University of Alberta

Gibberellin Biosynthesis during Germination and Young Seedling Growth of Pea

By

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Abstract

Seed germination and seedling growth are complex physiological processes that are controlled by multiple endogenous and environmental cues. Bioactive gibberellin (GA) is one of the major endogenous factors that control these developmental processes in a number of species, and its biosynthesis is tightly regulated. This thesis examined the developmental, tissue-specific and end-product regulation of GA biosynthesis during germination and young seedling growth of the large-seeded dicotyledonous model plant pea at the molecular and biochemical levels. During seed development, synthesis of bioactive GA_1 is minimized resulting in an accumulation of the immediate precursor GA_{20}, its 2β-hydroxylated catabolite, GA_{29}, and the transcripts of the *GA2ox* catabolic genes in the mature embryo. Following initial imbibition, de novo synthesis of bioactive GA_1 takes place both in the cotyledon (predominantly from the sequestered GA_{20}) and expanding embryonic axis of germinating pea seeds. During early seedling growth, the cotyledonary GA_{20} was shown to be transported to the growing axis (preferentially to the shoot) to support the synthesis of bioactive GA_1 in this tissue. Bioactive GA level appeared to regulate only the later part of the GA biosynthesis pathway in the root and shoot tissues of developing seedlings. In general, the expression profiles of the GA biosynthesis genes and endogenous GA levels indicated that GA biosynthesis during germination and early seedling growth is highly regulated, and each tissue modulates the transcript abundance of these genes, and in turn the GA pools for the production of bioactive GA required during these developmental processes.

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"Amesegnalehu" everyone!!

For my late mother

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Chapter 1

General Introduction

Seed Germination and Seedling Development

Seed germination incorporates those events that commence with the uptake of water by the quiescent dry seed and terminate with the elongation of the embryonic axis, usually the radicle (Bewley and Black, 1994). Therefore, germination *sensu stricto* does not include seedling growth which involves events such as mobilization of major storage reserves including carbohydrates, fats and oils, and proteins. Imbibition, resumption of metabolic activity, protein synthesis, and radicle extension (protrusion) are the major consecutive events of a germination process (Bewley, 1997), and have been distinguished as separate phases in crop species such as tomato and oat, but in pea the last three phases are difficult to separate (Sutcliffe and Bryant, 1977).

Water uptake by seeds (imbibition) is the initial step for germination to occur. The amount of water taken up by mature dry seeds during the imbibition phase is usually less than 2- to 3-fold of their dry weight (Bewley and Black, 1994). Bewley (1997) indicated that water uptake by germinating seeds follows a triphasic pattern. As the water potential of mature dry seed is much lower than the surrounding moist substrate, the initial water uptake (usually designated as phase I) is very rapid. Following the initial rapid water uptake phase, water influx into the seeds slows down reaching a lag or plateau phase, Phase II. The third phase is characterized by further

increase of water uptake, and is concurrent with radicle elongation. Though metabolic activities such as resumption of respiration commence during the first phase, major metabolic events occur in the second phase of water uptake.

Synthesis of nucleic acids and proteins is essential to support cellular metabolism in germinating seeds. Though residuals of most of the components necessary for protein synthesis are sequestered in mature dry seeds and used to direct the synthesis of protein (Comai and Harada, 1990), as germination proceeds protein synthesis becomes more dependent on the transcription of new mRNAs (Bewley and Black, 1994). Some of such newly synthesized mRNAs may be involved in the synthesis of germination specific proteins, which are necessary for the expansion of embryo axis.

Radicle extension through the seed coat occurs by cell elongation, and signals the termination of germination (Bewley, 1997). This process may or may not be accompanied by cell division. Therefore, cell division is mainly a post-germinative event, associated with axis growth and seedling establishment. In germinating seeds of some species such as maize, DNA synthesis may take place in two phases. The first phase occurs within the first few hours after imbibition (HAI), probably as DNA repair (Bewley and Black, 1994), and the second one occurs in the post germinative stage, and is associated with nuclear division (Osborne and Boubriak, 1994). However, in the storage tissues of dicot species, which do not undergo cell division, the amount of DNA may or may not change after imbibition (Bewley and Black, 1994). For example, in pea, the amount of DNA in the cotyledons remained unchanged both during and after

germination until senescence of the cotyledon when DNA level starts to decline (Bryant and Greenway, 1976).

Bewley (1997) outlined three possible reasons for the commencement of radicle growth. First, a negative osmotic potential in the radicle, as a result of accumulation of solutes probably from the hydrolysis of polymeric reserves in the radicle cells, causes an increase in the uptake of water to increase turgor and then cell extension. However, as indicated by Welbaum and Bradford (1990) and Bradford (1995), no consistent results have been observed regarding the changes in the osmotic potential of radicle cells in germinating seeds. Secondly, extensibility of the radicle cell wall (probably due to the relaxation and slippage of the polysaccharides that makes up the load-bearing network of the cell wall; Cosgrove, 1996) to enhance the growth potential of the embryo. Cell wall loosening proteins induce turgor-driven enlargement of cells by disrupting the non-covalent adhesion of matrix polysaccharides to cellulose microfibrils. The third possibility is weakening of the tissues surrounding the embryo axis, which confers mechanical resistance against radicle protrusion. The layers surrounding the embryo radicle may include diploid maternal tissues such as testa (seed coat) and perisperm, and the endosperm (usually triploid in angiosperms, with two-third of its genome originating from the mother plant) (Leubner-Metzger, 2003). For example, in tomato the micropylar endosperm confers a major part of the mechanical resistance, while the testa accounts for about 20% of the resistance during the early period of seed imbibition (Groot and Karssen, 1987). Expression of genes associated with cell wall loosening (such as those encoding expansin, xyloglucan

endotransglycosylase and endo-ß-mannanase mainly in the micropylar endosperm cap tissue of germinating tomato seeds; Chen and Bradford, 2000; Nonogaki et al., 2000; Chen et al., 2002) provided evidence for the possible role of specific cell wall hydrolases in weakening the endosperm. However, in non-endospermic species such as pea, at maturity the cotyledons are the sole storage organs and the embryo is enclosed by the testa, which confers no hindrance to radicle protrusion (Petruzzelli et al., 1995).

On the basis of morphological patterns of the cotyledons in germinating seeds, germination can be categorized as epigeal or hypogeal. In epigeal germination, displayed by species such as Arabidopsis and tomato, the hypocotyls extend and the cotyledon emerges above the germination medium to become foliate and photosynthetic, where as in hypogeal species, such as pea, the hypocotyls remain short and compact, and the cotyledons, which serve as a major storage organ, stay below the germination medium. Therefore, in hypogeal species, subsequent to radicle protrusion, stored reserves are mobilized from the cotyledon, until the cotyledon is depleted of nutrients, to support the continued growth of the embryo axis (seedling).

Pea (*Pisum sativum L.*), used by Mendel in his seminal experiments over 100 years ago, is a classical genetic model species. The availability of a number of gibberellin (GA) biosynthesis and response mutants has also made pea well-suited for studying GA biosynthesis and metabolism. Moreover, pea seeds have been used in germination studies for several reasons (Sutcliffe and Bryant, 1977). First, the axis of pea seeds is sufficiently large to be separated easily from cotyledons during early stages of germination, and a small number of seeds contain sufficient material for biochemical

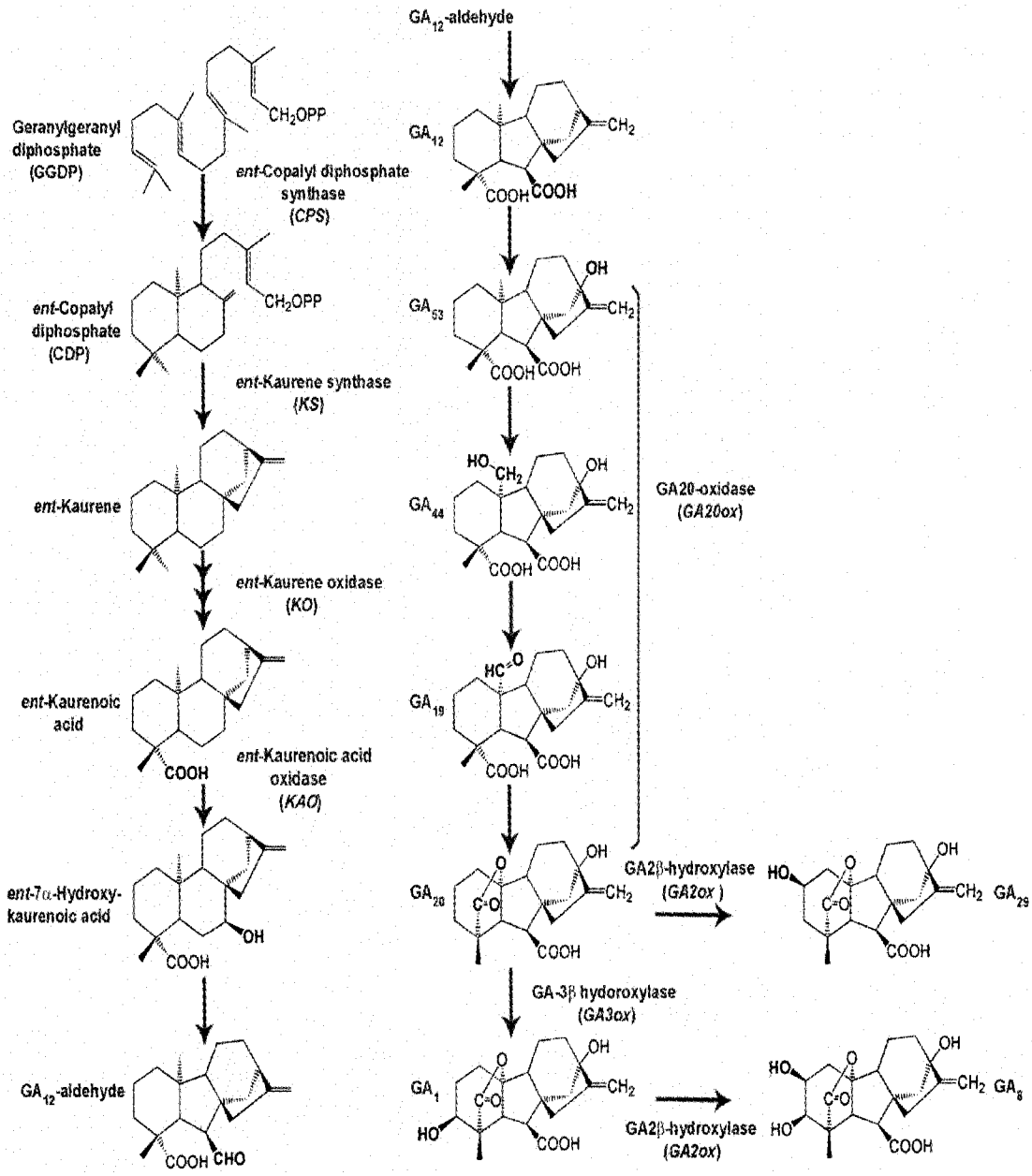
studies. Second, the seeds are quiescent (not dormant) at maturity and are available at all times of the year. As a crop plant, pea is one of the world's commercially important grain legumes.

Gibberellin Biosynthesis and Catabolism

Plant hormones are chemical signal molecules that are essential for normal plant growth and influence many aspects of plant growth and development processes. GAs, one of the major phytohormones, are tetracyclic diterpenoids consisting of 19 or 20 carbon atoms and control a wide range of plant growth and developmental processes including seed germination, leaf expansion, stem elongation, flower induction and seed development (Davies, 1995). In addition, they mediate interactions between plant developmental processes and environmental signals such as photoperiod and light quality (Hedden and Phillips, 2000). At present 136 different GAs are known in higher plants, fungi and bacteria (Sponsel and Hedden, 2004). However, the majority of GAs are either precursors or catabolites, and only a few have intrinsic biological activity.

On the basis of the nature of the enzymes involved, and the corresponding localization in the cell, the GA biosynthetic pathway can be separated into three different phases (Figure 1.1; Hedden and Phillips, 2000). The early pathway, from geranylgeranyl diphosphate (GGDP) to GA_{12}-aldehyde, encompasses the first two phases that are common to all species (Hedden and Phillips, 2000). It has been assumed that GGDP is synthesized from mevalonic acid (MVA); however it appears recently that it is formed primarily via the non-mevalonate pathway involving glyceraldehyde 3-

Figure 1.1. Gibberellin biosynthesis pathway in pea. Early in the pathway geranylgeranyl diphosphate is converted via *ent*-copalyl diphoshate to *ent*-kaurene, which is sequentially oxidized to GA_{12}-aldehyde. Further oxidation of GA_{12}-aldehyde produces GA_{12}, which is then 13-hydroxylated to GA_{53}; carbon 20 is sequentially oxidized by GA 20-oxidase from GA_{53}, via GA_{44} and GA_{19}, to GA_{20}, Finally, GA_{20} is oxidized by 3β-hydroxylase to the bioactive GA_1. GA_{20} and GA_1 may be inactivated by 2β-hydroxylases to GA_{29} and GA_8, respectively (pathway figure; Reinecke, 2006; unpublished).



phosphate and pyruvate (Hedden and Proebsting, 1999). The first phase of GA biosynthesis involves two-step cyclization by two diterpene cyclases, and takes place in the plastid. The first cyclization, involves the conversion of GGDP to *ent*-copalyl diphosphate (CDP) via protonation of the C-14-C-15 double bond, and is catalyzed by *ent*-copalyl diphosphate synthase (CPS). The second cyclization step involves the conversion of CDP to the tetracyclic hydrocarbon *ent*-kaurene by *ent*-kaurene synthase (KS). As GGDP is also the precursor for other compounds such as carotenoids, which are present at several orders of magnitude greater concentration than the GAs (Hedden and Proebsting, 1999), *ent*-kaurene synthesis is likely to be a point of control for the biosynthesis of GAs. GA-deficient mutants (that respond to exogenous GA) have been crucial in identifying genes encoding GA-biosynthesis enzymes, and in elucidating mechanisms regulating GA biosynthesis. To date, only one gene coding for CPS (*PsCPS1*; corresponding to the *LS* locus) has been cloned from pea (Ait-Ali et al., 1997). A mutation in the *LS* gene (*ls-1*) prevents normal mRNA splicing (but is not a null mutation), resulting in three different mutant mRNAs, all of which are predicted to encode a truncated protein with reduced activity (Ait-Ali et al., 1997).

In his review of growth retardants, Rademacher (2000) described that "Onium" compounds containing a positively charged ammonium, phosphonium or sulphonium group such as chlormequat-Cl, Mepiquat-Cl, AMO-1618 and Chlorphonium-Cl retard plant growth by blocking GA biosynthesis via inhibiting mainly the activity of CPS. Such inhibitors of CPS, which appear to mimic cationic intermediates in the cyclization of GGDP, bind tightly to the enzyme and then block the reaction (Rademacher, 2000).

The second phase of GA biosynthesis involves oxidation of *ent*-kaurene to GA_{12}-aldehyde by NADPH-dependent cytochrome P450 mono-oxygenases (*ent*-kaurene oxidase, *ent*-kaurenoic acid oxidase and GA_{12}-aldehyde synthase) located on the endoplasmic reticulum. *ent*-Kaurene oxidase catalyses the stepwise oxidation of *ent*-kaurene at C-19 via *ent*-kaurenol and *ent*-kaurenal to *ent*-kaurenoic acid, which is then hydroxylated by *ent*-kaurenoic acid oxidase to *ent*-7α-hydroxykaurenoic acid. The conversion of *ent*-7α-hydroxykaurenoic acid to GA_{12}-aldehyde involves contraction of the B ring from a C_6 to a C_5 structure with extrusion of C-7. Recently a gene encoding *ent*-kaurene oxidase (*PsKO1*; corresponding to the *LH* locus) and two genes encoding *ent*-kaurenoic acid oxidase (*PsKAO1* and *PsKAO2*; corresponding to the *NA* locus) have been isolated from pea (Davidson et al., 2003; Davidson et al., 2004).

Nitrogen containing heterocyclic compounds such as paclobutrazol, uniconazol, tetcyclasis, and ancymidol block GA biosynthesis and then retard plant growth by inhibiting the activity of *ent*-kaurene oxidases that catalyses the oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Graebe, 1987). All inhibitors of *ent*-kaurene oxidation are characterized by a common structural feature of a lone electron pair on the sp2-hybridized nitrogen of their heterocyclic ring. As this electron pair is localized at the periphery of the inhibitor molecule, and the target monoxygenase contain cytochrome P450, it appears likely that the lone electron pair of the growth retardant displace oxygen from its binding site at the protoheme iron, which in turn inhibits the functioning of *ent*-kaurene oxidase (Rademacher, 2000).

Conversion of GA_{12}-aldehyde to C-19 GAs takes place primarily in the cytosol via either early 13-hydroxylation, which produces GA_{20} and GA_1, or non-13-hydroxylation, which leads to the synthesis of GA_9 and GA_4, depending on species and tissues (Crozier et al., 2000). In general, enzymes catalyzing these reactions are soluble dioxygenases, which require 2-oxoglutarate as a co-substrate, and Fe^{2+} and ascorbate as cofactors for their activity (Hedden and Phillips, 2000). In pea, the third phase of GA biosynthesis is mainly through the early 13-hydroxylation pathway (Sponsel, 1995). GA_{12}-aldehyde is oxidized by GA 7-oxidase into GA_{12}, which in turn is converted to GA_{53} by GA 13-hydroxylase. Thereafter, successive oxidations at C-20, catalyzed by multifunctional GA 20-oxidases, result in the conversion of GA_{53} via GA_{44} and GA_{19} to a C-19 GA, GA_{20}, with the loss of CO_2. Two genes coding for GA 20-oxidases have been cloned in pea, *PsGA20ox1* (Martin et al., 1996; Garcia-Martinez et al., 1997) and *PsGA20ox2* (Lester et al., 1996). Expression of the *PsGA20ox1* cDNA as fusion protein in *Escherichia coli* produced a GA 20-oxidase activity converting $[^{14}C]GA_{12}$ to $[^{14}C]GA_9$ via GA_{15} and GA_{24}, and $[^{14}C]GA_{53}$ to $[^{14}C]GA_{20}$ via GA_{44} and GA_{19}.

The formation of biologically active GA_1 from its precursor, GA_{20} is accomplished by hydroxylation of C-3, which is catalyzed by GA 3β-hydroxylase. The *LE* gene of pea encodes a GA 3β-hydroxylase (Lester et al., 1997; Martin et al., 1997). To date three mutant *le* alleles (*le-1*, *le-2* and *le-3*) are known in pea, of which *le-1*, the mutation caused by substitution of the nucleic acid base A for G at position 685 relative to the start codon (leading to a threonine at position 229 rather than an alanine), is Mendel's *le* mutation. As this base substitution occurs near to an amino acid motif (His-

Thr-Asp) known to be conserved in dioxygenases and involved in iron binding, it appears that the mutation reduces affinity of the enzyme for GA substrates (Martin et al., 1997). Heterologous expression in *E. coli* demonstrated that the product of the cDNA from the *le-1* mutant reduced the conversion of ^{14}C-labeled GA_{20} to ^{14}C-labeled GA_1 by 92%, as compared to the product of *LE* cDNA (Martin et al., 1997).

2β-hydroxylation of bioactive GA_1 or its precursor, GA_{20}, leads to the formation of biologically inactive catabolites, GA_8 and GA_{29}, respectively. Martin et al. (1999) and Lester et al. (1999) cloned two genes encoding GA 2-oxidases in pea. *PsGA2ox1*; corresponds to the *SLN* locus, and the *sln* mutation produces seedlings with a slender or hyper-elongated phenotype. Recombinant expression of the *PsGA2ox1* cDNA in *E. coli* converted GA_1, GA_4, GA_9, GA_{20}, and GA_{29} to the corresponding 2β-hydroxylated products. The *PsGA2ox2* gene product also converted GA_1 to GA_8, but was much less effective in catalyzing the conversion GA_{20} to GA_{29}, and did not convert GA_{29} to GA_{29}-catabolite.

Previous studies with cell preparations have shown that acylcyclohexadiones such as prohexadione, trinexapac-ethyl, and daminozide inhibit most steps after GA_{12}-aldehyde (Rademacher, 2000). Due to their structural similarity with 2-oxoglutarate, a co-substrate for dioxygenases, these compounds compete for binding sites primarily at 3β- and 2β-hydroxylation steps and in turn inhibit enzyme activity (Griggs et al., 1991; Rademacher, 2000).

GA Response Pathway

After its synthesis, bioactive GA is perceived and its signal is transduced through a transduction cascade to trigger biological response. Even though photoaffinity-labeling experiments and binding studies have provided evidence for plasma membrane-bound and soluble-cytosolic or microsomal GA-binding proteins (Sun, 2000), it is only recently that a soluble GA receptor, GIBBERELLIN INSENSITIVE DWARF1 (GID1), has been isolated (Ueguchi-Tanaka et al., 2005). GID1 protein was found to share homology with the consensus sequence of the hormone sensitive lipase family, bind to biologically active GAs, and interact with the rice DELLA protein, SLR1, in a GA-dependent manner. Moreover, loss-of-function mutations in *GID1* resulted in a severe dwarf phenotype with no GA responsiveness.

Elucidation of GA signal transduction pathways has been in progress through isolation and characterization of genes responsible for GA-insensitive mutants (Ashikari et al., 2003). Genes coding for putative transcriptional regulators including *GAI* (*gibberellic-acid insensitive*) and *RGA* (*repressor of ga1-3*) in Arabidopsis, *SLN1* (*slender1*) in barley, *D8* (*dwarf 8*) in maize, *Rht* (*reduced height*) in wheat, and *SLR1* (*slender rice 1*) in rice have been isolated (Gomi and Matsuoka, 2003). These proteins, which are members of the GRAS (designated after GAI, RGA and SCARECROW) family of transcriptional regulators, are referred as DELLA proteins, because of the unique "DELLA" motifs in their N-terminal domain. The DELLA proteins are located in the nucleus and suppress downstream GA signaling, but are rapidly degraded in response to bioactive GAs.

Degradation of DELLA proteins, which leads to various GA responses *in planta*, takes place via the ubiquitin-proteasome pathway mediated by the SCF complex (Fleet and Sun, 2005). Evidence supporting this hypothesis came from the characterization of GA-insensitive dwarf mutants of rice (*gid2*) and Arabidopsis (*sly1*; *sleepy1*), which were found to accumulate DELLA proteins, SLR1 and RGA, respectively, in the presence of high amount of bioactive GA (GA treatment degraded SLR1 in wild type rice, Gomi and Matsuoka, 2003). *GID2* and *SLY1* genes were isolated recently and found to code for putative F-box proteins (substrate receptor subunits of SCF type E3 ubiquitin ligase) that interact with a rice Skp1 (Suppressor of kinetochore protein1), an adaptor protein that links Cullins with the F-box protein to form Skp1-cullin-F-box (SCF) complex (Gomi and Matsuoka, 2003). The SCF complex functions as an E3 ubiquitin ligase to degrade the SLR1 and RGA DELLA proteins. Furthermore, inhibition of barley DELLA protein (SLN1) degradation by specific proteasome and kinase inhibitors has been reported (Richards et al., 2001).

GA response pathway controls a wide range of growth and developmental processes. Therefore, some positive regulators of GA signaling such as a GA-regulated MYB transcription factor (GAMYB identified as activator of the expression of α-amylase in barley aleurone), D1 (*dwarf 1*) of rice, RSG (*REPRESSION OF SHOOT GROWTH*), and PHOR1 (*PHOTOPERIOD-RESPONSIVE1*) have also been identified as down stream components in a number of species (Olszewski et al., 2002; Gomi and Matsuoka, 2003). As GA promotes seed germination in many plant species, some GA response mutants have been shown to affect germination. For example, recessive *spy*

(spindly), *rgl2* (*rga like 2*), and *eaf1* (*early flowering1*) mutants of Arabidopsis are able to germinate in the presence of paclobutrazol (inhibitor of GA biosynthesis; normally blocks germination in Arabidopsis), therefore, SPY, RGL2 and EAF1 are likely to be negative regulators of GA signaling (Sun, 2000; Lee et al., 2002). *RGL2* transcript abundance was shown to rise rapidly following imbibition of wild-type Arabidopsis seeds and then decline as germination proceeds. Furthermore, the *rgl2* mutation rescued the seed germination defect of the GA biosynthesis mutant *ga1-3*, indicating that RGL2 is a GA-response negative regulator of seed germination (Lee et al., 2002). Jacobsen et al. (1996) cloned the *SPY* gene and found to encode a Ser/Thr *O*-linked N-acetylglucosamine transferase (OGT) that modifies proteins via glycosylation of Ser/Thr residues. In tomato, *procera* (*pro*) mutation was found to partially offset the negative effects of *gib-1* mutation on germination, suggesting PRO as a negative regulator of seed germination (Yamaguchi and Kamiya, 2001). However, the specific role of most of GA signaling components in germinating seeds has yet to be addressed.

The Role of GA during Seed Germination

GA promotes seed germination in many plant species. In the course of seed maturation, various events happen including sequestration of GA (Sponsel, 1983), and accumulation of storage products for use during germination and/or early seedling growth of the embryo. Mobilization of seed reserves in cereals following germination is one of the best characterized physiological roles of GAs. GA produced by the embryo of imbibed seeds induces aleurone layer cells to synthesize and secrete α-amylase and

other hydrolases which degrade stored endosperm starch and other polymeric reserves, providing nutrients for the developing seedling (Jacobsen et al., 1995).

Evidences for the role of GAs in promoting the germination of seeds from dicotyledonous species is emerging. The failure of wild-type Arabidopsis seeds to germinate in the presence of GA biosynthesis inhibitors such as paclobutrazol and uniconazole (Hilhorst and Karssen, 1988; Nambara et al., 1991), and the lack of germination of several GA-deficient mutants of Arabidopsis (*ga1-3* and *ga2-1*) and tomato (*gib1*) indicate the requirement of de novo GA biosynthesis upon imbibition for radicle emergence to occur.

Puncture force analysis revealed that, unlike that observed in the wild-type tomato seeds, the force to break the endosperm and the testa covering the radicle of the embryo in GA deficient *gib-1* mutant does not decrease with imbibition. However, incubation of the endosperm and testa of the *gib-1* mutant with wild-type embryos decreased the puncture force markedly (Groot and Karssen, 1987). Mechanical removal of tissues surrounding the embryo (testa plus endosperm) restored the ability of GA-deficient non-germinating mutants of Arabidopsis (Debeaujon and Koornneef, 2000) and tomato (Groot and Karssen, 1987) to germinate under optimal condition without exogenous GA treatment.

It has been suggested that an endogenous GA synthesized in the embryo of small seeded dicotyledons such as tomato and celery diffuses to the endosperm and induces germination by weakening the tissues surrounding the embryo (Jacobsen et al., 1976; Jacobsen and Pressman, 1979; Groot and Karssen, 1987). This hypothesis is in

agreement with the accumulation of GA biosynthesis gene transcripts (*AtKO1*,and *AtGA3ox1 and AtGA3ox2*) predominantly in the cortex of embryo axis (but not in the seed envelope surrounding it; Yamaguchi et al., 2001), and the expression of GA inducible genes coding for cell wall loosening proteins (including expansins, endo-ß-mannanase, xyloglucan endotransglycosylase, and ß-1,3-glucanase) mainly in the micropylar endosperm cap region (Chen and Bradford, 2000; Nonogaki et al., 2000; Chen et al., 2002; Leubner-Metzger, 2003).

GAs are also implicated in stimulating the growth potential of the embryo axis in Arabidopsis, as GA-deficient embryos showed reduced rates of growth (Karssen and Lacka, 1986). Cell specific expression patterns of GA 3-oxidase genes (*AtGA3ox1* and *AtGA3ox2*; Yamaguchi et al., 2001) suggested that the embryo axis is the major site for the synthesis of bioactive GA, and this embryo-synthesized bioactive GA may be involved in stimulating cell enlargement *in situ*. Indeed, Yamaguchi et al. (2001) observed enlarged cortical cells in the embryo axis, which appeared more vacuolated during initial growth of the embryo axis likely due to GA response. The expression of GA inducible expansin genes of tomato (*LeEXPs*) exclusively in the cortical tissue of a radicle (*LeEXP8*), and in the embryo (*LeEXP10*) of germinating seeds (Chen et al., 2001) supports the above notion.

However, GA-biosynthesis-inhibitor studies using compounds that inhibit both early (the cyclization of geranylgeranyl diphosphate to *ent*-copalyl diphosphate; Sponsel, 1983; oxidation of *ent*-kaurene to *ent*-kaurenoic acid; Graebe, 1986) and late (3-hydroxylation of GA_{20} to GA_1; Ross et al., 1993) steps in the GA pathway suggested

that de novo GA biosynthesis was not essential for pea seed germination, but was essential for the maintenance of normal seedling growth soon after germination (4 days after imbibition; DAI). Furthermore, the *na* mutation of the pea GA biosynthesis gene *NA* (*PsKAO1*; encodes *ent*-kaurenoic acid oxidase mainly expressed in vegetative tissues; Davidson et al., 2003) provides genetic evidence for the role of GAs in early pea vegetative growth. Pea seeds with the *na* mutation will germinate (the homolog *PsKAO2* is highly expressed in the developing seeds and results in normal GA levels in *na* seeds; Potts and Reid, 1983; Davidson et al., 2003), however, following germination, *na* dramatically decreases bioactive GA in the vegetative parts of the plant (Proebsting et al., 1992), results in severely dwarf plants with extremely short internodes (Potts and Reid, 1983) and reduced taproot length (Yaxley et al., 2001). These data suggest that after germination, de novo GA biosynthesis is required for normal pea seedling growth.

Regulation of GA Biosynthesis

Given the large numbers of enzymes and genes involved in the GA biosynthesis pathway, regulation of GA metabolism is likely to be complex (Hedden and Phillips, 2000). GA biosynthesis is regulated by a number of endogenous cues including developmental stage, tissue type, and GA action. Environmental signals such as light (duration and quality) and temperature also influence the metabolism of GAs (Hedden and Kamiya, 1997).

Developmental regulation

Imbibition of wild-type Arabidopsis seeds induces temporal up-regulation of several GA biosynthesis genes coding for enzymes catalyzing the synthesis of bioactive GA and its precursors (Yamaguchi et al., 1998; Ogawa et al., 2003), and a discernable increase in bioactive GA level (Ogawa et al., 2003). *AtKO1*, *AtGA20ox3* and *AtGA3ox1* genes were up regulated within 8 h of imbibition but declined afterwards. However, the transcript abundance of *AtGA3ox2*, which was found to be expressed only in young seedlings (Yamaguchi et al., 1998), displayed a marked increase just prior to radicle emergence, and this expression pattern of *AtGA3ox2* fit best with the increase in bioactive GA_4 level. The transcription of GA catabolism genes (*GA2ox* family; coding for enzymes that catalyze the deactivation of bioactive GAs or its immediate precursor) was found to be minimal in germinating seeds of Arabidopsis.

Following seed germination, the embryo axis continues to grow, and bioactive GAs play a role in promoting stem elongation, leaf expansion and root growth (Olszewski et al., 2002). During seedling growth, GA biosynthesis genes are expressed in both immature (growing tissues such as shoot and root tips) and mature tissues. *CPS* gene transcripts are abundant in rapidly growing tissues of Arabidopsis and pea (root tips, hypocotyls, shoot apices), and also in expanded leaves of Arabidopsis and mature internodes of pea (Silverstone et al., 1997; Ross et al., 2003). In pumpkin, the mRNAs of two *CPS* genes (*CmCPS1* and *CmCPS2*) are abundant in very young tissues and decline with age (Smith et al., 1998). In contrast, the expression of *KS* gene in pumpkin and Arabidopsis did not exhibit tissue specificity and did not change with age (Smith et

al., 1998; Yamaguchi et al., 1998), suggesting the more critical developmental regulation of *CPS* than *KS* to control *ent*-kaurene synthesis.

Dioxygenase gene family members carry out both anabolic and catabolic roles in GA metabolism and their pattern of regulation is specific to the different plant tissues and developmental processes. GA dioxygenase genes (*CmGA7ox*, *CmGA20ox3* and *CmGA3ox3*) are mainly expressed in the rapidly growing hypocotyls, cotyledons, root tips and shoot apices of pumpkin seedlings (Smith et al., 1998; Lange et al., 2005). Consistently, the highest amount of bioactive GA (GA_4) was detected in the rapidly growing hypocotyls and root tips of pumpkin (Lange et al., 2005). In pea, dioxygenase genes such as *PsGA20ox1*, *PsGA3ox1* and *PsGA2ox1* are expressed not only in immature (apical bud, young leaf and young internode) but also in mature tissues (mature internode and mature leaf) (Ross et al., 2003). However, mature tissues contain less GA_1 and GA_{20} than the immature tissues, probably due to the rapid 2β-oxidation of these GAs in mature tissues.

Regulation by GA action

Regulation of the late GA biosynthesis genes by the level of bioactive GAs is evident in a number of species (Hedden and Kamiya, 1997). For example, increasing the bioactive GA level by treatment with GA_3 substantially reduced the transcript levels of the *PsGA20ox1* gene in deseeded pericarps (van Huizen et al., 1997) and expanding shoots (consisting of leaves, stipules, stems and petioles; Martin et al., 1996) of pea. Furthermore, treatment of the apical buds of pea seedlings with exogenous GA_1

drastically decreased the mRNA level of *GA3ox* gene (Ait-Ali et al., 1999). Consistent with feedback regulation of *GA20ox* and *GA3ox* mRNA levels by bioactive GA, treatments with GA_3 and 2, 2- dimethyl GA_4 markedly decreased the conversion of [^{14}C]GA_{12} to putative [^{14}C]GA_{20}, oxidation of GA_{19} to GA_{20}, and 3β-hydroxylation of GA_{20} to GA_1 (Ozga et al., 1992; Martin et al., 1996). On the other hand, in GA deficient lines *ls-1* and *na*, the rate of 3β-hydroxylation of GA_{20} to GA_1 exhibited a large increase (Ross et al., 1999). Feedback regulation of GA biosynthesis has also been reported during the germination of Arabidopsis seeds (Yamaguchi et al., 1998). *AtGA3ox1* expression was up-regulated in the germinating seeds of *ga1-3* mutant, and this up-regulation was offset by treatment with exogenous GA_4.

The level of bioactive GA also controls the expression of GA catabolism genes. Elliott et al. (2001) showed reduced expression of both *PsGA2ox1* and *PsGA2ox2* in two different GA deficient mutants of pea, *ls-1* and *n*a whereby the reduced expression of *PsGA2ox1* in the *na* mutant was reversed by treatment with exogenous GA_1. Treatment of immature flower buds of Arabidopsis *ga1-2* mutant with GA_3 also caused an increase in the transcript abundance of *AtGA2ox1* and *AtGA2ox2* (Thomas et al., 1999).

Feedback regulation of the earlier part of the GA biosynthesis pathway does not appear to occur (Hedden and Phillips, 2000). The expression of *PsCPS1* (*LS*), *PsKO1* (*LH*), *PsKAO1* (*NA*) and *PsKAO2* in pea shoots remained similar between GA deficient mutants (*lh-1*, *lh-2*, *ls-1*, *na-1*) and their wild-types (Davidson et al., 2005). Treating both the wild-type and GA-deficient mutant peas with GA biosynthesis inhibitor,

paclobutrazol, did not affect the expression of these genes. Increasing the level of GA precursors early in the pathway does not also appear to exert regulatory role in the synthesis of bioactive GA. For example, overexpression of *AtCPS1* in Arabidopsis resulted in a 1008-fold increase in *ent*-kaurene synthesis (Fleet et al., 2003), with no effect on the expression of late GA biosynthesis genes (*AtGA20ox1* and *AtGA3ox1*) and the levels of GAs (GA_9, GA_4, GA_{34} and GA_{51}) down in the pathway. Similarly, overexpression of the other early GA biosynthesis gene, *ent*-kaurene oxidase (*KO*) did not alter the level of endogenous GAs, including the biologically active GA_4, late in the pathway (Swain et al., 2005).

Objectives

In order to test the general working hypothesis that GA biosynthesis during germination and early seedling growth of pea is regulated developmentally, tissue specifically and by bioactive GA levels, the following objectives were completed. To extend our inferences further, two distinctly different cultivars of pea ('Alaska', a model cultivar for tall [*LE*] vining pea; and 'Carneval', a model cultivar for semi-leafless, semi-dwarf field pea), both of which germinate readily upon imbibition under normal environmental conditions, were used as experimental materials.

1. To examine the developmental and end-product regulation of the first committed step of GA biosynthesis in germinating and developing seedlings.

2. To examine the temporal and spatial regulation of GA biosynthesis in the later part of the pathway during seed development, germination and young seedling growth.

3. To investigate the dynamics of GA metabolism in the cotyledons of germinating seeds, and transport to the expanding embryonic axis.

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Chapter 2

Regulation of GA Biosynthesis Genes during Germination and Young Seedling Growth of Pea (*Pisum sativum* L.)

Introduction

Seed germination incorporates those events that commence with the uptake of water by the quiescent dry seed (imbibition) and that terminate with the protrusion of the radicle from the seed coat (Bewley and Black, 1994). Subsequently, seed storage reserves are mobilized from the cotyledons to support the growth of the embryo axis (seedling). A critical role of GAs has been shown for promoting the germination and early seedling growth of small-seeded dicotyledonous plants such as *Arabidopsis thaliana* and tomato (*Lycopersicon esculentum* Mill.) (Koornneef and van der Veen, 1980; Groot and Karssen, 1987) and large seeded pumpkin (*Cucurbita maxima* L.; Lange et al., 2005). GAs are synthesized from geranylgeranyl diphosphate (GGDP) by a pair of interacting enzymes, *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS), to the tetracyclic hydrocarbon *ent*-kaurene (Duncan and West, 1981; Figure 1.1). CPS catalyses the first committed step in the GA biosynthesis pathway and *ent*-kaurene synthesis appears to be regulated by *CPS* at the mRNA level suggesting that the expression of *CPS* may act as a gatekeeper, controlling the flow of metabolites into the GA biosynthetic pathway (Smith et al., 1998; Yamaguchi et al., 1998; Hedden and Phillips, 2000).

Seeds from the CPS-impaired GA-deficient mutant of Arabidopsis (*ga1-3*) and tomato (*gib-1*) failed to germinate without exogenous GAs (Koornneef and van der Veen, 1980; Groot and Karssen, 1987). Weakening the seed envelope that confers mechanical restraint on radicle protrusion (Groot and Karssen, 1987; Debeaujon and Koornneef, 2000; Chen and Bradford, 2000) and facilitating embryo growth potential (Groot and Karssen, 1987) are two proposed roles for GAs in stimulating germination of small-seeded species. These GA-mediated events are regulated in part by the modulation of tissue- and cell-specific GA concentrations and by altering the ability of cells to respond to GA (Richards et al., 2001).

In Arabidopsis where most of the CPS activity is encoded by a single gene (*AtCPS1*; Sun and Kamiya, 1994), studies using histochemical localization of *AtCPS1* (promoter-GUS-reporter gene expression) and quantitative RT-PCR of *AtCPS1* mRNA, indicated that the expression of *AtCPS1* was in the provasculature of both cotyledons and embryo axes of germinating seeds (Yamaguchi et al., 2001), and in rapidly growing tissues of 1 to 5 d-old seedlings (Silverstone et al., 1997). In pumpkin, where at least two *CPS* genes are present, Northern blot analysis showed strict developmental and tissue-specific regulation of both genes during seedling growth (3-7 DAI; Smith et al., 1998).

Pea is characterized by hypogeal germination unlike the epigeal germination habit of pumpkin, tomato and Arabidopsis. In addition, pea seeds are non-endospermic at maturity as compared to the endospermic seeds of Arabidopsis and tomato. Experiments using a GA biosynthesis inhibitor that blocks CPS activity suggested that

normal CPS enzyme activity for de novo GA biosynthesis was not essential for pea seed germination, but for the maintenance of normal seedling growth soon after germination (epicotyl growth; Sponsel, 1983)

Ent-copalyl diphosphate synthesis in pea appears to be controlled by a single copy gene in pea (*PsCPS1*; corresponding to the *LS* locus; Ait-Ali et al., 1997). A mutation in the *LS* gene (*ls-1*) prevents normal mRNA splicing resulting in three different mutant mRNAs, all of which are predicted to encode a truncated protein with reduced enzyme activity (Ait-Ali et al., 1997). Plants homozygous for the *ls-1* mutation are dwarf with a large reduction in shoot growth (internode length is ca. 75% smaller than in wild-type plants; Reid and Potts, 1986), and a small reduction in root elongation (Yaxley et al., 2001). The *ls-1* mutation substantially reduced the levels of GAs in pea shoots, roots and developing seeds at contact point (Ait-Ali et al., 1997; Yaxley et al., 2001). *PsCPS1* is expressed in immature (apical buds) and mature shoot tissues, leaves and developing seeds of pea (Ait-Ali et al., 1997; Ross et al., 2003; Davidson et al., 2005). However, developmental regulation of *CPS* expression during pea seed germination and early seedling growth has not been studied to date.

Evidence is accumulating that bioactive GA levels are maintained by homeostatic mechanisms in plant tissues. One demonstrated mechanism is the modulation of the expression of GA biosynthesis genes by levels of bioactive GAs (Hedden and Phillips, 2000). Increasing bioactive GAs (GA_3, GA_1) significantly reduced the transcript levels (feed-back regulation) of *PsGA20ox1* and *PsGA3ox1* in actively growing pea shoots and pericarps (Martin et al., 1996; van Huizen et al., 1997;

Ait-Ali et al., 1999). Feed-forward regulation of *PsGA2ox1* and *PsGA2ox2*, with bioactive GAs regulating their own catabolism, has also been observed in pea shoots (Elliott et al., 2001). However, feed-back regulation of the early GA biosynthesis genes by bioactive GA at the mRNA level does not appear to take place. No feedback inhibition of *AtCPS* or *AtKO1* expression was observed in Arabidopsis seedlings when treated with bioactive GA (Silverstone et al., 1997; Helliwell et al., 1998). In pea shoot tissues from well-developed stems, the transcript levels of *PsCPS1*, *PsKO1*, *PsKAO1*, and *PsKAO2* were not altered by the level of bioactive GA present in various GA biosynthesis mutants (Davidson et al., 2005). The regulation of GA biosynthesis by bioactive GAs in the pea root has received less attention than the shoot, although the root responds differently to bioactive GAs than the shoot (exogenous GA strongly promotes shoot growth but has little effect on root elongation in pea; Tanimoto, 1990). Nevertheless, Elliot et al. (2001) did observe feed-back regulation of *PsGA3ox1* and feed-forward regulation of *PsGA2ox1* and *PsGA2ox2* in the roots of the GA biosynthesis mutant *ls-1* compared to the wild-type (*LS*).

Since de novo GA biosynthesis is not essential for pea seed germination but it is for the maintenance of normal seedling growth soon after germination, the expression patterns of *CPS* message in pea tissues during germination and early seedling growth would reflect the requirement for precursors early in the biosynthesis pathway and provide a developmental marker for the timing of events leading to de novo GA biosynthesis during these developmental stages. In this study, we examined the expression pattern of the *PsCPS1* during germination, pre-emergence and post-emergence

stages of two distinctly different genotypes of pea which germinate readily upon imbibition. We also determined if feed-back and feed-forward regulations of the early and latter part of the GA biosynthesis pathway occur similarly in shoots and roots of young pea seedlings at the mRNA level in response to bioactive GA. In addition, the effects of the GA biosynthesis inhibitor, prohexadione, on GA biosynthesis gene expression were characterized in these tissues. Real-time RT-PCR, the method of choice for sensitive, specific and reproducible quantification of mRNA (Bustin, 2000) was used to profile the transcript levels. Our data indicate that *CPS* expression is regulated in a developmental and tissue specific manner to produce precursors for GA biosynthesis required for embryo axis expansion and young seedling growth. Feed-back regulation of *CPS* and *PsGA20ox2* at the mRNA level by bioactive GA levels was not observed in either roots or shoots of young seedlings. However, transcription of *PsGA20ox1* and *PsGA3ox1* exhibited feed-back regulation, and transcription of *PsGA2ox1* and *PsGA2ox2* exhibited feed-forward regulation with bioactive GA levels in both seedling roots and shoots. Our data also support the hypothesis that part of the homeostatic mechanism to maintain lower levels of bioactive GAs in pea roots compared to the shoot is through regulation of the mRNA levels of the genes in the latter part of the GA biosynthesis pathway.

Materials and Methods

Plant Material

The pea (*Pisum sativum* L.) cultivars 'Alaska' (I_3) and 'Carneval' were used in this study (Figure 2.1). 'Alaska' was chosen as a model vining-type pea plant. It has

Figure 2.1. Two week-old seedlings of 'Alaska' (right) and 'Carneval' (left)

normal leaflet morphology (AF), wild-type internode length (*LE*), white flowers and green cotyledons at maturity, and it flowers at approximately the 10th node under long or short day conditions. 'Carneval' was chosen as a model for semi-dwarf (semi-leafless; *af*) field pea (*Pisum sativum* L.) which is used extensively in crop agriculture. 'Carneval' has white flowers and yellow cotyledons at maturity, flowers at about the 15 to 17th node under long day conditions and was found to contain Mendel's dwarfing gene, *le-1* (Appendix 1). Both cultivars readily germinate on imbibition with water at 15°C to 25°C.

Growth Conditions and Harvesting

Mature air-dry seeds of 'Alaska' (5.4% relative water content; RWC) and 'Carneval' (5.8% RWC) were planted at a depth of ca. 2.5 cm into moist sterilized sand in 3 L plastic pots (10 seeds per pot), and the pots were placed in a growth chamber (Conviron, Ashville, NC) at 22/20^{0}C (day/night) under a 16/8 h photoperiod with cool white fluorescent and incandescent lights (205.5 μE $m^{-2}s^{-1}$) until harvest. For germination and growth measurements, seeds or seedlings of each cultivar were harvested at 0.5, 1, 2, 3, 4, 6, 7 and 8 DAI from the sand medium, and separated into cotyledons and embryo axes (0.5 and 1 DAI) or into cotyledons, roots and shoots (2-8 DAI; 5-10 seeds or seedling per replication; 3 replications per time point). Seeds were scored as germinated when protrusion of radicle (2-5 mm) through the seed coat was visible. RWC of the cotyledons was determined by comparing the sample weights before and after drying for 72 h at 60°C, and are expressed on a fresh weight basis. For

RNA extraction, seedlings at 0.5, 1, 2, 4 and 6 DAI were separated either into cotyledons and embryo axes (0.5 and 1 DAI), or cotyledons, shoots and roots (2 and 4 DAI), or cotyledons, shoots, root tips (ca. 4 mm) and remainder of roots (6 DAI), and tissues were immediately frozen in liquid N_2 and stored at -80^{0}C until extraction. For examining the mRNA levels in the mature embryos (0 DAI), seeds of the two cultivars were immersed in ice: water (1:1, v/v) for 4 h to facilitate seed coat removal, and the embryos (cotyledon plus embryo axis) were immediately frozen in liquid nitrogen and stored at -80^{0}C until RNA extraction.

To investigate the effect of bioactive GA level on the regulation of mRNA levels of GA biosynthesis genes, mature air-dry seeds of 'Alaska' were imbibed for 24 h in darkness, after nicking the testa, on filter paper in 9 cm Petri plates (20 seeds per plate) with a 10 mL per plate aqueous solution of 0.1 mM GA_3, 9.9 mM prohexadione-calcium (Apogee; BASF, NC, USA), or water (control). The imbibed seeds were then planted into moist sterilized sand in 3 L plastic pots (10 seeds per pot), and placed in a growth chamber environment as described above. Seedlings were harvested at 4 and 6 DAI for growth measurements and RNA extraction.

RNA Isolation

Tissues were finely ground in liquid N_2, and 200 to 550 mg fresh weight (mature embryos, embryo axes, shoots, roots and root tips) or 100 to 250 mg fresh weight (cotyledons) subsamples were used for total RNA isolation using a modified TRIzol (Invitrogen, Carlsbad, CA) protocol. After initial extraction with the TRIzol reagent and

centrifugation, the supernatant was cleaned by chloroform partitioning (0.2 mL mL^{-1} TRIzol). The resulting supernatant fraction was then precipitated subsequently by isopropanol (0.25 mL mL^{-1} TRIzol) and high salt solution (1.2 M Na citrate and 0.8 M NaCl) to remove polysaccharides and proteoglycans, 4 M LiCl, and finally by a mixture of 3 M Na acetate (pH 5.2): 100% ethanol (1:20, v/v). The precipitate was dissolved in DEPC treated water. The integrity of the RNA was verified both electrophoretically and by the average 260 to 280 nm absorption ratio. The total RNA samples from all tissues were then digested with DNase (DNA-free kit, Ambion, Austin, TX), and the cotyledonary total RNA samples were further purified with RNeasy columns (Qiagen, Valencia, CA). Sample RNA concentration was determined in duplicate by A_{260} measurement, and then the samples were stored at -80^0C until quantitation by real-time RT-PCR.

Primers and Probes

Primers and probes for the target gene quantifying amplicon *GA3ox87* (used for *PsGA3ox1* quantification) and for the reference gene amplicon *18S62* (used for pea 18S rRNA quantification) were designed using Primer Express Software (Applied Biosystems, Foster City, CA) by Ozga et al. (2003; Table 2.1). Primers and probes for target gene quantifying amplicons *CPS-92* (spans nucleotides 1833 to 1924), *GA20ox1-104* (spans nucleotides 873 to 976), *GA20ox2-88* (spans nucleotides 305 to 392), *GA2ox1-73* (spans nucleotides 722 to 794) and *GA2ox2-83* (spans nucleotides 817 to

Table 2.1. Primer and probe sequences used in the quantification of relative mRNA levels, and validation of quantifying primer sets.

Gene	Type	Quantifying (5' to 3')	Validating (5' to 3')
		Amplicon: *CPS-92*	
PsCPS1	FP[a]	TGTTAGGAATGAAGATTTGAGGAAAGA	
	RP[b]	TCTTCATCCTCCGGGCAAT	
	Probe	TCGATGTTCGAGACTATT	
		Amplicon: *GA20ox1-104*	Amplicon: *GA20ox1-216*
PsGA20ox1	FP[a]	GCATTCCATTAGGCCAAATTTC	GATCAAGTTGGTGGCTTGCAA
	RP[b]	CCACTGCCCTATGTAAACAACTCTT	TGGGCTAACCACTTTATCACCTTT
	Probe	CCTTCATGGCTCTTTC	CCTTCATGGCTCTTTC
		Amplicon: *GA20ox2-88*	
PsGA20ox2	FP	AATACATCTTCTCTACCGTTGCAAAT	
	RP	TTGGCGGTGTTAAACAAGGTT	
	Probe	ACATACCCTCAGAGTTC	
		Amplicon: *GA3ox87*	Amplicon: *GA3ox126*
PsGA3ox1	FP	TTCGAGAACTCTGGCCTCAAG	(Ozga et al., 2003)
	RP	ATGTTCCTGCTAACTTTTTCATGGTT	
	Probe	ACAATATCACAGAATCTGGT	
		Amplicon: *GA2ox1-73*	Amplicon: *GA2ox126*
PsGA2ox1	FP	TTCCTCCTGATCATAGCTCCTTCT	CTCTTAGAGATGGTAGCTGGATTTCA
	RP	TTGAACCTCCCATTAGTCATAACCT	TTGCCAAAACTCTATGTCTCACACT
	Probe	GAGAATCACCAACATT	GAGAATCACCAACATT
		Amplicon: *GA2ox2-83*	
PsGA2ox2	FP	AACACAACAAAGCCTAGAATGTCAA	
	RP	ACCATCTTCGATAACGGGCTTAT	
	Probe	TGTATTTTGCAGCACCACC	
		Amplicon: *18S62*	
Ps18S	FP	ACGTCCCTGCCCTTTGTACA	
rRNA	RP	CACTTCACCGGACCATTCAAT	
	Probe	ACCGCCCGTCGCTCCTACCG	

[a] FP: Forward Primer. [b] RP: Reverse Primer

899) were designed based on reported sequences of pea *PsCPS1* (also named ent-kaurene synthase A gene, *LS*; GenBank accession number U63652; Ait-Ali et al., 1997), *PsGA20ox1* (GenBank accession number U70471; Garcia-Martinez et al., 1997), *PsGA20ox2* (GenBank accession number U58830; Lester et al., 1996), *PsGA2ox1* (GenBank accession number AF056935; Martin et al., 1999), and *PsGA2ox2* (GenBank accession number AF100954; Lester et al., 1999), respectively (Table 2.1). A second set of validating primers were designed for *GA20ox1* and *GA2ox1*: *GA20ox1-216* (spans nucleotides 832 to 1047; see Figure A.7) and *GA2ox1-126* (spans nucleotides 695 to 820; see Figure A.11), as described for *PsGA3ox1* (*GA3ox1-126*) quantitation (Ozga et al., 2003), and used as a control to test the specificity of the amplification of the target genes.

All probes were TaqMan MGB and labeled at the 5' end with fluorescent reporter dye 6-carboxyfluorescein (target gene probes) or VIC (*18S62* reference gene probe), and at the 3' end with non-fluorescent quencher (NFQ) dye (Applied Biosystems). A Gapped BLAST (Altschul et al., 1997) program (versions BLASTN 2.2.10) was used to search databases for sequences homologous to the amplicon sequences of each gene. To confirm the PCR product produced by the quantifying primers, RT-PCR amplification products were separated and identified using 1% agarose gel electrophoresis and ethidium bromide staining.

Real-Time RT-PCR Assay

Real-time RT-PCR assays were performed on a model 7700 sequence detector (Applied Biosystems) using a TaqMan One-Step RT-PCR Master Mix Reagent Kit (Applied Biosystems). For each 25 μL Taqman reaction, 5 μL (200 ng) of total RNA for target genes or 10 pg for 18S rRNA was mixed with 12.5 μL of 2X Master Mix (containing AmpliTaq Gold DNA Polymerase), 0.6 μL of 40X MultiScribe (reverse transcriptase and RNase inhibitor mix), 1.5 μL forward primer (5 μM; final concentration 300 nM), 1.5 μL reverse primer (5 μM; final concentration 300 nM), 0.5 μL probe (5 μM; final concentration 100 nM) and 3.4 μL DEPC treated water. Samples were subjected to thermal cycling conditions of reverse transcription at 48°C for 30 min, DNA polymerase activation at 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 s followed by anneal extension at 60°C for 1 min. PCR amplification of each sample was carried out in duplicate in 96-well optical reaction plates covered with optical caps (Applied Biosystems), and the average of the two sub-samples was used to calculate the sample transcript abundance. Total RNA extracts from each tissue were pooled across all time points per cultivar, and this pooled sample was run on each plate and was used as a control to correct for plate to plate amplification differences. A pooled sample from one real-time RT-PCR run was taken arbitrarily as the standard for normalizing the C_t values of samples in other runs as follows:

$$\textit{Normalized } C_t \textit{ value of sample} = (C_t \textit{ value of pooled sample in the standard run} / C_t \textit{ value of pooled sample in the sample run}) * C_t \textit{ value of sample}$$

The relative transcript abundance of the target genes in the individual plant sample was determined by $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001), where ΔC_t was the difference between the target sample C_t and average C_t of the reference sample. The reference sample for the *PsCPS1* developmental profile experiment (both cultivars) was 'Alaska' 6 DAI cotyledon (C_t = 29.64). The reference sample for the exogenous GA_3 and prohexadione experiments was *PsGA20ox2* from the prohexadione-treated 4 DAI shoots (C_t = 28.27). Two to 4 biological replicates of each plant sample were assayed.

Results and Discussion

Quantitation of Target Genes

The specificity of the RT-PCR products of the *PsCPS1, PsGA20ox1*, and *PsGA20ox2, PsGA2ox1*, and *PsGA2ox2* target gene quantifying amplicons was confirmed electrophoretically. The forward and reverse primer sets of each target gene amplicon (Table 2.1) produced a single product with a desired length of 92-bp (*CPS-92*), 104-bp (*GA20ox1-104*), 88-bp (*GA20ox2-88*), 73-bp (*GA2ox1-73*) and 83-bp (*GA2ox2-83*) (data not shown). The forward and reverse primer sets of *PsGA20ox1* and *PsGA2ox1* validating amplicons (Table 2.1) also produced a single product with a desired length of 216-bp (*GA20ox1-216*) and 126-bp (*GA2ox1-126*), respectively. The *GA3ox1-87* (quantifying amplicon) and *GA3ox1-126* (validating amplicon), and pea 18S small subunit nuclear ribosomal RNA forward and reverse primer sets (Table 2.1) were previously characterized by (Ozga et al., 2003).

The pea 18S rRNA amplicon was used as a loading control to estimate variation in input total RNA concentration across all samples within each cultivar. The average cycle threshold (C_t) value (± SD) for the 18S amplicon across 'Alaska', 'Carneval' and GA_3- or prohexadione-treated and untreated 'Alaska' tissue samples was 25.92 ± 0.69 (n = 84; coefficient of variation [CV] = 2.7%), 25.96 ± 0.86 (n = 82; CV = 3.3%) and 25.31 ± 0.84 (n = 46; CV = 3.3%), respectively. As the CV of the 18S amplicon C_t values was very low among all the samples assayed (2.7 to 3.3% CV), target gene mRNA values were not normalized to the 18S signal as described in Ozga et al. (2003). Similar trends in C_t values were obtained between quantifying and validating amplicons of *PsGA20ox1* and *PsGA2ox1* with the original fluorescent probes (Table 2.2). A similar confirmation of quantifying and validating amplicons for *PsGA3ox1* transcript abundance in pea tissue was reported by (Ozga et al., 2003). A BLAST search of all database sequences from GenBank, European Molecular Biology Laboratory (EMBL), DNA Data Bank of Japan and Protein Data Base (excluding expressed sequence tags [ESTs], sequence tagged sites, genome survey sequences, and phase 0, 1 or 2 high-throughput genomic sequences) found significant sequence alignment only between the quantifying and validating amplicons, *CPS-92* and *PsCPS1* (E values 9×10^{-44}), *GA20ox1-104* and *GA20ox1-216,* and *PsGA20ox1* (E values 10^{-117} to 7×10^{-51}), *GA20ox2-88* and *PsGA20ox2* (E value 2×10^{-41}), *GA2ox1-73* and *GA2ox1-126*, and *PsGA2ox1* (E values 2×10^{-64} to 10^{-32}), and *GA2ox2-83* and *PsGA2ox2* (E value 2×10^{-38}). No significant homology was detected to either the target genes from other plant species or other genes in the family of terpene cyclases or 2-oxoglutarate dependent

Table 2.2. C_t values generated by quantifying and validating primer sets of *PsGA20ox1* and *PsGA2ox1*

Samples	*PsGA20ox1*		*PsGA2ox1*	
	QP[a] (C_t)	VP[b] (C_t)	QP (C_t)	VP (C_t)
Shoot pool	23.8	27.2	26.0	29.1
'Carneval' shoot, 4 DAI	23.0 ± 0.5[c]	26.1 ± 0.5	24.0 ± 0.2	27.9 ± 0.4
'Carneval' shoot, 6 DAI	23.6 ± 0.3	26.8 ± 0.2	24.5 ± 0.1	28.1 ± 0.0

[a] QP = Quantifying primer set.

[b] VP = Validating primer set.

[c] Data are means ± SE, n = 2.

dioxygenases of pea. In a BLAST search of ESTs in GenBank, EMBL, and DNA Data Bank of Japan, the only significant sequence alignments were between the quantifying and validating amplicons, *GA20ox1-104* and *GA20ox1-216*, and three EST sequences from *Glycine max* (E values ranged from 10^{-75} to 2 x 10^{-29}) and one from *Lotus corniculatus* (E values ranged from 4 x 10^{-60} to 2 x 10^{-29}); *GA20ox2-88* and two ESTs from *Medicago truncatula* (E values 9 x 10^{-10}); *GA2ox1-73* and *GA2ox1-126* amplicons and two ESTs from *M. truncatula* (E values ranged from 10^{-49} to 7 x 10^{-25}); and *GA2ox2-83* and two ESTs from *M. truncatula* (E values 9 x 10^{-22}). Ozga et al. (2003) found that the *GA3ox1-87* (quantifying) and *GA3ox1-126* (validating) amplicons had significant homology to two EST sequences from *M. truncatula* and one from *G. max*. No pea ESTs were found to have significant sequence alignment to the *CPS-92* amplicon sequence.

PsCPS1 Gene Transcripts Present in the Mature Embryo

PsCPS1 transcripts were detected in the mature embryos (0 DAI) of both genotypes (*PsCPS1*, C_t = 27.2 ± 0.2 for 'Alaska' and 26.1 ± 0.1 for 'Carneval'; no template control, C_t = 40 ± 0; data are means ± SE, n = 2 to 3), which mainly reflects cotyledonary transcript levels at this stage (based on cotyledonary tissue weight as 98% of the mature embryo, and yields of total RNA from embryo axes and cotyledons). It is highly unlikely that 'Alaska' mature embryos imbibed a sufficient amount of water (0.7% increase in RWC of seed) during the 4 h period in ice water to sufficiently hydrate cells of the embryo to allow for de novo transcription; therefore, the results

suggest that the *PsCPS1* transcripts detected in the mature embryos of both genotypes were transcripts synthesized during seed development and sequestered in the embryo at the end of seed maturation. These findings are in agreement with those of Ait-Ali et al. (1997) and Davidson et al. (2005), since expression of *PsCPS1* was found to be high in the developing pea embryo at contact point (ca. 20 days after anthesis). Moreover, the activity of CPS enzyme (from cell free enzyme preparations) was high at the same stage of seed development (Ait-Ali et al., 1997). Indeed, mature dry seeds of pea contained *ent*-kaurene at ca. 0.4 pmol seed^{-1} in 'Torsdag' tall genotype, and significantly higher *ent*-kaurene levels (198 pmol seed^{-1}) were found in the dwarf mutant WB 5863 (derived from 'Torsdag') (Graebe et al., 1987). We also found higher levels of *PsCPS1* in the mature embryos of 'Carvenal" (dwarf; *le-1*) compared to 'Alaska' (Tall; *LE*; see below), although in a different background.

CPS Expression in Cotyledons of Hypogeal Species Differs from that in Epigeal Species

As the RWC of cotyledons increased to 50 to 55% (from 0 to 1 DAI; Figure 2.2A), cotyledonary *PsCPS1* transcript levels declined 3.5- to 4.3-fold in both cultivars (Figure 2.3C). A small but further decline in cotyledonary *PsCPS1* mRNA levels occurred during the later stages of germination to the active seedling growth stage (1 to 6 DAI; Figure 2.3C). Lower but significant levels of *PsCPS1* mRNA were still present in 6 DAI cotyledons, as indicated by Ct values for cotyledonary *PsCPS1* mRNA samples (C_t values of approximately 30 for the 6 DAI 'Alaska' cotyledon samples as compared to the no template control C_t value of 40). The presence of *PsCPS1* mRNA in

Figure 2.2. Germinating pea (*Pisum sativum* L.) seeds and actively growing seedlings of 'Alaska' (left in each pair) and 'Carneval' (right in each pair) from mature embryo (0 DAI) to 6 DAI (A). Root (B) and shoot (C) fresh and dry weights from 2 to 6 DAI. Data are means ± SE, n = 15 to 28.

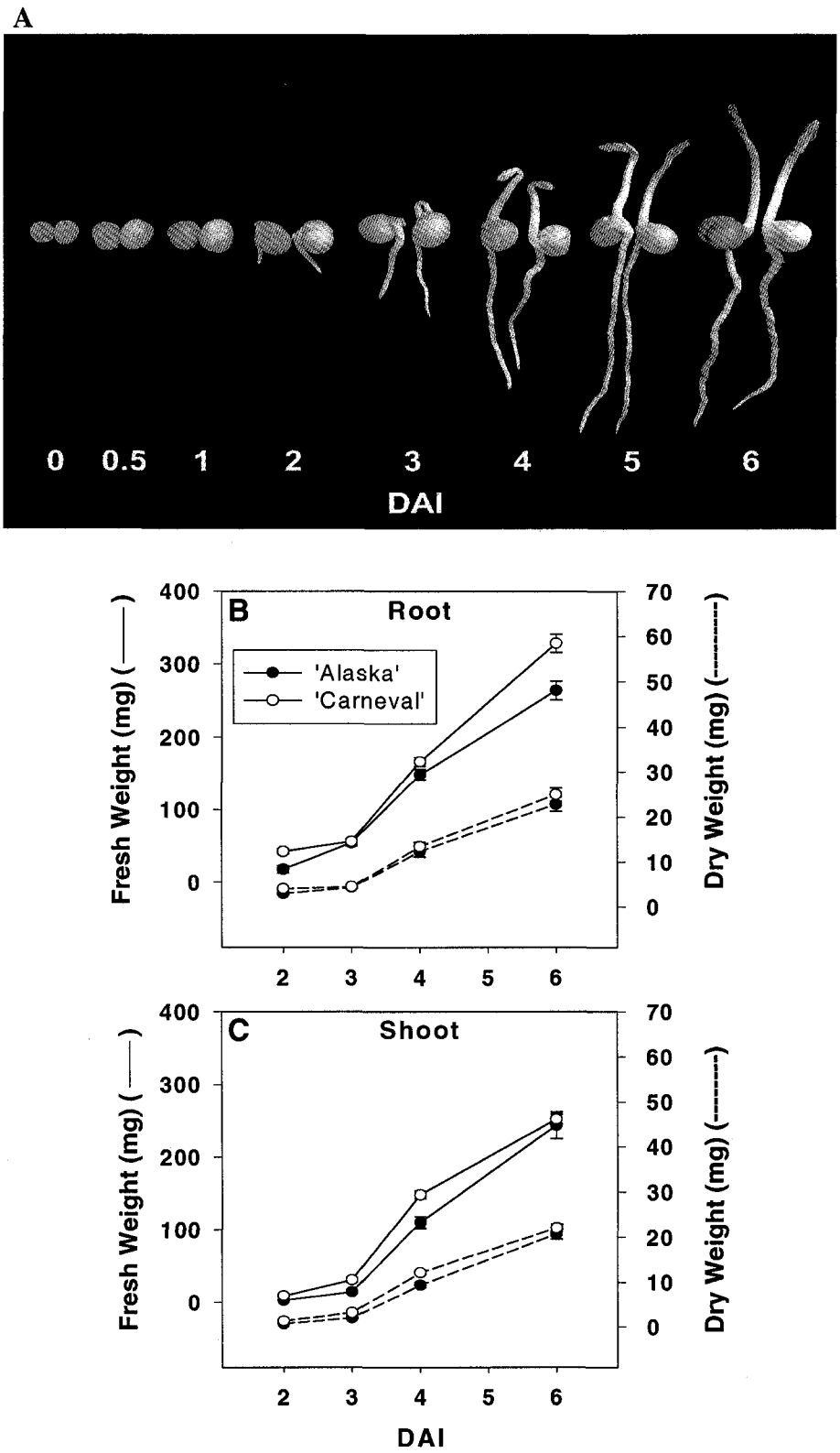
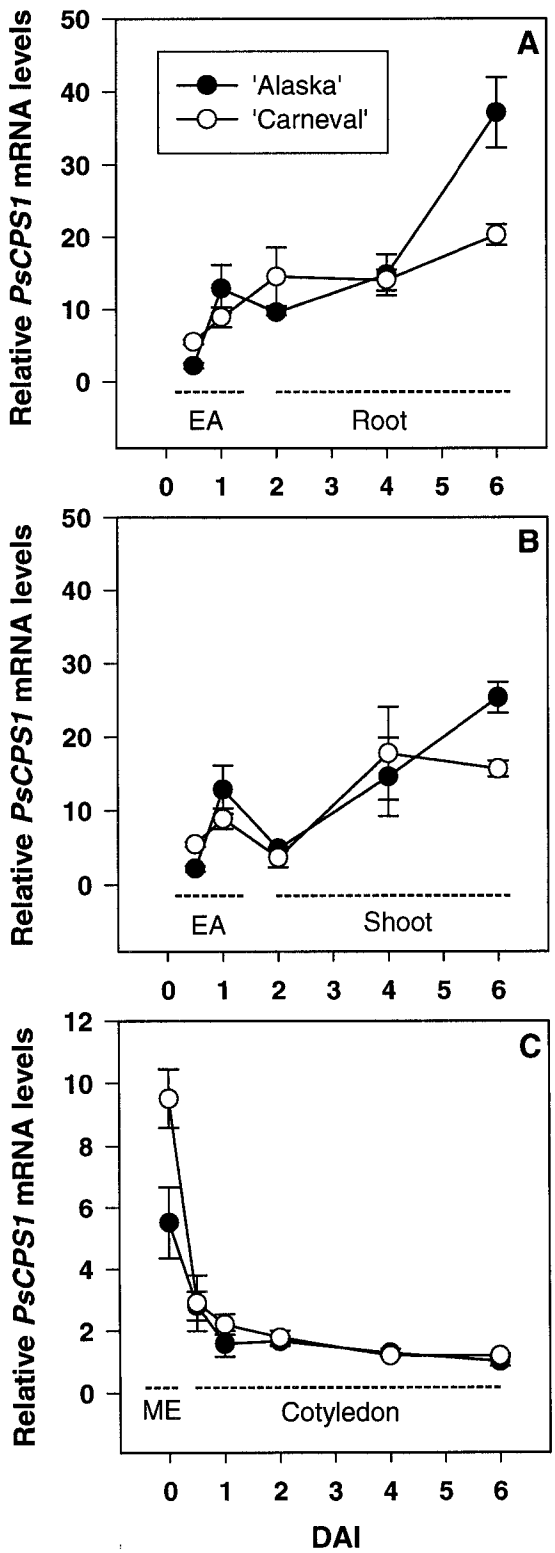


Figure 2.3. Relative mRNA levels of *PsCPS1* during seed germination and early seedling growth of 'Alaska' and 'Carneval' (A, B, C). Relative mRNA levels were determined in mature embryos (ME; C), embryo axes (EA, 0.5 and 1 DAI; A, B), roots (2-6 DAI; A), shoots (2-6 DAI; B), and cotyledons (0.5-6 DAI; C) of pea. The mRNA levels were compared across genotypes, developmental stages, and tissues using the average of 'Alaska' 6 DAI cotyledon samples for normalization. Data are means ± SE, n = 2 to 3.



the mature cotyledonary tissue of pea (cells of pea cotyledons are fixed post-mitotically with a normal life span of 2 to 3 weeks; Smith and Flinn, 1967) supports the previous reports that *CPS* transcripts are present not only in actively growing immature tissues (Silverstone et al., 1997; Ross et al., 2003) but also in non-growing mature tissues such as mature internodes of pea (Ross et al., 2003) and fully expanded leaves of Arabidopsis (Silverstone et al., 1997). The *PsCPS1* mRNA stored in the cotyledons of the mature embryo may be translated into protein to produce precursors for the latter part of the GA biosynthesis pathway during and immediately following imbibition of the seed. Consistent with this hypothesis, Graebe et al. (1992) observed that e*nt*-kaurene synthesis began in the pea cotyledons 24 h after imbibition and continued through 10 DAI, but the e*nt*-kaurene synthesis capacity of the shoots was significantly greater than that of the cotyledons from 4 to 10 DAI (per gram fresh weight [gfw]). These data, along with data on the patterns of GA biosynthesis gene expression in the latter part of the pathway (*GA20ox*, *GA3ox*, *GA2ox*; Ayele et al., 2005), suggest that the synthesis of bioactive GAs in pea cotyledons is likely limited to a short period following seed imbibition.

Germination in pea is hypogeal; the cotyledons remain in the germination medium and do not develop into green leaf-like structures that photosynthesize. Our data suggest that the cotyledons of hypogeal species carry over *CPS* transcripts synthesized during seed development for translation into protein upon imbibition of the seeds. In contrast, species such as pumpkin, Arabidopsis and sunflower that exhibit epigeal germination (the hypocotyls extend and the cotyledons emerge above the soil

and expand rapidly), cotyledonary *CPS* expression increases following imbibitions of the seeds. In pumpkin, *CmCPS1* was highly expressed in rapidly expanding cotyledons (3-4 DAI; Smith et al., 1998). Yamaguchi et al. (2001) also reported activity for the *AtCPS1* gene promoter in the provasculature of the cotyledons of 1-day imbibed *AtCPS1* promoter-GUS transgenic Arabidopsis seeds. Furthermore, over 90% of the CPS enzyme activity detected in 4 DAI sunflower seedlings occurred in the rapidly expanding cotyledons (compared to seedling roots and shoots; Shen-Miller and West, 1985). Therefore, the expression pattern of *CPS* genes in the cotyledonary tissue of embryos is related to the morphological development pattern of this organ. Cotyledonary *CPS* transcription takes place mainly during the period of rapid cotyledonary growth, which occurs during seed development in pea (hypogeal species) and during early seedling growth of Arabidopsis, sunflower, and pumpkin (epigeal species).

PsCPS1 Expression in the Embryo Axis and Young Seedling Shoots and Roots

As the embryo axes doubled in fresh weight from 0.5 to 1 DAI (Table 2.3), the abundance of *PsCPS1* transcripts increased in the embryo axes of both cultivars (5.8-fold in 'Alaska' and 1.6-fold in 'Carneval').

At 2 DAI, when shoot growth was 5- to 7-fold lower than root growth (shoot fresh weight equals 2.5 ± 0.5 for 'Alaska' and 9.0 ± 1.0 for 'Carneval', and root fresh weight equals 17.8 ± 5.0 for 'Alaska' and 42.1 ± 3.1 for 'Carneval'; data are means ± SE, n = 15 to 28) (Figures 2.3B and C), transcript abundance of *PsCPS1* was similarly

Table 2.3. Growth of embryo axes (0.5-1 DAI), and roots and shoots (6-8 DAI) of 'Alaska' and 'Carneval'[a]

DAI	Fresh weight (mg)		Dry weight (mg)		Length (mm)		No. of lateral roots	
	'Alaska'	'Carneval'	'Alaska'	'Carneval'	'Alaska'	'Carneval'	'Alaska'	'Carneval'
				Embryo axis				
0.5	4.1 ± 0.6[b]x[c]	5.6 ± 0.4x	2.0 ± 0.1x	1.8 ± 0.1x	3.2 ± 0.4x	3.9 ± 0.2x		
1	8.2 ± 0.8y	13.1 ± 0.8y	2.5 ± 0.1y	2.9 ± 0.1y	5.2 ± 0.3y	6.9 ± 0.3y		
				Root				
6	256.9 ± 9.9x	281.9 ± 10.9x	21.2 ± 2.2x	22.9 ± 1.1x	82.3 ± 2.9x	94.3 ± 4.2x	17.4 ± 1.1x	22.2 ± 0.8x
7	369.0 ± 14.7y	470.5 ± 15.7y	34.1 ± 2.4y	55.4 ± 4.4y	97.1 ± 2.9y	115.6 ± 4.4y	24.4 ± 1.2y	27.2 ± 1.3y
8	553.6 ± 27.9z	563.1 ± 31.4z	65.3 ± 4.6z	63.7 ± 5.8z	112.0 ± 4.1z	132.2 ± 6.1z	33.1 ± 1.4z	36.5 ± 1.2z
				Shoot				
6	224.7 ± 7.5x	243.4 ± 6.9x	22.3 ± 0.8x	23.3 ± 0.9x	50.2 ± 1.5x	49.4 ± 1.1x		
7	318.9 ± 10.9y	326.6 ± 8.2y	33.1 ± 1.3y	33.8 ± 0.8y	64.0 ± 1.7y	60.0 ± 1.3y		
8	431.6 ± 13.6z	384.7 ± 14.4z	47.6 ± 2.0z	41.9 ± 2.1z	80.8 ± 2.0z	69.2 ± 1.5z		

[a] By 2 DAI, 90% of 'Carneval' and 65% of 'Alaska' seeds exhibited radicle protrusion.

[b] Data are means ± SE, n = 15 to 30.

[c] Means followed by different letters (x, y, z) indicate significant difference among days after imbibition (DAI) within tissues and cultivars by LSD, $P \leq 0.05$.

lower in the shoot than the root (2- and 3.9-fold lower in 'Alaska' and 'Carneval', respectively; Figures 2.3A and B). As shoots grew from 2 to 4 DAI, *PsCPS1* transcript level increased (3-fold in 'Alaska' and 4.8-fold in 'Carneval'). From 4 to 6 DAI, *PsCPS1* transcript abundance further increased in the shoots of 'Alaska'. The capacity for synthesis of [^{14}C]*ent*-kaurene from [^{14}C]MVA in cell free enzyme extracts of 'Alaska' shoot tips increased from minimally detectable levels at 3 DAI to half-maximal levels by 5 DAI, then increased to maximal levels by 9 DAI (Ecklund and Moore, 1974). Graebe et al. (1987; 1992) also found that *ent*-kaurene synthesis was high in the shoots of pea from 4 to 10 DAI (not detected prior to 4 DAI in the shoots). These data suggest that expression of *PsCPS1* is under developmental regulation, increasing upon imbibition in the actively growing embryos axis. Translational or post-translational regulation of *PsCPS1* to produce active enzyme, at least in the shoots, appears to occur when the supply of GA precursors and bioactive GAs stored in the mature pea seed are depleted (by 4 DAI, when shoots first respond to GA biosynthesis inhibitors with reduced growth; Sponsel, 1983).

In 'Carneval', from 4 to 6 DAI, shoot-derived *PsCPS1* transcript abundance did not increase (Figure 2.3B), and by 8 DAI the shoots became significantly shorter than 'Alaska' (Table 2.3) due to the presence of the *le-1* mutation that reduces the synthesis of GA_1 by 92% (Martin et al., 1997). These data may suggest that *CPS* expression is regulated by endogenous bioactive GA; however, the differences in shoot-derived *CPS* abundance between 'Alaska' and 'Carneval' may only be a reflection of their different genetic backgrounds. Using Northern analysis, Davidson et al. (2005) found that *PsCPS1*

was consistently slightly higher in stem tissue from the GA-deficient *lh-2* mutant than the *LH* wild-type. However, no substantial increase in expression of *PsCPS1* was observed in stem tissue (from plants with a well developed shoot system) of GA-deficient mutants *lh-1*, *ls-1* and *na-1* compared to their respective wild-types, or in wild-type stem tissue from seeds treated with the GA biosynthesis inhibitor paclobutrazol.

In the root, soon after radicle protrusion (2 DAI), root-expressed *PsCPS1* transcript abundance was either at the same level in 'Alaska' or slightly higher in 'Carneval' than the levels observed in the pre-germinative 1-DAI embryo axes (Figure 2.3A). The transcript level of root-expressed *PsCPS1* did not change from 2 to 4 DAI in both cultivars, but as root growth continued through 6 DAI (Figure 2.2) the transcript levels increased 2.5-fold in 'Alaska' and 1.4-fold in 'Carneval' (Figure 2.3A). This increase in abundance of root-expressed *PsCPS1* transcripts from 4 to 6 DAI could be due to contributions of *CPS* transcripts from the lateral root primordia, which were apparent by 6 DAI (Table 2.3). Silverstone et al. (1997) found that GUS staining was confined exclusively to a small region that includes the root meristem in the main and lateral roots of 5-d-old *AtCPS1* promoter-GUS transgenic Arabidopsis seedlings. Indeed, as most of the lateral root primordia were localized around the base of 6 DAI tap root (Figure 2.2A), the similar levels of *PsCPS1* transcripts found in the 6 DAI root tips and the remainder of the roots (Figure 2.4) may reflect the inclusion of the laterals root initials in the later tissue.

Although similar levels of *PsCPS1* transcript were found in the seedling shoot and root (4 to 6 DAI; Figures 2.3A and B), *ent*-kaurene synthesis was found mainly to occur

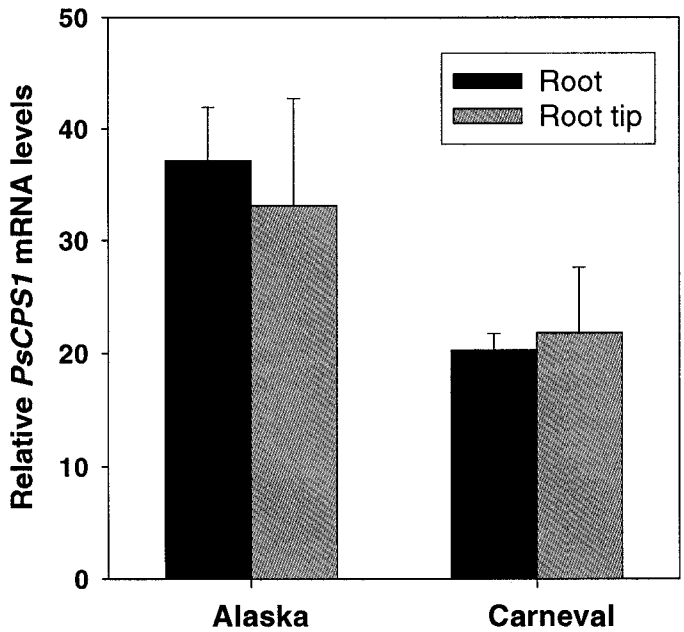


Figure 2.4. Relative mRNA levels of *PsCPS1* in 6 DAI roots and root tips (4 mm) of 'Alaska' and 'Carneval'. mRNA levels were compared across genotype and tissue using the average of 'Alaska' 6 DAI cotyledon samples for normalization. Data are means ± SE, n = 2 to 3.

in the shoots during early seedling growth (Graebe et al., 1987). It is possible that the minimal *ent*-kaurene synthesizing activity observed in the roots was sufficient to produce the level of *ent*-kaurene required for normal root growth, as roots require lower GA levels for optimal growth than the shoots (Tanimoto, 1990; Yaxley et al., 2001). Post-transcriptional regulation of *CPS* or post-translational regulation of the protein product may occur to reduce the abundance of the active protein in the root. Indeed, the pea *ls-1* mutation (*PsCPS1*), which dramatically reduced shoot elongation (350%; Yaxley et al., 2001) and shoot GA levels (GA_{19}, GA_{20}, GA_1 and GA_{29}; 3.5 to 13-fold; Ait-Ali et al., 1997), also resulted in a 5- to 267-fold decrease in root GA levels (GA_{19}, GA_{20}, GA_1, GA_{29} and GA_8) but only a slight reduction in taproot elongation (~7%) and a subtle effect on the root phenotype (Yaxley et al., 2001).

Effects of Prohexadione on GA Biosynthesis Gene Expression in Shoots and Roots

To further test whether expression of *CPS* and other genes in the GA biosynthesis pathway are regulated by bioactive GA levels in young pea seedling tissues, bioactive GA levels were modified and the transcript abundance of *PsCPS1* along with the late GA biosynthesis genes was monitored in both roots and shoots of pea.

Prohexadione (an acylcyclohexanedione-type GA biosynthesis inhibitor that inhibits both 2β- and 3β-hydroxylation of GA_{20} and 2β-hydroxylation of GA_1 by competing for 2-oxoglutarate, a required cofactor for both GA 2β- and 3β-hydroxylases; Rademacher, 2000) was used to reduce bioactive GA levels in the seedling tissue. Prohexadione treatment of the seed at imbibition reduced seedling shoot growth in fresh

weight and length by 4 DAI (34% and 32%, respectively, Table 2.4). The reduction in shoot growth was a direct effect of prohexadione on GA biosynthesis as the growth inhibition was reversed by GA_3 treatment (prohexadione plus GA_3 treatment; Table 2.4). By 4 DAI, prohexadione treatment only increased the expression of shoot-derived genes coding for enzymes that catalyze 2β-hydroxylation of GA_{20} (*PsGA2ox1*) and GA_1 (*PsGA2ox2*) (3- to 5-fold increase; Table 2.5). Another acylcyclohexanedione-type GA biosynthesis inhibitor, Primo (trinexapac-ethyl), was shown to inhibit the enzyme activity of *E. coli*-expressed *PsGA3ox1* and *PsGA2ox1* by 65% and 44%, respectively (King et al., 2004). These data, along with those of Brown et al. (1997) that showed that treatment with prohexadione caused marked accumulation of GA_{20} (220% of the control levels) and reduced level of GA_1 (86% of the control level) in peanut plants, suggest that the inhibition of both GA 2β- and 3β-hydroxylases by prohexadione in pea caused a build-up of GA_{20} in the shoot resulting in feed-forward regulation of *PsGA2ox1* by 4 DAI. Additionally, reduction of GAs including GA_{20} in GA-deficient *ls-1* plants resulted in reduced mRNA levels of both *PsGA2ox1* and *PsGA2ox2* in the internodes (but not apices) of shoots compared to the wild-type (Elliott et al., 2001). Although the enzyme coded by *PsGA2ox2* is considered mainly to use GA_1 as a substrate for 2β-hydroxylation (Lester et al., 1999), the increase in *PsGA2ox2* mRNA as a result of prohexadione treatment suggests that GA_{20} accumulation may also lead to feed-forward regulation of the transcription of this gene.

By 6 DAI, a 2- to 3-fold increase in the transcript abundance of *PsGA20ox1* and *PsGA3ox1* was observed in the shoots from prohexadione-treated seeds (Table 2.5).

Table 2.4. Effect of prohexadione or GA_3 on root and shoot growth of 'Alaska' seedlings.

DAI	Fresh weight (mg)				Length (mm)			
	Control	prohexadione	GA_3	prohexadione + GA_3	Control	prohexadione	GA_3	prohexadione + GA_3
				Root				
4	123.1 ± 7.0[a]w[b]	127.8 ± 4.4w	116.0 ± 5.3w	120.0 ± 7.0w	49.4 ± 2.4wx	52.0 ± 2.4wx	53.7 ± 1.9w	46.4 ± 2.3x
6	214.8 ± 14.9wx	229.0 ± 11.8wx	232.8 ± 12.0w	203.3 ± 9.7w	77.5 ± 2.8w	85.5 ± 3.0x	88.0 ± 1.6x	87.1 ± 2.5x
				Shoot				
4	104.0 ± 4.8w	69.0 ± 5.1x	115.8 ±7.0w	98.2 ± 6.1w	26.4 ± 1.0w	18.0 ± 1.1x	40.6 ± 3.0y	32.9 ± 2.2z
6	222.0 ± 10.9w	182.0 ± 8.9x	293.5 ± 17.1y	267.8 ± 10.7y	46.4 ± 1.4w	39.2 ± 1.4x	114.4 ± 4.6y	92.1 ± 2.5z

[a]Data are means ± SE, n = 14 to 20.

[b]Means followed by different letters (w, x, y, z) indicate significant difference among hormone treatments within tissues and days after imbibition by LSD, $P \leq 0.05$.

Table 2.5. Relative mRNA levels of early and late GA biosynthesis genes in roots and shoots of 4 and 6 DAI 'Alaska' seedlings in response to seed treatment with prohexadione or GA_3[a].

DAI	Treatment	*PsCPS1*	*PsGA20ox1*	*PsGA20ox2*	*PsGA3ox1*	*PsGA2ox1*	*PsGA2ox2*
				Root			
4	Control	5.2 ± 0.5[b]	36.5 ± 12.7	5.8 ± 1.2	123.8 ± 14.0	129.6 ± 3.8	72.7 ± 8.1
	prohexadione	4.3 ± 0.5	34.7 ± 3.8	5.1 ± 1.8	41.2 ± 14.5	321.1 ± 48.7	163.8 ± 8.3
	GA_3	3.3 ± 1.2	3.9 ± 1.1	2.4 ± 0.6	3.3 ± 0.2	207.1 ± 28.4	112.9 ± 22.4
6	Control	7.5 ± 1.7	11.7 ± 1.2	12.0 ± 0.3	53.7 ± 1.9	24.0 ± 4.7	53.2 ± 14.7
	prohexadione	5.3 ± 2.1	32.0 ± 5.7	9.6 ± 0.3	44.5 ± 6.9	95.1 ± 18.4	86.7 ± 8.4
	GA_3	5.9 ± 1.9	3.0 ± 0.6	7.5 ± 2.9	5.3 ± 0.4	35.0 ± 13.6	231.3 ± 27.6
				Shoot			
4	Control	12.4 ± 0.7	223.7 ± 58.4	1.3 ± 0.3	145.9 ± 0.2	11.1 ± 4.1	50.2 ± 6.7
	prohexadione	9.8 ± 1.3	214.7 ± 4.6	1.0 ± 0.1	150.7 ± 3.7	56.3 ± 8.8	133.2 ± 12.9
	GA_3	16.1 ± 3.3	5.6 ± 1.3	1.5 ± 0.3	30.7 ± 9.8	27.3 ± 0.7	71.0 ± 14.1
6	Control	18.1 ± 2.4	271.4 ± 20.7	3.4 ± 0.9	24.9 ± 2.6	1.8 ± 0.7	26.9 ± 11.7
	prohexadione	15.2 ± 2.6	701.5 ± 44.4	6.6 ± 1.3	73.4 ± 7.5	4.4 ± 0.4	59.1 ± 17.6
	GA_3	29.5 ± 1.5	24.8 ± 17.1	3.0 ± 0.5	65.0 ± 1.4	9.7 ± 1.4	54.2 ± 0.2

[a] mRNA levels were compared across developmental stages, tissues and gene family members using the average of the *PsGA20ox2* prohexadione-treated 4-DAI shoot samples as the reference for normalization.

[b] Data are means ± SE, n = 2 to 3, except for 6-DAI samples for *PsCPS1,* where n = 4.

These data, along with the decline of *PsGA2ox1* and *PsGA2ox2* transcript levels observed in prohexadione-treated 6-DAI shoots relative to 4 DAI, suggest that after 4 DAI, the pool of GA_{20} and GA_1 in the shoots of prohexadione-treated seeds was sufficiently low enough to stimulate feed-back regulation of *GA20ox* and *GA3ox* genes resulting in increased transcription of these GA biosynthesis genes. Likewise, the shoot apices of GA-deficient *ls-1* plants, exhibited higher levels of *PsGA20ox1* and *PsGA3ox1* mRNA compared to the wild-type (Elliott et al., 2001). Increased expression of specific *GA20ox* and *GA3ox* genes in potato leaves (Carrera et al., 1999) and the shoots and roots of pumpkin seedlings (Lange et al., 2005) in response to treatment with the GA biosynthesis inhibitors prohexadione and LAB 150978 (inhibits *ent*-kaurene oxidation), respectively, was also reported.

Treatment with prohexadione during seed imbibition did not affect seedling root growth by 6 DAI (length and fresh weight, Table 2.4). As pea roots contain substantially lower levels of GAs than the shoots (Ross, 1998; Yaxley et al., 2001), the markedly lower expression of *PsGA20ox1* and higher expression of *PsGA2ox1* in the control roots as compared to the control shoots (Table 2.5) are likely involved in the maintenance of lower root GA_{20} and GA_1 levels. Even though root growth was not affected, prohexadione treatment caused a greater than 2-fold increase in the transcript abundance of root-expressed genes coding for 2β-hydroxylation of GA_{20} and GA_1 (*PsGA2ox1* and *PsGA2ox2*) by 4 DAI (Table 2.5). The concomitant 3-fold decrease in the transcript level of the gene that codes for GA 3β-hydroxylase (*PsGA3ox1*; Table 2.5) in the root may suggest that prohexadione inhibited 2β-hydroxylation of GA_1 to

GA_8 resulting in a transient accumulation of GA_1 and in turn, feed-back regulation of *PsGA3ox1* (GA_3 treatment also decreased *PsGA3ox1* message levels by 4 DAI, see below). By 6 DAI, maintenance of higher *PsGA20ox1* mRNA levels in roots from prohexadione-treated seeds compared to the 6-DAI controls, and of lower *PsGA2ox2* transcript levels in roots from prohexadione-treated seeds compared to those at 4 DAI, was evident (Table 2.5). These data reflect a shift in mRNA expression for increased capacity to produce bioactive GA in response to the inhibition of GA biosynthesis by prohexadione in the root. However, the transcriptional repression of *PsGA3ox1* observed in 4 DAI roots but not in shoots from prohexadione-treated seeds, and *PsGA3ox1* transcriptional up-regulation in 6 DAI shoots but not in roots from prohexadione-treated seeds, suggest that part of the homeostatic mechanism of the root to maintain lower levels of bioactive GAs than in the shoot is through regulation of *PsGA3ox1* mRNA levels. Expression of *PsGA20ox2* in the young pea seedling shoots and roots was minimally or not affected by the prohexadione treatment (Table 2.5).

Effects of GA_3 on GA Biosynthesis Gene Expression in Shoots and Roots

Treatment with GA_3 stimulated shoot growth by 4 DAI in 'Alaska' seedlings (54% increase in length; Table 2.4) and resulted in feed-back regulation of *PsGA20ox1* and *PsGA3ox1* transcription, decreasing their mRNA abundance (Table 2.5). By 6 DAI, *PsGA3ox1* transcription was derepressed (mRNA levels increased) in shoots from GA_3-treated seeds but *PsGA20ox1* transcription was not (Table 2.5). Therefore, *PsGA20ox1* and *PsGA3ox1* message levels were not regulated in parallel by seed treatment with

bioactive GA, in this case, with derepression of *PsGA3ox1* expression preceding that of *PsGA20ox1* at 6 DAI. Previous studies have also shown that bioactive GAs (GA_3, GA_1) significantly reduced the transcript levels of *PsGA20ox1* and *PsGA3ox1* in actively growing pea shoots and pericarps (Martin et al., 1996; van Huizen et al., 1997; Ait-Ali et al., 1999). Reductions in the synthesis of GA_{20} and GA_1 in expanding pea shoot tissues in response to bioactive GA treatment have also been reported (Martin et al., 1996).

Between the two *GA2ox* gene family members, shoot-derived *PsGA2ox1* (codes for enzyme that 2β-hydroxylates GA_{20} and GA_1) showed a greater feed-forward regulation in response to GA_3 treatment (an increase of 2.5-fold at 4 DAI and 5.4-fold at 6 DAI; Table 2.5). *PsGA2ox2* message levels also exhibited feed-forward regulation (2-fold increase) by 6 DAI in the shoots. These data complement those of Elliott et al. (2001) where they observed reduced expression of both *PsGA2ox1* and *PsGA2ox2* in pea internodes, and *PsGA2ox1* in the leaflets of GA_1-deficient *ls-1* mutant. Thomas et al. (1999) also found elevated mRNA levels of *AtGA2ox1* and *AtGA2ox2* in GA_3-treated immature flower buds of the GA-deficient *ga1-2* Arabidopsis mutant.

Treatment with GA_3 (as also observed in the treatment with prohexadione) did not affect root growth (length, fresh weight, Table 2.4). Nevertheless, by 4 DAI, GA_3 application resulted in substantial reductions in *PsGA20ox1* and *PsGA3ox1* mRNA levels (9- to 38-fold) in the roots, which were maintained at low levels through 6 DAI (Table 2.5).

The mRNA levels of both *PsGA2ox* genes also increased (approximately 1.5-fold) in the roots of seeds treated with GA_3 by 4 DAI (Table 2.5). By 6 DAI, *PsGA2ox1* mRNA levels were lower in both control and roots from GA_3-treated seeds; however, roots from GA_3-treated seeds exhibited a greater than 3-fold increase in *PsGA2ox2* mRNA levels compared to the control. This feed-forward regulation of *PsGA2ox2* by GA_3 was greater in the roots (4.3-fold greater than control) than the shoots (2-fold greater than control) suggesting a greater requirement for 2β-hydroxylation to maintain lower levels of bioactive GAs in root tissues.

Application of GA_3 did not alter the transcript abundance of *PsGA20ox2* in the shoots, and only a slight decrease in abundance was observed in the roots.

Expression of PsCPS1

Although modulation of bioactive GA levels by application of GA_3 or the GA biosynthesis inhibitor, prohexadione, resulted in regulation of the transcription of late GA biosynthesis genes in young pea seedling shoots and roots (4 to 6 DAI), *PsCPS1* transcript abundance was, in general, unaffected (Table 2.5), with one exception. The expression of *PsCPS1* in the shoots of seeds treated with GA_3 increased approximately 2-fold from 4 to 6 DAI, coinciding with a greater increase in shoot growth than the control (Tables 2.4 and 2.5). These data from young pea seedling shoot tissues confirm those of Davidson et al (2005) who used shoot tissues from pea plants with well-developed shoot systems, extend these finding to pea roots, and show over time that bioactive GA does not feed-back regulate *CPS* at the mRNA level.

In summary, in pea, which exhibits a hypogeal germination habit, *PsCPS1* mRNA remains in the mature embryo from the maturation phase of seed development, and its expression during germination and seedling growth is tightly associated with the actively growing tissues of the embryo axis. By 1 DAI, the embryo axis is the major site for up-regulation of *PsCPS1*, and increased *PsCPS1* mRNA levels are maintained in the roots and shoots of young seedlings to produce precursors for GA biosynthesis required for growth. Therefore, significant developmental regulation of *CPS* occurs not only during seed development but also during germination and early seedling growth in pea.

CPS is not regulated, however, at the mRNA level by bioactive GA in either roots or shoots of the young pea seedling. The GA biosynthesis genes in the latter part of the pathway, *PsGA20ox1* and *PsGA3ox1*, exhibited feed-back regulation while *PsGA2ox1* and *PsGA2ox2* were feed-forward regulated by bioactive GA at the mRNA level in both roots and shoots of pea seedling. In general, feed-back regulation of *PsGA20ox2* by bioactive GA at the mRNA level was not observed in either roots or shoots of the young seedling. These data confirm the regulatory role of bioactive GA on the transcript levels of late but not early GA biosynthesis genes in root and shoot tissues of pea. Further, our data suggest that the inhibition of the activities of soluble-dioxygenases by prohexadione leads to a reduction in growth prior to feed-back regulation that increases the abundance of transcripts coding for production of GA_{20} and GA_1. Our data also suggest that part of the homeostatic mechanism of the root to maintain lower levels of bioactive GAs relative to the shoot is through regulation of the mRNA levels of the latter part of the GA biosynthesis pathway.

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Chapter 3

Developmental Regulation of Gibberellin Biosynthesis during Germination and Young Seedling Growth of Pea (*Pisum sativum* L.)

Introduction

Seed germination incorporates those events that commence with the uptake of water by the quiescent dry seed (imbibition) and terminate with the protrusion of the radicle from the seed coat (Bewley and Black, 1994). Subsequently, seed storage reserves are mobilized to support the growth of the embryo axis (seedling). Previous studies have shown that GAs play a critical role in promoting the germination of small-seeded dicotyledonous plants such as Arabidopsis (*Arabidopsis thaliana*) and tomato (*Lycopersicon esculentum* Mill.). Seeds from the GA deficient mutants of Arabidopsis (*ga1-3* and *ga2-1*) and tomato (*gib-1*) failed to germinate without exogenous GAs (Koornneef and van der Veen, 1980; Groot and Karssen, 1987). Moreover, GA biosynthesis inhibitors such as uniconazole and tetcyclasis inhibit germination of wild-type Arabidopsis seeds (Hilhorst and Karssen, 1988; Nambara et al., 1991). In these small-seeded species, two roles have been proposed for GAs in stimulating germination. One role is to weaken the aleurone and testa tissues that surround the embryo radicle to overcome the mechanical restraint conferred by these tissues (Groot and Karssen, 1987; Debeaujon and Koornneef, 2000). The second role is to increase the growth potential of the embryo axis, as GA-deficient embryos show reduced rate of growth (Groot and Karssen, 1987).

These GA-mediated events are regulated in part by the modulation of tissue- and cell-specific GA concentrations and by altering the ability of cells to respond to GA (Richards et al., 2001). Monitoring the expression of genes encoding enzymes involved in GA biosynthesis and catabolism is one approach to gain insight into the regulation of GAs. Analysis of whole seed extracts of Arabidopsis revealed up-regulation of GA biosynthesis genes that encode enzymes that produce bioactive GA, and the immediate precursor of bioactive GA (*GA3ox* genes, Yamaguchi et al., 1998; Ogawa et al., 2003 and *GA20ox* genes, Ogawa et al., 2003), with minimal transcription of *GA2ox* genes that encode enzymes which reduce the pool of bioactive GA or its immediate precursor during seed germination (Ogawa et al., 2003). This gene expression pattern led to a detectable increase in bioactive GA_4 in the germinating Arabidopsis seed (Ogawa et al., 2003). However, the tissue-specific regulation and coordination of the expression of these gene families and the developmental changes of endogenous GAs within the embryo during germination and early seedling growth are not well understood.

Even though a picture of the roles of GAs during germination and early seedling growth is starting to emerge for small-seeded dicots, little is known about the roles of GAs in these processes in large-seeded, non-endospermic and hypogeous dicotyledonous plants such as pea (*Pisum sativum* L.), which has been a model system to understand the role of GAs in other aspects of plant growth and development. GA biosynthesis has been recently investigated in young seedlings of the large-seeded dicot, pumpkin (*Cucurbita maxima*; epigeal species); however, the germination process was not the focus of this study (Lange et al., 2005).

In pea, the later part of GA biosynthesis is mainly through the early 13-hydroxylation pathway: $GA_{12}\rightarrow GA_{53}\rightarrow GA_{44}\rightarrow GA_{19}\rightarrow GA_{20}\rightarrow GA_1$ (Figure 1.1). GA 20-oxidase catalyzes the conversion of GA_{53} via GA_{44} and GA_{19} to GA_{20}, GA 3β-hydroxylase converts GA_{20} to bioactive GA_1, and GA 2β-hydroxylases convert GA_{20} to biologically inactive GA_{29} and GA_1 to biologically inactive GA_8 (Hedden and Phillips, 2000).

GA-biosynthesis-inhibitor studies suggested that de novo GA biosynthesis is not essential for pea seed germination, but for the maintenance of normal seedling growth soon after germination (Sponsel, 1983; Graebe, 1986; Ross et al., 1993). In support of this hypothesis, bioactive GA_1 was detected in the shoots of developing wild-type pea seedlings as early as 4 DAI (DAI; Ross et al., 1992). Furthermore, the *na* mutation of the pea GA biosynthesis gene *NA* (*PsKAO1*; encodes *ent*-kaurenoic acid oxidase mainly expressed in vegetative tissues; Davidson et al., 2003) does not inhibit germination (the homolog *PsKAO2* is highly expressed in the developing seeds and results in normal GA levels in *na* seeds; Potts and Reid, 1983; Davidson et al., 2003) but affects bioactive GA level, shoot and root growths in the post germinative phases (Potts and Reid, 1983; Proebsting et al., 1992; Yaxley et al., 2001).

Mature dry pea seeds (cv. Torsdag; *LE*) contained GA_{20} (11 ng g^{-1}), GA_{29} (126 ng g^{-1}), and GA_{29}-catabolite (11,595 ng g^{-1}), but GA_1 was not detected (Ross et al., 1993). GA_1 was detected in the shoots of 4-day-old pea plants (apical portion above the 1st fully expanded leaf; 1.1 ng $gfwt^{-1}$; cv. Torsdag; *LE*). These data, along with data from the GA-biosynthesis-inhibitor studies, suggest an expression pattern of GA

biosynthesis genes that is translated into an increase in bioactive GA_1 in shoots of young pea seedlings. However, the exact timing and tissue-specific nature of de novo GA biosynthesis during these early events in pea seedling growth in the shoots and the other seedling organs is not known. Since bioactive GAs in young seedlings of pea are low (Ross et al., 1993) and specific GAs can also be readily transported between tissues (Proebsting et al., 1992), profiling the expression patterns of the genes that code for the regulatory GA biosynthesis enzymes, along with quantitation of the endogenous GAs, in each organ of germinating seeds, and developing seedlings would greatly increase our understanding of the spatial and temporal regulation of GA biosynthesis during germination and early seedling growth.

The developmental and homeostatic regulation of the expression of the genes encoding GA 20-oxidase, GA 3β-hydroxylase, and GA 2β-hydroxylase (*GA20ox, GA3ox* and *GA2ox*) plays an important regulatory role in modulating the concentration of bioactive GAs (Hedden and Phillips, 2000). The following late GA biosynthesis genes have been identified in pea: two *GA20ox* genes (*PsGA20ox1*, expressed in vegetative tissues and developing seeds; Martin et al., 1996; Garcia-Martinez et al., 1997; van Huizen et al., 1997; and *PsGA20ox2*, expressed in developing seeds; Ait-Ali et al., 1997), one *GA3ox* gene (*PsGA3ox1*, expressed in vegetative tissues and developing seeds; Martin et al., 1997; Lester et al., 1997; Ozga et al., 2003) and two *GA2ox* genes (*PsGA2ox1* and *PsGA2ox2*, both expressed in vegetative tissues and developing seeds, with *PsGA2ox1* playing a major role in GA_{20} deactivation in pea tissues while *PsGA2ox2* likely being important for GA_1 deactivation in the shoot;

Martin et al., 1999; Lester et al., 1999). The coordination of the expression patterns of these key GA biosynthesis genes during germination and early seedling growth in pea is not known.

This study characterizes the expression pattern of these key GA biosynthesis genes (*PsGA20ox1* and *PsGA20ox2, PsGA3ox1,* and *PsGA2ox1* and *PsGA2ox2*) in mature embryos, embryo axes, cotyledons, shoots, and roots of pea from 0 to 6 DAI, and examines the coordination of gene expression among these GA gene family members during the critical stages for germination and seedling establishment in pea. Quantitation of gene expression was completed using real-time RT-PCR, the method of choice for sensitive, specific and reproducible quantification of mRNA (Bustin, 2000). The endogenous GAs that are products of enzymes coded by these GA biosynthesis genes were also quantitated over the same developmental period. To broaden our inferences on the role of these GA genes in these processes, we compared the GA biosynthesis gene expression patterns in two distinctly different cultivars of pea ('Alaska', a model cultivar for tall [*LE*] vining pea; and 'Carneval', a model cultivar for semi-leafless, semi-dwarf field pea), both of which germinate readily upon imbibition under normal environmental conditions. Our data suggest that the spatial and temporal coordination of the expression of these key regulatory GA biosynthesis genes in the developing seeds (resulting in sequestration of these mRNAs in the quiescent [mature] seed) and in germinating seeds is required to modulate the capacity to produce bioactive GA and in turn to bring about specific developmental programs within these tissues.

Materials and Methods

Plant Material

The pea cultivars (*Pisum sativum* L.) used in this study are model cultivars for vining type pea ('Alaska'; I_3) and field pea ('Carneval') as described previously (Chapter 2).

Growth Conditions and Harvesting

Mature air-dry seeds of 'Alaska' (5.4% RWC) and 'Carneval' (5.8% RWC) were planted at a depth of ca. 2.5 cm into moist sterilized sand in 3 L plastic pots (10 seeds per pot), the pots were placed in a growth chamber (Conviron, Ashville, NC) at 22/20^{0}C (day/night) in a 16/8 h photoperiod with cool white fluorescent and incandescent lights (205 μE $m^{-2}s^{-1}$) until harvest. For germination and growth measurements, seeds of each cultivar were harvested at 0.5, 1, 2, 3, 4 and 6 DAI from the sand medium, and separated into cotyledons and embryo axes (0.5 and 1 DAI) or into cotyledons, roots and shoots (2-6 DAI) (5-10 seeds or seedling per replication; 3 replications per time point). Seeds were scored as germinated when protrusion of radicle (2-5 mm) through the seed coat was visible. RWC of the cotyledons was determined by comparing the sample weights before and after drying for 72 h at 60°C, and are expressed on a fresh weight basis. For RNA extraction, seedlings at 0.5, 1, 2, 4 and 6 DAI were separated either into cotyledons and embryo axes (0.5 and 1 DAI), or cotyledons, shoots and roots (2 and 4 DAI), or cotyledons, shoots, root tips (ca. 4 mm) and remainder of roots (6 DAI), and immediately frozen in liquid N_2 and stored at -80^{0}C

until extraction. From 2 DAI, only germinated seeds were used. RNA was also extracted from developing seeds and mature embryos (0 DAI). Developing seeds were harvested at 20 DAA and stored at -80^{0}C until RNA extraction. In the case of mature embryos (0 DAI), seeds of the two cultivars were immersed in ice: water (1:1, w/v) for 4 h to facilitate seed coat removal, and the embryos (cotyledon plus embryo axis) were immediately frozen in liquid nitrogen and stored at -80^{0}C until RNA extraction.

RNA Isolation

Tissues were finely ground in liquid N_2, and 200 to 550 mg fresh weight (developing seeds, mature embryos, embryo axes, shoots, roots and root tips) or 100 to 250 mg fresh weight (cotyledons) subsamples were used for total RNA isolation using a modified TRIzol (Invitrogen, Carlsbad, CA) protocol as described previously (Chapter 2). After initial extraction with the TRIzol reagent and centrifugation, the supernatant was cleaned by chloroform partitioning (0.2 mL mL^{-1} TRIzol). The resulting supernatant fraction was then precipitated sequentially by isopropanol (0.25 mL mL^{-1} TRIzol) and high salt solution (1.2 M Na citrate and 0.8 M NaCl) to remove polysaccharides and proteoglycans, 4 M LiCl, and finally by a mixture of 3 M Na acetate (pH 5.2): 100% ethanol (1:20, v/v). The precipitate was dissolved in DEPC treated water. Verification of the RNA integrity, DNase digestion of the total RNA samples, purification of the seed and cotyledonary total RNA samples, and determination of sample RNA concentration was performed as described previously (Chapter 2).

Gene Expression Analysis

Primers and Probes

Primers and probes for the reference gene amplicon *18S-62* (used for pea 18S rRNA quantification), and target genes amplicons, *GA20ox1-104* (used for *PsGA20ox1* quantification), *GA20ox2-88* (used for *PsGA20ox2* quantification), *GA3ox1-87* (used for *PsGA3ox1* quantification), *GA2ox1-73* (used for *PsGA2ox1* quantification) and *GA2ox2-83* (used for *PsGA20ox2* quantification) were designed using Primer Express Software (Applied Biosystems) as described previously (Chapter 2).

All probes were TaqMan MGB and labeled at the 5' end with fluorescent reporter dye 6-carboxyfluorescein (target gene probes) or VIC (*18S62* reference gene probe), and at the 3' end with non-fluorescent quencher (NFQ) dye (Applied Biosystems). Search of databases for sequences homologous to the amplicon sequences of each gene, and confirmation of the PCR product produced by the quantifying primers was performed as described previously (Chapter 2).

Real-Time RT-PCR Assay

Real-time RT-PCR assays were performed on a model 7700 sequence detector (Applied Biosystems) using a TaqMan One-Step RT-PCR Master Mix Reagent Kit (Applied Biosystems) as a 25 µL (for the expression analysis of *PsGA20ox2* and *PsGA2ox2*) as described previously (Chapter 2) or 50 µL (for the analysis of *PsGA20ox1*, *PsGA3ox1* and *PsGA2ox1* expression) reactions. For each 50 µL reaction, 5 µL of sample RNA (200 ng of total RNA for *PsGA20ox1*, *PsGA3ox1* and *PsGA2ox1*

or 10 pg of total RNA for 18S rRNA quantitation) was mixed with 25 μL of 2X Master Mix (containing AmpliTaq Gold DNA polymerase), 1.25 μL of 40X MultiScribe (reverse transcriptase and RNase inhibitor mix), 3 μL forward primer (5 μM; final concentration 300 nM), 3 μL reverse primer (5 μM; final concentration 300 nM), 1 μL probe (5 μM; final concentration 100 nM) and 11.75 μL DEPC treated water. Samples were subjected to thermal cycling conditions of reverse transcription at 48°C for 30 min, DNA polymerase activation at 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 s followed by anneal extension at 60°C for 1 min. PCR amplification of each sample was carried out in duplicate in 96-well optical reaction plates covered with optical caps (Applied Biosystems), and the average of the two sub-samples was used to calculate the sample transcript abundance. Total RNA extracts from each tissue were pooled across all time points per cultivar, and this pooled sample was run on each plate and used as a control to correct for plate to plate amplification differences. A pooled sample from one real-time RT-PCR run was taken arbitrarily as the standard for normalizing the C_t values of samples in other runs as described previously (Chapter 2).

The relative transcript abundance of the target genes in the individual plant samples was determined by the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001) as described previously (Chapter 2), where ΔC_t was the difference between the target sample C_t and average C_t of the reference sample. Transcript levels were compared across all genes, genotypes, developmental stages and tissues using the average C_t value of *PsGA20ox1* from the mature embryo of 'Carneval' as the reference sample. Two to 3 biological replicates of each plant sample were assayed.

Analysis of Endogenous GA Levels

Endogenous GA levels in the developing seeds (20 DAA), mature embryos (cotyledon plus embryo axis), cotyledons and embryo axes (1 DAI), and cotyledons, shoots, and roots (2 and 4 DAI) were analyzed by Dr. Leonid Kurepin at the University of Calgary as described elsewhere (Jacobsen et al., 2002).

Results and Discussion

Quantitation of target genes

The specificity of the RT-PCR product of the target genes amplicons (*GA20ox1-104*, *GA20ox2-88*, *GA3ox1-87*, *GA2ox1-73*, *GA2ox2-83*) and the reference gene amplicon (*18S-62*; for pea 18S rRNA) were confirmed electrophoretically as described previously (Chapter 2).

The pea 18S rRNA amplicon was used as a loading control to estimate variation in input total RNA concentration across all samples within each cultivar as described previously (Chapter 2). The average cycle threshold (C_t) value (± SD) for the 18S amplicon in all 'Alaska' and 'Carneval' tissue samples was 25.92 ± 0.69 (n = 84; CV = 2.7%) and 25.96 ± 0.86 (n = 82; CV = 3.3%), respectively. As the CV of the 18S amplicon C_t values was very low among all the samples assayed (2.7 to 3.3% CV), target gene mRNA values were not normalized to the 18S signal.

Results on BLAST search for significant sequence homology between the amplicons of the target genes and other genes in all GenBank, European Molecular Biology Laboratory (EMBL), DNA Data Bank of Japan and Protein Data Base database

sequences (including expressed sequence tags [ESTs], sequence tagged sites, genome survey sequences, and phase 0, 1 or 2 high-throughput genomic sequences) are detailed in Chapter 2.

GA biosynthesis in developing seeds and mature embryos

High levels of *PsGA20ox2, PsGA2ox1* and *PsGA2ox2* mRNAs (Table 3.1; encoding the enzymes that convert GA_{53} to GA_{20} [GA 20-oxidase], and GA_{20} to GA_{29} and/or GA_1 to GA_8 by 2ß-hydroxylation, respectively; Figure 1.1) were detected in the developing seeds (20 days after anthesis; DAA), along with high levels of GA_{20} and GA_{29} (Table 3.2). In contrast, the transcript abundance of *PsGA20ox1* was low (ca. 3,000-fold lower than *PsGA20ox2*) in the developing seeds suggesting that *PsGA20ox2* expression is responsible for coding the majority of the GA 20-oxidase for production of GA_{20} in the developing seeds. *PsGA3ox1* mRNA levels were also low, and bioactive GA_1 as well as its immediate biologically inactive catabolite, GA_8, were not detected in the 20 DAA developing seeds (Tables 3.1 and 3.2). These data are consistent with previous gene expression studies (Lester et al., 1999; Ait-Ali et al., 1997), the feeding experiments that indicated the developing pea cotyledon as a site for 2ß-hydroxylation of GA_{20} into GA_{29} (Sponsel, 1983) and with an emerging hypothesis that bioactive GA is minimized in the developing embryo to allow for seed maturation processes to proceed. Curaba et al. (2004) found that the embryonic transcription regulators, *LEC2* and *FUS3,* involved in multiple aspects of Arabidopsis seed development (including repression of leaf traits and premature germination, and activation of seed storage

Table 3.1. Relative transcript levels of *PsGA20ox1*, *PsGA20ox2*, *PsGA3ox1*, *PsGA2ox1* and *PsGA2ox2* in developing seeds, mature embryos, and 0.5 and 1 DAI embryo axes and cotyledons[a].

	Developing Seeds	Mature Embryos	Cotyledons		Embryo Axes	
Gene	**20 DAA**	**0 DAI**	**0.5 DAI**	**1 DAI**	**0.5 DAI**	**1 DAI**
			'Alaska'			
PsGA20ox1	9.5 ± 4.8[b]	1.1 ± 0.3	2.1 ± 1.1	28.6 ± 17.3	66.8 ± 24.7	870.7 ± 106.3
PsGA20ox2	28848.3 ± 2623.8	43.3 ± 4.9	91.6 ± 29.3	145.4 ± 74.4	5.9 ± 1.0	25.3 ± 1.7
PsGA3ox1	301.1 ± 126	7.2 ± 0.3	8.0 ± 4.4	183.8 ± 76.1	354.8 ± 79.4	5361.8 ± 973.1
PsGA2ox1	15234.9 ± 2332.8	401.9 ± 7.1	533.2 ± 153.6	111.1 ± 14.9	14.0 ± 2.3	15.1 ± 7.0
PsGA2ox2	4973.6 ± 755.7	3313.2 ± 701.9	5518.2 ± 867.2	2193.9 ± 940.8	8235.6 ± 971.4	2250.7 ± 1145.5
			'Carneval'			
PsGA20ox1		1.0 ± 0.0	0.8 ± 0.7	14.9 ± 14.3	46.3 ± 22.5	768.0 ± 79.2
PsGA20ox2		338.3 ± 217.3	180.9 ± 143.6	1098.9 ± 472.5	4.9 ± 0.7	7.6 ± 0.7
PsGA3ox1		4.6 ± 0.6	48.9 ± 40.1	44.8 ± 32.1	689.2 ± 308.3	4312.4 ± 606.9
PsGA2ox1		2259.6 ± 433.4	549.6 ± 193.5	1441.6 ± 51.3	31.3 ± 14.4	31.8 ± 1.9
PsGA2ox2		19668.1 ± 5228.3	4038.0 ± 1849.6	2436.1 ± 1020.2	6896.0 ± 1672.5	1687.6 ± 309.1

[a] Transcript levels were compared across genes, genotypes, developmental stages and tissues using the average of mature embryo *PsGA20ox1* samples of 'Carneval' as a reference for normalization.

[b] Data are means ± SE, n = 2 to 3.

Table 3.2. Endogenous GAs in developing seeds, mature embryos, embryonic axes and cotyledons of 1 DAI seeds, and the cotyledons, shoots and roots of young seedlings of 'Alaska'.

Tissue	DAI	GA_{19}	GA_{20}	GA_{29}	GA_1	GA_8
		ng gfw^{-1}				
20 DAA seed		21.75 ± 3.86[a]	445.94 ± 96.33	188.71 ± 33.99	nd[b]	nd
Mature Embryo	0	1.80[c]	21.96 ± 3.95	84.01 ± 1.21	<0.29[d]	0.45 ± 0.22
Cotyledon	1	1.14 ± 0.10	9.84 ± 0.73	23.30 ± 1.42	1.46 ± 0.06	0.71 ± 0.15
Cotyledon	2	<0.28	3.22 ± 0.31	39.16 ± 18.51	nd	0.59 ± 0.02
Cotyledon	4	<0.39	<0.38	20.64 ± 0.66	nd	0.95 ± 0.23
Embryo Axis	1	3.68 ± 0.15	11.64 ± 2.20	148.56 ± 26.95	1.16 ± 0.05	10.13 ± 0.85
Shoot	2	2.54 ± 0.08	2.25 ± 0.29	145.78 ± 10.02	0.95 ± 0.07	14.80 ± 0.15
Shoot	4	2.04 ± 0.04	0.63 ± 0.01	93.18 ± 20.46	1.03 ± 0.03	14.27 ± 2.17
Root	2	2.68 ± 0.20	2.65 ± 0.18	137.73 ± 9.70	0.73 ± 0.01	10.75 ± 0.05
Root	4	1.29 ± 0.15	0.89 ± 0.04	67.54 ± 3.99	0.73 ± 0.06	6.22 ± 0.08
				pg $organ^{-1}$		
20 DAA seed		9570 ± 1700	196330 ± 42410	83080 ± 14960	nd	nd
Mature Embryo	0	420	5190 ± 933	19850 ± 285	<70	110 ± 52
Cotyledon pair	1	420 ± 36	3610 ± 267	8550 ± 520	540 ± 22	260 ± 56
Cotyledon pair	2	<110	1310 ± 126	15870 ± 7500	nd	240 ± 9
Cotyledon pair	4	<180	<170	9270 ± 295	nd	430 ± 102
Embryo Axis	1	40 ± 2	140 ± 26	1740 ± 316	10 ± 1	120 ± 10
Shoot	2	70 ± 2	60 ± 8	3940 ± 271	30 ± 2	400 ± 4
Shoot	4	150 ± 3	50 ± 1	6670 ± 1465	70 ± 2	1020 ± 156
Root	2	140 ± 10	130 ± 9	6940 ± 489	40 ± 0	540 ± 3
Root	4	120 ± 14	80 ± 4	6390 ± 378	70 ± 5	590 ± 8

[a] Data are means ± SE, n = 2; [b] Not detected in two samples; [c] 1 sample;

[d] GA not detected in 1 of 2 samples.

protein genes), down-regulated *AtGA3ox2* gene expression in the maturing embryo, and resulted in lowering the levels of bioactive GAs in the immature embryos (as determined using the *lec2* and *fus3* Arabidopsis mutants).

Further maturation of the embryo resulted in a large reduction in *PsGA20ox2* (665-fold) and *PsGA2ox1* (38-fold) but not *PsGA2ox2* mRNA levels, consistent with lower but significant levels of GA_{20} (20-fold) and GA_{29} (2.3-fold) detected in the embryo at maturity (Tables 3.1 and 3.2). The high levels of *PsGA2ox2* mRNA in the mature embryo as well as in the embryo axis of 0.5 DAI seeds (Table 3.1), and its preference for GA_1 as a substrate (Lester et al., 1999), suggest that one role for GA 2-oxidases during this developmental period could be to insure minimal levels of bioactive GA_1, in order to complete normal seed maturation and possibly to aid in prevention of precocious germination. Since synthesis of GA_1 and GA_8 in the embryo during the latter stage of pea seed development was very low or not detectable (Table 3.2; Sponsel, 1983), these GAs did not accumulate in the mature embryo (Table 3.2; Ross et al., 1993).

GA biosynthesis in the cotyledonary tissue during germination and early seedling growth

Transcripts of GA catabolic gene (*GA2ox*) family members (*PsGA2ox1* and *PsGA2ox2*) were in greater abundance than the GA biosynthesis genes (*GA20ox* and *GA3ox*; 7- to 19668-fold higher than *PsGA20ox1* and *PsGA20ox2*; 56- to 4322-fold higher than *PsGA3ox1*; Table 3.1) in the mature embryo in both cultivars, which mainly reflects cotyledonary transcript levels at this stage (based on cotyledonary tissue weight

as 98% of the mature embryo, and yields of total RNA from embryo axes and cotyledons).

During the first 0.5 DAI, mature air-dry seeds of 'Carneval' absorbed more water (1.7-fold) than 'Alaska' (Table 3.3; Figure 3.1). At 1 DAI, the RWC of cotyledons from both cultivars were similar (50% in 'Alaska' and 55% in 'Carneval'), and the RWC gradually increased from 1 to 6 DAI in both cultivars (Table 3.3). The transcript levels of cotyledon-expressed *GA20ox, GA3ox* and *GA2ox* genes remained relatively constant in 'Alaska' (Table 3.1; Figures 3.2C, 3.3C, and 3.4C) during the first 0.5 DAI, consistent with its slow rate of seed imbibition (27% RWC after 0.5 DAI, Table 3.3). However, 'Carneval' cotyledons, concomitant with their higher rate of water uptake (46% RWC within the first 0.5 DAI; Table 3.3), exhibited a 4 to 5-fold decrease in *PsGA2ox1* and *PsGA2ox2* mRNA levels (Table 3.1; Figure 3.4F). Similarly, when the RWC of 'Alaska' cotyledon increased to 50% by 1 DAI (Table 3.3), the transcript abundance of *PsGA2ox1* decreased (3.6-fold; Table 3.1) along with a decrease in the level of GA_{29} (3.6-fold; Table 3.2).

The decrease in 2β-hydroxylation of GA_{20} to GA_{29} together with the increase in transcription of *PsGA3ox1* (25-fold; Table 3.1) and production of GA_1 (5-fold; Table 3.2) in the cotyledons by 1 DAI suggest that the cotyledonary GA_{20} (decreased 2.3-fold by 1 DAI; Table 3.2) serves as substrate for *in situ* 3β-hydroxylation into bioactive GA_1. Although bioactive GAs have a well-defined role in coordinating mobilization of the reserve materials in cereals (Jacobsen et al., 1995), their role in mobilization of cotyledonary reserves in dicots is unclear. In addition, bioactive GAs in the cotyledons

Table 3.3. RWC of mature air-dry seeds, seeds after 4 h imbibition, and cotyledons at 0.5 to 6 DAI in 'Alaska' and 'Carneval'.

	(% RWC)	
	'Alaska'	'Carneval'
Mature air-dry seeds	5.4 ± 0.1[a]	5.8 ± 0.1
Seeds 4 HAI[b]	6.1 ± 0.1	23.2 ± 2.2
0.5 DAI	26.6 ± 4.8	45.7 ± 1.1
1 DAI	50.1 ± 1.8	54.6 ± 0.4
2 DAI	52.6 ± 1.0	55.9 ± 0.3
4 DAI	61.8 ± 0.5	62.3 ± 0.3
6 DAI	67.0 ± 0.5	68.2 ± 0.3

[a] Data are means ± SE, n = 15 to 30.

[b] Mature air-dry seeds were soaked in ice water for 4 h to facilitate removal of the seed coat.

Figure 3.1. Germinating pea (*Pisum sativum* L.) seeds and actively growing seedlings of 'Alaska' (left in each pair) and 'Carneval' (right in each pair) from mature embryo (0 DAI) to 6 DAI (A). Embryo axis (EA) fresh weight and length from 0.5 to 1 DAI (Table inserted in Figure 2B, C). Root fresh weight (B) and length (C), and shoot fresh weight (D) and length (E) from 2 to 6 DAI. Data are means ± SE, n = 15 to 28.

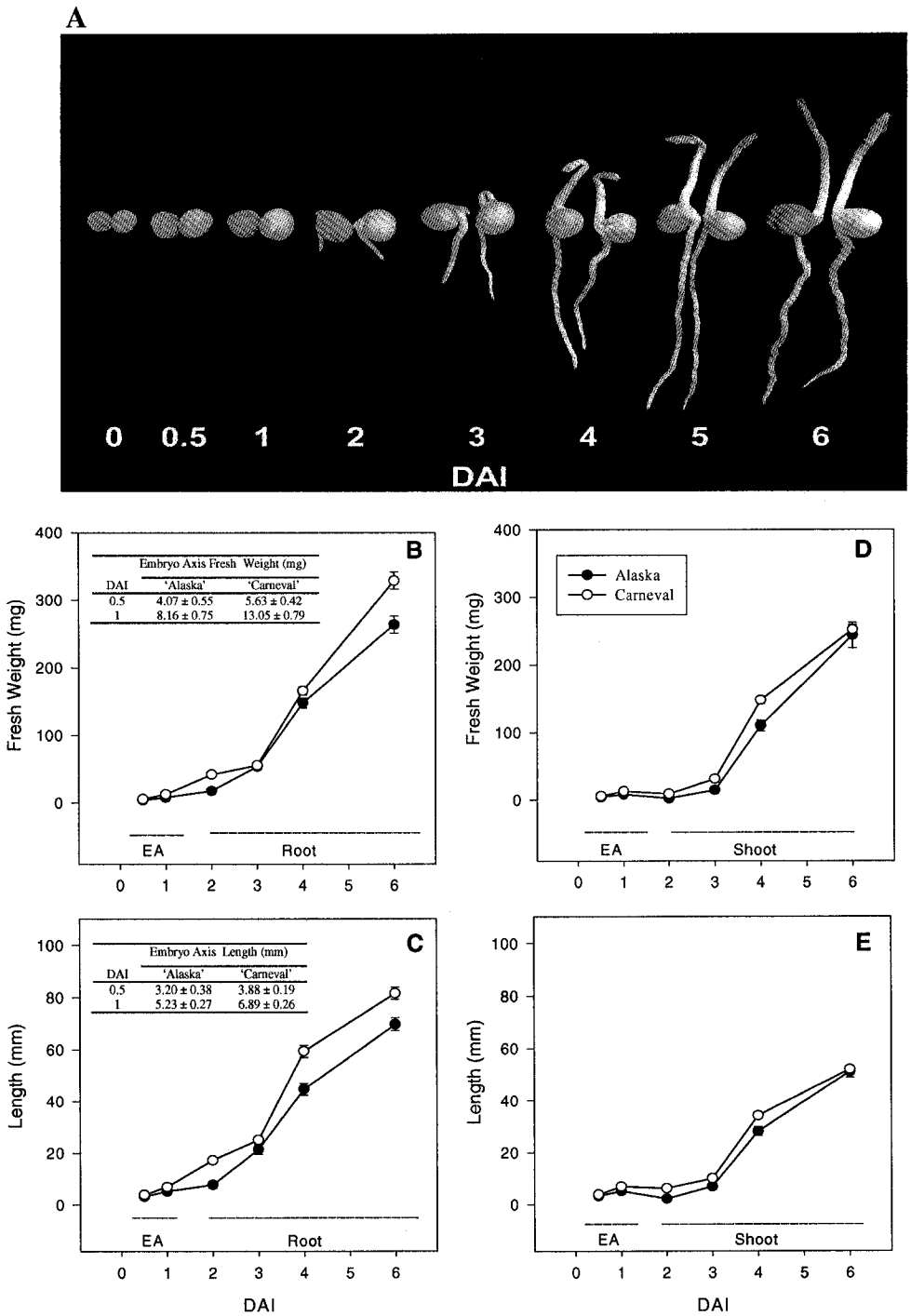


Figure 3.2. Relative transcript levels of *PsGA20ox1* and *PsGA20ox2* during seed germination and early seedling growth of 'Alaska' (A, B, C) and 'Carneval' (D, E, F). Relative transcript levels were determined in mature embryos (ME; C, F), embryo axes (EA, 0.5 and 1 DAI; A, B, D, E), roots (2-6 DAI; A, D), shoots (2-6 DAI; B, E), and cotyledons (0.5-6 DAI; C, F) of pea. Transcript levels were compared across genes, genotypes, developmental stages and tissues using the average of mature embryo *PsGA20ox1* samples of 'Carneval' as a reference for normalization. Data are means ± SE, n = 2 to 3.

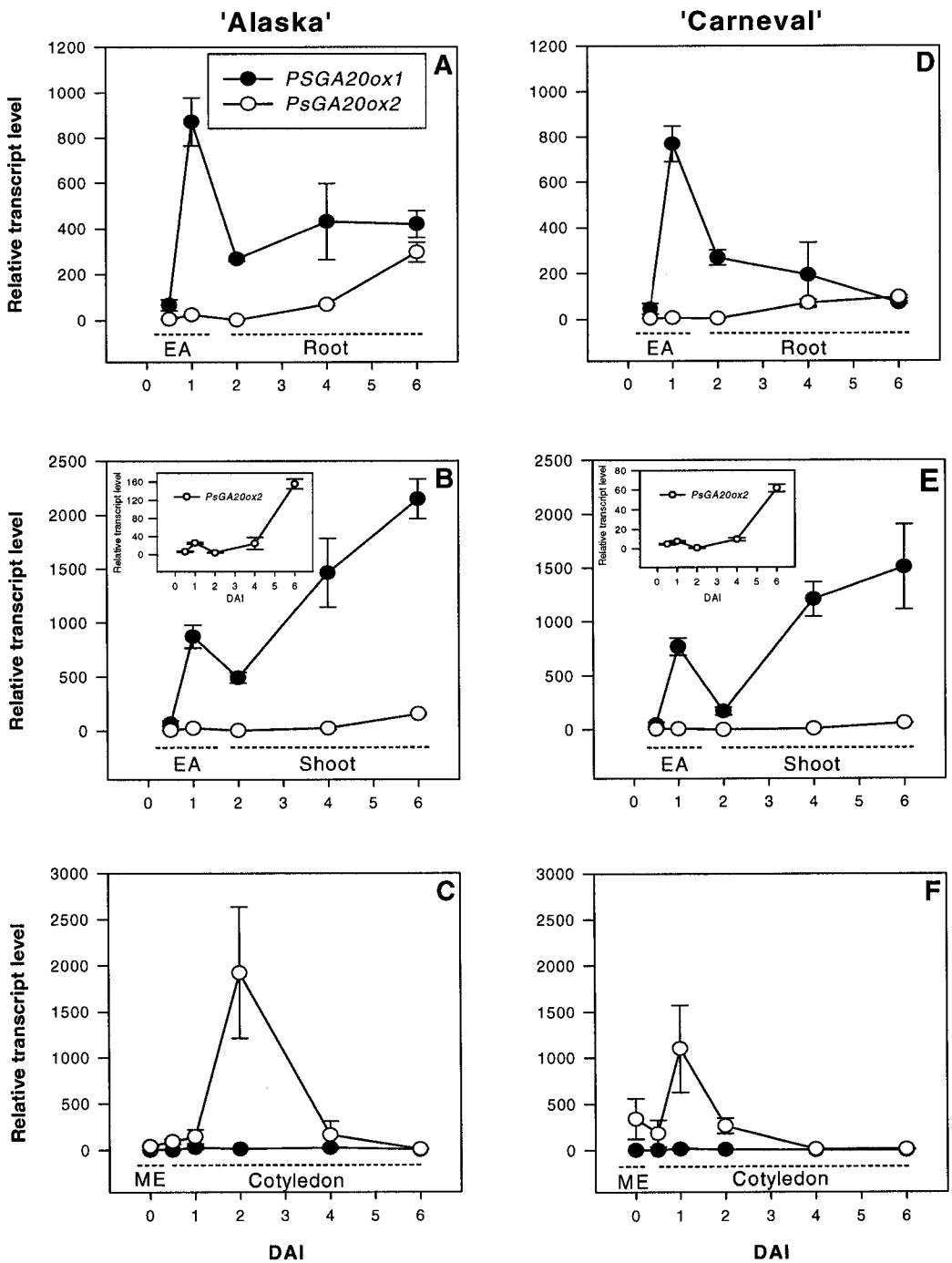


Figure 3.3. Relative transcript levels of *PsGA3ox1* during seed germination and early seedling growth of 'Alaska' and 'Carneval'. Relative mRNA transcript levels were determined in mature embryos (ME; C), embryo axes (EA, 0.5 and 1 DAI; A, B), roots (2-6 DAI; A), shoots (2-6 DAI; B), and cotyledons (0.5-6 DAI; C) of pea. Transcript levels were compared across genes, genotypes, developmental stages and tissues using the average of mature embryo *PsGA20ox1* samples of 'Carneval' as a reference for normalization. Data are means ± SE, n = 2 to 3.

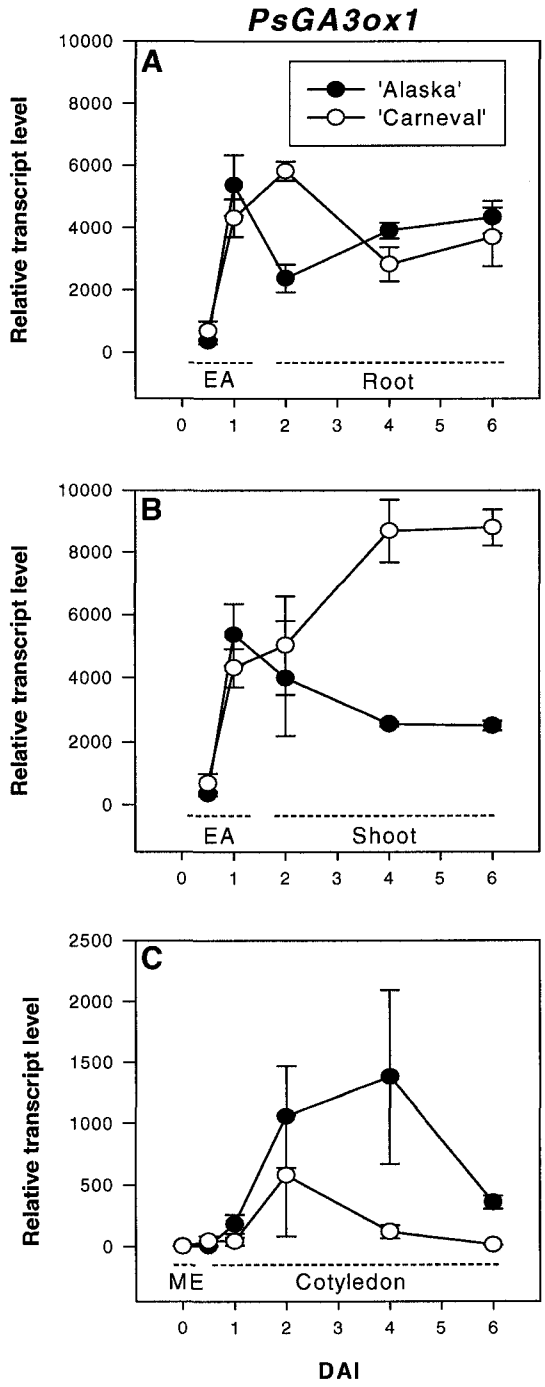
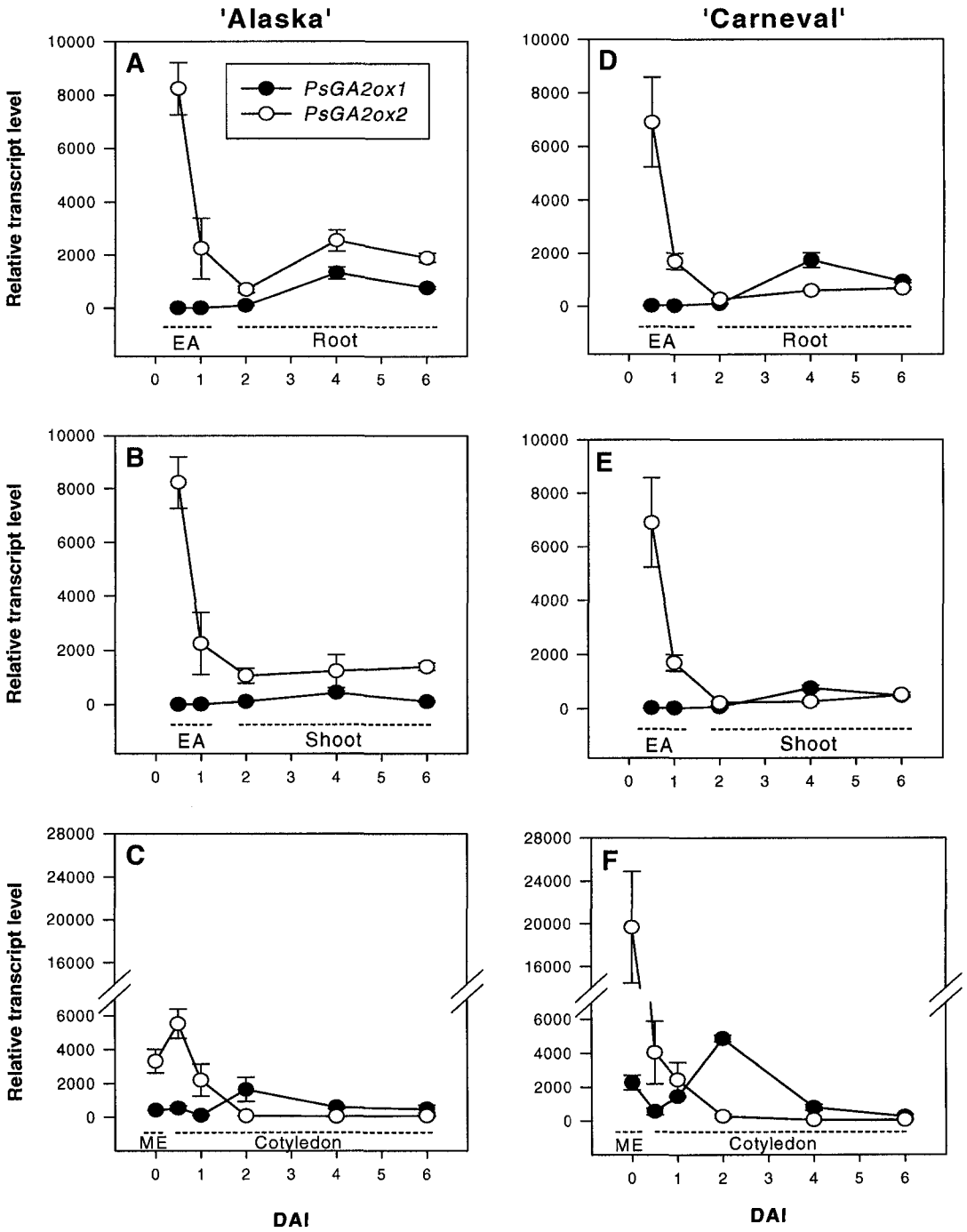


Figure 3.4. Relative transcript levels of *PsGA2ox1* and *PsGA2ox2* during seed germination and early seedling growth of 'Alaska' (A, B, C) and 'Carneval' (D, E, F). Relative transcript levels were determined in the mature embryos (ME; C, F), embryo axes (EA, 0.5 and 1 DAI; A, B, D, E), roots (2-6 DAI; A, D), shoots (2-6 DAI; B, E), and cotyledons (0.5-6 DAI; C, F) of pea. Transcript levels were compared across genes, genotypes, developmental stages and tissues using the average of mature embryo *PsGA20ox1* samples of 'Carneval' as a reference for normalization. Data are means ± SE, n = 2 to 3.



would not be involved in growth or photosynthetic processes as germination in pea is hypogeal (the cotyledons do not develop into green leaf-like structures that photosynthesize) and the cells of pea cotyledons are fixed post-mitotically with a normal life span of 2 to 3 weeks (Smith and Flinn, 1967). However, as specific cell wall loosening enzymes have been shown to be GA inducible during germination of other species (tomato and Arabidopsis; Chen et al., 2001; Ogawa et al., 2003), the bioactive GA_1 produced in the cotyledon could be necessary for promoting the formation of the large reticulum of intercellular spaces in the cotyledons of germinating pea seeds (Smith and Flinn, 1967), possibly to accommodate the high respiratory activity of the cotyledonary storage parenchyma cells during storage mobilization (Bain and Mercer, 1966a). Transport of GA_{20} from the cotyledon to the embryo axis to support synthesis of bioactive GAs for embryos axis expansion can also occur (Ross et al., 1995).

Radicle protrusion (occurs between 1 and 2 DAI; Figure 3.1A) was accompanied by an increase in the expression of cotyledonary *PsGA20ox2* (13-fold in 'Alaska' and 6-fold in 'Carneval'; Figure 3.2C) and *PsGA3ox1* (6-fold in 'Alaska' and 13-fold in 'Carneval'; Figure 3.3C) and a decrease in cotyledonary GA_{20} and GA_1 levels (Table 3.2). Although *GA20ox* and *GA3ox* genes have been shown to be feed-back regulated by bioactive GA levels (pea shoot apices of GA-deficient *ls-1* plants exhibited higher levels of *PsGA20ox1* and *PsGA3ox1* mRNA compared to the wild-type; Elliott et al., 2001), the sharp increase at 1 to 2 DAI, and subsequent decrease at 4 DAI, in cotyledonary *PsGA20ox2* mRNA levels (Figure 3.2C) is not consistent with stimulation of transcript levels as a result of feed-back regulation, as GA_{20} levels continued to

decrease from 1 to 4 DAI (Table 3.2) in this tissue. Indeed, *PsGA20ox2* was not subject to feed-back regulation by bioactive GA levels in the roots and shoots of pea seedlings (Chapter 2). Cotyledonary *PsGA3ox1* mRNA levels increased from 0.5 to 1 DAI, as the GA_1 content increased after 1 d of imbibition (in 'Alaska'; Tables 3.1 and 3.2). From 1 to 2 DAI, *PsGA3ox1* transcript abundance increased further as the GA_1 content decreased to undetectable levels, and both remained at similar levels through 4 DAI (Table 3.2; Figure 3.3C). Although feed-back regulation of *PsGA3ox1* transcription by bioactive GA is likely occurring in the cotyledons after 1 DAI ('Alaska'), the increase in cotyledonary *PsGA20ox2* and *PsGA3ox1* transcript levels observed during or soon after radicle protrusion (1-2 DAI) in both cultivars (Figure 3.3C) may suggest that a signal from the embryo axis may induce expression of GA biosynthesis genes in the cotyledon for production of bioactive GA in this tissue. Indeed, Bain and Mercer (1966b) found that the presence of the axis is required for 2 days following imbibition for complete subcellular organization of the cotyledon to occur.

GA biosynthesis in the embryo axis

Consistent with the initial rate of imbibition (Table 3.3), initiation of radicle protrusion was observed in 53% of the 'Carneval' seeds by 1 DAI, and the percentage of germinated seeds increased to 77% by 1.5 DAI, when only 27% of 'Alaska' seeds exhibited radicle emergence. The germination percentages further increased to 90% in 'Carneval' and 65% in 'Alaska' by 2 DAI.

From 0.5 to 1 DAI, the embryonic-axis fresh weight doubled, axis length increased by approximately 1.6 times in both cultivars (Figures 3.1B, C, see table inset), and transcript abundance markedly increased for embryo-axis-derived *PsGA20ox1* (13-fold in 'Alaska' and 17-fold in 'Carneval') and *PsGA3ox1* (15-fold in 'Alaska' and 6-fold in 'Carneval'; Table 3.1; Figures 3.2 and 3.3). The large increase in gene expression of these *GA20ox* and *GA3ox* biosynthesis genes, concomitant with a substantial decrease in *PsGA2ox2* transcript abundance (ca. 4-fold) and maintenance of low levels of *PsGA2ox1* mRNA levels (Table 3.1; Figure 3.4) from 0.5 to 1 DAI in the embryo axis, suggests increased capacity to synthesize and maintain bioactive GAs in this tissue for its expansion soon after imbibition. The presence of GA_{20}, GA_1 and GA_8 in the 1-DAI-embryo axis is supportive of this hypothesis, specifically since GA_1 was not detected in the developing embryo (20 DAA), and it was much less in the mature embryo than the 1-DAI-embryo axis (Table 3.2).

An increase in *GA20ox (AtGA20ox1* and *AtGA20ox3)* and *GA3ox* (*AtGA3ox1* and *AtGA3ox2)* transcript abundance and bioactive GA_4 level were also shown after imbibition of wild-type Arabidopsis seeds (Ogawa et al., 2003). However, as whole seed extracts were analyzed, their data does not address the tissue specificity of GA biosynthesis gene expression and GA production during the seed germination process.

GA biosynthesis in young seedling roots and shoots

After seed germination (ca. 2 DAI; Figure 3.1A), the plumules of the seedlings of both cultivars started to appear above the germination medium at 4 DAI, and by 5

DAI, the shoots of 73% of 'Alaska' and 88% of 'Carneval' seedlings had emerged. Complete emergence of all germinated seedlings took place by 6 DAI in both cultivars. Root growth in fresh weight and length was generally higher than shoot growth from 2 to 6 DAI in both cultivars (Figure 3.1). Both cultivars had similar average rates of shoot and root growth with a slower growth rate from 2 to 3 DAI followed by a higher rate of growth from 3 to 6 DAI (Figure 3.1).

In the shoot, *PsGA20ox1* transcript levels were more abundant (141-fold in 'Alaska' and 213-fold in 'Carneval') than *PsGA20ox2* at 2 DAI and they increased markedly from 2 to 6 DAI as the shoot rapidly elongated (Figures 3.1D, E and 3.2B, E). In contrast, by 2 DAI the GA_{20} levels in the shoots decreased 5-fold from those in the 1-DAI embryo axis, and GA_{20} levels continued to decrease from 2 to 4 DAI (4-fold; Table 3.2). Since *PsGA20ox1* transcription was shown to be feed-back regulated by bioactive GA (application of GA_3 reduced and application of GA biosynthesis inhibitor prohexadione increased *PsGA20ox1* mRNA levels; Chapter 2) in the shoots of 'Alaska' seedlings, the large increase in shoot-derived *PsGA20ox1* mRNA is likely due to a feed-back regulation mechanism as the result of low GA_{20} levels in the shoot tissues during this stage of rapid growth.

Further, as the expression of shoot-derived *PsGA2ox1* remained low (Figure 3.4B) and the GA_{29} concentration (ng $gfwt^{-1}$) decreased in the shoot from 2 to 4 DAI (Table 3.2), the reduction in shoot GA_{20} levels is not a result of increased 2β-hydroxylation of GA_{20} to GA_{29} in this tissue. Instead, the maintenance of *PsGA3ox1* transcripts and GA_1 concentration (ng $gfwt^{-1}$) in the shoots from 2 to 6 DAI in 'Alaska'

(which contains the wild-type *LE* gene; Figure 3.3B; Table 3.2) suggests that a significant portion of the pool of GA_{20} in the young shoot is used as a substrate for synthesis of GA_1 in situ. In addition, it is possible that the shoot-derived GA_{20} was transported to the root for synthesis of GA_1 (Proebsting et al., 1992). Shoot-derived *PsGA2ox2* transcript abundance was also maintained at the same level from 2 to 6 DAI in both cultivars (Figure 3.2B and E), and is in agreement with the maintenance of shoot GA_8 level in 'Alaska' from 2 to 4 DAI (Table 3.2).

In the root, by 2 DAI, growth was rapid and *PsGA20ox1* (60- to 120-fold higher than that of *PsGA20ox2*; Figure 3.2A, D) and *PsGA3ox1* (Figure 3.3A) transcripts were abundant. Similar to the shoot tissues, the GA_{20} concentration in the roots decreased during this rapid growth phase (Table 3.2). However, even though feed-back regulation by bioactive GA was also evident for *PsGA20ox1* (but not for *PsGA20ox2*) transcription in the roots of 'Alaska' seedlings (4-6 DAI; Chapter 2), as the roots continued to grow and mature (4-6 DAI), *PsGA20ox1* transcript levels either remained the same ('Alaska') or declined ('Carneval'), and those of *PsGA20ox2* increased to levels similar to that of *PsGA20ox1* by 6 DAI (Figure 3.2A, D). These data suggest that roots, which are more sensitive to GAs (Tanimoto, 1990) and contain lower levels of GA_1 and GA_8 than the shoots (ng $gfwt^{-1}$; Table 3.2), may partially limit bioactive GA_1 production by maintaining lower *GA20ox* transcript levels than that in the shoot (Figures 3.2A, B, D and E). These results are also similar to those of Lange et al. (2005) in 3- to 5-d-old pumpkin seedlings where *CmGA20ox3* expression was higher in the shoot tip and hypocotyl than in the root and root tip.

PsGA3ox1 (*LE*) transcripts were maintained at similar moderately high levels as the roots of both cultivars grew from 2 to 6 DAI (Figure 3.3A; Martin et al., 1997). Consistently, GA_1 was maintained at the same level from 2 to 4 DAI in 'Alaska' roots (Table 3.2). Yaxley et al. (2001) found that roots from 12 d-old pea plants isogenic for *LE* and *le-1* had similar levels of GA_1 and length. In addition, a second pair of isolines for the null mutation *le-2* (truncated *le-1*; Martin et al., 1997) and *le-1* had similar root GA_1 levels and length in 12 day-old plants (Yaxley et al. 2001). As a result Yaxley et al. (2001) proposed that another *GA3ox* gene exists in pea roots that can substitute for the loss of the wild-type *LE*. Our data is consistent with the hypothesis that *LE* contributes to the mRNA pool for *GA3ox*, but another *GA3ox* gene is likely also expressed in pea roots.

To further localize expression of GA biosynthesis genes in the root, we analyzed the expression pattern of the GA biosynthesis genes in root tips (ca. 4 mm, which constitutes about 2.4 to 3.3% of the total root fresh weight), and in the remaining part of the root from 6 DAI seedlings. *PsGA20ox2* transcript abundance was similar to that of *PsGA20ox1* in the more mature root tissue, but was markedly lower (12- to 24-fold lower) in the root tips in both cultivars (Table 3.4). In 'Alaska' pea roots, the first 2 to 3 mm of the root apex consists of the root cap and the meristem containing mostly dividing cells. Following this cell division zone is a zone consisting of mainly elongating and differentiating cells (from 2-3 to 11 mm or greater; Rost and Baum, 1988; Rost et al., 1988). The root-specific expression pattern of the *GA20ox* genes suggests that *PsGA20ox1* is the major gene for GA_{20} synthesis in the root tip containing

Table 3.4. Relative transcript levels of *PsGA20ox1, PsGA20ox2, PsGA3ox1, PsGA2ox1* and *PsGA2ox2* in 6 DAI roots and root tips of 'Alaska' and 'Carneval'.

Tissue	*PsGA20ox1*	*PsGA20ox2*	*PsGA3ox1*	*PsGA2ox1*	*PsGA2ox2*
			'Alaska'		
Root[a]	419.4[b] ± 58.4[c]	296.6 ± 43.1	4332.6 ± 523.0	771.0 ± 54.4	1896.8 ± 174.8
Root tip[d]	1031.4 ± 5.6	11.0 ± 5.4	4668.5 ± 1088.3	587.6 ± 34.6	3032.1 ± 1091.5
			'Carneval'		
Root	71.8 ± 5.7	96.3 ± 6.8	3700.2 ± 936.3	935.3 ± 52.6	665.7 ± 50.7
Root tip	82.3 ± 11.8	6.5 ± 2.3	2807.1 ± 132.2	1044.5 ± 5.9	1522.5 ± 97.4

[a] Remainder part of the 6 DAI root after 4 mm of its tip is removed.

[b] Transcript levels were compared across genes, genotypes, developmental stages and tissues using the average of mature embryo *PsGA20ox1* samples of 'Carneval' as a reference for normalization.

[c] Data are means ± SE, n = 2 to 3.

[d] 4 mm root tip.

mainly dividing cells while *PsGA20ox1* and *PsGA20ox2* transcripts contribute to the *GA20ox* transcript pool in more mature root cells.

Similar expression pattern of *PsGA3ox1* was evident between the root and root tip at 6 DAI (Table 3.4). These data are consistent with those in developing pumpkin seedlings, where expression of *CmGA3ox3* was similar between the root and root tip tissues (Lange et al., 2005).

Cultivar specific GA gene expression

In general, the relatively similar GA gene expression patterns between the two different genotypes studied indicates the general nature and importance of the spatial and temporal regulation of these GA biosynthesis genes to facilitate establishment of the pea seedling. Where the expression pattern of the *GA20ox*, *GA3ox* and *GA2ox* genes differed between the two genotypes is likely related to the *LE* (*PsGA3ox1*) gene. 'Carneval' carries the *le-1* mutation and the GA 3β-hydroxylase enzyme coded by the *le-1* gene is substantially less efficient than the wild-type enzyme (Lester et al., 1997; Martin et al., 1997). In 'Carneval' shoots, the *PsGA3ox1* mRNA levels increased approximately 3-fold over that observed in 'Alaska' (*LE*) shoots from 4 to 6 DAI (shoots were in the dark from 2-4 DAI as emergence from the potting media began at 4 DAI; Figure 3.3B). The greater abundance of *PsGA3ox1* transcripts in the shoots of the 'Carneval' may be a result of negative feedback control of *GA3ox* expression as observed in a number of other studies (Hedden and Phillips, 2000). Overall, our data are consistent with the view that the shoots are dependent on *PsGA3ox1* (*LE*) transcripts for

conversion of GA_{20} to bioactive GA_1 to stimulate shoot elongation in pea (Ingram et al., 1984; Ross et al., 1992).

Additionally, from 2 to 6 DAI, *PsGA2ox2* transcript abundance is lower in the actively growing shoots and roots of 'Carneval' (*le-1*) compared to 'Alaska' (*LE*). These data may suggest that one mechanism to compensate for the reduction in enzyme efficiency of the GA 3β-hydroxylase coded by the much less efficient *le-1* (*PsGA3ox1*; results in lower levels of GA_1 compared to wild-type *LE*, Ross et al., 1993) is to reduce the levels of *PsGA2ox2* transcripts, which has been implicated to play a major role in GA_1 deactivation in the pea shoot (Lester et al., 1999).

In summary, our data support the emerging hypothesis that bioactive GA is minimized in the developing embryo to allow for seed maturation processes to proceed. In addition, high *GA2ox* message levels in the mature quiescent embryo and in the embryo axis at 0.5 DAI, likely reflect a mechanism to maintain bioactive GA at minimal levels to prevent embryo axis expansion during the later phases of seed maturation and/or under non-optimal germination conditions. Within 1 DAI, a dramatic change in the expression patterns of these regulatory GA biosynthesis genes occurs in the embryo axis for increased capacity to produce bioactive GA for embryos axis expansion. In the cotyledons, the expression pattern of these suite of genes and endogenous GAs levels suggests that the pea cotyledons serve as a reservoir of GA_{20} (initially present and newly synthesized) that either may be used in situ (by GA 3β-hydroxylase initially present or synthesized de novo from *PsGA3ox1* mRNA), or be transported to the embryo axis, and serve as a substrate for production of bioactive GA_1.

As the young seedling initiates rapid growth (2 to 6 DAI), both shoots and roots display unique expression patterns for coordination of GA biosynthesis within and between these tissues. Over all, these data show that coordination of these key GA biosynthesis genes during germination and early seedling growth is highly regulated, and suggests that each tissue modulates the levels of these transcripts to maintain specific pools of the precursors and the biologically active GA, during seed maturation, germination and active growth phases (early seedling growth) of the plant.

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Chapter 4

Gibberellin Metabolism and Transport during Germination and Young Seedling Growth of Pea (*Pisum sativum* L.)

Introduction

Plant hormones are chemical signal molecules that influence many aspects of plant growth and development processes. In pea, mature seeds contain embryonic plants that are arrested in their development and that await the appropriate environmental conditions to initiate growth and development into seedlings. GAs, in part, controls the transition process from quiescence to active growth of the embryo axis. In plant species such as Arabidopsis and tomato, endogenous levels of GAs are a critical determinant for seed germination. Severely GA deficient mutants of Arabidopsis (*ga1-3* and *ga2-1*) and tomato (*gib-1*) failed to germinate without exogenous GAs (Koornneef and van der Veen, 1980; Groot and Karssen, 1987) and embryos of GA deficient *gib-1* mutant of tomato showed reduced growth rate (Groot and Karssen, 1987), suggesting the requirement of newly synthesized GAs for radicle protrusion in tomato and Arabidopsis.

Seed germination and/or early seedling growth in pea have also been suggested to be promoted by GAs (Brian and Hemming, 1955). However, GA biosynthesis inhibitor studies using compounds that inhibit *ent*-copalyl diphosphate synthase (Sponsel, 1983), the oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Graebe, 1986), and 3ß-hydroxylation of GA_{20} to GA_1 (Ross et al., 1993) suggested that de novo GA

biosynthesis was not essential for pea seed germination, but for the maintenance of normal seedling growth soon after germination. GAs have been shown to be sequestered at the end of pea seed maturation (Sponsel, 1983; Ross et al., 1993), and used for the maintenance of normal growth of the embryonic axis (Ross et al., 1993). Using enzymatic hydrolysis of mature pea seed extracts, Sponsel (1983) indicated that conjugated GAs in mature pea seeds are likely minor contributors to the free GA pool during early seedling growth processes. Previous studies on the relationship of the axis and the cotyledons in germinating seeds and seedlings of pea indicated that the presence of embryo axis is necessary for complete subcellular organization in the cotyledons (Bain and Mercer, 1966b). However, whether the embryo axis affects GA biosynthesis in the cotyledons of germinating seeds is not known.

In the later part of the GA biosynthesis pathway, GAs are mainly synthesized through the early 13-hydroxylation pathway in pea: $GA_{12}\rightarrow GA_{53}\rightarrow GA_{44}\rightarrow GA_{19}\rightarrow GA_{20}\rightarrow GA_1$ (Sponsel, 1995). The activity of GA 3β-hydroxylases (catalyze the conversion of GA_{20} to bioactive GA_1) and 2β-hydroxylases (catalyze the conversion of GA_{20} to biologically inactive GA_{29} or the bioactive GA_1 to biologically inactive GA_8) is critical in modulating the level of bioactive GA_1 (Hedden and Phillips, 2000). Results of in vivo and in vitro radiolabeled-GA feeding experiments suggest that tissue-specific localization of enzymes catalyzing the conversion of GA_{20} to GA_{29} and GA_{29} to GA_{29}-catabolite occurs in developing pea seeds, where GA_{20} is metabolized to GA_{29} in the cotyledons, and GA_{29} is transported to the testa where it is metabolized to GA_{29}-

catabolite (Sponsel, 1983). It is not known whether tissue-specific localization of GA metabolism occurs in the organs of germinating pea seedling.

Some evidence exists that GA are transported from the cotyledons to the embryo axis during germination and early seedling growth of pea. Ross et al. (1993) observed that application of prohexadione (inhibits primarily the 3β-hydroxylation of GA_{20}), but not paclobutrazol (inhibits the oxidation of *ent*-kaurene), to the seeds at the start of germination reduced internode length of *sln* plants (a null mutation in *GA2ox1*, inhibiting the 2β-hydroxylation of GA_{20} to GA_{29}), and GA_{20} applied to the dry seeds of *SLN* resulted in a phenocopy of the *sln* mutant. The authors concluded that cotyledonary GA_{20} is transported to the actively growing young shoots, where it is converted to bioactive GA_1 to stimulate internode elongation.

Auxins are also implicated in the processes of germination and early seedling growth (McDavid et al., 1972; Ogawa et al., 2003; Rampey et al., 2004), as well in stimulating GA biosynthesis in pea (van Huizen et al., 1997; Ross et al., 2000; Ozga and Reinecke, 2003). Ogawa et al.(2003) indicated that treatment of GA deficient *ga1-3* seeds of Arabidopsis with bioactive GA up-regulated genes encoding auxin transporter as well as cytochrome P450s (CYPs) protiens that are, as reported by Zhao et al. (2002), necessary for the formation of IAA via indole-3-acetaldoxime. IAA, required for cell division and elongation processes, has also been reported to be sequestered in a conjugated form in the mature seeds of pea (IAA-amide conjugates were 2-fold greater than free IAA; Bandurski and Schulze, 1977). Such bound IAA in seeds could release free IAA upon hydrolysis for use during seed germination and early seedling growth

processes (Epstein et al., 1980; Bialek and Cohen, 1989; Rampey et al., 2004). However, a detailed analysis of the type, amount and location of auxins in the embryo from the mature seed to the young seedling stage has not been completed.

In the present study, we tested the hypotheses that GA_{20} in pea cotyledons is transported to the expanding embryonic axis during germination and young seedling growth, and that the presence of the embryo axis is required for stimulation of GA biosynthesis gene expression in the cotyledons resulting in an increase in the level of bioactive GA. Our data suggest that GA_{20} is preferentially transported from the cotyledons to the shoot of the young pea seedlings. Once in the shoot of the embryonic axis, GA_{20} could be targeted for transport to the root. We also found that the embryo axis induces the expression of specific GA biosynthesis genes in imbibing cotyledons leading to increased metabolism of GA_{20} through GA_1 to GA_8.

Additionally, the endogenous levels and localization of two naturally occurring auxins, IAA and 4-Cl-IAA in the embryos of mature pea seeds and in young seedlings (4 DAI) were determined to understand the role of these auxins during germination and early seedling growth. Our data suggest that 4-Cl-IAA is preferentially sequestered in the mature seed for use *in situ* or for transport to the expanding axis during germination and early seedling growth processes. Cotyledonary IAA levels increased during imbibition (dry weight basis), suggesting de novo synthesis and/or hydrolysis of IAA from conjugates are important to stimulate processes required for germination and early seedling growth.

Materials and Methods

Growth Assay

The model pea (*Pisum sativum* L) cultivars used in this study are 'Alaska', a tall vining pea; and 'Carneval', a semi-dwarf field pea. Mature air-dry seeds of 'Alaska' (5.4% RWC) and 'Carneval' (5.8% RWC) were surface sterilized in 1.2% sodium hypochlorite solution for 25 min, rinsed five times with sterile deionized water, and then placed in a 9 cm sterile Petri plate (20 seeds per plate), on a sterile Whatman #1 filter paper wetted with 10 mL of sterile deionized water. After two days of imbibition in darkness, the testa was removed and seedlings with adequate shoot and root growth were aseptically transferred into a 15 cm sterile Petri plate (5 seedlings $plate^{-1}$) on a sterile Whatman #1 filter paper wetted with 10 mL sterile deionized water, and covered with a second layer of filter paper. The plates were sealed with parafilm then placed vertically (to obtain straight roots and shoots) until harvest in a growth chamber (Conviron, Ashville, NC) at 22/20^{0}C (day/night) in a 16/8 h photoperiod with cool white fluorescent and incandescent lights (205.5 $\mu E\ m^{-2}s^{-1}$).

For growth measurements, seedlings of each cultivar were harvested at 2 (from the smaller Petri plate), 3, 4, 5 and 6 (from the larger Petri plate) DAI, and separated into cotyledons, roots and shoots (7-10 seedlings per replication; 3 replications per time point). For RNA extraction and hormone analysis, two days after the transfer to the larger plates (4 DAI), seedlings were harvested, separated into shoots, roots and cotyledons, and immediately frozen in liquid N_2 and stored at -80^{0}C. To analyze gene expression and endogenous hormone levels in the mature embryo (cotyledon and

embryo axis combined), seeds of the two cultivars were immersed in ice: water (1:1, w/v) for 4 h to facilitate seed coat removal, and the embryos were immediately frozen in liquid nitrogen and stored at -80^{0}C until extraction for mRNA and hormone level analysis. In order to study whether the presence of the embryo axis is required to induce the expression of GA biosynthesis genes, and in turn, the metabolism of GAs in the cotyledons of germinating pea seeds, mature seeds of 'Alaska' were surface sterilized as described above and were kept immersed in sterile water for 2 h. After the 2 h imbibition period, the testa and embryo axis were removed without damage to the cotyledon using a scalpel. The cotyledon halves were placed onto filter paper moistened with 10 mL sterile water in a 9-cm plate for 46 h in the dark.

Shoot and root morphology studies

For assessment of lateral branching, mature air-dried seeds of the two cultivars were planted at a depth of ~ 2.5 cm into a 1:1 ratio of sand : Metro-Mix (W.R. Grace and Co., Toronto, Canada) in 3 L plastic pots (6 seeds per pot, 3 pots per cultivar), and the pots were in a growth chamber with conditions as described above. The number of lateral branches per shoot was assessed for 1 month after planting.

To assess the role of the shoot on growth and morphology of the root, seedlings of both cultivars were placed into sterile culture as described above. After 2 days of imbibition in darkness in 9 cm sterile Petri plate culture, seedlings were decapitated by removing the plumules without damage to the cotyledon using a scalpel (control plants were left intact) prior to transfer into 15-cm sterile Petri plates (6 seedlings per plate).

Decapitated seedlings of both 'Alaska' and 'Carneval' were either left untreated or treated with IAA or 4-Cl-IAA (1 μM or 10 μM in 0.1% Tween-80 solution, 1 μl applied directly to the stump) daily until harvest (6 DAI; with the first treatment started immediately after decapitation). To investigate the effect of shoot-derived GA on root response to IAA, decapitated seedlings of 'Carneval' (*le-1*) were treated with IAA plus GA_3 or 4-Cl-IAA plus GA_3 (10 μM in 0.1% Tween-80 solution, 1 μl applied to the stump). After treatment all Petri plates were placed in a growth chamber environment as described above. One set of decapitated seedlings of 'Carneval' were also kept in darkness until harvest. Roots were then harvested, and the necessary data recorded.

RNA Isolation

Tissues were finely ground in liquid N_2, and used for RNA isolation following a modified Trizol (Invitrogen, Carlsbad, CA) protocol as described previously (Chapter 2). The RNA fraction was subjected to further extraction with chloroform (0.2 mL mL^{-1} TRIzol), subsequently precipitated Isopropanol (0.25 mL mL^{-1} TRIzol) and high salt solution (1.2 M Na citrate and 0.8 M NaCl), 4 M LiCl, and finally by a mixture of 3 M Na acetate (pH 5.2): 100% ethanol (1:20, v/v). The precipitate was dissolved in DEPC treated water. Verification of the RNA integrity, DNase digestion of the total RNA samples, purification of the seed and cotyledonary total RNA samples, and determination of sample RNA concentration was performed as described previously (Chapter 2).

Gene Expression Analysis

Primers and Probes

Primers and probes for the quantifying amplicons (*GA20ox1-104* and *GA20ox2-88*, *GA3ox1-87*, *GA2ox2-73* and *GA2ox2-83*, and *18S62*) of the target genes (*PsGA20ox1* and *PsGA20ox2*, *PsGA3ox1*, and *PsGA2ox1* and *PsGA2ox2*) and reference gene (pea 18S rRNA) were designed as described previously (Chapter 2).

Real-Time RT-PCR Assay

Real-time RT-PCR assays for each target gene and 18S rRNA were performed on a model 7700 sequence detector (Applied Biosystems) using a TaqMan One-Step RT-PCR Master Mix Reagent Kit (Applied Biosystems) as a 50 μL (for *PsGA20ox1*, *PsGA3ox1* and *PsGA2ox1* expression analysis in the mature embryos and 4 DAI tissues) or 25 μL (for the expression analysis of *PsGA20ox2* and *PsGA2ox2* in the mature embryos and 4 DAI tissues, and for all the target genes in the 2 DAI cotyledons imbibed with or with no axis) reactions as described previously (Chapters 2 and 3). Thermal cycling conditions were 48°C for 30 for RT, 95°C for 10 min for *Taq* activation, and 40 cycles of 95°C for 15 s and 60°C for 1 min for PCR. Total RNA extracts from each tissue were pooled across all time points per cultivar, and this pooled sample was run on each plate and used as a control to correct for plate to plate amplification differences. A pooled sample in one real-time RT-PCR run was taken as the standard arbitrarily, and used for normalizing the C_t values of samples in other runs as described previously (Chapter 2).

The average of two assays of each sample was used to determine the relative transcript abundance of each target gene using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001), where ΔC_t was the difference between the target sample C_t and average C_t value of 'Alaska' mature embryo *PsGA20ox1* sample (C_t = 34.10). Two to 3 biological replicates of each plant sample were assayed.

Extraction and partitioning

Endogenous Auxin and GA levels

Mature embryos (cotyledon plus embryo axis), cotyledons, shoots, or roots (approximately 20 gfw per sample) were ground to a fine powder in a pre-cooled mortar and pestle, in liquid nitrogen. The fine powder was then homogenized with cold 80% (v/v) aqueous methanol containing 10 mg L^{-1} butylated hydroxytoluene (10 mL gfw^{-1}). Stable isotope labeled GAs, [^{2}H]GA_1 (300 ng), [^{2}H]GA_8 (300 ng), [^{2}H]GA_{19} (300 ng), [^{2}H]GA_{20} (291.9 ng), [^{2}H]GA_{29} (300 ng), [$^{13}C_6$]IAA (463.9 ng) and [$^2H_4$]4-Cl-IAA (406.3 ng), were added at homogenization of each extract as internal standards for recovery determination at the GC-MS step. The plant material was extracted overnight twice on a shaker (100 rpm) at 4^{0}C in darkness. The extracts were then centrifuged for 30 min at 10,000 *g*; the methanolic extracts were pooled and evaporated to the aqueous phase using a SpeedVac concentrator (Savant, Farmingdale, NY) without supplemental heating. The aqueous phase was then filtered through silylated glasswool, adjusted to pH 8 with 0.1 N NH_4OH, and partitioned four times against *n*-hexane (5 mL). The aqueous phase was then adjusted to pH 3 with 0.1 N HCl and partitioned five times

against ethyl acetate (5 mL). The combined ethyl acetate extract was reduced in volume using the SpeedVac concentrator, and hexane was added to drive off any acid residue. The reduced ethyl acetate extract was partitioned four times against 5 % (w/v) aqueous $NaHCO_3$ (2 mL). The pH of the combined $NaHCO_3$ extract was adjusted to pH 3 with 6 N HCl on ice, and then partitioned four times against ethyl acetate (5 mL). The ethyl acetate extracts were pooled and evaporated to complete dryness using the Speedvac concentrator. The ethyl acetate extract residue was then dissolved in H_2O (1 mL), loaded onto a Bond Elut C_{18} column (1g; Varian Inc., Harbor City, CA), which was preconditioned with methanol (2 mL) followed by H_2O (2 mL). The column was then washed with H_2O (2 mL) and eluted with MeOH (4 mL). The MeOH eluates were pooled, evaporated to dryness, resuspended in MeOH (1 mL) and loaded onto a Bond Elut DEA column (500 mg; Varian Inc., Harbor City, CA) that was preconditioned with MeOH (5 mL). The Bond Elut DEA column, which had a head of DEAE-Sephacel (1 mL of gel suspended in EtOH), was then eluted with 1 N AcOH in MeOH (5 mL). The eluates of the 1N AcOH in MeOH were combined and dried completely using the Speedvac concentrator (hexane was added in the drying process to eliminate residual acetic acid) prior to HPLC purification.

GA Metabolism and Transport

To study the metabolism of GA_{20} in the cotyledons of germinating seeds and its transport from the cotyledons to the embryo axis, seeds of 'Alaska' were surface sterilized and imbibed for the first 2 days in 9 cm sterile Petri plate prior to transfer into

the 15 cm Petri-plate culture as described above. [^{14}C]GA_{20} (specific activity of 54 μCi/μmol) was injected into one cotyledon of 2 DAI (imbibed with or with no axis) or 4 DAI seedlings of 'Alaska' at two spots (a total of 2.5 μl of 50% aqueous ethanol; a total of ca. 82000 dpm). The [^{14}C]GA_{20}-labelled cotyledon halves were incubated for 12 or 24 h on filter paper moistened with 10 mL sterile water in 15-cm Petri plate (5 cotyledons per plate) placed at 22/20^{0}C (day/night) in a 16/8 h photoperiod as described previously. The [^{14}C]GA_{20}-treated seedlings were incubated in 15-cm Petri plates as described above for 6, 12, 24 and 48 h. Incubation period started 4 h into the photoperiod for all treatments. After incubation with [^{14}C]GA_{20} substrate, the seedlings were separated into treated and untreated cotyledons, roots and shoots. Cotyledon halves and seedlings after separation into tissues were harvested onto dry ice and stored at -80^{0}C until extraction.

The [^{14}C]GA_{20} treated and untreated cotyledon halves, roots and shoots (5 tissues per sample of all tissues) were homogenized in cold 80% (v/v) methanol (10 mL per sample) using a polytron homogenizer in sylylated 30 ml corex tubes. 17-[^{14}C]GA_7 (ca. 11000 dpm) was added at homogenization of each sample extract as an external standards for recovery determination of radioactive metabolites at the HPLC step. The extracts were shaken overnight on a shaker (150 rpm) at 4^{0}C in darkness, and then centrifuged for 30 min at 10,000 *g*. The methanolic supernatant was removed, and the residue was resuspended in 5 ml of homogenization solvent and shaken for at least 4 h. The residue extracts were centrifuged for 30 min at 10,000 *g*, and the pooled methanolic extracts were evaporated to the aqueous phase using a SpeedVac concentrator. After

adjusting the pH of the aqueous extract to 8.0 with 0.1N NH_4OH, the extract was partitioned four times against *n*-hexane (5 ml) in silylated 20 ml glass scintillation vials. The aqueous phase was then adjusted to pH 3 with 0.1 N HCl and partitioned five times against ethyl acetate (5 mL). The combined ethyl acetate extract was reduced in volume using the SpeedVac concentrator, and partitioned four times against 5 % (w/v) aqueous $NaHCO_3$ (2 mL). The combined $NaHCO_3$ extract was transferred into 30 ml silylated pyrex tubes, and the pH was adjusted to 3 with 6 N HCl on ice, and then partitioned four times against ethyl acetate (5 mL). The ethyl acetate extracts were pooled and evaporated to complete dryness using a Speedvac concentrator prior to HPLC purification.

High Performance Liquid Chromatography

The ethyl acetate extract residues were dissolved in 400 µL of 20% (v/v) MeOH, filtered through a 0.45-µm nylon filter (Whatman International Ltd., Maidstone, England) and injected onto a 4.6 x 250 mm Spherisorb C_{18} column (5 µm; Beckman Instruments Inc., Fullerton, CA). The samples were eluted at 1 mL min^{-1} using the following linear gradient of methanol (solvent A) and 0.01% (v/v) aqueous TFA (solvent B): 20% (v/v) solvent A for 1 min, gradient to 100% (v/v) solvent A in 45 min, and isocratic 100% solvent A for 5 min. Radioactivity in the effluent of samples from the [^{14}C]GA_{20} metabolism study was monitored using a flow-through radiochemical detector (Beckman 171). Fractions eluting at the retention times of GA_8 (9.6 min), GA_{29} (10.7 min), GA_1 (16.1 min), GA_{29}-catabolite (16.4 min), IAA (17.8 min), 4-Cl-IAA

(23.3 min), GA_{20} (24.2 min), and GA_{19} (27.4 min) were collected and reduced to dryness using a SpeedVac concentrator. For the [^{14}C]GA_{20} metabolism study, pooled samples of the putative ^{14}C-GAs were methylated using diazomethane and rechromatographed on C_{18} HPLC (using the same solvent system).

Gas Chromatography-Mass Spectrometry

Fractions containing putative endogenous GAs, IAA and 4-Cl-IAA were re-suspended in 100 µL of 100% (v/v) methanol, and methylated by drop wise addition of diazomethane and incubation for 20 min at room temperature. The methylation step was repeated for a second time to ensure completion of the reaction. The solvent was evaporated under nitrogen at 40^{0}C. Conversion of the methylated samples to their methyl ester-trimethylsilyl ether derivatives was performed according to Gaskin and MacMillan (1991). The dried sample was taken up in 50 µL of pyridine and a 15 µL mixture of pyridine, hexamethyldisilazane and trimethylchlorosilane in the ratio of 5:6:4. The tightly capped vial was incubated at 100^{0}C for 10 min followed by drying in a stream of nitrogen at 40^{0}C. The sample residue was then brought up in 20 µL of dichloromethane (DCM) and 10 µL N-methyl-N-trimethylsilyltriflouroacetamide (MSTFA), tightly capped and heated to 100^{0}C for 45 s to dissolve the NH_4Cl. The silylated samples were stored in a dessicator over P_2O_5 at room temperature prior to GC-MS injection. After removal of the MSTFA and DCM under a stream of nitrogen at 40^{0}C, the sample was brought up in 5 µL of dry redistilled DCM prior to injection onto a Hewlett-Packard model 5890 series II *plus* gas chromatograph interfaced to a Hewlett-

Packard model 5972A mass selective detector. Samples (1 µl) were injected on-column onto a HP-5 MS capillary column (30 m x 0.25 mm x 0.25 µm film thickness; J&W Scientific Inc) with an initial column temperature at 50^0C for 2 min followed by temperature programming at 10^0C min^{-1} to 150^0C, and then 3 ^{0}C min^{-1} to 300^0C. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. Hormones were identified by selected ion monitoring (SIM) of three prominent ions (including the molecular ion, M^+) characteristic to the corresponding hormone. In addition, a solution of *n*-alkanes was co-injected with the samples, and retention times of the respective *n*-alkane peaks were recorded to obtain the Kovats Retention Index (KRI) values for further confirmation of the identity of the GAs studied. Endogenous hormone levels were calculated according to Gaskin and MacMillan (1991) where the protio- and deutero-ions monitored were corrected for the donation of natural isotopes to the peak area and quantification was based on the most prominent ion measured: protio-IAA-Me-TMS (202 ion); protio-4-Cl-IAA-Me-TMS (236 ion); protio-GA_{19}-Me-TMS (434 ion; KRI, 2627); protio-GA_{20}-Me-TMS (418 M^+ ; KRI, 2561); protio-GA_1-Me-TMS (506 M^+; KRI, 2710); protio-GA_8-Me-TMS (594 M^+; KRI, 2840); protio-GA_{29}-Me-TMS (506 M^+ ; KRI, 2717).

Results and Discussion

[^{14}C]GA_{20} metabolism in the cotyledons of germinating pea seeds

The metabolism of [^{14}C]GA_{20} in the cotyledons of germinating pea seeds and transport of [^{14}C]GA_{20} from the cotyledons to the embryo axis was studied over a 48 h

period. [^{14}C]GA_{20} and its ^{14}C-labeled metabolites that eluted at the same HPLC retention times as [^{14}C]GA_{20}, [^{14}C]GA_8, [^{14}C]GA_{29} and [^{14}C]GA_{29}-catabolite were detected in the treated pea tissues, and the methylated ^{14}C-GAs rechromatographed (under the same HPLC conditions) as one peak at the same retention time as their corresponding methyl-ester standards [GA_8 (13.7 min), GA_{29} (16.5 min), GA_1 (18.5 min), GA_{29}-catabolite (24.6 min) and GA_{20} (27.0 min)]. Within 6 h after [^{14}C]GA_{20} application, cotyledons from 2 DAI seedlings metabolized the majority of the labeled substrate (83%; Table 4.1). Though ^{14}C-GA_1 was not detected as a labeled metabolite in this study (likely due to a rapid turnover rate), its immediate 2β-hydroxylated metabolite, [^{14}C]GA_8 (9% of the [^{14}C]GA_{20} applied into the cotyledon), was detected in the 2 DAI cotyledons 6 h after radiolabel incubation (Table 4.1). A 3-fold increase in [^{14}C]GA_8 levels (ca. 26% of the [^{14}C]GA_{20} applied into the cotyledon) occurred 12 h after [^{14}C]GA_{20} application, and [^{14}C]GA_8 was the major free ^{14}C-GA metabolite detected at this time and after 24 h of incubation (Table 4.1). These data along with the expression of *PsGA3ox1* (Figure 4.1C) support the hypothesis that bioactive GA_1 is synthesized in the cotyledonary tissue of germinating pea seeds. This cotyledonary GA_1 may be involved in triggering the expression of GA-inducible cotyledonary genes encoding cell wall loosening enzymes (Chen et al., 2001; Ogawa et al., 2003). Such cell wall hydrolysis may take part in the formation of large reticulum of intercellular spaces observed during germination of pea seeds (Smith and Flinn, 1967) with possible function of accommodating high respiratory activity during reserve mobilization (Bain and Mercer, 1966a).

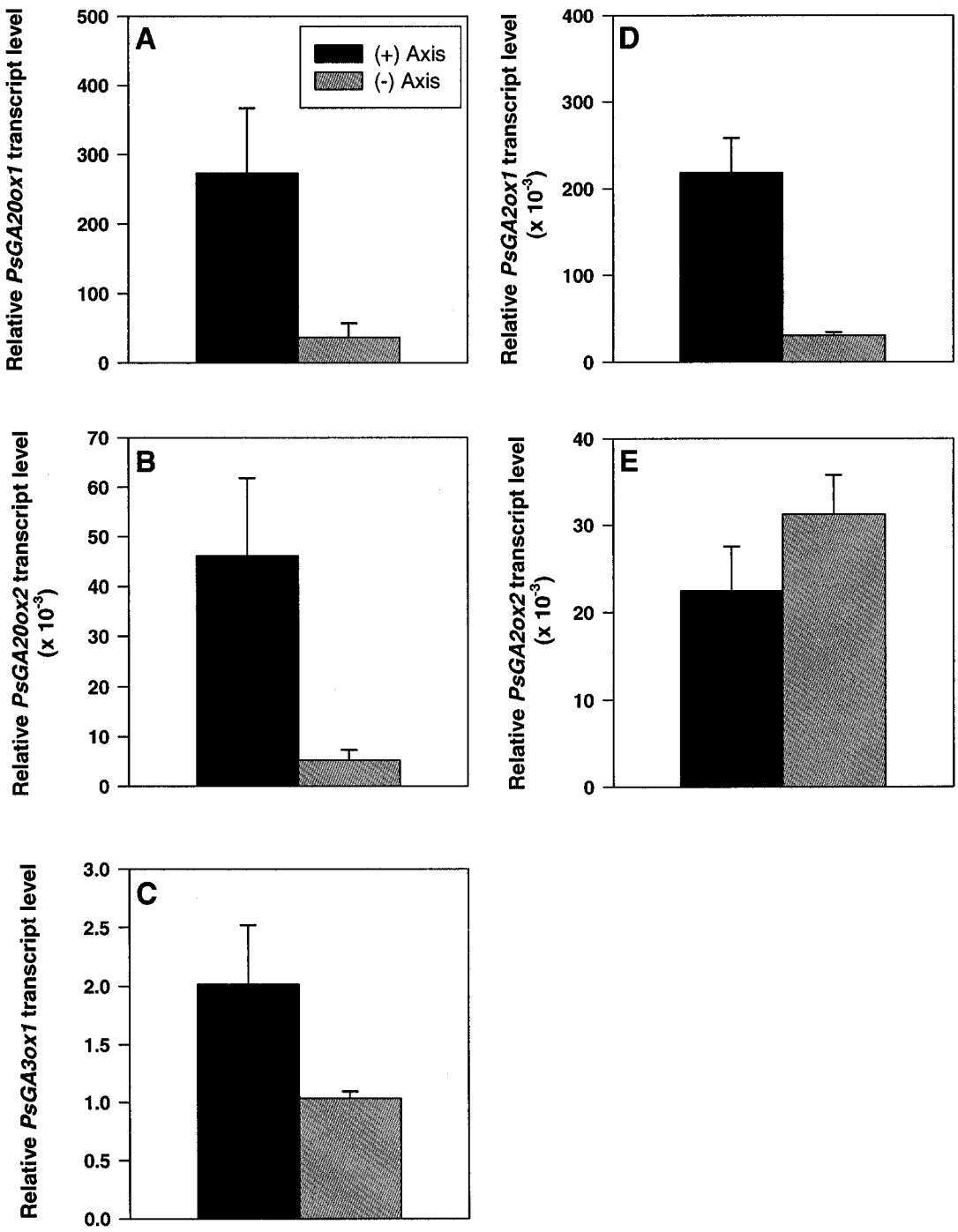
Table 4.1. Percentage of [^{14}C]GA_{20} metabolized to ^{14}C-GA metabolites in the cotyledons of 'Alaska' over a 48 h period[a].

Cotyledons	Incubation time (h)			
	6	12	24	48
	%[^{14}C]GA_{20} unmetabolized			
2 DAI (+	17.1[b] ± 3.1[c]	7.0 ± 2.8	1.8 ± 0.9	0.4 ± 0.4
2 DAI (- axis)	-	8.9 ± 4.1	13.5 ± 3.0	-
4 DAI (+	30.9 ± 4.9	9.4 ± 0.7	3.9 ± 1.4	1.3 ± 0.3
	%[^{14}C]GA_{20} metabolized to [^{14}C]GA_8			
2 DAI (+	8.6 ± 1.4	26.1 ± 11.6	17.9 ± 5.4	0.8 ± 0.2
2 DAI (- axis)	-	2.4 ± 2.4	2.4 ± 2.4	-
4 DAI (+	0 ± 0	0.5 ± 0.3	0.3 ± 0.3	0.7 ± 0.4
	%[^{14}C]GA_{20} metabolized to [^{14}C]GA_{29}			
2 DAI (+	8.4 ± 1.7	6.6 ± 0.9	8.4 ± 2.1	11.7 ± 8.0
2 DAI (- axis)	-	4.7 ± 4.7	5.6 ± 2.8	-
4 DAI (+	6.5 ± 0.6	16.5 ± 2.6	19.7 ± 1.5	15.8 ± 0.7
	%[^{14}C]GA_{20} metabolized to [^{14}C]GA_{29}-catabolite			
2 DAI (+	2.2 ± 0.5	0.9 ± 0.9	0.4 ± 0.4	4.3 ± 3.3
2 DAI (- axis)	-	0.1 ± 0.1	0.2 ± 0.1	-
4 DAI (+	0 ± 0	0.7 ± 0.2	2.8 ± 0.6	4.3 ± 0.3
	%[^{14}C]GA_{20} metabolized to [^{14}C]GA_{29} + [^{14}C]GA_{29}-catabolite			
2 DAI (+	10.7 ± 2.1	7.6 ± 0	8.7 ± 2.0	16.0 ± 11.3
2 DAI (- axis)	-	4.8 ± 4.6	5.8 ± 2.9	-
4 DAI (+	6.5 ± 0.6	17.1 ± 2.9	22.5 ± 1.2	20.1 ± 0.9
	%[^{14}C]GA_{20} metabolized to [^{14}C]GA_8 + [^{14}C]GA_{29} + [^{14}C]GA_{29}-catabolite			
2 DAI (+	19.3 ± 2.2	33.7 ± 11.6	26.6 ± 6.0	16.8 ± 11.1
2 DAI (- axis)	-	7.2 ± 3.9	8.2 ± 0.7	-
4 DAI (+	6.5 ± 0.6	17.6 ± 3.1	22.8 ± 1.5	16.7 ± 3.6

[a] ^{14}[C]GA_{20} (ca. 82000 dpm in a 2.5 µl of 50% aqueous ethanol) was injected into one cotyledon of a 2 or 4 DAI seedling (+ axis), or into a 2 DAI cotyledon in which the embryo axis was removed within 2 HAI (- axis).

[b] (^{14}[GAs] detected/^{14}[GA_{20}] applied) X 100; [c] Data are means ± SE, n=2 to 3.

Figure 4.1. Relative transcript levels of *PsGA20ox1* (A), *PsGA20ox2* (B), *PsGA3ox1* (C), *PsGA2ox1* (D) and *PsGA2ox2* (E) in 'Alaska' cotyledons imbibed for 2 days with or without the axis attached. Transcript levels were compared across genes and tissues using the average of cotyledon with no axis *PsGA3ox1* samples as a reference for normalization. Data are means ± SE, n = 3.



High levels of *PsGA2ox1* mRNA (codes for GA 2-oxidase that converts GA_{20} to GA_{29}) and conversion of [^{14}C]GA_{20} to [^{14}C]GA_{29} (up to 12% of the [^{14}C]GA_{20} applied into the cotyledon) were evident in the 2 DAI cotyledons (Figure 4.1D; Table 4.1). Only a small amount of GA_{29}-catabolite was detected in the cotyledon during the 48 h incubation period with [^{14}C]GA_{20} (Table 4.1). These data support the previous result that 2β-hydroxylation of GA_{20} to GA_{29} takes place in the cotyledon whilst conversion of GA_{29} to GA_{29}-catabolite in this tissue is minimal (Sponsel, 1983).

Cotyledons of 4 DAI pea seedlings exhibited a different pattern of [^{14}C]GA_{20} metabolism than those at 2 DAI. Four DAI cotyledons mainly converted [^{14}C]GA_{20} into [^{14}C]GA_{29} with little to no conversion of [^{14}C]GA_{20} to [^{14}C]GA_8 (Table 4.1; Figure 4.2). The endogenous profile of GAs in the cotyledons of the 4 DAI 'Alaska' seedlings (Table 4.2) is consistent with the [^{14}C]GA_{20} metabolic profile, where GA_{29} was the major GA detected with very low levels of GA_1 and GA_8, and with the high level of *PsGA2ox1* mRNA (Table 4.3) in this tissue. These data suggest that the production of bioactive GA_1 in the cotyledons of young pea seedlings is limited to a short duration during germination and early seedling growth.

The expression profiles of cotyledonary GA biosynthesis genes were relatively similar between the two cultivars studied, with the exceptions of *PsGA2ox1* and *PsGA2ox2* (Table 4.3). *PsGA2ox1* gene expression in the 4 DAI cotyledons of 'Carneval' was lower (6-fold) than that of 'Alaska', and is consistent with the lower (12-fold) endogenous GA_{29} level observed in the 4 DAI cotyledon of 'Carneval' (Table 4.2). Additionally, *PsGA2ox2* transcript abundance was lower in the 4 DAI cotyledons

Figure 4.2. [^{14}C]GA_{20} (A), and its metabolites, [^{14}C]GA_{29} (B), [^{14}C]GA_{29}-cat (C) and [^{14}C]GA_8 (D) detected over a 48 h period in the cotyledons imbibed in the presence of the embryo axis for 2 or 4 days. [^{14}C]GA_{20} (ca. 82000 dpm in 2.5 µl of 50% aqueous ethanol) was injected into one cotyledon of a 2 or 4 DAI seedlings. Data are means ± SE, n=2 to 3.

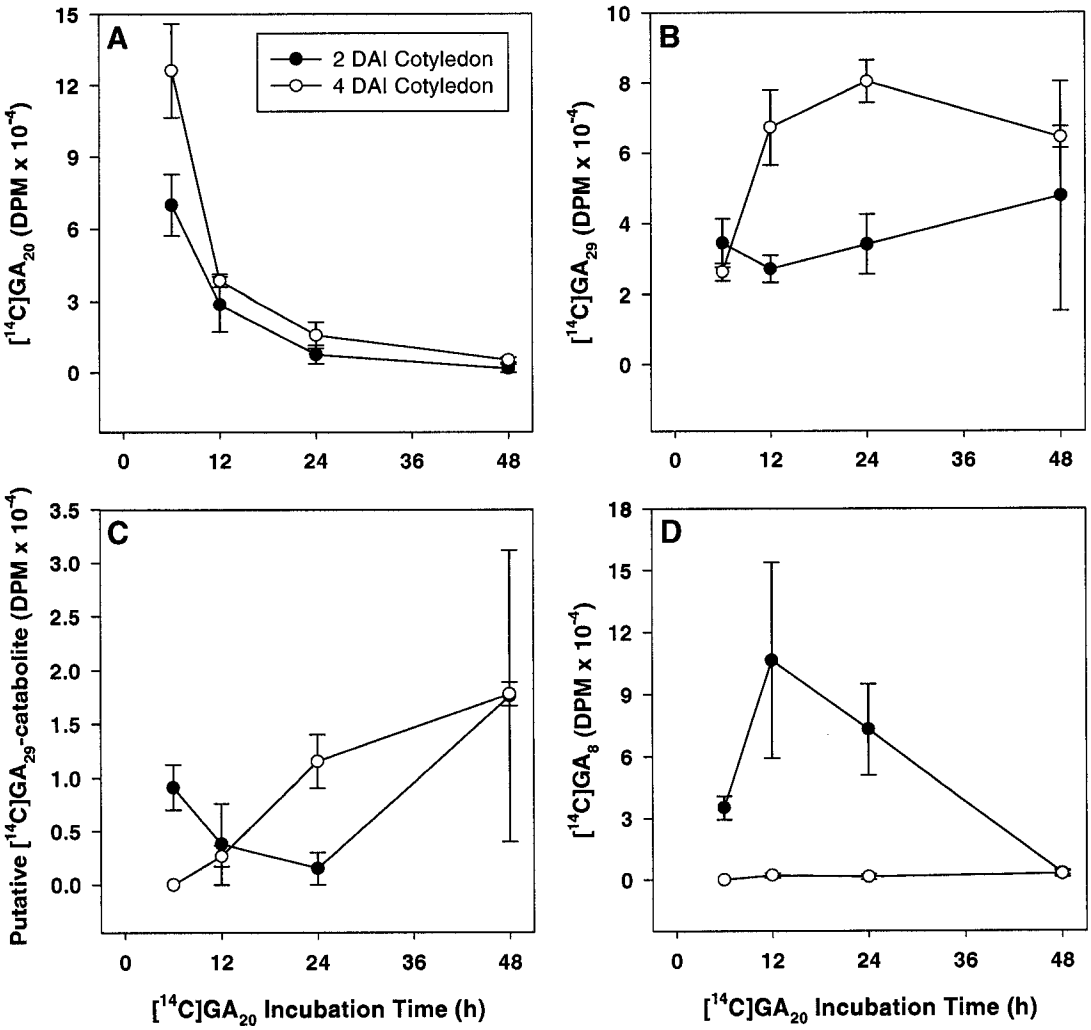


Table 4.2. Endogenous GA levels in mature embryos, and cotyledons, shoots and roots of 4 DAI seedlings of 'Alaska' and 'Carneval' grown in a Petri plate.

Cultivar	Tissue	Experiment No.	GA_{19}	GA_{20}	GA_{29}	GA_1	GA_8
					ng^{-1} gfw		
Alaska	Mature embryo	1	1.78	10.84	-	0.07	nd
	Cotyledon	1	0.05	0.87	339.48	nd[a]	0.54
		2	0.15	1.64	-	0.13	-
	Root	1	0.61	0.21	243.31	0.15	7.70
		2	1.18	-	365.96	0.27	-
	Shoot	1	0.20	0.18	381.45	0.01	17.90
Carneval	Mature embryo	1	1.18	13.44	277.92	0.12	-
	Cotyledon	1	0.15	0.54	27.81	nd	0.10
	Root	1	1.50	0.22	24.12	nd	0.30
		2	2.00	0.12	28.82	nd	0.27
	Shoot	1	0.32	0.13	47.81	nd	2.06
		2	0.38	0.12	51.21	nd	2.91
					pg^{-1} organ		
Alaska	Mature embryo	1	375.7	2285.4	-	15.7	nd
	Cotyledon pair	1	24.0	405.0	157702.0	nd	250.8
		2	68.9	758.2	-	62.0	-
	Root	1	32.0	11.0	12652.0	6.0	401.0
		2	61.4	-	19030.0	13.9	-
	Shoot	1	4.2	3.8	8010.0	0.3	376.0
Carneval	Mature embryo	1	328.1	3741.2	77373.3	33.6	-
	Cotyledon pair	1	62.0	215.3	11178.4	nd	41.0
	Root	1	68.1	9.9	1097.4	nd	12.1
		2	90.8	5.3	1311.2	nd	13.6
	Shoot	1	6.8	2.7	1008.8	nd	43.4
		2	7.9	2.5	1080.4	nd	61.4

[a] not detected

Table 4.3. Relative transcript levels of *PsGA20ox1*, *PsGA20ox2*, *PsGA3ox1*, *PsGA2ox1* and *PsGA2ox2* in cotyledons, shoots and roots of 4 DAI seedlings of 'Alaska' and 'Carneval' grown in a Petri plate[a].

Gene	Cotyledon	Shoot	Root
		'Alaska'	
PsGA20ox1	2.9 ± 1.3[b]	1930.5 ± 102.5	1653.6 ± 577.9
PsGA20ox2	499.1 ± 60.6	194.4 ± 89.2	745.3 ± 260.1
PsGA3ox1	187.1 ± 49.6	31738.3 ± 2587.5	34755.0 ± 856.0
PsGA2ox1	22670.9 ± 1860.5	5583.5 ± 439.5	5710.4 ± 605.0
PsGA2ox2	20110.3 ± 5929.0	1083.9 ± 219.7	3418.9 ± 330.2
		'Carneval'	
PsGA20ox1	1.0 ± 0.2	1004.0 ± 299.1	685.3 ± 191.1
PsGA20ox2	123.4 ± 14.9	136.8 ± 34.3	582.3 ± 128.3
PsGA3ox1	52.8 ± 19.7	14595.9 ± 2118.7	13791.5 ± 2139.8
PsGA2ox1	3676.5 ± 467.4	3830.1 ± 775.7	6762.7 ± 1339.0
PsGA2ox2	415.2 ± 102.7	358.5 ± 60.6	1934.0 ± 348.4

[a] Transcript levels were compared across genes, genotypes and tissues using the average of cotyledonary *PsGA20ox1* samples of 'Carneval' as a reference for normalization.

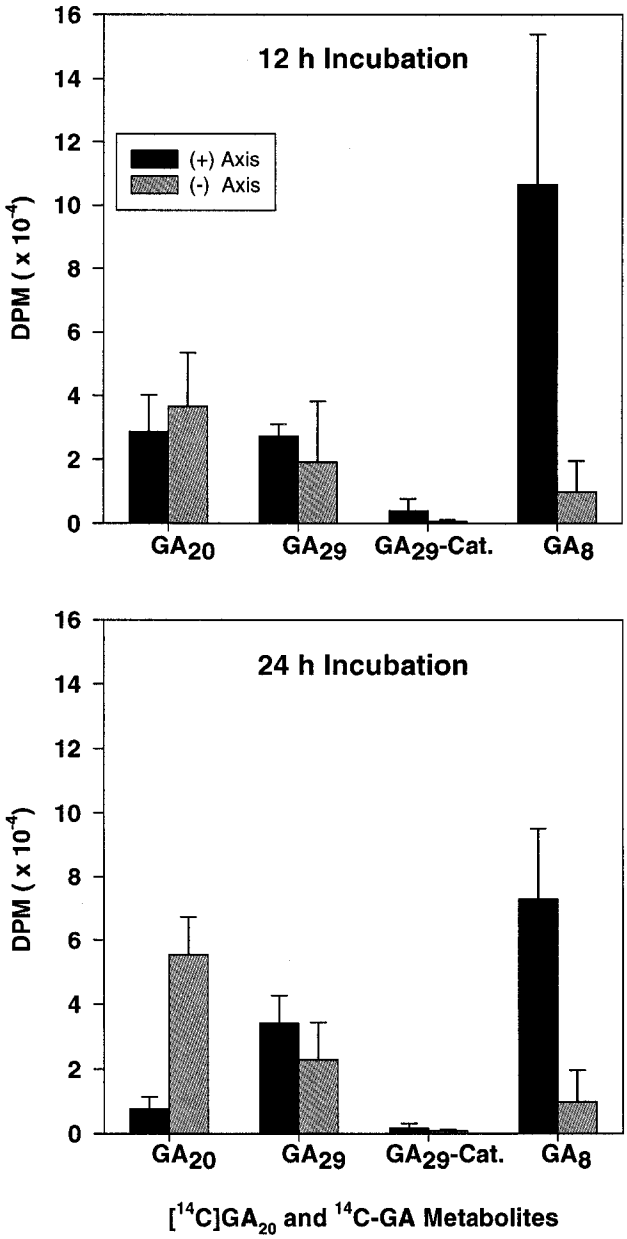
[b] Data are means ± SE, n = 2 to 3.

of 'Carneval' (*le-1*) compared to 'Alaska' (*LE*). These data may suggest that one mechanism to compensate for the reduction in the efficiency of GA 3β-hydroxylase coded by *le-1* (led to lower GA_1 levels in the shoots of 4 DAI seedlings; Ross et al., 1993; and also undetectabe GA_1 in the cotyledons; Table 4.2) is to reduce the levels of *PsGA2ox2* transcripts, which has been implicated to play a major role in GA_1 deactivation in the pea shoot (Lester et al., 1999). Such lower abundance of *PsGA2ox2* mRNA was also observed in the actively growing shoots and roots of 'Carneval' as compared to that of 'Alaska' (Chapter 3).

Effect of the embryo axis on GA biosynthesis in the Cotyledon

To examine whether the presence of the embryo axis is required to induce the expression of GA biosynthesis genes, and in turn, the metabolism of GAs in the cotyledons of germinating pea seeds, expressions of *PsGA20ox1*, *PsGA20ox2*, *PsGA3ox1*, *PsGA2ox1* and *PsGA2ox2*, and metabolism of $[^{14}C]GA_{20}$ were compared in the cotyledons of 'Alaska' imbibed for 2 days with or without the presence of the embryo axis (axis excised within 2 HAI). Removal of the embryo axis from the cotyledons 2 HAI reduced the transcript abundance of cotyledonary *PsGA20ox1* (3-fold), *PsGA20ox2* (9-fold), *PsGA3ox1* (2-fold), and *PsGA2ox1* (7-fold), but had no effect on *PsGA2ox2* transcript level (Figure 4.1) after 2 days of imbibition. Embryo axis removal also resulted in over 7-fold reduction in the conversion of $[^{14}C]GA_{20}$ to $[^{14}C]GA_8$ in the 2 DAI cotyledons (Figure 4.3). Since *PsGA2ox2* transcript levels were

Figure 4.3. [^{14}C]GA_{20}, and its metabolites, [^{14}C]GA_{29}, [^{14}C]GA_{29}-catabolite and [^{14}C]GA_8 detected over a 12 h (A) and 24 h (B) period in the cotyledons imbibed for 2 days with or without the axis attached. ^{14}[C]GA_{20} (ca. 82000 dpm in a 2.5 µl of 50% aqueous ethanol) was injected into one cotyledon of a 2 DAI seedling imbibed with the axis attached or into one cotyledon of the seeds imbibed without the axis (axis detached 2 HAI). Data are means ± SE, n=2 to 3.



not affected by embryo axis removal, the reduction in conversion of [^{14}C]GA_{20} to [^{14}C]GA_8 is likely due to the lower transcript abundance of cotyledonary *PsGA3ox1* (Figure 4.1), leading to reduced GA 3β-hydroxylase activity, and then less [^{14}C]GA_1 substrate for conversion to [^{14}C]GA_8.

GA transport from the cotyledon into the embryo axis

The growth of pea seedlings of both cultivars in a Petri-plate system was studied for 6 DAI. Root growth in fresh weight and length was greater than shoot growth from 2 to 6 DAI in both cultivars (Figure 4.4). In general, the growth profile of the root was linear in both cultivars from 2 to 6 DAI and shoot growth was slower from 2 to 4 DAI followed by increased rate of growth from 4 to 6 DAI (Figure 4.4).

[^{14}C]GA_{20} injected into the cotyledons of 4 DAI seedlings was transported to the shoot tissues (1.3% of the [^{14}C]GA_{20} applied to the cotyledons) by 12 h after application, where it was further metabolized to [^{14}C]GA_{29} (Table 4.4). No [^{14}C]GA_{20} was found in the root tissues of the young seedlings, however, [^{14}C]GA_{29} and [^{14}C]GA_{29}-catabolite were detected. It is possible that once in the shoot, some [^{14}C]GA_{20} may be targeted for transport to the root of the seedling, and subsequently metabolized to [^{14}C]GA_{29} and [^{14}C]GA_{29}-catabolite. These data are consistent with the findings of Proebsting et al. (1992), where grafting and radio-labeled feeding experiments suggested that GA_{20} is the major transported form of GA in pea. However, transport of [^{14}C]GA_{29} from the cotyledons to the embryo axis (both root and shoot

Figure 4.4. Growth of seedlings of 'Carneval' and Alaska in a Petri-plate system. Four day-old seedlings of 'Carneval' (left four) and 'Alaska' (right four) (A). Root fresh weights (B) and length (C), and shoot fresh weight (D) and length (E) from 2 to 6 DAI. Data are means ± SE, n = 24 to 30.

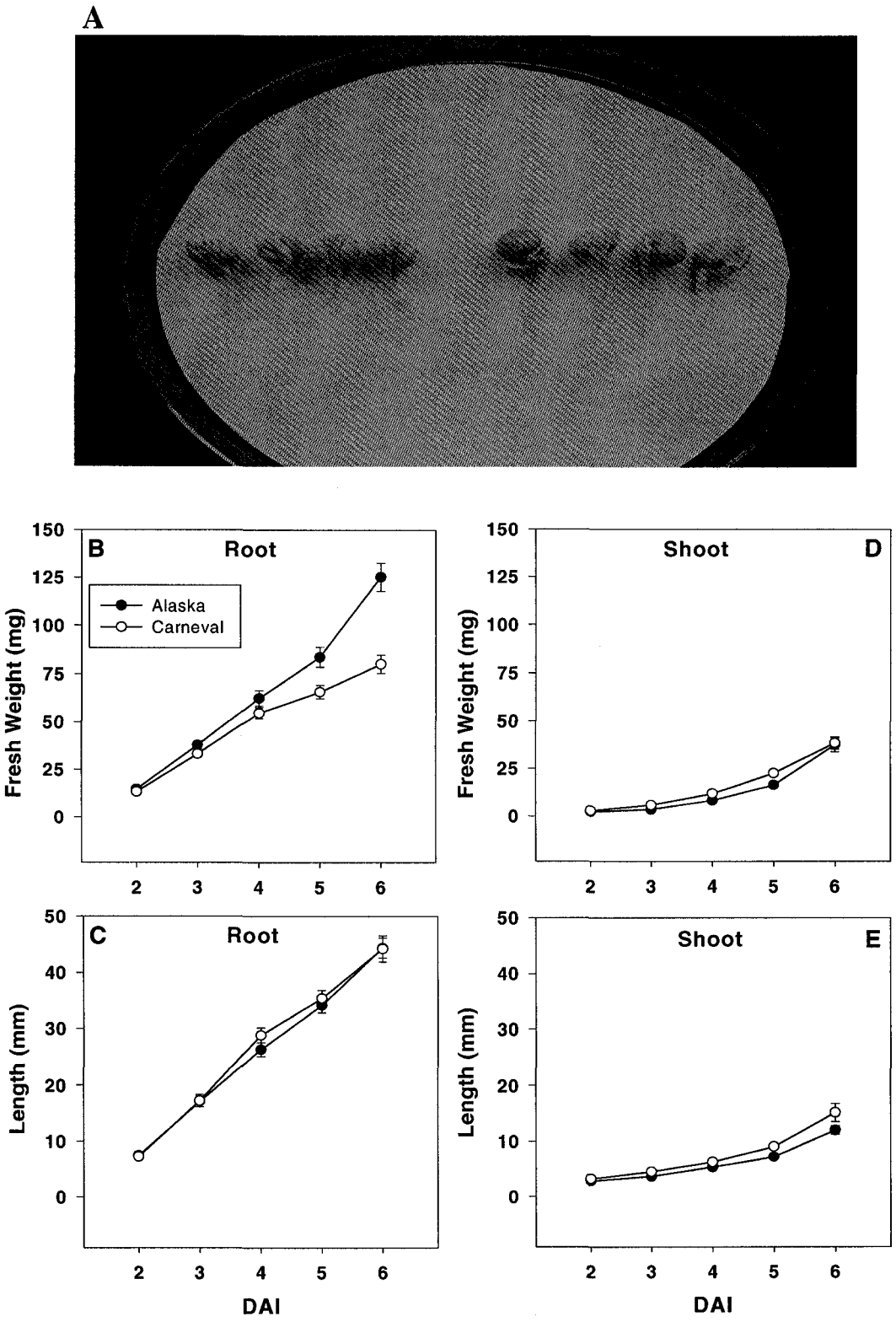


Table 4.4. [^{14}C]GA_{20} and its metabolites, [^{14}C]GA_{29}, [^{14}C]GA_{29}-catabolite, [^{14}C]GA_1 and [^{14}C]GA_8 detected in the roots and shoots of 4 DAI 'Alaska' seedlings over a 48 h period[a].

Incubation time (h)	[^{14}C]GA (DPM)				
	GA_{20}	GA_{29}	$GA_{29\text{-cat}}$	GA_1	GA_8
			Root		
12	nd[b]	2910 ± 325[c]	2156[d]	nd	nd
24	nd	2062 ± 256	nd	nd	nd
48	nd	5632 ± 1188	4053 ± 2866[e]	nd	nd
			Shoot		
12	5184[c]	5015 ± 2597	nd	nd	nd
24	2466 ± 1026[d]	8941 ± 574	nd	nd	nd
48	nd	18641 ± 6718	nd	nd	nd

[a] [^{14}C]GA_{20} (ca. 82000 dpm in a 2.5 µl of 50% aqueous ethanol) was injected in a 2.5 µl of 50% aqueous ethanol into one-half of the cotyledons imbibed for 4 d.

[b] [^{14}C]GAs not detected

[c] Data are means ± SE, n=2 to 3.

[d] One sample out of 3

[e] Two samples out of 3

tissue) can not be ruled out, as GA_{29} is hypothesized to be mobile in the developing seeds (Sponsel, 1983).

These [^{14}C]GA_{20} transport data, along with the presence of moderate levels of GA_{20} in the mature embryo (10-13 ng gfwt^{-1}; Table 4.2), as well as high levels of *PsGA20ox2* mRNA in 2 DAI cotyledons (Figure 4.1), support the hypothesis that GA_{20} sequestered during seed development and/or de novo synthesized in the imbibing cotyledons can be transported to the embryonic axis of germinating seeds to serve as a substrate for 3ß-hydroxylation to bioactive GA_1. The [^{14}C]GA_{20} transport data also suggest that the shoot is the major recipient of the cotyledonary GA_{20}. Indeed, GA_{20} (10 µg seed $^{-1}$) applied to the dry seed of wild type pea (cv. Torsdag) prior to sowing stimulated internode elongation, while treatment of dry seeds of the *sln* mutant prior to sowing with prohexadione (100 µg seed $^{-1}$; inhibitor of 3ß- hydroxylation), markedly reduced the length of internodes (between nodes 1 and 4; node counting started from the cotyledons as zero) during early seedling growth (Ross et al., 1993).

PsGA3ox1 (codes for the enzyme that converts GA_{20} to GA_1) was the most abundant GA biosynthesis gene in the rapidly expanding 4 DAI root and shoot tissues of both cultivars (Table 4.3). Even though endogenous GA_1 was detected only in the roots and shoots of 'Alaska' (Table 4.2), the presence of GA_8 along with the expression of *PsGA3ox1* in the corresponding tissues of 'Carneval' suggest the synthesis of GA_1 to stimulate growth-related processes in these tissues at this stage. The markedly lower level of *PsGA2ox2* mRNA in the roots and shoots of 'Carneval' compared to 'Alaska' (Table 4.3) is consistent with our data in Chapter 3, and again suggests that one

mechanism to compensate for the reduction in enzyme efficiency of the GA 3β-hydroxylase coded by the mutated *PsGA3ox1* (*le-1*) in 'Carneval' is to decrease the amount of GA 2β-hydroxylase available to convert the bioactive GA_1 to GA_8.

The relatively high levels of *PsGA2ox1* mRNA (Table 4.3) and endogenous GA_{29} (Table 4.2) in 4 DAI roots and shoots, along with the detection of [^{14}C]GA_{29} in the roots and shoots after [^{14}C]GA_{20} application to the cotyledons of 4 DAI seedlings (Table 4.4), suggests that conversion of GA_{20} to GA_{29} is a major mechanism to regulate the pool of GA_{20} in these tissues during early seedling growth.

Endogenous auxin levels

The endogenous level of two naturally occurring auxins, IAA and 4-Cl-IAA, in the mature embryos and tissues of 4 DAI seedlings of 'Alaska' and 'Carneval' were determined by GC-MS-SIM. Since 98.2% of the fresh weight (98.8 % of the dry weight) of the mature embryo is that of the cotyledons, the endogenous auxin levels in the mature embryo reflect that of the cotyledon at this stage. Rapidly growing pea seeds (~ 1 week post-anthesis) contain high levels of free IAA and 4-Cl-IAA (315-1570 ng/gfw of IAA and 239-720 ng/gfw of 4-Cl-IAA; Katayama et al., 1988; Magnus et al., 1997) that decrease to low levels as the seeds mature (Katayama et al., 1988). Therefore, the IAA and 4-Cl-IAA detected in the mature embryos (with no testa) of both cultivars (Table 4.5) represent the residuals of the two auxins sequestered at the end of seed maturation. The level of 4-Cl-IAA in the mature embryos was higher (4.7-

Table 4.5. IAA and 4-Cl-IAA levels in mature embryos, and cotyledons, roots and shoots of 4 DAI seedlings of 'Alaska' and 'Carneval' grown in a Petri plate.

Cultivar	Auxin	Experiment No.	Mature embryo	4 DAI		
				Cotyledon	Root	Shoot
				ng^{-1} gfw		
Alaska	IAA	1	3.45	2.86	18.61	15.52
		2	-	-	24.75	-
	4-Cl-IAA	1	16.29	1.97	9.64	1.32
Carneval	IAA	1	1.08	3.37	19.93	1.94
	4-Cl-IAA	1	10.30	1.60	1.37	0.16
				ng^{-1} gdw		
Alaska	IAA	1	3.67	6.64	219.72	105.15
		2	-	-	292.55	-
	4-Cl-IAA	1	17.37	4.57	113.95	8.94
Carneval	IAA	1	1.39	7.65	269.69	14.55
	4-Cl-IAA	1	13.26	3.63	18.54	1.20
				pg^{-1} seedling organ[a]		
Alaska	IAA	1	726.2	1318.1	967.8	326
		2	-	-	1287.2	-
	4-Cl-IAA	1	3434.4	911.3	75.3	27.7
Carneval	IAA	1	301.8	1354	907.0	41.0
	4-Cl-IAA	1	2868.6	644	62.2	3.5

[a] gfw basis; cotyledon values are per cotyledon pair

fold in 'Alaska' and 9.5-fold in 'Carneval') than IAA (Table 4.5), and was comparable to those reported in the mature seeds of 'Asaka' (Katayama et al., 1988). However, the IAA level was lower than that of Katayaman et al. (1988; 7-fold) and Bandurski and Schulze (1977; 86-fold) who analyzed the whole seed including the testa, suggesting that free IAA biosynthesis and/or IAA conjugate hydrolysis during seed development may take place partly in the testa.

The concentration of IAA in the cotyledons of both cultivars was maintained after 4 days of imbibition (Table 4.5), when the RWC of the cotyledon increased to 56 to 57% (implying that IAA content increased 1.8-fold in 'Alaska' and 5.5-fold in 'Carneval' in a gram dry weight [gdw] basis). These data indicate that IAA is either denovo synthesized or hydrolyzed from conjugates in the cotyledon during this period. In the mature air-dried seeds of 'Alaska', the level of peptidyl-IAA conjugates is over twice of the level of free IAA (Bandurski and Schulze, 1977), and enzymatic hydrolysis of IAA-L-Alanine, an IAA conjugate, to free IAA has been reported within 48 h after application into the stem segments of 6 to 7-d-old 'Alaska' seedlings (Hangarter and Good, 1981). It is therefore reasonable to suggest that endogenous IAA-amino acid conjugates present in the seeds of pea are contributors to the increase of cotyledonary free-IAA pool during germination and seedling growth (Epstein et al., 1980). Genetic and molecular evidence on the role of IAA conjugate as a source to the free-IAA pool during germination is also starting to emerge. Arabidopsis amidohydrolase genes have been shown to be expressed in the seeds and mature embryos (*ILL2* and *ILR1*) and in the tissues of 1 to 8-d-old seedling (*ILR1, IAR3, ILL1* and *ILL2*) of wild type

Arabidopsis (Rampey et al., 2004). Additionally, 4-d-imbibed seeds and/or 8-d-old seedlings of auxin-conjugate hydrolase triple mutant (*ilr1 iar3 ill2*) accumulated 5 to 8-fold higher amide-linked conjugates (IAA-Ala and IAA-Leu), and have shown 33 to 47% reduction in the level of free IAA.

The cotyledonary free-IAA pool can be used *in situ* or for transport to the growing embryo axis. Gladish et al. (2000) presented evidence that IAA applied to the cotyledons of 2 and 5-d-old pea seedlings was transported into the axes of developing seedlings within 6 h, where the majority (73 to 81%) of the transport was into the actively growing root.

Cotyledonary 4-Cl-IAA concentration, on the other hand, decreased during the 4 day imbibition period (8.3-fold in 'Alaska' and 6.4-fold in 'Carneval' in a gfw basis; 3.7-fold in both cultivars in a gdw basis, Table 4.5). The 4-Cl-IAA sequestered in the mature embryo may therefore be used to stimulate germination and initial growth processes of the embryonic axis. The higher level of 4-Cl-IAA (per organ basis) detected in the cotyledon than in the corresponding shoots and roots at 4 DAI may imply that the cotyledon serves as a reservoir of 4-Cl-IAA for transport to the axis to support its growth.

Though phenotypes associated with difference in auxin levels were not evident in shoots and roots of 4 DAI seedlings (Figure 4.4), the differences in shoot auxin content (8-fold greater in the shoots of 'Alaska' than in 'Carneval'; Table 4.5) between the two cultivars is interesting in the light of their root and shoot morphology at the later stages of development. A primary lateral branch with average length of 1.58 cm (± 0.14;

n = 18) was apparent at node no. 2 (node counting started from the cotyledons as zero) in 'Carneval' 2 weeks after planting (with no more lateral branching observed in the following 2 weeks under the same environmental conditions), but no lateral branches were evident in 'Alaska' during this period. Moreover, roots from either intact control or decapitated 6 DAI seedlings of 'Carneval' did not produce any visible lateral root primordia in the Petri-plate culture (Table 4.6).

As lateral root primordia were apparent by 6 DAI when 'Carneval' seedlings are grown in sand (Chapters 2 and 3), our observation may suggest that light affects the response of roots to IAA in stimulating the formation of lateral root primordia. However, 'Carneval' seedlings grown in darkness (before and after decapitation) in the same Petri-plate culture did not produce of lateral root primordia by 6 DAI (Table 4.6). Lateral root primordia (4.5 ± 0.8 per root; n = 16; Table 4.6) were evident on 'Alaska' roots at 6 DAI, and their numbers decreased markedly by decapitation of the plumule at 2 DAI (1.2 ± 0.6 per root; n = 16; Table 4.6), suggesting the transport of auxin from the shoot to the root, where it induces lateral root growth. Indeed, application of IAA or 4-Cl-IAA at 10^{-5} M directly to the stump of decapitated seedlings did increase the number of lateral roots primordia in 'Alaska', however, in 'Carneval' the exogenous IAA or 4-Cl-IAA did not induce the formation of lateral root primordia (Table 4.6). As endogenous GA_1 was detected only in the roots and shoots of 'Alaska' (Table 4.2), the difference between the two cultivars in the formation of lateral root primordia may suggest the role of GA in modulating IAA response of root cells. However, application of GA_3 (by itself or in combination with IAA or 4-Cl-IAA) at 10^{-5} M to the stump of

Table 4.6. Root fresh weight, length and number of lateral root primordia in control (intact), decapitated, and decapitated and hormone treated 6 DAI seedlings of 'Alaska' and 'Carneval' grown in a Petri plate[a]

Treatment	Fresh weight(mg)	Primary root length (mm)	Number of lateral root primordia
	'Alaska'		
Control	103.8 ± 10.6[b]	42.8 ± 4.2	4.5 ± 0.8
Decapitated	117.2 ± 14.0	44.1 ± 4.6	1.2 ± 0.6
Decapitated + IAA (10^{-6} M)	136.0 ± 9.5	48.4 ± 1.7	1.8 ± 0.4
Decapitated + IAA (10^{-5} M)	145.6 ± 9.3	47.2 ± 2.8	2.8 ± 0.7
Decapitated + 4-Cl-IAA (10^{-6} M)	121.3 ± 9.2	47.4 ± 3.3	1.3 ± 0.7
Decapitated + 4-Cl-IAA (10^{-5} M)	142.1 ± 10.5	47.1 ± 3.6	3.1 ± 0.7
	'Carneval'		
Control	103.6 ± 4.4	60.0 ± 2.0	0 ± 0
Decapitated	95.0 ± 5.2	49.4 ± 2.1	0 ± 0
Decapitated (dark)	102.2 ± 8.2	50.9 ± 2.1	0 ± 0
Decapitated + IAA (10^{-6} M)	96.7 ± 4.5	48.9 ± 1.7	0 ± 0
Decapitated + IAA (10^{-5} M)	104.7 ± 5.6	53.0 ± 2.1	0 ± 0
Decapitated + 4-Cl-IAA (10^{-6} M)	107.4 ± 5.4	48.7 ± 1.4	0 ± 0
Decapitated + 4-Cl-IAA (10^{-5} M)	99.4 ± 5.3	49.9 ± 1.7	0 ± 0
Decapitated + GA_3 (10^{-5} M)	104.9 ± 9.3	47.9 ± 2.7	0 ± 0
Decapitated + IAA + GA_3 (10^{-5} M)	82.6 ± 9.6	41.6 ± 4.4	0 ± 0
Decapitated + 4-Cl-IAA + GA_3(10^{-5} M)	103.2 ± 5.5	50.7 ± 2.3	0 ± 0

[a] Seedlings of both cultivars were decapitated at 2 DAI by removing the plumules without damage to the cotyledons.

[b] Data are means ± SE, n = 16 to 20.

decapitated 'Carneval' seedlings did not induce the formation of lateral root primordia on the roots of Petri-plate grown seedlings (Table 4.6). Length and fresh weight of the primary root of both cultivars remained unaffected by decapitation (Table 4.6; McDavid et al., 1972).

In general, the concentrations of IAA and 4-Cl-IAA in the roots of both cultivars were greater than that detected in the respective shoot tissues (Table 4.5). These data along with the greater root growth than the corresponding shoots support the view by Pengelly and Torrey (1982) that IAA level has effect on the contributions of cell division and cell enlargement to root growth. Indeed, root IAA content (Table 4.5) and root growth (Figure 4.4) were similar between the two cultivars. IAA levels (both per gfw and per organ basis) in the shoot and root tissues of both cultivars were markedly higher than that of 4-Cl-IAA (11.8 to 12.8-fold in 'Alaska' and 11.8 to14.6-fold in 'Carneval; Table 4.5), indicating that IAA is the major form of auxin in the rapidly expanding tissues of young pea seedlings.

In summary, results from our metabolism study support the hypothesis that synthesis of bioactive GA_1 from initially present or newly synthesized GA_{20} occurs in the non-expanding cotyledonary tissue of germinating pea seeds. Though [^{14}C]GA_1 was not detected, the decrease in the capacity of the cotyledonary tissue to metabolize [^{14}C]GA_{20} to [^{14}C]GA_8 as imbibition continued to 4 DAI suggests that the 3β-hydroxylase activity in the cotyledons is limited to a short window of time during germination. The embryo axis appears to control the metabolism of [^{14}C]GA_{20} in the cotyledons, as excision of the embryo axis within 2 HAI reduced the expression of GA

biosynthesis (*PsGA20ox1*, *PsGA20ox2* and *PsGA3ox1*) and catabolism (*PsGA2ox1*) genes and also the metabolism of [^{14}C]GA_{20} to [^{14}C]GA_8. [^{14}C]GA_{20} applied into the cotyledon of germinating seeds was also shown to be transported to the shoot. An increase in IAA and decrease in 4-Cl-IAA levels in cotyledons seeds imbibed for 4 days suggest that cotyledonary 4-Cl-IAA is metabolized during imbibition to stimulate the initial growth processes in germinating seeds. The difference in the concentration of shoot-derived IAA and 4-Cl-IAA between the two cultivars was correlated with the lateral branch morphology of the developing seedlings.

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Chapter 5

Summary and Conclusion

A critical role of GAs has been shown for promoting the germination and early seedling growth of small-seeded, endospermic and epigeous dicotyledonous plants such as Arabidopsis and tomato, and large seeded pumpkin. However, its role during the germination and young seedling growth of large seeded dicotyledonous species such as pea, which are non-endospermic and hypogeous, remains unclear. Knowledge on how, where and when GA is synthesized during these developmental processes is crucial in understanding its role. Therefore, this research was targeted to examine the temporal and spatial regulation of de novo GA biosynthesis during germination and young seedling growth in pea.

The first part of this study showed that expression of the *PsCPS1* gene (encoding CPS that catalyses the first committed step in the GA biosynthesis pathway) during germination and early seedling growth is tightly associated with the actively growing tissues, the embryo axis, root and shoot. Complementing these data with studies on CPS enzyme activity and time-course changes in endogenous *ent*-kurene level would extend our understanding about the developmental regulation of GA biosynthesis early in the pathway. *CPS* was shown not to be regulated at the mRNA level by bioactive GA either in the roots or shoots of developing seedlings. As active

GA level may also regulate the level and/or activity of enzymes, it would be of interest to extend this study to the enzyme level.

The second part of our study shows that initial imbibition of pea seeds led to a decrease in the level of cotyledonary GA_{20}, but an increase in the expression of *PsGA3ox1* and the levels of GA_1, indicating the use of GA_{20} stored in the cotyledons for *in situ* de novo synthesis of bioactive GA. Our data also show that embryo axis growth prior to radicle protrusion was accompanied by a marked increase in the mRNA levels of *PsGA20ox1* and *PsGA3ox1*, but by decrease/low levels of *PsGA2ox* genes, for an increase in the synthesis of active GA in this tissue. Expression patterns of these GA biosynthesis genes along with the endogenous GAs profiles in the root and shoot of the developing seedling support the key role for de novo GA biosynthesis during early post-germinative growth. As the GA oxidases form multigene families (Sponsel and Hedden, 2004), with the advancement of pea genomics, it is more likely that additional family members of these genes will be isolated, and provide an opportunity to extend our understanding of GA biosynthesis and its regulation in germinating seeds. Once GA is synthesized, its signal is likely to be targeted to a number of metabolic pathways, thus, examining the temporal and spatial expression of GA-regulated genes along with quantitative and qualitative analysis of GA-regulated metabolites would help in understanding the role of GA during germination and young seedling growth in pea.

Our GA metabolism study bridges the gene expression and GA profile studies, which are suggestive of bioactive GA synthesis in the non-expanding cotyledonary tissue of germinating pea seeds. This study further demonstrated that the maximal

activity of 3β-hydroxylase enzyme and production of bioactive GA in the cotyledons occurs during radicle protrusion, and these events of GA biosynthesis in the cotyledons are controlled by a signal from the embryo axis. Further study will be needed to examine and characterize the nature of this embryo-axis derived signal. From our study, it is clear that ^{14}C-GA_{20} was transported from the cotyledons to the shoot, and possibly to the root during early seedling growth. However, as ^{14}C-GA_{29} was detected along with ^{14}C-GA_{20} in the shoot, and also in the root tissues, it would be of interest to test whether GA_{29} is co-transported from the cotyledon and/or metabolized from GA_{20} after its transport.

Study on changes in the levels of the two naturally occurring auxins, IAA and 4-Cl-IAA, during germination and young seedling growth revealed an increase of IAA and a decrease of 4-Cl-IAA levels in the cotyledons, relative to their level prior to imbibition. Though this result suggests a difference in the roles of these two auxins during germination and early seedling growth, further characterization of their synthesis and metabolism is needed to get a better picture. Interaction between hormones might form part of the broad homeostatic mechanism that coordinates and modulates plant growth (Hedden and Phillips, 2000). Therefore, investigating the possible cross talk between GAs and auxins during the germination of pea seeds would contribute to our understanding of the complex mechanisms by which GA moderates seed germination.

In conclusion, the following paragraphs describe the working models established from this study (Figure 5.1 and Figure 5.2). GA_{20} is sequestered in the cotyledons at maturity. During imibibition, *PsGA20ox2* transcripts predominate in the cotyledonary

tissue for the de novo synthesis of GA 20-oxidase and production of GA_{20}. This pool of cotyledonary GA_{20} (initially present and newly synthesized) serves as a substrate for 3β-hydroxylation (by GA 3β-hydroxylase coded by *PsGA3ox1*) into bioactive GA_1 *in situ*, or it is transported to the embryo axis to support synthesis of bioactive GAs for embryo axis expansion. A signal from the expanding embryo axis appears to regulate the expression of *PsGA20ox2* and *PsGA3ox1*, and the production of GA_1.

Transcription of the gene (*PsCPS1*) that codes for *ent*-kaurene (precursor of GAs) is up-regulated in the axis of germinating pea seeds (before radicle protrusion), and in both shoot and root tissues during early seedling growth. *PsGA20ox1* transcripts code for the majority of GA 20-oxidase activity in the shoots, and both *PsGA20ox1* and *PsGA20ox2* are abundant in the root tissues by 6 DAI. *PsGA3ox1* transcripts are abundant in both the shoots and roots of young pea seedlings and *PsGA3ox1* likely codes for most of the GA3β-hydroxylase in the shoot, and also contribute to the pool of this enzyme in the root for the production of bioactive GA_1.

PsGA2ox1 and *PsGA2ox2* genes code for GA 2β-hydroxylases that convert GA_{20} to GA_{29} and GA_1 to GA_8, respectively. In preparation for germination and early seedling growth, the abundance of *PsGA2ox2* mRNA markedly decreases in the cotyledons and the embryo axes by 1 DAI for a reduced capacity to catabolize bioactive GA_1 to biologically inactive GA_8. Early in the post-germinative phase, *PsGA2ox1* and *PsGA2ox2* transcript abundance in the shoots and roots of the seedling are maintained at a low level to minimize the catabolism of GA_{20} to GA_{29} and GA_1 to GA_8 during this active growth phase.

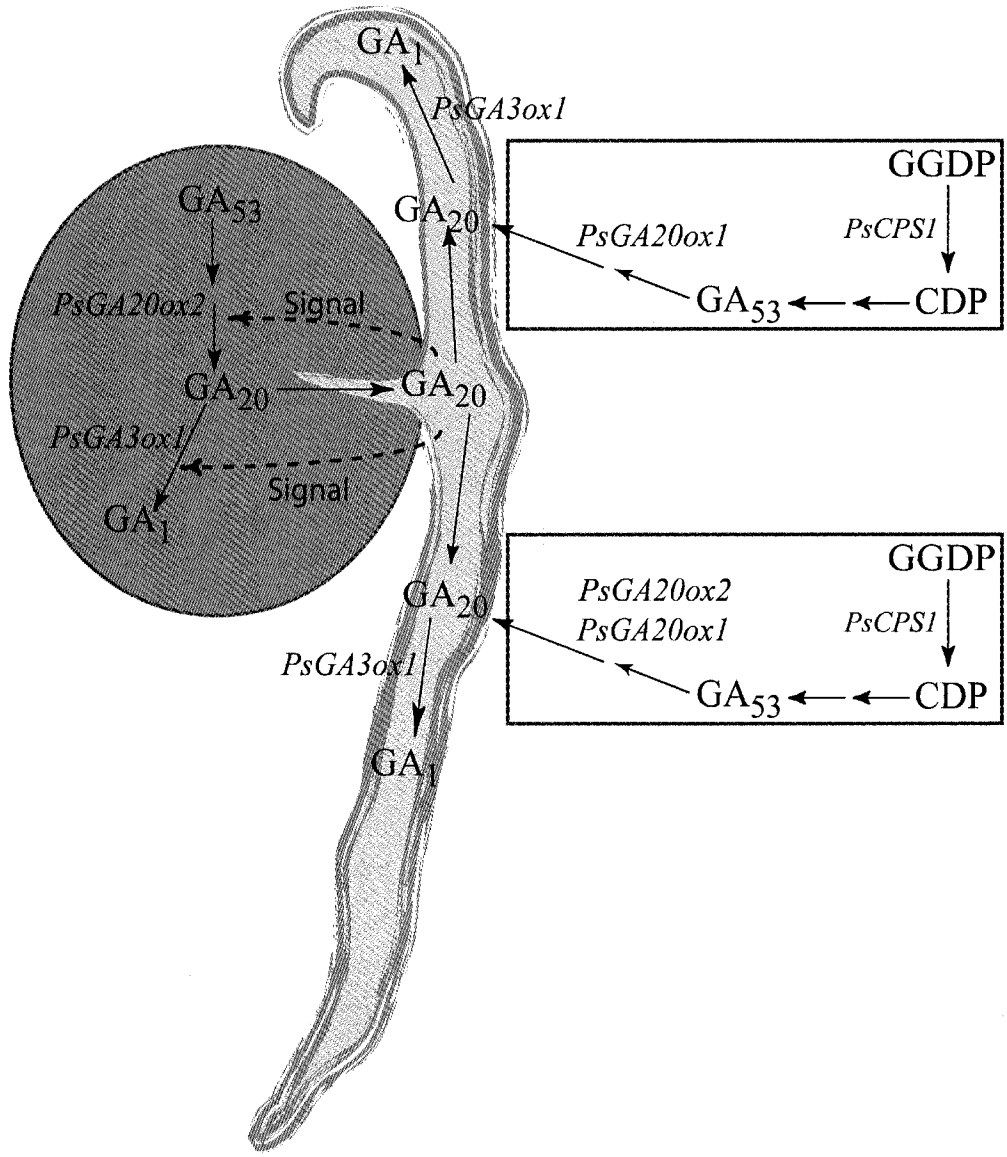

Figure 5.1. Model for GA biosynthesis during pea seed germination and young seedling growth.

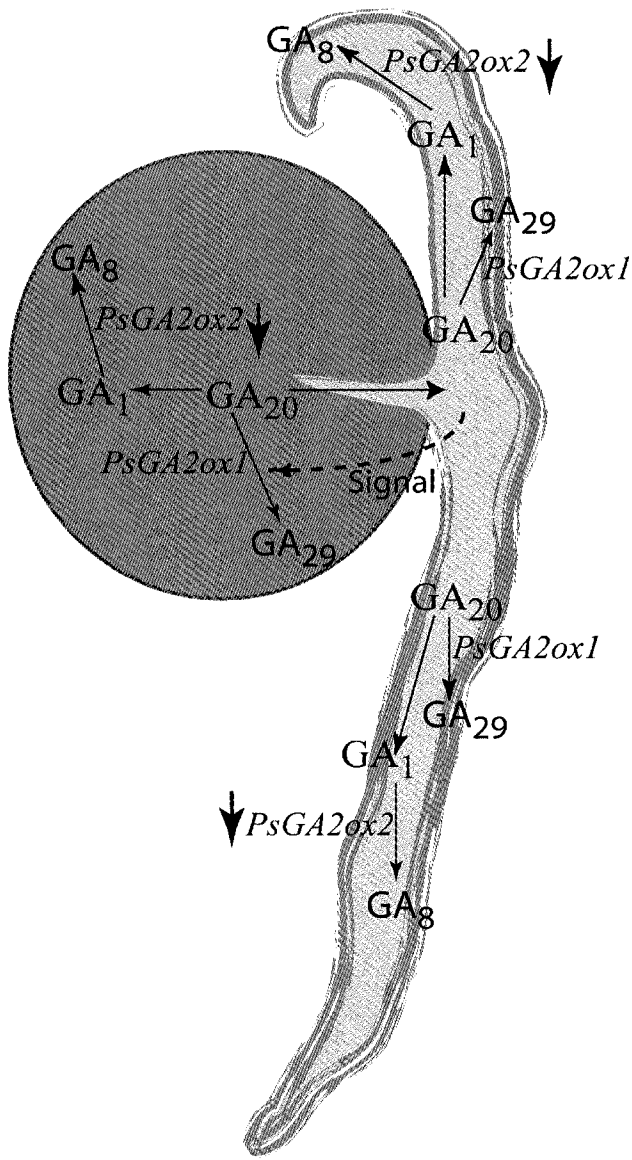

Figure 5.2. Model for GA catabolism during pea seed germination and young seedling growth.

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Appendix

Sequencing of *PsGA3ox1* Gene in 'Carneval'

RNA Isolation

Tissues were finely ground in liquid N_2, and 200 to 550 mg fresh weight (mature embryos, embryo axes, shoots, roots and root tips) or 100 to 250 mg fresh weight (cotyledons) sub-samples were used for total RNA isolation using a modified TRIzol (Invitrogen, Carlsbad, CA) protocol. After initial extraction with the TRIzol reagent and centrifugation, the supernatant was cleaned by chloroform partitioning (0.2 mL mL^{-1} TRIzol). The resulting fraction was then precipitated sequentially by isopropanol (0.25 mL mL^{-1} TRIzol) and high salt solution (1.2 M Na citrate and 0.8 M NaCl), 4 M LiCl, and finally by a mixture of 3 M Na acetate (pH 5.2): 100% ethanol (1:20, v/v) to remove polysaccharides and proteoglycans. The precipitate was dissolved in DEPC treated water. The integrity of the RNA was verified both electrophoretically and by the average OD_{260} to OD_{280} nm absorption ratio. The total RNA samples of all tissues were then digested with DNase (DNA-free kit, Ambion, Austin, TX), and the cotyledonary total RNA samples were further purified with RNeasy columns (Qiagen, Valencia, CA). Sample RNA concentration was determined in duplicate by A_{260} measurement, and then the samples were stored at -80^{0}C until quantitation by real-time RT-PCR.

RT-PCR

First strand cDNA was synthesized by reverse transcription of total RNA from the shoots of 4 DAI 'Carneval' seedlings (200 mg fresh weight). The reverse transcription reaction mix contained 3 μL of total RNA (0.968 μg μL^{-1}), 1 μL oligo (dT) primer (0.5 μg μL^{-1}; Invitrogen), 2 μL dNTP mix (10 mM; Invitrogen), 1 μL M-MLV reverse transcriptase (200 units μL^{-1}; Invitrogen), 4 μL 5X first strand buffer (supplied with the enzyme) and 2 μL DTT (0.1 M; Invitrogen) and deionized water to 20 μL. Samples were subjected to denaturation at 75°C for 5 min, incubation at 37°C for 1 h, and enzyme inactivation at 75°C for 10 min in a Perkin Elmer Thermal Cycler (model 2400, Applied Biosystems). The PCR forward primer (5'-TACTCACTATGCCTTCACTCTCCG-3') and reverse primer (5'-ACCTATTTAGCCCACTTGGACACTATT-3'), located at the start and end of the *PsGA3ox1* reading frame, were designed based on the reported pea *PsGA3ox1* (*LE*) sequence from Martin et al. (1997), and used to amplify a 1.28-kb *PsGA3ox1* cDNA fragment. PCR reactions included 1 μL reverse transcription reaction as template, 1 μL of each primer (10 pM μL^{-1}), 1 μL of dNTP mix (10 mM; Invitrogen), 0.4 μL of *Taq* DNA polymerase (5 units μl^{-1}; Invitrogen), 5 μL of 10X PCR buffer (with no $MgCl_2$) (supplied with the enzyme), 1 μL of $MgCl_2$ (50 mM; Invitrogen), and deionized water to 50 μL. The reaction mixtures were heated to 94°C for 5 min, cycled 40 times for 30 s at 94°C, 1 min at 50°C, and 1 min at 72°C, and extended for 10 min at 72°C in a Perkin Elmer Thermal Cycler (model 2400, Applied Biosystems).

Cloning

The amplified product was purified by 0.85% (w/v) agarose gel in 1X Tri acetic acid EDTA. Gel bands (< 210 mg) were excised over long wave UV light, the DNA fragments extracted with QIAEX II Gel Extraction Kit (Qiagen), and ligated in pBluescript KS+ (Stratagene, La Jolla, CA). Clones, grown from *E.coli* DH-5α competent cells at 37^{0}C overnight on selective agar plates containing 100 μg ampicillin per mL medium, were used to inoculate a 5 mL L-Broth culture medium (1% Bacto trypton, 0.5% Bacto-yeast extract, 1% NaCl) with ampicillin (100 μg/mL medium). The culture was then grown overnight at 37^{0}C with shaking. The plasmid vector was then purified from the bacterial clone with GenElute Plasmid Miniprep Kit (Sigma), digested to completion with EcoRV, and tailed with ddT by incubation with *Taq* DNA polymerase (1 unit/ μg plasmid/ 10 μL volume) using standard buffer conditions (100 mM KCl, 40 mM Tris-HCl pH 8.4, and 1 mM $MgCl_2$) in the presence of 2 mM dTTP for 2 h at 72 ^{0}C, followed by purification with spin column (Sigma). Ligation mix that contained 6 μL PCR product, 2 μL T-vector, 1 μL T4 DNA ligase (1 unit/μL; Gibco BRL), and 2 μL 5X DNA ligase buffer (supplied with the enzyme) in a volume of 12 μL, was incubated overnight at 4^{0}C. The ligation product was used to transform *E.coli* DH-5α competent cells. Clones were grown, as mentioned above, at 37^{0}C overnight on selective agar plates containing ampicillin (100 μg/mL medium), X-Gal (20 mg/mL in dimethyl sulfoxide, 40 μL per plate) and IPTG (0.1 M in water, 40 μL per plate). Culture tubes containing 5 mL of L-Broth medium with ampicillin (100 μg/mL medium) were inoculated with recombinant clones screened by the blue/white clone

selection method. Cultures were then grown overnight while shaking at 37^0C and the plasmid for sequencing was prepared from DH-5α clones with GenElute Plasmid Miniprep Kit (Sigma). The presence of the insert within the expected size (*ca.* 1.28-kb) and its correct orientation were revealed by plasmid digestion with *Kpn*I and *Xba*I restriction endonucleases, followed by agarose gel electrophoresis.

Sequence Analysis

DNA was sequenced at the DNA lab, Department of Biochemistry, University of Alberta (Edmonton) on automated sequencer (model 370, Applied Biosystems) using dye terminator chemistry. Gapped BLAST and PSI-BLAST (Altschul et al., 1997) programs (versions BLASTN 2.2.6, BLASTP 2.2.6 and BLASTX 2.2.6) were used to search for homologous sequences in the databases, and to generate DNA and amino acid sequence alignments.

Summary

To determine if the semi-dwarfing habit of 'Carneval' was the result of a mutation in the *PsGA3ox1* gene, *PsGA3ox1* was cloned from 'Carneval' shoots. One distinct electrophoretic band of about 1.28-kb (Figure A.1) was obtained by PCR amplification of the 4 DAI 'Carneval' shoot cDNA, and restriction analysis of the PCR product cloned into pBluescript KS+ showed an insert of the same size (*ca.* 1.28-kb). A BLAST search revealed 100% identity of the cDNA sequence at the nucleotide level (1125 bases) to the pea *le-1* gene (GenBank accession no. AF010167; Martin et al.,

1997). Sequence alignment of the cDNA cloned from 'Carneval' shoot with that of the pea *LE* gene (GenBank accession no. AF001219; Martin et al., 1997) showed substitution of A for G at base position 685, relative to the start codon, leading to a threonine at position 229 rather than an alanine (Figure A.2).

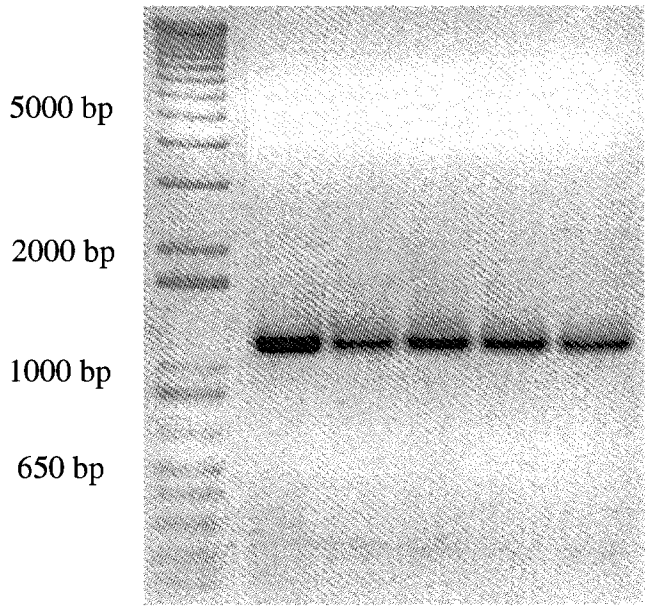

Figure A.1. Agarose gel electrophoresis of PCR products (replicated five times). PCR generated a single product of *ca.*1280 bp. Size markers are shown on the left hand side in base pairs.

Figure A.2. Alignment of deduced GA 3-oxidase amino acid sequence of 'Carneval' with those from pea *Le* (GenBank accession no. AF001219) and pea *le-1* (GenBank accession numbers T06244, AAC86820 and AAC49793). Amino acid substitutions are shaded.

```
Le              1  MPSLSEAYRAHPVHVNHKHPDFNSLQELPESYNWTHLDDHTLIDSNNIMKESTTTVPVID 60
le              1  MPSLSEAYRAHPVHVNHKHPDFNSLQELPESYNWTHLDDHTLIDSNNIMKESTTTVPVID 60
'Carneval'      1  MPSLSEAYRAHPVHVNHKHPDFNSLQELPESYNWTHLDDHTLIDSNNIMKESTTTVPVID 60

Le             61  LNDPNASKLIGLACKTWGVYQVMNHGIPLSLLEDIQWLGQTLFSLPSHQKHKATRSPDGV 120
le             61  LNDPNASKLIGLACKTWGVYQVMNHGIPLSLLEDIQWLGQTLFSLPSHQKHKATRSPDGV 120
'Carneval'     61  LNDPNASKLIGLACKTWGVYQVMNHGIPLSLLEDIQWLGQTLFSLPSHQKHKATRSPDGV 120

Le            121 SGYGIARISSFFPKLMWYEGFTIVGSPLDHFRELWPQDYTRFCDIVVQYDETMKKLAGTL 180
le            121 SGYGIARISSFFPKLMWYEGFTIVGSPLDHFRELWPQDYTRFCDIVVQYDETMKKLAGTL 180
'Carneval'    121 SGYGIARISSFFPKLMWYEGFTIVGSPLDHFRELWPQDYTRFCDIVVQYDETMKKLAGTL 180

Le            181 MCLMLDSLGITKEDIKWAGSKAQFEKACAALQLNSYPSCPDPDHAMGLAPHTDSTFLTIL 240
le            181 MCLMLDSLGITKEDIKWAGSKAQFEKACAALQLNSYPSCPDPDHAMGLAPHTDSTFLTIL 240
'Carneval'    181 MCLMLDSLGITKEDIKWAGSKAQFEKACAALQLNSYPSCPDPDHAMGLAPHTDSTFLTIL 240

Le            241 SQNDISGLQVNREGSGWITVPPLQGGLVVNVGDLFHILSNGLYPSVLHRVLVNRTRQRFS 300
le            241 SQNDISGLQVNREGSGWITVPPLQGGLVVNVGDLFHILSNGLYPSVLHRVLVNRTRQRFS 300
'Carneval'    241 SQNDISGLQVNREGSGWITVPPLQGGLVVNVGDLFHILSNGLYPSVLHRVLVNRTRQRFS 300

Le            301 VAYLYGPPSNVEICPHAKLIGPTKPPLYRSVTWNEYLGTKAKHFNKALSSVRLCTPINGL 360
le            301 VAYLYGPPSNVEICPHAKLIGPTKPPLYRSVTWNEYLGTKAKHFNKALSSVRLCTPINGL 360
'Carneval'    301 VAYLYGPPSNVEICPHAKLIGPTKPPLYRSVTWNEYLGTKAKHFNKALSSVRLCTPINGL 360

Le            361 FDVNDSNKNSVQVG 374
le            361 FDVNDSNKNSVQVG 374
'Carneval'    361 FDVNDSNKNSVQVG 374
```

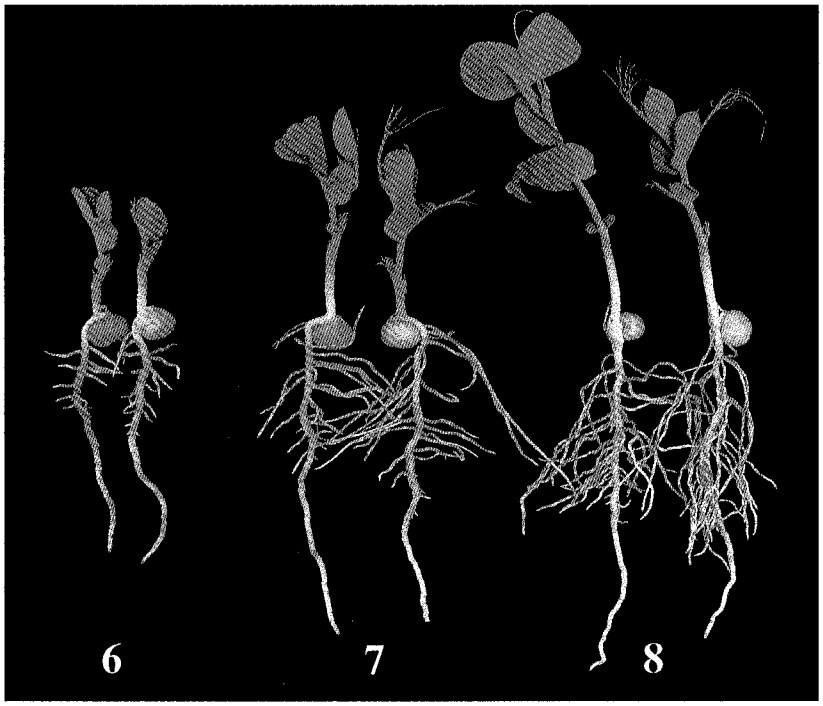

Figure A.3. Growth of seedlings of 'Alaska (left in each pair) and 'Carneval' (right in each pair) in sand from 6 to 8 DAI.

Figure A.4. Nucleotide sequence of *PsCPS1* (also named ent-kaurene synthase A gene, *LS*; GenBank accession number U63652; Ait-Ali et al., 1997) with forward and reverse primers (shaded and underlined with arrows) and probe (shaded and underlined) sequences used in quantifying the relative transcript levels.

```
   1  atgtttactc acttctccac ccactttcat cttccatctt cttcttctct
  51  cttctttctt catccattct acaaatcctc ttctttaggt gctgtgtcgt
 101  ttgtggctaa agacaaagag aaaagatgta gagctatatc caaatcaaga
 151  actcaggaat atgaaggagt atttcaaact aatgtagcaa cattaaagtt
 201  gagtgaaatt aatgtggaag atgtcatagt catagatgat gaagaggagc
 251  aagatattag ggtgggttta gtgaacaaaa taaaatcaat attgagttca
 301  ttggaagatg gagagataac tatatctgct tatgatactg cttgggttgc
 351  tcttgttgaa gatgttaatg ctattagcac ccctcaattt ccttctagtc
 401  tagagtggat tgcaaaaaat caacttcaag atggttcatg gggtgatagc
 451  cgattattct cggctcatga tcgaattatc aatacattgg catgtgtaat
 501  cgcgttacga tcatggaata tgcattcaga aaagtgtgac aaaggaatga
 551  tatttttag agagaatctt agcaagcttg agaatgagaa tgaggagcat
 601  atgccaattg ggtttgaagt tgctttccct tcactacttg aaggagctag
 651  agggattaaa ccattgatgt gcccaaatga ttctccaata ttgaaaaaca
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 751  cataaagtgc caacaacatt gttgcatagc ttggagggta tgtctggctt
 801  ggattggaaa caacttctaa aattgcaatc acaagatgga tcattcttgt
 851  tttctccatc ttccacagct tttgcattaa tgcaaactaa agatggaaat
 901  tgcctaaaat acttgaataa tgttgtcaaa aaattcaatg gaggagtccc
 951  aaatgtgtat ccggtggatt tatttgaaca tatttgggtg gttgatcgtc
1001  tcgaacgcct tggaatatct cgatttttc gacatgagat caaagattgt
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1101  aaattcaaat gttcaagata ttgatgacac agcaatggct ttcagattac
1151  ttagattaca tggtcaccaa gtttcagccc atgtgtttaa gcactttgag
1201  agaaatggtg aattcttctg ctttgctggt caatgcacac aagcagtgac
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1351  agagaagcaa atgaactcat agataagtgg atcataatga agaatctgcc
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1451  gcattgagac aagattttac attgatcaat atggtgctga aagcgatgta
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2251  gtacaaatgg tgtttcaaaa atgtcctaat gacattgatt ttaatgtcaa
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2551  a
```

```
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 151  gaaaagccat gcatgaatgt gccggaactt gatgttccac tcattgattt
 201  caaaaacttc ctttctggtg acccttttgc ggcaatggaa gcttctaaaa
 251  ccattggtga agcatgtgag aaacatgggt tttttcttgt ggttaatcat
 301  ggaattgata caaagttaat tgaacatgct catagttaca tgaatgattt
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 501  aaatattgtt aaagattacc tttccaacac attaggggaa gattttcaac
 551  aatttgggga ggtttaccaa gaatattgtg aagcaatgag caaactttca
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 901  aagagttgtt tacatagggc agtggtgaac aacaagacaa caagaaaatc
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1001  gtgaattggt gaatgatttg acaccaagga tctatccaga ttttacatgg
1051  ccaatgcttc ttgagtttac tcaaaaacat tatagagctg atatgaggac
1101  acttgaggca tttacaaaat ggattcaaca aaaacaaaac tagctctatt
1151  ttcaaccaca tgaataaaaa caaattatta gagccattga tatacaaccc
1201  tagtgaaaaa gtggccaatc atgtgaaagt acacttgtgt aattcatgag
1251  gcacattagt caagaataat gaagaattag ggttatgatt taaatggtta
1301  tgaagcatca ataagaaaaa tttgccacta attttggatt att
```

Figure A.5. Nucleotide sequence of *PsGA20ox1* (GenBank accession number U70471; Garcia-Martinez et al., 1997) with forward and reverse primers (shaded and underlined with arrows) and probe (shaded and underlined) sequences used in quantifying the relative transcript levels.

```
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  51  cacaccttag tttattcttt ctccacttga aaactctctc tctctctctc
 101  tagtttctac ttcaaatttt tacataaatt gcatttcaca aaaatactca
 151  ccaagtatac tcaaacatga aagtgctttg ttcttcaatg ctatttgcac
 201  ccccaaatgc aaatgaaagt ttcatgaatg aacaaaaaca atgtcttgat
 251  aatacatctt ctctaccgtt gcaaattaca aacatacctt cagagttcat
 301  atggccagat catgagaaac cttgtttaac accgccaaaa cttgaagttc
 351  cacctattga cttgaaagct tttctatctg atgatccaaa gtccatttca
 401  aatgcttgtt caaaggtgaa ccatgcatgc aaaaaacatg gttttttct
 451  tgttgttaac catggtgttg ataacaagct tatagctcaa gctcataagc
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 601  ctcatctaaa cttccatgga aggaaacact ctcttttcgt tactccgcgg
 651  atgaatcgtg cagaactgtt gaggactatt ttgtcaatat catgggagaa
 701  gatttcagac aatttgggat tgtctaccag aagtattgtg aagctatgag
 751  caatctctca cttgggataa tggagcttct tggtatgagc ttaggagttg
 801  gcaaggaata ttttagagaa ttttttgaag gaaatgaatc agtaatgaga
 851  ttgaattact atccaccgtg taaaaatcct gatttagcat tcggaactgg
 901  acctcattgt gaccctactt cactaaccat tctccaccaa gatcaagttg
 951  aaggtcttca agtgcttgtg gatggaatat ggcactcggt tgttcctaaa
1001  gaagatgctt ttgtggtcaa cattggtgac acatttatgg ctctatcaaa
1051  tggaatgttc aagagttgct tgcatagagc aattgtaaat gacaaaatag
1101  tgagaaaatc acttgcattt tttctatgtc caaatgaaga caaaatagtg
1151  actcctccaa aagagcttat tgacaaagag aatccaagga aatatccaaa
1201  cttcacatgg ccaagtttgc ttgaatttac acaaaaacat tacagggctg
1251  atgaaagaac acttgatgct ttttcaatgt ggttacaaga aaaaacaact
1301  acttagaggt gcctccacct ttgtcatatt gcaagcaaaa ttttaaattt
1351  tgttctattt taagttgagg aaaatgacat cacataaagt gaaaagaggg
1401  tgtggttttt attgagagaa gaagataata gaaaacacca aagaataggc
1451  caagtatgta ataagtgttg gaagaatcct aatcatgcat gcaaatggag
1501  gaaattaaac gttattcctc taagaataaa gagtacttca ataatgcttg
1551  ttgtataatc atgtttttct t
```

Figure A.6. Nucleotide sequence of *PsGA20ox2* (GenBank accession number U58830; Lester et al., 1996) with forward and reverse primers (shaded and underlined with arrows) and probe (shaded and underlined) sequences used in quantifying the relative transcript levels.

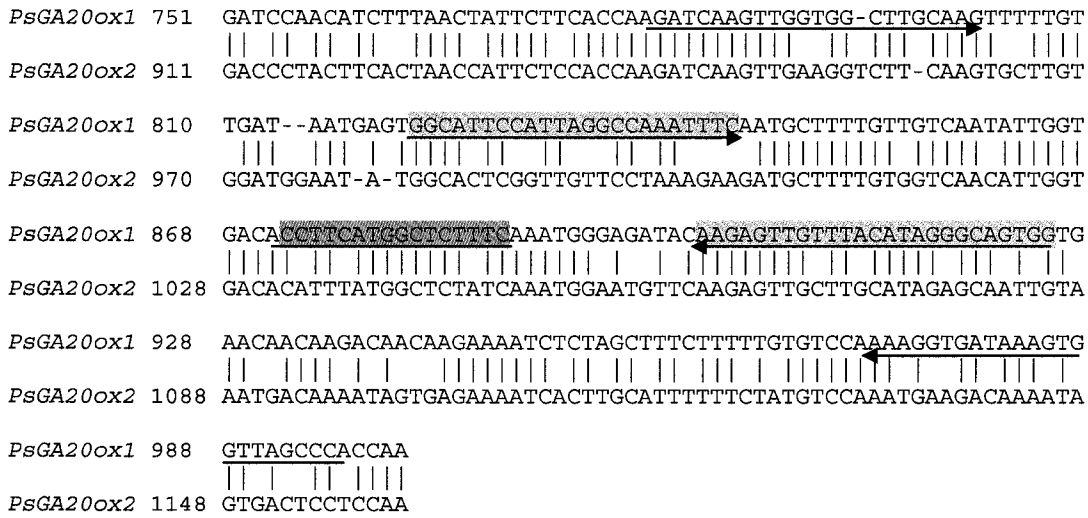

Figure A.7. Alignment of *PsGA20ox1* and *PsGA20ox2* nucleotide sequences with validating (unshaded and underlined with arrows) and quantifying (shaded and underlined with arrows) primers and probe (shaded and underlined) of *PsGA20ox1* located in the homologous region.

Figure A.8. Alignment of *PsGA20ox1* and *PsGA20ox2* nucleotide sequences with primers (shaded and underlined with arrows) and probes (shaded and underlined) of *PsGA20ox2* located in the homologous region.

```
   1  atggtgttac tatccaaacc aacttcagaa caatacacct atgttaggaa
  51  caacatgcca atcacatttt cttcatcaat ccctctcgtt gacctatcaa
 101  aaccagatgc aaaaaccctc atagtaaaag cttgtgaaga ttttggattc
 151  ttcaaagtta taaaccatgg tatccctttg gatgctatct cccaattgga
 201  atccgaggcc ttcaaattct tctctctccc tcaaacagag aaagaaaaag
 251  cagggcctgc aaatcctttt ggctatggta acaaacgtat tggactcaat
 301  ggtgatattg gttggattga atatcttctc ctcacaacca atcaagatca
 351  caatttctct ctttatggag aagacataca caaatttagg ggtttgttga
 401  aagattataa gtgtgcaatg aggaatatgg catgtgagat acttgatttg
 451  atggcagaag ggttaaagat acaaccaaag aatgtgttta gcaagcttgt
 501  gatggataaa cagagtgact gtctttttag ggttaatcat taccctgctt
 551  gccctgaatt agctatcaat ggtgagaatt tgattggctt tggagaacac
 601  actgaccctc aaattatttc aattttgagg tcaaataata cttcaggctt
 651  tcaaatttct cttagagatg gtagctggat ttcagttcct cctgatcata
 701  gctccttctt tatcaatgtt ggtgattctc ttcaggttat gactaatggg
 751  aggttcaaaa gtgtgagaca tagagttttg gcaaatggca tagacccaag
 801  gctgtctatg atttactttt gtggaccacc tttgagtgag aaaatagcac
 851  cattaccttc actcatgaaa ggaaaagaaa gtttgtataa agaatttaca
 901  tggtttgagt acaagagttc aacttatggt tcaaggttgg ctgataatag
 951  gcttggaaat tatgaaagga ttgctgccac ttaatattgg aggatcatag
1001  tgttgttcaa ttgtcaatag aaatggggat acaatatata tctataattc
1051  aatcaaaatc aacttcaaaa tacaatgttt gttaagatag agttgtatgt
1101  agtataacta tgataagttt ttttttatgt atttttcttt atggtacttt
1151  aaggttacat ttattaatta tgaggtgtca tatagatatg atagtattta
1201  ttccccttt gcatcaacac tcatgtaagt acttttctaa gtatatgaaa
1251  tatttcatat gtttgtgtt
```

Figure A.9. Nucleotide sequence of *PsGA2ox1* (GenBank accession number AF056935; Martin et al., 1999) with forward and reverse primers (shaded and underlined with arrows) and probe (shaded and underlined) sequences used in quantifying the relative transcript levels.

```
   1  atgataagaa caaagaaaac aaaggcagta ggaattccga cgatcgacct
  51  ttctctcgaa aggtcgcagt tgtcggaatt agtagtgaaa gcatgtgaag
 101  aatatggttt cttcaaggtg gtgaatcata gtgtaccaaa agaggttatt
 151  tctagattgg atgaggaagg aatagagttt ttctcaaaaa attcttcaga
 201  aaaacgtcaa gctggtactt ctactccttt tggttatggc tgcaaaaata
 251  ttggtcctaa tggtgataaa ggtgaacttg aatatcttct tcttcattcc
 301  aatcctattt caatctctga aagatccaaa accatagcaa aagatcaccc
 351  cataaagttc agttgcatcg tcaatgatta cataaaagca gttaaagact
 401  taacatgtga gattcttgag ctagcagcag aaggattgtg ggtccctgac
 451  aagtcctcac tcagcaaaat tatcaaagat gaacatagtg actcacttct
 501  tagaatcaat cactaccctc cagttaagaa actagggaat gataattggg
 551  acccatccaa aattcagaat agcaataata ataatattgg ttttggagaa
 601  cattctgacc ctcagatctt aactatctta agatccaaca acgtgggtgg
 651  tcttcagatt tcaactcacc atggtttgtg gatccctgtc cctcctgacc
 701  ccagtgaatt ctatgtcatg gttggtgatg cccttcaggt tttgacaaat
 751  ggaagatttg tgagtgtgag acatagagtt ttgacaaaca caacaaagcc
 801  tagaatgtca atgatgtatt ttgcagcacc acggttgaat tggttgataa
 851  gcccgttatc gaagatggtg acggcccata gcccatgttt atataggccc
 901  tttacatggg cccaatacaa acaagcagca tatgctctca gattaggaga
 951  tacacgtctt gatcaattca aggttcaaaa acaagaagat agcaatgatt
1001  ctcattctct atgacacttg aatggcattt gttgtcatag tttttctttt
1051  ggatcactta ttggttcttg gtttatatat agttttgata ttttttgggt
1101  tttgg
```

Figure A.10. Nucleotide sequence of *PsGA2ox2* (GenBank accession number AF100954; Lester et al., 1999) with forward and reverese primers (underlined) and probe (shaded and underlined) sequences used in quantifying the relative transcript levels.

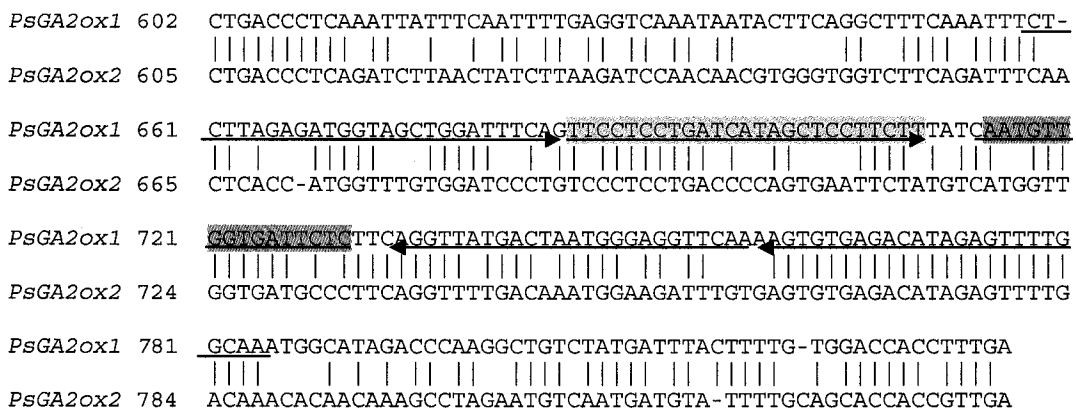

Figure A.11. Alignment of *PsGA2ox1* and *PsGA2ox2* nucleotide sequences with validating (unshaded and underlined with arrows) and quantifying (shaded and underlined with arrows) primers and probe (shaded and underlined) of *PsGA2ox1* located in the homologous region.

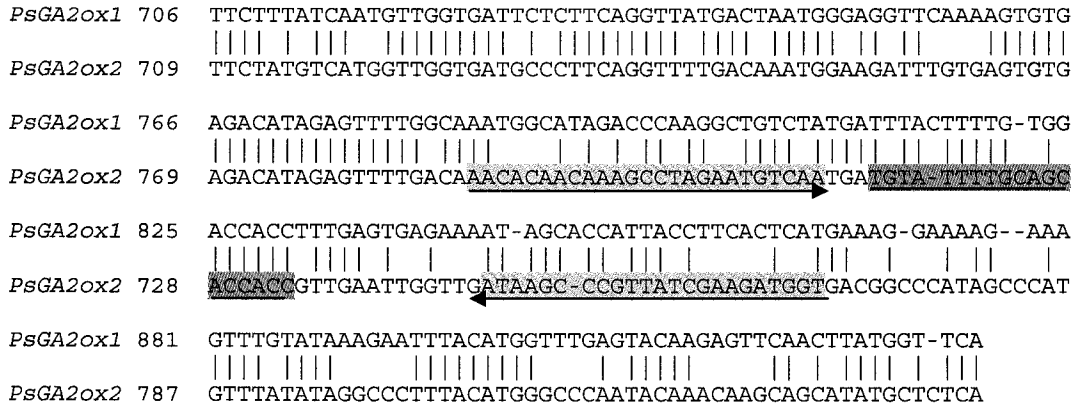

Figure A.12. Alignment of *PsGA2ox1* and *PsGA2ox2* nucleotide sequences with primers (shaded and underlined with arrows) and probes (shaded and underlined) of *PsGA2ox2* located in the homologous region.

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