University of Alberta

The Role of Hormones in Early Pea Fruit Growth and Carbohydrate Partitioning

by

Marilyn Johnstone 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**

in

Plant Science

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Spring 2004

Your file Votre référence
ISBN: 0-612-96495-7
Our file Notre référence
ISBN: 0-612-96495-7

Abstract

Plant hormones [gibberellins (GAs) and auxins] are thought to be involved in normal pea (*Pisum sativum*) fruit growth and development. This research focused on understanding the differing roles of two pea auxins, indole-3-acetic acid (IAA) and 4-chloroindole-3-acetic acid (4-Cl-IAA) in pea fruit growth and carbohydrate partitioning. GA_3 or 4-Cl-IAA (50 μM) applied alone to deseeded pericarp (ovary) tissue promoted elongation and applied simultaneously, showed a synergistic growth effect on this tissue. IAA was inhibitory to deseeded pericarp elongation both when applied alone and in conjunction with GA_3. We have demonstrated that 4-Cl-IAA and IAA stimulated similar rates of ethylene evolution and reconfirmed studies that show IAA inhibited pericarp growth through the action of ethylene. However, ethylene did not affect 4-Cl-IAA-treated (50 μM) pericarp growth. GA_3 and 4-Cl-IAA treatment to deseeded pericarps maintained pericarp starch, glucose, and sucrose levels comparible to pericarps with seeds. However, IAA and ethylene stimulated starch degradation and senescence in control and GA_3-treated deseeded pericarps. These data support the hypothesis that the two naturally occurring auxins, 4-Cl-IAA and IAA, play different roles during pea fruit development.

My dear family, thank you for letting me experiment in my life!

For Eric, Jessie, Cecil and Clayton

Acknowledgements

My graduate studies program has trained me be more critical of my work and the work of others. In addition, it has given me the tools and confidence to approach and tackle other opportunities outside of science. For giving me more than I ever knew I needed, I would like to thank my supervisor, Dr. Jocelyn Ozga. She has guided me through uncertainties and shared her experiences in a gentle and generous manner. My committee members, Dr. Dixon, and Dr. Deyholos have offered their professional guidance throughout my program. For technical help, Dr. Dennis Reinecke was available to answer my questions and give his advice when I needed to troubleshoot a problem. Proper maintenance of my experimental plants and growth chambers was so vital to my work. Bruce Alexander and his team were dependable and flexible to my needs, and just as important offered friendship to a fellow plant lover. My colleagues, both graduate students and technicians made me feel like a valuable presence in the department. Special thanks to Bernie Dansereau, Gabe Botar, Jane Pankuch, Abdul Faraj, Jenny Thistleton, Belay Ayele and the members of the Ozga research group. Finally, thank you to the people who have personally supported me with their friendship and love, my friends Carla Starchuk and Liana Tempany, my dear Eric Labonté, and my family.

Table of Contents

Table of Figures

List of Abbreviations

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
ACS	1-aminocyclopropane-1-carboxylate synthase
ACO	1-aminocyclopropane-1-carboxylate oxidase
ADP	adenosine diphosphate
ANOVA	analysis of variance
CEND	carboxyl-terminal end
CTR	constitutive triple response
2,4-D	2,4-dichlorophenoxyacetic acid
DAA	days after anthesis
DMSO	dimethylsulphoxide
dwt	dry weight
eq.	equivalent
EIN2	ethylene insensitive
ERF	ethylene response factor
ERS	ethylene response sensor
ETO	ethylene overproducing mutant
ETR	ethylene triple response
fwt	fresh weight
4-Cl-IAA	4-chloroindole-3-acetic acid
GA	gibberellin
GC	gas chromatography
gin-1	glucose insensitive mutant
GOPOD	glucose oxidase and peroxidase
h	hour
IAA	indole-3-acetic acid
MACC	malonyl 1-aminocyclopropane-1-carboxylate
MAPK	mitogen activated protein kinase
1-MCP	1-methylcyclopropene

List of Abbreviations

NBD	2, 5-norbornadiene
ppm	parts per million
Ps-ACS	*Pisum sativum* 1-aminocyclopropane-1-carboxylate synthase
PsGA20ox1	*Pisum sativum* gibberellin 20-oxidase
PsGA3ox1	*Pisum sativum* gibberellin 3ß-hydroxylase
SAM	S-adenosyl-methionine
SE	standard error
Ser/Thr	serine/threonine
SP	split-pericarp
SPNS	deseeded split pericarp
SS	sucrose synthase
STS	silver thiosulphate
TIBA	2,3,5-triiodobenzoic acid

Chapter 1: Introduction

Economic Importance of Field Pea in Canada

Pulse crops are important to the Canadian economy. According to the Food and Agriculture Organization of the United Nations, Canada produced 14% of the world's total dry peas in 2002 [900,000 tons; Fig. 1; (FAO, 2002)]. Western Canadian prairie farmers use pulses in their crop rotation to help maintain nitrogen in the soil and reduce the risk of pests in their other crops, often wheat and canola. Field pea seed is used extensively as an inexpensive, high quality source of plant protein in feed ingredients for cattle, swine, poultry and fish (Pulse Canada, 2003).

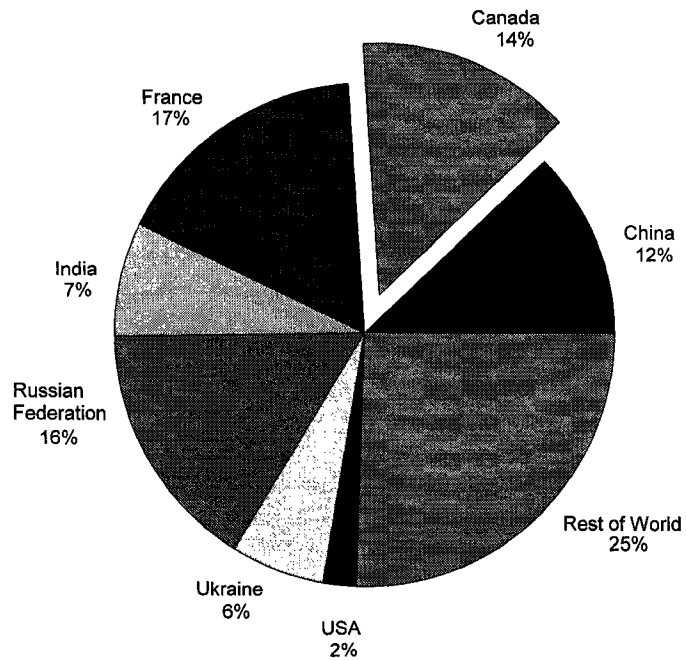


Figure 1.1: World Pea Production [total dry peas: 9 871 849 Mt. (FAO, 2002)]

Pea Fruit Development

Pea plants (*Pisum sativum* L.) are self-pollinators, where pollination and fertilization take place approximately 24-36 h prior to anthesis (Cooper, 1938). Following fertilization of the ovules, ovary (pod; pericarp) growth (length) follows a sigmoidal pattern, where initial growth is slow, followed by a rapid linear growth phase (2 to 5 DAA), then a slower phase where maximum length is reached by 8 days after anthesis [DAA; (Eeuwens and Schwabe, 1975; Ozga et al., 2002)]. In addition, pericarp diameter increases from 6 to 12 DAA to accommodate the developing seed (Ozga et al., 2002). Cell division in the pericarp increases from anthesis (0 DAA) to 2 DAA, followed by cell expansion from 2 to 7 DAA (Vercher and Carbonell, 1991; Ozga et al., 2002).

In developing pea fruit, the pericarp is the primary sink for photoassimilates following pollination and fertilization of the ovules (up to 10 DAA) until seed development advances to a point that the seeds become the major terminal sink (Flinn and Pate, 1970). This is reflected in organ growth, where the rapid rate of pericarp growth precedes that of the seeds. Although the major sink tissue in the early developing pea fruit is the pericarp, the presence of the seeds is required for maintenance of this sink potential (Ozga et al., 1992).

The Role of Gibberellins and Auxins in Pea Fruit Development

Gibberellins (GAs) and auxins (IAA, 4-Cl-IAA) are two classes of plant hormones involved in plant growth and development. A unique auxin, 4-chloro-indole acetic acid (4-Cl-IAA), has been found in nine species of the Fabaceae family including *Pisum sativum.* In *P. sativum,* both 4-Cl-IAA and IAA are present in seeds, pericarps, and roots (Magnus et al., 1997), but only 4-Cl-IAA can stimulate pericarp growth (Reinecke and Ozga, 1995). In pollinated deseeded ovaries, GA_3 and 4-Cl-IAA promoted pericarp elongation similarly (Ozga and Reinecke, 1999). Simultaneous application of GA_3 and 4-Cl-IAA at 9 nmol each to pollinated deseeded ovaries stimulated pericarp elongation significantly more than either hormone applied alone (Ozga and Reinecke, 1999). GA_3 and 4-Cl-IAA also stimulate growth in non-pollinated ovaries, however, in this tissue, GA_3 is more biologically active with respect to growth (Ozga and Reinecke, 1999). The synergistic effect on growth was not observed when GA_3 and 4-Cl-IAA were applied simultaneously to non-pollinated pericarps (Ozga and Reinecke, 1999). These data show that the receptivity of the pericarp tissue to hormones, specifically auxin (4-Cl-IAA) changes during pericarp development from pre-pollination to post-pollination.

The discovery by our research group that both GAs and auxin (4-Cl-IAA) can replace the seed requirement for pericarp growth has led to a more detailed study of the GA biosynthesis pathway in the pea pericarp. Pea

plants metabolize GAs by the early 13-hydroxylation pathway: $GA_{12} \rightarrow GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20} \rightarrow GA_1$ (Sponsel, 1995).

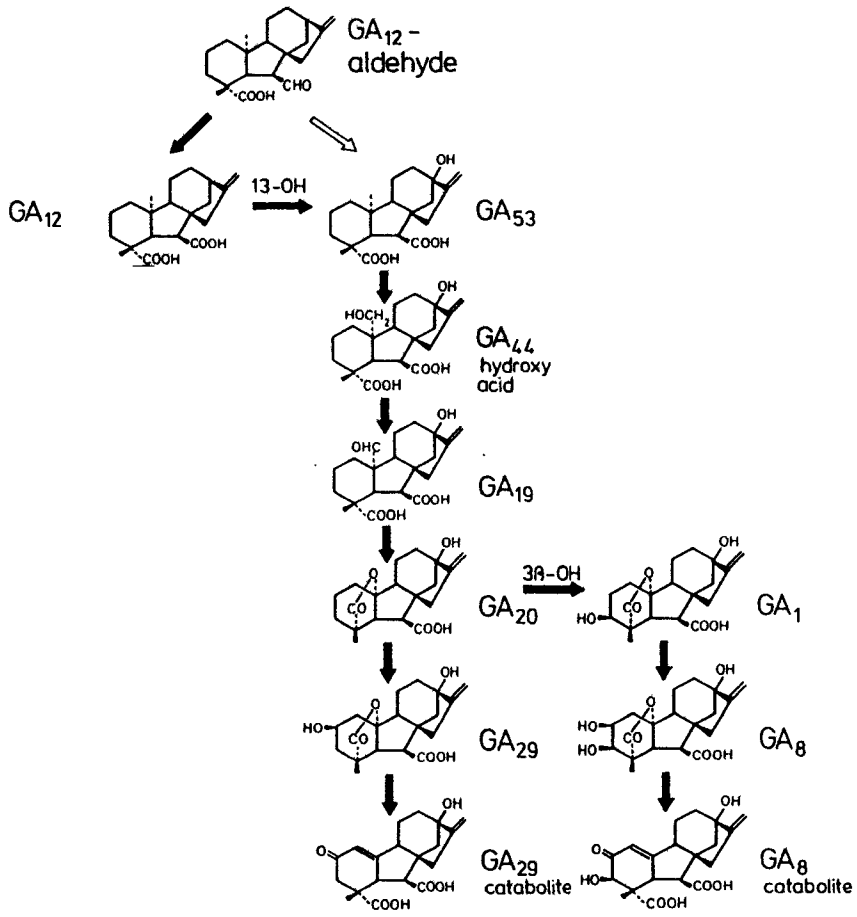


Figure 1.2: GA biosynthesis pathway in *Pisum sativum* (Graebe, 1987).

The enzyme, GA 20-oxidase, is responsible for the oxidation of carbon 20 (C-20 of the *ent*-gibberellane ring) $GA_{53} \rightarrow GA_{44} \rightarrow GA_{19}$, and the loss of C-20,

$GA_{19} \rightarrow GA_{20}$. GA 3-ß hydroxylase is responsible for hydroxylation of GA_{20} at the 3 position, producing GA_1, which is the biologically active GA in pea stem internodes (Phinny, 1984). The regulation of transcript levels of GA 20-oxidase (*GA20ox*; (van Huizen et al., 1997) and GA 3-ß hydroxylase [*GA3ox*; (Ozga et al., 2003)] by seeds and hormones was studied in pea pericarp. In 2 DAA pericarps with seeds, pericarp *GA20ox* and *GA3ox* mRNA levels were maintained over the treatment period (36 and 24 h, respectively). However, when the seeds were removed, pericarp *GA20ox* and *GA3ox* mRNA levels decreased to minimal levels. When the deseeded pericarps were treated with 4-Cl-IAA, *GA20ox* and *GA3ox* transcript levels substantially increased compared to deseeded pericarps. Gibberellin metabolism studies with the split pea pericarp system showed that when seeds were present, GA_{19} was metabolized to GA_{20} (van Huizen et al., 1995). However, when seeds were removed this conversion was inhibited. When 4-Cl-IAA was applied to deseeded pericarps, the conversion of GA_{19} to GA_{20} was significantly higher than in pericarps with seeds up to eight hours after treatment (van Huizen et al., 1995). These results suggest that 4-Cl-IAA may be a seed-derived factor that stimulates GA biosynthesis in the pericarp, leading to increased production of the bioactive gibberellin, GA_1, and growth.

Reinecke et al. (1995) compared the growth promoting properties of 4-, 5-, 6-, and 7- chloro-IAAs and the corresponding fluoro-IAA analogs on

pollinated deseeded pericarp growth. Results showed that the substituent at the 4-position of the indole ring was important for biological activity in this system (Reinecke et al., 1995). Further experiments characterizing the physicochemical properties of 4-Cl-IAA and its 4-substituted analogs showed that the 4-substituent's size and lipophilicity were associated with auxin's growth-promoting activity on pea pericarp (Reinecke et al., 1999). In addition, only the biologically active auxins stimulated *GA20ox* and *GA3ox* mRNA levels in deseeded pericarps (Ngo et al., 2002; Ozga et al., 2003).

The Role of Ethylene in Early Pea Fruit Development

The ethylene biosynthesis pathway in plants is well documented (Figure 1.3). S-adenosyl-methionine (SAM) is formed from methionine by SAM synthetase (Kende, 1993). The major rate limiting step of ethylene biosynthesis is the formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from SAM by ACC synthase (ACS). ACC is oxidized by ACC oxidase (ACO) to form ethylene, CO_2, O_2, and HCN (Kende, 1993). To decrease ethylene production, ACC can be malonylated to form MACC.

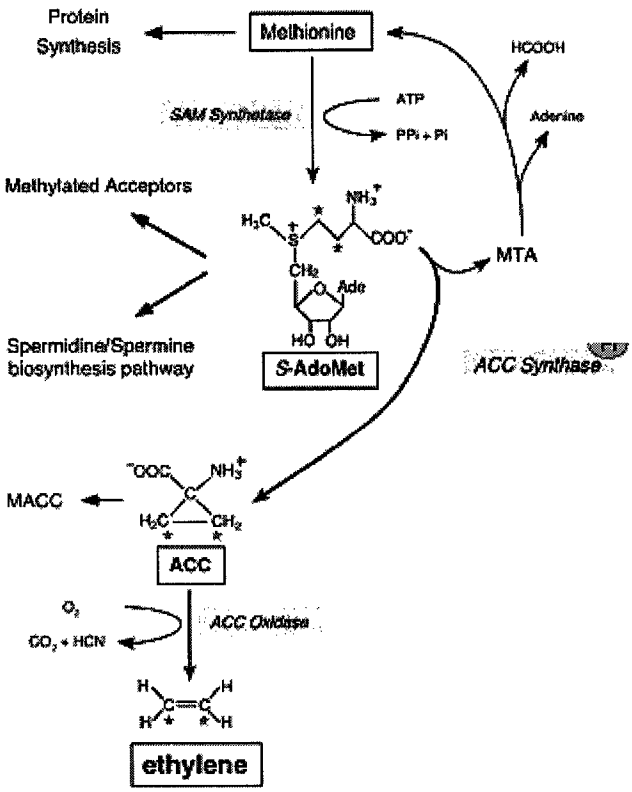


Figure 1.3: Ethylene biosynthesis model (modified from Wang 2002).

Pollinated pea ovaries evolved ethylene at a low and steady rate from 1 to 4 DAA (0.2 to 0.5 nL C_2H_4 g^{-1} h^{-1}) (Orzáeaz et al., 1999). In contrast, the mean rate of ethylene production in non-pollinated ovaries (will senesce after the equivalent to 4 DAA) increased from 0.5 to 1.75 nL C_2H_4 g^{-1} h^{-1} during this period. In general the level of ACO mRNA in the pericarp paralleled the rate of ethylene production. In non-pollinated ovaries, ACO mRNA levels

increased substantially by the equivalent to 4 DAA. Application of GA_3 to non-pollinated ovaries at the equivalent to 0 DAA lead to a progressive decrease in ACO mRNA levels from the equivalent of 1 to 4 DAA.

Orzáeaz and Granell (Orzáeaz and Granell, 1997) tested non-pollinated pea ovary sensitivity to GA_3 and ethylene. Non-pollinated pea flowers were emasculated at –2 DAA, treated once with a hormone solution (300 μM GA_3), and then left to grow for five days. After this period, ovaries that had grown over 50% more than their original length were scored. All of the non-pollinated ovaries from the equivalent of –1 to 2 DAA were able to respond to GA_3. When the tissue was treated with GA_3 at the equivalent to 3 DAA, less than half the non-pollinated ovaries grew more than 50% of their original length. By the equivalent to 4 DAA, the non-pollinated ovaries were totally unresponsive to GA_3 treatment. However, when ovaries were treated with a combination of STS and GA, the ethylene biosynthesis inhibitor expanded their window of responsiveness to GA_3 up to the equivalent of 4 DAA. In addition, exogenous ethylene at 10 ppm totally inhibited GA_3-induced growth in the equivalent to 0 and 1 DAA non-pollinated ovaries. This data shows that exogenously applied ethylene mediates the tissue's responsiveness to GA_3 in emasculated ovaries.

Auxins are also known to stimulate ethylene biosynthesis (Kende, 1993). The levels of ACS transcript (*Ps-ACS2*) and enzyme activity

increased in less than one hour after treatment with IAA in etiolated stem internodes of 5 to 6 day-old pea seedlings (Peck and Kende, 1995). Two hours after IAA treatment, ACO activity and transcript levels began to increase. The IAA-induced increase in ACO activity and transcript levels was blocked by treatment with 2,5-norbornadiene (NBD, an ethylene action inhibitor), which indicates that ACO was stimulated by auxin-induced ethylene.

The different isoforms of ACS (Ps-ACS1 and Ps-ACS2 share only 49% amino acid identity) were differentially regulated by auxin in pea (Peck and Kende, 1998). Using etiolated pea stems, Peck and Kende (1998) showed indole-3-acetic acid (100 mM IAA) induction of Ps-ACS1 and Ps-ACS2 transcript levels. Ps-ACS1 increased from nearly undetectable levels within 15 minutes of auxin application and continued to increase over the next 4 hours. Ps-ACS2 transcript was biphasic where the first maximum was at 30 minutes after auxin application, followed by a decrease then a second higher peak reached at 4 hours. Wounding of the stem only induced Ps-ACS2 transcript levels, with mRNA levels increasing 10 minutes after wounding followed by an increase in ethylene levels at 26 minutes. Ethylene repressed auxin-induced mRNA levels of Ps-ACS1 and Ps-ACS2 by 4 hours, which was consistent with reduced ACS activities measured within the same experiment (Peck and Kende, 1998).

As stated previously, in pollinated deseeded pericarps, 4-Cl-IAA stimulates growth and IAA does not. Indeed, higher concentrations of exogenous IAA promote pericarp senescence compared to a non-hormone control (SPNS; Reinecke et al., 1995). This promotion of senescence can be reversed by treatment with STS (silver thiosulfate; an ethylene action inhibitor) suggesting auxin-induced ethylene production is responsible for the stimulation of pericarp senescence (Reinecke and Ozga, 1995). When IAA and GA_3 were applied simultaneously (50 μM) to 2 DAA pericarps immediately after deseeding, the pericarp elongation was inhibited and subsequently the tissue senesced (Reinecke and Ozga, 1995) Application of STS prior to the hormone treatments reversed the inhibitory effect of IAA on GA_3-induced growth (Reinecke and Ozga, 1995). These results suggest that IAA-induced ethylene inhibited GA_3-induced pericarp elongation.

Since 4-Cl-IAA can also stimulate ethylene release in pea shoots (Ahmad et al., 1987), it is important to determine if 4-Cl-IAA and IAA differentially affect ethylene biosynthesis in the pea pericarp.

4-Cl-IAA's stimulatory effect on pericarp growth, both alone and in conjunction with GA_3, as opposed to IAA's inhibitory effect, may also partially reside in the ability of 4-Cl-IAA to block ethylene signal transduction. Deseeded pericarps treated with 50 μM 4-Cl-IAA plus IAA grow similarly to those treated with 4-Cl-IAA alone (Reinecke, unpublished data). Therefore, it

is possible that one of the effects of 4-Cl-IAA is to block the ethylene signal transduction pathway.

Most of our current knowledge of ethylene signal transduction is from epistasis experiments involving *Arabidopsis* mutants in ethylene response. Ethylene's effect on dicotyledonous seedlings is known as the triple response which consists of a shortening and radial swelling of the hypocotyl, an inhibition of root elongation and an exaggeration of the curvature of the apical hook (Guzman and Ecker, 1990). The first ethylene mutants discovered were isolated using the triple response phenomenon.

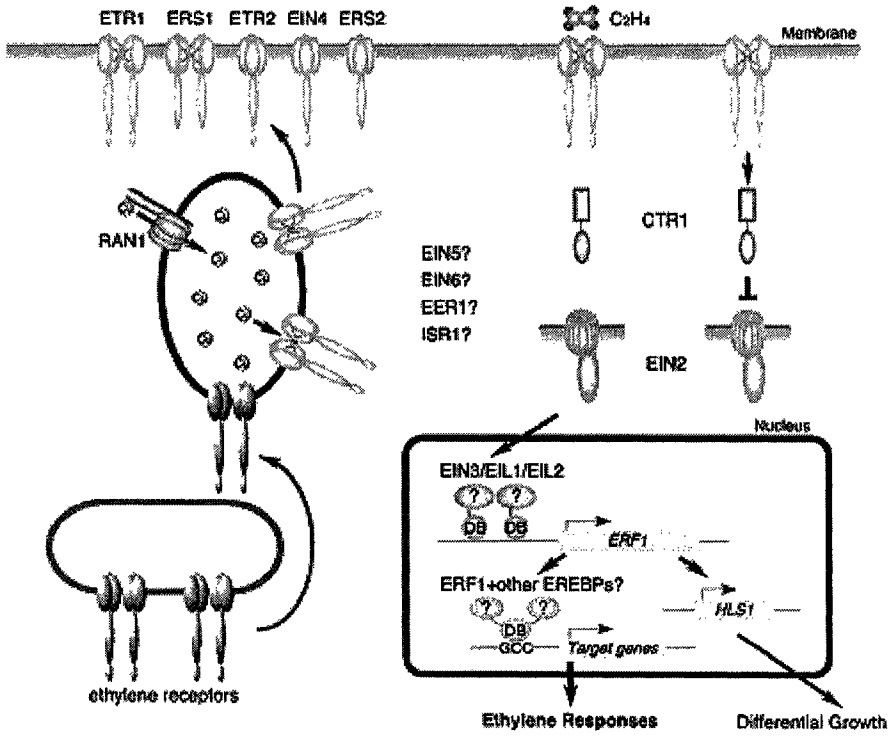


Figure 1.4: Ethylene Signal Transduction in Arabidopsis (Wang et al. 2002)

Ethylene is perceived by a family of membrane-bound receptors which share homology with bacterial two-component histidine kinases. The "two-component" system refers to a histidine protein kinase, which contains a conserved kinase core, and a response regulator protein, which contains a conserved regulatory domain. The histidine kinase transfers a phosphoryl group to the response regulator. Phosphotransfer to the response regulator results in activation of a downstream effector protein that continues the signal transduction (Stock et al., 2000). Five ethylene receptors have been identified in Arabidopsis: ETR1, ETR2, ERS1, ERS2 and EIN4. Only ETR1, ETR2 and EIN 4 have a receiver domain with similarities to bacterial histidine kinases. ERS1 and ERS 2 are thought to use the receiver domains of other proteins by forming heterodimers with them. Structural similarities of the receiver domain further subdivide the receptors into ETR1- or ETR2-like subfamilies. The ETR1-like subfamily, consisting of ETR1 and ERS1, has three membrane-spanning regions at the N-terminal end where ethylene binding occurs. Copper is a required cofactor for ethylene binding to take place (Hirayama et al., 1999). The C-terminal end contains a well-conserved histidine kinase domain. The ETR2-like subfamily is less understood, but includes ETR2, EIN4, and ERS2. In the absence of an ethylene signal,

ethylene receptors activate CTR1 which in turn negatively regulates the downstream ethylene response pathway.

Using ethylene triple response epistasis analysis, Kieber et al. (Kieber et al., 1993) were able to isolate and position CTR1 downstream of the receptors. In a yeast two-hybrid system and protein association assays, ETR1 and ERS1 both interacted with CTR1 (Clarke et al., 1998). The ctr1 gene belongs to the Raf family of Ser/Thr protein kinases that initiate MAP-kinase signaling cascades in mammals (Clarke et al., 1998). CTR1 is inactivated by the ethylene receptors upon ethylene binding, indicating that it is a negative regulator of downstream signaling events.

Arabidopsis triple response screening also identified ethylene-insensitive mutants ein2, and ein3. EIN2 is a central component in the linear pathway of ethylene signal transduction identified through triple response screening. Analysis of the sequence of EIN2 revealed that the NH_2-terminal contained regions of extreme hydrophobicity, whereas the COOH-terminal amino acids are mostly hydrophilic (Alonso et al., 1999). The hydrophobic portion of the protein behaves as an integral membrane protein and shares sequence similarity to the Nramp family (metal transporters) of proteins (Alonso et al., 1999). EIN2 was expressed in several heterologous systems, but no metal-transporting capacity was observed (Alonso et al., 1999). The cellular location of endogenous EIN2 protein is still unknown. Manipulation of

the full length EIN2 protein and NH_2-terminal hydrophobic Nramp-related domain in transgenic plants did not show constitutive ethylene responses or hypersensitivity to ethylene (Alonso et al., 1999). Only transgenic plants that expressed the COOH end of EIN2 (CEND) showed constitutive ethylene response phenotypes at maturity (Alonso et al., 1999). These results suggest that the NH_2-terminal domain is necessary for sensing the ethylene signal, and the COOH-terminal portion is required for transducing the signal (Alonso et al., 1999). Interestingly, EIN2 mutants have been isolated in screens for auxin transport inhibitor resistance (Fujita and Syono, 1996), cytokinin response (Su and Howell, 1992), and abscisic acid (ABA) hypersensitivity (Beaudoin et al., 2000; Ghassemian et al., 2000). Further downstream of EIN2 in the ethylene signal transduction pathway is the transcription factor, EIN3 and the response factor ERF1 (Wang et al., 2002). To date, the only reported auxin interaction in the ethylene signal transduction pathway is at the level of EIN2 as stated above.

Hormonal Regulation of Sink Potential in Pea Fruit

Movement of photoassimilates from sites of synthesis in the leaf (source tissue) to the sites of accumulation or rapid turnover (sink tissue) can be regulated at many points. It is generally accepted that the concentration gradient of photoassimilates between the source and the sink tissue is the

primary determinant of the rate of transport and the pattern of partitioning (Brenner and Cheikh, 1995). Hormones may serve as modulators of many of the specific rate limiting components that affect this concentration gradient between source and sink tissues (Brenner and Cheikh, 1995).

In developing pea fruit, the ovary (pericarp) is the primary sink for photoassimilates following pollination and fertilization of the ovules (2 to 8 DAA) until seed development advances to a point that the seeds become the major terminal sink (Flinn and Pate, 1970). Fruit set and development of non-pollinated pea ovaries can be induced by application of GA_3 to the ovaries (Garcia-Martinez and Carbonell, 1980). During non-pollinated pea fruit set, GA_3 increased [^{14}C]-sucrose accumulation in pea ovaries by regulating phloem transport (Peretó and Beltrán, 1987). Radioactively labeled sucrose was applied in an aqueous solution to the major leaflet adjacent to the flower. The first measurement of [^{14}C]-sucrose accumulation two hours after treatment at the leaf showed that the GA_3 treated non-pollinated ovaries had accumulated more radioactively labeled sucrose than the controls and that the rate of uptake increased continuously for four hours after treatment (Peretó and Beltrán, 1987). [^{3}H]-GA_1 was applied to the ovaries and detected after 50 minutes in the major leaflet adjacent to the flower. Differences in [^{14}C]-sucrose accumulation between control and GA_3 treated ovaries were only detected after one hour of GA_3 application to the ovaries (Peretó and Beltrán,

1987). The timing between the increased sucrose accumulation and the movement of GA_3 from the ovary to the leaflet supports the role of GA_3 in phloem loading at the source (leaflet). Application of GA_3 to the leaflet will increase phloem loading by stimulating leaf photosynthesis if treatments are applied to intact plants at least several hours before measurement of photosynthesis (Brenner and Cheikh, 1995). GA_3 also has the ability to increase phloem loading at the source leaflet separately from its effect on photosynthesis. In *Vicia faba* L., all leaves were removed except one source and one sink leaf. GA_3 application (10 μM) to the source leaf increased export of [^{14}C] to the sink leaf and roots within 35 minutes of application. The enhanced rate of sucrose export was not due to an enhanced rate of photosynthesis or to changes in the starch/sucrose ratio within the source leaf; rather to an increased proportion of sucrose exported (Aloni et al., 1986). GA_3 also has the ability to increase phloem unloading at the sink. [^{14}C]-sucrose was applied to the major leaflet for transport to the ovary tissues. Just before it reached the ovaries, GA_3 was applied to them. In comparison to the untreated ovaries and the surrounding tissues, GA_3 significantly stimulated [C^{14}]-sucrose accumulation in the ovaries (Peretó and Beltrán, 1987). These experiments show that, *in vivo*, gibberellins increase phloem loading of sucrose at the source (major leaflet) and unloading at the sink (non-pollinated ovary).

Once sucrose has reached the sink tissue, the cells must break it down into hexose components for maintenance of the source-sink sucrose gradient and growth of the tissue. Invertases and sucrose synthase (SS) are involved in the immediate metabolism of sucrose. Following anthesis (0 DAA), the activities of soluble acid (vacuolar) invertase and neutral (cytosolic) invertase increased and correlated with the maximum rates of pea pericarp growth (Estruch and Beltrán, 1991). Pericarp cell-wall invertase and sucrose synthase activity remained low and unchanged from 0 to 8 DAA (Estruch and Beltrán, 1991) . The higher activity of the inter-cellular pericarp sucrose metabolizing enzymes suggests that phloem unloading of sucrose into the pericarp tissue occur symplasmically via the plasmodesmata, rather than apoplasmically during early pericarp development. These data are consistent with data from studies on other fruits where symplasmic phloem unloading into young developing fruit tissue is the norm (Patrick, 1997)

Estruch and Beltrán (Estruch and Beltrán, 1991) also found that daily GA_3 (20 µL, 300 mM) treatment to non-pollinated ovaries from the equivalent of 2 DAA increased pericarp cytosolic, vacuolar, and cell wall-bound invertase activities by the equivalent of 4 DAA. The peak in GA-induced invertase activities also coincided with the peak in GA-treated ovary growth rate (at equivalent of 4 DAA). In shorter term studies, GA_3 treatment increased cytosolic and cell wall-bound invertase activities within 90 minutes after GA

application to non-pollinated ovaries. GA_3 treatment maintained vacuolar invertase activity in non-pollinated ovaries compared to the loss of activity observed in the untreated controls. Cytosolic and cell-wall bound invertases could be used by the plant as a rapid way to increase phloem unloading of sucrose into the pericarps via symplasmic and apoplasmic routes, whereas vacuolar invertase is likely more important for longer-term modulating of cellular sucrose levels.

When non-pollinated ovaries were treated with 2,4-dichlorophenoxyacetic acid (2,4-D, a synthetic auxin) at the equivalent to 2 DAA, again, vacuolar, cytosolic, and cell wall invertase activities increased by the equivalent to 4 DAA (Estruch and Beltrán, 1991). However, in shorter term studies with GA_3 or 2,4-D- treated non-pollinated ovaries, only GA_3 stimulated cell-wall invertase activities (within 1 h after application; Estruch and Beltran, 1991).

GA (Peretó and Beltrán, 1987) stimulation of phloem unloading within the pea fruit (and possibly auxin) could partially be through coordination of invertase activities to maintain "sink strength" in both short term and longer term development.

Ethylene may also have a role in pea pericarp sugar sensing. Recent work using an *Arabidopsis* ethylene-overproducing mutant, *eto1-1* and an ethylene insensitive mutant, *etr1-1* showed that when ethylene is consistently

present that the plant is glucose insensitive (Smeekens, 2000). Conversely, when the plant is unable to detect ethylene, as in *etr1-1*, the plant shows a glucose hypersensitive phenotype (Smeekens, 2000). The specific mechanism by which ethylene regulates glucose sensitivity in *Arabidopsis* is not clear. However, ethylene may function to suppress glucose down regulation of glucose-responsive genes, thereby allowing hexose build-up in sink organs such as pericarps and seeds.

The main goal of my thesis is to understand further the roles of GA, auxin, and ethylene in early pea fruit development. Specifically:

Objective 1: To test if the difference in the activity (with respect to growth) of 4-Cl-IAA and IAA in pea pericarp tissue is due to a differential effect on ethylene biosynthesis or action.

Objective 2: To test if ethylene is involved in the differential activity of 4-Cl-IAA with GA_3 (synergistic growth of pericarp) and IAA with GA_3 (inhibits GA_3-induced growth of pericarp).

Objective 3: To further understand how hormones (GA, auxin, and ethylene) affect carbohydrate partitioning into pea fruit.

Literature Cited

Ahmad A, Andersen A, Engvild K (1987) Rooting, growth and ethylene evolution of pea cuttings in response to chloroindole auxins. Physiologia Plantarum **69:** 137-140

Aloni B, Daie J, Wyse RE (1986) Enhancement of [^{14}C]-Sucrose Export from Source Leaves of *Vicia faba* by Gibberellic Acid. Plant Physiology **82:** 962-966

Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR (1999) EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. Science **284:** 2148-2152

Beaudoin N, Serizet C, Gosti F, Giraudat J (2000) Interactions between abscisic acid and ethylene signaling cascades. The Plant Cell **12**

Brenner ML, Cheikh N (1995) Hormones in photosynthate partitioning and seed filling. *In* PJ Davies, ed, Plant Hormones Physiology, Biochemistry and Molecular Biology. Kluwer Academic Publishers, Dordrecht, pp 649-670

Clarke JD, Liu Y, Klessig DF, Dong X (1998) Uncoupling PR gene expression from NPR1 and bacterial resistance: Characterization of the dominant Arabidopsis cpr6-1 mutant. Plant Cell **10:** 557-569

Cooper DC (1938) Embryology of *Pisum sativum*. Botanical Gazette **100:** 123-132

Eeuwens CJ, Schwabe WW (1975) Seed and pod wall development in *Pisum sativum,* L. in relation to extracted and applied hormones. Journal of Experimental Botany **26:** 1-14

Estruch JJ, Beltrán JP (1991) Changes in invertase activities precede ovary growth induced by gibberellic acid in *Pisum sativum*. Physiologia Plantarum **81:** 319-326

FAO (2002) (Food and Agriculture Organization of the United Nations) FAOSTAT statistics database - Agriculture Rome, Italy http://apps.fao.org/cgi-bin/nph-db.pl?sub-set=agriculture

Flinn AM, Pate JS (1970) A quantitative study of carbon transfer from pod and subtending leaf to the ripening seeds of the field pea (*Pisum arvense* L.). Journal of Experimental Botany: 71-82

Fujita H, Syono K (1996) Genetic analysis of the effects of polar auxin transport inhibitors on root growth in *Arabidopsis thaliana*. Plant and Cell Physiology **37:** 1094-1101

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

Ghassemian M, Nambara E, Cutler S, Kawaide H, Kamiya Y, McCourt P (2000) Regulation of abscisic acid signaling by the ethylene response pathway in *Arabidopsis*. 12

Graebe JE (1987) Gibberellin Biosynthesis and Control. Annual Review of Plant Physiology **38:** 419-465

Guzman P, Ecker JR (1990) Exploiting the Triple Response of Arabidopsis to Identify Ethylene-Related Mutants. Plant Cell **2:** 513-524

Hirayama T, Kieber JJ, Hirayama N, Kogan M, Guzman P, Nourizadeh S, Alonso JM, Dailey WP, Dancis A, Ecker JR (1999) RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in *Arabidopsis*. Cell **97:** 383-393

Kende H (1993) Ethylene Biosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology **44:** 283-307

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR (1993) CTR1, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the Raf family of protein kinases. Cell **72:** 427-441

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

Ngo P, Ozga JA, Reinecke DM (2002) Specificity of auxin regulation of gibberellin 20-oxidase gene expression in pea pericarp. Plant Molecular Biology **49:** 439-448

Orzáeaz D, Blay R, Granell A (1999) Programme of senescence in petals and carpels of *Pisum sativum* L. Planta **208:** 220-226

Orzáeaz D, Granell A (1997) DNA fragmentation is regulated by ethylene during carpel senescence in *Pisum sativum*. The Plant Journal **11:** 137-144

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

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

Ozga JA, van Huizen R, Reinecke DM (2002) Hormone and Seed-Specific Regulation of Pea Fruit Growth. Plant Physiology **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 Physiology **131:** 1137-1146

Patrick JW (1997) Phloem unloading: Sieve element unloading and post-sieve element transport. Annual Review of Plant Physiology and Plant Molecular Biology **48:** 191-222

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

Peck SC, Kende H (1998) 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 Molecular Biology **38:** 977-982

Peretó JG, Beltrán JP (1987) Hormone-directed sucrose transport during fruit set induced by gibberellins in *Pisum sativum*. Physiologia Plantarum **69:** 356-360

Phinny BO (1984) Gibberellin A_1, dwarfism and the control of shoot elongation in higher plants. Society for Experimental Biology Seminar Series **23:** 17-41

Pulse Canada (2003) Pulse Canada - A Most Unusual Year. Bi-Annual publication. Manitoba, Canada http://www.pulsecanada.com/pdf/feb03.pdf

Reinecke DM, Ozga JA (1995). IAA and 4-Cl-IAA Effect on Pea Pericarp Growth, 15th International Conference on Plant Growth Substances, Minneapolis, MN, # 447.

Reinecke DM, Ozga JA, Ilic N, Magnus V, Kojic-Prodic B (1999) Molecular properties of 4-substituted indole-3-acetic acids affecting pea pericarp elongation. Plant Growth Regulation **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

Smeekens S (2000) Sugar-induced signal transduction in plants. Annual Review of Plant Physiology and Plant Molecular Biology **51:** 49-81

Sponsel VM (1995) The biosynthesis and metabolism of gibberellins in higher plants. *In* PJ Davies, ed, Plant Hormones: Physiology, Biochemistry and Molecular Biology, Ed 2nd. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 66-97

Stock AM, Robinson VL, Goudreau PN (2000) Two-Component Signal Transduction. Annual Review of Biochemistry **69:** 183-215

Su WP, Howell SH (1992) A single genetic-locus, *ckr*1, defines *Arabidopsis* mutants in which root-growth is resistant to low concentrations of cytokinin. Plant Physiology **99:** 1569-1574

van Huizen R, Ozga JA, Reinecke DM (1997) Seed and hormonal regulation of gibberellin 20-oxidase expression in pea pericarp. Plant Physiology **115:** 123-128

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 Physiology **109:** 1213-1217

Vercher Y, Carbonell J (1991) Changes in the structure of ovary tissues and in the ultrastructure of mesocarp cells during ovary senescence or fruit

development induced by plant growth substances in *Pisum sativum*. Physiologia Plantarum **81:** 518-526

Wang KL-C, Li H, Ecker JR (2002) Ethylene Biosynthesis and Signaling Networks. The Plant Cell **Supplement:** S131-S151

Chapter 2: Interaction of Auxins, Gibberellin, and Ethylene in Pea Fruit Growth

Introduction

Pea (*Pisum sativum* I_3-Alaska type) fruit development is regulated by its developing seeds. During the linear phase of pericarp growth, seeds are essential for pericarp elongation (Ozga et al., 1992). Removal or abortion of seeds inhibits pericarp growth. Developmental coordination between the seeds and the pericarp is likely moderated by phytohormones (Ozga and Reinecke, 2003).

Gibberellins (bioactive GA_1 and GA_3) (Garcia-Martinez et al., 1991; Rodrigo et al., 1997) and auxins, indole-3-acetic acid (IAA) and 4-chloroindole-3-acetic acid (4-Cl-IAA) are naturally occurring in pea fruit (Magnus et al., 1997). 4-Cl-IAA (50 μM) applied to 2 DAA pollinated deseeded pea pericarps will promote elongation. In combination with GA_3, 4-Cl-IAA stimulates synergistic growth in deseeded pea pericarps (Ozga and Reinecke, 1999). Exogenous IAA at 50 μM is inhibitory to deseeded pericarp growth both when applied alone (Reinecke et al., 1995) and in conjunction with gibberellin (GA_3) (Reinecke and Ozga, 1995). However, pretreatment of deseeded pericarps with silver thiosulfate (STS, an ethylene action inhibitor) will reverse the inhibitory effect of IAA on GA_3- promoted growth (Reinecke and Ozga, 1995).

Pea plants metabolize gibberellins by the early 13-hydroxylation pathway: $GA_{12} \rightarrow GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20} \rightarrow GA_1$ (Sponsel, 1995). Using the split and deseeded pea pericarp system, van Huizen et al. (1995, 1997) were able to show that 4-Cl-IAA stimulates expression of the gene that codes for the enzyme that converts $GA_{19} \rightarrow GA_{20}$ (*PsGA20ox1)* and conversion of $GA_{19} \rightarrow GA_{20}$. Follow-up studies by Ozga et al. (2003) showed that 4-Cl-IAA also stimulated message of *PsGA3ox1*, which codes for the

enzyme that converts GA_{20} to biologically active GA_1. IAA was ineffective in stimulating *PsGA20ox1* and *PsGA3ox1* (Ngo et al., 2002; Ozga et al., 2003). Both auxins have shown the ability to stimulate ethylene biosynthesis. 4-Cl-IAA induces ethylene production in pea shoot cuttings (Ahmad et al., 1987). IAA is known to stimulate 1-aminocyclopropane-1-carboxylate synthase (ACS; the rate limiting enzyme in the ethylene biosynthesis pathway) message levels and enzyme activity in etiolated internodes of 5 to 6 day-old pea seedlings, (Kende, 1993; Peck and Kende, 1998). However, IAA and/or 4-Cl-IAA stimulation of ethylene biosynthesis in pea fruit tissue has not been reported.

This study explores the role of ethylene as an agent involved in the differential activity of the two naturally occurring auxins, IAA and 4-Cl-IAA, in pea pericarp growth. Firstly, IAA- and 4-Cl-IAA-induced ethylene production in pea pericarp tissue was determined. Secondly, the effect of IAA on GA_3-induced pericarp growth was confirmed and the ethylene releasing agent, Ethephon, was used to determine if ethylene could mimic IAA's effects on pericarp growth. Lastly, the effect of ethylene on 4-Cl-IAA-induced growth was studied. We found that both auxins induced ethylene evolution in pea pericarps with a similar production profile. We confirmed that GA_3-induced pericarp growth was inhibited by both IAA and ethylene, but not 4-Cl-IAA, and, IAA's inhibitory effect could be reversed by the ethylene action inhibitor, STS. Ethylene could mimic IAA effects on pericarp growth; however, it did not affect 4-Cl-IAA-induced pericarp growth. These data suggest that IAA-induced ethylene production inhibits pericarp growth and promotes senescence of the tissue. The role of the other naturally occurring auxin, 4-Cl-IAA, is opposite to that of IAA. 4-Cl-IAA stimulates pericarp growth even though it stimulates similar levels of ethylene in the pericarp tissue. The stimulatory role of 4-Cl-IAA on pericarp growth is likely two fold, one is the stimulation of growth promoting signal transduction pathways, and the second is to reduce sensitivity of the tissue to the ethylene which promotes senescence and inhibits GA response.

Materials and Methods

Plant Material

Five pea seeds (*Pisum sativum* L.) line I_3 (Alaska-type) per pot were planted in 2L soil containing Metro-mix 290 and sand (1:1) and grown in a Conviron growth chamber at 19/17°C (day/night) under a 16-hour photoperiod (400 $\mu E \cdot m^{-2} \cdot s^{-1}$ from cool white fluorescent and incandescent lights). Plants were thinned when 15 cm tall to three uniform seedlings per pot. During plant development all lateral side shoots and the 1st and 2nd flowering nodes were removed. For treatments, one fruit per plant between the 3rd and 5th flowering nodes was used. Subsequent flowers and lateral buds were removed as they developed, the terminal apical meristem remained intact and the pericarp stayed attached to the plant during all experiments.

Growth Studies

Two days after anthesis (DAA) pericarps (fruits) were either left intact (intact treatment), split down the dorsal suture (SP treatment) or split with removal of the seeds (SPNS and hormone treatments) as described by Ozga et al. (1992). GA_3, 4-Cl-IAA and/or IAA (50 µM in 0.1% [v/v] aqueous Tween 80) were applied immediately after deseeding to the inner pericarp wall (endocarp) and daily thereafter to 6 DAA (30 µL, 2 and 3 DAA; and 40 µL, 4, 5 and 6 DAA). Ethephon (30 µL; 250 or 1000 ppm) was applied once immediately after splitting and deseeding the pericarp. When Ethephon was applied in combination with other hormone treatments, a 30 µL aliquot (250 or 1000 ppm) was applied one time, 90 minutes after the initial auxin and/or GA_3 solutions. For treatments requiring silver thiosulphate (STS; 1 mM), a 20 µL aliquot was applied once as a pre-treatment immediately after splitting and deseeding the fruit. Hormone solutions were applied 30 minutes after STS application. For growth experiments, the pericarps were enclosed in a clear plastic bag to maintain moisture. Fruits were harvested at 9 DAA (7 days after initial treatment) and measured for length, fresh and dry weight.

Ethylene Analysis

Ethylene evolution from intact, SP, or deseeded pericarps treated with Ethephon, auxins and/or GA_3 was investigated over a 24 h period. Intact, split pericarp (SP), and split and deseeded pericarps (SPNS) were treated once with 30 µL 0.1% (v/v) aqueous Tween 80 and remained attached to the plant for the incubation period of 4, 8, 12 or 24 hours. Pericarps were open to the atmosphere during incubation on-plant. After incubation, the pericarps were harvested to measure the rate of ethylene evolution. The number of pericarps per vial was 1 to 4. Ethephon (30 µL; 250 or 1000 ppm)-treated pericarps remained attached to the plant for 1and 2 (SPNS or Ethephon 1000 ppm treatments), 4, 8, 12, and 24 hour (SPNS, Ethephon 250 and 1000 ppm treatments) incubation times. Pericarps were open to the atmosphere during the incubation on-plant. After incubation, pericarps were harvested to measure the rate of ethylene evolution. Relative ethylene evolution was calculated by subtracting the ethylene evolved (nL ·g fwt^{-1}· h^{-1}) from the SPNS control pericarps from that of the hormone-treated pericarps. For the experiment testing sensitivity of deseeded pericarps to IAA treatment both on and off-plant, 2 DAA pericarps were split and deseeded on-plant and immediately treated with 30 µL of 50 mM IAA. The 12 hour on-plant incubation pericarps remained attached to the plant and open to the atmosphere. At the same time, the 12 hour pericarp off-plant incubation group was immediately detached from the plant after IAA-treatment and placed in an open-ended 7 mL glass vial in the growth chamber. After 12 hours of incubation in the growth chamber, the on-plant pericarps were harvested and the off-plant pericarps were collected to measure the rate of ethylene evolution. To test hormone induction of ethylene evolution in deseeded pea pericarps, auxins (IAA or 4-Cl-IAA) and GA_3 (30 µL, 50 mM hormone in 0.1% [v/v] aqueous Tween 80), were applied to 2 DAA deseeded pericarps and incubated on-plant for 4, 8, 12, or 24 hours. Treated pericarps remained attached to the plant. Pericarps were harvested after the incubation period to measure the rate of ethylene evolution. In the auxin reapplication

experiment, 2 DAA pericarps were treated with 30 µL of 50 µM IAA, 4-Cl-IAA, or 0.1% Tween 80 at 0 h. One group was again treated at 24 hours. Harvests included in the experiment were at 12, 24 and 36 hours.

Gas Chromotography

For ethylene quantitation, a 1 mL gas sample from the headspace of the vial was withdrawn using a Hamilton gas-tight syringe and injected on to a 2.9 m · 6.35 mm O.D 80/100 mesh Porapak N column attached to a Hewlett Packard 5890 GC fitted with a flame-ionization detector. The injection and detection ports were set at 250°C and the column temperature was isothermal at 120°C. Nitrogen was used as the carrier gas with a flow rate of 30 mL min^{-1}. Ethylene content was determined by comparing the sample to an equal volume of 0.96 ppm ethylene primary standard.

Statistical Analysis

Growth experiments were analyzed using SAS Proc GLM model I ANOVA ($P \leq 0.0001$) and mean separation was performed using the least significant difference (LSD) test ($P < 0.05$).

Results:

Hormone-induced pericarp growth

Pericarp growth response to 50 µM exogenous auxin differed depending on the specific auxin. 4-Cl-IAA stimulated pericarp growth; however, IAA-treated pericarps did not grow significantly more than the SPNS control. When applied in combination, 4-Cl-IAA synergistically enhanced GA_3-treated pericarp growth, whereas IAA significantly inhibited GA_3-stimulated pericarp growth GA_3 (Figure 2.1 A and B).

When STS was applied as a pre-treatment to GA_3 + IAA-treated pericarps, it reversed the inhibitory effect of IAA on GA_3-treated fruit growth (Figure 2.2 A and B). STS alone did not enhance pericarp growth more than

the control (Figure 2.2 A and B). To test if ethylene could mimic IAA's effect on GA_3-induced pericarp growth, Ethephon (ethylene-releasing agent) was applied to pericarps. Ethephon at 250 and 1000 ppm significantly inhibited 0.1% (v/v) aqueous Tween 80-treated and GA_3-treated deseeded pericarp growth (Figure 2.3). However, the pericarp response was more consistent at 1000 ppm (Data not shown). IAA inhibits GA_3-stimulated growth via ethylene as shown by pretreatment with STS on GA_3 + IAA-treated pericarps.

The growth of 4-Cl-IAA-treated deseeded pericarps was not negatively affected by Ethephon (1000 ppm) (Figure 2.4 A and B). An STS pre-treatment was also able to reverse the negative effect of Ethephon (1000 ppm) on GA_3-treated pericarp growth (Figure 2.4 A and B).

Ethylene evolution in control and Ethephon-treated fruit.

Intact pericarps (2 DAA) released low amounts (3.8 to 1.0 nL·g $fwt^{-1}·h^{-1}$) of ethylene over the 24 hour treatment period (Figure 2.5 A). Splitting of the pericarp resulted in a small increase in the rate of ethylene evolution 4 h after the splitting event (21.8 nL·g $fwt^{-1}·h^{-1}$), then decreased to rates similar to that of the intact pericarp (Figure 2.5 A). Splitting and deseeding of the pericarp (SPNS) triggered a much higher rate of ethylene evolution by 4 hours (129.4 nL·g $fwt^{-1}·h^{-1}$) compared to intact and SP-treated pericarps (Figure 2.5 A). By 24 hours the SPNS rate had decreased to 57.5 nL·g $fwt^{-1}·h^{-1}$, still 10-fold higher than SP, and 50-fold higher than intact fruit. Intact and SP treatments had grown (fresh weight) two-fold more than SPNS by 24 hours (Figure 2.5 B).

Ethephon (1000 ppm) stimulated a burst of ethylene evolution six-fold above SPNS rates (589.0 nL·g $fwt^{-1}·h^{-1}$) one hour after application, then decreased rapidly to two-fold above SPNS rates (243.5 nL·g $fwt^{-1}·h^{-1}$) by 8 hours and maintained this rate until 24 hours (Figure 2.5 C). The concentration of Ethephon in the treatment was reflected in the rate of ethylene evolution from the pericarp. At four hours, pericarps treated with 1000 ppm Ethephon had a rate of 498.5 nL·g $fwt^{-1}·h^{-1}$, and those treated with

250 ppm had a rate of 191.0 nL·g $fwt^{-1}·h^{-1}$, a ratio of 2.6:1 (Figure 2.5 C). Ethephon treated deseeded pericarps did not grow (fresh weight) more than SPNS controls (Figure 2.5 D).

Twelve hours after treatment, IAA stimulated ethylene evolution (184 nL·g $fwt^{-1}·h^{-1}$) from pericarps that were attached to the plant (on-plant) significantly more than control treated (on-plant) pericarps (55 nL·g $fwt^{-1}·h^{-1}$) (Figure 2.6 A). Pericarp removal from the plant during the treatment time (12 h) stimulated fresh weight loss and ethylene evolution in SPNS pericarps (senescence) and IAA-application to these pericarps had minimal effects on these parameters (Figure 2.6 A and B). Therefore, deseeded pericarps (in a pre-senescence state) must be attached to the plant in order to observe IAA-induced changes in ethylene evolution.

IAA and 4-Cl-IAA stimulated similar rates of ethylene evolution in 2 DAA deseeded pericarps (Figure 2.7 A and C). Addition of GA_3 to the auxin treatment did not change the ethylene evolution profile compared to auxin alone (Figure 2.7 A and C). GA_3 stimulation of ethylene evolution in deseeded pericarp is equal, or less than SPNS-treatment (Figure 2.7 E). Deseeded pericarps were still sensitive to IAA or 4-Cl-IAA reapplication at 24 hours after initial treatment; 4-Cl-IAA stimulated a higher rate of ethylene evolution at 36 hours compared to IAA-treated pericarps (Figure 2.8). By 36 hours, 4-Cl-IAA-treated deseeded pericarp growth (fresh weight) was 3-fold higher than IAA-treated deseeded pericarps (data not shown).

Figure 2.1: Effect of exogenous natural pea hormones (GA_3, IAA, 4-Cl-IAA) on pericarp growth.

A: Representative pericarps harvested at 7 DAA (five days after initial treatment). Deseeded pericarps were treated daily for 5 days with 50 μM of GA_3, IAA, GA_3 + IAA, 4-Cl-IAA, or GA_3 + 4-Cl-IAA or 0.1% (v/v) aqueous Tween 80 for SPNS treatment.

B: Growth (fresh weight) of hormone-treated pericarps. Deseeded pericarps were treated with hormone solutions as described in A and harvested 9 DAA (7 days after initial treatment) Data are means ± SE, n = 7 to 38.

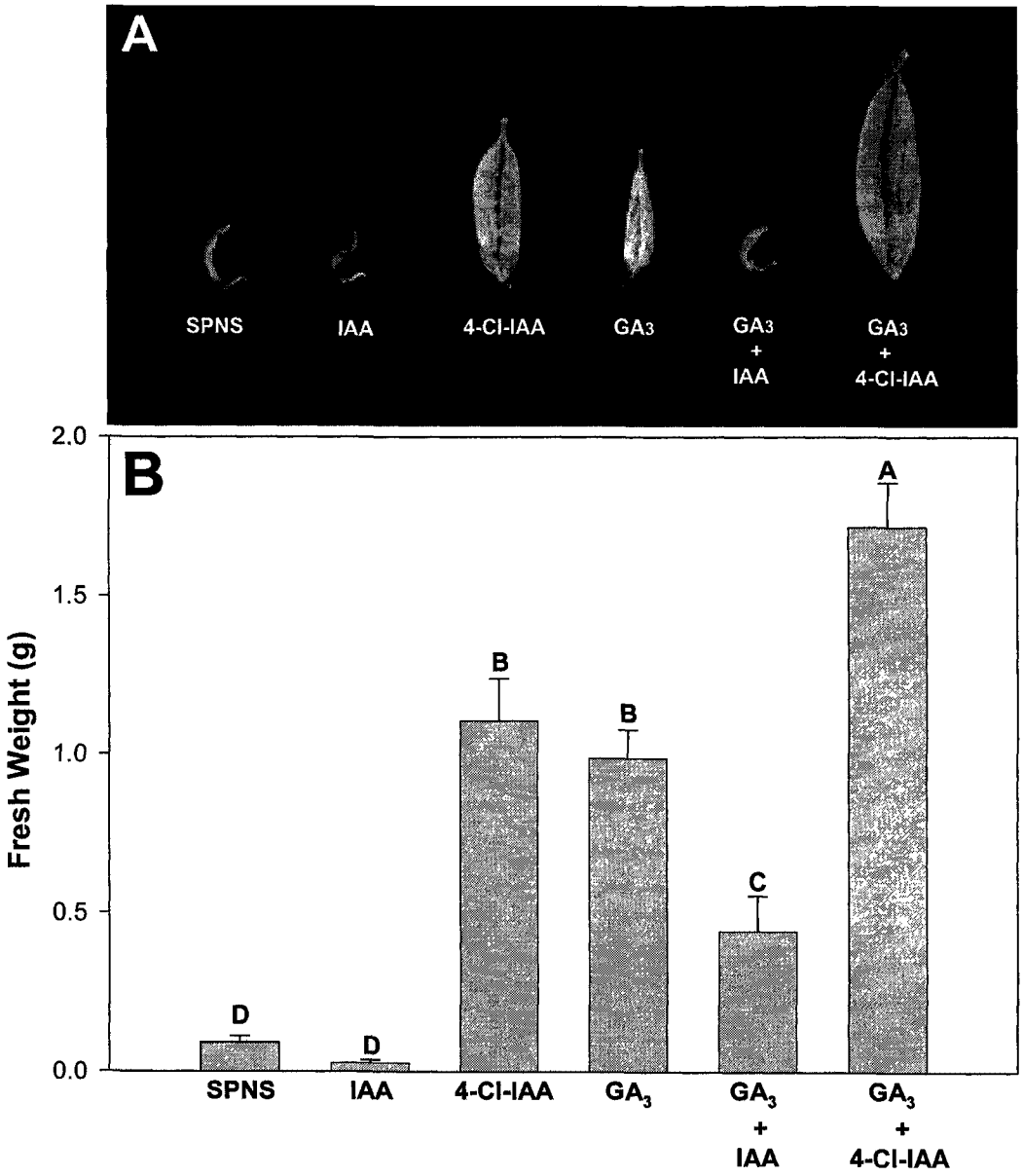


Figure 2.2: Effect of the ethylene action inhibitor, STS, on control and hormone-treated pea pericarp growth.

A: Representative pericarps harvested at 7 DAA (five days after initial treatment). Deseeded pericarps were treated daily for 5 days with 50 μM of GA_3, IAA, GA_3 + IAA or 0.1% (v/v) aqueous Tween 80 (SPNS). For STS treated pericarps, STS (20 μl, 1 mM) was applied once on the first day of experiment (STS) or once as a pre-treatment 30 minutes prior to hormone treatment (GA_3 + IAA + STS).

B: Growth (fresh weight) of hormone-treated pericarps. Deseeded pericarps were treated with hormone solutions as described in A and harvested at 9 DAA (7 days after initial treatment) Data are means ± SE, n = 8 to 38.

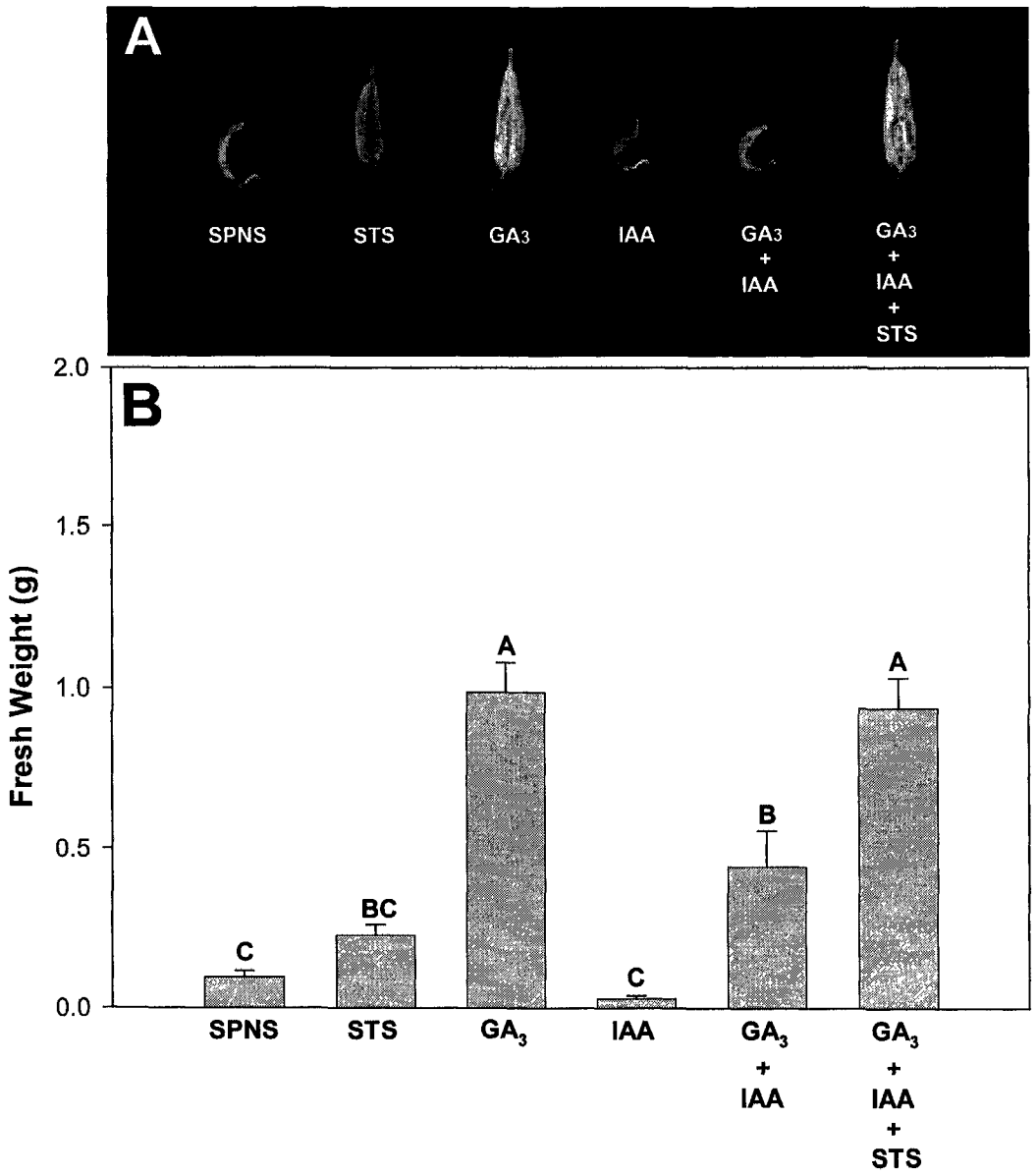


Figure 2.3: Ethephon inhibition of GA_3-induced pericarp growth.

Deseeded pericarps were treated with 50 µM of GA_3, or 0.1% (v/v) aqueous Tween 80 (SPNS) daily for five days and harvested at 9 DAA (7 days after initial treatment). Ethephon was applied only one time to the deseeded pericarps either immediately (Ethephon 250 or 1000 ppm) or 90 minutes after GA_3 (GA_3 + Ethephon 250 ppm and GA_3 + Ethephon 1000 ppm). Data are means ± SE, n = 4 to 8.

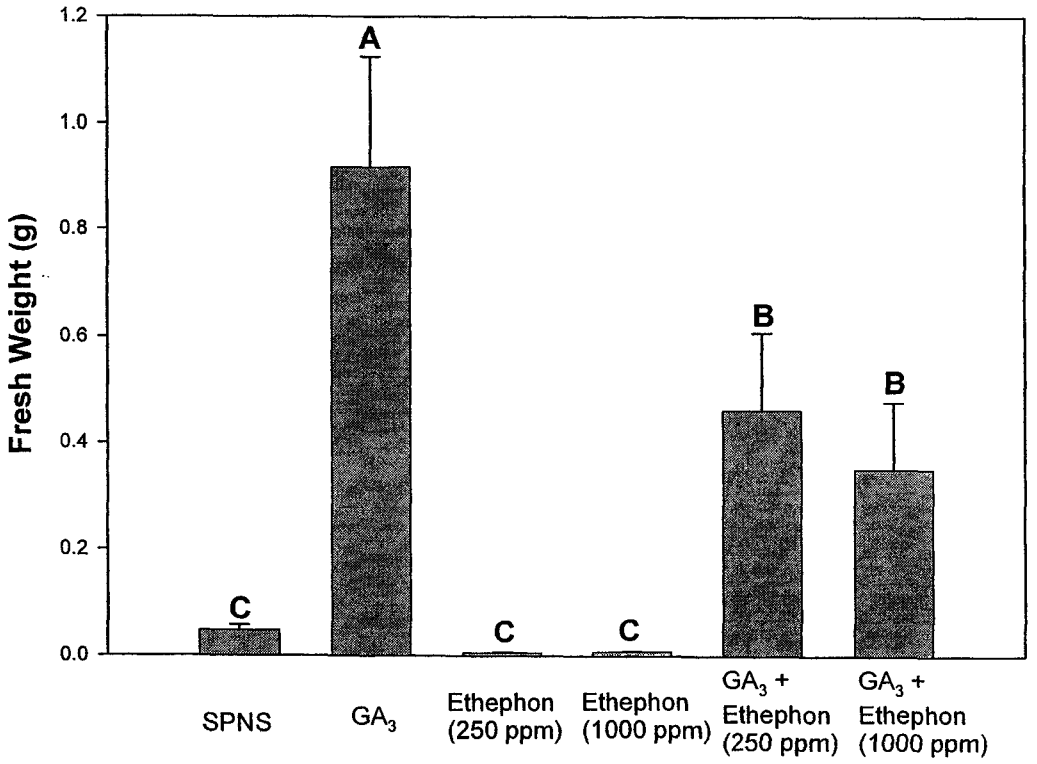


Figure 2.4: The effect of Ethephon on GA_3 and 4-Cl-IAA-induced pericarp growth.

A: Representative pericarps harvested at 7 DAA (five days after initial treatment). Deseeded pericarps were treated with 50 µM of GA_3, IAA, GA_3 + IAA, or 0.1% (v/v) aqueous Tween 80 (SPNS). Ethephon (1000 ppm) was applied once, either immediately after pericarp splitting and deseeding or 90 minutes after 4-Cl-IAA or GA_3 application (GA_3 + Ethephon, 4-Cl-IAA + Ethephon) on day one of experiment. STS (1 mM) was applied as a pre-treatment (GA_3 + Ethephon + STS, GA_3 + IAA + STS).

B: Growth (fresh weight) of hormone-treated pericarps. Deseeded pericarps were treated with hormone solutions as described in A and harvested at 9 DAA (7 days after initial treatment) Data are means ± SE, n = 8 to 38.

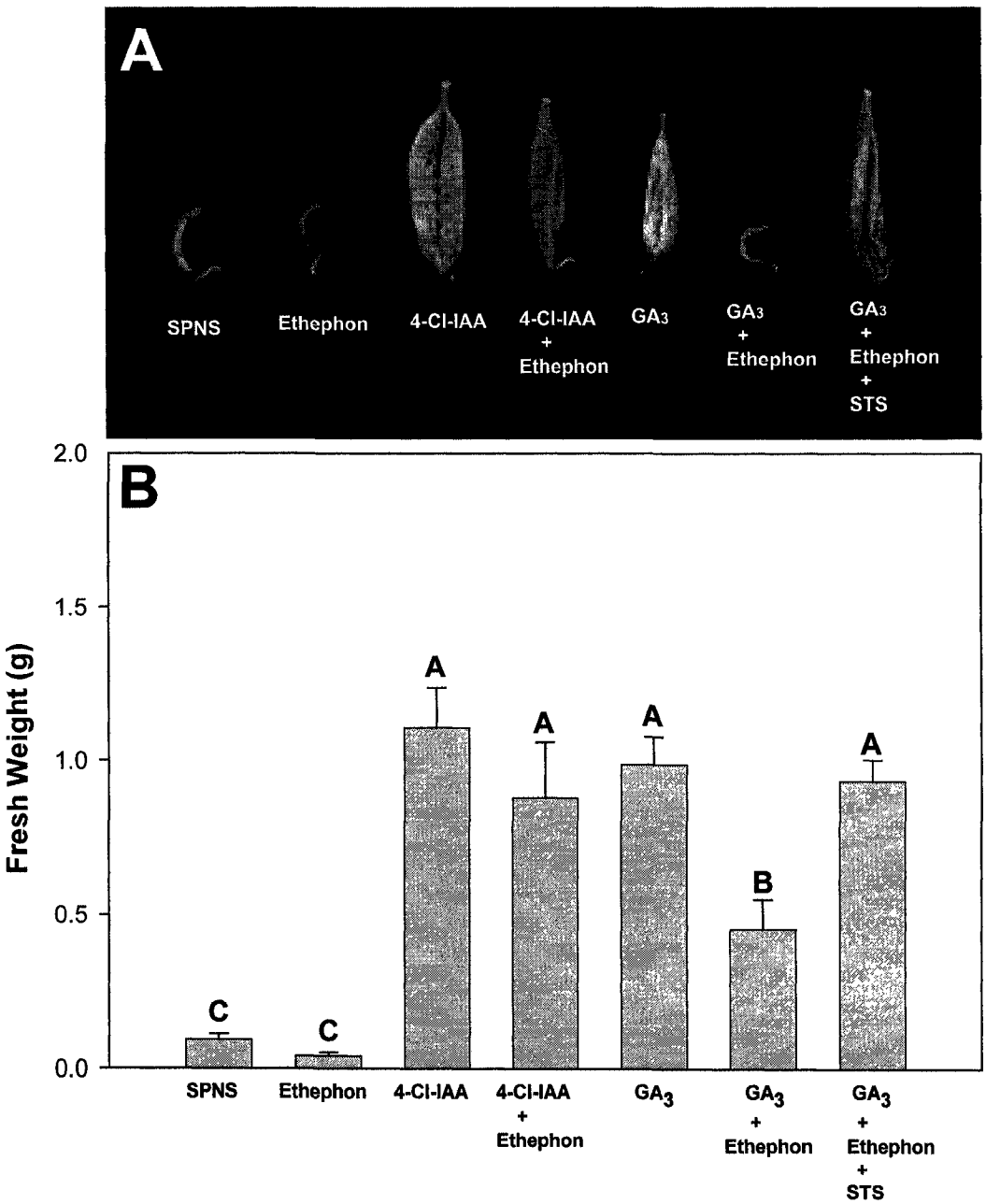


Figure 2.5: Rate of ethylene evolution and growth (fresh weight) of Intact, SP, SPNS pericarps (A and B), and Ethephon-treated deseeded pericarps (C and D).

Two DAA pericarps were left intact (Intact treatment), split along the dorsal suture (SP), or split and deseeded (SPNS). Ethylene evolution (A) and pericarp growth (fresh weight; B) were monitored and over a 24 hour period. Data are means ± SE, n = 4 to 19.

Two DAA pericarps were split and deseeded and immediately treated with Ethephon (250 or 1000 ppm) or 0.1% (v/v) aqueous Tween-80 (SPNS). Ethylene evolution (C) and growth (fresh weight; D) from the pericarps were monitored over a 24 hour period. Data is presented as relative ethylene evolution where the rate of ethylene evolution from SPNS pericarps is subtracted from that of the Ethephon-treated pericarps. Data are means ± SE, n = 4.

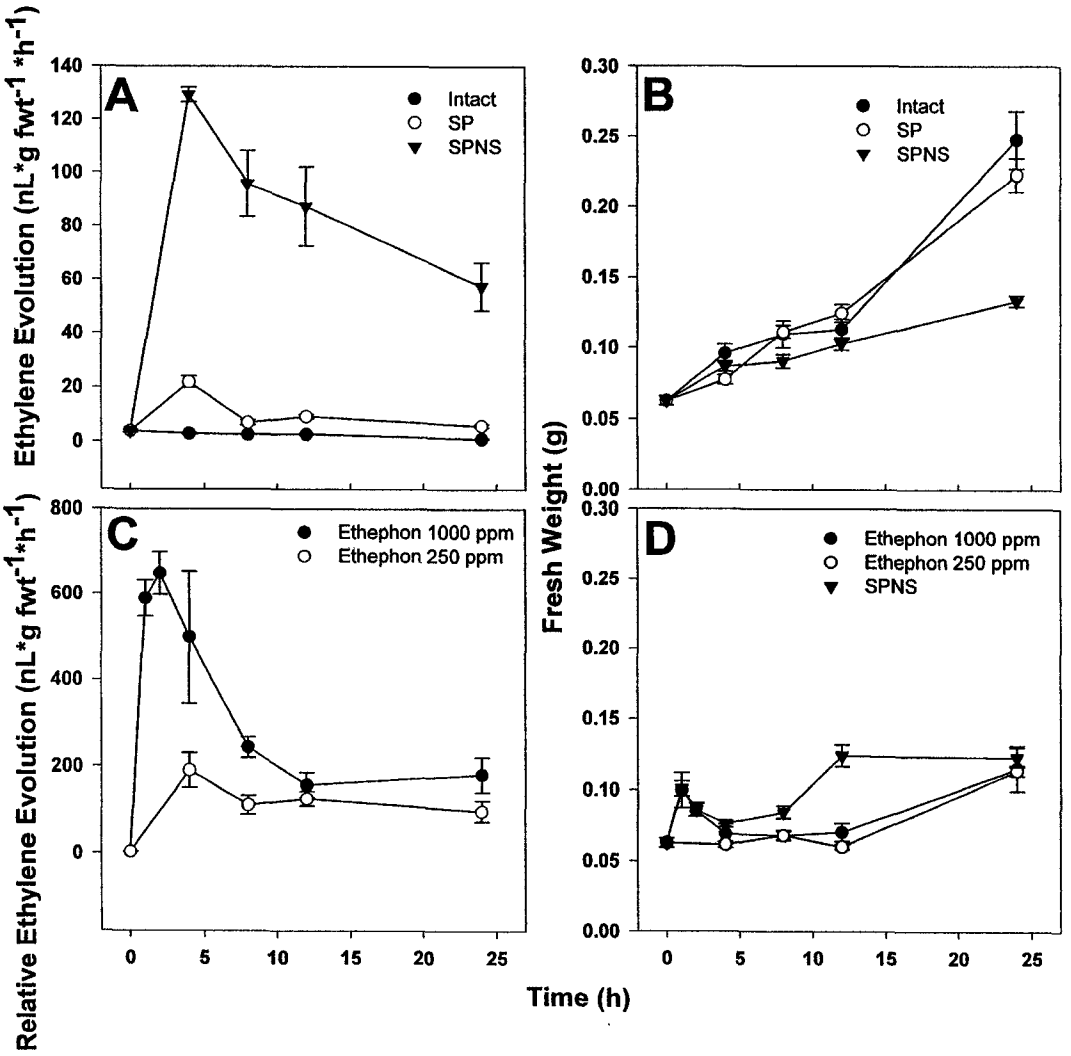


Figure 2.6: Ethylene evolution and growth (fresh weight) of IAA and SPNS-treated pericarps 12 hours after hormone treatment.

A: Two DAA pericarps were split and deseeded and treated immediately with either 50 µM IAA or 0.1% (v/v) aqueous Tween 80 (SPNS). Deseeded pericarps remained attached to the plant (on-plant), or were detached from the plant and placed in an open-ended 7 mL vial (off-plant) for a 12 h incubation period. After 12 hours, pericarps were collected for ethylene evolution analysis. Data are means ± SE, n = 7 to 10.

B: Growth (fresh weight) of IAA- and SPNS-treated pericarps 12 hours after treatment. Data are means ± SE, n = 7 to 10.

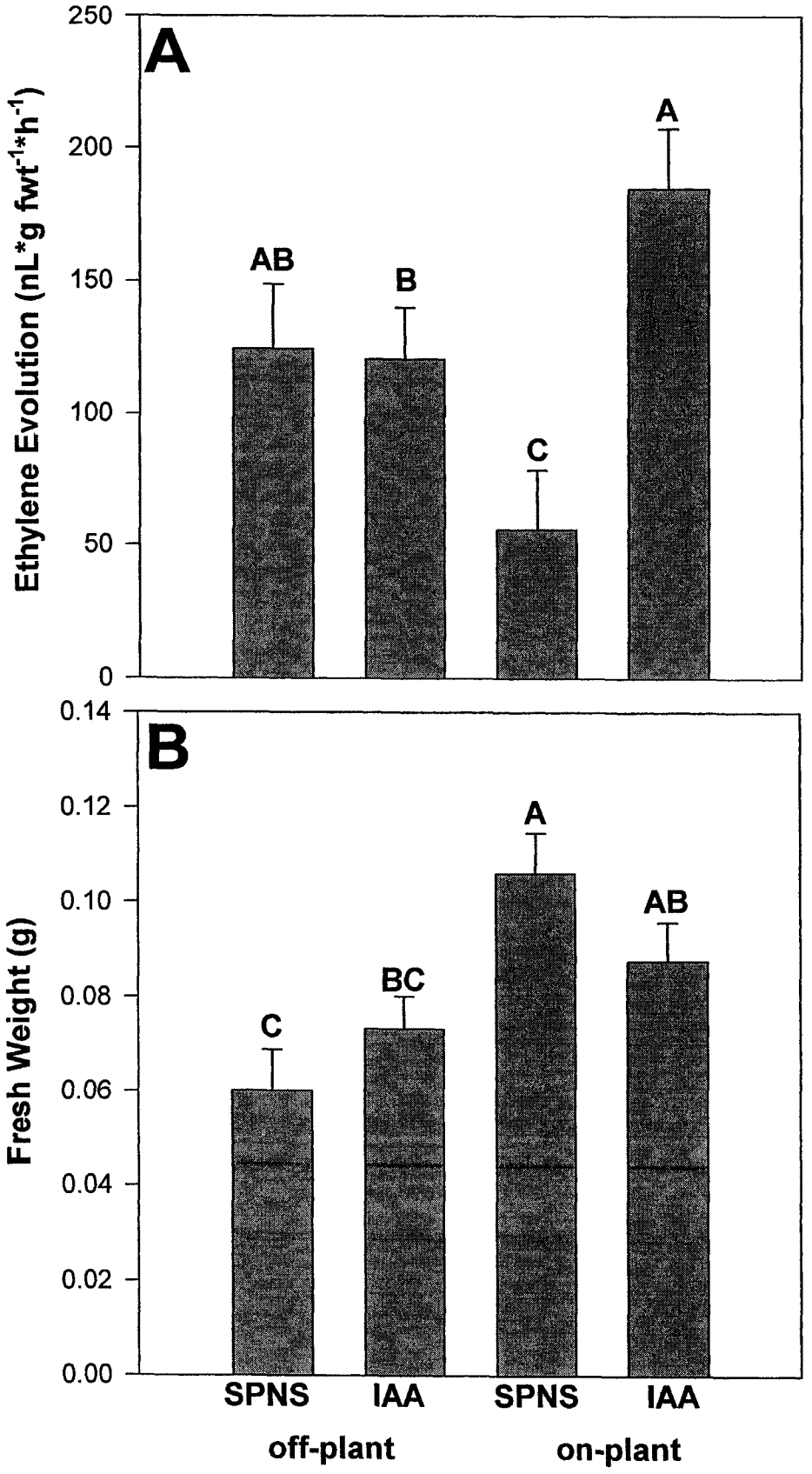


Figure 2.7: Relative ethylene evolution and pericarp growth in hormone and control-treated pericarps.

Two DAA pericarps were split, deseeded and immediately treated with 50 µM IAA or IAA + GA_3 (A and B); 4-Cl-IAA or 4-Cl-IAA + GA_3 (C and D); GA_3 (E and F); or 0.1% (v/v) Tween 80 (SPNS). Subsequently, pericarps were harvested at 4, 8, 12, and 24 hours. Data is presented as the relative rate of ethylene evolution where the rate of ethylene evolution of the SPNS pericarps is subtracted from the hormone-treated pericarps. Data are means ± SE, n = 6 to 8.

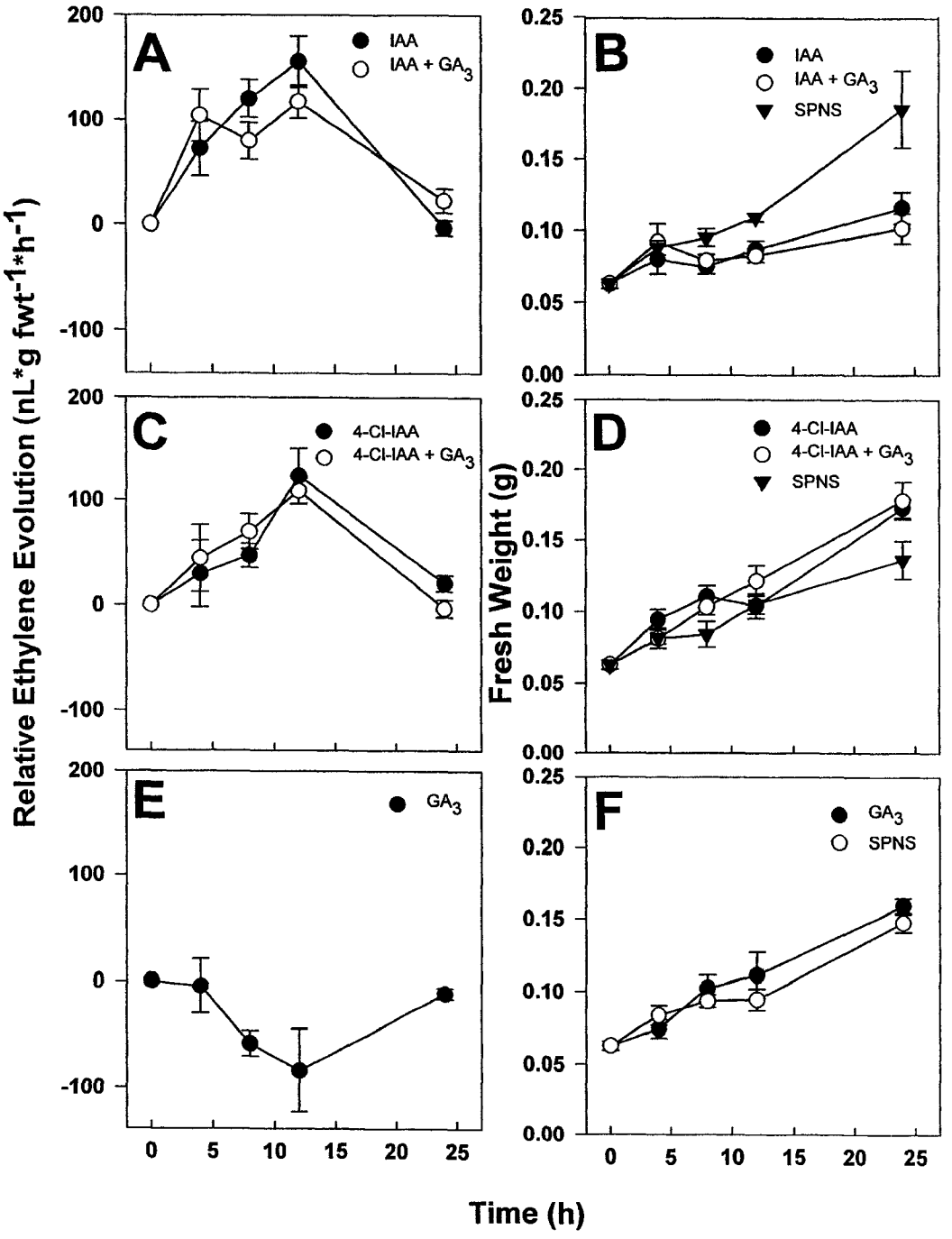
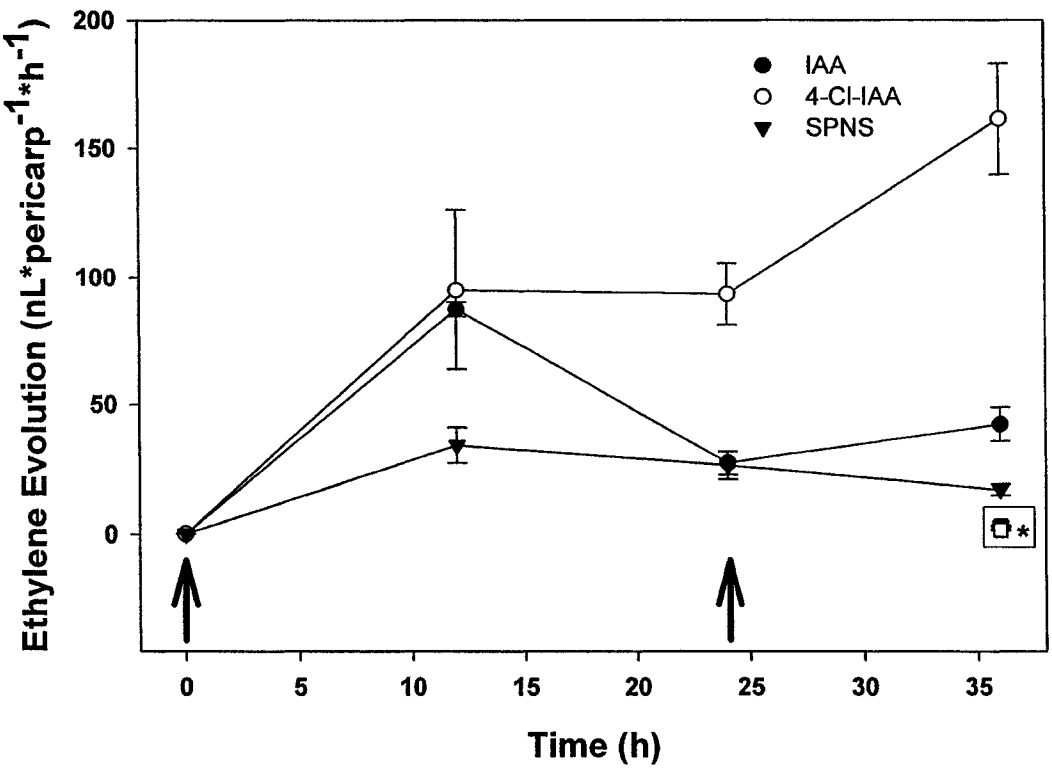


Figure 2.8: Sensitivity of pericarp tissue to reapplication of auxin (IAA or 4-Cl-IAA) 24 hours after initial treatment.

Two DAA pericarps were split, deseeded and treated at 0 h and 24 h after splitting with 50 μM IAA, 4-Cl-IAA, or 0.1% (v/v) aqueous Tween 80 (SPNS). Pericarps were harvested at 12, 24, and 36 hours after initial treatment and assayed for ethylene production. Arrow indicates time of application.

* Two DAA pericarps were split, deseeded and treated immediately (0h) with 50 μM IAA, 4-Cl-IAA, or 0.1% (v/v) aqueous Tween 80 (SPNS) then were harvested and assayed at 36 h for ethylene evolution. These data are plotted on Figure 8 within the box at the 36 h time point. At 36 hours, data for treatments with one treatment application: IAA = 3.1 ± 0.1; 4-Cl-IAA = 3.1 ± 0.4; SPNS = 1.6 ± 0.2 ($nL \cdot pericarp^{-1} \cdot h^{-1}$). Data are means ± SE, n = 3 to 4.



Discussion

In pollinated deseeded fruit, 4-Cl-IAA and GA_3 promoted pericarp elongation both alone and synergistically when applied simultaneously (Reinecke et al., 1995; Ozga and Reinecke, 1999). The other naturally occurring auxin, IAA, inhibited deseeded pericarp growth, as well as the stimulatory effect of GA_3 on this tissue (Reinecke and Ozga, 1995). STS (ethylene action inhibitor silver thiosulfate) treatment to deseeded pericarps delayed pericarp senescence but did not stimulate growth (Reinecke et al., 1995). STS also blocked the IAA-induced inhibition of pericarp growth but did not result in IAA-stimulation of pericarp growth (Reinecke and Ozga, 1995; Reinecke et al., 1995). However, application of STS to GA_3 + IAA-treated deseeded pericarps reversed the inhibitory effect of IAA on GA_3-stimulated growth (Reinecke and Ozga, 1995). These data suggest that the main effects of exogenous IAA on deseeded pea pericarp growth are through IAA-induced ethylene, and they differ from that of 4-Cl-IAA. Therefore, we investigated the role of ethylene in early pea pericarp development.

Although ethylene in fruit ripening has been extensively studied, little is known about the role of ethylene in early fruit development. A study of the coordination of pollination and corolla senescence in pea suggested that the ethylene produced by the ovary at anthesis is a developmental event that is independent of pollination and fertilization of the ovary, since a similar amount of ethylene was produced from both pollinated and non-pollinated ovaries (Orzáeaz et al., 1999). Following pollination, intact pollinated pericarps naturally released low amounts of ethylene [Anthesis ~ 0.8; 1 DAA ~0.5; 2 DAA ~0.25 (nL C_2H_4 g fwt^{-1} h^{-1})] (Orzáeaz et al., 1999). In non-pollinated pericarps, ethylene production started to increase at the equivalent to 2 DAA (~1 nL $g^{-1}h^{-1}$; ~4X above pollinated control) and continued to do so through 4 DAA (~1.6 nL $g^{-1}h^{-}1$; five times above pollinated control), when senescence of the tissue is evident (Orzáeaz et al., 1999). Ethylene (10 µL L^{-1} C_2H_4 in air) immediately induced carpel senescence in non-pollinated pea fruit, even when the fruit was treated with GA_3 (20 µL, 0.3 mM) (Orzáeaz and Granell,

1997). These data suggest that ethylene acts as a natural senescence hormone in pea pericarp tissue when the fruit lacks fertilized ovules (seeds).

In the pea split-pericarp system, splitting of the pericarp (SP) increased ethylene evolution at 4 hours (Figure 2.5 A), likely in response to wounding (Peck and Kende, 1998). Splitting and removal of seeds from the pericarp (SPNS) significantly stimulated ethylene evolution above that observed in SP (Figure 5 A). Since SPNS tissue did not grow significantly compared to the SP and intact pericarps (Figure 2.5 B), the higher levels of ethylene in the SPNS treatment are probably due to a combination of wound and senescing ethylene (Orzáeaz and Granell, 1997; Peck and Kende, 1998; Orzáeaz et al., 1999).

Ethephon (2-chloroethylphosphonic acid) was used as a synthetic source of ethylene (Warner and Leopold, 1969) in the treatment of deseeded pericarps. Both concentrations of Ethephon tested (1000 and 250 ppm) produced sustained levels of ethylene throughout the 24 h experimental period (Figure 2.5 C). The magnitude of ethylene production by 250 ppm was more similar to that produced by 50 µM IAA (Figure 2.5 C, Figure 2.7 A). However, pericarp ethylene-response (with respect to growth) was much more consistent with 1000 ppm than 250 ppm Ethephon (data not shown); therefore, this concentration was used for the remaining experiments.

The profile of ethylene evolution from Ethephon (1000 and 250 ppm; greatest within 4 h of application) was different from auxin-induced ethylene production, which peaked at 12 hours after application (Figure 2.6 C and Figure 2.7 A and C). Regardless, Ethephon (at 1000 and 250 ppm) mimicked IAA's inhibitory effect on pericarp growth (Figure 2.4) when applied alone or in combination with GA_3 (Figure 2.4). Ethephon's affect was also reversed by application of STS (Figure 2.4). These data confirmed the hypothesis that IAA inhibition of deseeded pericarp growth is through IAA-induced ethylene. Further, IAA-induced ethylene acts to inhibit GA response in deseeded pericarp tissue. The difference between the action of IAA and 4-Cl-IAA was investigated by monitoring their effect on ethylene biosynthesis.

Classical phytohormone activity experiments have tested treatments on excised plant tissue such as pea stem segments (Law and Hamilton, 1984). We tested the possibility that we could use excised pericarps for auxin-induced ethylene evolution experiments. However, once the pericarps are removed from the mother plant, tissue senescence is accelerated. Twelve hours after pericarp removal, deseeded pericarps (SPNS) exhibited significant fresh weight loss and increased ethylene evolution compared to those attached to the mother plant (Figure 2.6 A and B). Therefore, in order to observe IAA-induced changes in ethylene evolution using the split-pericarp system, deseeded pericarps must be attached to the plant.

Even though 4-Cl-IAA promoted pericarp growth and IAA did not, 4-Cl-IAA stimulated similar ethylene levels and developmental profile of ethylene production as IAA in deseeded pericarps. Deseeded pericarp tissue remained responsive to 4-Cl-IAA and to a lesser extent, IAA, in the production of ethylene at least through 24 h after deseeding (Figure 2.8). Both auxins likely stimulated the regulatory enzyme 1-aminocyclopropane-1-carboxylate synthase (ACS) (Peck and Kende, 1995, 1998). Therefore, the difference between the two endogenous auxins does not lie in their ability to stimulate ethylene biosynthesis. Interestingly, when 4-Cl-IAA and IAA are applied simultaneously, the pericarp growth response is the same as 4-Cl-IAA alone (Reinecke and Ozga, 1995). In addition, pericarps which are treated with 4-Cl-IAA do not respond negatively to ethylene (Figure 2.4 A and B). These data suggest that ethylene perception or signal transduction may be uniquely altered by 4-Cl-IAA.

Ethylene is perceived by a family of five receptors that are similar to the two-component histidine kinases (Chang and Shockey, 1999). The receptors activate the constitutive triple response (CTR1) protein which is a negative regulator of ethylene response (Kieber et al., 1993). The transition protein called ethylene insensitive 2 (EIN2) relates the ethylene signal downstream from CTR1 to the transcription factors. The structure of EIN2 suggests that it is an integral membrane protein that acts as a bifunctional

signal transducer (Alonso et al., 1999). EIN2 mutants have been isolated in screenings for auxin transport inhibitor resistance in *Arabidopsis* roots (Fujita and Syono, 1996). Currently, EIN2 is the only point in the ethylene signal transduction pathway that has implicated an interaction with auxin (Wang et al., 2002). Future experiments to elucidate the mechanism by which 4-Cl-IAA blocks the perception of ethylene could focus on ethylene receptor expression, or the link between EIN2 and auxin in pea.

In pea fruit, seeds are required for pericarp elongation (Ozga et al., 1992; van Huizen et al., 1997; Ozga et al., 2003). GA_3 and 4-Cl-IAA can substitute for the seeds and stimulate pericarp growth (Ozga and Reinecke, 1999). An analysis of free 4-Cl-IAA in intact pericarps showed that the concentration from 3 to 6 DAA in the seeds (144.5 to 231 ng g^{-1} fwt) was much higher than in the pericarp (3.5 to 6 ng g^{-1} fwt) (Magnus et al., 1997). Previous work with 4-Cl-IAA suggests that the increase in growth observed in pericarp tissue is due in part to 4-Cl-IAA's unique ability to stimulate the expression of both *PsGA20ox1* (van Huizen et al., 1997) and *PsGA3ox1* (Ozga et al., 2003), thereby stimulating GA biosynthesis in this tissue. One of the roles of 4-Cl-IAA could be as a seed-derived factor which promotes GA biosynthesis in the pericarp, thereby stimulating growth (Ozga and Reinecke, 2003); the other, as a factor that blocks ethylene action resulting in a higher response to the GA produced.

The levels of free IAA in intact pea pericarp [3 DAA = 29; 6 DAA = 17 (ng g^{-1} fwt)] and seeds [3 DAA = 392.5; 6 DAA = 1495 (nL C_2H_4 g fwt^{-1} h^{-1})] from 3 to 6 DAA are higher than 4-Cl-IAA (Magnus et al., 1997). The level of IAA required to stimulate ethylene biosynthesis in plant tissue varies with type and developmental stage of the tissue (Peck et al., 1998). Further, tissue receptivity to ethylene will determine the extent of the response to ethylene (Peck et al., 1998). When pea stem tissue was treated with an auxin transport inhibitor (TIBA), epinasty occurred above the point of application, and lateral bud release below this point (Law and Davies, 1990). IAA may have accumulated to a sufficient concentration above the point and stimulated

ethylene release to cause the epinasty. A 50 μM application of IAA was high enough to stimulate ethylene biosynthesis in deseeded pea fruit (Figure 2.7 A) and accelerate senescence (Figure 2.7 B). By regulating the concentration of IAA in the young pea fruit, the plant could stimulate ethylene release, and thereby senescence if developmental problems occur within the ovary. In non-pollinated pea fruit, ethylene stimulates tissue senescence (Orzáeaz and Granell, 1997). One role of ethylene in pollinated pea fruit may be to regulate seed or fruit abortion. The plant could modify the concentration of IAA in its tissue to stimulate ethylene biosynthesis. 4-Cl-IAA could be used by the plant to control the specific tissue that is affected by the release of ethylene.

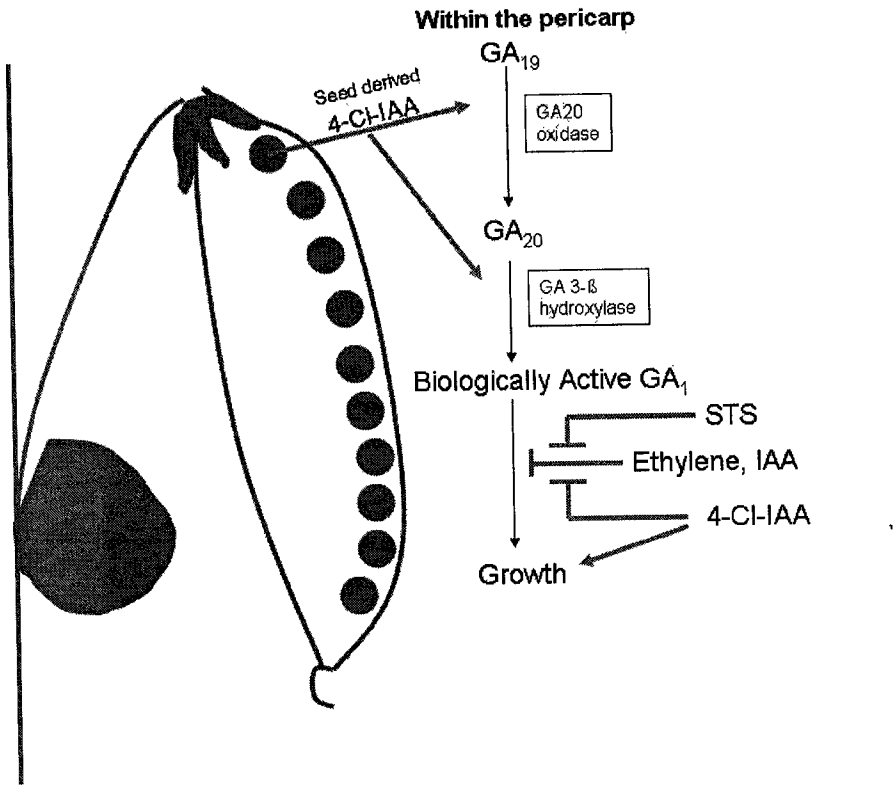
GA_3 did not alter the auxin-induced ethylene production profile for 4-Cl-IAA or IAA in deseeded pericarps (Figure 2.7 A and C). Without the addition of auxin to the pericarp tissue, GA_3-treated deseeded pericarp produced low levels of ethylene (Figure 2.7 E). GA_3-treated non-pollinated pea ovaries showed a progressive decrease in 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) mRNA levels up to the equivalent to 4 DAA (Orzáeaz et al., 1999). Although ACO is not the major regulatory enzyme for ethylene biosynthesis, it is responsible for the conversion of ACC to ethylene. Ethylene was shown to increase ACO transcript message in etiolated pea internodes (Peck and Kende, 1995); therefore, the progressive decrease in ACO transcript levels in the GA_3-treated non-pollinated pea ovaries may reflect the lower ethylene production by GA_3-treated tissue.

In summary, the similarity of the profiles of ethylene evolution in 4-Cl-IAA- and IAA-treated deseeded pericarps does not support the idea that the difference in the action of these two auxins is due to a differential effect on ethylene biosynthesis in pericarp tissue. One possible distinction between 4-Cl-IAA and IAA effects on pericarp growth is their differential ability to modify the tissue's ethylene perception or signal transduction. However, this alone cannot explain 4-Cl-IAA's ability to stimulate growth (and the lack of effect by IAA) since STS (ethylene action inhibitor) did not act to stimulate growth in IAA-treated deseeded pericarps (Reinecke and Ozga, 1995). In addition,

since IAA-induced ethylene blocks GA response in deseeded pericarps and 4-Cl-IAA blocks ethylene action, it is possible that 4-Cl-IAA from the seeds is transported to the pericaps and stimulates GA biosynthesis and also acts to block ethylene action, thereby promoting GA response in this tissue. A model of our proposed interactions of GA, auxin, and ethylene is diagrammed in Figure 9.

Figure 2.9: Hormone interactions in early pea fruit development

Model developed from the following research:
(Ozga et al., 1992; Reinecke and Ozga, 1995; van Huizen et al., 1995; van Huizen et al., 1997; Ozga and Reinecke, 1999; Ozga et al., 2002; Ozga and Reinecke, 2003; Ozga et al., 2003)



Literature Cited

Ahmad A, Andersen A, Engvild K (1987) Rooting, growth and ethylene evolution of pea cuttings in response to chloroindole auxins. Physiologia Plantarum **69:** 137-140

Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR (1999) EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. Science **284:** 2148-2152

Chang C, Shockey JA (1999) The ethylene-response pathway: signal perception to gene regulation. Current Opinion in Plant Biology **2:** 352-358

Fujita H, Syono K (1996) Genetic analysis of the effects of polar auxin transport inhibitors on root growth in *Arabidopsis thaliana*. Plant and Cell Physiology **37:** 1094-1101

Garcia-Martinez JL, Santes CM, Croker SJ, Hedden P (1991) Identification, quantitation and distribution of gibberellins in fruits of *Pisum sativum* L. cv. Alaska during pod development. Planta **184:** 523-530

Kende H (1993) Ethylene Biosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology **44:** 283-307

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR (1993) CTR1, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the Raf family of protein kinases. Cell **72:** 427-441

Law DM, Davies PJ (1990) Comparative Indole-3-Acetic Acid levels in the Slender Pea and Other Pea Phenotypes. Plant Physiology **93:** 1539-1543

Law DM, Hamilton RH (1984) Effects of Gibberellic Acid on Endogenous Indole-3-Acetic Acid and Indoleacetyl Aspartic Acid Levels in a Dwarf Pea. Plant Physiology **75:** 255-256

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

Ngo P, Ozga JA, Reinecke DM (2002) Specificity of auxin regulation of gibberellin 20-oxidase gene expression in pea pericarp. Plant Molecular Biology **49:** 439-448

Orzáeaz D, Blay R, Granell A (1999) Programme of senescence in petals and carpels of *Pisum sativum* L. Planta **208:** 220-226

Orzáeaz D, Granell A (1997) DNA fragmentation is regulated by ethylene during carpel senescence in *Pisum sativum*. The Plant Journal **11:** 137-144

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

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

Ozga JA, Reinecke DM (2003) Hormonal Interactions in Fruit Development. Journal of Plant Growth Regulation **22:** 73-81

Ozga JA, van Huizen R, Reinecke DM (2002) Hormone and Seed-Specific Regulation of Pea Fruit Growth. Plant Physiology **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 Physiology **131:** 1137-1146

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

Peck SC, Kende H (1998) 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 Molecular Biology **38:** 977-982

Peck SC, Pawlowski K, Kende H (1998) Asymmetric responsiveness to ethylene mediates cell elongation in the apical hook of peas. The Plant Cell **10:** 713-719

Reinecke DM, Ozga JA (1995). IAA and 4-Cl-IAA Effect on Pea Pericarp Growth, 15th International Conference on Plant Growth Substances, Minneapolis, MN, # 447.

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

Rodrigo MJ, Garcia-Martinez JL, Santes CM, Gaskin P, Hedden P (1997) The role of gibberellins A_1 and A_3 in fruit growth of *Pisum sativum* L. and the identification of gibberellins A_4 and A_7 in young seeds. Planta **201:** 446-455

Sponsel VM (1995) The biosynthesis and metabolism of gibberellins in higher plants. *In* PJ Davies, ed, Plant Hormones: Physiology, Biochemistry and Molecular Biology, Ed 2nd. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 66-97

van Huizen R, Ozga JA, Reinecke DM (1997) Seed and hormonal regulation of gibberellin 20-oxidase expression in pea pericarp. Plant Physiology **115:** 123-128

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 Physiology **109:** 1213-1217

Wang KL-C, Li H, Ecker JR (2002) Ethylene Biosynthesis and Signaling Networks. The Plant Cell **Supplement:** S131-S151

Warner HL, Leopold AC (1969) Ethylene Evolution from 2-Chloroethylphosphonic Acid. Plant Physiology **44:** 156-158

Chapter 3: Carbohydrate Partitioning in Young Pea Fruit

Introduction

In pea (*Pisum sativum*), early fruit development is dominated by ovary (pericarp) growth. However, the presence of developing seeds is required for pericarp elongation, since the removal or destruction of the seeds results in slowing of pericarp growth and eventual abscission (Ozga et al., 1992). Developmental coordination between the pericarp and seeds is likely moderated by phytohormones (Ozga and Reinecke, 2003)

Developing pea seeds and pericarps contain GAs (Rodrigo et al., 1997) and auxins (4-chloroindole-3-acetic acid [4-Cl-IAA] and indole-3-acetic acid [IAA]) (Magnus et al., 1997). During early pericarp growth, application of GA and 4-Cl-IAA to pollinated deseeded pericarps can substitute for seeds (Ozga and Reinecke, 1999) and stimulate growth. The other auxin, IAA is inhibitory to pericarp growth (Reinecke et al., 1995).

Pea plants metabolize gibberellins by the early 13-hydroxylation pathway: $GA_{12} \rightarrow GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20} \rightarrow GA_1$ (Sponsel, 1995). Using the pea split-pericarp system, van Huizen et al. (van Huizen et al., 1995; van Huizen et al., 1997) were able to show that 4-Cl-IAA stimulates expression of the gene that codes for the multi-functional enzyme that converts $GA_{12} \rightarrow GA_{20}$ (*PsGA20ox1)* and conversion of $GA_{19} \rightarrow GA_{20}$. Follow-up studies by Ozga et al. (2003) showed that 4-Cl-IAA also stimulated message of *PsGA3ox1*, which codes for the enzyme that converts GA_{20} to biologically active GA_1. IAA was ineffective in stimulating *PsGA20ox1 and PsGA3ox1* (Ngo et al., 2002; Ozga et al., 2003)

The pericarp is the primary sink for photoassimilates during early fruit development (Flinn and Pate, 1970). Sieve tubes (Peretó and Beltrán, 1987) and companion cell complexes carry sucrose by bulk flow from the leaflets to the developing pea ovary where they are unloaded either through the

symplasmic space by plasmodesmata, or into the apoplasmic space through specific transporters (Lalonde et al., 2003). At the site of unloading, invertase and sucrose synthase cleave sucrose into hexoses to maintain the sink strength of the tissue (Lalonde et al., 2003). Hexoses may be accumulated in vacuoles for temporary storage (Lalonde et al., 2003), or move into the chloroplast to be used for starch synthesis (Heldt, 1997). In a temporary storage organ, such as the young pea ovary, starch is stored in the chloroplasts ready to be mobilized to the surrounding pericarp tissue or to the seeds at the appropriate time (Heldt, 1997).

Pea pericarp growth is rapid from 2 to 5 DAA, and growth in length is essentially complete by 8 DAA (Ozga et al., 2002). Pericarp diameter increases from 6 to 12 DAA to accommodate the developing seeds (Ozga et al., 2003). As the pericarp rate of growth slows, the seed growth rate increases (rapid linear increase from 8 to 20 DAA; Ozga et al., 2003). During this time, pericarps transition from a sink to a source tissue for photoassimilates for the developing seeds (Flinn and Pate, 1970).

In order to understand the role of hormones in the coordination of seed and ovary development in pea, a detailed developmental profile of the carbohydrate status of pericarps and seeds and the effects of the hormones on these profiles is required. In this study, starch, sucrose and glucose levels were monitored in the developing pericarps and seeds from 0 to 20 DAA. The effects of gibberellin, auxins and ethylene on carbohydrate partitioning in the pericarp were determined during the period when pericarps act as the major sink for carbohydrates in the fruit (0 to 7 DAA).

We found that carbohydrate levels changed in the pericarps and seeds during key growth periods of pea fruit development. GA_3 and 4-Cl-IAA treatment of deseeded pericarps maintained pericarp starch, glucose, and sucrose levels comparable to pericarps with seeds. However, IAA and ethylene stimulated starch degradation and senescence in deseeded pericarps. IAA and ethylene also inhibited the starch maintenance effect of GA_3, but the ethylene action inhibitor, silver thiosulphate (STS), reversed this

effect. Ethylene did not affect the carbohydrate status in 4-Cl-IAA-induced deseeded pericarps. These data support the hypothesis that 4-Cl-IAA and IAA play different roles during pea fruit development. Specifically, GA and 4-Cl-IAA act to stimulate fruit growth and development, and IAA and ethylene act to inhibit it.

Materials and Methods

Plant Material

Five pea seeds (*Pisum sativum* L.) line I_3 (Alaska-type) per pot were planted in 2L soil containing Metro-mix 290 and sand (1:1) and grown in a Conviron growth chamber at 19/17°C (day/night) under a 16-hour photoperiod (400 μE · m^{-2} · s^{-1} from cool white fluorescent and incandescent lights). Plants were thinned when 15 cm tall to three uniform seedlings per pot. During plant development all lateral side shoots and the 1st and 2nd flowering nodes were removed. For treatments, one fruit per plant between the 3rd and 5th flowering nodes was used. Subsequent flowers and lateral buds were removed as they developed, the terminal apical meristem remained intact and the pericarp stayed attached to the plant during all experiments.

Growth Studies

For the intact pea fruit carbohydrate profile, fruits were tagged at anthesis and the developmental stage was determined by counting the days from tagging. All pericarps and seeds were harvested during late morning from one growth chamber. For treated fruit carbohydrate profile, two days after anthesis (DAA) pericarps (fruits) were either left intact (intact treatment), split down the dorsal suture (SP treatment) or split with removal of the seeds (SPNS and hormone treatments) as described by Ozga et al. (1992). GA_3, 4-Cl-IAA and/or IAA (50 μM in 0.1% [v/v] aqueous Tween 80) were applied immediately after deseeding to the inner pericarp wall (endocarp) and daily thereafter to 6 DAA (30 μL, 2 and 3 DAA; and 40 μL, 4, 5 and 6 DAA). Ethephon (30 μL; 250 or 1000 ppm) was applied once immediately after splitting and deseeding the

pericarp. When Ethephon was applied in combination with other hormone treatments, a 30 µL aliquot (250 or 1000 ppm) was applied one time, 90 minutes after the initial auxin and/or GA_3 solutions. For treatments requiring STS, a 20 µL aliquot (1mM) was applied once as a pre-treatment immediately after splitting and deseeding the fruit. Hormone solutions were applied 30 minutes after STS application. For all experiments, the pericarps were enclosed in a clear plastic bag to maintain moisture. Hormone-treated fruits were harvested during late morning at 3, 4, 5, 6, and 7 DAA (1, 2, 3, 4, and 5 days after initial treatment) and stored at -80°C for carbohydrate analysis.

Carbohydrate Analysis

Frozen pericarps and seeds were lyophilized. Lyophilized pericarps and seeds (minimum of 2 whole or ½ pericarps, or seeds from 2 fruits) were ground in liquid N_2 using a mortar and pestle to a fine powder with the exception of pericarp tissue that was 10 DAA and older which also contained some fibre pieces (of sclerenchyma tissue) after thoroughly grinding that would not completely break down to a powder. A 2 – 114 mg (pericarp) or 40 – 103 mg (seeds) dry weight sub-sample was extracted with 10 mL of 80% ethanol. The extract was placed at room temperature for 1 hour, then immersed in a 75-80°C water bath for five minutes, and then centrifuged at 3000 rpm for 10 – 15 minutes. The supernatant was removed and the pellet re-extracted with an additional 10 mL of 80% ethanol. After removal of the supernatant, 2 mL of dimethylsulphoxide (DMSO) was added to the pellet and the pellet was stored at -20°C prior to starch analysis. The supernatants were pooled, dried under vacuum, and the residue was resuspended in hexane (4 mL), followed by 4 mL water for organic solvent partitioning to remove chlorophyll and lipids. The sample was partitioned 3 times (4 mL each) with hexane and the aqueous phase was dried under vacuum and stored at -20°C until glucose and sucrose determination.

The dried sample was rehydrated with water (1 to 8 mL) before the glucose assay. A 50 µL aliquot of the sample was used. Invertase [(1500

$U*mL^{-1}$) Sigma I 4504] was added to one of a pair of aliquots to hydrolyze sucrose in the sample. The samples were incubated for 15 minutes at room temperature. Next, 1.5 mL of glucose oxidase/peroxidase (GOPOD 600 $U*L^{-1}$ glucose oxidase, 325 $U*L^{-1}$ peroxidase – Megazyme International Ireland) was added and the samples were incubated in closed disposable culture tubes for 20 minutes in a 50 °C water bath. The optical absorbance of each sample was read in a spectrophotometer (Varian Cary 1E UV-Visible) using 1.5 mL disposable cuvettes at 510 nm and compared to a glucose standard curve made from a dilution series (100%, 80%, 50%, 40%, 25%, 20% of glucose standard) of $1g*L^{-1}$ glucose stock. To test the efficiency of invertase, a 1 $g*L^{-1}$ sucrose stock standard was used. Each assay sample was run in duplicate. Sucrose was determined by subtracting total glucose of the buffer-treated sample from total glucose of the invertase-treated sample.

Total starch was determined based on the amyloglucosidase/α amylase method (Megazyme International Ireland ltd.). Three mL of α-amylase (50 $U*mL^{-1}$) was added to each tube, the contents were vortexed and incubated in boiling water for six minutes. After the incubation, 50 µL of amyloglucosidase (200 $U*mL^{-1}$) and 4 mL of 200 mM sodium acetate buffer (pH 4.5) were added to each sample. The samples were vortexed and incubated for 30 minutes at 50 °C. After the incubation, the volume of each sample was adjusted to 12 mL volume with water. An aliquot of 1 mL was taken from each tube and centrifuged (3000 rpm for 10 minutes). From that one mL, a second aliquot of 50 uL was used for the glucose analysis as described in the soluble sugar assay.

Results

Pericarp and Seed Growth

Intact pericarp growth (length, width, fresh weight and dry weight) was rapid from 2 to 9 DAA, and then slowed from 10 to 20 DAA (Figure 3.1, Figure 3.2 B and C). Additionally, pericarps increased in diameter from 7 to 12 DAA to accommodate the rapidly developing seeds (Figure 3.1 B and C). Seed growth (fresh and dry weight) increased rapidly from 8 to 20 DAA (Figure 3.2 B and C).

Starch profiles of pericarps and seeds of intact pea fruit

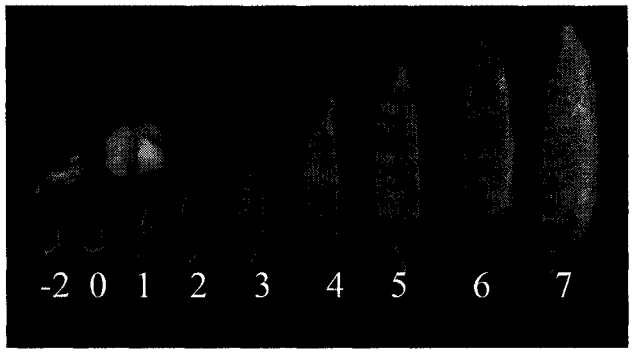
Pericarp starch levels on an mg dry weight basis were minimal at anthesis (0 DAA; 0.28 µMol glucose eq.*mg dwt^{-1}), increased by 2.7-fold after 24 h (1 DAA; 0.76 µMol glucose eq.*mg dwt^{-1}), and were maintained at this level from 1 to 4 DAA (Figure 3.3 B). Pericarp starch levels then increased by 1.5-fold from 4 to 5 DAA obtaining the highest starch levels per mg dry weight observed from 0 to 20 DAA, and remained at this level until 8 DAA (1 to 1.2 µMol glucose eq.*mg dwt^{-1}; Figure 3.3 A). From 8 to 12 DAA, the pericarp transitioned from a sink organ to a source organ for the seeds (characterized by a decrease in pericarp growth and rapid increase in seed growth) (Figure 3.2 B). During this transition period (8 to 12 DAA), pericarp starch levels decreased by 2.5-fold to 0.4 µMol glucose eq.*mg dwt^{-1}), and were maintained at this level until 16 DAA (Figure 3.3 A). From 16 to 20 DAA, pericarp starch levels decreased by half to 0.2 µMol glucose eq.*mg dwt^{-1} (Figure 3.3 A).

On the per organ level, pericarp starch levels were minimal from 0 to 3 DAA (0.75 µMol glucose eq.*$pericarp^{-1}$), then increased linearly from 4 to 8 DAA to reach a maximum of 390 µMol glucose eq.*$pericarp^{-1}$ at 8 DAA (Figure 3.3 B). During the pericarp sink-source transition period (8 to 12 DAA), pericarp starch decreased two-fold to 189 µMol glucose eq*$pericarp^{-1}$ by 12 DAA and remained at this level until 20 DAA (Figure 3.3 B).

Figure 3.1: Intact pea pericarp development

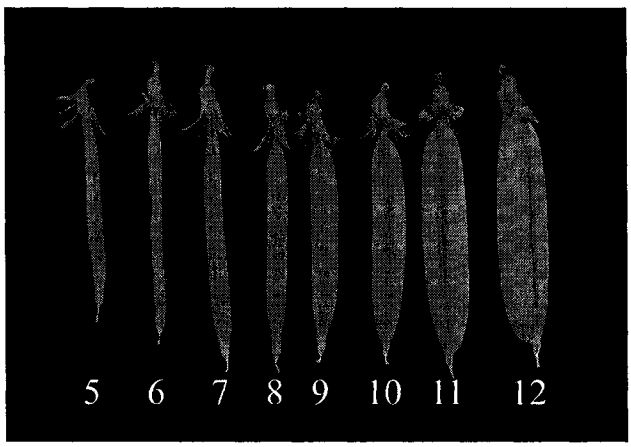
Development of pericarps during the rapid phases of growth in length, width (-2, 0, and 1 to 7 DAA; flower bud and flower at anthesis are -2 and 0 DAA, respectively; A) and diameter (5 to 12 DAA; B). Growth of pericarp in length, width, and diameter (-2 to 20 DAA; C). Data are means ± SE, n = 4 to 12.

A.



DAA

B.



DAA

C.

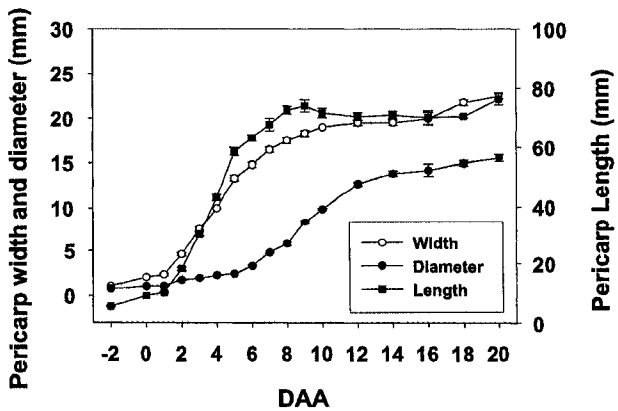


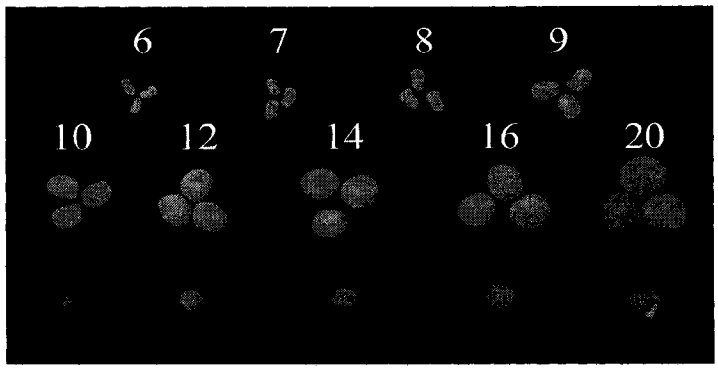
Figure 3.2: Pericarp and Seed Growth

A: Seed development from 6 DAA to cotyledon contact point (20 DAA: from left to right, row 1, 6 to 9 DAA seeds; row 2, 10, 12, 14, 16, and 20 DAA seeds; and row 3, embryos dissected from 10, 12, 14, 16, and 20 DAA seeds).

B: Growth in fresh weight of pericarps and seeds from -2 to 20 DAA. Data are means ± SE, n = 4 to 12 for pericarp fresh weight; n = 2 to 4 for seed fresh weight.

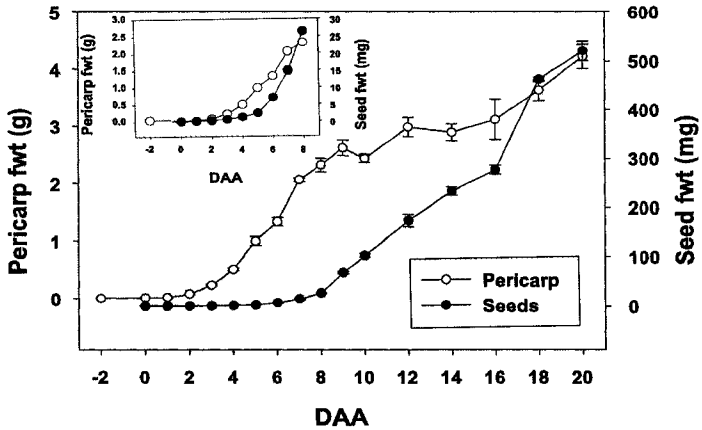
C: Growth in dry weight of pericarps and seeds from 0 to 20 DAA. Data are means ± SE, n = 4 to 12 for pericarp dry weight; n = 6 for seed fresh weight.

A.

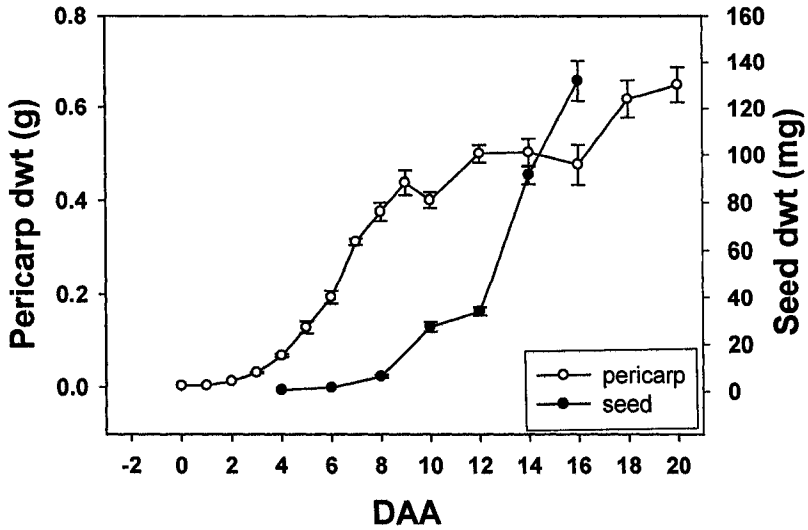


DAA

B.



C.



Seed starch level (on an mg per dwt basis) increased linearly from 4 DAA (0.23 µMol glucose eq.*mg dwt^{-1}) to 20 DAA (1.39 µMol glucose eq.*mg dwt^{-1}; Figure 3.3 A). On a per organ level, seed starch levels remained relatively low from 4 to 8 DAA (0.1 to 4 µMol glucose eq.*$seed^{-1}$), then increased by nearly 7-fold from 8 to 12 DAA (4 to 28 µMol glucose eq.*$seed^{-1}$; Figure 3.3 B) as the seed developed into a sink organ. From 12 to 20 DAA, seed starch increased linearly to reach a level of 330 µMol glucose eq*$seed^{-1}$ by 20 DAA (Figure 3.3 B).

Sucrose levels in pericarps and seeds of intact fruit

During the early stages of pericarp growth (0 to 4 DAA), sucrose levels were minimal (0.05 µMol glucose eq.*mg dwt^{-1}; 1.4 µMol glucose eq.*$pericarp^{-1}$; Figure 3.3 C and D). At 5 DAA, coincident with the maximum rate of pericarp growth in length (Figure 3.1 C), pericarp sucrose levels increased by 5-fold to 0.21 µMol glucose*mg dwt^{-1} (or 16-fold to 27 µMol glucose eq.*$pericarp^{-1}$) and these levels were maintained within the same range until 20 DAA when expressed per mg dwt (Figure 3.3 C). When expressed per organ, pericarp sucrose levels increased to 100 µMol glucose eq.*$pericarp^{-1}$ by 7 DAA and, in general, this level was maintained to 20 DAA (Figure 3.3 D).

Seed sucrose levels were also low at 4 DAA (0.08 µMol glucose eq.*mg dwt^{-1}) and increased 3.4-fold by 6 DAA and remained at this level, in general, until 20 DAA (Figure 3.3 C). When expressed per organ, seed sucrose levels were relatively low from 4 to 8 DAA (0.04 - 2 µMol glucose eq.*$seed^{-1}$), then increased 3.3-fold from 8 to 12 DAA to 6.5 µMol glucose eq.*$seed^{-1}$ (Figure 3.3 D). Seed sucrose levels increased linearly from 12 to 20 DAA to 81 µMol glucose eq.*$seed^{-1}$ by 20 DAA (Figure 3.3 D).

Glucose levels in pericarps and seeds of intact fruit

In pericarps, glucose levels were low from 0 to 2 DAA (0.1 - 0.6 µMol glucose eq.*mg dwt^{-1}), then increased linearly from 2 to 10 DAA to 0.75 µMol glucose eq.*mg dwt^{-1} by 10 DAA (Figure 3.3 E). Pericarp glucose levels were maintained between 0.50 to 0.75 µMol glucose eq.*mg dwt^{-1} from 12 to 20 DAA. On a per organ basis, pericarp glucose levels were low from 0 to 3 DAA (0.3 to 4.6 µMol glucose eq.*$pericarp^{-1}$), then increased rapidly from 4 to 7 DAA (from 18 to 190 µMol glucose eq.*$pericarp^{-1}$), then at a slower rate from 8 to 20 DAA (from 208 to 391 µMol glucose eq.*$pericarp^{-1}$; Figure 3.3 F). Seed glucose levels remained low throughout the developmental period studied (4 to 20 DAA; 0.01 to 0.03 µMol glucose eq.*mg dwt^{-1}; 0.005 to 6.9 µMol glucose eq.*$seed^{-1}$; Figure 3.3 E and F).

SP and SPNS control treatments

When pericarps were split 2 DAA and the seeds remained attached to the pericarp (SP treatment), pericarps increased in fresh weight by 20-fold from 2 to 7 DAA (0.06 to 1.18 g) (Figure 3.4 A). However, when the seeds were removed immediately after pericarp splitting, pericarp growth was inhibited and subsequently the tissue senesced (fresh weight loss due to senescence was evident by 6 DAA; Figure 3.4 B). Starch levels in SP pericarps were maintained between 0.5-1 µMol glucose eq.*mg dwt^{-1} from 2 to 7 DAA. SP pericarp glucose levels increased 11-fold from 2 to 5 DAA (0.1 to 0.7 µMol glucose eq.*mg dwt^{-1}) and remained at 0.7 µMol glucose eq.*mg dwt^{-1} until 7 DAA. SP pericarp sucrose levels were consistently lower than intact pericarp levels throughout the experiment; however, the increase in sucrose levels between 4 and 5 DAA in intact pericarps was also observed in SP pericarps (0.05 to 0.08 µMol glucose eq.*mg dwt^{-1}; Figure 3.3 C and D, Figure 3.4 A)
Seed removal (SPNS treatment) did not affect pericarp starch levels within the first 24 h after seed removal (3 DAA; Figure 3.4 B). However, after 48 h (4 DAA), pericarp starch levels declined to near zero and remained at this level until pericarp senescence (6-7 DAA; Figure 3.4 B). SPNS pericarp

glucose levels increased only three-fold from 2 to 5 DAA (0.1 to 0.4 µMol glucose eq.*mg dwt^{-1}), then decreased to 0.16 µMol glucose eq.*mg dwt^{-1} by 7 DAA. In addition, a significant increase in glucose levels was not concurrent with starch degradation from 3 to 4 DAA (Figure 3.4 B). Sucrose levels in SPNS pericarps were generally lower than in SP from 4 to 7 DAA (SP, average 0.07 ± 7.8124e-3 (SE) µMol glucose eq.*mg dwt^{-1} and SPNS average 0.0360 ± 8.1088e-3 (SE) µMol glucose eq.*mg dwt^{-1}; Figure 3.4 A and B).

IAA and Ethephon treatments

Application of IAA (50 µM) to deseeded pericarps did not stimulate pericarp growth; instead, pericarp senescence was stimulated compared to SPNS controls. Fresh weight decreases started earlier between 4 and 5 DAA (0.09 to 0.07 g) for IAA-treated pericarps compared to SPNS controls between 5 and 6 DAA (0.18 to 0.12 g) (Figure 3.4 B and C).

Twenty four hours after the initial IAA treatment, pericarp starch levels decreased 7-fold (0.67 to 0.13 µMol glucose eq.*mg dwt-1; Figure 3.4 C). By 48 h, pericarp starch levels further decreased to near zero and remained at this level until 7 DAA. Concomitant with starch degradation, pericarp glucose levels increased five-fold (0.06 to 0.32 µMol glucose*mg dwt^{-1}) in IAA-treated deseeded pericarps 24 h (3 DAA) after initial treatment and remained elevated for an additional 24 h (4 DAA), then gradually decreased to near zero by 7 DAA (Figure 3.4 C).

Application of Ethephon (1000 ppm) to deseeded pericarps stimulated pericarp senescence more rapidly than IAA. Ethephon-treated pericarps decreased in fresh weight by 4 DAA (0.1 to 0.08 g), compared to IAA at 5 DAA (0.09 to 0.07 g) (Figure 3.4 C and D). Ethephon-treated deseeded pericarps (1000 ppm; single application on first day of experiment), exhibited a similar carbohydrate profile as that of IAA-treated deseeded pericarps (Figure 3.4 D). Pericarp starch levels decreased 7-fold (0.67 to 0.13 µMol glucose eq.*mg dwt^{-1}) 24 h after the Ethephon treatment. By 48 h, pericarp

starch levels further decreased to near zero and remained at this level until 7 DAA. Pericarp glucose levels increased eight-fold (0.06 to 0.52 µMol glucose*mg dwt^{-1}) in deseeded pericarps 24 h (3 DAA) after Ethephon application, and glucose levels remained elevated for an additional 24 h (4 DAA), then gradually decreased to near zero by 7 DAA (Figure 3.4 C).

Sucrose levels for IAA- and ethephon-treated deseeded pericarp were maintained between 0.02 to 0.04 µMol glucose eq.*mg dwt^{-1} with one exception (Figure 3.4 C and D). Twenty four h after the initial (IAA) or only hormone (ethephon) application, pericarp sucrose levels increased two-fold in IAA-treated deseeded pericarps (0.04 to 0.08 µMol glucose eq.*mg dwt^{-1}) and decreased 14-fold in Ethephon-treated deseeded pericarps (0.04 to 0.003 µMol glucose eq.*mg dwt^{-1}). By 7 DAA, all carbohydrate levels measured in IAA- and Ethephon-treated deseeded pericarps were near zero, and pericarp fresh weight was lower than at 2 DAA (Figure 3.4 C and D).

GA_3, STS, and GA_3 combination hormone treatments

GA_3 (50 µM) treatment stimulated deseeded pericarp growth (12-fold increase in fresh weight from 2 DAA to 7 DAA; Figure 3.5 A). GA_3-treated deseeded pericarps maintained starch levels between 0.4 to 1 µMol glucose eq.*mg dwt-1 from 2 to 7 DAA (Figure 3.5 A). Pericarp glucose levels linearly increased (12-fold) from 2 to 7 DAA (Figure 3.5 A). Sucrose levels in GA_3-treated deseeded pericarps were 1.6 and 5.8 fold higher than SPNS control pericarps at 24 (3 DAA) and 48 h (4 DAA) after initial treatment, respectively, then decreased to that of SPNS from 5 to 7 DAA (Figure 3.5 A and Figure 3.4 B).

Application of IAA (daily application) or Ethephon (single application on day 1) to GA_3-treated deseeded pericarps inhibited the action of GA_3 on pericarp growth and starch maintenance (Figure 3.5 B and C). Pericarp starch levels dramatically decreased within the first 24 h after initial (IAA) or only (Ethephon) compound application and remained at these low levels until

7 DAA, similar to the IAA- and Ethephon-treated deseeded pericarps (Figure 3.5 B and C, Figure 3.4 C and D).

Interestingly, when IAA was applied to GA_3-treated deseeded pericarps, glucose levels did not increase concomitantly with starch degradation but after a 24 h delay (4 DAA), then decreased to minimal levels by 6 DAA (Figure 3.5 B). When Ethephon was applied to GA_3-treated deseeded pericarps, the glucose profile was similar but levels were lower than in Ethephon-treated deseeded pericarps (Figure 3.5 C and Figure 3.4 D). Sucrose levels were lower 24 h after the initial (IAA) or only (Ethephon) application to GA_3-treated deseeded pericarps (IAA + GA_3, 0.03 µMol glucose eq.*mg dwt^{-1}; Ethephon + GA_3, 0.05 µMol glucose eq.*mg dwt^{-1}; GA_3, 0.09 µMol glucose eq.*mg dwt^{-1}) but within the same range as GA_3-treated deseeded pericarps thereafter (Figure 3.5 A, B and C).

STS application (ethylene action inhibitor) to deseeded pericarps did not stimulate pericarp growth but did slow pericarp senescence (pericarps maintained fresh weight through 7 DAA; Figure 3.5 D). Although STS treatment did not inhibit starch degradation in deseeded pericarps, by 4 DAA, STS-treated deseeded pericarps had 6.5 times more starch than SPNS controls (0.2 compared to 0.03 µMol glucose eq.*mg dwt^{-1}) (Figure 3.5 D and Figure 3.4 B). Similar to the SPNS control, a concomitant increase in glucose levels was not observed with starch degradation in STS-treated deseeded pericarps.

When STS was applied as a pre-treatment to GA_3 plus IAA-, or GA_3 plus Ethephon-treated deseeded pericarps, the effects of IAA and Ethephon on GA_3-induced pericarp growth, starch content, and glucose profiles in deseeded pericarps were eliminated (Figure 3.5 E and F).

Figure 3.3: Starch (A and B), sucrose (C and D), and glucose (E and F) profiles of pericarps and seeds of intact fruit from 0 to 20 DAA.

Data are means ± SE, n = 4 to 6 (except 4 and 7 DAA seed, where n = 1)

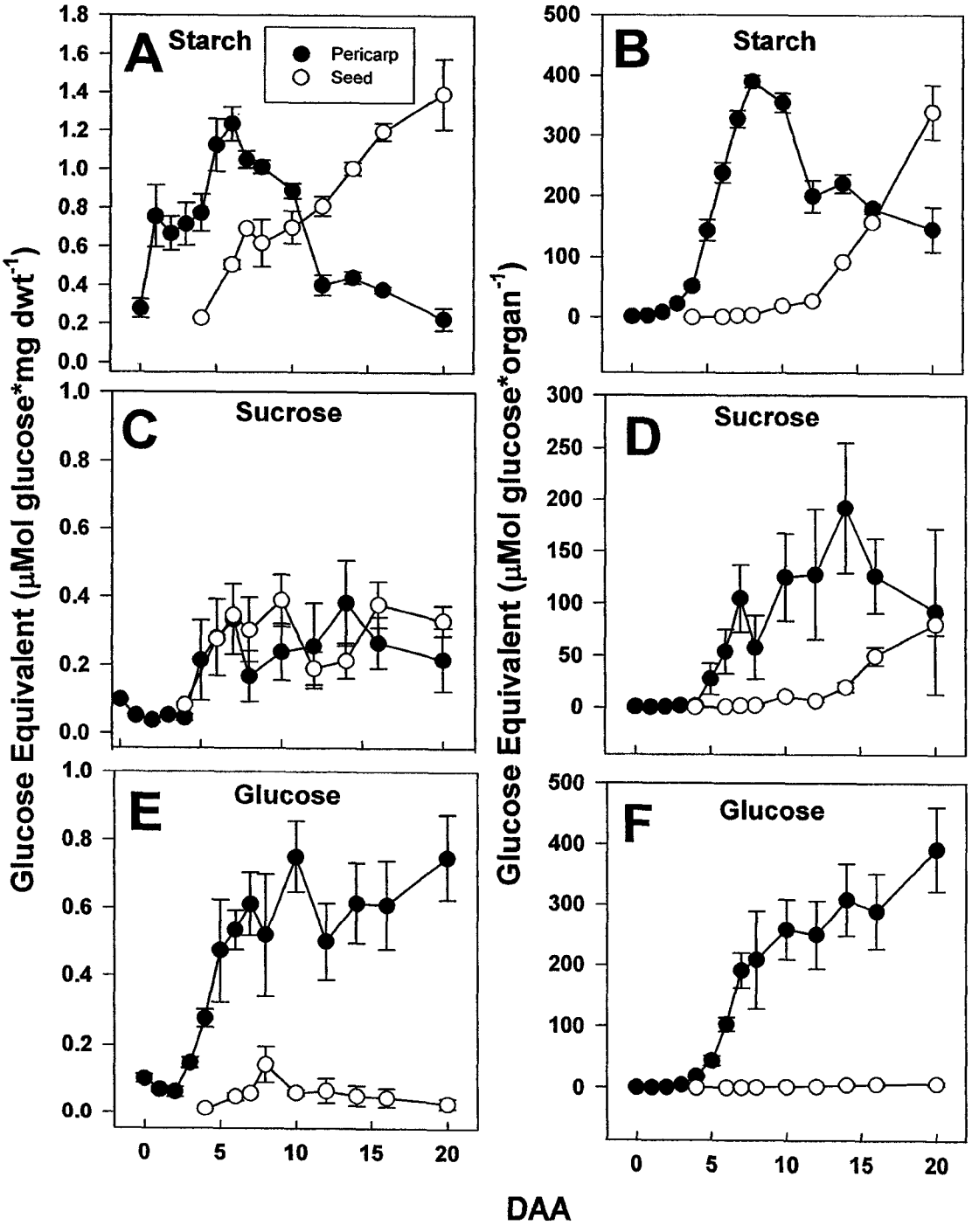


Figure 3.4: Starch, sucrose, glucose and fresh weight profiles of split-pericarps with seeds (SP; A), deseeded split-pericarp (SPNS; B), and deseeded pericarps treated with IAA (C) or Ethephon (D).

Two DAA pericarps were split (SP) or split and deseeded and immediately treated with 0.1% (v/v) aqueous Tween 80 (SP, SPNS) or IAA (50 μM) or Ethephon (1000 ppm) and once daily thereafter from 3 to 6 DAA. Data are means ± SE; n = 2 to 6 for carbohydrate data; n = 8 for fresh weight data.

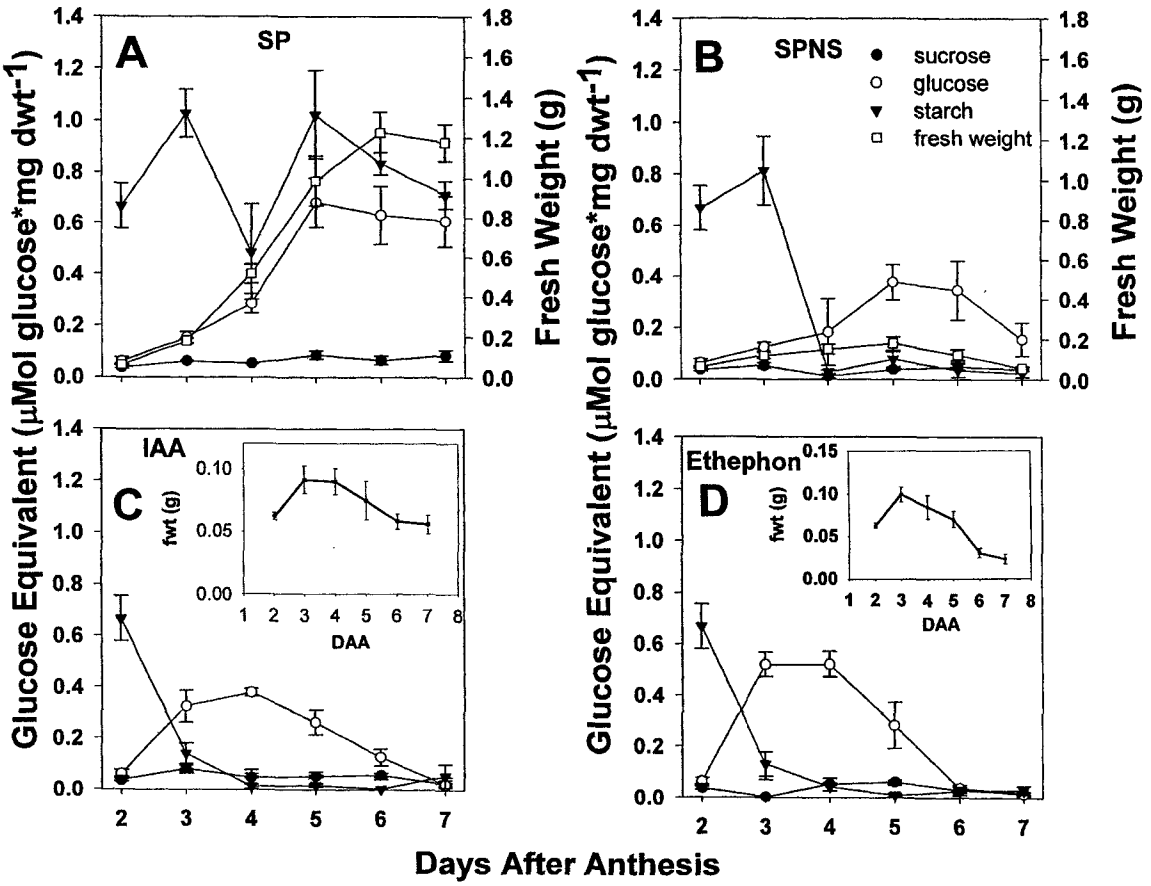
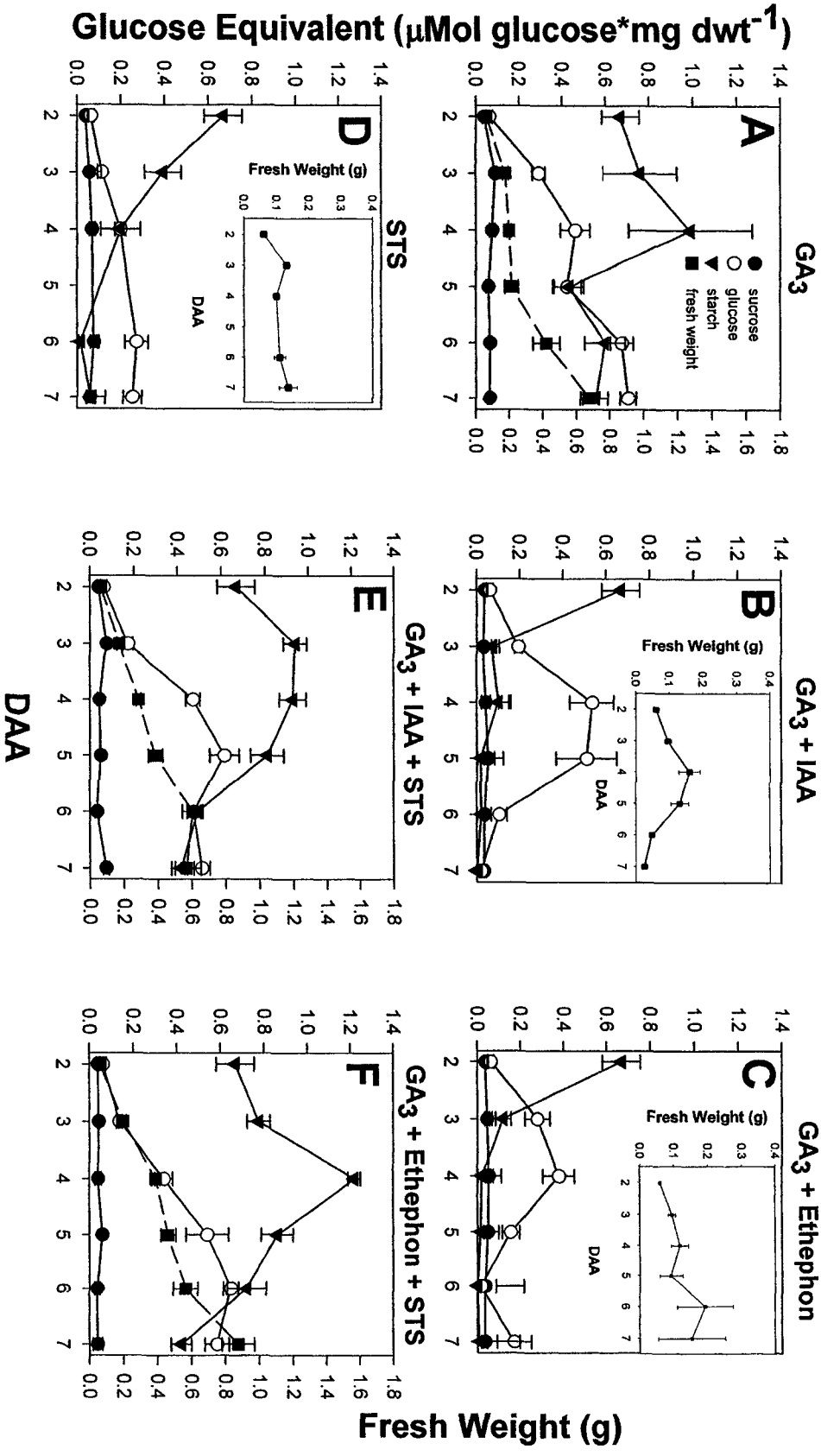


Figure 3.5: Starch, sucrose, glucose and fresh weight profiles of deseeded pericarps treated with GA_3 (A), GA_3 + IAA (B), GA_3 + Ethephon (C), STS (D), GA_3 + IAA + STS (E), and GA_3 + Ethephon + STS (F).

Two DAA pericarps were split and deseeded and immediately treated with 50 µM of GA_3, GA_3 + IAA or 0.1% (v/v) aqueous Tween 80 (SPNS) and daily thereafter for 5 days. Ethephon (1000 ppm) was applied only one time to the deseeded pericarps 90 minutes after the initial GA_3 treatment (GA_3 + Ethephon 1000 ppm, GA_3 + Ethephon + STS). STS (20 µl, 1 mM) was applied once on the first day of experiment (STS) or once as a pre-treatment 30 minutes prior to hormone treatment (GA_3 + IAA + STS and GA_3 + Ethephon + STS). Data are means ± SE; n = 2 to 4 for carbohydrate data; n = 8 for fresh weight data.



4-Cl-IAA and 4-Cl-IAA combination hormone treatments

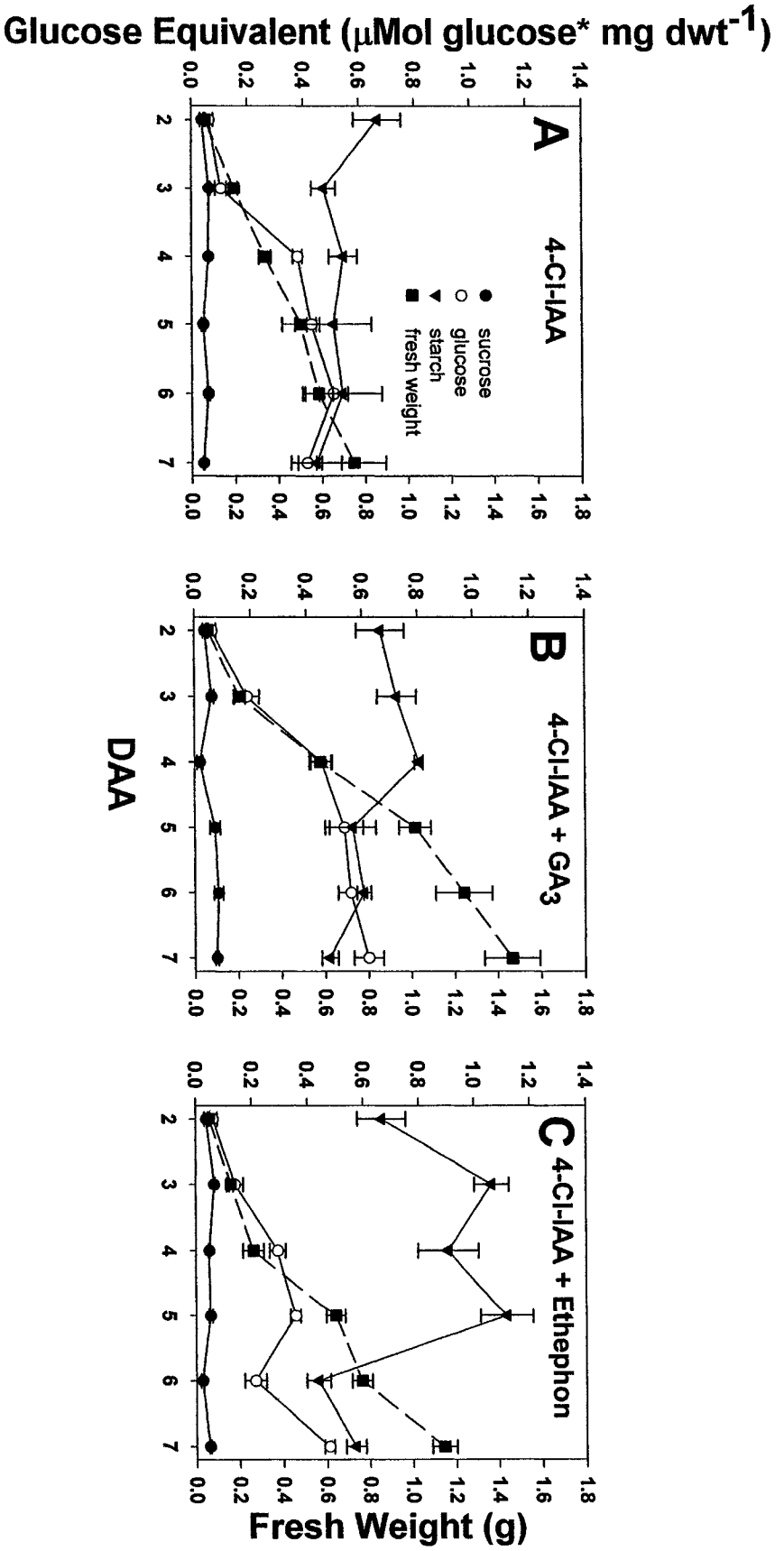
Similar to GA_3, 4-Cl-IAA stimulated pericarp growth, maintained starch levels, and increased glucose levels in deseeded pericarps (Figure 3.6 A). However, starch levels were lower in 4-Cl-IAA-treated than in GA_3-treated deseeded pericarps 24 (3 DAA) to 48 (4 DAA) h after initial hormone application. Pericarp glucose levels increased from 2 to 6 DAA, with the largest increase occurring between 3 and 4 DAA (Figure 3.6 A). Pericarp sucrose levels were maintained between 0.04 to 0.06 µMol glucose eq.*mg dwt^{-1} from 2 to 7 DAA (Figure 3.6 A).

When deseeded pericarps were treated with 4-Cl-IAA plus GA_3, growth was greater than deseeded pericarps treated with either 4-Cl-IAA or GA_3 alone (Figure 3.6 B). Pericarp starch levels were higher than 4-Cl-IAA-treated pericarps 24 to 48 h after initial hormone treatment, but not greater than GA_3-treated pericarps (Figure 3.5 A, Figure 3.6 A and C). Pericarp glucose and sucrose profiles were similar to 4-Cl-IAA- and GA_3-treated deseeded pericarps (Figure 3.5 A, Figure 3.6 A and C).

Application of Ethephon to 4-Cl-IAA-treated deseeded pericarps did not inhibit pericarp growth as observed for GA_3-treated deseeded pericarps (Figure 3.6 C and Figure 3.5 C). Pericarp starch levels were elevated from 3 to 5 DAA and glucose levels reduced from 4 to 6 DAA compared to deseeded pericarp treated with 4-Cl-IAA only (Figure 3.5 A and Figure 3.6 C). Sucrose levels varied from 0.02 to 0.08 µMol glucose eq.*mg dwt^{-1} (Figure 3.6 C).

Figure 3.6: Starch, sucrose, glucose and fresh weight profiles of deseeded pericarps treated with 4-Cl-IAA (A), 4-Cl-IAA + GA_3 (B), and 4-Cl-IAA + Ethephon (C).

Two DAA pericarps were split and deseeded and immediately treated with 50 µM of 50 µM 4-Cl-IAA, or 4-Cl-IAA + GA_3, or 0.1% (v/v) aqueous Tween 80 (SPNS) and daily thereafter for 5 days. Ethephon (1000 ppm) was applied only one time to the deseeded pericarps 90 minutes after the initial 4-Cl-IAA treatment. Data are the means ± SE; n = 3 to 4 for carbohydrate data; n = 8 for fresh weight data.



Discussion

The nature of phloem unloading along the path from the source tissue to the sink tissue is dynamic and, by responding to the prevailing source/sink balance, regulates photoassimilate supply to the terminal sink (Patrick, 1997). In developing pea fruit, the ovary (pericarp) is the primary sink for photoassimilates following pollination and fertilization of the ovules (2 to 8 DAA) until seed development advances to a point that the seeds become the major terminal sink. For most sinks, phloem unloading follows symplasmic routes (Patrick, 1997). During early pea pericarp development (0 to 8 DAA), Estruch and Beltrán (1991) monitored the activity of the sucrose metabolizing enzymes, invertase (cell wall-bound, cytosolic, and vacuolar) and sucrose synthase. Following anthesis (0 DAA), the activities of soluble acid (vacuolar) invertase and neutral (cytosolic) invertase increased and correlated with the maximum rates of pericarp growth. Pericarp cell-wall invertase and sucrose synthase activity remained low and unchanged from 0 to 8 DAA (Estruch and Beltrán, 1991). The higher activity of the inter-cellular pea pericarp sucrose metabolizing enzymes suggests that phloem unloading of sucrose into the pericarp tissue occurs symplasmically via the plasmodesmata, rather than apoplasmically during early pericarp development. These data are consistent with data from studies on other fruits where symplasmic phloem unloading into young developing fruit tissue usually occurs (Patrick, 1997).

During this early developmental period that included the rapid phase of pericarp growth in length (2-8 DAA), starch formed a temporary reserve that accumulated in the pericarp up to 8 DAA (Figure 3.3 A and B). Sucrose and glucose also accumulated in the pericarp during this rapid growth phase (Figure 3.3 C, D, E and F). A similar profile was observed in *P. sativum* cv. Finale, where starch levels (mg/pericarp) increased up to 10 DAA, followed by a gradual decrease during seed filling (15 to 20 DAA). Pericarp soluble carbohydrate (sucrose, glucose and fructose) levels (mg/pericarp) increased from less than 10 mg*pericarp^{-1} at 5 DAA to 75 mg/pericarp by 10 DAA (Rochat and Boutin, 1989).

Studies monitoring carbon transfer from the leaflets, stipules, and the pericarp of the first reproductive node of field pea (*Pisum arvense* L.) to the seeds showed that prior to 10 DAA, the pericarp did not export surplus carbon to the seeds (Flinn and Pate, 1970). The pericarp transitioned from a sink to a source of photoassimilates for the developing seeds by 10 DAA (Flinn and Pate, 1970). From 10 to 25 DAA, the pericarp supplied about 25% of the total carbon required by the developing seeds by contributing recycled respired carbon from the seeds (about 45%), fixing CO_2 from the atmosphere (about 10%) and mobilizing dry matter (likely starch) accumulated earlier in the life of the pod (about 45%). From 6 to 28 DAA, the leaflets and stipules subtending the same flowering node contributed about 40% of the total carbon required by the developing seeds. The remaining 35% of the carbon requirement for the developing seeds was acquired from sources external to this flowering node (Flinn and Pate, 1970).

The timing of the pericarp transition from a sink to a source organ for the seeds in this study (between 8 to 12 DAA) was similar to that observed by Flinn and Pate (1970). During the transition period (8-12 DAA), the growth rate of the pericarp slowed from a peak of 0.7 g $fwt^{-1}day^{-1}$ at 7 DAA to 0.3 g $fwt^{-1}day^{-1}$ by 12 DAA. Concurrently, pericarp starch levels also decreased (2.5-fold, 1.0 to 0.4 µMol glucose eq.*mg dwt^{-1}; 1.75-fold, 390 to 200 µMol glucose eq.*$pericarp^{-1}$, respectively; Figure 3.3 A and B). Meanwhile, during the pericarp transition phase (8 to 12 DAA), seed growth rate (11.5 to 35.9 mg*day^{-1}) and starch levels (0.6 to 0.8 µMol glucose eq.*mg dwt^{-1} and 4 to 27.5 µMol glucose eq.*$seed^{-1}$) increased (Figure 3.3 A and B).

In tomato fruit, the transition from starch to soluble sugar accumulation is accompanied by changes in the post sieve element pathway (Offler and Horder, 1992). During starch accumulation in the tomato outer pericarp, the plasmodesmata can accommodate sucrose transfer symplasmically (Offler and Horder, 1992). As the fruit develops, hexose accumulation occurs directly by the transfer and hydrolysis of sucrose into the apoplast, and then hexose uptake through the plasma membrane (Offler and Horder, 1992). During this later

period, the symplasmic route to the phloem parenchyma cells is maintained (Johnson et al., 1988; Offler and Horder, 1992) but structural restriction to symplasmic transfer is imposed by a significant decrease in plasmodesmatal cross-sectional area (Johnson et al., 1988; Offler and Horder, 1992).

Although detailed studies on symplasmic/apoplasmic transport have not been reported for pea fruit, phytohormones are likely signals to coordinate the pericarp's transition from sink to source tissue. One important enzyme expressed in both pea pericarps and seeds is *PsGA3ox1* which converts GA_{20} to biologically active GA_1. *PsGA3ox1* message levels peaked at 8 DAA and rapidly dropped from 8 to 12 DAA (Ozga et al., 2003), coinciding with the transition from sink to source tissue in the pericarps (Figure A.2 to A.7). The role of hormones has been implicated in regulation of plasmodesmatal conductance through calcium-related signaling pathways (Baluska et al., 2001) by altering callose deposition/hydrolysis and physiochemical states of key cytoskeleton components located around and in the neck regions of the plasmodesmata (Botha et al., 2000). Exogenous GA_3 has been shown to stimulate phloem unloading in non-pollinated pea pericarps (Peretó and Beltrán, 1987). One possibility is that the drop in pericarp *PsGA3ox1* levels from 8 to 12 DAA reduced the levels of biologically active GA_1, thereby reducing the extent of symplasmic transport of sucrose to the pea pericarp.

The increase in sucrose phosphate synthase activity which synthesizes sucrose in source and sink tissues (ap Rees, 1989) was observed by 8 DAA in pea pericarps. At the same time a decrease in pericarp soluble (vacuolar) acid invertase and soluble (cytosolic) neutral invertase activities (Estruch and Beltrán, 1991) occurred. These changes in the pericarp's sucrose synthesis and metabolism enzyme activities reflect a change in sucrose metabolism. These changes may be dictated by a reduction in symplasmic phloem unloading into the pericarp tissue coincident with an increase in phloem loading into the vascular tissue for transport of sucrose to the developing seeds, similar to what was observed in tomato (Offler and Horder, 1992).

As pericarp starch is mobilized to the seeds during the rapid phase of seed development, pericarp cellular integrity must be maintained during starch breakdown and therefore at least the initial stages of starch degradation must occur in the plastids. Studies of the sub-cellular distribution of the enzymes of starch metabolism in leaves suggest that starch is mobilized by the combined activities of endoamylase and starch phosphorylase (Stitt, 1984; Stitt and Steup, 1985). Characterization of the products of starch degradation in isolated chloroplasts revealed that glucose 1-phosphate produced by phosphorylase was metabolized within the chloroplast to triose phosphates and 3-phosphoglycerate, whereas the other products, glucose and maltose, were probably exported to the cytoplasm depending on the status of the cell. Both endoamylase and starch phosphorylase could be targets for phytohormone control during pericarp transitioning.

Reflective of a source tissue actively involved in phloem loading, pericarps from 15 to 20 DAA maintained lower starch levels than those prior to the sink/source transition. Sucrose and glucose levels (µMol/mg dwt) were maintained at levels similar to those observed immediately prior to the sink/source transition (Figure 3.3 A, C, and E).

In legume seeds, including pea, embryos are not vascularly connected to the maternal tissue. During early embryo development (0 to 8 DAA), the embryonic cells divide in a nutrient rich apoplastic liquid (endosperm) supplied by the surrounding seed coat. In *P. sativum* cv. Greenfeast, during the pre-storage phase (6 DAA) a few starch grains were observed in the testa (seed coat) in the cells underlying the outer epidermal layer and ovular cells adjacent to it (Hardham, 1976). In this study, seed starch levels between 4 to 8 DAA increased 36-fold (0.1 to 4 µMol glucose eq.*seed^{-1}); however, the amount of starch per seed was only 1.2% (µMol glucose eq.*seed^{-1}) that of seeds at 20 DAA (Figure 3 B). Sucrose levels per seed were relatively low from 4 to 8 DAA (Figure 3.3 C and D). Another pea cultivar, cv. Finale also had low starch and sucrose amounts (mg*seed^{-1}) in the embryo during the pre-storage phase of seed development (10 DAA) (Rochat and Boutin, 1989).

In the legume *Vicia fava*, the seed coat sieve elements also unload sucrose into the apoplastic space between the seed coat and the developing embryo (Weber et al., 1995). During the seed pre-storage phase in this species, seed coats had a much higher amount (μMol*g fwt^{-1}) of sucrose than either the apoplast or the cotyledons. However, hexose levels (μMol*g fwt^{-1}) were much higher in the apoplast and cotyledons compared to the seed coat (Weber et al., 1995). A developmental profile showed that cell wall-bound invertase enzyme activity increased from 12 DAA and peaked between 15 to 22 DAA (pre-storage phase), and decreased to low levels between 30 and 40 DAA (seed filling phase). Cell wall-bound invertase enzyme activity was found in the seed coat and not the embryo from 15 to18 DAA (Weber et al., 1995). One cell wall-bound invertase gene was expressed during the pre-storage phase in the thin-walled parenchyma of the seed coat, a region know to be the site of photoassimilate unloading (Weber et al., 1995). Weber et al. (1995) proposed the following model for sucrose metabolism in seed tissues during the pre-storage phase. After unloading from the seed coat, sucrose is hydrolyzed by cell wall-bound invertases. The hexose products are then loaded into the cotyledons and control carbohydrate partitioning by modulating the sucrose synthase/sucrose-phosphate synthase pathway [the high ratio of hexoses to sucrose within the cotyledons during the pre-storage phase postulated to maintain a low sucrose synthase/sucrose-phosphate synthase ratio and inhibit the synthesis of the main storage protein, legumin B; (Weber et al., 1995)]. Sucrose-phosphate synthase (forms sucrose 6-phosphate from UDP-glucose and fructose 6-phosphate) activity increased from 15 to 22 DAA (pre-storage phase) in the cotyledons (Weber et al., 1995). Weber et al. (1995) proposed that hexoses transferred into the embryo during the pre-storage phase are reformed into sucrose 6-phosphate by sucrose-phosphate synthase.

Weber et al. (1995) observed the degradation of the thin-walled parenchyma cells that express the invertase prior to the initiation of the seed storage phase and postulated that this event initiated the storage phase (characterized by a switch to a low hexoses/ sucrose ratio) in the seed of *V. fava*.

It is possible that the same event may initiate the seed storage phase in *P. sativum*, as the increase in seed sucrose levels (per organ basis) from 8 to 12 DAA (2 to 6.5 µMol glucose eq.*seed^{-1}; Figure 3.3 D) signals a change in sucrose metabolism in the seed prior to the initiation of the seed storage phase 12 to 14 DAA (Figure 3.3 D).

During pea seed filling (10-24 DAA) in nine cultivars including 'Alaska Sweet', sucrose synthase activity in the seed coat and in the embryo showed a significant positive, linear correlation to the relative rate of starch synthesis – a key indicator of sink strength (Dejardin et al., 1997). By cleaving sucrose, sucrose synthase in the seed coat and the embryo could maintain sucrose unloading into the seed during the seed-filling stage. The increase in starch accumulation in *P. sativum* cv. Alaska seeds by 14 DAA (Figure 3.3 A and B) is likely correlated with an increase in sucrose synthase activity in the seed coat and embryo of the seed. The low amount of glucose in the seed (Figure 3.3 E and F) during seed filling (14-20 DAA) also suggests that sucrose is unloaded, cleaved by sucrose synthase, and the resultant products, UDP-glucose and fructose, are used for starch synthesis in the cotyledons and metabolic processes within the embryo.

Seed *PsGA3ox1* levels increased 8-fold from 3 to 4 DAA [10 to 80 fg *mg fwt^{-1}, respectively; (Ozga et al., 2003)], preceding the initiation of endosperm development by approximately 1 day (Hardham, 1976). Seed starch, sucrose, and glucose levels increased [7-fold, 11-fold, and 14-fold, respectively (per seed basis); Figure 3.3 B, D and E] from 4 to 6 DAA following the increase in *PsGA3ox1* levels. Seed *PsGA3ox1* levels remained between 53 to 100 fg*mg fwt^{-1} from 4 to 14 DAA; by 20 DAA *PsGA3ox1* levels decreased to 23 fg*mg fwt^{-1} (Ozga et al., 2003). Seed starch was maintained between 0.2 to 1 µMol glucose eq.*mg dwt^{-1} and 0.1 to 27 µMol glucose eq.*seed^{-1} from 4 to 12 DAA (Figure 3 A and B) then increased linearly from 14 to 20 DAA (Figure 3.3 A and B). Sucrose was maintained between 0.3 and 10 µMol glucose eq.*seed^{-1} from 4 to 12 DAA then increased linearly from 14 to 20 DAA (Figure 3.3 D). These data suggest

that GA could trigger endosperm development and maintenance of pre-storage phase carbohydrate status in the seed.

Although the major sink tissue in the fruit was the pericarp from 0 to 8 DAA, the presence of the seeds is required for pericarp growth (Ozga et al., 1992) and sink status for photoassimilates. When seeds are removed, pericarp growth was inhibited, starch levels dropped to near zero by 48 hours, and the pericarps senesced [(Ozga et al., 1992) Figure 3.4 B]. The 24h delay in starch degradation in deseeded pericarps might be due to endogenous growth factors that are still present in the pericarps immediately following seed removal (Figure 3.4 A and B). Starch degradation in the SPNS pericarps from 3 to 4 DAA did not result in a concomitant accumulation of glucose in the tissue. The majority of the starch could be degraded by starch phosphorylase instead of α-amylase resulting in an abundance of glucose-1-phosphate, instead of glucose (Figure 4 B).

GA_3 can replace the requirement of the seeds for maintenance of pericarps growth and sink capacity during early pericarp development (Ozga et al., 1992). GA_3-treated deseeded pericarps maintained starch and glucose levels (μMol glucose eq.*mg dwt-1) within the same range as SP or intact pericarps (2 to 7 DAA). GA_3 increased sucrose levels in deseeded pericarps compared to the SPNS control at 24 h (3 DAA) and 48 h (4 DAA) after initial treatment (Figure 3.5 A and Figure 3.4 B). Peretó and Beltrán (1987) also found that GA_3 treatment significantly stimulated [C^{14}]-sucrose accumulation in non-pollinated pea ovaries by increasing phloem unloading at the sink. The increase in phloem unloading by exogenous GA_3 could be a result of its effect on invertase activity. Estruch and Beltrán (1991) found that daily GA (20 μL, 300 mM) treatment to non-pollinated ovaries from the equivalent of 2 DAA increased pericarp cytosolic, vacuolar, and cell wall-bound invertase activities by the equivalent to 4 DAA. The peak in GA-induced invertase activities also coincided with the peak in GA-treated ovary growth rate (at the equivalent of 4 DAA). In shorter term studies, GA treatment increased cytosolic and cell wall-bound invertase activities within 90 min after GA application to non-pollinated ovaries. GA treatment maintained vacuolar invertase activity in non-pollinated ovaries

compared to the loss of activity observed in the untreated controls. Increasing cytosolic and cell-wall bound invertase activities via GA may be a rapid way to increase phloem unloading of sucrose into the pericarps via symplasmic and apoplasmic routes, whereas vacuolar invertase is likely more important for longer-term modulation of cellular sucrose levels.

4-Cl-IAA is a naturally occurring auxin in pea seeds and pericarps (Magnus et al., 1997) that stimulates pericarp GA biosynthesis, specifically, the conversion of GA_{19} to GA_{20} (van Huizen et al., 1995) and the message level of *PsGA3ox1* (Ozga et al., 2003). Similar to GA_3, 4-Cl-IAA maintained starch levels and increased glucose levels in deseeded pericarps (Figure 6 A). However, starch levels were lower in 4-Cl-IAA-treated compared to GA_3-treated deseeded pericarps at 24h (3 DAA) and 48 h (4 DAA) after initial hormone application, suggesting that 4-Cl-IAA is not equivalent to GA in this process. When non-pollinated ovaries were treated with 2,4-dichlorophenoxyacetic acid (2,4-D, a synthetic auxin) at the equivalent to 2 DAA, vacuolar, cytosolic, and cell wall invertase activities increased by the equivalent to 4 DAA (Estruch and Beltrán, 1991). However, in shorter term studies with GA_3 or 2,4-D- treated non-pollinated ovaries, only GA_3 stimulated cell-wall invertase activities [within 1 h after application; (Estruch and Beltrán, 1991)]. These data suggest that the naturally occurring auxin, 4-Cl-IAA, is also important for maintenance of sink potential in the pea pericarp.

When 4-Cl-IAA and GA_3 were applied in combination, growth (fresh weight) was greater than either treatment applied alone [(Ozga and Reinecke, 1999), Figure 6 B]. Starch levels (per mg dwt) were higher than 4-Cl-IAA-treated deseeded pericarps, but not greater than GA_3-treated deseeded pericarps (Figure 3.6 A and B, Figure 3.5 A). Likely, the 4-Cl-IAA plus GA_3 treatment stimulated an increase in phloem unloading of sucrose into the pericarp compared to either applied alone and this additional carbohydrate was used to enhance pericarp growth instead of increase starch levels.

IAA (50 mM) inhibited deseeded pericarp growth both alone and in combination with GA_3 [(Reinecke and Ozga, 1995; Reinecke et al., 1995); Figure

3.4 C and Figure 3.5 B]. IAA also enhanced starch degradation in deseeded pericarps within 24h after initial application (Figure 3.4 C). One of auxin's known effects is to stimulate ethylene biosynthesis via 1-aminocyclopropane-1-carboxylic acid synthase (ACS) [(Kende, 1993); Chapter 2]. Silver thiosulphate (STS - an ethylene action inhibitor) is able to block the inhibitory effect of IAA on GA_3-treated deseeded pericarps [(Reinecke and Ozga, 1995); Figure 3.5 E and F], strongly suggesting that IAA-induced ethylene is causing the growth inhibition. Ethylene [produced from Ethephon (1000 ppm)] and IAA-induced production of ethylene both stimulate starch degradation earlier than deseeded pericarp controls (2 to 7 DAA) (Figure 3.4 C and D). Ethylene inhibited the starch maintenance effects of GA_3 application, and this effect was reversed by the ethylene action inhibitor silver thiosulphate (STS) (Figure 3.5 E and F). One role for endogenous ethylene in fruit development may be to moderate the growth stimulating effect of GA possibly resulting in pericarp senescence or changing a sink tissue into a source tissue for photoassimilates.

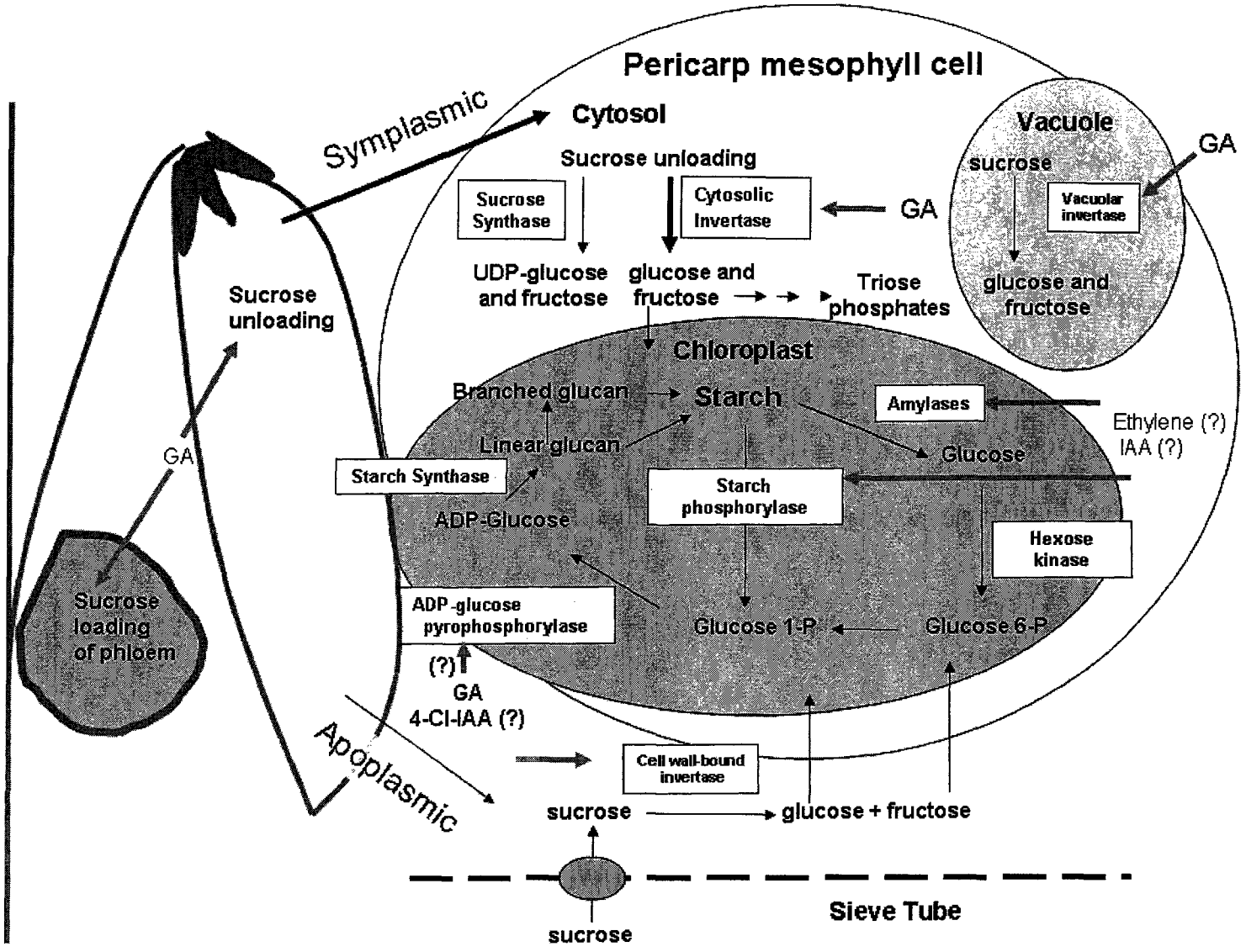
In regards to sugar sensing, the *Arabidopsis* glucose insensitive mutant (gin1) can be phenocopied by treating wild-type seedlings with 1-aminocyclopropane-1-carboxylic acid (ACC) (Zhou et al., 1998). Hexokinase-dependent pathways (of which gin1 is a factor) coordinate regulation of many developmental transitions in *Arabidopsis.* Another possible role for endogenous ethylene in fruit development may be to modulate the pericarp's level or sensitivity to hexoses.

Application of Ethephon (1000 ppm) to 4-Cl-IAA-treated deseeded pericarps did not inhibit pericarp growth (Figure 3.6 C). Starch, sucrose and glucose profiles were similar to or greater than 4-Cl-IAA-treated pericarps. These results show that 4-Cl-IAA blocks the effect of ethylene on starch degradation in pea pericarps and confirms the hypothesis that 4-Cl-IAA and IAA play different roles during pea fruit development. Specifically, that 4-Cl-IAA acts to stimulate fruit growth and development, and IAA via ethylene acts to inhibit it.

Pea fruit growth is a coordinated dynamic event between the pericarp and the seeds. We propose the following model of hormone regulation of

carbohydrate partitioning during early pea fruit development (Figure 3.7). Firstly, photoassimilates are imported into the pericarps under the control of the developing seeds. Seed-derived 4-Cl-IAA stimulates GA biosynthesis within the pericarp leading to an increase in biologically active GA_1 and promotion of GA tissue sensitivity (by blocking ethylene action). The higher pericarp GA_1 level and tissue sensitivity increase sucrose unloading into the pericarp, in part, by stimulating pericarp invertase activities. The transition from the pericarps as a sink to a source tissue for photoassimilates for the seed could be regulated by a decrease in GA_1, possibly in conjunction with an increase in ethylene. Further experiments monitoring the dynamic flow of photoassimilates within the fruit could uncover how phytohormones are directing sucrose unloading.

Figure 3.7: Model of the role of hormones in carbohydrate partitioning in pre-transition pea pericarps



Literature Cited

ap Rees T (1989) Hexose phosphate metabolism by nonphotosynthetic tissues of higher plants. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 14. Academic Press, London, pp 1-34

Baluska F, Cvrckova F, Kendrick Jones J, Volkmann D (2001) Sink plasmodesmata as gateways for phloem unloading. Myosin VIII and calreticulin as molecular determinants of sink strength. Plant physiology **126:** 39-46

Botha CEJ, Cross RHM, van Bel AJE, Peter CE (2000) Phloem loading in the sucrose export-defective (SXD-1) mutant maize is limited by callose deposition of plasmodesmata in bundle sheath vascular parenchyma interface. Protoplasma **214:** 65-72

Dejardin A, Rochat C, Wuilleme S, Boutin JP (1997) Contribution of sucrose synthase, ADP-glucose pyrophosphorylase and starch synthase to starch synthesis in developing pea seeds. Plant Cell Environment **20:** 1421-1430

Estruch JJ, Beltrán JP (1991) Changes in invertase activities precede ovary growth induced by gibberellic acid in *Pisum sativum*. Physiologia Plantarum **81:** 319-326

Flinn AM, Pate JS (1970) A quantitative study of carbon transfer from pod and subtending leaf to the ripening seeds of the field pea (*Pisum arvense* L.). Journal of Experimental Botany: 71-82

Hardham AR (1976) Structural aspects of the pathways of nutrient flow to the developing embryo and cotyledons of *Pisum sativum* L. Australian Journal of Botany **24:** 711-721

Heldt H-W (1997) Polysaccharides. *In* Plant Biochemistry and Molecular Biology. Oxford University Press Inc., New York, pp 219-246

Johnson C, Hall JL, Ho LC (1988) Pathways of uptake and accumulation of sugars in tomato fruit. Annals of Botany **61:** 593-603

Kende H (1993) Ethylene biosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology **44:** 283-307

Lalonde S, Tegeder M, Throne-Holst M, Frommer WB, Patrick JW (2003) Phloem loading and unloading of sugars and amino acids. Plant, Cell and Environment **26:** 37-56

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

Ngo P, Ozga JA, Reinecke DM (2002) Specificity of auxin regulation of gibberellin 20-oxidase gene expression in pea pericarp. Plant Molecular Biology **49:** 439-448

Offler CE, Horder BA (1992) The cellular pathway of short distance transfer of photosynthates in developing tomato fruit. Plant Physiology **99S:** 241

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

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

Ozga JA, Reinecke DM (2003) Hormonal Interactions in Fruit Development. Journal of Plant Growth Regulation **22:** 73-81

Ozga JA, van Huizen R, Reinecke DM (2002) Hormone and seed-specific regulation of pea fruit growth. Plant Physiology **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 Physiology **131:** 1137-1146

Patrick JW (1997) Phloem unloading: Sieve element unloading and post-sieve element transport. Annual Review of Plant Physiology and Plant Molecular Biology **48:** 191-222

Peretó JG, Beltrán JP (1987) Hormone-directed sucrose transport during fruit set induced by gibberellins in *Pisum sativum*. Physiologia Plantarum **69:** 356-360

Reinecke DM, Ozga JA (1995). IAA and 4-Cl-IAA effect on pea pericarp growth, 15th International Conference on Plant Growth Substances, Minneapolis, MN, 447.

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

Rochat C, Boutin J-P (1989) Carbohydrates and nitrogenous compounds changes in the hull and in the seed during the pod development of pea. Plant Physiology and Biochemistry **27:** 881-887

Rodrigo MJ, Garcia-Martinez JL, Santes CM, Gaskin P, Hedden P (1997) The role of gibberellins A_1 and A_3 in fruit growth of *Pisum sativum* L. and the identification of gibberellins A_4 and A_7 in young seeds. Planta **201:** 446-455

Sponsel VM (1995) The biosynthesis and metabolism of gibberellins in higher plants. *In* PJ Davies, ed, Plant Hormones: Physiology, Biochemistry and Molecular Biology, Ed 2nd. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 66-97

Stitt M (1984) Degradation of starch in chloroplasts: a buffer to sucrose metabolism. *In* DH Lewis, ed, Storage Carbohydrates in Vascular Plants. Cambridge University Press, Cambridge, pp 205-229

Stitt M, Steup M (1985) Starch and sucrose degradation. *In* R Douce, D Day, eds, Encyclopedia of Plant Physiology, Vol 18. Springer-Verlag, Berlin, pp 347-390

van Huizen R, Ozga JA, Reinecke DM (1997) Seed and Hormonal Regulation of Gibberellin 20-Oxidase Expression in Pea Pericarp. Plant Physiology **115:** 123-128

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 Physiology **109:** 1213-1217

Weber H, Borisjuk L, Heim U, Buchner P, Wobus U (1995) Seed coat-associated invertases of fava bean control both unloading and storage functions: cloning of cDNAs and cell type-specific expression. Plant Cell **7:** 1835-1846

Zhou L, Jang JC, Jones TL, Sheen J (1998) Glucose and ethylene signal transduction crosstalk revealed by an *Arabidopsis* glucose-insensitive mutant. Proceedings of the National Academy of Sciences of the United States of America **95:** 10294-10299

Chapter 4: Summary and Conclusions

The objective of this research was to determine why 4-Cl-IAA is active and IAA is not active in stimulating pericarp growth. Specifically:

1. to test if the difference in the activity (with respect to growth) of 4-Cl-IAA and IAA in pea pericarp tissue is due to a differential effect on ethylene biosynthesis or action.

2. to test if ethylene is involved in the differential activity of 4-Cl-IAA with GA_3 (synergistic growth of pericarp) and IAA with GA_3 (inhibits GA_3-induced growth of pericarp).

3. to further understand how hormones (GA, auxin, and ethylene) affect carbohydrate partitioning into pea fruit.

Previous research has shown that pea fruit development is regulated by seeds (Ozga et al., 1992). Further experiments with the split-pericarp system have indicated that exogenous GA_3 and 4-Cl-IAA can replace the requirement of the seeds for pericarp elongation (Ozga and Reinecke, 1999). However, exogenous IAA was inhibitory when applied by itself or in combination with GA_3 (Reinecke and Ozga, 1995).

Chapter 2 of this thesis explored the effect of 4-Cl-IAA and IAA on ethylene evolution and reconfirmed their differential effect on pea pericarp growth (Reinecke and Ozga, 1995; Ozga and Reinecke, 1999). Both 4-Cl-IAA and IAA stimulated ethylene evolution similarly in deseeded pericarps. IAA and IAA + GA_3 treatment inhibited pericarp growth through the action of ethylene. Direct application of ethylene was also found to inhibit GA_3-stimulated pericarp growth. However, ethylene evolution due to 4-Cl-IAA

treatment when applied separately or in combination with GA_3 showed no inhibitory effect on pericarp growth. The enhanced rate of ethylene production due to 4-Cl-IAA-treatment does not have an effect on its stimulation of pea pericarp growth.

Further experiments on the expression of genes involved in ethylene biosynthesis would help in understanding the role of ethylene in early pea fruit growth. A study on genes encoding 1-aminocyclopropane-1-carboxylate synthase (ACS) isoforms in etiolated pea seedlings showed the differential response of ethylene biosynthesis to wounding and auxin treatment (Peck and Kende, 1998). Previously cloned ACS gene sequences of pea seedlings could be used to identify ACS isolates that are expressed in pea fruit. The pea fruit ACS gene(s) could be monitored at both the activity and expression levels to corroborate ethylene evolution rates observed in Chapter 2 experiments.

Silver thiosulphate (STS) was used as a synthetic ethylene action inhibitor to test the effect of ethylene on pea fruit growth. However, STS may have some other additional effects on tissue growth besides blocking ethylene action. Thus, the results observed when deseeded pericarps were treated with STS, could be reconfirmed by similarly conducted experiments using a different ethylene action inhibitor, such as 1-methylcyclopropene (1-MCP).

Based on the observations made in Chapter 2, further studies will be needed to examine the role of ethylene in pea fruit development. At present, ethylene signal transduction understanding is based mainly on ethylene insensitive or hypersensitive mutants of *Arabidopsis* sp. (McCourt, 1999). Though peas are distantly related to *Arabidopsis* (Fabaceae vs. Brassicaceae) (Woodland, 1997), results from *Arabidopsis* could be used to screen for similar genes in pea. Already, pea equivalents for the ethylene receptor gene have been identified in pea ovaries (Orzáeaz et al., 1999). 4-

Cl-IAA and IAA have similar effects in pea stem tissue, unlike in pea fruit (Reinecke, 1999). Using transgenic pea plants that involve specific expression of genes from the ethylene signal transduction pathway in the fruit, we could devise experiments to help in the understanding of ethylene's role in pea fruit development.

In this study with pea fruit, we were interested in how phytohormones could regulate sink strength in the pea pericarp. Analysis of carbohydrate status in intact pericarps revealed a decrease in pericarp starch accumulation around 8 DAA (Chapter 3), followed by an increase in glucose levels. By using 2 to 7 DAA deseeded pericarps, we tested the effect of GA_3, IAA, 4-Cl-IAA, Ethephon and the ethylene action inhibitor, STS on starch, sucrose and glucose accumulation. GA_3 and 4-Cl-IAA maintained pericarp starch levels throughout the experiment. However, IAA and Ethephon immediately stimulated starch degradation when applied alone or with GA_3. STS prevented IAA and Ethephon's inhibitory effect on GA_3-treated growth.

Current experiments in our research group to examine how bioactive GA influences early pea fruit growth include gene expression studies and manipulation of genes in the gibberellin biosynthesis pathway. Pericarp starch levels were compared to mRNA levels of gibberellin 3ß-hydroxylase, the enzyme that converts GA_{20} to bioactive GA_1 (Appendix). The profile of starch accumulation and mRNA levels were similar. 'Alaska' pea plants that overexpress or underexpress gibberellin 3 ß-hydroxylase in the fruit have been engineered by our group. Further experiments with these transgenic peas would help us to understand the action of gibberellin on starch, sucrose and glucose levels of the pericarp.

The stage when pericarps transition from a sink to a source of photoassimilates for the seeds is a key part of fruit development. Further studies to monitor the role of phytohormones at this stage could lead to the manipulation of the timing of the transition. The experiments from Chapter 3

suggest that ethylene could be used as a signal for starch degradation within the pericarp, and its transfer to the developing seed. The use of ethylene action inhibitors to manipulate ethylene perception by the fruit would allow us to better understand the role of ethylene during this transition stage.

In conclusion, pea fruit development is a dynamic event where phytohormones influence fruit growth. Ethylene is an inhibitor of pea fruit growth and 4-Cl-IAA and IAA stimulate ethylene biosynthesis similarly. 4-Cl-IAA, unlike IAA is modifying the pericarp's sensitivity to ethylene possibly by preventing ethylene perception or ethylene signal transduction. 4-Cl-IAA has additional growth promoting effects separate from blocking the inhibitory effect of ethylene, since application of STS did not stimulate pericarp growth. 4-Cl-IAA could be transported from the seeds to the pericarp to block ethylene action and stimulate GA biosynthesis, thereby promoting GA response in this tissue.

Literature Cited

McCourt P (1999) Genetic Analysis of Hormone Signaling. Annual Review of Plant Physiology and Plant Molecular Biology **50:** 219-243

Orzáeaz D, Blay R, Granell A (1999) Programme of senescence in petals and carpels of *Pisum sativum* L. Planta **208:** 220-226

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

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

Peck SC, Kende H (1998) 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 Molecular Biology **38:** 977-982

Reinecke DM (1999) 4-Chlorindole-3-acetic acid and plant growth. Plant Growth Regulation **27:** 3-13

Reinecke DM, Ozga JA (1995). IAA and 4-Cl-IAA Effect on Pea Pericarp Growth, 15th International Conference on Plant Growth Substances, Minneapolis, MN, # 447.

Woodland DW (1997) Contemporary Plant Systematics, Ed 2nd. Andrews University Press, Berrien Springs, Michigan

Appendix

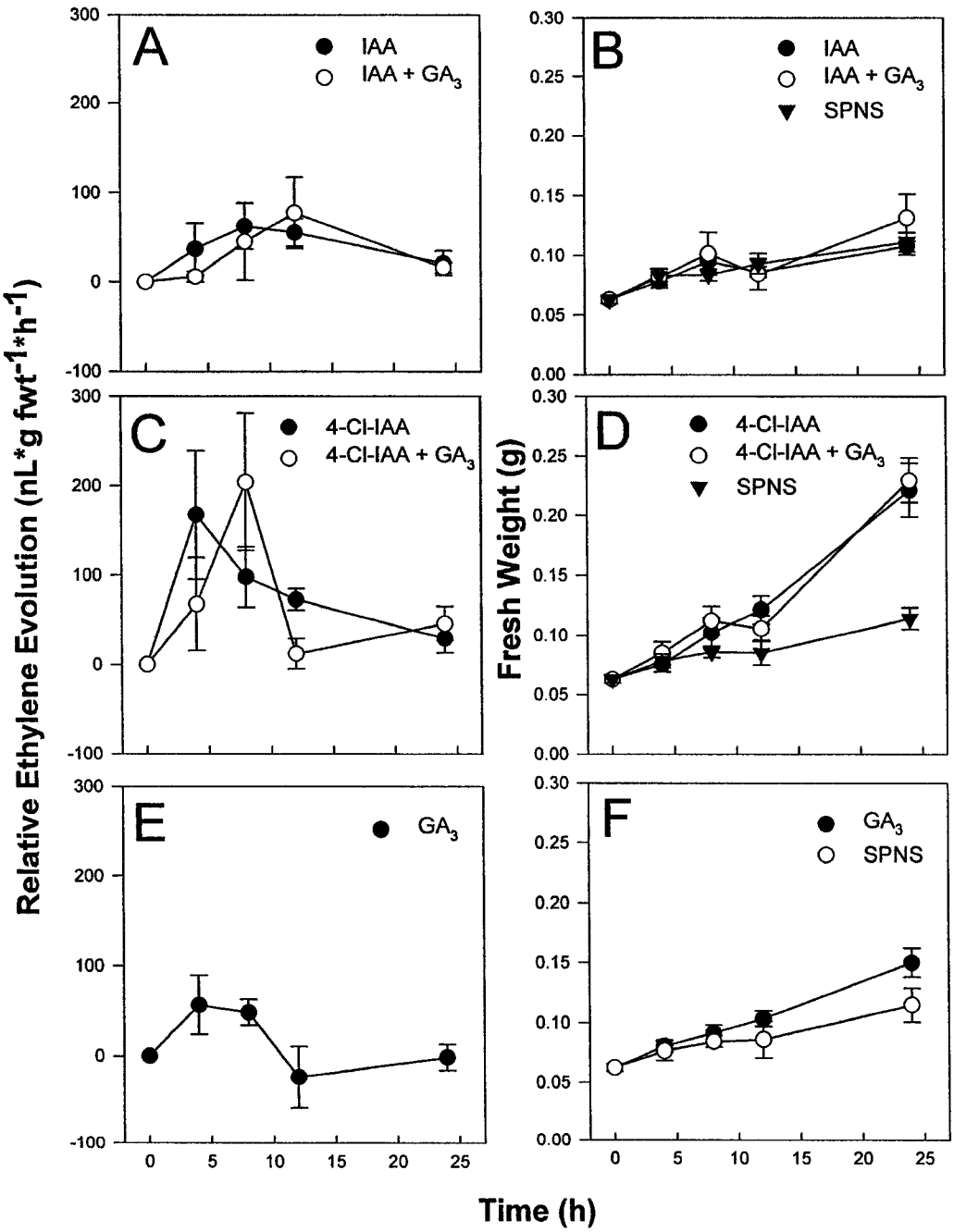
Ethylene evolution and pericarp growth in hormone-treated pericarps bagged during the incubation period on the plant.

Pea plants (*Pisum sativum* L.) line I_3 (Alaska-type) were grown as described in Chapter 2. To test hormone induction of ethylene evolution in deseeded pea pericarps, auxins (IAA or 4-Cl-IAA and GA_3 (30 µL, 50 mM hormone in 0.1% [v/v] aqueous Tween 80), were applied to 2 DAA deseeded pericarps and incubated on-plant for 4, 8, 12, or 24 hours. Treated pericarps remained attached to the plant and enclosed in a plastic bag. Pericarps were harvested after the incubation period to measure the rate of ethylene evolution. A 1 mL gas sample from the headspace of the vial was tested for ethylene quantitation on a GC fitted with a flame ionization detector, as described in Chapter 2. Ethylene content was determined by comparing the sample an equal volume of 0.96ppm ethylene primary standard.

Variable results were obtained from these experiments, probably due to the uncontrolled atmosphere surrounding the tissue in the bag.

Figure A.1: Relative ethylene evolution and pericarp growth in hormone-treated pericarps bagged during the incubation period on the plant

Two DAA pericarps were split, deseeded and immediately treated with 50 μM IAA or IAA + GA_3 (A and B); 4-Cl-IAA or 4-Cl-IAA + GA_3 (C and D); or GA_3 (E and F). After hormone application, pericarps were bagged. Subsequently, pericarps were harvested at 4, 8, 12, and 24 hours. Data is presented as the relative rate of ethylene evolution where the rate of ethylene evolution of the SPNS pericarps is subtracted from the hormone-treated pericarps. Data are means ± 4 to 8.



Comparison of GA 3ß-hydroxylase mRNA levels from Ozga et al. (2003) with starch, sucrose and glucose levels in the pericarp and seed (data from Chapter 3)

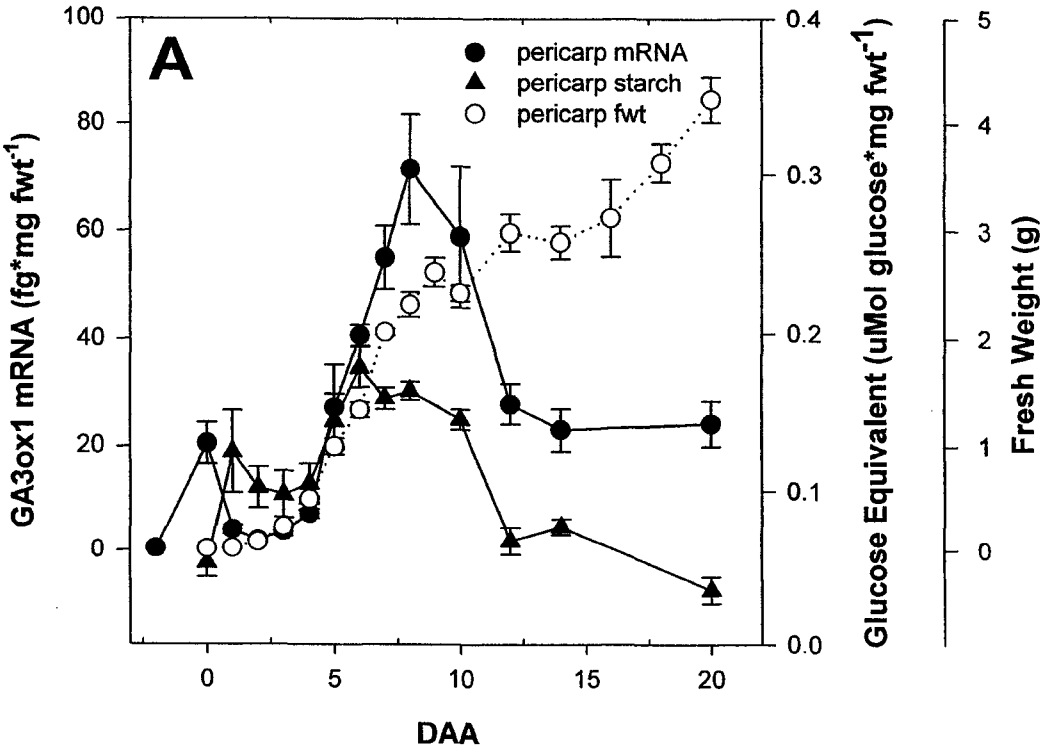
All GA3ß-hydroxylase mRNA data from:

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 Physiology **131:** 1137-1146

Figure A.2: Starch, GA3ox1 mRNA and Fresh Weight profiles of intact pericarps from -2 to 20 DAA

Starch and Fresh Weight: Starch levels from 0 to 20 DAA in pericarps expressed as µMol glucose eq.*mg fwt (A) or µMol glucose equivalent*pericarp^{-1} (B). Data are means ± SE, n = 4 to 6; pericarp fresh weight n = 4 (From Chapter 3).

Ps GA3ox1 mRNA modified from Ozga et al., 2003: Developmental regulation of *PsGA3ox1* mRNA levels in pea pericarps. *PsGA3ox1* mRNA levels from -2 to 20 DAA in pericarps expressed as femtogram milligram fresh weight^{-1} (A) and femtogram pericarp^{-1}(B). Data are means ± SE, n = 2 to 6.



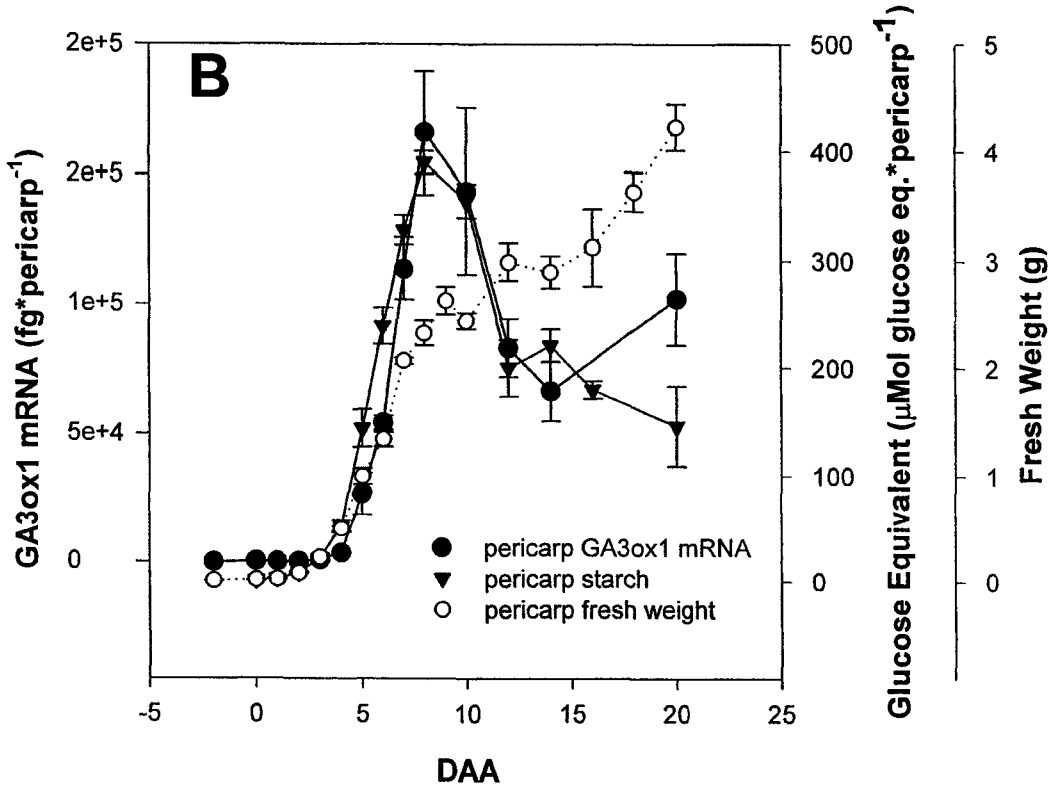
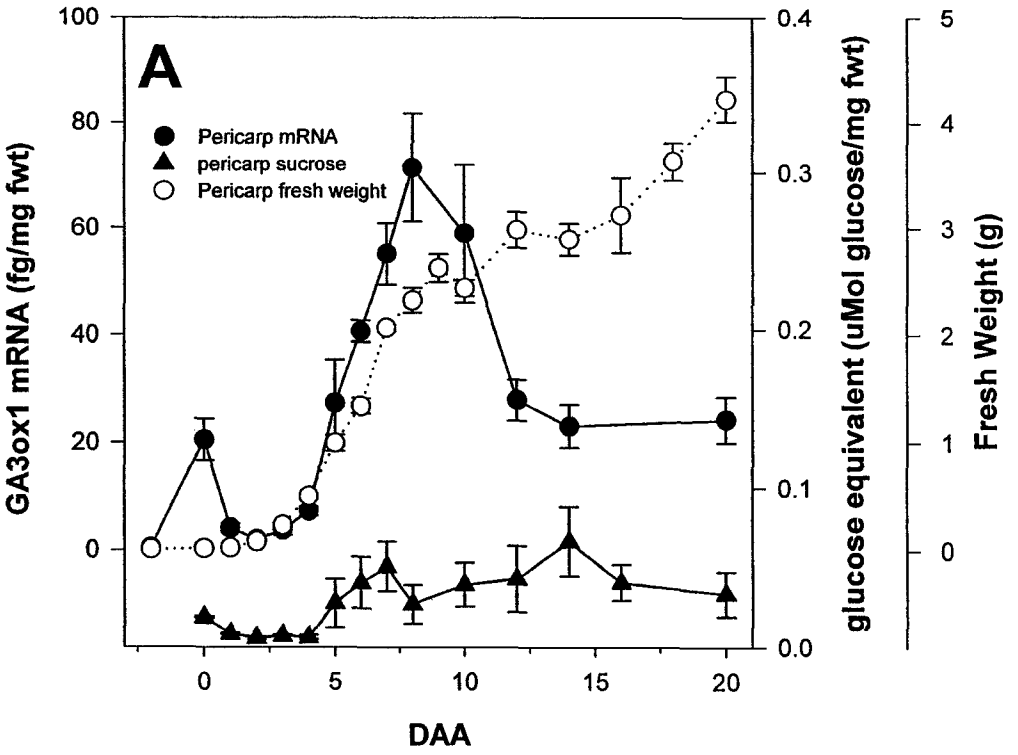


Figure A.3: Sucrose, *PsGA3ox1* mRNA, and fresh weight of pericarps from -2 to 20 DAA

Sucrose and Fresh Weight: Sucrose levels from 0 to 20 DAA in pericarps expressed as µMol glucose eq.*mg fwt (A) or µMol glucose equivalent*pericarp^{-1} (B). Data are means ± SE, n = 4 to 6; pericarp fresh weight n = 4 (From Chapter 3).

Ps GA3ox1 mRNA modified from Ozga et al., 2003: Developmental regulation of *PsGA3ox1* mRNA levels in pea pericarps. *PsGA3ox1* mRNA levels from -2 to 20 DAA in pericarps expressed as femtogram milligram fresh weight^{-1} (A) and femtogram pericarp^{-1}(B). Data are means ± SE, n = 2 to 6.



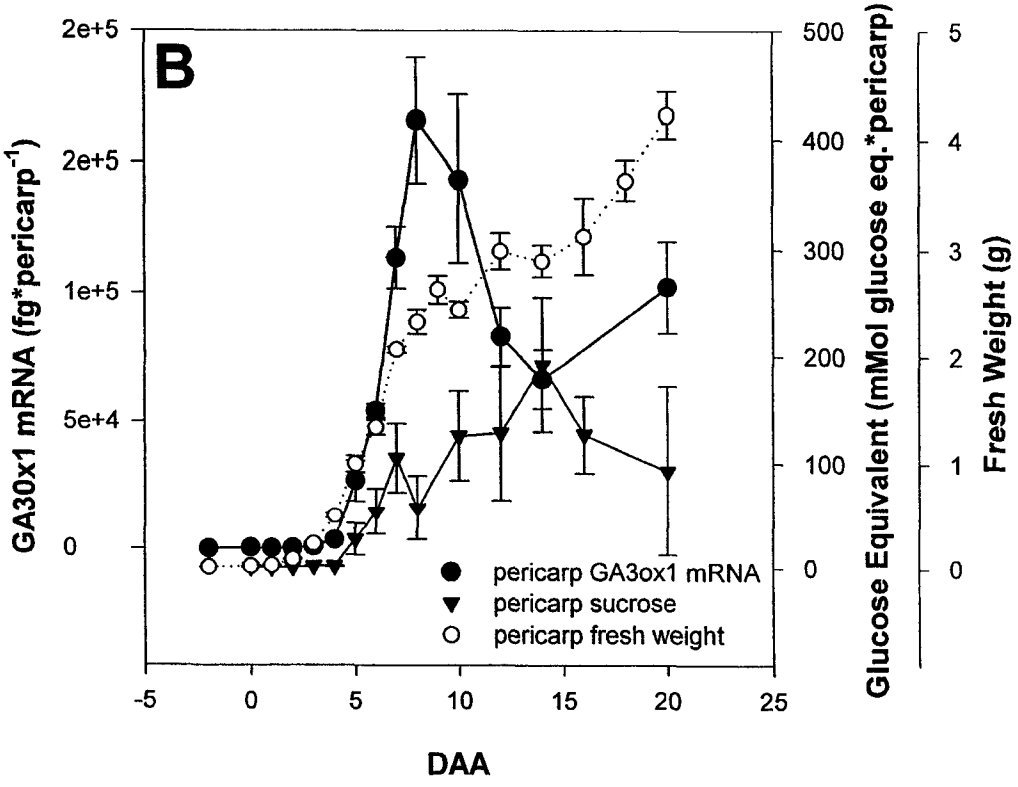
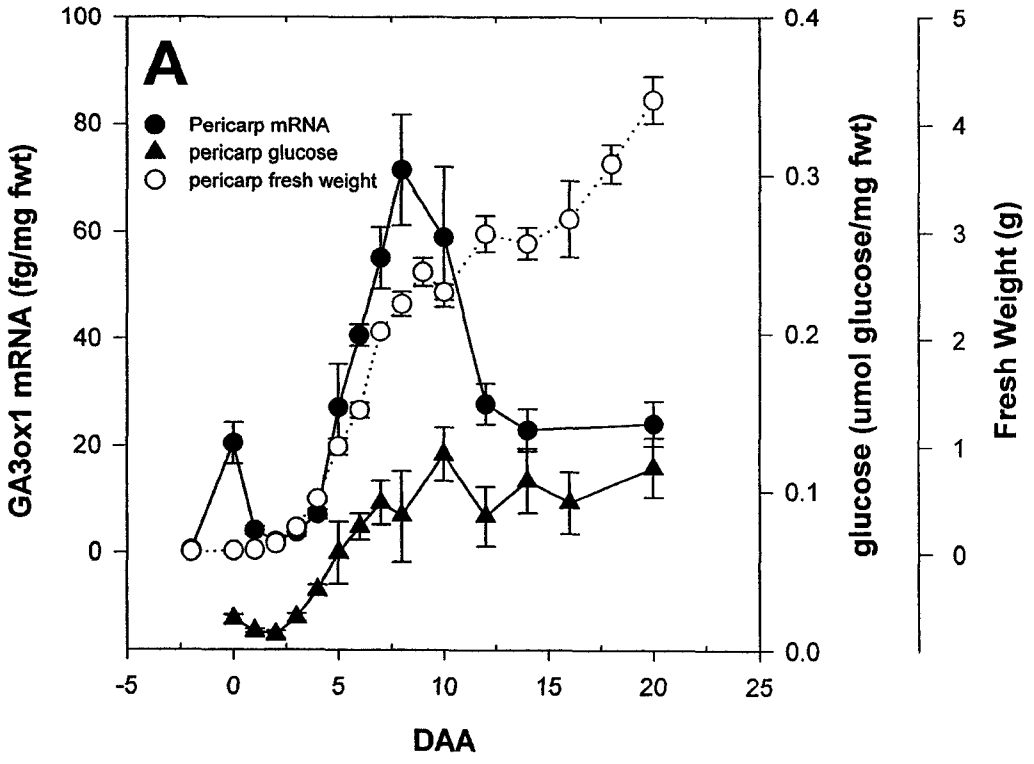


Figure A.4: Glucose, *PsGA3ox1* mRNA, and fresh weight of pericarps from -2 to 20 DAA

Glucose and Fresh Weight: Glucose levels from 0 to 20 DAA in pericarps expressed as µMol glucose*mg fwt (A) or µMol glucose*pericarp^{-1} (B). Data are means ± SE, n = 4 to 6; pericarp fresh weight n = 4 (From Chapter 3).

Ps GA3ox1 mRNA modified from Ozga et al., 2003: Developmental regulation of *PsGA3ox1* mRNA levels in pea pericarps. *PsGA3ox1* mRNA levels from -2 to 20 DAA in pericarps expressed as femtogram milligram fresh weight^{-1} (A) and femtogram pericarp^{-1}(B). Data are means ± SE, n = 2 to 6.



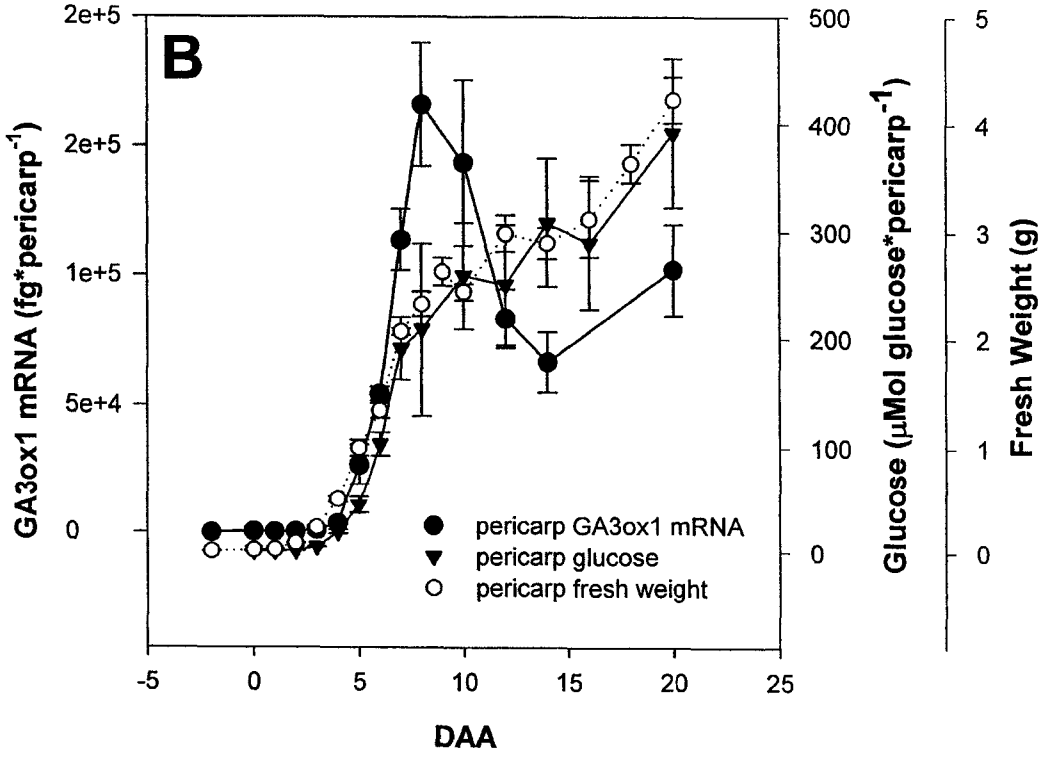
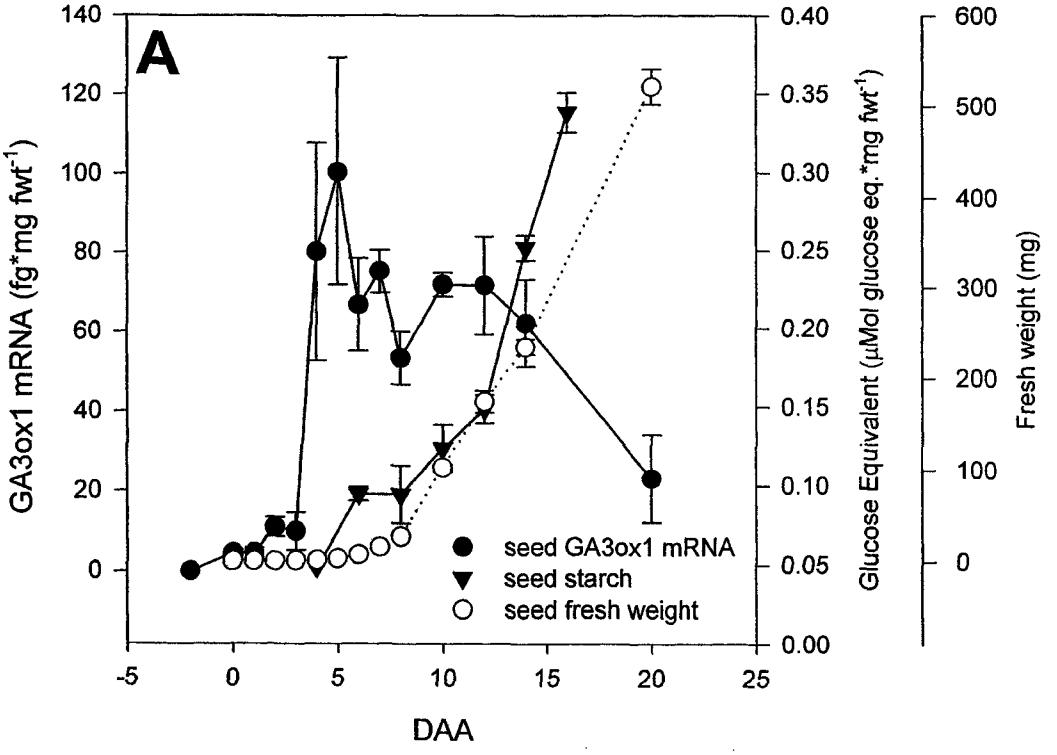


Figure A.5: Starch, GA3ox1 mRNA and fresh weight profiles of seeds from 4 to 20 DAA

Starch and Fresh Weight: Starch levels from 4 to 20 DAA in pericarps expressed as µMol glucose eq.*mg fwt (A) or µMol glucose eq.*pericarp^{-1} (B). Fresh weight from 0 to 20 DAA. Data are means ± SE, n = 4 to 6 (except 4 and 7 DAA, where n = 1); seed fresh weight n = 6 (From Chapter 3).

Ps GA3ox1 mRNA modified from Ozga et al., 2003: Developmental regulation of *PsGA3ox1* mRNA levels in pea pericarps. *PsGA3ox1* mRNA levels from -2 to 20 DAA in pericarps expressed as femtogram milligram fresh weight^{-1} (A) and femtogram pericarp^{-1}(B). Data are means ± SE, n = 2 to 6.



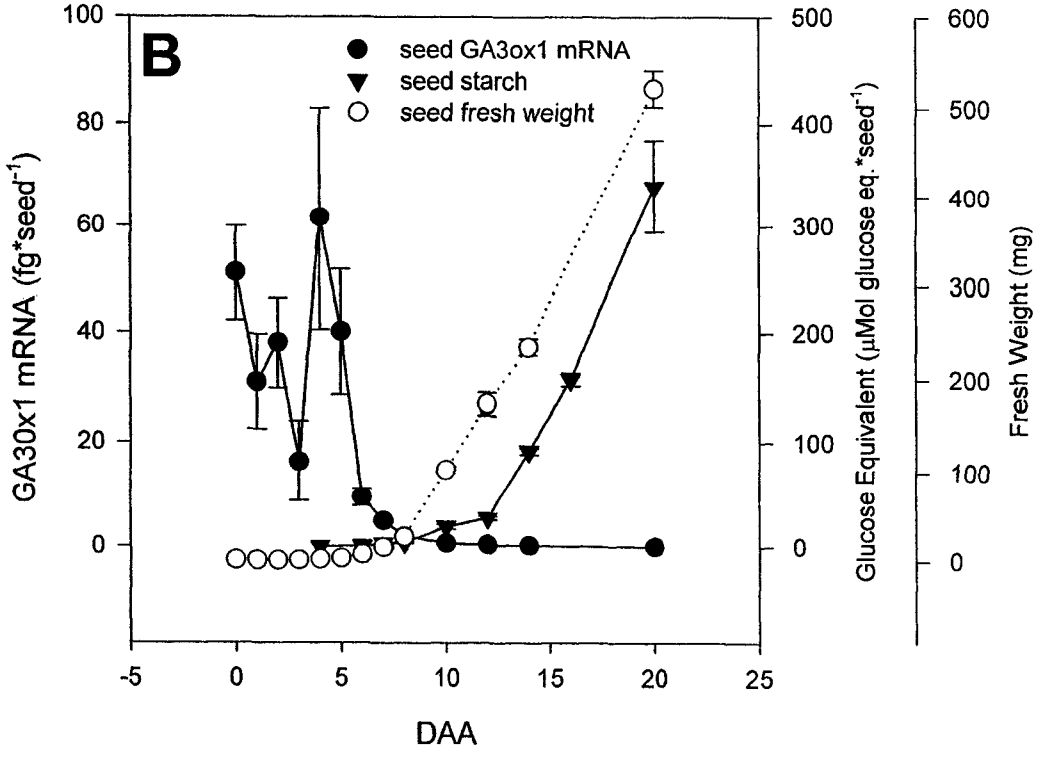
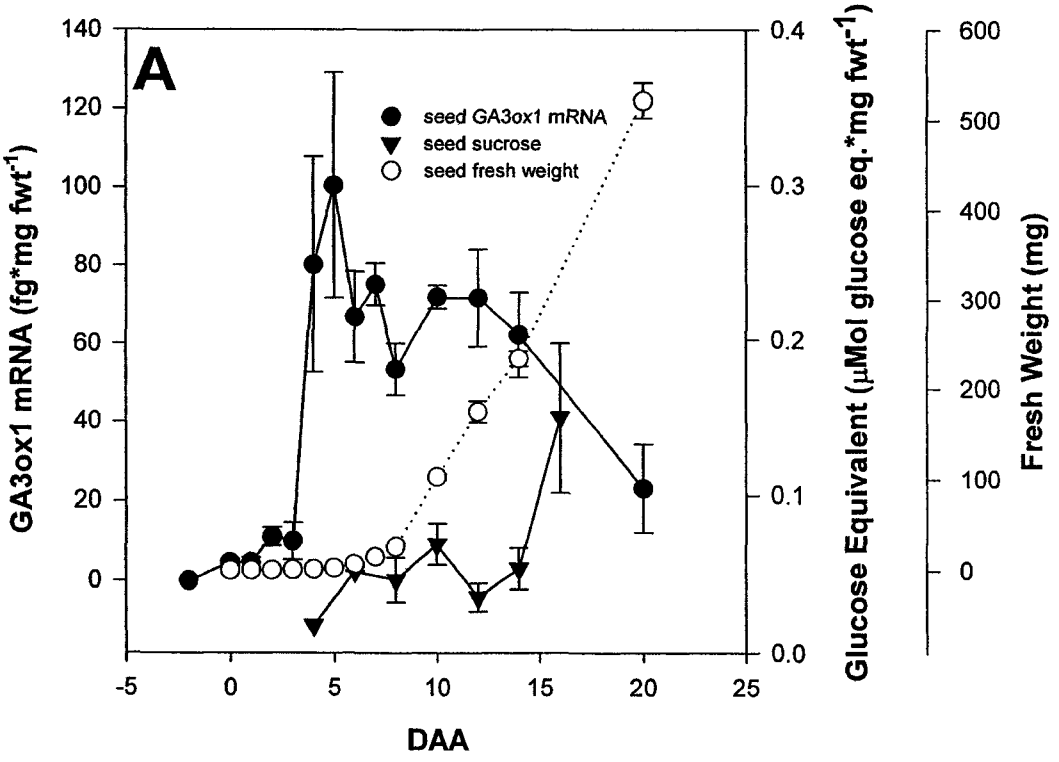


Figure A.6: Sucrose, GA3ox1 mRNA, and fresh weight profiles of seeds from 4 to 20 DAA

Sucrose and Fresh Weight: Sucrose levels from 4 to 20 DAA in pericarps expressed as µMol glucose eq.*mg fwt (A) or µMol glucose eq.*pericarp^{-1} (B). Fresh weight from 0 to 20 DAA. Data are means ± SE, n = 4 to 6 (except 4 and 7 DAA, where n = 1); seed fresh weight n = 6 (From Chapter 3).

Ps GA3ox1 mRNA modified from Ozga et al., 2003: Developmental regulation of *PsGA3ox1* mRNA levels in pea pericarps. *PsGA3ox1* mRNA levels from -2 to 20 DAA in pericarps expressed as femtogram milligram fresh weight^{-1} (A) and femtogram pericarp^{-1}(B). Data are means ± SE, n = 2 to 6.



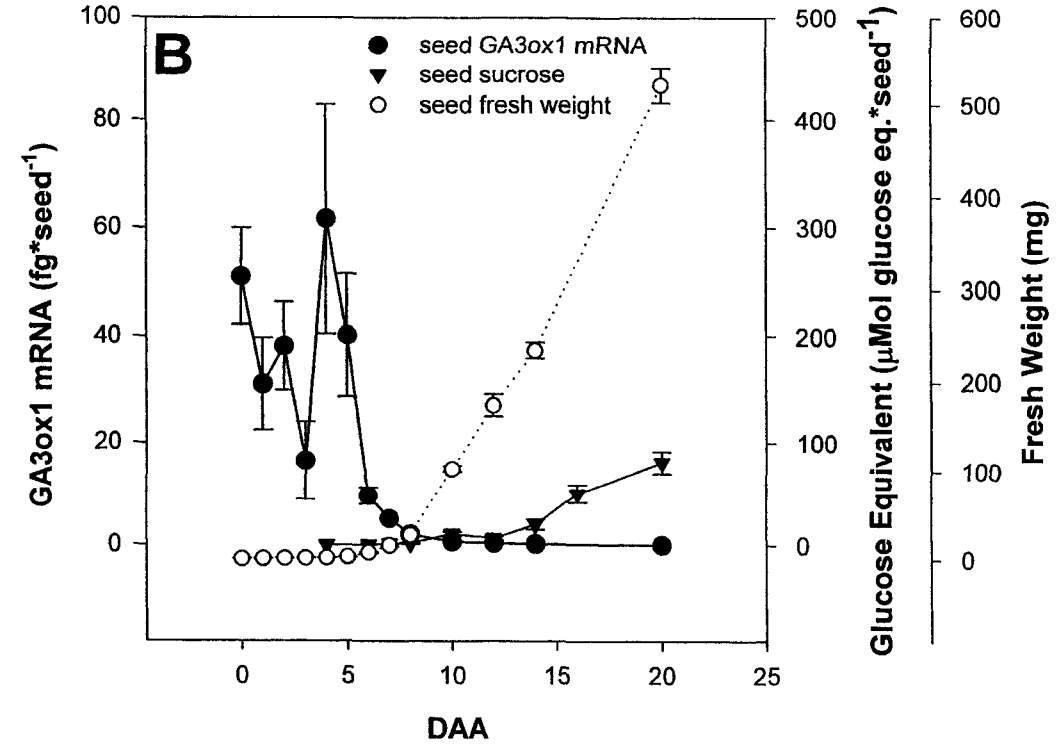


Figure A.7: Glucose, GA3ox1 mRNA, and fresh weight profiles of seeds from 4 to 20 DAA

Glucose and Fresh Weight: Glucose levels from 4 to 20 DAA in pericarps expressed as µMol glucose*mg fwt (A) or µMol glucose*pericarp^{-1} (B). Fresh weight is from 0 to 20 DAA. Data are means ± SE, n = 4 to 6 (except 4 and 7 DAA, where n = 1); seed fresh weight n = 6 (From Chapter 3).

Ps GA3ox1 mRNA modified from Ozga et al., 2003: Developmental regulation of *PsGA3ox1* mRNA levels in pea pericarps. *PsGA3ox1* mRNA levels from -2 to 20 DAA in pericarps expressed as femtogram milligram fresh weight^{-1} (A) and femtogram pericarp^{-1}(B). Data are means ± SE, n = 2 to 6.