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

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Abstract

To explore the coordination and regulation of GA biosynthesis during germination and early seedling growth in pea, the developmental expression profile of *PsCPS1* [that codes for *ent*-copalyl diphosphate synthase (CPS), which catalyses the first committed step in gibberellin (GA) biosynthesis], and regulation of expression of *PsCPS1* and the late GA biosynthesis genes were determined using real-time reverse transcription polymerase chain reaction (RT-PCR). Mature embryos contained *PsCPS1* transcripts prior to germination, suggesting that *PsCPS1* mRNAs synthesized during seed development were sequestered in the mature embryo. *PsCPS1* transcripts were detected in all tissues examined during germination and young seedling growth (cotyledons, embryo axis, shoots, and roots from 0.5 to 6 days after imbibition [DAI]) and were under tight developmental regulation. *PsCPS1* transcript abundance decreased upon imbibition in the post-mitotic cotyledons and remained low to 6 DAI. In contrast, *PsCPS1* transcript abundance increased in the actively growing embryo axes, and the shoots and roots of the young seedlings. *PsCPS1* did not exhibit feed-back regulation at the mRNA level in the roots or shoots of seedlings following modulation of bioactive GA levels (using GA_3 or prohexadione), whereas the late GA biosynthesis genes showed either feed-back (*PsGA20ox1* and *PsGA3ox1*) or feed-forward (*PsGA2ox1* and *PsGA2ox2*) regulation, with one exception, *PsGA20ox2*, which was not regulated in this manner. Our data also suggest that at least part of the homeostatic mechanism of the root to maintain lower levels of bioactive GAs than in the shoot is through regulation of the mRNA levels of the genes in the latter part of the GA biosynthesis pathway.

Key words: Gene expression; Gibberellin biosynthesis; *Pisum sativum* L.; Seed germination; Seedling growth; Feed-back regulation; Feed-forward regulation.

Introduction

Seed germination incorporates those events that commence with the uptake of water by the quiescent dry seed (imbibition) and that terminate with

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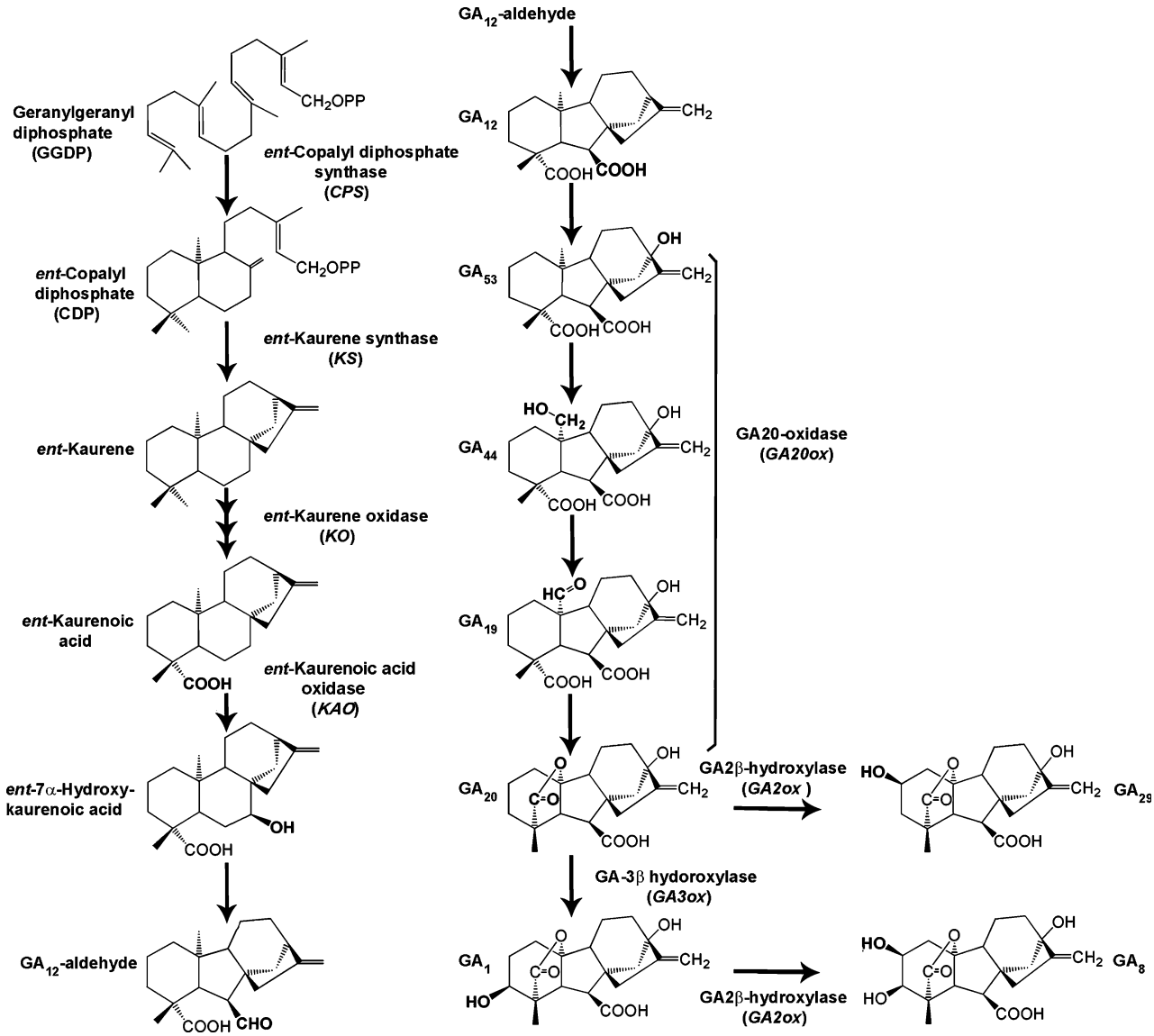

Figure 1. The early 13-hydroxylation gibberellin biosynthesis pathway of pea from geranylgeranyl diphosphate (GGDP) through GA_1 (the growth-effector GA of the pea seedling). The biologically inactive GAs, GA_{29}, and GA_8 are also shown. Abbreviations for selected genes in the pathway are noted in parentheses.

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 gibberellins (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 (*Curcurbita maxima* L.; Lange and others 2005). Early in the GA biosynthesis pathway, *ent*-kaurene is formed from geranylgeranyl diphosphate (GGDP) in a two-step process catalyzed by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS; Duncan and West 1981; Figure 1). *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 (Yamaguchi and others 1998; Smith and others 1998; Hedden and Phillips 2000).

Seeds from the CPS-impaired GA-deficient mutant of *Arabidopsis* (*ga1-3*) and tomato (*gal-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 (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 possibly altering the ability of cells to respond to GA (Richards and others 2001).

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 [N,N,N-trimethyl-1-methyl-(2′,6′,6′trimethylcyclohex-2′en-1′-yl)prop-2-enylammonium iodide] suggested that normal CPS enzyme activity for *de novo* GA biosynthesis was not essential for pea seed germination, but rather for the maintenance of normal seedling growth soon after germination (epicotyl growth; Sponsel 1983).

ent-Copalyl diphosphate synthesis appears to be controlled by a single copy gene in pea (*PsCPS1*; corresponding to the *LS* locus; Ait-Ali and others 1997). Pea plants carrying a mutation in the *LS* gene (*ls-1*) are dwarf with a large reduction in shoot growth (internode lengths approximately 75% smaller than in wild-type plants; Reid and Potts 1986) and a small reduction in root elongation (Yaxley and others 2001). The *ls-1* mutation substantially reduced the levels of GAs in pea shoots, roots, and developing seeds at contact point (Ait-Ali and others 1997; Yaxley and others 2001). Although *PsCPS1* expression has been studied in various pea tissues (Ross and others 2003; Ait-Ali and others 1997; Davidson and others 2005), 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 (presumably via feed-back regulation) of *PsGA20ox1* and *PsGA3ox1* in actively growing pea shoots and pericarps (Martin and others 1996; van Huizen, and others 1997; Ait-Ali and others 1999; Figure 1). Feed-forward regulation of *PsGA2ox1* and *PsGA2ox2*, with bioactive GAs regulating their own catabolism, has also been observed in pea shoots (Elliott and others 2001). However, feed-back regulation of the early GA biosynthesis genes by bioactive GA at the mRNA level does not appear to take place in pea shoots from well-developed stems (Davidson and others 2005). The regulation of GA biosynthesis by bioactive GAs in the pea root has received less attention than the shoot, although the root responds dramatically differently to bioactive GAs than the shoot (exogenous GA strongly promotes shoot growth but it has little effect on root elongation in pea; Tanimoto 1990). Because *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 seed 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 *PsCPS1* during germination, pre-emergence and post-emergence stages of two distinctly different genotypes of pea both of which germinate readily upon imbibition. We also determined if feed-back and feed-forward regulation occurs similarly in young shoots and roots of pea seedlings at the mRNA level in response to applied GA_3 in both the early and latter part of the GA biosynthesis pathway. In addition, the effects of the GA biosynthesis inhibitor, prohexadione, on GA biosynthesis gene expression were characterized in 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'. 'Carneval' is semi-dwarf in vine length (*le-1*) and semi-leafless (*af*; *afila*, leaflets are replaced by tendrils of normal anatomy), has white flowers and yellow cotyledons at maturity, and flowers at about the 15th to 17th node under long day conditions. 'Alaska' has normal leaflet morphology (*AF*), wild-type internode length (*LE*), and white flowers and green cotyledons at maturity; it flowers at approximately the 10th node under long or short day conditions. 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 approximately 2.5 cm into moist sterilized sand in 3-liter plastic pots (10 seeds per pot), and the pots were placed in a growth chamber (Conviron, Ashville, NC) at 22/20°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 days after imbibition (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; 15–30 seeds or seedling per time point). Seeds were scored as germinated when protrusion of the radicle (2–5 mm) through the seed coat was visible. Relative water content of the cotyledons was determined by comparing the sample weights before and after drying for 72 h at 60°C, and is 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 (approximately 4 mm) and remainder of roots (6 DAI), and tissues were immediately frozen in liquid N_2 and stored at −80°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°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-liter 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 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, by 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 260–280 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°C until quantitation by real-time reverse transcription polymerase chain reaction (RT-PCR).

Primers and Probes

Primers and probes for the target gene quantifying amplicon *GA3ox-87* (used for *PsGA3ox1* quantification) and for the reference gene amplicon *18S-62* (used for pea 18S rRNA quantification) were designed using Primer Express Software (Applied Biosystems, Foster City, CA) by Ozga and others (2003; Table 1). Primers and probes for target gene quantifying amplicons *CPS-92* (spans nucleotides 1833–1924), *GA20ox1-104* (spans nucleotides 873–976), *GA20ox2-88* (spans nucleotides 305–392), *GA2ox1-73* (spans nucleotides 722–794) and *GA2ox2-83* (spans nucleotides 817–899) were designed based on reported sequences of pea *PsCPS1* (also named *ent*-kaurene synthase A gene, *LS*; GenBank accession number U63652; Ait-Ali and others 1997), *PsGA20ox1* (GenBank accession number U70471; Garcia-Martinez and others 1997), *PsGA20ox2* (GenBank accession number U58830; Lester and others 1996), *PsGA2ox1* (GenBank accession number AF056935; Martin and others 1999), and *PsGA2ox2* (GenBank accession number AF100954; Lester and others 1999), respectively (Table 1). To test the specificity of the quantitation primers, a second set of validating primers were designed for *GA20ox1* and *GA2ox1*: *GA20ox1-216* (spans nucleotides 832–1047) and *GA2ox1-126* (spans nucleotides 695–820), as described for *PsGA3ox1* (*GA3ox1-126*) quantitation using real-time RT-PCR (Ozga and others 2003).

All probes were TaqMan MGB and labeled at the 5′ end with fluorescent reporter dye 6-carboxyfluorescein (target gene probes) or VIC (*18S-62* reference gene probe), and at the 3′ end with non-fluorescent quencher (NFQ) dye (Applied Biosystems). To

Table 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[z]	TGTTAGGAATGAAGATTTGAGGAAAGA	
	RP[y]	TCTTCATCCTCCGGGCAAT	
	Probe	TCGATGTTCGAGACTATT	
		Amplicon: *GA20ox1-104*	Amplicon: *GA20ox1-216*
PsGA20ox1	FP	GCATTCCATTAGGCCAAATTTC	GATCAAGTTGGTGGCTTGCAA
	RP	CCACTGCCCTATGTAAACAACTCTT	TGGGCTAACCACTTTATCACCTTT
	Probe	CCTTCATGGCTCTTTC	CCTTCATGGCTCTTTC
		Amplicon: *GA20ox2-88*	
PsGA20ox2	FP	AATACATCTTCTCTACCGTTGCAAAT	
	RP	TTGGCGGTGTTAAACAAGGTT	
	Probe	ACATACCCTCAGAGTTC	
		Amplicon: *GA3ox-87*	Amplicon: *GA3ox-126* (Ozga and others 2003)
PsGA3ox1	FP	TTCGAGAACTCTGGCCTCAAG	
	RP	ATGTTCCTGCTAACTTTTTCATGGTT	
	Probe	ACAATATCACAGAATCTGGT	
		Amplicon: *GA2ox1-73*	Amplicon: *GA2ox-126*
PsGA2ox1	FP	TTCCTCCTGATCATAGCTCCTTCT	CTCTTAGAGATGGTAGCTGGATTTCA
	RP	TTGAACCTCCCATTAGTCATAACCT	TTGCCAAAACTCTATGTCTCACACT
	Probe	GAGAATCACCAACATT	GAGAATCACCAACATT
		Amplicon: *GA2ox2-83*	
PsGA2ox2	FP	AACACAACAAAGCCTAGAATGTCAA	
	RP	ACCATCTTCGATAACGGGCTTAT	
	Probe	TGTATTTTGCAGCACCACC	
		Amplicon: *18S-62*	
Ps18S rRNA	FP	ACGTCCCTGCCCTTTGTACA	
	RP	CACTTCACCGGACCATTCAAT	
	Probe	ACCGCCCGTCGCTCCTACCG	

[z]*FP: Forward primer.* [y] *RP: Reverse primer.*

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 40× 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. Polymerase chain reaction 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 subsamples 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:

Table 2. C_t Values Generated by Quantifying and Validating Primer Sets of *PsGA20ox1* and *PsGA2ox1*

	PsGA20ox1		*PsGA2ox1*	
Samples	QP[z] (C_t)	VP[y] (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[w]	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

[z] *QP = quantifying primer set.*
[y] *VP = validating primer set.*
[w] *Data are means ± SE, n = 2.*

Normalized C_t value of sample = (C_t value of pooled sample in the standard run/C_t value of pooled sample in the sample run) * C_t value of sample.

The relative transcript abundance of the target genes in the individual plant samples 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 four 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, PsGA20ox2, PsGA2ox1,* and *PsGA2ox2* target gene quantifying amplicons was confirmed electrophoretically. The forward and reverse primer sets of each target gene amplicon (Table 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 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 1) were previously characterized by Ozga and others (2003).

Addition of RNase to samples of total RNA prior to the RT-PCR step resulted in no amplification of the PCR product for *PsGA2ox1* (C_t = 40, same as no template control) compared to the control (no RNAase added; C_t = 22.2). These data show that genomic DNA was not being amplified from the total RNA extracts.

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 coefficient of variation of the 18S amplicon C_t values was very low among all the samples assayed (2.7%–3.3% CV), target gene mRNA values were not normalized to the 18S signal as described in Ozga and others (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). A similar confirmation of quantifying and validating amplicons for *PsGA3ox1* transcript abundance in pea tissue was reported by Ozga and others (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 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 2×10^{-29}) and one from *Lotus corniculatus* (E values ranged from 4×10^{-60} to 2×10^{-29}); *GA20ox2-88* and two ESTs from *Medicago truncatula* (E values 9×10^{-10}); *GA2ox1-73* and *GA2ox1-126* amplicons and two ESTs from *M. truncatula* (E values ranged from 10^{-49} to 7×10^{-25}); and *GA2ox2-83* and two ESTs from *M. truncatula* (E values 9×10^{-22}). No 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*, $C_t = 27.2 \pm 0.2$ for 'Alaska' and 26.1 ± 0.1 for 'Carneval'; no template control, $C_t = 40 \pm 0$; data are means ± SE, $n = 2$ to 3). 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 and others (1997) and Davidson and others (2005), because expression of *PsCPS1* was found to be high in the developing pea embryo at contact point (approximately 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 and others 1997). Indeed, mature dry seeds of pea contained *ent*-kaurene at approximately 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 and others 1987). Although not in the same genetic background, we also found higher levels of *PsCPS1* in the mature embryos of 'Carvenal' (dwarf; *le-1*) compared to 'Alaska' (Tall; *LE*; see below).

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 2A), cotyledonary *PsCPS1* transcript levels declined 3.5- to 4.3-fold in both cultivars (Figure 3C). A small but further decline in cotyledonary *PsCPS1* mRNA levels occurred during the later stages of germination through to the active seedling growth stage (1 to 6 DAI; Figure 3C). Lower but still appreciable levels of *PsCPS1* mRNA were present in 6 DAI cotyledons, as indicated by C_t 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 the mature cotyledonary tissue of pea (cells of pea cotyledons are fixed post-mitotically with a normal life span of 2–3 weeks; Smith and Flinn 1967) supports the previous reports that *CPS* transcripts are present not only in actively growing immature tissues (Silverstone and others 1997; Ross and others 2003) but also in non-growing mature tissues, such as mature internodes of pea (Ross and others 2003) and fully expanded leaves of *Arabidopsis* (Silverstone and others 1997). The *PsCPS1* mRNA transcripts 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. However, it is also possible that the *PsCPS1* mRNA detected in the mature embryo is residual RNA (not full length). Consistent with the first hypothesis, Graebe and others (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). These data, along with data on the patterns of GA biosynthesis gene expression in the latter part of the pathway (*GA20ox, GA3ox, GA2ox*; Ayele and others 2005), suggest that the synthesis of bioactive GAs in pea cotyledons of germinating seeds 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 this hypogeal species carry over *CPS* transcripts synthesized during seed development for translation into protein upon imbibition of the seeds. In contrast, in species such as pumpkin and *Arabidopsis*,

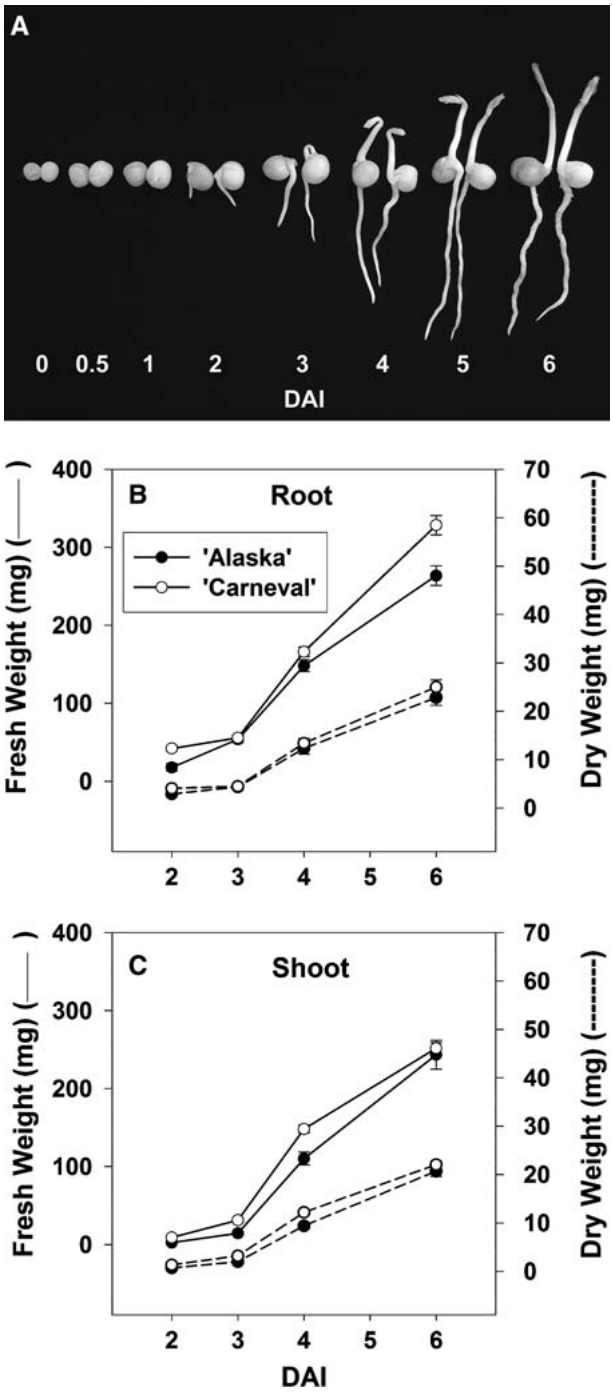

Figure 2. Germinating pea (*Pisum sativum* L.) seeds and actively growing seedlings of 'Alaska' (left in each pair) and 'Carneval' (right in each pair) from mature air-dried seed (0 DAI) to 6 DAI (A). Root (B) and shoot (C) fresh and dry weights from 2 to 6 DAI. Data are means ± SE, n = 15 to 28.

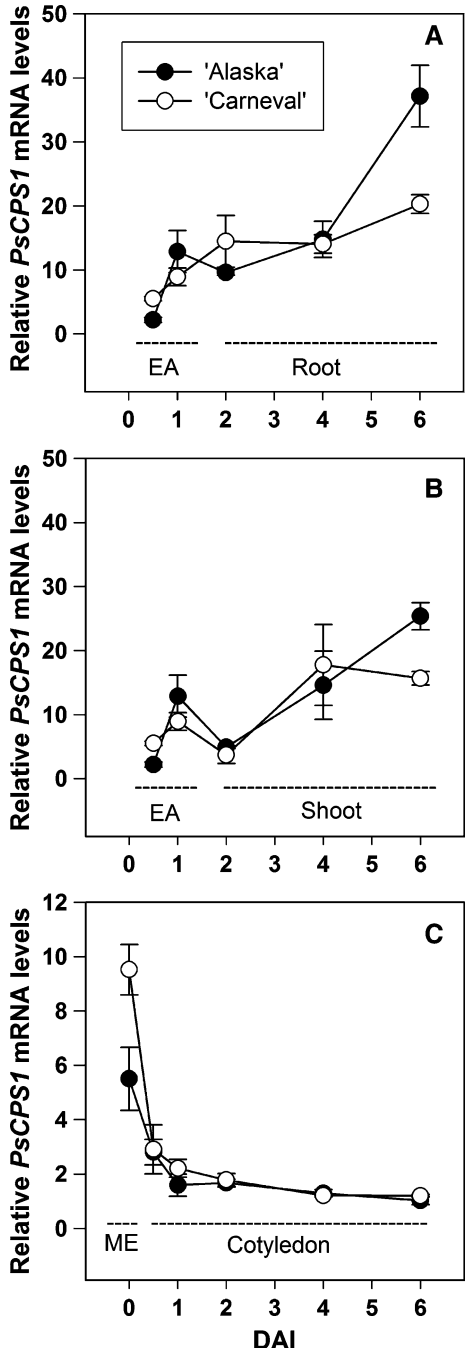

Figure 3. Relative mRNA levels of *PsCPS1* during seed germination and early seedling growth of 'Alaska' and 'Carneval'. Relative mRNA levels were determined in mature embryos (ME; C), embryo axes (EA, 0.5 and 1 DAI; A, B), roots (2–6 DAI; A), shoots (2–6 DAI; B), and cotyledons (0.5–6 DAI; C) of pea.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

which exhibit epigeal germination (the hypocotyls extend and the cotyledons emerge above the soil and expand rapidly), cotyledonary *CPS* expression increases following imbibition of the seeds. In pumpkin, *CmCPS1* was highly expressed in rapidly expanding cotyledons (3–4 DAI; Smith and others

Table 3. Growth of Embryo Axes (0.5–1 DAI), and Roots and Shoots (6–8 DAI) of 'Alaska' and 'Carneval'[z]

	Fresh weight (mg)		Dry weight (mg)		Length (mm)		No. of lateral roots	
DAI	'Alaska'	'Carneval'	'Alaska'	'Carneval'	'Alaska'	'Carneval'	'Alaska'	'Carneval'
Embryo axis								
0.5	4.1 ± 0.6[y]a[x]	5.6 ± 0.4a	2.0 ± 0.1a	1.8 ± 0.1a	3.2 ± 0.4a	3.9 ± 0.2a		
1	8.2 ± 0.8b	13.1 ± 0.8b	2.5 ± 0.1b	2.9 ± 0.1b	5.2 ± 0.3b	6.9 ± 0.3b		
Root								
6	256.9 ± 9.9a	281.9 ± 10.9a	21.2 ± 2.2a	22.9 ± 1.1a	82.3 ± 2.9a	94.3 ± 4.2a	17.4 ± 1.1a	22.2 ± 0.8a
7	369.0 ± 14.7b	470.5 ± 15.7b	34.1 ± 2.4b	55.4 ± 4.4b	97.1 ± 2.9b	115.6 ± 4.4b	24.4 ± 1.2b	27.2 ± 1.3b
8	553.6 ± 27.9c	563.1 ± 31.4c	65.3 ± 4.6c	63.7 ± 5.8b	112.0 ± 4.1c	132.2 ± 6.1c	33.1 ± 1.4c	36.5 ± 1.2c
Shoot								
6	224.7 ± 7.5a	246.4 ± 6.4a	22.3 ± 0.8a	23.5 ± 0.9a	50.2 ± 1.5a	49.6 ± 1.1a		
7	318.9 ± 10.9b	326.6 ± 8.2b	33.1 ± 1.3b	33.8 ± 0.8b	64.0 ± 1.7b	60.0 ± 1.3b		
8	431.6 ± 13.6c	384.7 ± 14.4c	47.6 ± 2.0c	41.9 ± 2.1c	80.8 ± 2.0c	69.2 ± 1.5c		

[z]*By 2 DAI, 90% of 'Carneval' and 65% of 'Alaska' seeds exhibited radicle protrusion.*
[y]*Data are means ± SE, n = 15 to 30.*
[x]*Means followed by different letters (a,b,c) indicate significant difference among days after imbibition (DAI) within tissues and cultivars by LSD, $p \leq 0.05$.*

1998). Yamaguchi and others (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 (epigeal species) 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* and pumpkin (epigeal species).

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

Because the abundance of *PsCPS1* transcripts in the cotyledonary and embryo axis tissues at 0.5 DAI is relatively similar (Figure 3), it is likely that the *PsCPS1* transcripts in the mature embryo (based on cotyledonary tissue weight as 98% of the mature embryo, and yields of total RNA from embryo axes and cotyledons at 0.5 DAI) mainly reflect cotyledonary transcript levels at this stage. As the embryo axes doubled in fresh weight from 0.5 to 1 DAI (Table 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'; Figure 3).

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) (Figure 2B and 2C), transcript abundance of *PsCPS1* was similarly lower in the shoot than the root (2- and 3.9-fold lower in 'Alaska' and 'Carneval', respectively; Figure 3A and 3B). 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 and others (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 transcription is under developmental regulation, increasing upon imbibition in the actively growing embryo 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 3B), and by 8 DAI the shoots became significantly shorter than 'Alaska' (Table 3) due to the presence of the *le-1* mutation that reduces the synthesis of GA_1 by 92% (Martin and others 1997). These data could suggest that *CPS* transcription 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 and others (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, transcript level of root-expressed *PsCPS1* did not change from 2 to 4 DAI, but as root growth continued through 6 DAI (Figure 2) the transcript levels increased (2.5-fold in 'Alaska' and 1.4-fold in 'Carneval'; Figure 3A). Silverstone and others (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-day-old *AtCPS1* promoter-GUS transgenic *Arabidopsis* seedlings. In the present study, lateral root primordia were observed at 6 DAI and they were localized around the base of the tap root (Figure 2A; Table 3). The higher root *PsCPS1* mRNA levels at 6 DAI compared to 4 DAI (Figure 3A), and the similar relative abundance of *PsCPS1* transcripts found in the 6 DAI root tips and the remainder of the roots ('Alaska', 33.1 ± 9.6 and 37.1 ± 4.8 and 'Carneval', 21.8 ± 5.8 and 20.3 ± 1.5, root tips and remainder of the root, respectively), may reflect the inclusion of the lateral root initials in the later tissue.

Although similar levels of *PsCPS1* transcript were found in the seedling shoot and root (4 to 6 DAI; Figure 3A and 3B), *ent*-kaurene synthesis occurred mainly in the shoots during early seedling growth (Graebe and others 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 apparently require lower GA levels for optimal growth than the shoots (Tanimoto 1990; Yaxley and others 2001). Post-transcriptional regulation of *CPS* or post-translational regulation of the protein product may occur, thereby reducing the abundance of the active protein in the root. Indeed, the pea *ls-1* mutation (*PsCPS1*), which dramatically reduced shoot elongation (350%; Yaxley and others 2001) and shoot GA levels (GA_{19}, GA_{20}, GA_1, and GA_{29}; 3.5- to 13-fold; Ait-Ali and others 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 in a slight reduction in taproot elongation (~7%) and a subtle effect on the root phenotype.

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

To further test whether transcription of *CPS* and other genes in the GA biosynthesis pathway are regulated by levels of bioactive GA in young pea seedling tissues, bioactive GA levels were modified with prohexadione, and the expression 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 to the seed at imbibition reduced seedling shoot fresh weight and length by 4 DAI (34% and 32%, respectively, Table 4). The reduction in shoot growth was most likely a direct effect of prohexadione on GA biosynthesis as the growth inhibition was reversed by GA_3 treatment (Table 4). By 4 DAI, prohexadione treatment only increased the mRNA abundance of shoot-derived genes coding for enzymes that catalyze 2β-hydroxylation of GA_{20} (*PsGA2ox1*) and GA_1 (*PsGA2ox2*) (3–5-fold increase; Table 5). Another acylcyclohexanedione-type GA biosynthesis inhibitor, Primo (trinexapac-ethyl), was shown to inhibit the enzyme activity of *Escherichia coli*–expressed *PsGA3ox1* and *PsGA2ox1* by 65% and 44%, respectively (derived from pea; King and others 2004). These published data along with those of Brown and others (1997) that showed that treatment with prohexadione caused marked accumulation of GA_{20} and reduced level of GA_1 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} and reduction of GA_1 in the shoot (and reduced shoot growth). It is assumed that only bioactive GAs (that is, GA_1) can feed-forward regulate the expression of *GA2ox* genes (Elliott and others 2001); however, the increase in *PsGA2ox1* and *PsGA2ox2* mRNA abundance with prohexadione treatment by 4 DAI suggests that GA_{20} may also stimulate feed-forward regulation of *GA2ox* gene expression. Further experiments are

Table 4. Effect of Prohexadione or GA_3 on Root and Shoot Growth of 'Alaska' Seedlings

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

Data are means ± SE, n = 14 to 20.
[y]Means followed by different letters (a,b,c,d) indicate significant difference among hormone treatments within tissues and days after imbibition by LSD, $p \leq 0.05$.

required to test the possibility that GA_{20} acts directly (not through conversion to bioactive GA_1) to feed-forward regulate *GA2ox* genes in pea. Other published work has shown that a reduction of GAs including GA_{20} and GA_1 in GA-deficient pea *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 (Elliot and others 2001).

By 6 DAI, a 2- to 3-fold increase in the transcript abundance of *PsGA20ox1*, *PsGA20ox2*, and *PsGA3ox1* was observed in the shoots from prohexadione-treated seeds (Table 5). These data, along with the decline of *PsGA2ox1* and *PsGA2ox2* transcript levels observed in 6-DAI shoots relative to 4 DAI, suggest that after 4 DAI, the pool of GA_1 in the shoots of prohexadione-treated seeds was sufficiently low enough to stimulate feed-back regulation of both *GA20ox* and *GA3ox* genes resulting in increased transcription of these GA biosynthesis genes. In a somewhat similar analogy, the shoot apices of GA-deficient *ls-1* plants exhibited higher levels of *PsGA20ox1* and *PsGA3ox1* mRNA compared to the wild-type (Elliott and others 2001).

Increased expression of specific *GA20ox* and *GA3ox* genes in potato leaves (Carrera and others 1999) and the shoots and roots of pumpkin seedlings (Lange and others 2005) in response to treatment with the GA biosynthesis inhibitors prohexadione and LAB 150978 (inhibits *ent*-kaurene oxidation), respectively, has also been reported.

Treatment with prohexadione during seed imbibition did not affect seedling root growth by 6 DAI (Table 4). With growth of the pea seedling, roots contain substantially lower levels of GAs than the shoots (Ross 1998; Yaxley and others 2001), the markedly lower expression of *PsGA20ox1* and higher expression of *PsGA2ox1* in the control roots as compared to the control shoots (Table 5) are likely causal factors 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 5). The concomitant 3-fold decrease in the transcript level of the gene that codes for GA 3β-hydroxylase (*PsGA3ox1*; Table 5) in the root suggests that prohexadione may also have inhibited 2β-hydroxylation of GA_1 to GA_8 thereby resulting in a transient accumulation of GA_1 and initiating GA_1-induced feed-back regulation of *PsGA3ox1* (GA_3 treatment also decreased *PsGA3ox1* message levels by 4 DAI, see below). At 6 DAI, the presence of higher

Table 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[z]

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

[z]*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.*
[y]*Data are means ± SE, n = 2 to 3, except for 6-DAI samples for PsCPS1, where n = 4.*
[x]*Means followed by different letters (a,b,c) indicate significant difference among hormone treatments within genes, tissues and days after imbibition by LSD, $p \leq 0.05$.*

PsGA20ox1 mRNA levels in roots from prohexadione-treated seeds compared to the 6-DAI controls (Table 5) may reflect a shift in mRNA expression for increased capacity of the root to produce growth-active GA_1 via GA_{20}. However, root growth was not significantly affected by these changes in mRNA expression by 6 DAI.

When the seeds were treated with prohexadione, the greater shift in mRNA expression for increased GA biosynthesis at 6 DAI in the shoots (increased mRNA levels for *PsGA20ox1*, *PsGA20ox2*, and *PsGA3ox1*) compared to the roots (only *PsGA20ox1* mRNA levels increased) likely reflects the different requirements of these tissues for bioactive GA for growth (Table 5).

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

Treatment with GA_3 stimulated shoot growth in length by 4 DAI in 'Alaska' seedlings (54% increase; Table 4). Transcripts of *PsGA20ox1* and *PsGA3ox1* were relatively high in abundance in the shoots (controls) at 4 DAI and GA_3 application to the seeds reduced both *PsGA20ox1* and *PsGA3ox1* mRNA levels in this tissue by 4 DAI (Table 5). In 6 DAI-shoots, *PsGA20ox1* mRNA levels from the control treatment were unchanged from those at 4 DAI, and GA_3 treatment reduced *PsGA20ox1* mRNA levels similarly to that observed at 4 DAI. However, from 4 to 6 DAI, shoot *PsGA3ox1* transcript abundance decreased 6-fold in the control seedlings (Table 5). Further reduction in *PsGA3ox1* transcript abundance by GA_3 treatment was not observed in 6-DAI-shoots (Table 5) suggesting that feed-back regulation at the mRNA level by bioactive GA is dependent on transcript abundance and/or stage of development of the tissue. 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 and others 1996; van Huizen and others 1997; Ait-Ali and others 1999). Reduction in the synthesis of GA_{20} and GA_1 in expanding pea shoot tissues in response to application of bioactive GA (2,2-dimethyl GA_4) has also been reported (Martin and others 1996).

In the shoots, of the two *GA2ox* gene family members studied (code for enzymes that 2β-hydroxylate GA_{20} and GA_1) only *PsGA2ox1* mRNA abundance significantly increased (2.5-fold at 4 DAI) in response to seed-applied GA_3 treatment (Table 5; LSD at $p \leq 0.05$) relative to the controls. Elliott and others (2001) observed reduced expression of both *PsGA2ox1* and *PsGA2ox2* in pea internodes and *PsGA2ox1* in the leaflets of GA_1-deficient *ls-1* mutant. Thomas and others (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 did not, in general, affect root growth (Table 4). But, by 4 DAI, GA_3 applied to the seed reduced *PsGA20ox1* and *PsGA3ox1* mRNA levels in the roots by 9- to 38-fold, respectively, and

the reduced mRNA levels for *PsGA3ox1*, relative to the control, were maintained through 6 DAI (Table 5; LSD at $p \leq 0.05$). The mRNA levels for *PsGA2ox1* were not affected in the roots by seed-applied GA_3 treatment (Table 5; LSD at $p \leq 0.05$). However, roots from GA_3-treated seeds exhibited a 4.3-fold increase in *PsGA2ox2* mRNA levels compared to the control at 6 DAI. Significant feed-forward regulation of *PsGA2ox2* by GA_3 (LSD at $p \leq 0.05$) was only observed in the roots suggesting that root tissue is more sensitive for deactivating bioactive GA than the shoot tissue.

Application of GA_3 (and, in general, prohexadione) to the seeds did not alter the transcript abundance of *PsGA20ox2* in the shoots or roots.

Expression of *PsCPS1*

Although application of GA_3 or the late-stage GA biosynthesis inhibitor, prohexadione, modified the mRNA levels of several late-stage GA biosynthesis genes in young pea seedling shoots and roots (Table 5), the transcript levels of the very early gene, *PsCPS1*, were unaffected in the roots (Table 5). For shoots, though, seed-applied GA_3 elevated *PsCPS1* transcript abundance 1.6-fold at 6 DAI compared to the control (Table 5). Shoot growth in both fresh weight and length was also significantly greater in the seedlings from GA_3-treated seeds than the control at 6 DAI (Table 4). These data may reflect developmental control of *CPS* expression in rapidly expanding tissue. In general, our data from young pea seedling shoot tissues confirm those of Davidson and others (2005), who used shoot tissues from pea plants with well-developed shoot systems, extend these findings to pea roots, and show over time that bioactive GA levels do not feed-back regulate *CPS* at the mRNA level.

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 very closely associated with the actively growing tissues of young seedlings over the next 5 days, presumably producing precursors for the downstream GA biosynthesis of endogenous GA_1. Thus, developmental regulation of *PsCPS1* occurs not only during seed development but also during germination and early seedling growth in pea.

PsCPS1 is not regulated, however, at the mRNA level by applied GA_3 or prohexadione in the roots or by prohexadione in the shoots (Table 5). Two GA biosynthesis genes, *PsGA20ox1* and *PsGA3ox1*, exhibited feed-back regulation (mRNA levels decreased in response to GA_3 application) for both roots and shoots (Table 5). In contrast, *PsGA2ox1* was feed-forward regulated by applied GA_3 at the mRNA level in the shoots and *PsGA2ox2* in the roots of pea seedlings. In general, feed-back regulation of *PsGA20ox2* by bioactive GA levels 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 GA soluble-dioxygenase enzyme activities 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 than that in the shoot is through regulation of the mRNA levels of the latter part of the GA biosynthesis pathway.

REFERENCES

Ait-Ali T, Frances S, Weller JL, Reid JB, Kendrick RE, and others, 1999. Regulation of gibberellin 20-oxidase and gibberellin 3β-hydroxylase transcript accumulation during de-etiolation of pea seedlings. Plant Physiol 121:783–791.

Ait-Ali T, Swain SM, Reid JB, Sun T-P, Kamiya Y. 1997. The *LS* locus of pea encodes the gibberellin biosynthesis enzyme *ent*-kaurene synthase A. Plant J 11:443–454.

Ayele BT, Ozga JA, Reinecke DM. 2005. Developmental regulation of key GA biosynthesis genes during germination and young seedling growth of pea (*Pisum sativum L.*). Plant Biology 2005, Seattle, Washington, USA, Abstract # 293.

Bewley JD, Black M. 1994. Seeds: Physiology of Development and Germination, New York, USA: Plenum Press. pp 1–31.

Brown RGS, Kawaide H, Yang Y-Y, Rademacher W, Kamiya Y. 1997. Daminozide and prohexadione have similar modes of action as inhibitors of the late stages of gibberellin metabolism. Physiol Plant 101:309–313.

Carrera E, Jackson SD, Prat S. 1999. Feed-back control and diurnal regulation of gibberellin 20-oxidase transcript levels in potato. Plant Physiol 119:765–773.

Chen F, Bradford KJ. 2000. Expression of an expansin is associated with endosperm weakening during tomato seed germination. Plant Physiol 124:1265–1274.

Davidson SE, Swain SM, Reid JB. 2005. Regulation of the early GA biosynthesis pathway in pea. Planta 222:1010–1019.

Debeaujon I, Koornneef M. 2000. Gibberellin requirement for Arabidopsis seed germination is determined by both testa characteristics and embryonic abscisic acid. Plant Physiol 122:415–424.

Duncan JD, West CA. 1981. Properties of kaurene synthetase from *Marah macrocarpus* endosperm: evidence for the participation of separate but interacting enzymes. Plant Physiol 68:1128–1134.

Ecklund PR, Moore TC. 1974. Correlations of growth rate and de-etiolation with rate of *ent*-kaurene biosynthesis in pea (*Pisum sativum* L.). Plant Physiol 53:5–10.

Elliott RC, Ross JJ, Smith JJ, Lester DR, Reid JB. 2001. Feed-forward regulation of gibberellin deactivation in pea. J Plant Growth Regul 20:87–94.

Garcia-Martinez JL, Lopez-Diaz I, Sanchez-Beltran MJ, Phillips AL, Ward DA, and others, 1997. Isolation and transcript analysis of gibberellin 20-oxidase genes in pea and bean in relation to fruit development. Plant Mol Biol 33:1073–1084.

Graebe JE, Böse G, Grosselindemann E, Hedden P, Aach H, and others, 1992. The biosynthesis of *ent*-kaurene in germinating seeds and the function of 2-oxoglutarate in gibberellin biosynthesis. In: Karssen CM, Van Loon LC, Vreugdenhil D, editors. Progress in Plant Growth Regulation, Dordrecht, The Netherlands: Kluwer Academic Publishers. pp 545–554.

Graebe JE, Grosselindemann E, Stöckl D, Zander M. 1987. Gibberellin biosynthesis in cell-free systems and germinating seeds. In: Lilov D, Vassilev G, Christov C, Andonova T, editors., Plant Growth Regulators, Proceedings of the 4th International Symposium on Plant Growth Regulators, Popov Institute of Plant Physiology, Sofia, Bulgaria, pp 29–38.

Groot SPC, Karssen CM. 1987. Gibberellins regulate seed germination in tomato by endosperm weakening: a study with gibberellin deficient mutants. Planta 171:525–531.

Hedden P, Phillips AL. 2000. Gibberellin metabolism: new insights revealed by genes. Trends Plant Sci 5:523–530.

King RW, Junttila O, Mander LN, Beck EJ. 2004. Gibberellin structure and function: biological activity and competitive inhibition of gibberellin 2- and 3-oxidases. Physiol Plant 120:287–297.

Koornneef M, van der Veen JH. 1980. Induction and analysis of gibberellin sensitive mutants in *Arabidopsis thaliana* (L.) Heynh. Theor Appl Genet 58:257–263.

Lange T, Kappler J, Fischer A, Frisse A, Padeffke T, and others, 2005. Gibberellin biosynthesis in developing pumpkin seedlings. Plant Physiol 139:213–223.

Lester DR, Ross JJ, Ait-Ali T, Martin DN, Reid JB. 1996. A gibberellin 20-oxidase cDNA (accession no. 458830) from pea seed. Plant Physiol 111:1353.

Lester DR, Ross JJ, Smith JJ, Elliott RC, Reid JB. 1999. Gibberellin 2-oxidation and the SLN gene of *Pisum sativum*. Plant J 19:65–73.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408.

Martin DN, Proebsting WM, Hedden P. 1997. Mendel's dwarfing gene: cDNAs from the *Le* alleles and function of the expressed proteins. Proc Natl Acad Sci USA 94:8907–8911.

Martin DN, Proebsting WM, Hedden P. 1999. The *SLENDER* gene of pea encodes a gibberellin 2-oxidase. Plant Physiol 121:775–781.

Martin DN, Proebsting WM, Parks TD, Dougherty WG, Lange T, and others, 1996. Feed-back regulation of gibberellin biosynthesis and gene expression in *Pisum sativum* L. Planta 200:159–166.

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

Rademacher W. 2000. Growth retardants: effects on gibberellin biosynthesis and other metabolic pathways. Annu Rev Plant Physiol Plant Mol Biol 51:501–531.

Reid JB, Potts WC. 1986. Internode length in *Pisum*. Two further mutants, *lh* and *ls*, with reduced gibberellin synthesis, and a gibberellin insensitive mutant, *lk*. Physiol Plant 66:417–426.

Richards DE, King KE, Ait-Ali T, Harberd NP. 2001. How gibberellin regulates plant growth and development: a molecular genetic analysis of gibberellin signaling. Annu Rev Plant Physiol Plant Mol Biol 52:67–88.

Ross JJ. 1998. Effects of auxin transport inhibitors on gibberellins in pea. J Plant Growth Regul 17:141–146.

Ross JJ, Davidson SE, Wolbang CM, Bayly-Stark E, Smith JJ, and others, 2003. Developmental regulation of the gibberellin pathway in pea shoots. Functional Plant Biol 30:83–89.

Shen-Miller J, West CA. 1985. Distribution of *ent*-kaurene synthetase in *Helianthus annus* and *Marah macrocarpus*. Phytochemistry 24:461–464.

Silverstone AL, Chang C-W, Krol E, Sun T-P. 1997. Developmental regulation of the gibberellin biosynthetic gene *GA1* in *Arabidopsis thaliana*. Plant J 12:9–19.

Smith DL, Flinn AM. 1967. Histology and histochemistry of the cotyledons of *Pisum arvense* L. during germination. Planta 74:72–85.

Smith MW, Yamaguchi S, Ait-Ali T, Kamiya Y. 1998. The first step of gibberellin biosynthesis in pumpkin is catalyzed by at least two copalyl diphosphate synthases encoded by differentially regulated genes. Plant Physiol 118:1411–1419.

Sponsel VM. 1983. The localization, metabolism and biological activity of gibberellins in maturing and germinating seeds of *Pisum sativum* cv. Progress No. 9. Planta 159:454–468.

Tanimoto E. 1990. Gibberellin requirement for the normal growth of roots. In: Takahashi N, Phinney BO, MacMillan J, editors. Gibberellins, New York, USA: Springer-Verlag. pp 229–240.

Thomas SG, Phillips AL, Hedden P. 1999. Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. Proc Natl Acad Sci USA 96:4698–4703.

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

Yamaguchi S, Kamiya Y, Sun T-P. 2001. Distinct cell-specific expression patterns of early and late gibberellin biosynthetic genes during *Arabidopsis* seed germination. Plant J 28:443–453.

Yamaguchi S, Smith MW, Brown RGS, Kamiya Y, Sun T-P. 1998. Phytochrome regulation and differential expression of gibberellin 3ß-hydroxylase genes in germinating Arabidopsis seeds. Plant Cell 10:2115–2126.

Yaxley JR, Ross JJ, Sherriff LJ, Reid JB. 2001. Gibberellin biosynthesis mutations and root development in pea. Plant Physiol 125:627–633.