seeds, n= 6-9 in pericarp tissues.

Table A7. Effect of seed removal and hormone treatment on *PsACS1* transcript abundance in the pericarps of pollinated pea ovaries.

Treatment	Time after treatment (h)[a]			
	0	**2**	**8**	**12**
Intact	27 ± 6[b]	29 ± 6	29 ± 5	19 ± 4
SPNS	137 ± 20	145± 21	76 ± 17	57 ± 8
SP		324 ± 79	648 ± 102	544 ± 20
4-Cl-IAA		269213 ± 19071	11987 ± 2580	3955 ± 1510
IAA		5767 ± 1563	93 ± 44	42 ± 8
Ethephon		186 ± 41	36 ± 5	102 ± 2
4-Cl-IAA+Ethephon			17176 ± 3288	8356 ± 2352
IAA+Ethephon			69 ± 21	34 ± 11
STS+IAA		5163 ± 3161	25 ± 1	18 ± 3
STS		46 ± 10	30 ± 9	20 ± 6
IAA+4-Cl-IAA		340795 ± 15682	27275 ± 6891	4912 ± 508

[a] Two DAA pericarps were either left intact, split (SP), or split and deseeded (SPNS) and treated 12 h after deseeding with 4-Cl-IAA (50 μM), IAA (50 μM), or ethephon (1000 mg L^{-1}) in 0.1% aqueous Tween 80 (30 μL) alone or in combination. Auxin or auxin-ethephon combinations were IAA plus 4-Cl-IAA, 4-Cl-IAA plus ethephon, or IAA plus ethephon in 0.1% aqueous Tween 80 (30 μL total). When fruits were treated with both auxin and ethephon, ethephon was applied 90 min after the auxin treatment and samples were collected based on the time after auxin treatment. Because of the delayed ethephon application, the auxin plus ethephon-treated pericarps were not studied at the 2 h time-point. Deseeded pericarps were also pretreated with STS (1 mM in 0.1% aqueous Tween 80, 30 μL) at pericarp splitting and deseeding (STS treatment), and IAA (50 μM in 0.1% aqueous Tween 80, 30 μL) was applied to STS pretreated pericarps (IAA plus STS treatment). The SP and SPNS controls were treated with 0.1% aqueous Tween 80 (30 μL). All the samples were collected with respect to the time after hormone treatment.

[b] Data are means ± SE, n=3 to 8, with the exception of STS + IAA 2 h treatment, where n=2.

SUPPLEMENTARY MATERIALS AND METHODS

Preparation of MOPS buffers

MOPS extraction buffer: 8.37 g of MOPS (final concentration 400 mM) was dissolved in 50 mL of deionized water. Into this solution, 10 mL of glycerol (10% v/v final concentration) and 0.594 g of ascorbic acid sodium salt (30 mM final concentration) were added. After mixing the solution, the pH was adjusted to 7.2, and the volume was adjusted to 100 mL with deionized water.

MOPS reaction buffer: 1.046 g of MOPS (50 mM final concentration) was dissolved in 50 mL of deionized water. Into this solution, 10 mL of glycerol (10% v/v final concentration), 0.099 g of ascorbic acid sodium salt (5 mM final concentration), 0.168 g of sodium bicarbonate (20 mM final concentration), 0.0003 g of iron sulphate (0.2 mL of 10 mM stock; 0.02 mM final concentration), 0.010 g of ACC (1 mM final concentration) and 0.015 g of DTT (1 mM final concentration) were added. After mixing the solution, the pH was adjusted to 7.2, and the volume was adjusted to 100 mL with deionized water.

Appendix B

Supplementary information for Chapter 3

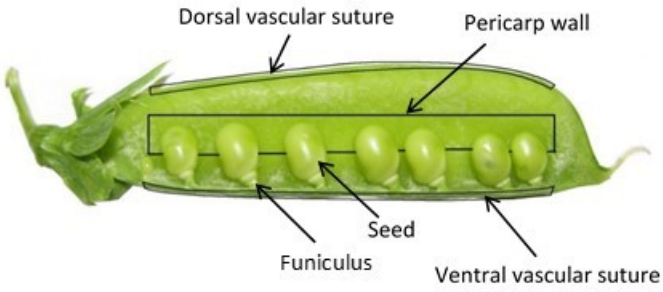


Figure B1. Pea fruit tissues selected for transcript abundance analysis of auxin receptors over fruit development. Pericarp tissues of 0 to 10 days after anthesis (DAA) fruits were dissected into pericarp wall, dorsal vascular suture, and ventral vascular suture tissues. Seeds were also collected separately. Representative fruit is approximately at 9 DAA.

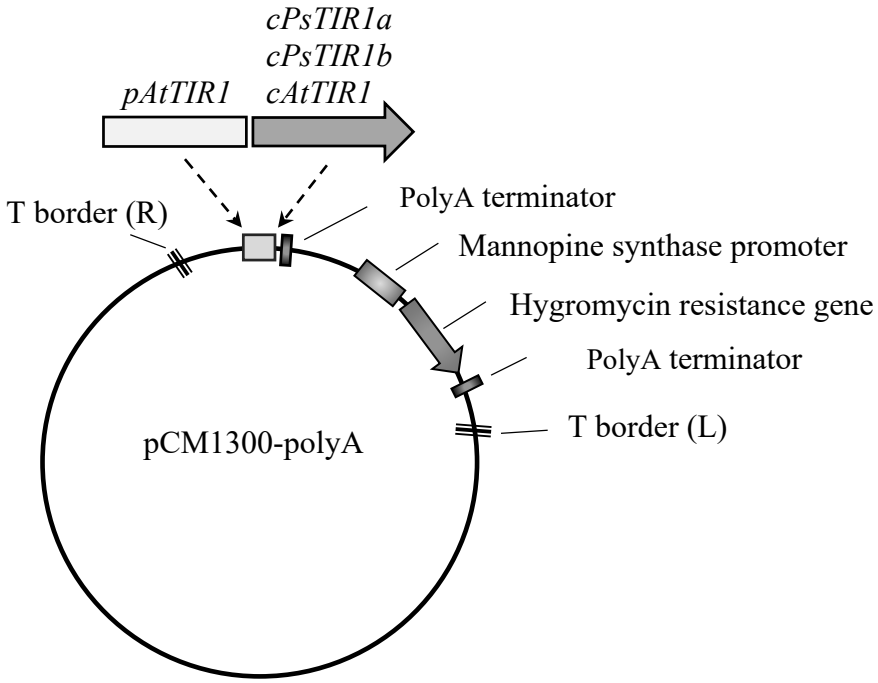


Figure B2. Schematic diagram of the *pAtTIR1::PsTIR1a*, *pAtTIR1::cPsTIR1b*, and *pAtTIR1::cAtTIR1* gene construct used for *Agrobacterium*-mediated transformation of *PsTIR1a, PsTIR1b,* and *AtTIR1* into *Arabidopsis tir1-10* or *tir1-10 afb2-3* plants. The complete coding sequence (CDS) of *the TIR1* genes was placed under the *AtTIR1* promoter in pCM1300-polyA transformation vector, which is a modified version of pCAMBIA1300 containing a mannopine synthase promoter to drive the expression of the hygromycin resistance gene. Towards the end of the pUC18 polylinker, a *CaMV 35S* polyadenylation sequence (PolyA terminator) has been incorporated. The pCM1300-polyA vector was kindly provided by Dr. Enrico Scarpella, University of Alberta.

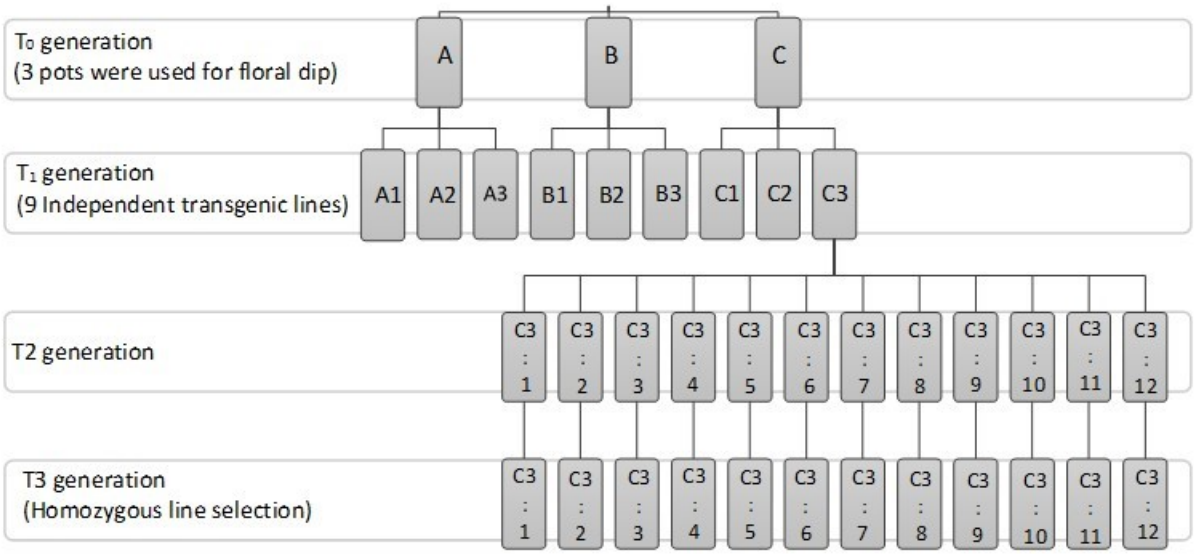


Figure B3. Selection of transgenic *Arabidopsis* plants homozygous for the transgene. Three pots of *Attir1-10* or *Attir1-10 afb2-3* mutants were used for each floral dip transformation of a transgene construct (*pAtTIR1::cPsTIR1a*, *pAtTIR1::cPsTIR1b* or *pAtTIR1::cAtTIR1*). Nine independent transformants were selected at T1 generation which are heterozygous for the transgene. The T1 plants were allowed to self-pollinate, and 12 progenies (T2) were selected from each line (line C3 is shown as an example). Due to the segregation of transgene, the T2 plants are homozygous, hemizygous, or null for the transgene. About 30 to 60 T3 seeds of each T2 line were grown on half-strength MS medium with 25 mg/L hygromycin to determine if the T2 plant is homozygous for the insert. If all the progenies (T3 plants) of a particular T2 line is hygromycin resistant, that T2 line is considered to be homozygous for the insert.

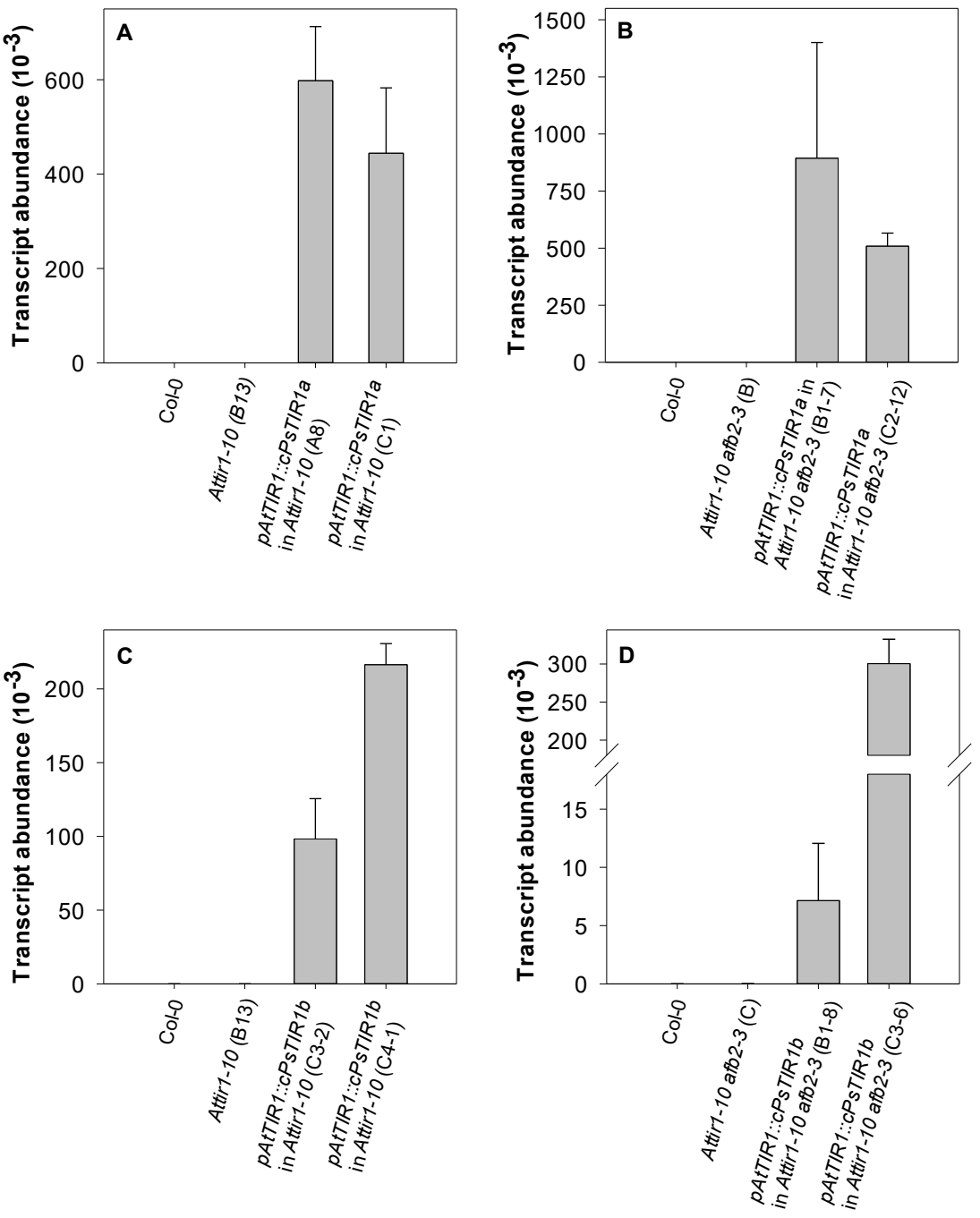


Figure B4. Relative expression of AtTIR1 promoter driven pea auxin receptors *PsTIR1a* (*pAtTIR1::cPsTIR1a*; **A** and **B**) and *PsTIR1b* (*pAtTIR1::cPsTIR1b*; **C** and **D**) in *Attir1-10* (**A** and **C**) and *Attir1-10 afb2-3* (**B** and **D**) double mutants. The transgene construct was introduced into the mutants by floral dip transformation. Transgene expression was analyzed in the leaves of about a month old T_4 generation plants homozygous for the transgene. Data are means ± SD (n=3-4). Each sample is composed of leaves from about three to six plants.

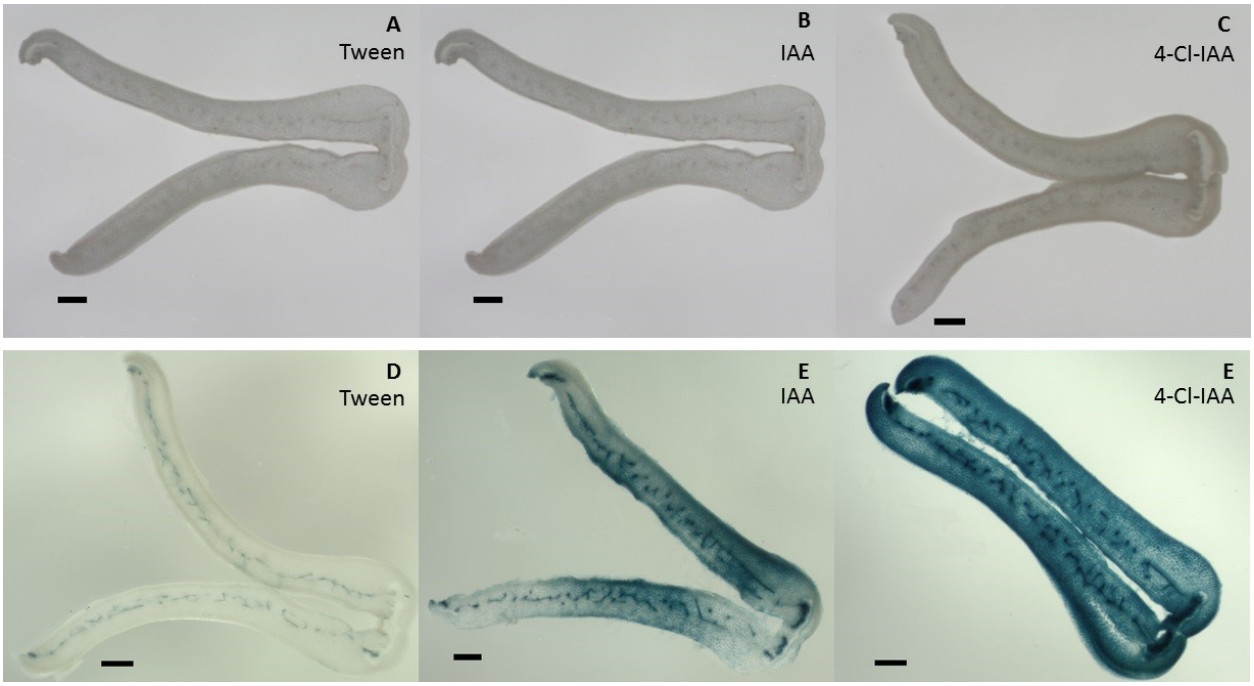


Figure B5. Pericarp GUS staining in WT plants (**A** to **C**) and transgenic plants expressing *DR5::GUS* (**D** to **E**) treated with 0.1% aqueous Tween 80 (**A** and **D**), IAA (50 μM; **B** and **E**) or 4-Cl-IAA (50 μM; **C** and **E**). Two days after anthesis pollinated pericarps were split and deseeded (SPNS) 12 h prior to treatment. Samples were collected at 8h after treatments. Scale bars = 500 μm

```
   1 ATGAATTATT TTCCAGACGA GGTAATAGAA CATGTGTTTG ACTATGTGGT
  51 GTCACATAGC GACAGAAACA GTTTGTCTTT GGTATGCAAA AGTTGGTATA
 101 GAATAGAGGG ATTTACAAGG AAAAGGGTGT TCATAGGAAA CTGTTACTCT
 151 ATTAGTCCTG AGAGGTTGGT AGAGAGGTTT CCTGATTTCA AATCTTTAAC
 201 TCTAAAGGGA AAACCTCATT TTGCTGACTT CAGTTTGGTT CCTCATGGTT
 251 GGGGTGGTTT TGTTTATCCA TGGATTGAAG CTCTTGCTAA GAGTAGAGTT
 301 GGGTTGGAGG AGCTTAGGTT GAAGAGGATG GTTGTGTCAG ATGAGAGCCT
 351 GGAGCTACTG TCTCGTTCTT TCATGAATTT TAAGTCTTTA GTTCTTGTTA
 401 GCTGTGAAGG GTTCACCACT GATGGACTTG CTGCTGTAGC TGCAAATTGC
 451 AGGTCTCTTA GGGAGCTAGA TTTGCAAGAG AATGAAGTTG AAGATCACAA
 501 AGGACAGTGG CTAAGTTGTT TTCCGGAAAA CTGTACATCA CTCGTCGCTC
 551 TTAATTTTGC TTGCCTTAAA GGAGAGATTA ACGTGGGAGC ACTTGAGAGA
 601 CTTGTGGCAA GATCACCTAA CCTCAAGACT CTAAGGTTAA ACCGTTCCGT
 651 GCCGGCTGAT GCACTTCAAA GGATACTAAT GCGAGCGCCT CAAATAGCAG
 701 ATTTGGGTAT TGGATCATTT ATCCATGATC TCAATTCAGA GGCCTACATA
 751 AAGCTTAAGA ATACCATTCT TAGATGCCGG TCAATAACGA GTTTGTCCGG
 801 ATTTTTGGAA GTGGCTCCTT TTAGCCTTGC TGCTGTGTAT CCAATTTGCC
 851 GGAACTTAAC ATCCTTGAAC TTGAGCTATG CAGCAAGCAT TCAGGGCGCT
 901 GAGCTTATTA AACTTATTCG CCATTGCGGC AAACTACAGC GCTTATGGAT
 951 AATGGATTGC ATTGGAGACA AAGGACTAGT TGCTGTAGCT ACTATATGTA
1001 AAGAGTTGCA AGAATTGAGG GTATTTCCAT CGGCACCATT TGGAAATCAA
1051 GCAGCTGTTA CCGAAGTAGG ACTTGTTGCG ATATCAAAGG GATGCCCAAA
1101 GCTCCACTCG TTACTCTACT TCTGCCACCA GATGACAAAT GCTGCTCTCA
1151 TAACAGTAGC CAAGAACTGT CCAAATTTTA TCCGATTTAG GTTATGCATC
1201 CTCGATGCAA CAAAACCTGA CTCCGACACA ATGCAGCCAC TGGATGAAGG
1251 TTTTGGGGCA ATCGTACAGT CATGCAAACG ACTGAGGCGG CTATCACTCT
1301 CCGGTCAGTT GACCGACCAG GTCTTCCTTT ACATTGGAAT GTACGCGGAG
1351 CAGCTTGAAA TGCTATCTAT TGCTTTTGCT GGCGAGAGTG ACAAGGGAAT
1401 GCTCTATGTA TTGAATGGTT GCAAAAAGCT TCGCAAGCTC GAGATAAGAG
1451 ACTGCCCTTT CGGCGACACA GCACTTCTGA CAGACGTAGG GAAGTATGAA
1501 ACAATGCGAT CCCTTTGGAT GTCGTCGTGT GAGGTGACTG TAGGAGCATG
1551 CAAGACATTG GCGAAGAAGA TGCCGAGTTT GAATGTGGAG ATCTTCAATG
1601 AAAGTGAACA AGCAGATTGT TATGTGGAAG ATGGGCAAAG AGTGGAGAAG
1651 ATGTATTTGT ATCGTTCTGT GGCTGGTAAA AGGGAAGATG CACCAGAATA
1701 TGTATGGACT CTGTAG
```

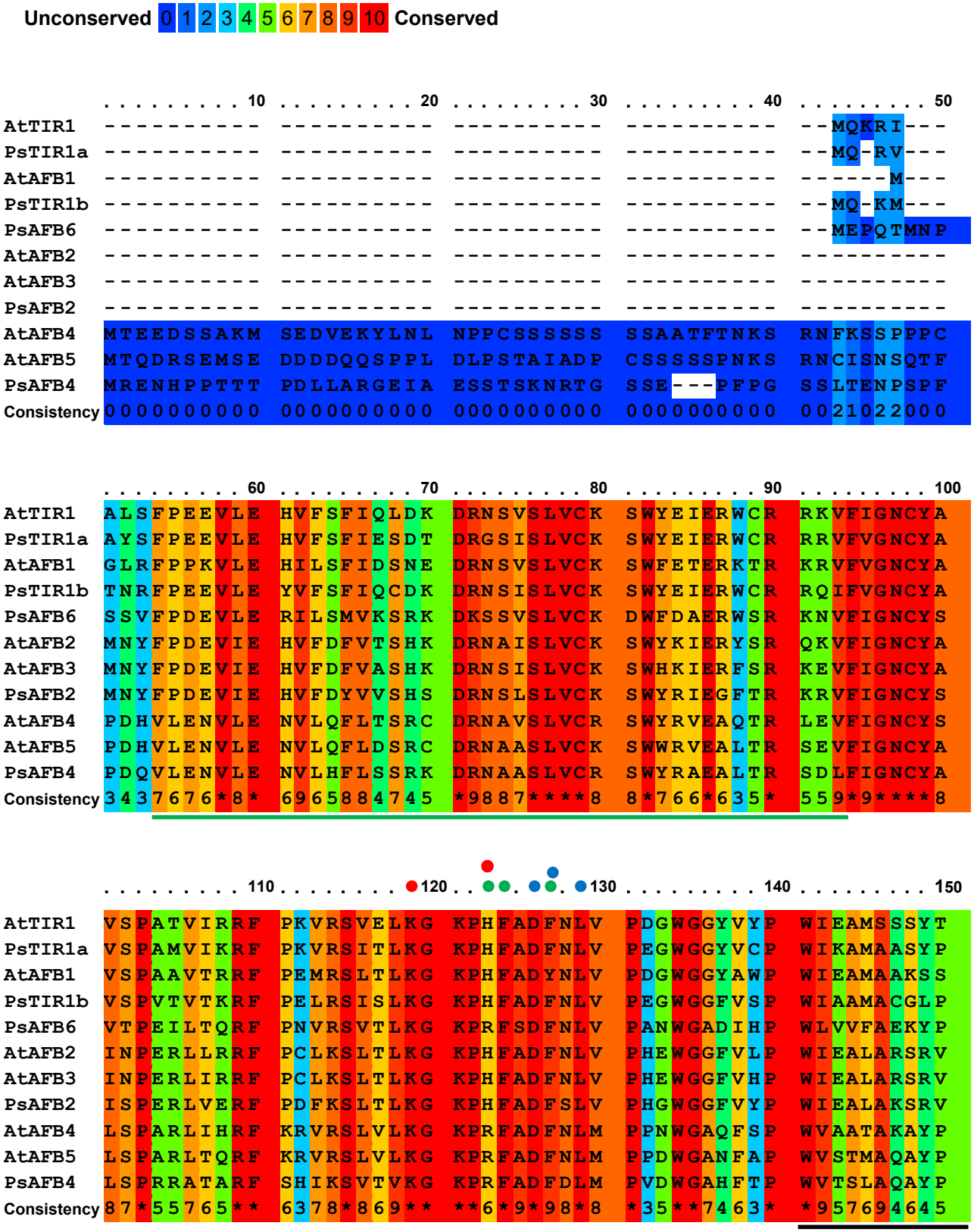
Figure B6. The complete coding sequence of the auxin receptor *PsAFB2* from *P. sativum* L. cv. I3 (Alaska-type). The 615th nucleotide position in the original PsAFB2 sequence (Nadeau, 2009) has been corrected from G to A based on resequencing results. Forward and reverse qRT-PCR primers, and the probe binding sites, are in green, blue and red fonts, respectively. The gray highlighted region represents the F-box domain. The leucine-rich repeat domain coding region is marked with bold and italicized fonts.

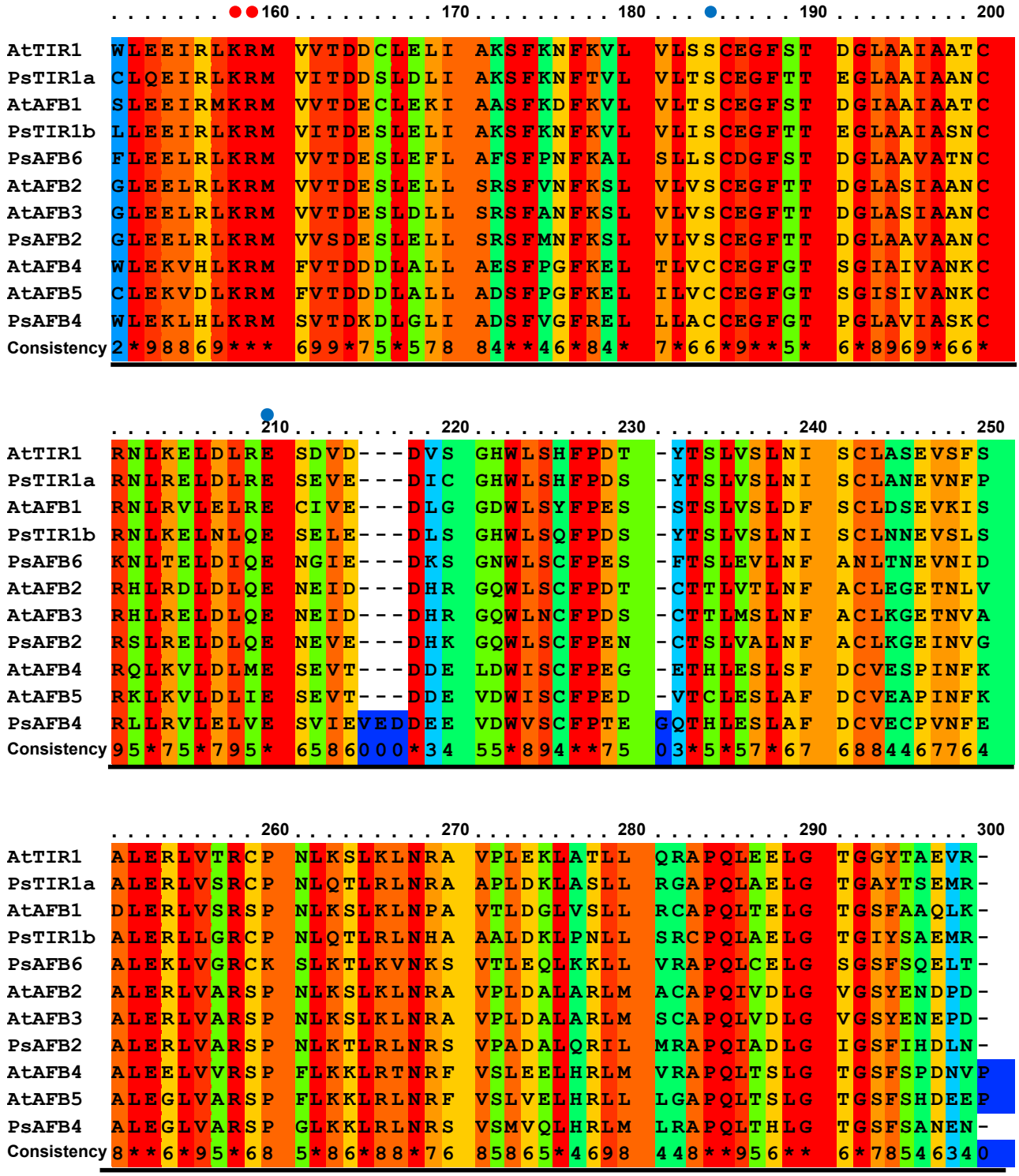
```
   1 ATGCAGAGAG TAGCATACTC GTTCCCTGAG GAAGTACTCG AGCACGTTTT
  51 TTCGTTCATT GAATCGGACA CAGACAGAGG CTCGATCTCG CTGGTATGCA
 101 AATCGTGGTA CGAGATTGAG CGGTGGTGCA GACGGCGGGT GTTCGTCGGG
 151 AACTGCTACG CGGTGAGTCC GGCGATGGTG ATAAAACGGT TTCCGAAAGT
 201 GAGATCTATC ACACTGAAAG GGAAACCGCA CTTTGCGGAC TTCAATTTGG
 251 TACCGGAAGG TTGGGGTGGT TATGTATGTC CTTGGATCAA AGCTATGGCT
 301 GCTAGTTATC CGTGTTTGCA AGAGATTAGG CTTAAGAGAA TGGTTATCAC
 351 TGATGATTCT TTGGACCTTA TCGCTAAATC GTTCAAGAAT TTCACGGTTT
 401 TGGTGCTTAC TTCTTGTGAA GGTTTCACTA CGGAAGGACT TGCTGCCATT
 451 GCTGCCAATT GCAGGAATTT GAGGGAGTTG GACTTGCGGG AGAGTGAAGT
 501 GGAGGACATT TGCGGTCATT GGCTAAGCCA TTTTCCTGAT TCATACACAT
 551 CATTGGTTTC ACTAAACATC TCTTGCTTGG CAAACGAGGT GAATTTTCCT
 601 GCACTAGAGC GCCTGGTTAG TAGGTGTCCT AACTTGCAGA CGCTCAGGCT
 651 CAATCGTGCC GCACCCCTTG ATAAGCTTGC CAGCCTTCTT AGGGGGGCTC
 701 CACAGCTTGC TGAGTTAGGC ACTGGAGCCT ACACGTCTGA GATGCGGCCC
 751 GAGGTCTTTT CAAATCTGGC GGCGGCATTT TCTGGGTGCA TGCAAATGAA
 801 GAGCTTGTCT GGCTTTTGGG ATGTCCTCCC ATCCTACCTG CCTGCCGTTT
 851 ATCCTGTATG CTCCAGACTC ACATCACTAA ACTTAAGTTA TGCTACGATC
 901 CAAAGCCCGG ATCTTATCAA ACTTGTTGGT GAATGTGAGA GTTTGCAGCG
 951 GTTGTGGGTG TTGGATTACA TTGAAGATGC CGGCCTTGAC ATGCTTGCTG
1001 CATCATGTAA GGATCTGAGG GAGTTAAGGG TGTTTCCATC TAATCCATTT
1051 GGGCTGGAAC CAAATGTAGC ATTGACAGAG CAGGGCCTTG TTTCTGTGTC
1101 TGAAGGTTGC CCCAAGCTCC ATTCGGTTCT ATATTTTTGT CGTCAAATGA
1151 CCAATGCTGC CCTAACTATA ATTGCTCGGA ACAGGCCTAA TTTGACTCGT
1201 TTTAGATTAT GTATTATTGA GCCTCGCACT CCTGACTATC TTACTCGTCA
1251 ACCTCTGGAT GTTGGATTTG GAGCTATTGT TGAGCAATGT AAGAGTCTTC
1301 AGCGCCTTTC CCTCTCAGGG CTTCTCACCG ACCGTGTTTT TGAGTATATT
1351 GGGACTTATG GGAAGAAGCT TGAGATGCTT TCTGTAGCTT TTGCCGGAGA
1401 AAGTGATTTG GGACTCCATC ACGTCCTCTC CGGGTGTGAC AACCTTAGGA
1451 AGTTGGAAAT CAGGGACTGC CCCTTTGGTG ACAAAGCTCT TTTAGCCAAT
1501 GCTGCAAAAT TGGAGACAAT GCGATCCCTT TGGATGTCCT CTTGCCATGT
1551 GAGTTATGGA GCATGTAAAC TGTTGGGTCT GAAGTTGCCG AGACTCAATG
1601 TTGAAGTCAT TGATGAAAGA GGACCTCCAG ATTCAAGGCC AGATAATAGT
1651 CCCGTTGAGA AGCTTTATAT ATACAGGACA ATTTCTGGGC CTAGATTGGA
1701 CATGCCTGGT TATGTATGGA CAATGGAAGA TGATTCTGCA TATCCTGAAT
1751 GA
```

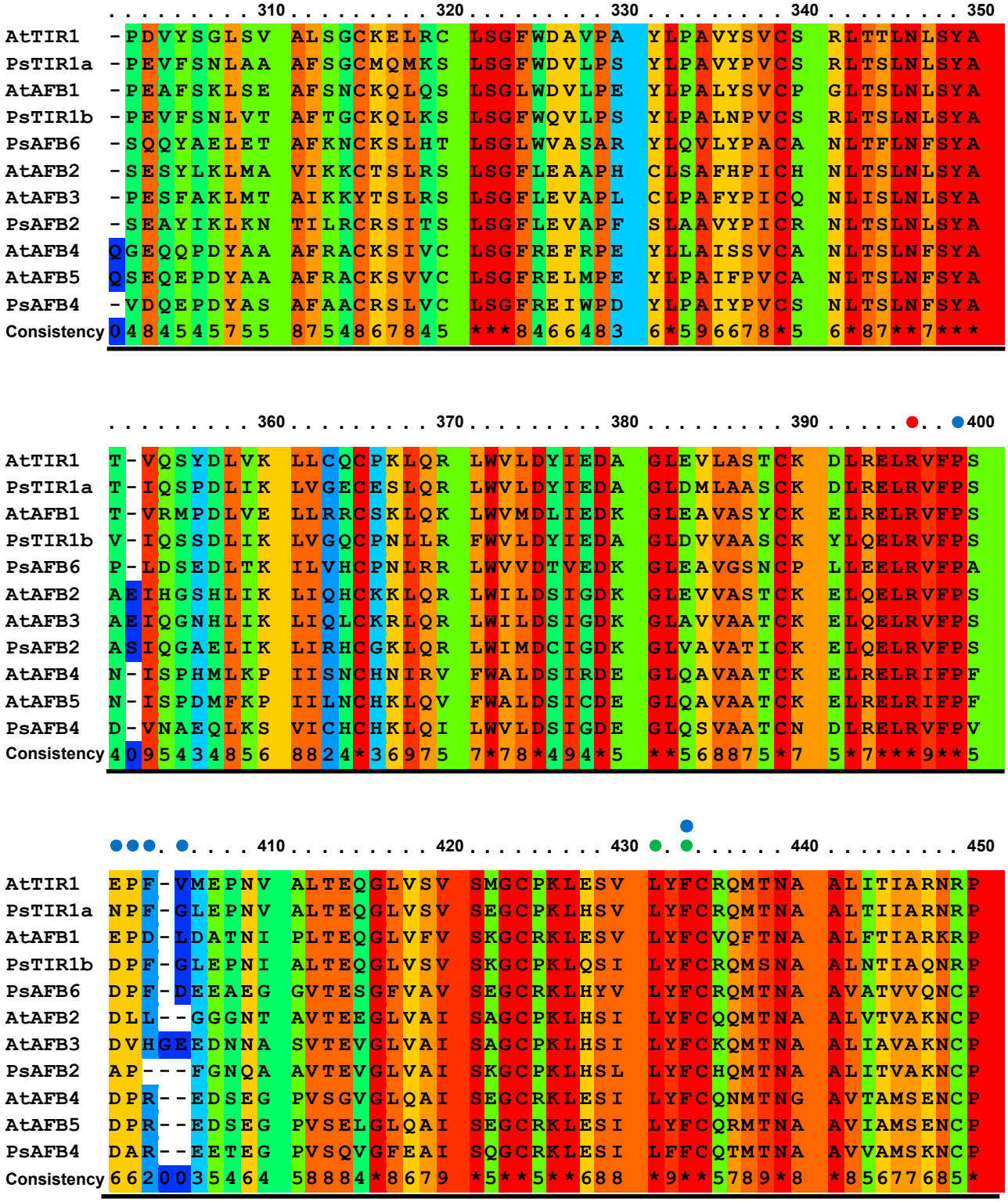
Figure B7. The complete coding sequence of the auxin receptor *PsTIR1a* from *P. sativum* L. cv. I_3 (Alaska-type). Forward and reverse qRT-PCR primers, and the probe binding sites, are in green, blue and red fonts, respectively. The gray highlighted region represents the F-box domain. The leucine-rich repeat domain coding region is marked with bold and italicized fonts.

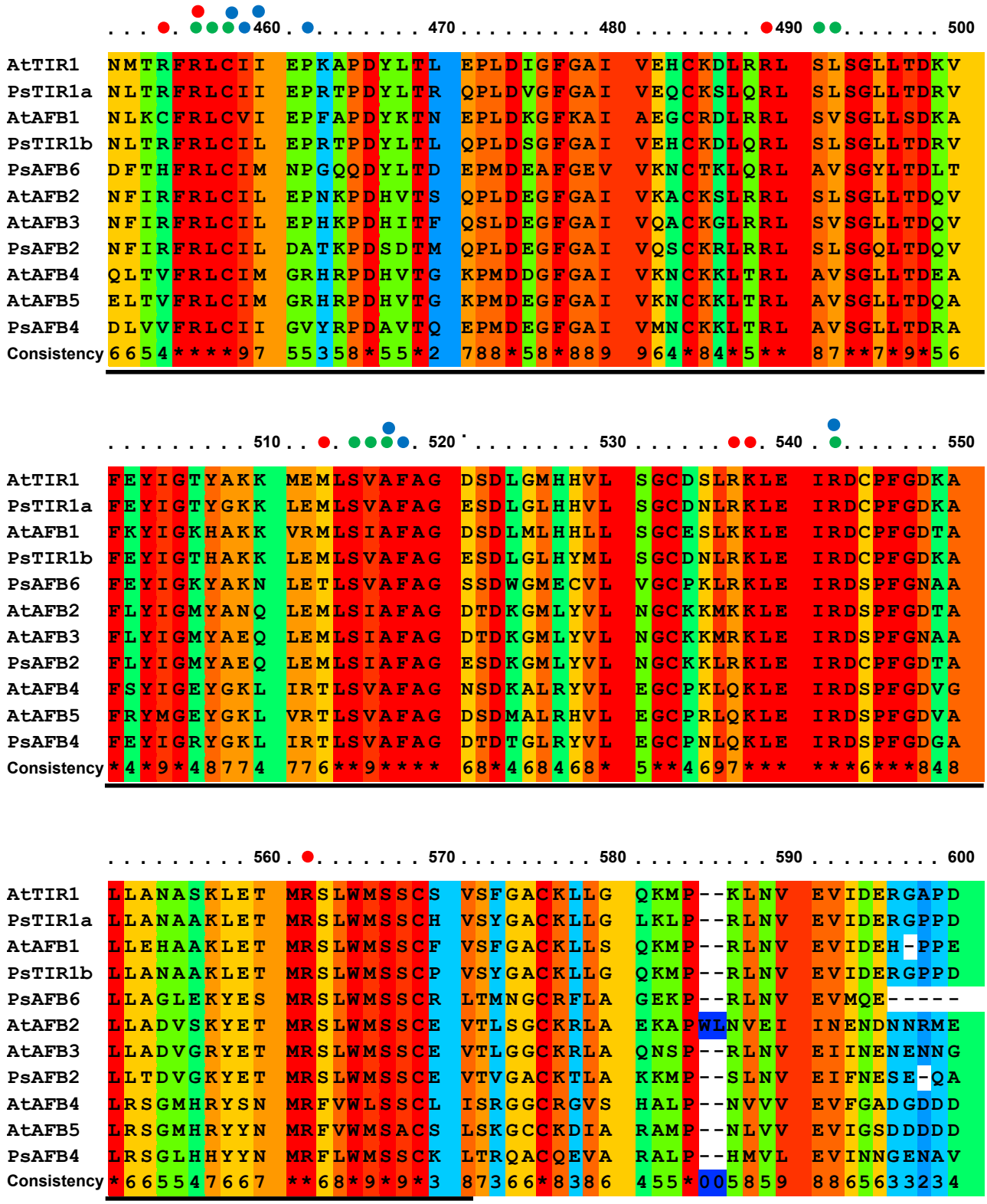
```
   1 ATGCAGAAAA TGACGAACCG GTTCCCCGAG GAAGTTCTCG AGTACGTGTT
  51 CTCGTTCATT CAATGTGACA AAGACCGAAA CTCCATCTCT CTAGTGTGTA
 101 AGTCGTGGTA CGAGATCGAA CGGTGGTGCA GGAGGCAGAT TTTCGTCGGA
 151 AACTGCTACG CTGTTAGTCC TGTCACGGTG ACGAAACGGT TCCCGGAGTT
 201 GAGATCCATT TCGTTGAAAG GGAAACCGCA CTTTGCGGAC TTCAATTTGG
 251 TGCCTGAAGG TTGGGGAGGT TTCGTTAGTC CGTGGATCGC GGCTATGGCT
 301 TGTGGTTTGC CGTTGCTCGA AGAGATTCGG CTCAAGAGGA TGGTTATAAC
 351 GGATGAGAGC TTGGAGCTTA TTGCTAAATC GTTTAAGAAT TTTAAAGTTT
 401 TGGTTCTTAT TTCGTGTGAG GGTTTCACTA CGGAAGGACT TGCCGCCATT
 451 GCTTCTAATT GCAGGAATTT GAAGGAATTG AACTTGCAGG AGAGTGAACT
 501 GGAGGACCTT AGTGGGCATT GGCTAAGCCA ATTCCCTGAT TCTTACACAT
 551 CCTTAGTTTC ACTTAACATC TCTTGCTTGA ACAATGAGGT GAGTTTGTCC
 601 GCCCTAGAGC GCCTGCTTGG TAGGTGTCCT AACCTCCAGA CTCTTCGTCT
 651 CAATCATGCT GCAGCCCTTG ATAAACTGCC GAACTTACTT AGCCGATGTC
 701 CTCAGCTGGC TGAGTTAGGC ACAGGAATCT ACTCAGCTGA GATGCGCCCA
 751 GAGGTCTTCT CAAACCTTGT AACAGCATTT ACTGGGTGCA AGCAATTGAA
 801 GAGCTTGTCT GGTTTTTGGC AAGTTCTGCC ATCCTACCTT CCGGCACTAA
 851 ATCCGGTCTG CTCTAGGCTA ACATCACTAA ACCTGAGTTA TGCTGTCATT
 901 CAAAGCTCTG ATCTTATCAA GCTTGTCGGT CAATGCCCCA ATTTGTTGCG
 951 GTTTTGGGTG CTTGATTACA TAGAAGATGC TGGTCTTGAT GTCGTTGCTG
1001 CTTCATGCAA GTATCTACAG GAGTTGAGGG TGTTTCCTTC TGATCCATTT
1051 GGTTTAGAAC CAAATATTGC GTTGACAGAA CAGGGTCTTG TCTCTGTATC
1101 TAAAGGCTGC CCAAAGCTCC AGTCCATTCT ATATTTTTGT CGTCAAATGT
1151 CTAACGCCGC TCTAAATACT ATTGCGCAGA ATAGGCCTAA TTTGACTCGC
1201 TTTCGCCTGT GTATTCTGGA GCCTCGAACT CCTGACTATC TTACCCTTCA
1251 ACCACTGGAT TCTGGTTTTG GAGCTATTGT AGAGCATTGT AAGGATCTTC
1301 AGCGCCTTTC CCTTTCCGGC CTTCTAACTG ATCGTGTTTT TGAGTATATT
1351 GGAACTCATG CGAAGAAGCT GGAGATGCTA TCTGTTGCCT TTGCCGGAGA
1401 GAGCGATTTG GGTCTCCATT ATATGCTGTC CGGGTGTGAT AATCTTAGGA
1451 AGCTAGAGAT CAGGGACTGC CCCTTTGGCG ACAAGGCTCT CTTGGCCAAT
1501 GCGGCAAAAC TCGAGACAAT GCGATCCCTT TGGATGTCCT CTTGCCCAGT
1551 GAGTTATGGA GCCTGTAAGC TACTGGGTCA GAAAATGCCC AGACTTAATG
1601 TTGAAGTCAT TGATGAAAGA GGACCTCCAG ATTCAAGGCC AGACAGTTGT
1651 CCTGTTGAGA AGCTCTATAT ATACAGGAGT ACTGCTGGGC CAAGGCTGGA
1701 CATGCCTGGT TTTGTCTGGA CAATGGAGGA TGATTCCTCC CTGCGCAGTG
1751 TATAG
```

Figure B8. The complete coding sequence of the auxin receptor *PsTIR1b* from *P. sativum* L. cv. I_3 (Alaska-type). Forward and reverse qRT-PCR primers, and the probe binding sites, are in green, blue and red fonts, respectively. The gray highlighted region represents the F-box domain. The leucine-rich repeat domain coding region is marked with bold and italicized fonts.


Unconserved 0 1 2 3 4 5 6 7 8 9 10 Conserved
AtTIR1
PsTIR1a
AtAFB1
PsTIR1b
PsAFB6
AtAFB2
AtAFB3
PsAFB2
AtAFB4
AtAFB5
PsAFB4
Consistency


160 170 180 190 200
AtTIR1 WLEEIRLKRM VVTDDCLELI AKSFKNFKVL VLSSCEGFST DGLAAIAATC
PsTIR1a CLQEIRLKRM VITDDSLDLI AKSFKNFTVL VLTSCEGFTT EGLAAIAANC
AtAFB1 SLEEIRMKRM VVTDECLEKI AASFKDFKVL VLTSCEGFST DGIAAIAATC
PsTIR1b LLEEIRLKRM VITDESLELI AKSFKNFKVL VLISCEGFTT EGLAAIASNC
PsAFB6 FLEELRLKRM VVTDESLEFL AFSFPNFKAL SLLSCDGFST DGLAAVATNC
AtAFB2 GLEELRLKRM VVTDESLELL SRSFVNFKSL VLVSCEGFTT DGLASIAANC
AtAFB3 GLEELRLKRM VVTDESLDLL SRSFANFKSL VLVSCEGFTT DGLASIAANC
PsAFB2 GLEELRLKRM VVSDESLELL SRSFMNFKSL VLVSCEGFTT DGLAAVAANC
AtAFB4 WLEKVHLKRM FVTDDDLALL AESFPGFKEL TLVCCEGFGT SGIAIVANKC
AtAFB5 CLEKVDLKRM FVTDDDLALL ADSFPGFKEL ILVCCEGFGT SGISIVANKC
PsAFB4 WLEKLHLKRM SVTDKDLGLI ADSFVGFREL LLACCEGFGT PGLAVIASKC
Consistency 2*9886 9***6 99*75*578 84**46*84* 7*66*9**5* 6*8969*66*
210 220 230 240 250
AtTIR1 RNLKELDLRE SDVD---DVS GHWLSHFPDT -YTSLVSLNI SCLASEVSFS
PsTIR1a RNLRELDLRE SEVE---DIC GHWLSHFPDS -YTSLVSLNI SCLANEVNFP
AtAFB1 RNLRVLELRE CIVE---DLG GDWLSYFPES -STSLVSLDF SCLDSEVKIS
PsTIR1b RNLKELNLQE SELE---DLS GHWLSQFPDS -YTSLVSLNI SCLNNEVSLS
PsAFB6 KNLTELDIQE NGIE---DKS GNWLSCFPES -FTSLEVLNF ANLTNEVNID
AtAFB2 RHLRDLDLQE NEID---DHR GQWLSCFPDT -CTTLVTLNF ACLEGETNLV
AtAFB3 RHLRELDLQE NEID---DHR GQWLNCFPDS -CTTLMSLNF ACLKGETNVA
PsAFB2 RSLRELDLQE NEVE---DHK GQWLSCFPEN -CTSLVALNF ACLKGEINVG
AtAFB4 RQLKVLDLME SEVT---DDE LDWISCFPEG -ETHLESLSF DCVESPINFK
AtAFB5 RKLKVLDLIE SEVT---DDE VDWISCFPED -VTCLESLAF DCVEAPINFK
PsAFB4 RLLRVLELVE SVIEVEDDEE VDWVSCFPTE GQTHLESLAF DCVECPVNFE
Consistency 95*75*795* 6586000*34 55*894**75 03*5*57*67 6884467764
260 270 280 290 300
AtTIR1 ALERLVTRCP NLKSLKLNRA VPLEKLATLL QRAPQLEELG TGGYTAEVR-
PsTIR1a ALERLVSRCP NLQTLRLNRA APLDKLASLL RGAPQLAELG TGAYTSEMR-
AtAFB1 DLERLVSRSP NLKSLKLNPA VTLDGLVSLL RCAPQLTELG TGSFAAQLK-
PsTIR1b ALERLLGRCP NLQTLRLNHA AALDKLPNLL SRCPQLAELG TGIYSAEMR-
PsAFB6 ALEKLVGRCK SLKTLKVNKS VTLEQLKKLL VRAPQLCELG SGSFSQELT-
AtAFB2 ALERLVARSP NLKSLKLNRA VPLDALARLM ACAPQIVDLG VGSYENDPD-
AtAFB3 ALERLVARSP NLKSLKLNRA VPLDALARLM SCAPQLVDLG VGSYENEPD-
PsAFB2 ALERLVARSP NLKTLRLNRS VPADALQRIL MRAPQIADLG IGSFIHDLN-
AtAFB4 ALEELVVRSP FLKKLRTNRF VSLEELHRLM VRAPQLTSLG TGSFSPDNVP
AtAFB5 ALEGLVARSP FLKKLRLNRF VSLVELHRLL LGAPQLTSLG TGSFSHDEEP
PsAFB4 ALEGLVARSP GLKKLRLNRS VSMVQLHRLM LRAPQLTHLG TGSFSANEN-
Consistency 8**6*95*68 5*86*88*76 85865*4698 448**956** 6*7854634 0


310 320 330 340 350
AtTIR1 -PDVYSGLSV ALSGCKELRC LSGFWDAVPA YLPAVYSVCS RLTTLNLSYA
PsTIR1a -PEVFSNLAA AFSGCMQMKS LSGFWDVLPS YLPAVYPVCS RLTSLNLSYA
AtAFB1 -PEAFSKLSE AFSNCKQLQS LSGLWDVLPE YLPALYSVCP GLTSLNLSYA
PsTIR1b -PEVFSNLVT AFTGCKQLKS LSGFWQVLPS YLPALNPVCS RLTSLNLSYA
PsAFB6 -SQQYAELET AFKNCKSLHT LSGLWVASAR YLQVLYPACA NLTFLNFSYA
AtAFB2 -SESYLKLMA VIKKCTSLRS LSGFLEAAPH CLSAFHPICH NLTSLNLSYA
AtAFB3 -PESFAKLMT AIKKYTSLRS LSGFLEVAPL CLPAFYPICQ NLISLNLSYA
PsAFB2 -SEAYIKLKN TILRCRSITS LSGFLEVAPF SLAAVYPICR NLTSLNLSYA
AtAFB4 QGEQQPDYAA AFRACKSIVC LSGFREFRPE YLLAISSVCA NLTSLNFSYA
AtAFB5 QSEQEPDYAA AFRACKSVVC LSGFRELMPE YLPAIFPVCA NLTSLNFSYA
PsAFB4 -VDQEPDYAS AFAACRSLVC LSGFREIWPD YLPAIYPVCS NLTSLNFSYA
Consistency 048454575 5 875486784 5 ***846648 3 6*596678*5 6*87**7***
360 370 380 390 400
AtTIR1 T-VQSYDLVK LLCQCPKLQR LWVLDYIEDA GLEVLASTCK DLRELRVFPS
PsTIR1a T-IQSPDLIK LVGECESLQR LWVLDYIEDA GLDMLAASCK DLRELRVFPS
AtAFB1 T-VRMPDLVE LLRRCSKLQK LWVMDLIEDK GLEAVASYCK ELRELRVFPS
PsTIR1b V-IQSSDLIK LVGQCPNLLR FWVLDYIEDA GLDVVAASCK YLQELRVFPS
PsAFB6 P-LDSEDLTK ILVHCPNLRR LWVVDTVEDK GLEAVGSNCP LLEELRVFPA
AtAFB2 AEIHGSHLIK LIQHCKKLQR LWILDSIGDK GLEVVASTCK ELQELRVFPS
AtAFB3 AEIQGNHLIK LIQLCKRLQR LWILDSIGDK GLAVVAATCK ELQELRVFPS
PsAFB2 ASIQGAELIK LIRHCGKLQR LWIMDCIGDK GLVAVATICK ELQELRVFPS
AtAFB4 N-ISPHMLKP IISNCHNIRV FWALDSIRDE GLQAVAATCK ELRELRIFPF
AtAFB5 N-ISPDMFKP IILNCHKLQV FWALDSICDE GLQAVAATCK ELRELRIFPF
PsAFB4 D-VNAEQLKS VICHCHKLQI LWVLDSIGDE GLQSVAATCN DLRELRVFPV
Consistency 409543485 6 8824*369 75 7*78*494*5 **568875*7 5*7***9**5
410 420 430 440 450
AtTIR1 EPF-VMEPNV ALTEQGLVSV SMGCPKLESV LYFCRQMTNA ALITIARNRP
PsTIR1a NPF-GLEPNV ALTEQGLVSV SEGCPKLHSV LYFCRQMTNA ALTIIARNRP
AtAFB1 EPD-LDATNI PLTEQGLVFV SKGCRKLESV LYFCVQFTNA ALFTIARKRP
PsTIR1b DPF-GLEPNI ALTEQGLVSV SKGCPKLQSI LYFCRQMSNA ALNTIAQNRP
PsAFB6 DPF-DEEAEG GVTESGFVAV SEGCRKLHYV LYFCRQMTNA AVATVVQNCP
AtAFB2 DLL--GGGNT AVTEEGLVAI SAGCPKLHSI LYFCQQMTNA ALVTVAKNCP
AtAFB3 DVHGEEDNNA SVTEVGLVAI SAGCPKLHSI LYFCKQMTNA ALIAVAKNCP
PsAFB2 AP---FGNQA AVTEVGLVAI SKGCPKLHSL LYFCHQMTNA ALITVAKNCP
AtAFB4 DPR--EDSEG PVSGVGLQAI SEGCRKLESI LYFCQNMTNG AVTAMSENCP
AtAFB5 DPR--EDSEG PVSELGLQAI SEGCRKLESI LYFCQRMTNA AVIAMSENCP
PsAFB4 DAR--EETEG PVSQVGFEAI SQGCRKLESI LFFCQTMTNA AVVAMSKNCP
Consistency 662003546 4 5888 4*867 9 *5**5**688 *9**5789*8 *856776 85*


460 470 480 490 500
AtTIR1 NMTRFRLCII EPKAPDYLTL EPLDIGFGAI VEHCKDLRRL SLSGLLTDKV
PsTIR1a NLTRFRLCII EPRTPDYLTR QPLDVGFGAI VEQCKSLQRL SLSGLLTDRV
AtAFB1 NLKCFRLCVI EPFAPDYKTN EPLDKGFKAI AEGCRDLRRL SVSGLLSDKA
PsTIR1b NLTRFRLCIL EPRTPDYLTL QPLDSGFGAI VEHCKDLQRL SLSGLLTDRV
PsAFB6 DFTHFRLCIM NPGQQDYLTD EPMDEAFGEV VKNCTKLQRL AVSGYLTDLT
AtAFB2 NFIRFRLCIL EPNKPDHVTS QPLDEGFGAI VKACKSLRRL SLSGLLTDQV
AtAFB3 NFIRFRLCIL EPHKPDHITF QSLDEGFGAI VQACKGLRRL SVSGLLTDQV
PsAFB2 NFIRFRLCIL DATKPDSDTM QPLDEGFGAI VQSCKRLRRL SLSGQLTDQV
AtAFB4 QLTVFRLCIM GRHRPDHVTG KPMDDGFGAI VKNCKKLTRL AVSGLLTDEA
AtAFB5 ELTVFRLCIM GRHRPDHVTG KPMDEGFGAI VKNCKKLTRL AVSGLLTDQA
PsAFB4 DLVVFRLCII GVYRPDAVTQ EPMDEGFGAI VMNCKKLTRL AVSGLLTDRA
Consistency 6654****97 55358*55*2 788*58*889 964*84*5** 87**7*9*56
510 520 530 540 550
AtTIR1 FEYIGTYAKK MEMLSVAFAG DSDLGMHHVL SGCDSLRKLE IRDCPFGDKA
PsTIR1a FEYIGTYGKK LEMLSVAFAG ESDLGLHHVL SGCDNLRKLE IRDCPFGDKA
AtAFB1 FKYIGKHAKK VRMLSIAFAG DSDLMLHHLL SGCESLKKLE IRDCPFGDTA
PsTIR1b FEYIGTHAKK LEMLSVAFAG ESDLGLHYML SGCDNLRKLE IRDCPFGDKA
PsAFB6 FEYIGKYAKN LETLSVAFAG SSDWGMECVL VGCPKLRKLE IRDSPFGNAA
AtAFB2 FLYIGMYANQ LEMLSIAFAG DTDKGMLYVL NGCKKMKKLE IRDSPFGDTA
AtAFB3 FLYIGMYAEQ LEMLSIAFAG DTDKGMLYVL NGCKKMRKLE IRDSPFGNAA
PsAFB2 FLYIGMYAEQ LEMLSIAFAG ESDKGMLYVL NGCKKLRKLE IRDCPFGDTA
AtAFB4 FSYIGEYGKL IRTLSVAFAG NSDKALRYVL EGCPKLQKLE IRDSPFGDVG
AtAFB5 FRYMGEYGKL VRTLSVAFAG DSDMALRHVL EGCPRLQKLE IRDSPFGDVA
PsAFB4 FEYIGRYGKL IRTLSVAFAG DTDTGLRYVL EGCPNLQKLE IRDSPFGDGA
Consistency *4*9*48774 776**9**** 68*46846 8* 5**4697*** ***6***848
560 570 580 590 600
AtTIR1 LLANASKLET MRSLWMSSCS VSFGACKLLG QKMP--KLNV EVIDERGAPD
PsTIR1a LLANAAKLET MRSLWMSSCH VSYGACKLLG LKLP--RLNV EVIDERGPPD
AtAFB1 LLEHAAKLET MRSLWMSSCF VSFGACKLLS QKMP--RLNV EVIDEH-PPE
PsTIR1b LLANAAKLET MRSLWMSSCP VSYGACKLLG QKMP--RLNV EVIDERGPPD
PsAFB6 LLAGLEKYES MRSLWMSSCR LTMNGCRFLA GEKP--RLNV EVMQE-----
AtAFB2 LLADVSKYET MRSLWMSSCE VTLSGCKRLA EKAPWLNVEI INENDNNRME
AtAFB3 LLADVGRYET MRSLWMSSCE VTLGGCKRLA QNSP--RLNV EIINENENNG
PsAFB2 LLTDVGKYET MRSLWMSSCE VTVGACKTLA KKMP--SLNV EIFNESE-QA
AtAFB4 LRSGMHRYSN MRFVWLSSCL ISRGGCRGVS HALP--NVVV EVFGADGDDD
AtAFB5 LRSGMHRYYN MRFVWMSACS LSKGCCKDIA RAMP--NLVV EVIGSDDDDD
PsAFB4 LRSGLHHYYN MRFLWMSSCK LTRQACQEVA RALP--HMVL EVINNGENAV
Consistency *66554766 7 **68*9*9*3 873 66*8386 455*005859 8865633234

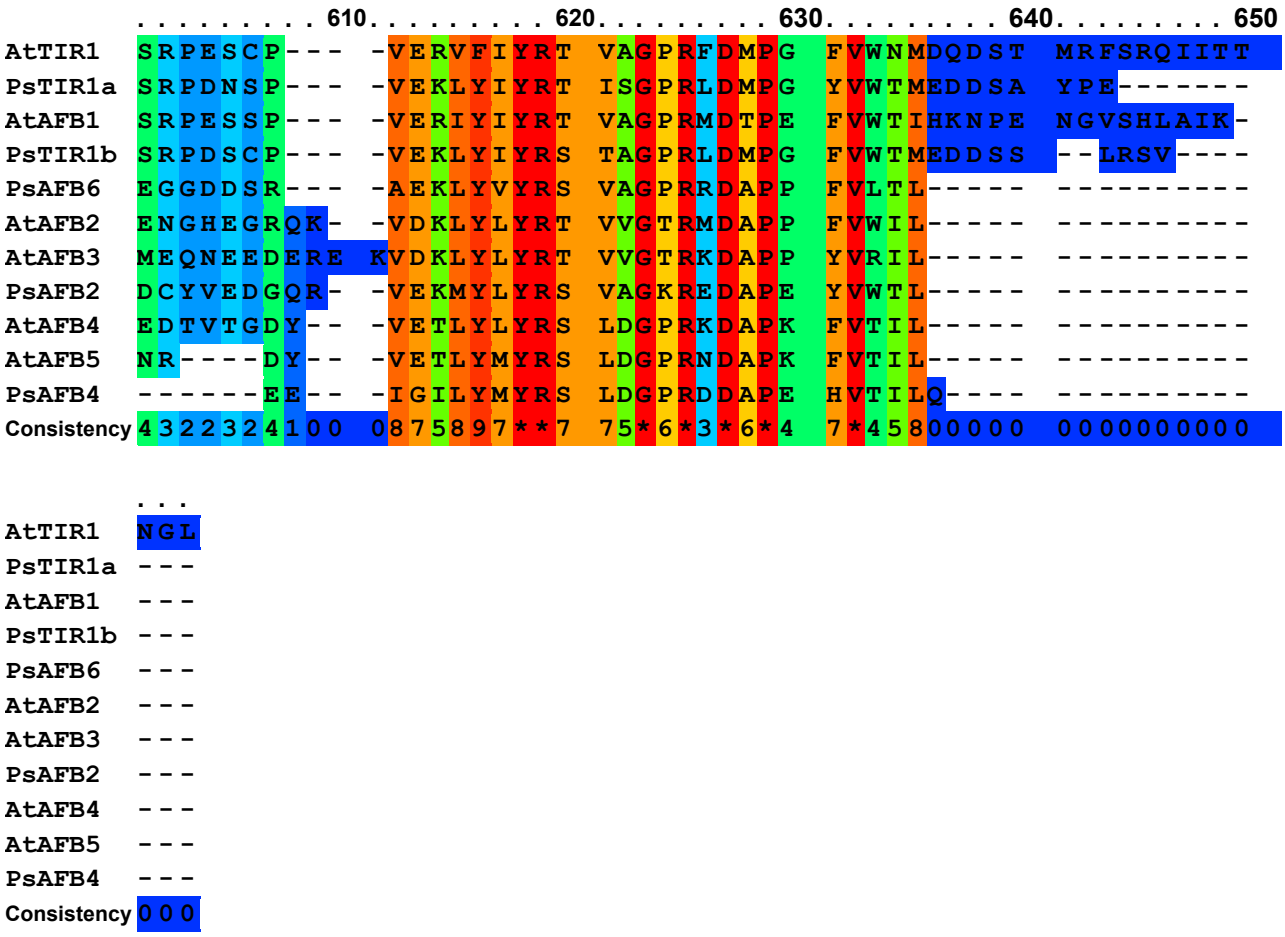


Figure B9. Alignment of the predicted pea (*P. sativum* L. cv. I_3 Alaska-type) TIR1/AFB auxin receptor protein sequences PsTIR1a, PsTIR1b, PsAFB2, PsAFB4, and PsAFB6 with the auxin receptor proteins of *Arabidopsis thaliana* (At). Sequences were aligned using the PRALINE multiple sequence alignment tool under default parameters (www.ibi.vu.nl/programs/pralinewww). Color coding shows the amino acid conservation as shown in the legend at the beginning of the sequence alignment. Green, blue and red circles show the auxin, IAA7 and $InsP_6$ contacting amino acid residues of AtTIR1 (Tan et al., 2007). The dotted green line denotes the F-box domain, and the solid black lines denote the leucine-rich repeat domains with respect to AtTIR1 (Dharmasiri et al., 2005).

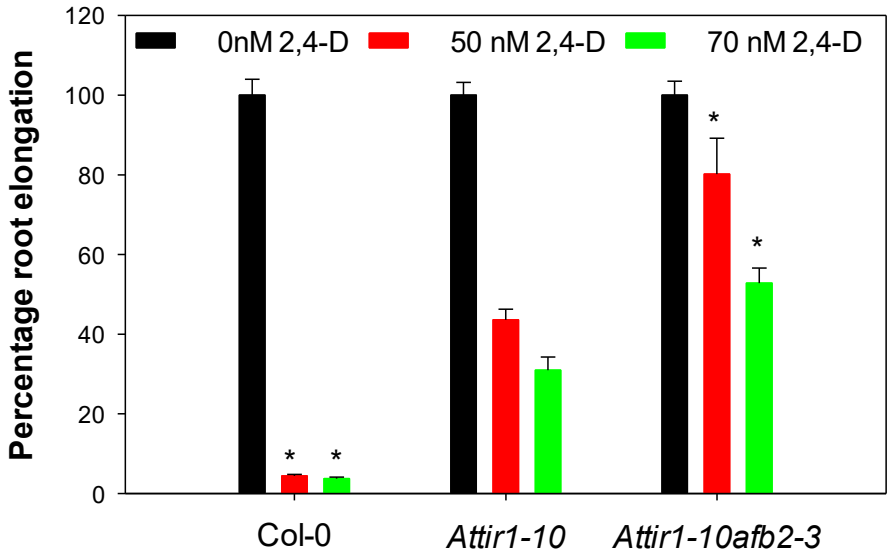


Figure B10. Root elongation of *Arabidopsis tir1-10* and *tir1-10 afb2-3* mutants grown in the presence of 2,4-D. Four-day-old seedlings were transferred to media containing 0, 50, or 70 nM 2,4-D, and grown for three days before root length measurement. Root elongation of each genotype in 2,4-D is expressed as a percentage compared to the same genotype in the media without auxin. Data are means ± SE (n=8), Comparisons were made using a two-tailed student's T-test.

* Means are significantly different from that of *tir1-10* single mutants within 2,4-D concentration, $P<0.01$.

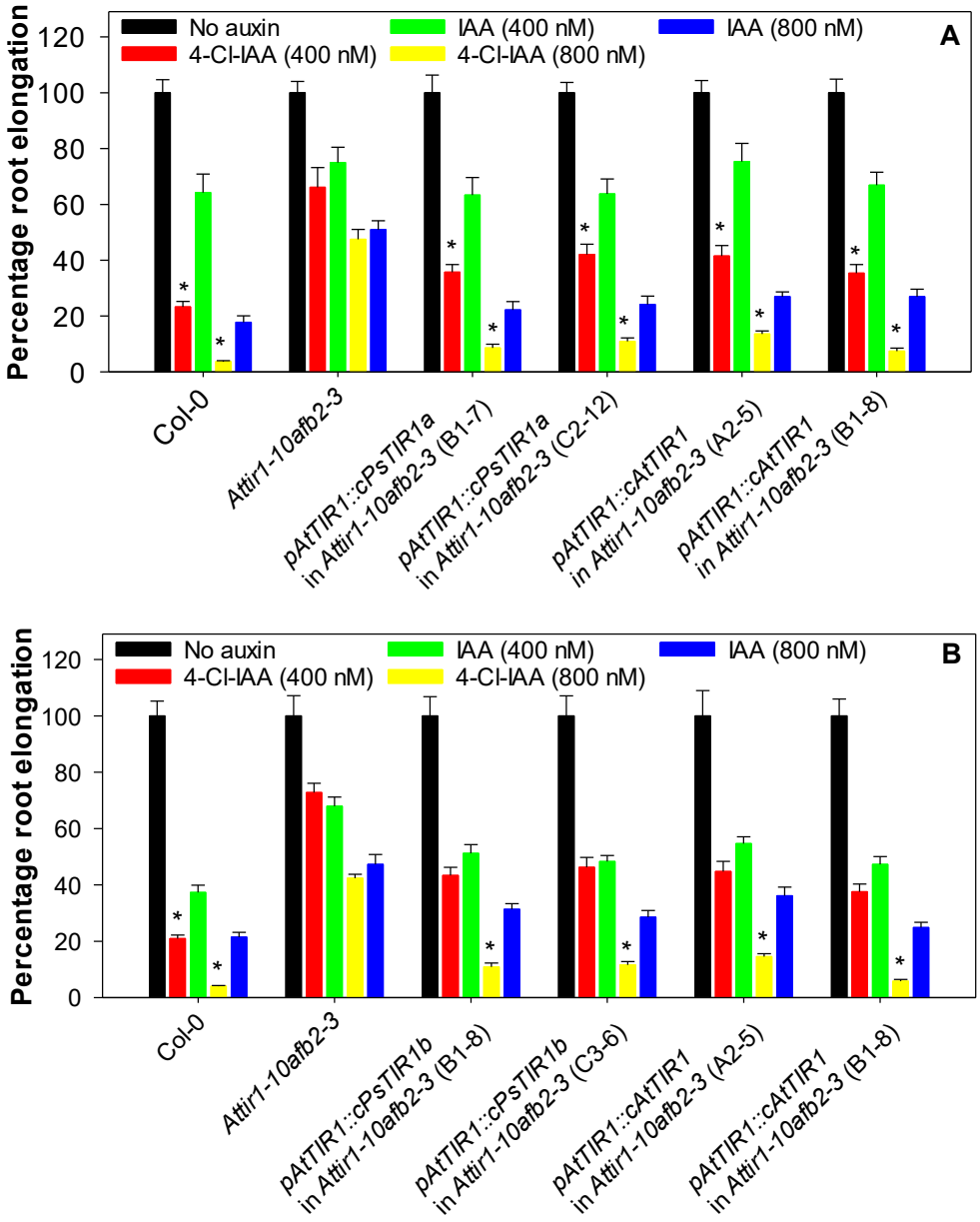


Figure B11. The effect of IAA or 4-Cl-IAA on the root elongation of *Arabidopsis Attir1-10afb2-3* double mutants expressing *PsTIR1a* (**A**) and *PsTIR1b* (**B**). Four-day-old Col-0, *tir1-10 afb2-3* and *tir1-10 afb2-3* expressing *pAtTIR1::cAtTIR1*, *pAtTIR1::cPsTIR1a* (**A**) or *pAtTIR1::cPsTIR1b* (**B**) were transferred to media containing 0, 400 and 800 nM IAA or 4-Cl-IAA and grown for three days prior to measurement of root elongation. Root elongation of each genotype in auxin is expressed as a percentage compared to the same genotype in the media without auxin. Data are means ± SE (n=8). Comparisons were made using a two-tailed student's T-test.

* 4-Cl-IAA means are significantly different from that of IAA within the same auxin concentration, $P<0.01$

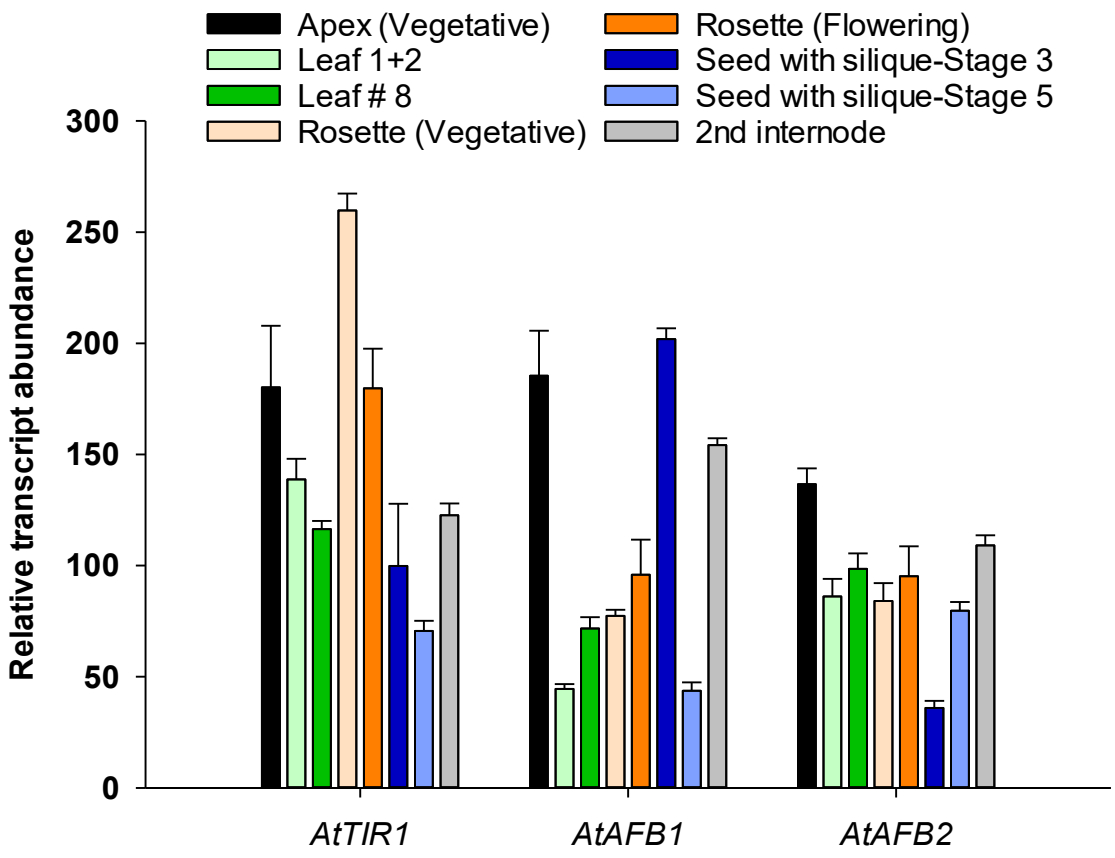


Figure B12. Tissue and developmental variation in *Arabidopsis AtTIR1*, *AtAFB1* and *AtAFB2* transcript abundance as assessed using microarray analysis by Schmid et al. (2005). Data was accessed through Arabidopsis eFP browser (Winter et al., 2007).

Leaf 1+2 and vegetative shoot apex are from seven days old plants. Rosette leaf # 8 is from 17 days old plants. Vegetative rosette and entire rosette after transition to flowering (but before bolting) are from 14 and 21 days old plants, respectively. Siliques with seeds stage 3 (mid globular to early heart embryo) and silique with seeds stage 5 (late heart to mid torpedo embryo) are from 8-week-old plants. The second internode is from 21+ days old plants. Shoot apex, leaf, internode and entire rosette after transition to flowering are samples collected from plants grown under continuous light. Siliques are from long-day-grown plants. Vegetative rosette is from short-day-grown plants (Schmid et al., 2005).

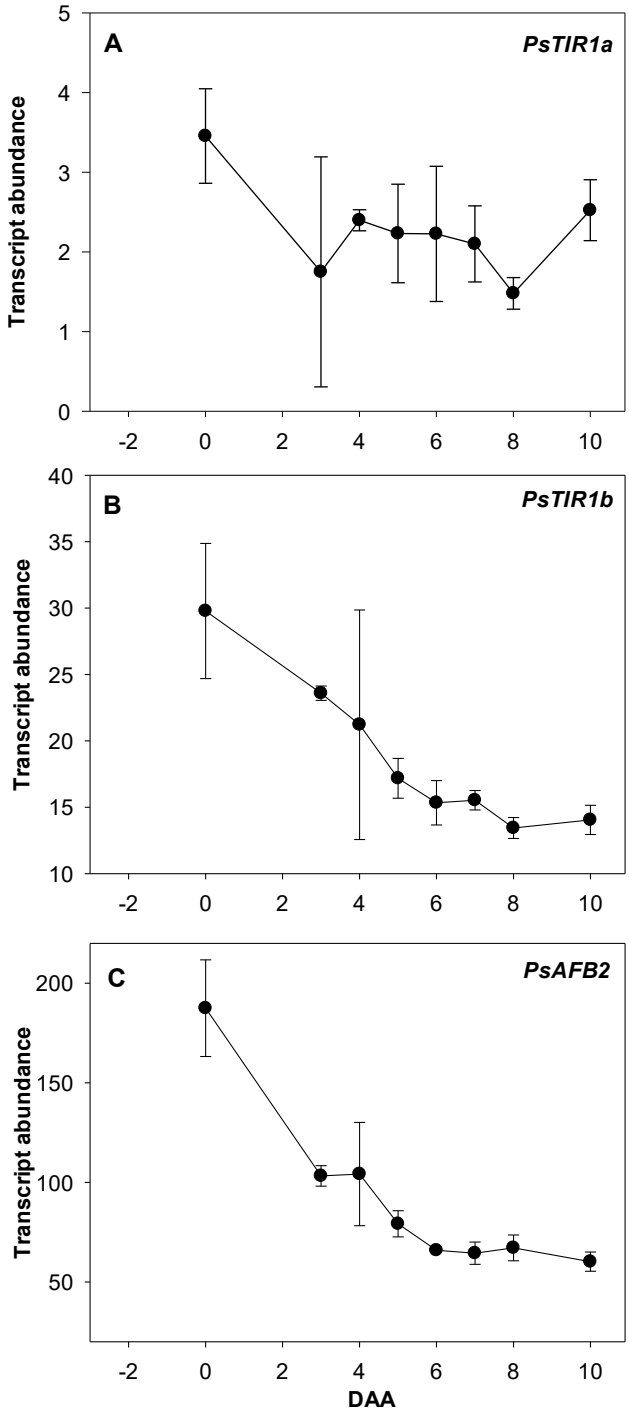


Figure B13. Relative transcript abundance of *PsTIR1a* (**A**), *PsTIR1b* (**B**), and *PsAFB2* (**C**) in the seeds of pollinated pea fruits from 0 to 10 days after anthesis (DAA). Data are means ± SD, n=3 with the exception of 3 and 4 DAA seeds where n=2.

Table B1. Primers used for PCR amplification of full-length CDS of *PsTIR1a*, *PsTIR1b*, *PsAFB2*, *AtTIR1* and the promoter of *AtTIR1*.

Target	**Primer**	**Sequence**
PsTIR1a CDS	TIR1seq-Set2-FW	GGCGTTTCAGATCTAACTCGAACTCT
	TIR1seq-Set2-RV	GATCAGATTCCGTATCTAGTAGCTCAGCC
PsTIR1b CDS	PsAFB1-seqSet1-FW	ACACTTTAAGCTCGTTAATGGTGTC
	PsAFB1-seqSet2-RV	TGGCATTAAATATCTGCCACA
PsAFB2 CDS	PsAFB2-seqSet1-FW	TTCCATGAAAGTTGTAGAATTTGG
	PsAFB2-3′ UTRSet1-RV	ACTCAACATATTAATGAAGTGCCATC
AtTIR1 CDS	AtTIR1-sqF1	ATGCAGAAGCGAATAGCC
	AtTIR1-sqR1	TTATAATCCGTTAGTAGTAATGATTT
AtTIR1 Promoter	pAtT1-sqF1	TTGCAAACCCATCGAACGTT
	AtPM-TIR1set1-RV	CGTACCATGACTTGCACACC

Table B2. Reaction composition for PCR reactions with Qiagen LongRange PCR kit (Qiagen) used for the amplification of *PsTIR1a* and *PsAFB6* full-length coding sequences.

Component	**Amount (µL)**	**Final concentration**
LongRange PCR Buffer with Mg^{2+}, 10x	5	1x; 2.5 mM Mg^{2+}
dNTP mix (10 mM each)	2.5	500 µM of each dNTP
PsAFB6 Primerpair1TR/F (20 µM)	6.25	2.5 µM
PsAFB6 Primerpair1TR/R (20 µM)	6.25	2.5 µM
LongRange PCR Enzyme Mix	0.5	2.25 units
Template cDNA (7.5 ng/µL)	6.7	50.25 ng
Water	22.8	
TOTAL	50	

Table B3. Primers and probes used for qRT-PCR quantitation of *PsTIR1a*, *PsTIR1b*, *PsAFB2*, and *AtPP2AA3* transcript abundance. [a]

Gene	qRT-PCR Efficiency (%)	Primer /Probe	Sequence
PsTIR1a	93.6	Forward	TGCACTAGAGCGCCTGGTTA
		Reverse	TGTGGAGCCCCCCTAAGAA
		Probe	ACTTGCAGACGCTCAGGCTCAATCG
PsTIR1b	96	Forward	TGTCCTAACCTCCAGACTCTTCGT
		Reverse	GCTGAGGACATCGGCTAAGTAAGT
		Probe	ATCATGCTGCAGCCCTTGATAAACTGCC
PsAFB2	95	Forward	CATCCTCGATGCAACAAAACC
		Reverse	GACTGTACGATTGCCCCAAAA
		Probe	ACTCCGACACAATGCAGCCACTGGA
AtPP2AA3	99.1	Forward	AGCATGGCCGTATCATGTTCT
		Reverse	TGGCCAAAATGATGCAATCTC
		Probe	CACAACCGCTTGGTCGACTATCGGAAT

[a] The probes are 6-FAM fluorescent dye labeled at 5’ end and double-quenched with Iowa Black FQ (IBFQ) quencher at the 3’ end and ZEN quencher in the middle.

Table B4. Primers used for PCR verification of homozygous T-DNA insertion lines. [a]

Mutant	Primer	Primer sequence
tir1-10 (SALK_090445)	LP	CACGTGTCATCATCAGAATCG
	RP	ATTTCCCACCTCAGGAGATTC
afb2-3 (SALK_137151)	LP	TCAACGGTCAAGATCCATCTC
	RP	CTGCAATTAGCGGCAATAGAG
All SALK mutants	LBb1.3 (T-DNA boarder primer)	ATTTTGCCGATTTCGGAAC

[a] Each primer pair was used together with T-DNA border primer LBb1.3 as described in O'Malley and Ecker, (2010). PCR reactions were done with Phire Plant Direct PCR kit (Thermo Scientific) as per the manufacturer recommendations.

Table B5. Primers used for the incorporation of restriction endonuclease sites into the ends of the *PsTIR1a* and *PsTIR1b* auxin receptor CDS and *AtTIR1* promoter region for the construction of the transformation vector in the pCM1300-polyA vector backbone.

Target	Primer	Primer sequence [a]
PsTIR1a CDS	cPsT1a-Sma1F	AAACCCGGG ATGCAGAGAGTAGCATACTCGTTC
	cPsT1a-Sac1R	AAAGAGCTC TCATTCAGGATATGCAGAATCATC
PsTIR1b CDS	cPsT1b-Sma1F	AAACCCGGG ATGCAGAAAATGACGAACCG
	cPsT1b-Sac1R	AAAGAGCTCCTATACACTGCGCAGGGAGG
AtTIR1 CDS	cAtT1-Sma1F	AAACCCGGGATGCAGAAGCGAATAGCC
	cAtT1-Sac1R	AAAGAGCTCTTATAATCCGTTAGTAGTAATGATTT
AtTIR1 Promoter	pAtT1-Sal1F	AAAGTCGACTTGCAAACCCATCGAACGTT
	pAtT1-Sma1R	AAACCCGGG TGCGGCCAAATAACCTCGA

[a] The selected restriction recognition sequences are underlined within the primer sequence.

Table B6. Primers used for the verification of *PsTIR1a*, *PsTIR1b* and *AtTIR1* transgene construct in *Arabidopsis*. [a]

Construct	Primer	Primer sequence
pAtTIR1::*cPsTIR1a*	pAtTIR1-sqF3	CCTTTTCTCTTCTCTTCCCACT
	PsTIR1-qRTset1-RV	GTCCCTGATTTCCAACTTCCTAAG
pAtTIR1::*cPsTIR1b*	pAtTIR1-sqF3	CCTTTTCTCTTCTCTTCCCACT
	cPsAFB1/TIR2-qPCR set2-RV	GCTGAGGACATCGGCTAAGTAAGT
pAtTIR1::*cAtTIR1*	cAtTIR1-sqF2	GGTCAGTTTCTCTGCTCTGGA
	pCM1300plyA-sqR1	GATTTGTAGAGAGAGACTGGTGATT

[a] In the plants transformed with pea auxin receptors, the primer pair was chosen as the forward primer binds to the *AtTIR1* promoter and the reverse primer binds to the CDS of the transgene. In plants transformed with *AtTIR1*, the forward primer binds to the *AtTIR1* CDS and the reverse primer binds to the *CaMV 35S* terminator.

Table B7. Relative transcript abundance of *PsTIR1a*, *PsTIR1b* and *PsAFB2* in the pericarp wall, dorsal and ventral vascular sutures, and ovules/seeds of pollinated and non-pollinated fruits (average of 1 to 3 DAA tissues).

Gene	Pollinated				Non-pollinated [a]			
	Seeds	**Pericarp wall**	**Dorsal suture**	**Ventral suture**	**Ovule**	**Pericarp wall**	**Dorsal suture**	**Ventral suture**
PsTIR1a	2.5±0.6[b]	2.5±0.1	3.5±0.2	3.1±0.2	3.3±0.1	3.8±0.1	4.5±0.1	3.7±0.1
PsTIR1b	23.5±0.2	28.6±1.2	28±1.3	23.9±0.9	36.6±2.2	53.4±2.3	58.1±2.8	51.7±2.6
PsAFB2	130.3±16.3	90±9.5	101.7±12.7	79.7 ± 5.1	155.6±7.1	114.3±9.4	115.7±8.7	92.6±3

[a] In non-pollinated fruits, flowers were emasculated at -2 DAA to prevent pollination.

[b] Data are means ± SE, n=4 in ovules/seeds, n= 6-9 in pericarp tissues.

SUPPLEMENTARY MATERIALS AND METHODS

Analysis of free and conjugated IAA

Following is the free and conjugate IAA analysis protocol provided by National Research Council, Saskatoon, Canada (NRCC SK).

Chemicals and calibration curves

The IAA conjugate IAA-Glu was synthesized and prepared at the NRCC SK. IAA-Leu, IAA-Ala, IAA-Asp and IAA were purchased from Sigma–Aldrich. Deuterated forms of the hormones, which were used as internal standards: d3-IAA-Leu, d3-IAA-Ala, d3-IAA-Asp and d3-IAA-Glu were synthesized and prepared at NRCC SK; d5-IAA was purchased from Cambridge Isotope Laboratories (Andover, MA). Calibration curves were created for all compounds of interest. Quality control samples were run along with the tissue samples.

Instrumentation

Analysis was performed on a UPLC/ESI-MS/MS utilizing a Waters ACQUITY UPLC system, equipped with a binary solvent delivery manager and a sample manager coupled to a Waters Micromass Quattro Premier XE quadrupole tandem mass spectrometer via a Z-spray interface. MassLynx and QuanLynx (Micromass, Manchester, UK) were used for data acquisition and data analysis.

Extraction and purification

An acidified aliquot (100 µL) containing all the internal standards, each at a concentration of 0.2 ng μL^{-1}, was added to 1 mL weighed homogenized liquid sample. 3 mL of isopropanol: water: glacial acetic acid (80:19:1, v/v/v) were further added, and the samples were agitated in the dark for 14-16 h at 4 ºC. Samples were then centrifuged and the supernatant was isolated and dried on a Büchi Syncore Polyvap (Büchi, Switzerland). Further, they were reconstituted in 100 µL acidified methanol, adjusted to 1 mL with acidified water, and then partitioned against 2 mL hexane. After 30 min, the aqueous layer was isolated and dried as above. Dry samples were reconstituted in 800 µL acidified methanol and adjusted to 1 mL with acidified water. The reconstituted samples were passed through equilibrated Sep-Pak

C18 cartridges (Waters, Mississauga, ON, Canada), and then the final eluate was split in two equal portions. One portion (#1) was dried completely (and stored) while the other portion was dried down to the aqueous phase on a LABCONCO centrivap concentrator (Labconco Corporation, Kansas City, MO, USA). The second portion was partitioned against ethyl acetate (2 mL) and further purified using an Oasis WAX cartridge (Waters, Mississauga, ON, Canada). The GA enriched fraction (#2) was eluted with 2 mL acetonitrile: water (80:20, v/v) and then dried on a centrivap as described above. An internal standard blank was prepared with 100 µL of the deuterated internal standards mixture. A quality control standard was prepared by adding 100 µL of a mixture containing all the analytes of interest, each at a concentration of 0.2 ng μL^{-1}, to 100 µL of the internal standard mix. Finally, fractions #1 and #2, blanks, and quality controls were reconstituted in a solution of 40% methanol (v/v), containing 0.5% acetic acid and 0.1 ng μL^{-1} of each of the recovery standards.

Hormone quantification by HPLC-ESI-MS/MS

The procedure for quantification of auxins in plant tissue was performed as described in detail in Lulsdorf et al. (2013). Samples were injected onto an ACQUITY UPLC® HSS C18 SB column (2.1x100 mm, 1.8 µm) with an in-line filter and separated by a gradient elution of water containing 0.02% formic acid against an increasing percentage of a mixture of acetonitrile and methanol (50:50, v/v).

Briefly, the analysis utilizes the Multiple Reaction Monitoring (MRM) function of the MassLynx v4.1 (Waters Inc) control software. The resulting chromatographic traces are quantified off-line by the QuanLynx v4.1 software (Waters Inc) wherein each trace is integrated and the resulting ratio of signals (non-deuterated/internal standard) is compared with a previously constructed calibration curve to yield the amount of analyte present (ng per sample). Calibration curves were generated from the MRM signals obtained from standard solutions based on the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard, as described by Ross et al. (2004). The quality control samples, internal standard blanks and solvent blanks were also prepared and analyzed along each batch of tissue samples.

Appendix C

FUNCTIONAL CHARACTERIZATION OF PsAFB2 AUXIN RECEPTOR IN ARABIDOPSIS

C. 1 INTRODUCTION

The *Arabidopsis* AFB2 protein is a functional auxin receptor (Dharmasiri et al., 2005; Parry et al., 2009). In pull-down assays and yeast-two hybrid assays, AtAFB2 interacts with Aux/IAA co-receptor proteins in an auxin-dependent manner (Parry et al., 2009; Calderón Villalobos et al., 2012). The role of AtAFB2 in auxin perception has been further verified by the phenotypic characterization of *AtAFB2* mutants. A T-DNA insertion in the *AtAFB2* promoter region causes reduced *AFB2* gene expression in *Atafb2-3* plants. In *Atafb2-5* plants, a nucleotide substitution replaces glycine 70 with arginine (Parry et al., 2009). Both *afb2-3* and *afb2-5* plants show slightly increased root elongation compared to that of Col-0 plants in root growth assays with 2,4-D (Parry et al., 2009). In addition, *Attir1-1 afb2-3* double mutant seedlings showed increased root elongation in the presence of 2,4-D compared to that of *Attir1-1* seedlings, which further confirms the role of *afb2-3* as an auxin receptor. However, seedlings expressing *AtAFB2* (expression driven by the *AtTIR1* promoter; expressed as an *AtAFB2::GUS* translational fusion) in *Attir1-1* did not exhibit an auxin sensitive root elongation phenotype (Parry et al., 2009). Based on this observation, it has been speculated that the role of AtAFB2 is not identical to that of AtTIR1, and AtAFB2 may have specialized functions (Parry et al., 2009).

In this experiment, the pea auxin receptor PsAFB2 was functionally characterized in *Arabidopsis*. The *Attir1-10 afb2-3* double mutants were chosen as the mutant background, assuming that their stronger auxin resistant phenotype (Chapter 3) would allow the detection of even a slight restoration of auxin sensitivity by PsAFB2. As *AtAFB2* expression did not recover the auxin sensitivity of root elongation in *Attir1-1* plants (Parry et al., 2009), the *Attir1-10 afb2-3* double mutant expressing *AtTIR1* was analyzed as the control.

C. 2 MATERIALS AND METHODS

All the *Arabidopsis thaliana* lines used in this study were of Columbia (Col-0) ecotype background. Transgenic *Arabidopsis* plants were created as described in Chapter 3. In brief, the pCM1300-polyA (Chapter 3) vector was used as the plant transformation vector. Complete coding sequence (CDS) of *PsAFB2* or *AtTIR1* was placed downstream of AtTIR1 promoter (-1 bp to - 3008 bp region from the start codon of *AtTIR1*). Primers used for the amplification of complete CDS and promoter regions are given in Table C1. The constructs were transformed into *Attir1-10 afb2-3* double mutants by floral-dip transformation. Two independent transgenic lines were selected for final analysis as described in Chapter 3. The selected lines are homozygous for the insert and appeared to contain a single insertion. Expression of the *PsAFB2* transgene in the transgenic *Arabidopsis* plants was verified by qRT-PCR as described in Chapter 3 with the *PsAFB2* qRT-PCR primers and the probe (Appendix Table B1). Root elongation assays were done as described in Chapter 3, using the 2,4-D concentrations of 0, 70 and 90 nM.

C. 3 RESULTS AND DISCUSSION

A qRT-PCR analysis confirmed the expression of *PsAFB2* in the two *Arabidopsis Attir1-10 afb2-3* double mutant lines with *PsAFB2* transgene (Fig. C1). The root elongation of *Attir1-10 afb2-3* seedlings was clearly resistant to 2,4-D compared to the wild-type plants (Fig. C2). Expression of *Arabidopsis AtTIR1* increased the auxin sensitivity of *Attir1-10 afb2-3* double mutants resulting in reduced root elongation in the 2,4-D medium. The plants expressing PsAFB2 also showed a reduced root length at 90 nM 2,4-D concentration (Fig. C2). At 70 nM 2,4-D concentration, only one of the two transgenic lines expressing *PsAFB2* was clearly 2,4-D resistant compared to *Attir1-10 afb2-3* double mutants. The increased auxin sensitivity of the mutants expressing *PsAFB2* indicates that PsAFB2 also acts as an auxin receptor similar to AtTIR1.

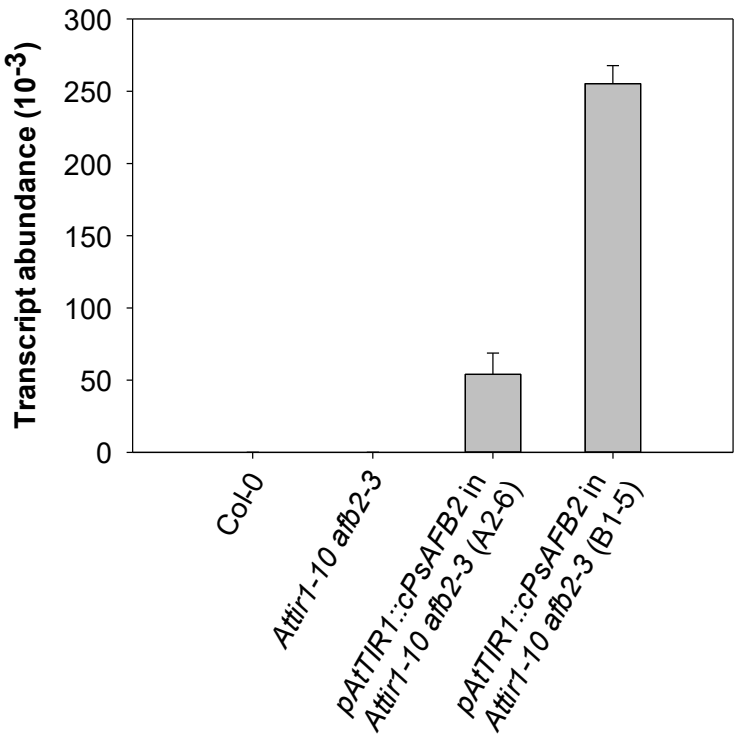


Figure C1. Relative transcript abundance of the pea auxin receptor gene *PsAFB2* (driven by the *AtTIR1* promoter*; pAtTIR1::cPsAFB2*) in *Attir1-10 afb2-3* double mutant. The transgene construct was introduced into *Attir1-10 afb2-3* by floral-dip transformation, and two independent transgenic lines were selected for the final analysis. Transgene expression was analyzed in the leaves of about a month old T_4 generation plants homozygous for the transgene. Data are means ± SD (n=4). Each sample is composed of leaves from about three to six plants.

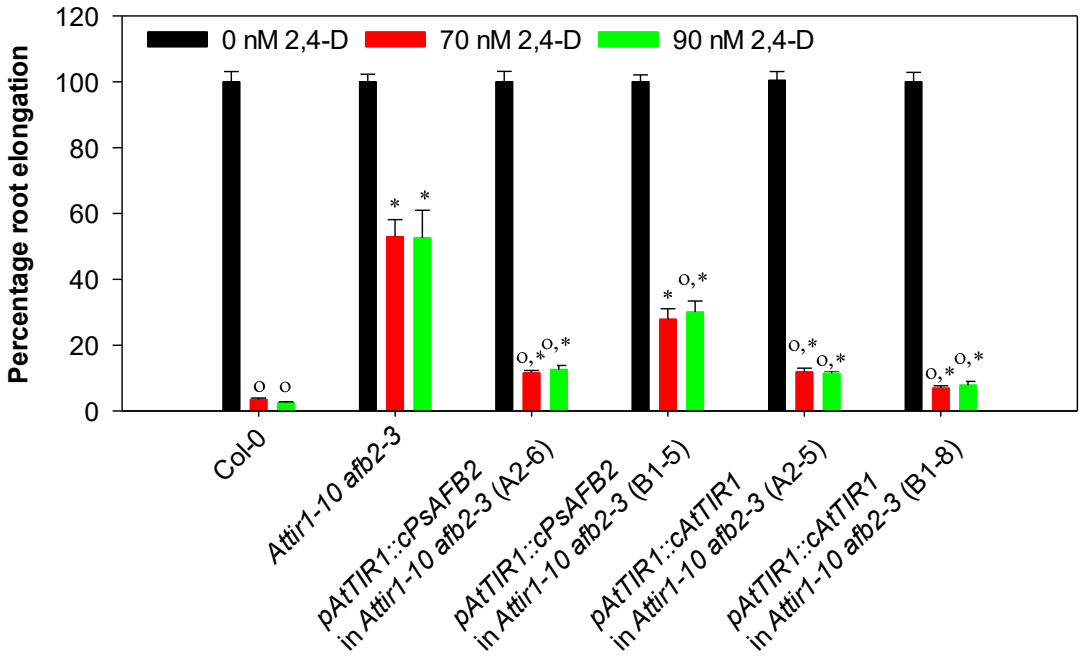


Figure C2. Functional characterization of pea PsAFB2 auxin receptors in *Arabidopsis tir1-10 afb2-3* double mutant background. Four-day-old seedlings of Col-0, *Attir1-10 afb2-3* double mutants, and transgenic plants expressing *pAtTIR1::cAtTIR1* or *pAtTIR1::cPsAFB2* in *Attir1-10 afb2-3* double mutant background were transferred to media containing 0, 70 or 90 nM 2, 4-D. After the transfer, seedlings were grown for three days prior to measurement of root elongation. Root elongation of each genotype in auxin is expressed as a percentage compared to the same genotype in the media without auxin. Data are means ± SE (n=8). Comparisons were made using a two-tailed student's T-test.

* Means are significantly different from that of Col-0 within respective auxin treatment, P<0.01;

º Means are significantly different from that of *Attir1-10* afb2-3 double mutants at same auxin concentration, P<0.01

Table C1. Primers used for the incorporation of restriction endonuclease sites when creating *PsAFB2* and *AtTIR1* transformation vectors. [a]

Target	Primer	Primer sequence
PsAFB2 CDS	cPsA2-Sma1F	AAACCCGGGATGAATTATTTTCCAGACGAGGT
	cPsA2-Sac1R	AAAGAGCTC CTACAGAGTCCATACATATTCTGGTG
AtTIR1 CDS	cAtT1-Sma1F	AAACCCGGGATGCAGAAGCGAATAGCC
	cAtT1-Sac1R	AAAGAGCTCTTATAATCCGTTAGTAGTAATGATTT
AtTIR1 Promoter	pAtT1-Sal1F	AAAGTCGACTTGCAAACCCATCGAACGTT
	pAtT1-Sma1R	AAACCCGGG TGCGGCCAAATAACCTCGA

[a] Restriction endonuclease sites were incorporated into the ends of auxin receptor CDS and *AtTIR1* promoter region with primers containing added restriction recognition sequences (underlined) for cloning them into pCM1300-polyA.

Appendix D

Supplementary information for Chapter 4

```
   1 ATGAGAGAAA ACCATCCTCC AACAACAACA CCAGATCTAC TAGCAAGAGG
  51 CGAGATCGCC GAATCATCCA CCTCAAAAAA CCGAACCGGT TCATCCGAAC
 101 CATTCCCCGG TTCATCTCTT ACCGAAAACC CATCCCCCTT CCCAGACCAA
 151 GTCCTCGAGA ACGTTCTAGA AAACGTCCTC CACTTCCTCT CCTCCCGCAA
 201 AGACCGCAAC GCCGCCTCAT TGGTCTGCCG TTCCTGGTAC CGTGCGGAAG
 251 CCCTCACTCG ATCCGACCTC TTCATCGGTA ACTGCTACGC ACTCTCCCCA
 301 CGGCGAGCCA CCGCGCGTTT CAGCCACATC AAGTCGGTTA CCGTCAAGGG
 351 AAAACCTAGG TTTGCGGATT TCGATCTGAT GCCGGTTGAT TGGGGGGCCC
 401 ACTTCACTCC TTGGGTTACT TCACTTGCTC AGGCTTACCC TTGGCTTGAA
 451 AAGCTTCACC TTAAACGTAT GTCTGTTACC GATAAAGATC TCGGCCTCAT
 501 CGCCGATTCG TTCGTCGGTT TTCGTGAGCT TTTACTTGCT TGCTGTGAAG
 551 GGTTTGGTAC CCCTGGTCTT GCTGTTATTG CTTCTAAGTG CAGATTGCTG
 601 AGGGTGCTGG AACTGGTGGA ATCTGTGATT GAGGTTGAGG ATGATGAAGA
 651 GGTGGATTGG GTATCTTGTT TTCCGACTGA GGGACAAACT CATTTGGAGT
 701 CTCTTGCCTT TGATTGTGTT GAGTGCCCTG TGAATTTCGA GGCATTGGAA
 751 GGGCTGGTGG CTAGATCGCC TGGTTTGAAG AAGCTTAGAT TGAACCGATC
 801 TGTTTCGATG GTTCAGCTTC ATCGGCTCAT GCTTCGTGCT CCTCAGCCTCA
 851 CACATCTTGG AACGGGTTCG TTCAGTGCTA ATGAGAATGT TGATCAAGAG
 901 CCTGATTATG CATCTGCTTT TGCAGCTTGC AGATCGTTGG TTTGTTTGTC
 951 TGGATTTAGG GAGATTTGGC CGGATTACCT TCCTGCTATT TATCCAGTTT
1001 GCTCAAACCT CACTTCGTTG AATTTTAGTT ATGCTGATGT TAATGCGGAA
1051 CAGCTCAAGT CTGTTATATG CCACTGTCAT AAGTTGCAGA TATTGTGGGT
1101 CCTTGATTCA ATAGGCGATG AAGGCCTTCA GTCAGTGGCT GCAACTTGCA
1151 ATGACTTGCG CGAGCTTCGA GTTTTTCCTG TGGATGCGAG GGAGGAAACT
1201 GAAGGTCCTG TTTCTCAGGT TGGTTTTGAA GCCATTTCAC AGGGTTGTAG
1251 AAAATTAGAA TCGATTTTGT TTTTCTGCCA GACAATGACG AACGCAGCTG
1301 TGGTTGCAAT GTCGAAGAAC TGTCCCGATC TTGTGGTGTT TCGTCTCTGT
1351 ATAATTGGGG TGTATCGTCC CGATGCTGTG ACACAAGAAC CCATGGATGA
1401 GGGTTTTGGT GCTATTGTTA TGAACTGCAA GAAGCTCACC CGACTTGCTG
1451 TATCTGGTCT GCTGACTGAT CGAGCTTTCG AATACATCGG AAGATATGGG
1501 AAATTGATTA GGACACTATC AGTTGCCTTT GCCGGAGACA CTGACACAGG
1551 CCTTAGGTAT GTACTCGAAG GGTGCCCTAA TTTGCAGAAA CTTGAAATCA
1601 GGGACAGTCC CTTCGGGGAC GGAGCTTTGC GATCCGGTTT GCATCATTAC
1651 TATAACATGA GATTCCTTTG GATGTCGTCC TGTAAATTGA CACGTCAAGC
1701 TTGCCAAGAA GTTGCACGAG CATTGCCTCA CATGGTTTTG GAAGTTATCA
1751 ACAATGGCGA AAATGCAGTT GAGGAGATTG GAATACTGTA CATGTATCGG
1801 TCTCTTGATG GGCCGCGAGA TGATGCTCCA GAACATGTTA CCATTCTGCA
1851 GTAG
```

Figure D1. The complete coding sequence of the auxin receptor *PsAFB4* from *P. sativum* L. cv. I_3 (Alaska-type). Forward and reverse qRT-PCR primers, and the probe binding sites, are in green, blue and red fonts, respectively. The gray highlighted region represents the F-box domain. The leucine-rich repeat domain coding region is marked with bold and italicized fonts.

```
   1 ATGGAACCAC AAACCATGAA TCCCAGTTCA GTCTTTCCAG ATGAAGTGCT
  51 GGAGAGAATT CTCAGCATGG TGAAGTCACG CAAAGACAAG AGTTCGGTTT
 101 CATTGGTTTG CAAAGACTGG TTCGACGCTG AAAGATGGTC GAGAAAGAAT
 151 GTGTTCATAG GTAACTGTTA TTCCGTTACA CCAGAGATCT TGACTCAAAG
 201 ATTTCCGAAT GTTCGAAGTG TTACATTGAA AGGGAAGCCA CGTTTCTCTG
 251 ATTTCAACTT GGTTCCTGCT AATTGGGGTG CTGATATTCA TCCATGGCTT
 301 GTTGTTTTCG CTGAAAAGTA CCCTTTTCTT GAAGAGTTAA GGCTTAAGAG
 351 AATGGTTGTT ACTGATGAGA GTTTAGAGTT TCTGGCTTTT TCGTTTCCGA
 401 ATTTTAAAGC TCTTTCTCTT TTGAGCTGTG ATGGATTTAG CACTGATGGT
 451 TTAGCTGCTG TTGCTACTAA TTGCAAGAAC TTAACTGAGC TTGACATACA
 501 AGAGAATGGT ATCGAAGACA AAAGCGGTAA CTGGTTGAGT TGCTTCCCAG
 551 AAAGCTTTAC ATCATTGGAA GTGTTGAACT TTGCCAACCT AACCAATGAA
 601 GTAAACATCG ACGCGCTAGA GAAACTTGTT GGTAGGTGCA AATCATTGAA
 651 GACTTTGAAG GTTAACAAAA GCGTAACGCT GGAACAGTTG AAAAAACTTC
 701 TTGTTCGCGC CCCTCAGTTA TGTGAGCTTG GCAGTGGCTC ATTTTCGCAA
 751 GAGCTGACAT CTCAGCAGTA TGCAGAGCTC GAAACCGCGT TCAAAAATTG
 801 TAAAAGCCTT CACACCCTGT CTGGTTTATG GGTGGCTTCA GCGCGATATC
 851 TTCAAGTTCT ATACCCTGCG TGCGCGAATC TGACTTTTTT GAATTTTAGC
 901 TATGCTCCTC TTGACAGTGA AGATCTTACC AAGATTCTTG TTCACTGTCC
 951 TAATCTTCGA CGTCTTTGGG TTGTTGACAC CGTTGAAGAC AAGGGACTTG
1001 AAGCGGTTGG ATCGAACTGT CCATTGCTTG AGGAACTGCG TGTTTTTCCT
1051 GCAGATCCGT TTGACGAGGA AGCTGAAGGC GGGGTGACTG AATCGGGGTT
1101 TGTTGCTGTC TCTGAAGGAT GCCGGAAGCT TCACTATGTT CTCTACTTTT
1151 GTCGTCAAAT GACCAATGCT GCTGTCGCTA CCGTAGTCCA AAACTGCCCC
1201 GACTTTACTC ATTTCCGCCT CTGCATAATG AACCCTGGCC AGCAAGATTA
1251 CCTGACGGAC GAACCTATGG ACGAGGCCTT CGGAGAAGTT GTTAAGAACT
1301 GCACTAAACT TCAGAGGCTC GCTGTATCAG GTTATCTAAC GGACCTCACA
1351 TTCGAGTATA TAGGAAAGTA TGCCAAAAAC TTGGAAACGC TTTCGGTGGC
1401 TTTTGCAGGA AGCAGTGATT GGGGAATGGA GTGTGTACTG GTCGGATGTC
1451 CGAAACTGAG AAAACTCGAG ATAAGAGACA GTCCATTCGG AAATGCAGCG
1501 CTTTTGGCAG GTTTGGAGAA GTACGAGTCG ATGAGGTCAC TTTGGATGTC
1551 GTCCTGCAGA CTGACGATGA ATGGATGTAG ATTTTTGGCA GGAGAAAAGC
1601 CGAGGTTGAA TGTCGAAGTA ATGCAGGAAG AAGGAGGCGA TGATAGTCGG
1651 GCCGAAAAAC TTTATGTTTA TCGATCTGTT GCCGGGCCAA GAAGGGATGC
1701 ACCTCCTTTT GTTCTCACTC TCTGA
```

Figure D2. The complete coding sequence of the auxin receptor *PsAFB6* from *P. sativum* L. cv. I_3 (Alaska-type). Forward and reverse qRT-PCR primers, and the probe binding sites, are in green, blue and red fonts, respectively. The gray highlighted region represents the F-box domain. The leucine-rich repeat domain coding region is marked with bold and italicized fonts.

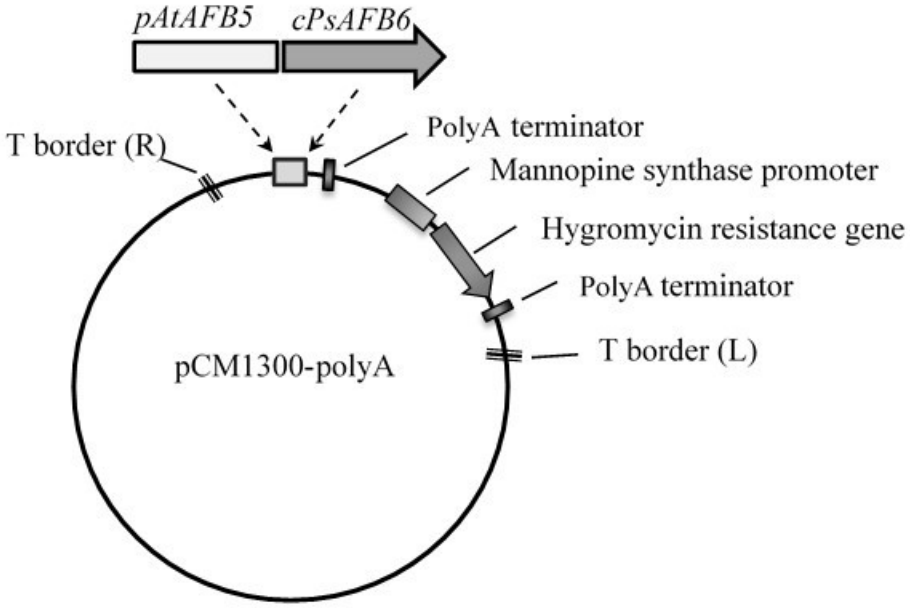


Figure D3. Schematic diagram of the *pAtTIR1::PsAFB6* gene construct used for *Agrobacterium*-mediated transformation of *PsAFB6* into *Arabidopsis tir1-10 or tir1-10 afb2-3* plants. The complete coding sequence of the *TIR1* genes was placed under the *AtAFB5* promoter in pCM1300-polyA transformation vector, which is a modified version of pCAMBIA1300 (Chapter 3).

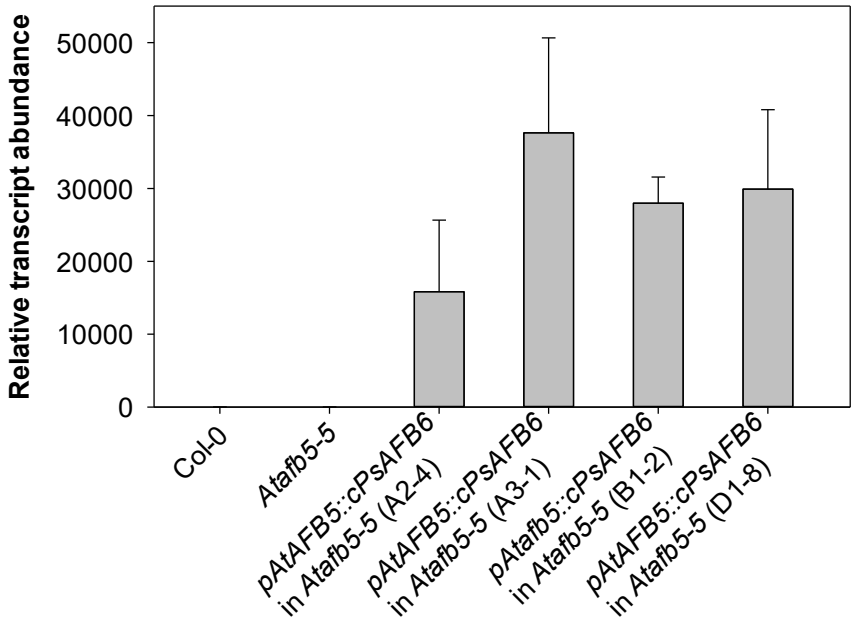


Figure D4. Relative expression of pea *PsAFB6* transgene in *Arabidopsis Atafb5-5* plants. The pea *PsAFB6* gene driven by *Arabidopsis AFB5* promoter (*pAtAFB5::cPsAFB6*) was introduced into *Atafb5-5* mutants by floral dip transformation. Expression of the transgene was analyzed in the leaves of about a month old plants. Data are means ± SD; n=3-4. Each sample is composed of leaves from about three to six plants.

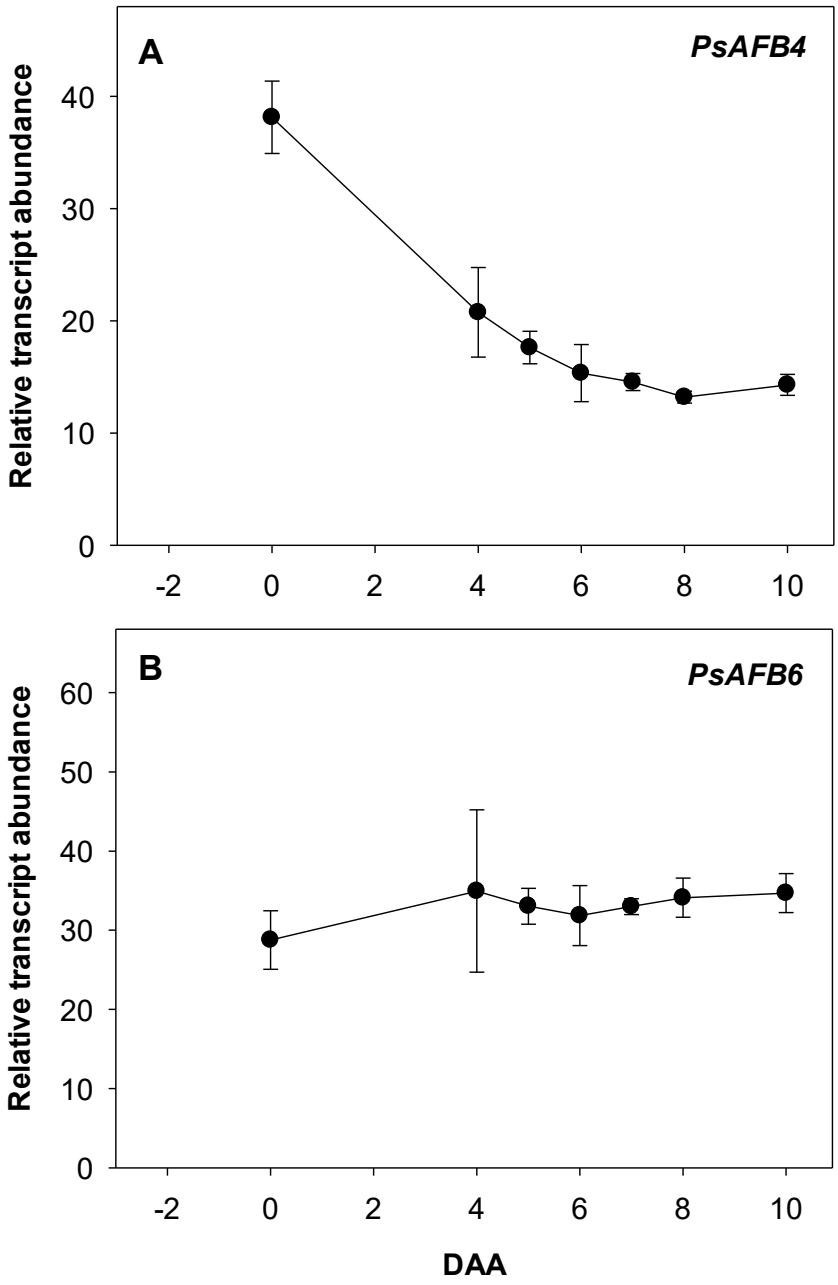


Figure D5. Relative transcript abundance of *PsAFB4* (**A**) and *PsAFB6* (**B**) in the seeds of 0 to 10 days after anthesis (DAA) pollinated pea fruits. Data are means ± SD, n=3 with the exception of 3 and 4 DAA where n=2.

A

```
   1 ATTGAGGAGG CTATTTACAC TTTTCTCTCT CTTCTTCTCT ACTTCTATAT
  51 AGCTAAACCA AGCTACATTG TAATACAAGA GAACATACAC ACACACCCTT
 101 AAGGAGAAAC TAACAAATAT TTTCAGTTTG AGAGAGAAGG GAAAGTTAAA
 151 AGAGTGTTTG GATTTCAGTG AGAAGCCAAA AATGGATACT TTGATTGGAA
 201 AGGAGAATGG TTTGAACATG AGGGAGACTG AGCTTTGTCT TGGTTTGCCT
 251 GGCGGAGGCG GCGGCGGCGG CGAGGTTGAA ACTCCTAGAG CTGCTGGAAA
 301 GAGAGGTTTC TCTGAGACAG TTGATTTGAA GCTGAATATT CAGACCAAGG
 351 AAGATCTGAA TGAGAATCTG AAGAATGTTT CAAAGGAGAA GAGCATGCTT
 401 AAGGACCCTG CCAAGCCACC AGCTAAGACT CAAGTAGTTG GTTGGCCACC
 451 AGTGAGGTCA TACAGAAAGA ACATGATGGC ACAAAAGGTT AACAATTCTG
 501 AGGAGAATGA GAAGACAACA AGTAGCACTA CTGCTGGTGC ATTTGTTAAG
 551 GTTTCCATGG ATGGAGCACC TTACCTTCGT AAGGTCGACT TAACAATGTA
 601 CAAAACCTAC AAAGATTTAT CCGATGCCTT AGCCAAAATG TTCAGCTCCT
 651 TCACCACTGG TAACTATGGG GCCCAAGGAA TGATAGACTT CATGAATGAA
 701 AGCAAGTTGA TGGATCTTCT TAACAGCTCT GAGTATGTGC CAACCTATGA
 751 AGATAAGGAT GGCGACTGGA TGCTCGTGGG AGATGTCCCA TGGGAGATGT
 801 TTGTTGAATC ATGCAAGCGT CTTCGCATAA TGAAAGGATC AGAAGCTATT
 851 GGGCTTGCAC CAAGAGCAAT GGAGAAATGC AAAAACAGAA GCTAAATACA
 901 TATCTTATAT GAAAGTCCTG TACCAAAGTG GACCTATAAG AAAAGATATA
 951 TATGAAGCTA GCACTACTTG TGGATTATTG GAGTGTTGTT AGTTTGGATG
1001 TGTCTTCAGT GTTTTTTGTT TTGTTCGTAT ATAGATTCAA CTTTAATTTT
1051 CAAGTATTTG TAATCGACAT GAAGCAATAA ACTTAACCAA ACCACCTATA
1101 TACTATATAG TTTGCCTTGT TTAATTATTA CTATTATTAT TACTATTATG
1151 TGATTGCTTC CATGCTGTCA TGTTTGTTGT GTGTGCTATA TATTATATAT
1201 ACTTTGAATT TTAATGTATA GATTCTTCTT GTTATATTTC ATTTATTTAG
1251 TAATGTTTTA ATTCATTTGT GTACTGAAAA TGATCAAAAT GTCTCAGCTG
1301 GATGAAGAGA TTTGTTAAAT ATTTGTAAGT GTGGGAAGGG AACAAAGCCA
1351 TGTGCATGTT GATGGAGTTT TGTTTATGGC CATTATAGTA GTCTTTGTCA
1401 TTTAGTTTTG TAATATCAAA TTCGCATTAA TTACTGGCTG AGTGTAGGGT
1451 TGGTGAGTGA ACACACAGTT GGAATATCAA TTTATATATA TGTGTACATT
1501 GATCAATGAC ATTATTAATG TTATTAAGGG TCAATGATGC ATATGCATAT
1551 GAATAAAAGT ATAA
```

B

```
   1 MDTLIGKENG LNMRETELCL GLPGGGGGGG EVETPRAAGK RGFSETVDLK
  51 LNIQTKEDLN ENLKNVSKEK SMLKDPAKPP AKTQVVGWPP VRSYRKNMMA
 101 QKVNNSEENE KTTSSTTAGA FVKVSMDGAP YLRKVDLTMY KTYKDLSDAL
 151 AKMFSSFTTG NYGAQGMIDF MNESKLMDLL NSSEYVPTYE DKDGDWMLVG
 201 DVPWEMFVES CKRLRIMKGS EAIGLAPRAM EKCKNRS
```

Figure D6. **A)** Predicted mRNA sequence of PsAux/IAA7 of *P. sativum* L. Predicted start and stop codons are in bold and underlined. The sequence was predicted by analyzing 10 different *P. sativum* L entries in NCBI Transcriptome Shotgun Assembly database. **B)** Predicted amino acid sequence of PsAux/IAA7

Table D1. Primers and probes used for qRT-PCR of *PsAFB4* and *PsAFB6*. [a]

Gene	qRT-PCR Efficiency (%)	Primer /Probe	Sequence
PsAFB4	100	Forward	GATCTGTTTCGATGGTTCAGCTT
		Reverse	AACCCGTTCCAAGATGTGTGA
		Probe	CGGCTCATGCTTCGTGCTCCTCAG
PsAFB6	102.3	Forward	TCGCTACCGTAGTCCAAAACTG
		Reverse	TGCTGGCCAGGGTTCATTA
		Probe	CCCGACTTTACTCATTTCCGCCTCTGC
AtPP2AA3	99.1	Forward	AGCATGGCCGTATCATGTTCT
		Reverse	TGGCCAAAATGATGCAATCTC
		Probe	CACAACCGCTTGGTCGACTATCGGAAT

[a] The probes are 6-FAM fluorescent dye labeled at 5′ end and double-quenched with Iowa Black FQ (IBFQ) quencher at the 3′ end and ZEN quencher in the middle.

Table D2. Primers used for amplification of full-length CDS of *PsAFB4*, *PsAFB6*, and the promoter of *AtAFB5*.

Target	**Primer**	**Sequence**
PsAFB6 CDS	PsAFB6- TR/FP	5′-ATGGAACCACAAACCATGAATCCCAG-3′
	PsAFB6-TR/RP	5′-TCAGAGAGTGAGAACAAAAGGAGGTGC-3′
PsAFB4 CDS	PsAFB4-seqSet1-FW	5′- ATGAGAGAAAACCATCCTCCAAC-3′
	PsAFB4-seqSet1-RV	5′- CTTTGCGGCTTTCCATCAC-3′
AtAFB5 Promoter	pAtAFB5-sqF1	5′- TTGGTAGCAGGAGAGTTGTTCA-3′
	pAtAFB5-sqR1	5′- AAAGAGAGAGGCTTTGGATTCAG-3′

Table D3. Additional primers used for sequencing of cloned *PsAFB4* and *PsAFB6* coding sequences and *AtAFB5* promoter.

Target	Primer	Sequence
AtAFB5 promoter	pAtA5-sqF3	5′-GCTTCTCAATCTTCCTAGGCC-3′
AtAFB5 promoter	pAtA5-sqR3	5′-AGGTGGTAAGTGGTAACCGA-3′
AtAFB5 promoter	pAtA5-sqF4	5′-TTGATGGAGACAAGAAAGCAGA-3′
PsAFB4 CDS	AFB4-seq-FW-set3	5′-TTGTGTTGAGTGCCCTGT-3′
pCM1300plA vector	pCM1300plA-seqF1	5′-CGGGCCTCTTCGCTATTAC-3′
pCM1300plA vector	pCM1300plA-seqR1	5′-GATTTGTAGAGAGAGACTGGTGATT-3′
pCR8 vector	GW1	5′-GTTGCAACAAATTGATGAGCAATGC-3′
pCR8 vector	GW2	5′-GTTGCAACAAATTGATGAGCAATTA-3′
pCR8 vector	M13-FW (-20)	5′-GTAAAACGACGGCCAG-3′
pCR8 vector	M13-RV	5′-CAGGAAACAGCTATGAC-3′

Table D4. Primers used for the incorporation of restriction sites to the *PsAFB6* coding sequence and the *AtAFB5* promoter. [a]

Target	Primer	Primer sequence
PsAFB6 CDS	cPsA6-BamH1F	5′-AAAGGATCCATGGAACCACAAACCATGAATC-3′
	cPsA6-Kpn1R	5′-AAAGGTACCTCAGAGAGTGAGAACAAAAGGAGG-3′
AtAFB5 Promoter	pAtA5-Sal1F	5′-AAAGTCGACTTGGTAGCAGGAGAGTTGTTCA-3′
	pAtA5-BamH1R	5′-AAAGGATCCAAAGAGAGAGGCTTTGGATTCAG-3′

[a] Endonuclease restriction sites were incorporated into the ends of the *PsAFB6* CDS and *AtAFB5* promoter region with primers containing the selected restriction recognition sequences (underlined) for the construction of transformation vector in the pCM1300-polyA vector backbone.

Table D5. Relative transcript abundance of *PsAFB4* and *PsAFB6* in pericarp wall, dorsal and ventral vascular sutures and ovules/seeds of pollinated and non-pollinated fruits (average of 1 to 3 days after anthesis tissues).

Gene	Pollinated				Non-pollinated[a]			
	Seeds	**Pericarp wall**	**Dorsal suture**	**Ventral suture**	**Ovule**	**Pericarp wall**	**Dorsal suture**	**Ventral suture**
PsAFB4	24.9±2.8[b]	27±0.9	30.3±1.5	26.8±1.1	34.6±1.7	35.7±2.4	38.8±3.0	34±1.5
PsAFB6	31.5±0.6	24.2 ± 1.5	23.3±0.7	21.8±0.9	27.6±1.2	53.4 ± 4.9	51.7±7.5	55.8±6.5

[a] In non-pollinated fruits, flowers were emasculated at -2 DAA to prevent pollination.

[b] Data are means ± SE, n=4 in ovules/seeds, n= 6-9 in pericarp tissues.

Appendix E

AMINO ACID SEQUENCES USED IN THE PHYLOGENETIC TREE CREATION

E.1. AMINO ACID SEQUENCES USED FOR THE 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE (ACS) PHYLOGENETIC TREE (Fig. A10)

E.1.1 *Arabidopsis thaliana* (At) protein sequences

>AtACS1 (GenBank: NP_191710.1)
MSQGACENQLLSKLALSDKHGEASPYFHGWKAYDNNPFHPTHNPQGVIQMGLAENQLCSD
LIKEWIKENPQASICTAEGIDSFSDIAVFQDYHGLKQFRQAIATFMERARGGRVRFEAER
VVMSGGATGANETIMFCLADPGDAFLVPTPYYAAFDRDLRWRTGVRIIPVECSSSNNFQI
TKQALESAYLKAQETGIKIKGLIISNPLGTSLDRETLESLVSFINDKQIHLVCDEIYAAT
VFAEPGFISVAEIIQEMYYVNRDLIHIVYSLSKDMGLPGFRVGVVYSYNDVVVSCARRMS
SFGLVSSQTQSFLAAMLSDQSFVDNFLVEVSKRVAKRHHMFTEGLEEMGISCLRSNAGLF
VLMDLRHMLKDQTFDSEMALWRVIINKVKINVSPGSSFHCSEPGWFRVCFANMDEDTLQI
ALERIKDFVVGDRANKNKNCNCICNNKRENKKRKSFQKNLKLSLSSMRYEEHVRSPKLMS
PHSPLLRA

>AtACS2 (GenBank: NP_171655.1)
MGLPGKNKGAVLSKIATNNQHGENSEYFDGWKAYDKDPFHLSRNPHGIIQMGLAENQLCL
DLIKDWVKENPEASICTLEGIHQFSDIANFQDYHGLKKFRQAIAHFMGKARGGRVTFDPE
RVVMSGGATGANETIMFCLADPGDVFLIPSPYYAAFDRDLRWRTGVEIIPVPCSSSDNFK
LTVDAAEWAYKKAQESNKKVKGLILTNPSNPLGTMLDKDTLTNLVRFVTRKNIHLVVDEI
YAATVFAGGDFVSVAEVVNDVDISEVNVDLIHIVYSLSKDMGLPGFRVGIVYSFNDSVVS
CARKMSSFGLVSSQTQLMLASMLSDDQFVDNFLMESSRRLGIRHKVFTTGIKKADIACLT
SNAGLFAWMDLRHLLRDRNSFESEIELWHIIIDRVKLNVSPGSSFRCTEPGWFRICFANM
DDDTLHVALGRIQDFVSKNKNKIVEKASENDQVIQNKSAKKLKWTQTNLRLSFRRLYEDG
LSSPGIMSPHSPLLRA

>AtACS4 (GenBank: NP_179866.1)
MVQLSRKATCNSHGQVSSYFLGWEEYEKNPYDVTKNPQGIIQMGLAENQLCFDLLESWLA
QNTDAACFKRDGQSVFRELALFQDYHGLSSFKNAFADFMSENRGNRVSFDSNNLVLTAGA
TSANETLMFCLADPGDAFLLPTPYYPGFDRDLKWRTGVEIVPIQSSSTNGFRITKLALEE
AYEQAKKLDLNVKGILITNPSNPLGTTTTQTELNILFDFITKNKNIHLVSDEIYSGTVFN
SSEFISVMEILKNNQLENTDVLNRVHIVCSLSKDLGLPGFRVGAIYSNDKDVISAATKMS
SFGLVSSQTQYLLSSLLSDKKFTKNYLRENQKRLKNRQRKLVLGLEAIGIKCLKSNAGLF
CWVDMRPLLRSKTFEAEMDLWKKIVYEVKLNISPGSSCHCEEPGWFRVCFANMIDETLKL
ALKRLKMLVDDENSSRRCQKSKSERLNGSRKKTMSNVSNWVFRLSFHDREAEER

>AtACS5 (GenBank: NP_201381.1)
MKQLSTKVTSNGHGQDSSYFLGWEEYEKNPYDEIKNPNGMIQMGLAENQLCFDLIESWLT
KNPDAASLKRNGQSIFRELALFQDYHGMPEFKKAMAEFMEEIRGNRVTFDPKKIVLAAGS
TSANETLMFCLAEPGDAFLLPTPYYPGFDRDLKWRTGAEIVPIHCSSSNGFQITESALQQ
AYQQAQKLDLKVKGVLVTNPSNPLGTALTRRELNLLVDFITSKNIHLISDEIYSGTMFGF
EQFISVMDVLKDKKLEDTEVSKRVHVVYSLSKDLGLPGFRVGAIYSNDEMIVSAATKMSS
FGLVSSQTQYLLSALLSDKKFTSQYLEENQKRLKSRQRRLVSGLESAGITCLRSNAGLFC
WVDMRHLLDTNTFEAELDLWKKIVYNVKLNISPGSSCHCTEPGWFRVCFANMSEDTLDLA
LKRLKTFVESTDCGRMISRSSHERLKSLRKKTVSNWVFRVSWTDRVPDER

>AtACS6 (GenBank: NP_192867.1)
MVAFATEKKQDLNLLSKIASGDGHGENSSYFDGWKAYEENPFHPIDRPDGVIQMGLAENQ
LCGDLMRKWVLKHPEASICTSEGVNQFSDIAIFQDYHGLPEFRQAVAKFMEKTRNNKVKF
DPDRIVMSGGATGAHETVAFCLANPGDGFLVPTPYYPGFDRDLRWRTGVNLVPVTCHSSN
GFKITVEALEAAYENARKSNIPVKGLLVTNPSNPLGTTLDRECLKSLVNFTNDKGIHLIA
DEIYAATTFGQSEFISVAEVIEEIEDCNRDLIHIVYSLSKDMGLPGLRVGIVYSYNDRVV
QIARKMSSFGLVSSQTQHLIAKMLSDEEFVDEFIRESKLRLAARHAEITTGLDGLGIGWL
KAKAGLFLWMDLRNLLKTATFDSETELWRVIVHQVKLNVSPGGSFHCHEPGWFRVCFANM
DHKTMETALERIRVFTSQLEEETKPMAATTMMAKKKKKCWQSNLRLSFSDTRRFDDGFFS
PHSPVPPSPLVRAQT

>AtACS7 (GenBank: NP_194350.1)
MGLPLMMERSSNNNNVELSRVAVSDTHGEDSPYFAGWKAYDENPYDESHNPSGVIQMGLA
ENQVSFDLLETYLEKKNPEGSMWGSKGAPGFRENALFQDYHGLKTFRQAMASFMEQIRGG
KARFDPDRIVLTAGATAANELLTFILADPNDALLVPTPYYPGFDRDLRWRTGVKIVPIHC
DSSNHFQITPEALESAYQTARDANIRVRGVLITNPSNPLGATVQKKVLEDLLDFCVRKNI
HLVSDEIYSGSVFHASEFTSVAEIVENIDDVSVKERVHIVYSLSKDLGLPGFRVGTIYSY
NDNVVRTARRMSSFTLVSSQTQHMLASMLSDEEFTEKYIRINRERLRRRYDTIVEGLKKA
GIECLKGNAGLFCWMNLGFLLEKKTKDGELQLWDVILKELNLNISPGSSCHCSEVGWFRV
CFANMSENTLEIALKRIHEFMDRRRRF

>AtACS8 (GenBank: NP_195491.1)
MGLLSKKASCNTHGQDSSYFWGWEEYEKNPYDEIKNPDGIIQMGLAENQLSFDLIESWLA
KNPDAANFQREGQSIFRELALFQDYHGLPSFKNAMADFMSENRGNRVSFNPNKLVLTAGA
TPANETLMFCLADPGDAFLLPTPYYPGFDRDLKWRTGAEIVPIQCKSANGFRITKVALEE
AYEQAQKLNLKVKGVLITNPSNPLGTTTTRTELNHLLDFISRKKIHLISDEIYSGTVFTN
PGFISVMEVLKDRKLENTDVFDRVHIVYSLSKDLGLPGFRVGVIYSNDDFVVSAATKMSS
FGLISSQTQYLLSALLSDKTFTKNYLEENQIRLKNRHKKLVSGLEAAGIECLKSNAGLFC
WVDMRHLLKSNTFEAEIELWKKIVYEVKLNISPGSSCHCNEPGWFRVCFANLSEETLKVA
LDRLKRFVDGPSPTRRSQSEHQRLKNLRKMKVSNWVFRLSFHDREPEER

>AtACS9 (GenBank: NP_190539.1)
MKQLSRKVTSNAHGQDSSYFLGWEEYEKNPYDEIKNPNGIIQMGLAENQLCFDLIETWLA
KNPDAAGLKKDGQSIFKELALFQDYHGLPEFKKALAEFMEEIRGNRVTFDPSKIVLAAGS
TSANETLMFCLAEPGDAFLLPTPYYPGFDRDLKWRTGAEIVPIHCSSSNGFQITESALQQ
AYQQAQKLDLKVKGVLVTNPSNPLGTMLTRRELNLLVDFITSKNIHLISDEIYSGTVFGF
EQFVSVMDVLKDKNLENSEVSKRVHIVYSLSKDLGLPGFRVGAIYSNDEMVVSAATKMSS

FGLVSSQTQYLLSALLSDKKFTSTYLDENQKRLKIRQKKLVSGLEAAGITCLKSNAGLFC
WVDMRHLLDTNTFEAELELWKKIVYDVKLNISPGSSCHCTEPGWFRVCFANMSEDTLDLA
MKRLKEYVESTDSRRVISKSSHDRIKSLRKRTVSNWVFRVSWTDRVPDER

>AtACS10 (GenBank: NP_564804.1)
MTRTEPNRSRSSNSDSDKNSGNVGGGRTTGMRVIVPLQGVVQGRGGLFLGSVIPCAFFYF
LQFYLKRNRKNDESDNSGEQNSSASSSSSPNSGLPDPTRSQSAGHLTELTGLPRSLSRIL
LSPRNSGGAVSVSGRVNCVLKGGDSSPYYVGQKRVEDDPYDELGNPDGVIQLGLAQNNKL
SLDDWVLENPKEAISDGLSISGIASYEPSDGLLELKMAVAGFMTEATKNSVTFDPSQLVL
TSGASSAIEILSFCLADSGNAFLVPTPCSPGYDRDVKWRTGVDIIHVPCRSADNFNMSMV
VLDRAFYQAKKRGVRIRGIIISNPSNPMGSLLSRENLYALLDFARERNIHIISNEIFAGS
VHGEEGEFVSMAEIVDTEENIDRERVHIVYDLSKDLSFRGLRSAAIYSFNESVLSASRKL
TTLSPVSSPTQHLLISAISNPKNVQRFVKTNRQRLQSIYTELVEGLKELGIECTRSNGGF
YCWADMRGLISSYSEKGEIELWNKLLNIGKINVIPGSCCHCIEPGWFRICFSNLSERDVP
VVMNRIRKVCETCKSQN

>AtACS11 (GenBank: NP_567330.1)
MLSSKVVGDSHGQDSSYFLGWQEYEKNPFHESFNTSGIVQMGLAENQLSFDLIEKWLEEH
PEVLGLKKNDESVFRQLALFQDYHGLPAFKDAMAKFMGKIRENKVKFDTNKMVLTAGSTS
ANETLMFCLANPGDAFLIPAPYYPGFDRDLKWRTGVEIVPIHCVSSNGYKITEDALEDAY
ERALKHNLNVKGVLITNPSNPLGTSTTREELDLLLTFTSTKKIHMVSDEIYSGTVFDSPE
FTSVLEVAKDKNMGLDGKIHVVYSLSKDLGLPGFRVGLIYSNNEKVVSAATKMSSFGLIS
SQTQHLLANLLSDERFTTNYLEENKKRLRERKDRLVSGLKEAGISCLKSNAGLFCWVDLR
HLLKSNTFEAEHSLWTKIVCEVGLNISPGSSCHCDEPGWFRVCFANMSDQTMEVAMDRVK
GFVDNNNGGKQKRTMWDTRRRSLINKWVSKLSSVTCESER

>AtACS12 (GenBank: NP_199982.2)
MRLIVPLRGVIQGRGGLFVGSLIPCCLFYFLQLYLKRRRPPPSDPTDLPRTFSRTNLFSR
GNSIGRVRVSSRAVPVAKPSDSPYYIGLERVKTDPYDRITNTDGIIQLGLAESTLCFDLL
QRWMSENLMESMMQSDDGEFDISSIAMYKPFEGLLELRVAFADFMSRIMGGNVSFDPSNM
VITAGGTPAIEVLAFCLADHGNAFLIPTPYYPGFDRDIKFRTGVELIPVHCRSSDNFTVT
VSALEQALNQARKRGSKVSGILFSNPSNPVGNILSRETLCDILRFAQEKNIHVISDEIFA
GSVYGDKEFVSMAEIAGSGEFDKTRVHIIYGLSKDLSIPGFRAGVIYSFHEDVVNAAKKL
MRFSSVPVLVQRILISLLSDVRFIEGYMAAHRQRIRDKHIRFVEGLKQLGIPCAESGGGL
YCWVDMSSLLTSYSEKGELELFEKLLTVAKINATPGTACYCIEPGWFRCCFTALADEDIP
VIMERIRQLAESFRS

>AtALAAT2 (GenBank: NP_001185380.1)
MRRFLINQAKGLVDHSRRQHHHKSPSFLSPQPRPLASSPPALSRFFSSTSEMSASDSTSS
LPVTLDSINPKVLKCEYAVRGEIVNIAQKLQEDLKTNKDAYPFDEIIYCNIGNPQSLGQL
PIKFFREVLALCDHASLLDESETHGLFSTDSIDRAWRILDHIPGRATGAYSHSQGIKGLR
DVIAAGIEARDGFPADPNDIFLTDGASPAVTIKTKTLSHRVRVHMMMQLLLSSEKDGILS
PIPQYPLYSASIALHGGSLVPYYLDEATGWGLEISDLKKQLEEARSKGISVRALVVINPG
NPTGQVLAEENQRDIVNFCKQEGLVLLADEVYQENVYVPDKKFHSFKKVARSLGYGEKDI
SLVSFQSVSKGYYGECGKRGGYMEVTGFTSDVREQIYKMASVNLCSNISGQILASLVMSP
PKPGDDSYDSYMAERDGILSSMAKRAKTLEDALNSLEGVTCNRAEGAMYLFPRINLPQKA
IEAAEAEKTAPDAFYCKRLLNATGVVVVPGSGFGQVPGTWHFRCTILPQEDKIPAIVNRL

TEFHKSFMDEFRN

>AtALAAT1 (GenBank: NP_173173.3)
MRRFVIGQAKNLIDQSRRRQLHHHKNLSFVSLIPPFSAPSDSSSRHLSSSSSSDMSASDS
SSSLPVTLDTINPKVIKCEYAVRGEIVNIAQKLQEDLKTNKDAYPFDEIIYCNIGNPQSL
GQQPITFFREVLALCSYTALLDESATHGLFSSDSIERAWKILDQIPGRATGAYSHSQGIK
GLRDAIADGIEARDGFPADPNDIFMTDGASPGVHMMMQLLITSEKDGILCPIPQYPLYSA
SIALHGGTLVPYYLDEASGWGLEISELKKQLEDARSKGITVRALAVINPGNPTGQVLSEE
NQRDVVKFCKQEGLVLLADEVYQENVYVPDKKFHSFKKVARSMGYGEKDLALVSFQSVSK
GYYGECGKRGGYMEVTGFTSDVREQIYKMASVNLCSNISGQILASLIMSPPKPGDDSYES
YIAEKDGILSSLARRAKTLEEALNKLEGVTCNRAEGAMYLFPCLHLPQKAIAAAEAEKTA
PDNFYCKRLLKATGIVVVPGSGFRQVPGTWHFRCTILPQEDKIPAIVDRLTAFHQSFMDE
FRD

E.1.2 *Hevea brasiliensis* (Hb) protein sequences

>HbACS1 (GenBank: AJT35543.1)
MVFKLNNQLLSKIATGNGHGEDSPYFDGWKAYDEDPYHPTKNPKGVIQMGLAENQLCFDL
IQEWLKNNPKASICTPEGAEEFRDIAIFQDYHGLPEFRNAVAKFMAKGRGNRVTFDPDRI
VMSGGATGAHEMVAFCLADPGEAFLVPTPYYPGFDRDLRWRTGVQLIPVECESSNQFKVT
RKALEDAYDTAQLDNIRVKGLLITNPSNPLGTILDRETLKSIVSFVNEKNIHLVCDEIYA
ATVFSQPDFVSISEIIEEEVECNLDLIHIVYSLSKDMGFPGFRVGIIYSYNDAVVSCARK
MSSFGLVSSQTQHLIASMLSDDEFVEKFIMQNKKRLATRYSSFTNGLAQVGIKCLKTSNA
GLFVWMDLRRLLKEQTVEGEIALWRVIINNVKLNVSPGSSFRCTEPGWFRVCFANMDDQT
MEVALSRIKTFILKSKEAKMSVKKLCWQGSLKLSFSSRIYDEIISPHSPIPHSPLVRART

>HbACS2 (GenBank: AJT35544.1)
MVFKLNNQLLSKIATGKGHGEDSPYFDGWKAYDNDPYHPTKNPDGVIQMGLAENQLCFDL
IQEWLKNNPKASICTTEGAEEFRDIAIFQDYHGLEEFRIAIAKFMAKGRGDRVTFDPDRI
VMSGGATGAHEMIAFCLADPGDAFLVPTPYYPGFDRDLRWRTGVQLFPVDCESSNYFKIT
REALEYAYEMAQLDNIRVKGLLITNPSNPLGTILDRETLKSIVNFINEKNIHLVCDEIYA
ATVFSQPEFVSISEIIEEEVECNLDLIHIVYSLSKDMGFPGFRVGIVYSYNDAVVSCARK
MSSFGLVSSQTQHLIASMLSDDEFVDNFIMQSKKRLASRYSSFTKGLAQVGIKCLKTSNA
GLFVWMDLRRLLKEQTVAGELALWRVIINDVKLNVSPGSSFHCTEQGWFRVCFANMDDQT
VEVALSRIKTFMLKNKEAMMPVKKLRWQGSLKLSFSSRIYDDFIMSPHSPFPQSPLVQAR
N

>HbACS3 (GenBank: AJT35545.1)
MVSGQLLSRIATNEGHGENSPYFDGWKEYDGNPFHPTDNPDGVIQMGLAENQLSFDLITD
WIKQNPEASICTAEGVDKFKDIANFQDYHGLREFREAIAKFMGMVRGGKVTFDPDRVVMG
GGATGANELIMFCLADPGDAFLVPSPYYPAFDRDLTWRTGVQIIPVDCYSSNNFQVTKDA
LESAYDKAQDAGINVKGLIIANPSNPLGTILDRETLKDLVSFINERNIHLVADEIYAATI
FSSPSFISVAEIIQEMDCNRDLIHIVYSLSKDMGLPGFRVGIVYSYNDAVVNCGRRMSSF
GLVSSQTQYMLASMLSDEEFVKNFLAESSRRLNKRHNMFTKGLEQVGISCLKGNAGLYVW
MDLRHLLKEPTFEGEMALWRVIIYQVKLNVSPGSSFHCKEPGWFRVCFANMDDETVEAAL
KRIRAFVCKGKEDQEMPTKSSKRWQKNLRLSFSARRFEEGVMLSPHMMSPHSPIPHSPLV

RAK

>HbACS4 (GenBank: AJT35546.1)
MVSGGQLLSKIATNEGHGENSPYFDGWKAYDRNPFHPTDNPDGVIQMGLAENQLSFDLIR
DWIKQNPEASICTVDGVHKFKDIANFQDYHGLPEFRKAIAKFMGMVRGGRVTFDPDRVVM
GGGATGANELIMFCLADHGDAFLVPTPYYPAFDRDLTWRTGVQIIPVDSCSFNKFQVTKN
ALEAAYDRAQGAGINVKGLIIANPSNPLGTVLERETLKDLVSFINERNIHLVVDEIYAAT
IFSSSSFISVAEIIEEMDCNRNLIHIVYSLSKDMGLPGFRVGIVYSYNDEVVRCGRKMSS
FGLVSSQTQYLLARMLSDEEFVKNFLAESSRRLNKRHSMFTKGLEQVGINCLKGNAGLYV
WMDLHHLLKEPTFEGEMTLWRVIIDQVRLNVSPGSSFHCKEPGWFRVCFANMDDQTVEAA
LKRIRAFVCKGKEDHQEMPTKNKQRWQRNLRLSFSARRFEEGVKSPHVMSPHSPIPHSPL
VRAK

>HbACS5 (GenBank: AJT35547.1)
MRLLSRKATCNTHGQDSSYFLGWEEYEKNPYDEIKNPTGIIQMGLAENQLSFDLLESWLA
NNPDAAVFKKDGQSIFRELALFQDYHGLPAFKKALVDFMAEIRGNRVTFDQNKIVLTAGA
TSANETLMFCLAEPGEAFLLPTPYYPGFDRDLKWRTGVEIVPIQCASSNGFQITAPALEE
AYLEAQRRNLRVKGVLVTNPSNPLGTTMSRSELNLLVNFITAKGIHLISDEIYSGTVFSS
PGFISIMEVLKDRKCENTEVWKRVHIVYSLSKDLGLPGFRVGAIYSNDEMVVSAATKMSS
FGLVSSQTQYLLSALLSDNKFTKNYISENQRRLKQRQKLLVKGLEKAGISCLKSNAGLFC
WVNMKHLLRSNTFEAEMELWKKIVYDVNLNISPGSSCHCTEPGWFRVCFANMSEETLKLA
MDRLKSFVDSISKTTSHQMVKNSRKKYLTKWVFRLSFHDRGPKER

>HbACS6 (GenBank: AJT35548.1)
MAIEIEQPTVGLSKVAVSETHGEDSPYFAGWKAYDEDPYDESENPSGVIQMGLAENQVSF
DLLEEYLEKHSEASTWGKGAPGFREHALFQDYHGLKSFRQAIASFMEQIRGGRAKFDPDR
VVLTAGATAANELLTFILADPGDALLVPTPYYPGFDRDLRWRTGVKIVPIHCDSSNNFQV
TPQALEAAYKDAEVMNIKVRGVLITNPSNPLGATIQRSVLEEILDFVTRKNIHLVSDEIY
SGSAFSSSEFVSIAEILEARGYKDSERVHIVYSLSKDLGLPGFRVGTIYSYNDKVVTTAR
RMSSFTLISSQTQHLLASMLSDQEFTKNYIKINRERLRKRYEMIIEGLRNAGIECLKGNA
GLFCWMNLSPLLKTQTREGELTLWKSIIRDLKLNISPGSSCHCSEPGWFRVCFANMSEQT
LEVALKRIHNFMDQRKTETN

>HbACS7 (GenBank: AJT35549.1)
MGLEIEQPTVGLSKVAVSETHGEDSPYFAGWKAYDEDPYDELENPSGVIQMGLAENQVSF
DLLEEYLEKHSEASTWGQGATGFRENALFQDYHGLKSFRQAMASFMEQIRGGRAKFDPDR
VVLTAGATAANELLIFILADPGDALLVPIPYYPGFDRDLRWRTGVKIVPIHCDSSNNFQV
TPQALEAAYKDAEAMNIKVRGVLITNPSNPLGATIQRSVLEDILDFATQKNIHLVSDEIY
SGSTFSSSEFVSIAEILEARGYKDSERVHIVYSLSKDLGLPGFRVGTIYSYNDKVVTTAR
KMSSFTLISSQTQHLLATMLSDKVFTKNYIKINRERLRKRYEMIIQGLRSAGIECLKGNA
GLFCWMNLSPLLKTPTKEGELSLWNSIIHDLKLNISPGSSCHCSEPGWFRVCFANMSEQT
LEVALKRIHNFMEQKKTETN

>HbACS8 (GenBank: AJT35550.1)
MTLTQIRTQSKEPEHPPRKPTGATGMRLIVPLQGVVQGRGGLILGSLIPCALFYFFHLYL
KRHRSANPSSNPPSPSASSPNLADIPRTASRSNLSSRGSFGPGRASISTRAMSIAKPNDS
PYYIGLDKVSGNPYDRTSNPDGIIQLGLSENRLCLDLIEKWMAKNLRDSIVGTDGDDLNI

NGITTYQPFDGLMELKVAMANFMSRVVGKVVSFDASQMVLTAGATPAVEILSFCLADHGN
AFLVPTPYYPGFDRDMRWRTGVELVPVHCRSTDNFILSVTALEQAYNHARKRGLKVRGLL
ISNPSNPVGNLLPRERLFDILNFAQEKNIHIISDEIFAGSVYGEDEFVSMAQILEEEDFD
KNRVHIIYGLSKDLSLPGFRVGAIYSYNENVLTAAKRLTRFSSISAPSQRLLASMLSDAS
FIEEYIETNKKRIGNIYGLFVEGFRRLGIRCMESSAGLYCWADMGKLIPSYSEKGELDLW
DKLLNIAKINVTPGSAFHCIEPGWFRCCFTTLTEDDIPVVIERIRKVAETCKSPG

>HbACS9 (GenBank: AJT35551.1)
MTRTRFFRSRTAEPDEDTIPTTYRSGGGGTAMRIIVPLQGVVQGRGGLFLGSVIPCALFY
FFQLYLKRNRNDQADSDDSNSQNQAPPSRSGSEGQLNELSVFTRSTSRNLVSPRSPSGRA
YVSSRANGIVKSGDSPYFVGLRKVIEDPYDESANPNGVIQLGLAENKLTLDLVEEWLVEN
AKPAILGGGGEELNISGIATYQPVDGLIELKVAVAGFMSQIMENAVSFNPSQIVLTAGAT
PAIETLIFCLADAGNAFLVPTPYYPGFDRDVKWRTGVEIVPVPCRSADNFSLSITALDRA
FNQAKKRGLKVRGVIISNPSNPVGNLLDRETLYSLLDFAREKNIHIVCNEIFAGSTHGSE
EFVSMAELIDLEDSDRDRVHIVYGLSNDLSLPGFRVGVLYSSNENVLAAAKKLTRFSSIS
APTQRLLISMLSDTKFVQIFIRINRERLQNMYVKFALGLKQLGIKCTKGSGGFYCWADMS
ELISSYSEKGELELWEKLLNTAKLNSTPGSSCHCIEPGWFRFCFTTLTERDIPVVMDRIQ
KIAETCKSCS

E.1.3 *Solanum lycopersicum (Sl)* protein sequences

>SlACS1a (GenBank: NP_001233922.2)
MVSISKNNQKQQLLSKIATNDGHGENSPYFDGWKAYANNPFHLTDNPTGVIQMGLAENQL
CFDLIQEWVVNNPKASICTVEGAENFQDIAIFQDYHGLPEFRQAVARFMEKVRGDRVTFD
PNRIVMSGGATGAHEMLAFCLADPGDAFLVPTPYYPGFDRDLRWRTGVQLFPVVCESCND
FKVTTKALEEAYEKAQQSNIKIKGLLINNPSNPLGTLLDKDTLRDIVTFINSKNIHLVCD
EIYAATVFDQPRFISVSEIVEDMIECNKDLIHIVYSLSKDLGFPGFRVGIVYSYNDTVVN
IARKMSSFGLVSTQTQHLLASMLSDEVFIDKFIAESSERLGERQGMFTKGLAEVGISTLK
SNAGLFFWMDLRRLLKEATFDSELELWRIIINEVKLNVSPGCSFHCSEPGWFRVCFANMD
DETMRIALKRISYFVLQPKGLNNIAAIKKQCSRRKLQISLSFRRLDHEFMNSPAHSPMNS
PLVRT

>SlACS2 (GenBank: NP_001234178.2)
MGFEIAKTNSILSKLATNEEHGENSPYFDGWKAYDSDPFHPLKNPNGVIQMGLAENQLCL
DLIEDWIKRNPKGSICSEGIKSFKAIANFQDYHGLPEFRKAIAKFMEKTRGGRVRFDPER
VVMAGGATGANETIIFCLADPGDAFLVPSPYYPAFNRDLRWRTGVQLIPIHCESSNNFKI
TSKAVKEAYENAQKSNIKVKGLILTNPSNPLGTTLDKDTLKSVLSFTNQHNIHLVCDEIY
AATVFDTPQFVSIAEILDEQEMTYCNKDLVHIVYSLSKDMGLPGFRVGIIYSFNDDVVNC
ARKMSSFGLVSTQTQYFLAAMLSDEKFVDNFLRESAMRLGKRHKHFTNGLEVVGIKCLKN
NAGLFCWMDLRPLLRESTFDSEMSLWRVIINDVKLNVSPGSSFECQEPGWFRVCFANMDD
GTVDIALARIRRFVGVEKSGDKSSSMEKKQQWKKNNLRLSFSKRMYDESVLSPLSSPIPP
SPLVR

>SlACS3 (GenBank: NP_001234026.2)
MKLLSEKATCNSHGQDSSYFLGWQEYEKNPYDEIQNPKGIIQMGLAENQLSFDLLESWLA
QNPDAAGFKRNGESIFRELALFQDYHGLPAFKNAMTKFMSEIRGNRVSFDSNNLVLTAGA

TSANETLMFCLANQGDAFLLPTPYYPGFDRDLKWRTGAEIVPIHCSSSNGFRITESALEE
AYLDAKKRNLKVKGVLVTNPSNPLGTTLNRNELELLLTFIDEKGIHLISDEIYSGTVFNS
PGFVSVMEVLIEKNYMKTRVWERVHIVYSLSKDLGLPGFRIGAIYSNDEMVVSAATKMSS
FGLVSSQTQYLLSCMLSDKKFTKKYISENQKRLKKRHAMLVKGLKSAGINCLESNAGLFC
WVDMRHLLSSNNFDAEMDLWKKIVYDVGLNISPGSSCHCTEPGWFRVCFANMSEDTLDLA
MRRIKDFVESTAPNATNHQNQQQSNANSKKKSFSKWVFRLSFNDRQRER

>SlACS4 (GenBank: NP_001234280.1)
MDLETSEISNYKSSVVLSKLASNEQHGENSPYFDGWKAYDNDPFHLVNNLNGVIQMGLAE
NQLSVDLIEEWIKRNPKASICTNDGIESFRRIANFQDYHGLPEFTNAIAKFMEKTRGGKV
KFDAKRVVMAGGATGANETLILCLADPGDAFLVPTPYYPGFNRDLRWRSGVQLLPISCKS
CNNFKITIEAIEEAYEKGQQANVKIKGLILTNPCNPLGTILDRDTLKKISTFTNEHNIHL
VCDEIYAATVFNSPKFVSIAEIINEDNCINKDLVHIVSSLSKDLGFPGFRVGIVYSFNDD
VVNCARKMSSFGLVSTQTQHLLAFMLSDDEFVEEFLIESAKRLRERYEKFTRGLEEIGIK
CLESNAGVYCWMDLRSLLKEATLDAEMSLWKLIINEVKLNVSPGSSFNCSEVGWFRVCFA
NIDDQTMEIALARIRMFMDAYNNVNKNGVMKNKHNGRGTTYDLTPQMGSTMKMLLA

>SlACS5 (GenBank: AAK72430.1)
MLKMLSKMAMCKSHGQDSSYFIGWQEYEKNPYDPLQNPSGIIQMGLAENQLSFDLIESWL
TRNQDVIQFRENGGSMFRDLALFQDYHGLQAFKNVLVSLMAEIRRKKVKFDPKNLVLTAG
STSANETLIFCLAEPGEALLIPTPYYPGFDRDLKWRTGAEIVPIQCYSSNNFRITESALE
EAYEQAQKRNLTVKGVFITNPSNPLGTTMSRNELNILITFAMTKNIHIVSDEIYAGTVFD
SPKFVSIIEALIDRKLEKSKMWNQVHIVSSLSKDLGLPGFRVGMIYSNNETLIAAATKMS
SFGLISSQTQYLLSKILGDRRFIKRYVKQNKKQLIHRREMLASGLANSGIECLDSNAGLF
CFVDMRDLLNSNTFEAEMELWKKIISNVGLNVSPGSSCHCSEPGWFRVCFANMSKETLDL
AMQRINNFVNFDRDIHRQQPLRFVTGAGSRRRRTIANWVVKFSSGDGRRDR

>SlACS6 (GenBank: NP_001234164.2)
MGLISKIATNDGHGENSAYFDGWKAYENDPFHPTQNPNGVIQMGLAENQLCFDLIQEWIV
NNPKASICTYEGVQDFQDTAIFQDYHGLPEFRKAVARFMEKVRGDRVRFDPERIVMSGGA
TGAHESLAFCLADPGDAFLVPTPYYPGFDRDLRWRTGVQLFPVVCESSNNFKVTKEALEE
AYSKAQESNIKVKGLLINNPSNPLGTILDKETLKDILRFINDKNIHLVCDEIYAATAFSQ
PSFISISEVKSEVVGCNDDLVHIVYSLSKDLGFPGFRVGIIYSYNDAVVNIARKMSSFGL
VSTQTQRLIASMLLDTIFVEDFIAKSSMRLLQRYGLFTKGLGQVGITTLKSNAGLFIWMD
LRRFLENSTFDDELKLWHIIIDKVKLNVSPGCSFHCSEPGWFRVCFANMDDATMKIALRR
IRHFVYLQPNKGVEVATKKQYCRTRSKLEISLSFRRLDDFMNSPHSPMSSPMVQARN

>SlACS7 (GenBank: NP_001234346.1)
MKLLSKKAMCNSHGQDSSYFLGWEEYQKNPYDEIRNPKGIIQMGLAENQLSFDLLESWLT
LNPDASAFKRNGHSIFRELSLFQDYHGLPAFKDALVQFMSEIRGNKVSFDSNKLVLTAGA
TSANETLMFCLADPGHAFLLPTPYYPGFDRDLKWRTGAEIVPIQCTSSNGFRITESALEE
AYTEAERRNLRVKGVLVTNPSNPLGTTLTKKELQLLLTFVSTKQIHLISDEIYSGTVFNS
PKFVSVMEVLIENNYMYTDVWDRVHIVYSLSKDLGLPGFRVGAIYSNDDRVVSAATKMSS
FGLISSQTQYLLSALLSDKKFTKNYVSENQKRLKKRHEMLVGGLKQIGIRCLESNAGLFC
WVDMRHLLSSNTFDGEMELWKKIVYEVGLNISAGSSCHCTEPGWFRACFANMSEDTLNIA
IQRLKAFVDSRVNNKDDIQNQQQCSNKKKSFSKWVFRLSFNERQRER

>SlACS8 (GenBank: NP_001234160.2)
MKLLSDMASCNSHGQDSSYFLGWQEYEKNPYDEIQNPKGIIQMGLAENQLSFDLLESWLA
QNPDAAGFKRNGESIFRELALFQDYHGLPDFKNALVQFMSEIRGNKVTFNPNKLVLTAGA
TSANETLMFCLANPGDAFLLPTPYYPGFDRDLKWRTGAEIVPIQCTSSNGFRITQSALEE
SYKLAKTRNLRVKGILVTNPSNPLGTTLTRNELELLVSFVAEKGIHLISDEIYSGTVFNS
PKFVSVMEVLIENNYMYTEVWDRVHIVYSLSKDLGLPGFRIGAIYSNDAVIVSAATKMSS
FGLISSQTQYLLSAMLTDKKFTKKYISENQKRLKKRHAMLVKGLESSGISCLESNAGLFC
WVDMRHLLKTNTFEAEIELWKKIVYEVRLNISPGSSCHCTEPGWFRACFANMSEDTLNLA
IQRIKSFVDSSDVIGINVDQSNQTNQNTSTSPKKKLFAKWGFRLSFNDRER

>SlACS10 (GenBank: XP_004245687.1)
MTRSRNRSPTRTTTISTGGAGGRDGGGATTAMRVIVPLQGVVQGRGGLFLGSVIPCALFY
FWQLYLKRNRSSGGDNNGESTAPARSPSSTHLPEVSSGSGLQRVHSRLLLSPKGTTGQSQ
VSARANSIISKQIDSSPYYVGLKRASEDPYDESSNPDGVIQLGLAENKLSLDLVQEWLAE
NVSRWMMTQDSSITGIATYQPFDGLLELKVAVGEFMSQALERSVSFSPSQMVLTGGATPA
LEILSFCLADPGNAFLVPSPYYPDLDRDVKWRTGVEIIPVPCRSADNFNLSIDALDRAFN
QAKKRGLKVRGIIISNPSNPVGNIFSRETLYNLLDFTTEKNIHVISNEILAGSTYGNEEF
VSMAEIIDSEDFDRSRVHIVYGLSKDLSLPGFRVGVIYSCNENVLAAAKKLTRFSSISAP
TQHLIIQMLSDAKFVQQFIKKNRERLRRMSSLFVSGLKQLGIECTRSSGGFYCWADMSRL
IRSYNEKGEIELWDNLLNVAKINATPGSSCHCVEPGWFRLCFSTLSEKDISAVMQRIQKV
LELRKSLS

>SlACS12 (GenBank: XP_004234155.1)
MTSAGQLKPNLDSENDTGKASPGDSGGTSGMRLVVPLQGVVQGRGGLILGSLIPCALFYF
LQFYLKRHRTTPSSSNPPSPSTSSPNLSDLQRSSSRLNLSTRGSVGRVFLSSRASLVAAP
NDSPYYVGMDRFRADPYDELDNPDGVIDLGIAENRLSLDLIEKWISSNVNIGSGGDGLNI
NGILTYQPFDGMAELKVAMSGFMSQVMGEKVSFDPSRMVLTSGATPAIEVLCFCLADHGN
ALLVPTPYYPGFDRDIRWRTGVDLIPVYCRSSDAFMVDITTLDQAFNHARKRGKKVRGIL
ISNPSNPVGNILNREMLYRILDFAREKNIHVISDEIFAGSNYGGEEFVSIAEILDEDDAD
RDRVHIIYGLSKDLSVPGFRLGVLYSFNENVVAASKKLTRFCAASAPTQSLLVAMLSDAG
FIKDYMRTNRERLRKVFDLFVAGLKQLGIECMKSSAGLYCWVNMSGLIGPYNEKGELELW
EKLLNVAKINVTPGSACHCIEPGWFRCCFSTVEEKGIPVVMERIRKVVELVRPSD

>SlACC (GenBank: XP_010313742.1)
MAIVELSKVANSDTHGEDSPYFAGWKAYDEDPFDEVHNPCGVIQMGLAENQVSFDMVEDY
LEKHSKTINCGSGISNFRENALFQDYHGLYSFRKSMAKFMEKIRGGRAKFNPDRVVITAG
ATAANELLTFILADPGDALLVPTPYYPGFDRDLRWRTGVKIIPIHCNSSNNFQVTPQALE
SAYEEAKFNKIKVRGILITNPSNPLGATIQRSNLEDILDFVVRKNIHLISDEIYSGSAFC
SSKFVSIAEVLKSRNDVDSERVHIVYSLSKDLGLPGFRVGTIYSYNDNVVTTARRMSSFT
LISSQTQQLLASMLSNEEFTTNYIKTNRDRLRKRYEKIIDGLKRSGIECLKGNAGLFCWM
NLSQLLEKSTKECELKLWNSILCEVKLNISPGSSCHCSEPGWFRVCFANMSEQTLEIALK
RLHNFMQRRTQNRY

>SlACSu1 (GenBank: XP_004252649.1)
MGFISNINNIELLSKVATNNGHGENSAYFDGWKAYEIDPFHPTKNPNGVIQMGLAENQLC
FDLIQEWVVNNQKSSICTAGGCEEFKEIAIYQDYHGLPEFRRGVASFMSKVRGDKIKFDE
ERIVMSGGATGAHELLAFCLADPGEAFLVPTPYYPGFDRDLSWRTGVQLFPIICESCNNF

KVTKKALEDAYNKAQQSNITIKGLLLNNPSNPLGTILDMEALKDTIRFINDKNIHLICDE
IYAATVFNVPKFISISEIIISEDVQCNLDLIHIVYSLSKDLGFPGFRVGIIYSYNDVVTK
CARKMSSFGLVSTQTQYLISNMLLDDTFIEKFVVESRERLEKRHGVFTKGLENIGINTLE
SNAGLFCWMDLRSLLEKNTFESEIKLWRMIINDVKLNVSPGCSFHCCEPGWFRVCFANMD
DDTMRIALRRIEIFVVQYKGINNIIEEGIGESLPITPGPVSLRSIYEKIDRRR

>SlACSu2 (GenBank: XP_004242974.1)
MAIEIEKRSTVVGLSNVATSDTHGEDSPYFAGWKAYDENPFDEVHNPSGVIQMGLAENQV
SFDLLEEYLEKQKDDGVAEISRFRENALFQDYHGLVCFRKAMASFMEKIRGGRARFDPDR
VVITAGATAANELLTFILADPGDALLLPTPYYPGFDRDLRWRTGVKIVPVHCDSSNNFQV
TPRALEDAYKEAESNNIKVRGVLITNPSNPLGATVQRCVLEEILEFVTRKNIHLVSDEIY
SGSAFCCSEFVSIAEILESRNYKDSERVHIVYSLSKDLGLPGFRVGTIYSYNDKVVTTAR
RMSSFTLISSQTQQLLASMLSDETFTENYIKKNRERLRMRYEMMIEGLRSAGIECLRGNA
GLFCWMNLTSLLEKPTKECELQVWNTILNQVKLNISPGSSCHCSEPGWFRVCFANMTENT
LQIALKRIHHFMETRATLQKY

>SlALAAT2_like (GenBank: XP_004241856.1)
MRRFISHKTINLISKTTTSTISSSSSPNSPSLSPNSPLNSLLRFFSALPAYSPDSMASDY
SATHITVDNINPKVLKCEYAVRGEIVSIAQTLQQRLKDNPGSHPFDEILYCNIGNPQALA
QQPITFFREVLALCDHPSILDKSETQGLFSADAIERAFQILEQIPGRATGAYSHSQGIKG
LRDTIASGMGARDGFPADPNDLFLTDGASPAVHMMMQLLIRSENDGILCPIPQYPLYSAS
IALHGGTLVPYYLDEQTGWGLEISELEHQLKTAKSKGIDVRALVVINPGNPTGQVLGEAN
QREIVEFCRKEGLVLLADEVYQENVYVPDKKFHSFKKVSRSMGYGETDITLVSFQSVSKG
FYGECGKRGGYMEITGFSPEVREQIYKLASVNLCSNISGQILASLIMSPPKVGDESYESF
SAEKEAILSSLARRAKTLEDALNSLEGVTCNRAEGAMYLFPCINLPDKAIKAAEAAKTAP
DAFYAKHLLNATGIVVVPGSGFRQVPGTWHFRCTILPQEEKIPAIVSRLTEFHKKFMDEF
RG

E.2 AMINO ACID SEQUENCES USED FOR THE 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE (ACO) PHYLOGENETIC TREE (Fig. A11)

E.2.1 *Arabidopsis thaliana* (At) protein sequences

>AtACO1 (GenBank: NP_179549.1)
MVLIKEREMEIPVIDFAELDGEKRSKTMSLLDHACDKWGFFMVDNHGIDKELMEKVKKMI
NSHYEEHLKEKFYQSEMVKALSEGKTSDADWESSFFISHKPTSNICQIPNISEELSKTMD
EYVCQLHKFAERLSKLMCENLGLDQEDIMNAFSGPKGPAFGTKVAKYPECPRPELMRGLR
EHTDAGGIILLLQDDQVPGLEFFKDGKWVPIPPSKNNTIFVNTGDQLEILSNGRYKSVVH
RVMTVKHGSRLSIATFYNPAGDAIISPAPKLLYPSGYRFQDYLKLYSTTKFGDKGPRLET
MKKMGNADSA

>AtACO2 (GenBank: NP_176428.1)
MEKNMKFPVVDLSKLNGEERDQTMALINEACENWGFFEIVNHGLPHDLMDKIEKMTKDHY
KTCQEQKFNDMLKSKGLDNLETEVEDVDWESTFYVRHLPQSNLNDISDVSDEYRTAMKDF
GKRLENLAEDLLDLLCENLGLEKGYLKKVFHGTKGPTFGTKVSNYPPCPKPEMIKGLRAH

TDAGGIILLFQDDKVSGLQLLKDGDWIDVPPLNHSIVINLGDQLEVITNGKYKSVLHRVV
TQQEGNRMSVASFYNPGSDAEISPATSLVEKDSEYPSFVFDDYMKLYAGVKFQPKEPRFA
AMKNASAVTELNPTAAVETF

>AtACO3 (GenBank: NP_172665.1)
MEMNIKFPVIDLSKLNGEERDQTMALIDDACQNWGFFELVNHGLPYDLMDNIERMTKEHY
KKHMEQKFKEMLRSKGLDTLETEVEDVDWESTFYLHHLPQSNLYDIPDMSNEYRLAMKDF
GKRLEILAEELLDLLCENLGLEKGYLKKVFHGTTGPTFATKLSNYPPCPKPEMIKGLRAH
TDAGGLILLFQDDKVSGLQLLKDGDWVDVPPLKHSIVINLGDQLEVITNGKYKSVMHRVM
TQKEGNRMSIASFYNPGSDAEISPATSLVDKDSKYPSFVFDDYMKLYAGLKFQAKEPRFE
AMKNAEAAADLNPVAVVETF

>AtACO4 (GenBank: NP_171994.1)
MESFPIINLEKLNGEERAITMEKIKDACENWGFFECVNHGISLELLDKVEKMTKEHYKKC
MEERFKESIKNRGLDSLRSEVNDVDWESTFYLKHLPVSNISDVPDLDDDYRTLMKDFAGK
IEKLSEELLDLLCENLGLEKGYLKKVFYGSKRPTFGTKVSNYPPCPNPDLVKGLRAHTDA
GGIILLFQDDKVSGLQLLKDGEWVDVPPVKHSIVVNLGDQLEVITNGKYKSVEHRVLSQT
DGEGRMSIASFYNPGSDSVIFPAPELIGKEAEKEKKENYPRFVFEDYMKLYSAVKFQAKE
PRFEAMKAMETTVANNVGPLATA

>AtACO5 (GenBank: NP_565154.1)
MAIPVIDFSKLNGEEREKTLSEIARACEEWGFFQLVNHGIPLELLNKVKKLSSDCYKTER
EEAFKTSNPVKLLNELVQKNSGEKLENVDWEDVFTLLDHNQNEWPSNIKETMGEYREEVR
KLASKMMEVMDENLGLPKGYIKKAFNEGMEDGEETAFFGTKVSHYPPCPHPELVNGLRAH
TDAGGVVLLFQDDEYDGLQVLKDGEWIDVQPLPNAIVINTGDQIEVLSNGRYKSAWHRVL
AREEGNRRSIASFYNPSYKAAIGPAAVAEEEGSEKKYPKFVFGDYMDVYANQKFMPKEPR
FLAVKSL

>AtACOH4 (GenBank: NP_171839.1)
MESTDRSSQAKAFDEAKIGVKGLVDSGITEIPALFRATPATLASLKSPPPPKHLTIPTVD
LKGASVVEKIGEAAEKWGLFHLVNHGIPVEVLERMIQGIRGFHEQEPEAKKRFYSRDHTR
DVLYFSNHDLQNSEAASWRDTLGCYTAPEPPRLEDLPAVCGEIMLEYSKEIMSLGERLFE
LLSEALGLNSHHLKDMDCAKSQYMVGQHYPPCPQPDLTIGINKHTDISFLTVLLQDNVGG
LQVFHEQYWIDVTPVPGALVINIGDFLQLITNDKFISAEHRVIANGSSEPRTSVAIVFST
FMRAYSRVYGPIKDLLSAENPAKYRDCTLTEFSTIFSSKTLDAPKLHHFKI

>AtACOH8 (GenBank: NP_191699.1)
MDSTATVASDRLSQLNSFEETMTGVKGLVDSGIKEVPAMFREPPAILASRKPPLALQFTI
PTIDLNGGVVYYKNQDSVTRRSMVEKIGDAAEKWGFFQVVNHGIPLDVLEKVKEGIRAFH
EQDAELKKRFYSRDHTRKMVYYSNLDLFTAMKASWRDTMCAYMAPDPPTSEDLPEVCGEI
MMEYAKEIMNLGELIFELLSEALGLNNSNHLKDMDCSKSLVLFGQYYPPCPQPDHTLGLS
KHTDFSFLTIVLQGNLGGLQVLHDKQYWIDIPPVPGALVVNLGDLLQLISNGKFISVEHR
VIANRAAEPRISVPCFFSTVMRESHRVYGPIKELLSEQNPPKYRDTTISEFASMYASKEI
NTSALLRLEI

>AtACOH9 (GenBank: NP_199157.1)
MTEKSAELVRLNELKAFVSTKAGVKGLVDTKITEVPRIFHIPSSSTLSNNKPSDIFGLNL

TVPIIDLGDGNTSAARNVLVSKIKEAAENWGFFQVINHGIPLTVLKDIKQGVRRFHEEDP
EVKKQYFATDFNTRFAYNTNFDIHYSSPMNWKDSFTCYTCPQDPLKPEEIPLACRDVVIE
YSKHVMELGGLLFQLLSEALGLDSEILKNMDCLKGLLMLCHYYPPCPQPDLTLGISKHTD
NSFITILLQDQIGGLQVLHQDSWVDVTPVPGALVISIGDFMQLITNDKFLSMEHRVRANR
DGPRISVACFVSSGVFPNSTVYGPIKELLSDENPAKYRDITIPEYTVGYLASIFDGKSHL
SKFRI

>AtACOH10 (GenBank: NP_199158.1)
MTENSEKIDRLNDLTTFISTKTGVKGLVDAEITEVPSMFHVPSSILSNNRPSDISGLNLT
VPIIDLGDRNTSSRNVVISKIKDAAENWGFFQVINHDVPLTVLEEIKESVRRFHEQDPVV
KNQYLPTDNNKRFVYNNDFDLYHSSPLNWRDSFTCYIAPDPPNPEEIPLACRSAVIEYTK
HVMELGAVLFQLLSEALGLDSETLKRIDCLKGLFMLCHYYPPCPQPDLTLGISKHTDNSF
LTLLLQDQIGGLQVLHEDYWVDVPPVPGALVVNIGDFMQLITNDKFLSVEHRVRPNKDRP
RISVACFFSSSLSPNSTVYGPIKDLLSDENPAKYKDITIPEYTAGFLASIFDEKSYLTNY
MI

>AtFe2OGl_ O80850 (GenBank: NP_180641.1)
MAGNYDRAGEVKAFDQMKIGVKGLVDAGITKVPRIFHHQDVAVTNPKPSSTLEIPTIDVG
GGVFESTVTRKSVIAKVRAAVEKFGFFQVINHGIPLEVMESMKDGIRGFHEQDSEVKKTF
YSRDITKKVKYNTNFDLYSSQAANWRDTLTMVMAPDVPQAGDLPVICREIMLEYSKRMMK
LGELIFELLSEALGLKPNHLKELNCAKSLSLLSHYYPPCPEPDRTFGISSHTDISFITIL
LQDHIGGLQVLHDGYWIDVPPNPEALIVNLGDLLQLITNDKFVSVEHRVLANRGEEPRIS
SASFFMHTIPNEQVYGPMKELLSKQNPPKYRNTTTTEMARHYLARGLDGTSPLLHFRI

>AtFe2OGl_ O80851 (GenBank: NP_180642.1)
MEATYDRASEVKAFDELKIGVKGLLDAGVTQIPRIFHHPHLNLTDSNLLLSSTTMVIPTI
DLKGGVFDEYTVTRESVIAMIRDAVERFGFFQVINHGISNDVMEKMKDGIRGFHEQDSDV
RKKFYTRDVTKTVKYNSNFDLYSSPSANWRDTLSCFMAPDVPETEDLPDICGEIMLEYAK
RVMKLGELIFELLSEALGLNPNHLKEMDCTKGLLMLSHYYPPCPEPGLTFGTSPHSDRSF
LTILLQDHIGGLQVRQNGYWVDVPPVPGALLVNLGDLLQLMTNDQFVSVEHRVLANKGEK
PRISVASFFVHPLPSLRVYGPIKELLSEQNLPKYRDTTVTEYTSHYMARGLYGNSVLLDF
KI

>At2OADIOXputative (GenBank: NP_180115.1)
MAENYDRASELKAFDEMKIGVKGLVDAGVTKVPRIFHNPHVNVANPKPTSTVVMIPTIDL
GGVFESTVVRESVVAKVKDAMEKFGFFQAINHGVPLDVMEKMINGIRRFHDQDPEVRKMF
YTRDKTKKLKYHSNADLYESPAASWRDTLSCVMAPDVPKAQDLPEVCGEIMLEYSKEVMK
LAELMFEILSEALGLSPNHLKEMDCAKGLWMLCHCFPPCPEPNRTFGGAQHTDRSFLTIL
LNDNNGGLQVLYDGYWIDVPPNPEALIFNVGDFLQLISNDKFVSMEHRILANGGEEPRIS
VACFFVHTFTSPSSRVYGPIKELLSELNPPKYRDTTSESSNHYVARKPNGNSSLDHLRI

>AtLDOX (GenBank: NP_194019.1)
MVAVERVESLAKSGIISIPKEYIRPKEELESINDVFLEEKKEDGPQVPTIDLKNIESDDE
KIRENCIEELKKASLDWGVMHLINHGIPADLMERVKKAGEEFFSLSVEEKEKYANDQATG
KIQGYGSKLANNASGQLEWEDYFFHLAYPEEKRDLSIWPKTPSDYIEATSEYAKCLRLLA
TKVFKALSVGLGLEPDRLEKEVGGLEELLLQMKINYYPKCPQPELALGVEAHTDVSALTF
ILHNMVPGLQLFYEGKWVTAKCVPDSIVMHIGDTLEILSNGKYKSILHRGLVNKEKVRIS

WAVFCEPPKDKIVLKPLPEMVSVESPAKFPPRTFAQHIEHKLFGKEQEELVSEKND

E.2.2 *Trifolium repens* (Tr) protein sequences

>TrACO1 (GenBank: AAD28196.2)
MENFPIVDMGKLNTEERKSTMEKIKDACENWGFFELVNHGISIEMMDKVEKLTKDHYKKC
MEQRFKEMVSSKGLECVQSEINDLDWESTFFLRHLPFSNISEIPDLDDDYRKIMKEFAQK
LENLAEELLDLLCENLGLEKGYLKKVFYGSKGPNFGTKVSNYPPCPKPDLIKGLRAHTDA
GGIILLFQDDKVSGLQLLKDDQWIDVPPMRHSIVINLGDQLEVITNGKYKSVMHRVIAQT
DGARMSLASFYNPSDDAIISPAPTLLKENETTSEIYPKFVFDDYMKLYMGLKFQAKEPRF
EAMMKAMSSVDVGPVVSI

>TrACO2 (GenBank: AAD28197.2)
MENFPIINLENLNGEERKATMEKIKDACENWGFFELVNHGISHDLMDTVERLTKEHYRIC
MEQRFKDLVANKGLEAVQTEVKDMDWESTFHLRHLPESNISEVPDLTDEYRKAMKEFALK
LEKLAEELLDLLCENLGLEKGYLKKAFYGSKGPTFGTKVANYPPCPKPDLVKGLRAHTDA
GGIILLFQDDKVSGLQLLKDGKWVDVPPMHHSIVINLGDQLEVITNGKYKSVEHRVIAQS
DGTRMSIASFYNPGSDAVIYPATTLIEENNEVYPKFVFEDYMNLYAGLKFQAKEPRFEAF
KESSNVKLGPIATV

>TrACO3 (GenBank: AAD28198.2)
MMNSPIISLERLNGVERKDTMEKIKDACQNWGFFELVNHGIPHDLMDTLERLTKEHYRKC
MEQRFKELVSSKGLDAVQTEVKDMDWESTFHVRHLPESNISELPDLSDEYRKVMKEFSLR
LEKLAEELLDLLCENLGLEKGYLKKAFYGSRGPTFGTKVANYPQCPNPELVKGLRAHTDA
GGIILLFQDDKVSGLQLLKDDEWIDVPPMRHSIVVNLGDQLEVITNGKYKSVEHRVIAQT
NGTRMSIASFYNPGSDAVIYPAPELLEKETEEKTNVYPKFVFEEYMKIYAALKFQAKEPR
FEALKA

>TrACO4 (GenBank: ABB97399.1)
MMNSPIISLERLNGVERKDTMEKIKDACENWGFFELVNHGIPHDLMDTLERLTKEHYRKC
MEQRFKELVSSKGLDAVQTEVKDMDWESTFHVRHLPESNISELPDLSDEYRKVMKEFSVR
EENLTDELLDLLCENLGLEKGYLGKAFYGSRGPTFGTKVPHYPPCPNPEVGKGLPAHTDS
GGIFLLFQEDKGSGLHLLRDDERIAPPPMLHSFEVNLRDQLEVITNGKYKSVEHRVIAQT
NGTRMSIASFYNPGSDAVIYPAPELLEKETEEKTNVYPKFVFEEYMKIYAALKFQAKEPR
FEALKA

E.2.3 *Malus domestica* (Md) protein sequences

>MdACO1 (GenBank: Q00985.1)
MATFPVVDLSLVNGEERAATLEKINDACENWGFFELVNHGMSTELLDTVEKMTKDHYKKT
MEQRFKEMVAAKGLDDVQSEIHDLDWESTFFLRHLPSSNISEIPDLEEEYRKTMKEFAVE
LEKLAEKLLDLLCENLGLEKGYLKKVFYGSKGPNFGTKVSNYPPCPKPDLIKGLRAHSDA
GGIILLFQDDKVSGLQLLKDGEWVDVPPMHHSIVINLGDQIEVITNGKYKSVMHRVIAQS
DGTRMSIASFYNPGNDSFISPAPAVLEKKTEDAPTYPKFVFDDYMKLYSGLKFQAKEPRF
EAMKAKESTPVATA

>MdACO2 (GenBank: O48882.1)
MATFPVVDMDLINGEERAATLEKINDACENWGFFELVNHGISTELLDTVEKMNKDHYKKT
MEQRFKEMVAAKGLEAVQSEIHYLDWESTFFLRHLPSSNISEIPDLEEDYRKTMKEFAVE
LEKLAEKLLDLLCENLGLEKGYLKKAFYGSKGPNFGTKVSNYPPCPKPDLIKGLRAHTDA
GGIILLFQDDKVSGLQLLKDGEWMDVPPVHHSIVINLGDQIEVITNGKYKSIMHRVIAQS
DGTRMSIASFYNPGDDAFISPAPALLEEKSEVSPTYPKFLFDDYMKLYSGLKFQAKEPRF
EAMKARETTPVETARGLRAVRWNTTKRNQN

>MdACO3 (GenBank: BAC53656.1)
MENFPVINLESLNGEGRKATMEKIKDACENWGFFELVSHGIPTEFLDTVERLTKEHYKQC
LEQRFKELVASKGLEGVQTEVKDMDWESTFHLRHLPQSNISEVPDLKDEYRNVMKEFALK
LEKLAEQLLDLLCENLGLEQGYLKKAFYGTKGPTFGTKVSNYPPCPNPDLIKGLRAHTDA
GGLILLFQDDKVSGLQLLKDGEWVDVPPMRHSIVINLGDQLEVITNGKYKSVEHRVIAQT
DGTRMSIASFYNPGSDAVIYPAPTLVEKEAEEKNQVYPKSVFEDYMKLYAGVKFEAKEPR
FEAMKAVEIKASFGLGPVISTA

>MdLDOX_like (GenBank: XP_008356542.1)
MVSSDSVNSRVETLAGSGISTIPKEYIRPKDELVNIGDIFEQEKNNEGPQVPTIDLKEIE
SDNEKVRAKCREKLKKAAVDWGVMHLVNHGISDELMDKVRKAGKAFFDLPIEQKEKYAND
QASGKIQGYGSKLANNASGQLEWEDYFFHCVYPEDKRDLSIWPQTPADYIEATAEYAKQL
RELATKVLKVLSLGLGLDEGRLEKEVGGLEELLLQMKINYYPKCPQPELALGVEAHTDVS
ALTFILHNMVPGLQLFYEGKWVTAKCVPNSIVMHIGDTLEILSNGKYKSILHRGMVNKEK
VRISWAVFCEPPKEKIILKPLPETVSEDEPAMFPPRTFAEHIQHKLFRKSQEALLPK

E.2.4 *Solanum lycopersicum* (Sl) protein sequences

>SlACO1 (GenBank: NP_001234024.2)
MENFPIINLEKLNGDERANTMEMIKDACENWGFFELVNHGIPHEVMDTVEKMTKGHYKKC
MEQRFKELVASKGLEAVQAEVTDLDWESTFFLRHLPTSNISQVPDLDEEYREVMRDFAKR
LEKLAEELLDLLCENLGLEKGYLKNAFYGSKGPNFGTKVSNYPPCPKPDLIKGLRAHTDA
GGIILLFQDDKVSGLQLLKDEQWIDVPPMRHSIVVNLGDQLEVITNGKYKSVLHRVIAQT
DGTRMSLASFYNPGSDAVIYPAKTLVEKEAEESTQVYPKFVFDDYMKLYAGLKFQAKEPR
FEAMKAMESDPIASA

>SlACO2 (GenBank: P07920.1)
MENFPIINLEKLNGAERVATMEKINDACENWGFFELVNHGIPHEVMDTVEKLTKGHYKKC
MEQRFKELVAKKGLEGVEVEVTDMDWESTFFLRHLPSSNISQLPDLDDVYREVMRDFRKR
LEKLAEELLDLLCENLGLEKSYLKNTFYGSKGPNFGTKVSNYPPCPKPDLIKGLRAHTDA
GGIILLFQDDKVSGLQLLKDGRWIDVPPMRHSIVVNLGDQLEVITNGKYKSVMHRVIAQK
DGTRMSLASFYNPGNDALIYPAPALVDKEAEEHNKQVYPKFMFDDYMKLYANLKFQAKEP
RFEAMKAMESDPIAIA

>SlACO3 (GenBank: NP_001233928.1)
MENFPIINLENLNGDERAKTMEMIKDACENWGFFELVNHGIPHEVMDTVEKLTKGHYKKC
MEQRFKELVASKGLEAVQAEVTDLDWESTFFLRHLPTSNISQVPDLDEEYREVMRDFAKR
LEKLAEELLDLLCENLGLEKGYLKNAFYGSKGPNFGTKVSNYPPCPKPDLIKGLRAHTDA

GGIILLFQDDKVSGLQLLKDEQWIDVPPMRHSIVVNLGDQLEVITNGKYKSVMHRVIAQT
DGTRMSLASFYNPGNDAVIYPAPSLIEESKQVYPKFVFDDYMKLYAGLKFQPKEPRFEAM
KAMEANVELVDQIASA

>SlACO4 (GenBank: BAA34924.1)
MESNFPVVDMGLLQTEKRPEAMDKIKDACENWGFFELVNHGISHELLDAVENLTKGHYKK
CMEQRFKEMVASKGLEAVQTEIDDLDWESTFFLKHLPVSNVYEVPDLDDEYRKVMKDFAL
KLEKLAENLLDLLCENLGLEKGYLKKAFYGSKGPTFGTKVSNYPPCPKPDLIKGLRAHTD
AGGIILLFQDDKVSGLQLLKDGNWIDVPPMKHSIVINLGDQLEVITNGRYKSIEHRVIAQ
QDGTRMSIASFYNPGSDAVIFPAPELIEKTEEDIKLKYPKFVFEDYMKLYAGLKFQAKEP
RFEAMKAVETTVNLGPIETV

>SlACO5 (GenBank: NP_001234037.1)
MEMPVIDFSKLEGEERCATMSLLHQACEKWGFFMIENHGIDSYLMDNVKQFVNQHYEANM
KKRFYESELPMSLEKNGNISNTDWESTFFVWHRPASNIYEIQGLSKELCKAVDDYIDQLI
KLAENLSELMCENLGLAKSYIKEAFSGSKGPSVGTKVAIYPQCPRPDLVRGLREHTDAGG
IILLLQDEQVPGLEFFKDGHWVNIPPSKNNRLFVNIGDQIEILTNGMYKSIRHRVMAEKD
GNRLSIATFYNPAGEAIISPASKLLYPCHLRFQDYLNLYSKTKFAEKGPRFESAKRLANG
H

E.2.5 *Petunia x hybrida* (Ph) protein sequences

>PhACO1 (GenBank: Q08506.1)
MENFPIISLDKVNGVERAATMEMIKDACENWGFFELVNHGIPREVMDTVEKMTKGHYKKC
MEQRFKELVASKALEGVQAEVTDMDWESTFFLKHLPISNISEVPDLDEEYREVMRDFAKR
LEKLAEELLDLLCENLGLEKGYLKNAFYGSKGPNFGTKVSNYPPCPKPDLIKGLRAHTDA
GGIILLFQDDKVSGLQLLKDGQWIDVPPMRHSIVVNLGDQLEVITNGKYKSVMHRVIAQK
DGARMSLASFYNPGSDAVIYPAPALVEKEAEENKQVYPKFVFDDYMKLYAGLKFQAKEPR
FEAMKAMETDVKMDPIATV

>PhACO3 (GenBank: Q08507.1)
MENFPIINLEKLNGSERDATMEMIKDACENWGFFELVNHGIPHEVMDTVEKLTKGHYKKC
MEQRFKELVASKGLEAVQAEVTDLDWESTFFLRHLPVSNISEVPDLDDEYREVMRDFAKR
LEKLAEELLDLLCENLGLEKGYLKKAFYGSKGPNFGTKVSNYPPCPKPDLIKGLRAHTDA
GGIILLFQDDKVSGLQLLKDGQWIDVPPMRHSIVVNLGDQLEVITNGKYKSVLHRVIAQT
DGTRMSLASFYNPGSDAVIYPAPTLVEKEADQECKQVYPKFVFDDYMKLYAGLKFQAKEP
RFEAMKAREADVKSDPIATA

>PhACO4 (GenBank: Q08508.1)
MENFPIINLENLCGAERDATMEMIKDACENWGFFELVNHGIPHEVMDTVEKFTKGHYKKC
MEQRFKELVASKGLEAVQAEVTDLDWESTFFLRHLPVSNISEVPDLDDEYREVMRDFAKR
LEKLAEELLDLLCENLGLEKGYLKKAFYGSKGPNFGTKVSNYPPCPKPDLIKGLRAHTDA
GGIILLFQDDKVSGLQLLKDDQWIDVPPMRHSIVINLGDQLEVITNGKYKSVPHRVIAQT
DGTRMSLASFYNPASDAVIYPAPALVERDAEENKQIYPKFVFDDYMKLYARLKFQAKEPR
FEAMKAMEADVKIDPVATV

>PhLDOX (GenBank: P51092.1)
MVNAVVTTPSRVESLAKSGIQAIPKEYVRPQEELNGIGNIFEEEKKDEGPQVPTIDLKEI
DSEDKEIREKCHQLKKAAMEWGVMHLVNHGISDELINRVKVAGETFFDQPVEEKEKYAND
QANGNVQGYGSKLANSACGQLEWEDYFFHCAFPEDKRDLSIWPKNPTDYTPATSEYAKQI
RALATKILTVLSIGLGLEEGRLEKEVGGMEDLLLQMKINYYPKCPQPELALGVEAHTDVS
ALTFILHNMVPGLQLFYEGQWVTAKCVPNSIIMHIGDTIEILSNGKYKSILHRGVVNKEK
VRFSWAIFCEPPKEKIILKPLPETVTEAEPPRFPPRTFAQHMAHKLFRKDDKDAAVEHKV
FNEDELDTAAEHKVLKKDNQDAVAENKDIKEDEQCGPAEHKDIKEDGQGAAAENKVFKEN
NQDVAAEESK

E.2.6 *Medicago truncatula* (Mt) protein sequences

>MtACO_G7KH99(GenBank: XP_003616884.1)
MMMNFPIISLEKLNGVERKDTMEKIKDACENWGFFELVNHGIPHDLMDTLERLTKEHYRK
CMEHRFKEVISSKGLDVVQTEVKDMDWESTFHVRHLPESNISEIPDLSDEYRKVMKEFSL
RLEKLAEELLDLLCENLGLEKGYLKKAFYGSRGPTFGTKVANYPQCPNPELVKGLRAHTD
AGGIILLFQDDKVSGLQLLKDGQWVDVPPMRHSIVVNLGDQLEVITNGKYKSVEHRVIAQ
TNGTRMSIASFYNPGSDAVIYPAPELLEKQTEEKHNVYPKFVFEEYMKIYAALKFHAKEP
RFEALKESNVNLGPIAIV

>MtACO_G7J9K5(GenBank: XP_003601593.1)
MDNFPIINLENLNGDERKATMEKIKDACENWGFFELVNHGIPHDLMDTVERLTKEHYRKC
MEQRFKELVSSKGLEAVQTEVKDMDWESTFHLRHLPESNISEIPDLSDEYRKSMKEFALK
LETLAEELLDLLCENLGLEKGYLKKALYGSKGPTFGTKVANYPPCPKPDLVKGLRAHTDA
GGIILLFQDDKVSGLQLLKDGNWVDVPPMHHSIVINLGDQLEVITNGKYKSVEHRVVAQT
DGTRMSIASFYNPGSDAVIYPAPTLIEENNEIYPKFVFEDYMNLYARLKFQAKEPRFEAF
KESNVNLGPIATV

>MtACO_G7ILQ8 (GenBank: XP_003594161.1)
MENFPVVDMGKLNTEERKATMEMIKDACENWGFFECVNHSISIELMDKVEKLTKEHYKKC
MEQRFKEMVASKGLECVQSEINDLDWESTFFLRHLPSSNISEIPDLDEDYRKTMKEFAEK
LEKLAEELLDLLCENLGLEKGYLKKVFYGSKGPNFGTKVSNYPPCPKPDLIKGLRAHTDA
GGIILLFQDDKVSGLQLLKDDQWIDVPPMPHSIVINLGDQLEVITNGKYKSVMHRVIAQT
DGARMSIASFYNPGNDAVISPASTLLKEDETSEIYPKFIFDDYMKLYMGLKFQAKEPRFE
AMMKAMSSVEVGPVVTI

>MtACO_A0A072V1X2 (GenBank: XP_013461308.1)
MAVPVIDFSKLNGEERAKTLAQIANGCEEWGFFQLINHGISEELLERVKKVSSEFYKLER
EENFKNSKTVKLLNDIAEKKSSEKLENVDWEDVITLLDDNEWPENTPSFRETMSEYRSEL
KKLAVSLTEVMDENLGLPKGYIKKALNDGEGDNAFFGTKVSHYPPCPHPELVNGLRAHTD
AGGVILLFQDDKVGGLQMLKDGEWLDVQPLPNAIVINTGDQIEVLSNGRYKSCWHRVLTF
TEGTRRSIASFYNPPLKATISPAPQLAEKDNQQVDDTYPKFVFGDYMSVYAEQKFLPKEP
RFRAVKAI

>MtACO_G7KJU7 (GenBank: XP_003620934.1)
MAIPVIDFSTLNGDKRGETMALLHEACQKWGCFLIENHDIEGKLMEKVKKVINSYYEENL

KESFYQSEIAKRLEKKENTCDVDWESSFFIWHRPTSNIRKIPNLSEDLCQTMDEYIDKLV
QVAETLSQMMSENLGLEKDYIKKAFSGNNNNGPAMGTKVAKYPECPYPELVRGLREHTDA
GGIILLLQDDKVPGLEFFKDGKWIEIPPSKNNAIFVNTGDQIEVLSNGLYKSVVHRVMPD
KNGSRLSIASFYNPVGEAIISPAPKLLYPSNYCYGDYLELYGKTKFGDKGPRFESIKNKA
NGN

>MtLDOX (GenBank: XP_003611189.1)
MGTVAQRVESLALSGISSIPKEYVRPKEELANIGNIFDEEKKEGPQVPTIDLKEINSSDE
IVRGKCREKLKKAAEEWGVMHLVNHGISDDLINRLKKAGETFFELPVEEKEKYANDQSSG
KIQGYGSKLANNASGQLEWEDYFFHCIFPEDKRDLSIWPKTPADYTKVTSEYAKELRVLA
SKIMEVLSLELGLEGGRLEKEAGGMEELLLQMKINYYPICPQPELALGVEAHTDVSSLTF
LLHNMVPGLQLFYEGKWVTAKCVPDSILMHIGDTIEILSNGKYKSILHRGLVNKEKVRIS
WAVFCEPPKEKIILKPLPELVTEKEPARFPPRTFAQHIHHKLFRKDEEEKKDDPKK

E.3 AMINO ACID SEQUENCES USED FOR THE TRANSPORT INHIBITOR RESPONSE 1/ AUXIN SIGNALING F-BOX (TIR1/AFB) PHYLOGENETIC TREE (Fig. 3.1)

E.3.1 *Arabidopsis thaliana* (At) protein sequences

>AtTIR1 (GenBank: NP_567135.1)
MQKRIALSFPEEVLEHVFSFIQLDKDRNSVSLVCKSWYEIERWCRRKVFIGNCYAVSPAT
VIRRFPKVRSVELKGKPHFADFNLVPDGWGGYVYPWIEAMSSSYTWLEEIRLKRMVVTDD
CLELIAKSFKNFKVLVLSSCEGFSTDGLAAIAATCRNLKELDLRESDVDDVSGHWLSHFP
DTYTSLVSLNISCLASEVSFSALERLVTRCPNLKSLKLNRAVPLEKLATLLQRAPQLEEL
GTGGYTAEVRPDVYSGLSVALSGCKELRCLSGFWDAVPAYLPAVYSVCSRLTTLNLSYAT
VQSYDLVKLLCQCPKLQRLWVLDYIEDAGLEVLASTCKDLRELRVFPSEPFVMEPNVALT
EQGLVSVSMGCPKLESVLYFCRQMTNAALITIARNRPNMTRFRLCIIEPKAPDYLTLEPL
DIGFGAIVEHCKDLRRLSLSGLLTDKVFEYIGTYAKKMEMLSVAFAGDSDLGMHHVLSGC
DSLRKLEIRDCPFGDKALLANASKLETMRSLWMSSCSVSFGACKLLGQKMPKLNVEVIDE
RGAPDSRPESCPVERVFIYRTVAGPRFDMPGFVWNMDQDSTMRFSRQIITTNGL

>AtAFB1 (GenBank: NP_567255.1)
MGLRFPPKVLEHILSFIDSNEDRNSVSLVCKSWFETERKTRKRVFVGNCYAVSPAAVTRR
FPEMRSLTLKGKPHFADYNLVPDGWGGYAWPWIEAMAAKSSSLEEIRMKRMVVTDECLEK
IAASFKDFKVLVLTSCEGFSTDGIAAIAATCRNLRVLELRECIVEDLGGDWLSYFPESST
SLVSLDFSCLDSEVKISDLERLVSRSPNLKSLKLNPAVTLDGLVSLLRCAPQLTELGTGS
FAAQLKPEAFSKLSEAFSNCKQLQSLSGLWDVLPEYLPALYSVCPGLTSLNLSYATVRMP
DLVELLRRCSKLQKLWVMDLIEDKGLEAVASYCKELRELRVFPSEPDLDATNIPLTEQGL
VFVSKGCRKLESVLYFCVQFTNAALFTIARKRPNLKCFRLCVIEPFAPDYKTNEPLDKGF
KAIAEGCRDLRRLSVSGLLSDKAFKYIGKHAKKVRMLSIAFAGDSDLMLHHLLSGCESLK
KLEIRDCPFGDTALLEHAAKLETMRSLWMSSCFVSFGACKLLSQKMPRLNVEVIDEHPPE
SRPESSPVERIYIYRTVAGPRMDTPEFVWTIHKNPENGVSHLAIK

>AtAFB2 (GenBank: NP_566800.1)
MNYFPDEVIEHVFDFVTSHKDRNAISLVCKSWYKIERYSRQKVFIGNCYAINPERLLRRF
PCLKSLTLKGKPHFADFNLVPHEWGGFVLPWIEALARSRVGLEELRLKRMVVTDESLELL
SRSFVNFKSLVLVSCEGFTTDGLASIAANCRHLRDLDLQENEIDDHRGQWLSCFPDTCTT
LVTLNFACLEGETNLVALERLVARSPNLKSLKLNRAVPLDALARLMACAPQIVDLGVGSY
ENDPDSESYLKLMAVIKKCTSLRSLSGFLEAAPHCLSAFHPICHNLTSLNLSYAAEIHGS
HLIKLIQHCKKLQRLWILDSIGDKGLEVVASTCKELQELRVFPSDLLGGGNTAVTEEGLV
AISAGCPKLHSILYFCQQMTNAALVTVAKNCPNFIRFRLCILEPNKPDHVTSQPLDEGFG
AIVKACKSLRRLSLSGLLTDQVFLYIGMYANQLEMLSIAFAGDTDKGMLYVLNGCKKMKK
LEIRDSPFGDTALLADVSKYETMRSLWMSSCEVTLSGCKRLAEKAPWLNVEIINENDNNR
MEENGHEGRQKVDKLYLYRTVVGTRMDAPPFVWIL

>AtAFB3 (GenBank: NP_563915.1)
MNYFPDEVIEHVFDFVASHKDRNSISLVCKSWHKIERFSRKEVFIGNCYAINPERLIRRF
PCLKSLTLKGKPHFADFNLVPHEWGGFVHPWIEALARSRVGLEELRLKRMVVTDESLDLL
SRSFANFKSLVLVSCEGFTTDGLASIAANCRHLRELDLQENEIDDHRGQWLNCFPDSCTT
LMSLNFACLKGETNVAALERLVARSPNLKSLKLNRAVPLDALARLMSCAPQLVDLGVGSY
ENEPDPESFAKLMTAIKKYTSLRSLSGFLEVAPLCLPAFYPICQNLISLNLSYAAEIQGN
HLIKLIQLCKRLQRLWILDSIGDKGLAVVAATCKELQELRVFPSDVHGEEDNNASVTEVG
LVAISAGCPKLHSILYFCKQMTNAALIAVAKNCPNFIRFRLCILEPHKPDHITFQSLDEG
FGAIVQACKGLRRLSVSGLLTDQVFLYIGMYAEQLEMLSIAFAGDTDKGMLYVLNGCKKM
RKLEIRDSPFGNAALLADVGRYETMRSLWMSSCEVTLGGCKRLAQNSPRLNVEIINENEN
NGMEQNEEDEREKVDKLYLYRTVVGTRKDAPPYVRIL

>AtAFB4 (GenBank: NP_567702.2)
MTEEDSSAKMSEDVEKYLNLNPPCSSSSSSSSAATFTNKSRNFKSSPPPCPDHVLENVLE
NVLQFLTSRCDRNAVSLVCRSWYRVEAQTRLEVFIGNCYSLSPARLIHRFKRVRSLVLKG
KPRFADFNLMPPNWGAQFSPWVAATAKAYPWLEKVHLKRMFVTDDDLALLAESFPGFKEL
TLVCCEGFGTSGIAIVANKCRQLKVLDLMESEVTDDELDWISCFPEGETHLESLSFDCVE
SPINFKALEELVVRSPFLKKLRTNRFVSLEELHRLMVRAPQLTSLGTGSFSPDNVPQGEQ
QPDYAAAFRACKSIVCLSGFREFRPEYLLAISSVCANLTSLNFSYANISPHMLKPIISNC
HNIRVFWALDSIRDEGLQAVAATCKELRELRIFPFDPREDSEGPVSGVGLQAISEGCRKL
ESILYFCQNMTNGAVTAMSENCPQLTVFRLCIMGRHRPDHVTGKPMDDGFGAIVKNCKKL
TRLAVSGLLTDEAFSYIGEYGKLIRTLSVAFAGNSDKALRYVLEGCPKLQKLEIRDSPFG
DVGLRSGMHRYSNMRFVWLSSCLISRGGCRGVSHALPNVVVEVFGADGDDDEDTVTGDYV
ETLYLYRSLDGPRKDAPKFVTIL

>AtAFB5 (GenBank: NP_568718.1)
MTQDRSEMSEDDDDQQSPPLDLPSTAIADPCSSSSSPNKSRNCISNSQTFPDHVLENVLE
NVLQFLDSRCDRNAASLVCKSWWRVEALTRSEVFIGNCYALSPARLTQRFKRVRSLVLKG
KPRFADFNLMPPDWGANFAPWVSTMAQAYPCLEKVDLKRMFVTDDDLALLADSFPGFKEL
ILVCCEGFGTSGISIVANKCRKLKVLDLIESEVTDDEVDWISCFPEDVTCLESLAFDCVE
APINFKALEGLVARSPFLKKLRLNRFVSLVELHRLLLGAPQLTSLGTGSFSHDEEPQSEQ
EPDYAAAFRACKSVVCLSGFRELMPEYLPAIFPVCANLTSLNFSYANISPDMFKPIILNC
HKLQVFWALDSICDEGLQAVAATCKELRELRIFPFDPREDSEGPVSELGLQAISEGCRKL
ESILYFCQRMTNAAVIAMSENCPELTVFRLCIMGRHRPDHVTGKPMDEGFGAIVKNCKKL
TRLAVSGLLTDQAFRYMGEYGKLVRTLSVAFAGDSDMALRHVLEGCPRLQKLEIRDSPFG

DVALRSGMHRYYNMRFVWMSACSLSKGCCKDIARAMPNLVVEVIGSDDDDDNRDYVETLY
MYRSLDGPRNDAPKFVTIL

>AtEBF1 (GenBank: NP_565597.1)
MSQIFSFAGENDFYRRGAIYPNPKDASLLLSLGSFADVYFPPSKRSRVVAPTIFSAFEKK
PVSIDVLPDECLFEIFRRLSGPQERSACAFVSKQWLTLVSSIRQKEIDVPSKITEDGDDC
EGCLSRSLDGKKATDVRLAAIAVGTAGRGGLGKLSIRGSNSAKVSDLGLRSIGRSCPSLG
SLSLWNVSTITDNGLLEIAEGCAQLEKLELNRCSTITDKGLVAIAKSCPNLTELTLEACS
RIGDEGLLAIARSCSKLKSVSIKNCPLVRDQGIASLLSNTTCSLAKLKLQMLNVTDVSLA
VVGHYGLSITDLVLAGLSHVSEKGFWVMGNGVGLQKLNSLTITACQGVTDMGLESVGKGC
PNMKKAIISKSPLLSDNGLVSFAKASLSLESLQLEECHRVTQFGFFGSLLNCGEKLKAFS
LVNCLSIRDLTTGLPASSHCSALRSLSIRNCPGFGDANLAAIGKLCPQLEDIDLCGLKGI
TESGFLHLIQSSLVKINFSGCSNLTDRVISAITARNGWTLEVLNIDGCSNITDASLVSIA
ANCQILSDLDISKCAISDSGIQALASSDKLKLQILSVAGCSMVTDKSLPAIVGLGSTLLG
LNLQQCRSISNSTVDFLVERLYKCDILS

>AtEBF2 (GenBank: NP_197917.1)
MSGIFRFSGDEDCLLGGSMYLSPGSCPGVYYPARKRLRVAATSFYSGFEEKQTSIDVLPE
ECLFEILRRLPSGQERSACACVSKHWLNLLSSISRSEVNESSVQDVEEGEGFLSRSLEGK
KATDLRLAAIAVGTSSRGGLGKLQIRGSGFESKVTDVGLGAVAHGCPSLRIVSLWNLPAV
SDLGLSEIARSCPMIEKLDLSRCPGITDSGLVAIAENCVNLSDLTIDSCSGVGNEGLRAI
ARRCVNLRSISIRSCPRIGDQGVAFLLAQAGSYLTKVKLQMLNVSGLSLAVIGHYGAAVT
DLVLHGLQGVNEKGFWVMGNAKGLKKLKSLSVMSCRGMTDVGLEAVGNGCPDLKHVSLNK
CLLVSGKGLVALAKSALSLESLKLEECHRINQFGLMGFLMNCGSKLKAFSLANCLGISDF
NSESSLPSPSCSSLRSLSIRCCPGFGDASLAFLGKFCHQLQDVELCGLNGVTDAGVRELL
QSNNVGLVKVNLSECINVSDNTVSAISVCHGRTLESLNLDGCKNITNASLVAVAKNCYSV
NDLDISNTLVSDHGIKALASSPNHLNLQVLSIGGCSSITDKSKACIQKLGRTLLGLNIQR
CGRISSSTVDTLLENLWRCDILY

E.3.2 *Malus domestica* (Md) protein sequences

>MdTIR1 (GenBank: XP_008386617.1)
MPKMANSFPEEVLEHVFSFIQTDQDRNSISTVCKSWYEIERWCRRRIFIGNCYAVSPRMV
IRRFPDIRSIELKGKPHFADFNLVPEGWGGYVYPWIAAMASAYPWLEEIKLKRMVVTDET
LELIAKSFKNFKLLVLSSCEGFSTDGLAAIAANCRNLRELDLHESDVEDLSGHWLSHFPD
TYTSLVSLNIACLGSELSFSALERLVGRCPNLRSLRLNRSVPLDRLANLLRQAPRLSELG
TGAYSAELRPDIFTILSGAFSRCKELKSLSGFWDVVPPYLTAIYSICPGLTSLNLSYSNI
QSPDLVKLVSECPNLQRLWVLDYIEDVGLNALAESCKDLRELRVFPSDPFVVEPNVSLTE
QGLISVSEGCPKLHSVLYFCRQMSNDALITIARNQPNFTCFRLCIIEPKIPDYLTLQPLD
VGFGAIVENCKNLRRLSISGLLTDRVFDYIGTYGKNLEMLSVAFAGDSDLGLHHILSGCD
NLRKLEIRDCPFGDKALLANAAKLETMRSLWMSSCSVSYGACKLLGQKLPRLNVEVIDER
GHPESRPENSPVEKLYIYRSVAGPRFDMPGFVWTMGKDSTLRIT

>MdTIR101 (GenBank: XP_008368919.1)
MREMQRMVNSFPEEVLEYVFSFIQTDQDRNSISTVCKSWYEIERWCRRRIFIGNCYAVSP
RMLIRRFPDVRSIELKGKPHFADFNLVPEGWGGYVYPWIAAMASAYPWLEEIKLKRMVVT
DETLELIAKSFKNMKLLVLSSCEGFSTDGLASIAANCRNLRELDLHESDVEDLSGHWLSH

FPDTYTSLVSLNIACLGSELSFSALERLVARCPNLRSLRLNRSVPLDRLANLLRLAPRLT
ELGTGAYSAELRPDVFAILSRAISGCKELKSLSGFWDVVPTYLPAIYSICPGLTSLNLSY
STIQSPDLVKLVSQCPNLQRLWVLDYIEDVGLDALAESCKDLRELRVFPSDPFVVEPNVS
LTEQGLISVSEGCPKLHSVLYFCRQMSNDALITIARNQPNFTCFRLCIIEPKIPDYLTLQ
PLDVGFGAIVENCKNLRRLSVSGLLTDQAFDYIGTYGKKLEMLSLAFAGDSDLGLHHILS
GCDNLRKLEIRDCPFGDKALLANAAKLETMRSLWMSSCSVSYGACKLLGQRLPRLNVEVI
DERGHPESRPESSPVEKLYIYRSVAGPRFDMPGFVWTMDEDSALRVT

>MdAFB2 (GenBank: XP_008379063.1)
MNYFPDEVVEHIFESVTSQKDRNAVSLVCKSWFRIERFSRERVFIGNCYAISPERVIERF
PGLKSLTLKGKPHFADFNLVPHEWGGFLQPWIEALADGRVGLEELRLKRMVVSDESLELL
SRLFPNFKSLVLVSCEGFTTEGLAAIAANCRFLKELDLQENDIDDHRGHWLSCFPESSTS
LVSLNFACLKGEINLAALERLVARSPDLKVLRLNRAVPPDTLQKVLTQAPQLVDLGTGSY
VLDSDSDTYNKLKATILKCKSIKSLSGFLEVGPRCLPSIYPILENLTSLNLSYASGVHGS
ELIELIRQCVKLQRLWILDCIGDKGLGVVAKSCKELQELRVFPSDPFAAGHASVTEEGLV
AISAGCPKLHSLLYFCQQMTNAALITVAKNCPNFIRFRLCILDPTKPDAVTMQPLDEGFG
AIVQACKKIRRLSLSGLLTDQVFLYIGMYAEQLEMLSIAFAGDSDKGMLYVLNGCKKLRK
LEIRDSPFGNKALLRDVGKYEAMRSLWMSSCEVTLGGCKALAKKMPRLNVEIINENDQMD
LDDEQRVEKMYLYRTLVGKRRDTPEFVWTL

>MdAFB102 (GenBank: XP_008341250.1)
MNYFPDEVIEQIFKAVTSQKDRNAVSLVCKSWYKIERFSRDRVFIGNCYAISPERVIERF
PGLKSLTLKGKPHFADFNLVPHEWGGFLQPWIEALADGRVGLEELRLKRMIVSDESLELL
SRSFPNFKSLVLVSCEGFTTDGLAAIAANCRLLKELDLQENDIDDHRGHWLSCFPESSTS
LVSLNFACLKGEINLPALERLVGRSPNLKVLRLNRAVPPDTLQKVLAQAPQLVDLGTGSY
XLDPDSDTYNXLKATVVKCKSIKSLSGFLEVGPRCLPSIYPILENLTSLNLSYAPGVHGS
ELIELIRQCVKLQRLWILDCIGDKGLGVVASTCKELQELRVFPSDPFAXGHASVTEEGLV
AISXGCPKLHSLLYFCQQMTNAALITVAKNCPNFIRFRLCILDPTKPDAVTMRPLDEGFG
AIVQACKNIRRLSLXGLLTDQVFLYIGMYAEQLEMLSIAFAGDSDNGMLYVLNGCKKLRK
LEIRDSPFGNEALLKDVGKYETMRSLWMSSCEVTLGGCKALAKEMPRLNVEIINESDQME
SGVDDEQRVEKMYLYRTLVGKRRDTPEFVWTL

>MdAFB5 (GenBank: XP_008338910.1)
MSEDPSIPSSSSSQMSEDDDRSPPLDLIDGPIPSSKSRNCSGVSGSGVGTSVEYTVPYPD
QVLENVLENVLCFLTSRHDRSSASLVCKSWYRAEALTRSELFIGNCYAVAPRRATARFTR
VRAVTIKGKPRFADFNLMPANWGAHLAPWVSSMAKAYPWLEKLCLKRMSVTDDDLALLAE
SFAGFKELVLVCCDGFGTSGLAVLASKCRQLRVLDLTETEVMDDDVDWISCFPESQTCLE
SLMFDCVECHVNFEALEKLVARSPSLKKLRLNRYVSIGQLYRLMVRAPQLTHLGTGSFNL
AEGMAQGDQELDQGDQELDYVSAFAACKSLVCLSGFRDILLDYIPVIYPVCSNLTALNFS
YANITAEQLKPVISQCHKLQSFWVLDSICDEGLKAVAATCKELRELRVFPVNAQEDAEGP
VSEVGLEAISEGCRKLRSILYFCQRMTNAAVIAMSKNCSELVVFRLCIMGRRRPDHVTGE
SMDEGFGAIVMNCKKLTRLAVSGLLTDRAFSYFGQYGKLVRTLSVAFAGDSDSGLKYVLE
GCPKLQKLEIRDSPFGDTALRSGLHHYYNMRFLWMSSCSLTRQGCWAIARELPRLVVEVM
KSEEGGEMGDNFDILYMYRSLEGPRDDIPQFVDILRPGI

>MdAFB105 (GenBank: XP_008338910.1)
MSEDPSIPSSSSSQMSEDDDRSPPLDLIDGPIPSSKSRNCSGVSGSGVGTSVEYTVPYPD

QVLENVLENVLCFLTSRHDRSSASLVCKSWYRAEALTRSELFIGNCYAVAPRRATARFTR
VRAVTIKGKPRFADFNLMPANWGAHLAPWVSSMAKAYPWLEKLCLKRMSVTDDDLALLAE
SFAGFKELVLVCCDGFGTSGLAVLASKCRQLRVLDLTETEVMDDDVDWISCFPESQTCLE
SLMFDCVECHVNFEALEKLVARSPSLKKLRLNRYVSIGQLYRLMVRAPQLTHLGTGSFNL
AEGMAQGDQELDQGDQELDYVSAFAACKSLVCLSGFRDILLDYIPVIYPVCSNLTALNFS
YANITAEQLKPVISQCHKLQSFWVLDSICDEGLKAVAATCKELRELRVFPVNAQEDAEGP
VSEVGLEAISEGCRKLRSILYFCQRMTNAAVIAMSKNCSELVVFRLCIMGRRRPDHVTGE
SMDEGFGAIVMNCKKLTRLAVSGLLTDRAFSYFGQYGKLVRTLSVAFAGDSDSGLKYVLE
GCPKLQKLEIRDSPFGDTALRSGLHHYYNMRFLWMSSCSLTRQGCWAIARELPRLVVEVM
KSEEGGEMGDNFDILYMYRSLEGPRDDIPQFVDILRPGI

>MdAFB6 (GenBank: XP_008383826.1)
MDPKRKKSGLHDPEDEYRVRSVFPDEVLERVLGMVESGKDRGSVSLVCKDWYDAERWSRR
HVFIGNCYATSPEIVSRRFRNIRSVTLKGKPRFADFNLVPDHWGSDVRPWVEAFAAEYPL
LEELRLKRMTVTDESLEMLGVCFGGFKALSLVSCDGFSTDGLAAIATHSKNLIELDIQEN
QIDEKSGSWLGCFPETFTSLEILNFASLNSDVDFDALERLVKRCISLKVLKVNKNVTLEQ
LQRLLTNAPQLLELGTGSFAVQEIAACQNSQFLRAFSNCKNLHTLSGLWEATTLYLPVVY
PACTTNLTFLNLSYAAALQSWDLAELVAYCPRLRRLWVLDTVEDQGLKAVGSNCPLLEEL
RVFPSDPDGDGIIRGVTESGFVAVSHGCRKLXYILYFCWQMTNAAVATVAQNCPDITHFR
LCIMIPGQPDYMTNEPMDEAFGAVAKNCTKLQRLSVSGLLTDXAFEYIGKYAKNLEKLSV
AFAGKSDLGMQCVLKGCPKLKKLEIRDCPFGNAAFLSDIEKYESMRSLWMSACNVTINAC
RLLASQMPRLNVEVMKDEGDDDCVADKVYVYRSVAGPRRDAPPFVLTF

>MdAFB106 (GenBank: XP_008364135.1)
MDSQRKKVLEQVLNLVTSPKDRSSVSLVCKDWYDVERHSRRHVFIGNCYSVSPEIVTRRF
PNIRSVTLKGRPRFSDFNLVPPNWGGDVRPWLDVFAXEYPQLEELRLKRMTVSDESLEFL
AASFADFKALSLLSCDGFSTDGLEAIATDCKNLTELDIQENDIDDKSGDWLSCFPENFTS
MEILNFSCLNSDVDFDALESLVSRCKSLKVLKVNKTVTLDQLRSLLNHAPQLLELGTGSF
WQELTPSQHSELERVFSNCKNLHALSGLWQATALYLPVLYPVCTNLTFLNLSYAATLQSW
DLAKLVANCPHLRRLWVLDTVEDKGLEAVGSNCPLLEELRVFPSSPDGDDIVDGVTESGF
VAVSNGCPRLRYVLYFCWQMTNAAVATVAQNCPDITHFRLCIMTPAKPDHLTGEPMDEAF
GAVVKTCXKLERLSVSGLLTDLTFEYIGKYAKNLEYLSVAFAGDSDQGMQYVMNGCPKLK
KLEIRDCPFGXXALLSGLEKYESMRSLWMSACXVTMNACRSLASKVRRLNVEVMKDDENG
DDSQADKVYVYRSIAGPRKDAPAFVLTF

E.3.3 *Solanum lycopersicum (Sl)* protein sequences

>SlTIR1 (GenBank: NP_001234673.1)
MAYSFPEEVLEHVFSFLTTDKDRNAVSVVCKSWYEIERWCRRRIFVGNCYAVSPRIMIRR
FPEVRSVELKGKPHFADFNLVPEGWGAYVYPWILAMSRSYPWLEEIKLKRMVITDESLEL
ISKSFKNFKVLVLSSCDGFTTDGLAAIAANCRNLRKLDLGESEVEDLSGHWLSHFPDNCT
SLVSLNIACLASEVSLLALERLVTRSPNLTTLKINRAVPLERLPNLLRRTSQLVKFGTGV
FSADVRSDFFSNLTEAFSSCKQLKCLSGFWDVVPAYLPALYPVCSRLTSLNLSYATCQNP
DLGKLISQCHNLRRLWVLDYIEDTGLEELAANCKDLQELRVFPSDPFAAEPNTTLTEQGL
VAVSDGCPKLQSVLYFCRQMTNAALVTIARNRPNMIRFRLCIIEPRTPDYLTLGSFDAGF
GAIVENCKELRRLSLSGLLTDRVFEYIGAHAKKLEMLSIAFAGDSDLGLHHVLSGCDSLR

KLEIRDCPFGDKALLANAAKLETMRSLWMSSCSVSFEACKMLAQKMPRLNVEVIDERGPP
DTRPESCPVEKLYIYRTVAGRRFDTPGYVWTMDEDAAVSLT

>SlAFB4 (GenBank: NP_001234722.1)
MSGSDNPSEMSEDEERPCPSDLTGGVTAKARNCCFNAAVTGGGGGIFNFSPHPDQVLENV
LENVLCFLTDRRDRNAASLVSKSWYRAEALTRSEVFIGNCYAVSPTRVTTRFKRVTSVAI
KGKPRFADFSLLPPDWGAHFTPWASVLGDSYRGLEKLYLKRMSISDDDLGLLARCFPNFK
ELVLVCCEGFGTSGLAIVARDCRQIRVLDLIESEVSDDEVDWISYFPXNKTCLESLTFDC
VECPIDFEALEKLVIRSPSLKRLRLNRFVSITQLYRLMIRAPQLTNLGTGSXGASTVTDE
PDPDYASAFAACKSMVCLSGFREIAPEYLPAIYPVCGNLTSLNLSYGANINTEQFKSVIS
RCHKLQVLWVFDSVCDEGLEAVAATCKDLRGIRVFPIEAREDADAPVSEVGLLAISEGCR
KLKSILYFCQKMTNAAVIAMSKNCPDLVVFRLCIMGRHLPDHVTNEPMDEGFGAIVKNCK
KLTRLAVSGLLTDRAFSYIGQYGKLVRTLSVAFAGNSDLALKYVLEGCPKLQKLEIRDCP
FGDLSLRSGLHHYYNMRFLWLSSCRVTLQGCQEIARQLPRLVVEVISGDDEEGSETNEHV
NTLYMYRSLDGPRADVPSFVQIL

>SlAFB6 (GenBank: XP_004232795.1)
MNPSLKKPRESVDLSKMSELSQSAFPDEVLEKVLSLVQSHKDRNSASLVCKDWYNAERWT
RTKLFIGNCYSVTPEIVARRFPKIKSVTLKGKPRFSDFNLVPENWGADIQAWLDVFAKVY
PFLEELRLKRMAVTDESLEFLAKSFLGFKALSLLSCDGFSTDGIGSIAAHCKNLTELDIQ
ENGMDDISGSWLSCFPDDFTSLEVLNFASMNTEISKDALERLVGRCKSLRVLKVNKNVTL
PQLQRLLVRAPQLMELGTGCFLPDQLTSRQYEELESAFSNCKHLHSLSGFWEANRRYLPS
LYAACASLTFLNLSYETIRSGELSKLLLHCPNLRRLWVLDTVNDKGLEAVGTSCPLLEEL
RVFPADPFEEDMDHGVTESGFIAVSAGCPKLQYVLYFCWQMTNAAVATIVHNCPNFTHFR
LCIMSPGQPDYLTNEPMDEAFGAVVKTCKKLQRLSVSGRLTDLTFEYIGKYAKNLETLSV
AFSGGTDWGMQCVLDGCSKLRKLEIRDSPFGNAALLSGMGKYESMRCLWMSACRVTMNGC
RILARERPRLNVEVIKDEHSDDYADKLYVYRSVAGPRRDAPPFVVTL

>SlEBF1 (GenBank: NP_001234858.1)
MPTLVNYSGDDEFYSGGSFCSADLGLMLSLGHADVYCPPRKRARISGPFVVEDRSKDPSL
EVLPDECLFEILRRLPGGRERGAAACVSKRWLTVLSSVKNSEICRSKSYNNLNDAIMISK
DEDLEVECDGYLTRCVEGKKATDIRLAAIAVGTSTRGGLGKLSIRGSNSVRGITNVGLSA
VAHGCPSLRVLSLWNVPSIGDEGLLEVARECHSLEKLDLSHCRSISNKGLVAIAENCPSL
TSLTIESCPNIGNEGLQAVGKYCTKLQSLTIKDCPLVGDQGVASLLSSGASMLTKVKLHG
LNITDFSLAVIGHYGKLITSLNLCSLRNVSQKGFWVMGNAQGLQSLVSLTITLCQGATDV
GLEAVGKGCPNLKYMCIRKCCFVSDGGLVAFAKEAGSLESLILEECNRITQVGILNAVSN
CRKLKSLSLVKCMGIKDLALQTSMLSPCESLRSLSIRSCPGFGSSSLAMVGKLCPKLHQL
DLSGLCGITDAGLLPLLENCEGLVKVNLSDCLNLTDQVVLSLAMRHGETLELLNLDGCRK
VTDASLVAIADYCPLLIDLDVSKSAITDSGVAALSRGVQVNLQVLSLSGCSMVSNKSVLS
LKKLGENLLGLNLQHCSVSCSSVELLVEALWRCDILS

>SlEBF2 (GenBank: NP_001293065.1)
MSKVFNFSGDHGGTVYPSPKESSLFLSLRNHVDVYFPPCKRSRVAVPFVFSEKKHKLSSI
DVLPDECLFEVLRRLSDGKDRSASACVSKRWLMLLSSIRGDETVISNPNPSLETEERSIQ
TALVKSVDCVKKGEVVDSNAAEVAEAESQDIEGEGHLSRCLDGKKATDVRLAAIAVGTPG
HGGLGKLSIRGSNPIRGVTDTGLKVIARGCPSLRALSLWNVSSVSDEGLTEIAQGCHLLE
KLDLCQCPAITDMSLMAIAKNCPNLTSLTIESCSKIGNETLQAVGRFCPKLKFVSLKNCP

LIGDQGIASLFSSAGHVLTKVKLHALNISDIALAVIGHYGIAITDIALIGLQNINERGFW
VMGNGQGLQKLRSLAITACHGVTDLGLEALGKGCPNLKLFCLRKCTILSDNGLVAFAKGS
VALENLQLEECHRITQAGFVGVLLSCGEKLKVLSMVKCFGVKELACRFPSVLPCNSLQSL
SIRNCPGVGNATLAIMGRLCPKLTHLELSGLLQVTDEGLFPLVQSCEAGLVKVNLSGCVN
VTDRSVSFITELHGGSLESLNVDECRYVTDMTLLAISNNCWLLKELDVSKCGITDSGVAS
LASTVRLNLQILSLSGCSMLSDKSVPFLQKLGQTLMGLNIQHCNGVSSSCVDLLLEQLWR
CDILS

E.3.3 *Pisum sativum* (Ps) protein sequences

>PsTIR1a (GenBank: KX954124)
MQRVAYSFPEEVLEHVFSFIESDTDRGSISLVCKSWYEIERWCRRRVFVGNCYAVSPAMV
IKRFPKVRSITLKGKPHFADFNLVPEGWGGYVCPWIKAMAASYPCLQEIRLKRMVITDDS
LDLIAKSFKNFTVLVLTSCEGFTTEGLAAIAANCRNLRELDLRESEVEDICGHWLSHFPD
SYTSLVSLNISCLANEVNFPALERLVSRCPNLQTLRLNRAAPLDKLASLLRGAPQLAELG
TGAYTSEMRPEVFSNLAAAFSGCMQMKSLSGFWDVLPSYLPAVYPVCSRLTSLNLSYATI
QSPDLIKLVGECESLQRLWVLDYIEDAGLDMLAASCKDLRELRVFPSNPFGLEPNVALTE
QGLVSVSEGCPKLHSVLYFCRQMTNAALTIIARNRPNLTRFRLCIIEPRTPDYLTRQPLD
VGFGAIVEQCKSLQRLSLSGLLTDRVFEYIGTYGKKLEMLSVAFAGESDLGLHHVLSGCD
NLRKLEIRDCPFGDKALLANAAKLETMRSLWMSSCHVSYGACKLLGLKLPRLNVEVIDER
GPPDSRPDNSPVEKLYIYRTISGPRLDMPGYVWTMEDDSAYPE

>PsTIR1b (GenBank: KX954125)
MQKMTNRFPEEVLEYVFSFIQCDKDRNSISLVCKSWYEIERWCRRQIFVGNCYAVSPVTV
TKRFPELRSISLKGKPHFADFNLVPEGWGGFVSPWIAAMACGLPLLEEIRLKRMVITDES
LELIAKSFKNFKVLVLISCEGFTTEGLAAIASNCRNLKELNLQESELEDLSGHWLSQFPD
SYTSLVSLNISCLNNEVSLSALERLLGRCPNLQTLRLNHAAALDKLPNLLSRCPQLAELG
TGIYSAEMRPEVFSNLVTAFTGCKQLKSLSGFWQVLPSYLPALNPVCSRLTSLNLSYAVI
QSSDLIKLVGQCPNLLRFWVLDYIEDAGLDVVAASCKYLQELRVFPSDPFGLEPNIALTE
QGLVSVSKGCPKLQSILYFCRQMSNAALNTIAQNRPNLTRFRLCILEPRTPDYLTLQPLD
SGFGAIVEHCKDLQRLSLSGLLTDRVFEYIGTHAKKLEMLSVAFAGESDLGLHYMLSGCD
NLRKLEIRDCPFGDKALLANAAKLETMRSLWMSSCPVSYGACKLLGQKMPRLNVEVIDER
GPPDSRPDSCPVEKLYIYRSTAGPRLDMPGFVWTMEDDSSLRSV

>PsAFB2
MNYFPDEVIEHVFDYVVSHSDRNSLSLVCKSWYRIEGFTRKRVFIGNCYSISPERLVERF
PDFKSLTLKGKPHFADFSLVPHGWGGFVYPWIEALAKSRVGLEELRLKRMVVSDESLELL
SRSFMNFKSLVLVSCEGFTTDGLAAVAANCRSLRELDLQENEVEDHKGQWLSCFPENCTS
LVALNFACLKGEINVGALERLVARSPNLKTLRLNRSVPADALQRILMRAPQIADLGIGSF
IHDLNSEAYIKLKNTILRCRSITSLSGFLEVAPFSLAAVYPICRNLTSLNLSYAASIQGA
ELIKLIRHCGKLQRLWIMDCIGDKGLVAVATICKELQELRVFPSAPFGNQAAVTEVGLVA
ISKGCPKLHSLLYFCHQMTNAALITVAKNCPNFIRFRLCILDATKPDSDTMQPLDEGFGA
IVQSCKRLRRLSLSGQLTDQVFLYIGMYAEQLEMLSIAFAGESDKGMLYVLNGCKKLRKL
EIRDCPFGDTALLTDVGKYETMRSLWMSSCEVTVGACKTLAKKMPSLNVEIFNESEQADC
YVEDGQRVEKMYLYRSVAGKREDAPEYVWTL

>PsAFB4 (GenBank: KX954126)
MRENHPPTTTPDLLARGEIAESSTSKNRTGSSEPFPGSSLTENPSPFPDQVLENVLENVL
HFLSSRKDRNAASLVCRSWYRAEALTRSDLFIGNCYALSPRRATARFSHIKSVTVKGKPR
FADFDLMPVDWGAHFTPWVTSLAQAYPWLEKLHLKRMSVTDKDLGLIADSFVGFRELLLA
CCEGFGTPGLAVIASKCRLLRVLELVESVIEVEDDEEVDWVSCFPTEGQTHLESLAFDCV
ECPVNFEALEGLVARSPGLKKLRLNRSVSMVQLHRLMLRAPQLTHLGTGSFSANENVDQE
PDYASAFAACRSLVCLSGFREIWPDYLPAIYPVCSNLTSLNFSYADVNAEQLKSVICHCH
KLQILWVLDSIGDEGLQSVAATCNDLRELRVFPVDAREETEGPVSQVGFEAISQGCRKLE
SILFFCQTMTNAAVVAMSKNCPDLVVFRLCIIGVYRPDAVTQEPMDEGFGAIVMNCKKLT
RLAVSGLLTDRAFEYIGRYGKLIRTLSVAFAGDTDTGLRYVLEGCPNLQKLEIRDSPFGD
GALRSGLHHYYNMRFLWMSSCKLTRQACQEVARALPHMVLEVINNGENAVEEIGILYMYR
SLDGPRDDAPEHVTILQ

>PsAFB6
MEPQTMNPSSVFPDEVLERILSMVKSRKDKSSVSLVCKDWFDAERWSRKNVFIGNCYSVT
PEILTQRFPNVRSVTLKGKPRFSDFNLVPANWGADIHPWLVVFAEKYPFLEELRLKRMVV
TDESLEFLAFSFPNFKALSLLSCDGFSTDGLAAVATNCKNLTELDIQENGIEDKSGNWLS
CFPESFTSLEVLNFANLTNEVNIDALEKLVGRCKSLKTLKVNKSVTLEQLKKLLVRAPQL
CELGSGSFSQELTSQQYAELETAFKNCKSLHTLSGLWVASARYLQVLYPACANLTFLNFS
YAPLDSEDLTKILVHCPNLRRLWVVDTVEDKGLEAVGSNCPLLEELRVFPADPFDEEAEG
GVTESGFVAVSEGCRKLHYVLYFCRQMTNAAVATVVQNCPDFTHFRLCIMNPGQQDYLTD
EPMDEAFGEVVKNCTKLQRLAVSGYLTDLTFEYIGKYAKNLETLSVAFAGSSDWGMECVL
VGCPKLRKLEIRDSPFGNAALLAGLEKYESMRSLWMSSCRLTMNGCRFLAGEKPRLNVEV
MQEEGGDDSRAEKLYVYRSVAGPRRDAPPFVLTL

>PsEBF1 (GenBank: KX261620)
MSQVFGFSGDNFCHGGSIYTNPKEASFFLSLGPQVDVYYPPQKRSRVSVPFVFDGEWFEQ
KQKTTIDSLPDECLFEIFRRLPVGEERSACACVSKRWLMLLSSICKSEICSNKNTSDGEN
KMEGDSQEFGGEGYLSRSLEGKKATDVRLAAIAVGTASRGGLGKLSIRGNNSNRGVTTLG
LKAVASGCPSLKVLSLWNVSSVGDEGLIEIANGCQQLEKLDLCRCPSISDKALIAVAKNC
PNLTELSLESCPNIHNEGLQAIGKLCSNLKSISIKDCAGVGDQGIAGLFSSTSLLLTKVK
LQALTVSDLSLAVIGHYGKAVTDLVLNFLPNVSERGFWVMGNANGLHKLKSLTIASCRGV
TDVGLEAVGKGCPNLKSVHLQKCAFLSDNGLISFTKAAISLESLKLEECHRITQFGFFGV
LFNCGAKLKALSLVSCYGIKDLDLELSPSSPCESLRSLTICNCPGFGNATLSVLGKLCPQ
LQQVELTGLKGVTDAGLLPLLESSEAGLVKVNLSGCVNLTDKVVSSLVNLHGWTLEILNL
EGCKNISNASLIAMAEHCQLLCDLDVSMCAISDAGIAALAHAKQINLQILSLSGCTLVTD
RSLPALRKLGRTLLGLNVQHCNSISSSAVEMLVELLWRCDILS

>PsEBF2 (GenBank: KX261621)
MPALVNHGGDDELYPGGLVDLGGRLRTASSNVDVYSSPTKRARISVPFSFGDLEAESEVD
VDYKSSVEILPDECLFEIFRRLSSGKERSSCACVSRKWLMLVSGICKDEIETVVKTISSD
ESHEDVEGDGYLTRRLEGKKATDVRLAAIAVGTSWRGGLGKLSIRGNNSVRGVTNRGLSA
VANGCPSLRSLSLWNVSSVGDKGLCEIGKRCHMLEKLDLCHSRWITNKGLIAIAEGCPNL
TTLNIESCSKIGNEGLQAVAKLCPNLHSISIKDCPLVGDHGVSSLLSSASELSRVKLQIL
NITDFSLAVIGHYGKSITNLVLSGLRNVNERGFWVMGVAQGLQKLVSLTITSCHGVTDAS
IEAIGKGCPNLKQMCLRRCCFVSDSGLVAFAKSAGSLENLHLEECNRFTQSGIIGAISSI

KAKLKSLTLVKCMGIKDIEVEVSMLSPCESLRSVTIKNCHGIGSASLAVIGKLCPQLQYV
DLTGLYGITDAGLLPLLENCEAGLVKVNLTGCWNLTDNIVSAMTRLHGGTLEVLNLDGCW
NITDASLVAIADNCLLLNDLDVSRCAITDAGISVLSDAIQLSLQVLSMSGCSEISNNCMP
FLKKLSQNLLGLNLQNCIGIGSNTIELLVESLWRCDILA