

Seed and Hormonal Regulation of Gibberellin 20-Oxidase Expression in Pea Pericarp¹

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To understand further how seeds, auxin (4-chloroindole-3-acetic acid [4-Cl-IAA]), and gibberellins (GAs) regulate GA biosynthesis in pea (*Pisum sativum* L.) pericarp at the molecular level, we studied the expression of GA 20-oxidase in this tissue using northern-blot analysis. Pericarp GA 20-oxidase mRNA levels were highest from prepollination (–2 d after anthesis [DAA]) through anthesis (0 DAA), then decreased 3-fold by 2 DAA, and remained at these levels through 6 DAA. The effects of seeds and hormones (4-Cl-IAA and GA₃) on the expression of GA 20-oxidase in pea pericarp were investigated over a 36-h treatment period. GA 20-oxidase mRNA levels in 2 DAA pericarp with seeds remained relatively stable throughout the treatment period; however, when the seeds were removed the pericarp transcript levels declined. When 2 DAA de-seeded pericarps were treated with 4-Cl-IAA, a significant increase in GA 20-oxidase mRNA levels was detected within 2 h and transcript levels remained elevated for up to 12 h after 4-Cl-IAA application. GA₃ significantly decreased GA 20-oxidase mRNA levels in de-seeded pericarp within 2 h of application. These data suggest that the previously reported conversion of GA₁₉ to GA₂₀ in pea pericarp is controlled by seeds, 4-Cl-IAA, and GA₃ at least in part by regulating GA 20-oxidase mRNA levels in this tissue.

In pea (*Pisum sativum* L.) normal pericarp growth requires the presence of seeds (Eeuwens and Schwabe, 1975). Removal or destruction of the seeds 2 to 3 DAA results in the slowing of pericarp growth and subsequent abscission (Eeuwens and Schwabe, 1975; Ozga et al., 1992). The effect of seeds on fruit development is assumed to involve plant hormones (Eeuwens and Schwabe, 1975; Gillaspay et al., 1993). Developing pea seeds contain GAs (biologically active GA₁ and GA₃; Garcia-Martinez et al., 1991; Santes et al., 1995) and auxins (4-Cl-IAA and IAA; Marumo et al., 1968; Ozga et al., 1993), and the requirement of seeds for pericarp growth can be replaced by the application of GAs (Eeuwens and Schwabe, 1975) or 4-Cl-IAA (Reinecke et al., 1995).

Results from metabolism studies obtained using the pea split-pericarp assay indicate that seeds and 4-Cl-IAA regulate a key step in the early 13-hydroxylation GA biosynthesis pathway in the pericarp, the conversion of GA₁₉ to

GA₂₀ (Ozga et al., 1992; van Huizen et al., 1995). In these studies pea pericarps were capable of converting [¹⁴C]GA₁₂ or [¹⁴C]GA₁₉ to [¹⁴C]GA₂₀ when seeds were present; however, seed removal resulted in minimal or no accumulation of [¹⁴C]GA₂₀ in the pericarp. The application of 4-Cl-IAA to de-seeded pericarp stimulated both the conversion of [¹⁴C]GA₁₉ to [¹⁴C]GA₂₀ and pericarp growth (van Huizen et al., 1995).

Recently, cDNA clones encoding GA 20-oxidases from pumpkin (*Cucurbita pepo*; Lange et al., 1994), Arabidopsis (*Arabidopsis thaliana*; Phillips et al., 1995; Xu et al., 1995), spinach (*Spinacia oleracea*; Wu et al., 1996), and pea (Garcia-Martinez et al., 1995; Martin et al., 1996) have been isolated. Heterologous expression of the pumpkin, Arabidopsis, spinach, and pea GA 20-oxidase cDNAs in *Escherichia coli* has shown that their fusion proteins catalyze the biosynthetic sequence GA₅₃ → GA₄₄ → GA₁₉ → GA₂₀. These data suggest that a single protein is responsible for the sequential oxidation and elimination of C-20.

Evidence for the photoperiodic regulation of GA 20-oxidase expression has been reported in spinach and Arabidopsis (Xu et al., 1995; Wu et al., 1996). GA 20-oxidase transcript levels were enhanced in the leaves of Arabidopsis after plants were transferred from short to long days (Xu et al., 1995). In spinach shoot tips GA 20-oxidase levels were found to increase in long-day conditions and decrease in short-day conditions (Wu et al., 1996). The same pattern of photoperiodic regulation was observed for the activities of the enzymes oxidizing GA₅₃ and GA₁₉ in spinach plants (Gilmour et al., 1986).

The application of GA was found to reduce the expression of GA 20-oxidase in Arabidopsis leaves (Xu et al., 1995) and three GA 20-oxidase genes in flowering shoots of the *ga1-2* mutant of Arabidopsis (Phillips et al., 1995). Martin et al. (1996) reported a decrease in GA 20-oxidase transcript levels coupled with an increase in endogenous GA₁₉ levels and a decrease in GA₂₀ levels in pea shoots after application of the highly biologically active GA, 2,2-dimethyl GA₄ to wild-type (tall; I₃ Alaska-type) pea plants. These data suggest that end-product repression occurs in the GA biosynthesis pathway in vegetative tissues.

To understand further how seeds, auxin, and GAs regulate GA biosynthesis in pea pericarp at the molecular

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Abbreviations: 4-Cl-IAA, 4-chloroindole-3-acetic acid; DAA, days after anthesis; SP, split pericarp with seeds; SPNS, split pericarp no seeds.

level, we studied the gene expression of GA 20-oxidase in this tissue using northern-blot analysis. Our data suggest that the conversion of GA₁₉ to GA₂₀ in pea pericarp is controlled by seeds, 4-Cl-IAA, and GA₃ at least in part by regulating GA 20-oxidase mRNA levels in this tissue.

MATERIALS AND METHODS

Plant Materials and Treatments

Plants of pea (*Pisum sativum* L., line I₃ Alaska-type) were grown in a 16-h photoperiod as previously described (van Huizen et al., 1995). Pericarps from intact ovaries were collected at -2, 0, 2, 3, 4, 5, and 6 DAA from the first to fifth flowering node for experiments monitoring GA 20-oxidase expression during early pea fruit development. For the remaining experiments one fruit per plant (at the third to fifth flowering node) was treated, and subsequent flowers were removed as they developed. Terminal apical meristems were intact and the pericarp remained attached to the plant during the entire experiment.

To remove the seeds the split-pericarp technique described by Ozga et al. (1992) was used. Pericarps of 2 DAA (15–22 mm) ovaries (pericarp plus seeds) were split down the dorsal suture 1 h prior to the 8-h dark period, and seeds were either undisturbed (SP treatment) or removed immediately (SPNS treatment). Pericarps were treated with GA₃ and/or 4-Cl-IAA (30 μL, 50 μM in 0.1% Tween 80) 12 h after deseeding, harvested 2, 4, 8, 12, and 24 h after hormone treatment, and placed immediately into liquid N₂ and stored at -70°C until RNA extraction. The controls were treated with 0.1% Tween 80 12 h after splitting (SP) or splitting and deseeding (SPNS) and harvested as described above. All treatments were applied to the inside surface (endocarp) of the pericarp. High humidity was maintained by enclosing the fruits in clear plastic bags for the duration of the experiment. Statistical analysis was performed using the General Linear Modeling program of SAS 6.10 (SAS Institute, Inc., Cary, NC) following a completely randomized design.

RNA Isolation and PCR Cloning

For each sample 3 pods or approximately 30 seeds were ground in liquid N₂ and a 0.3- to 0.5-g subsample was used for RNA extraction. Total RNA was extracted following the Trizol (GIBCO-BRL) procedure based on the method of Chomczynski and Sacchi (1987), with two additional chloroform extractions after the first to remove polysaccharides.

To amplify a 692-bp fragment of the GA 20-oxidase from pea (Garcia-Martinez et al., 1995; Martin et al., 1996) a sense 2-degenerate primer (A): 5'-GA[TC]GA[GA]AA[GA]CC-[AT]TG[TC]ATGAA-3', corresponding to the residues DEKPCMN, and an antisense degenerate primer (B): 5'-TT[TA]GG[CT]CT[TAG]AT[TAG]GA[AG]TGCCA-3', corresponding to the residues WHSIRPN, were synthesized.

The first-strand cDNA was reverse-transcribed from 0.2 μg of total RNA from 6 DAA seeds or from 2 DAA pericarp with 200 units of SuperScript RNase H⁻ Reverse Tran-

scriptase (GIBCO-BRL) in a 20-μL volume containing 1× first-strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂), 0.5 μM primer A, 25 μM DTT, and 25 μM of all four of the deoxyribonucleotide triphosphates for 60 min at 37°C. Four microliters from the first-strand cDNA reaction were amplified in a 50-μL volume containing 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), 1 μM primers A and B, 2.5 units of *Taq* DNA polymerase (GIBCO-BRL), 250 μM of all four of the deoxyribonucleotide triphosphates, and 4 mM MgCl₂. The reaction mixture was heated to 94°C for 5 min, and then subjected to 40 cycles of 94°C for 1 min, 56°C for 2 min, and 72°C for 1 min. A final extension was performed at 72°C for 5 min.

The products were separated by agarose gel electrophoresis and analyzed using ethidium bromide staining. The PCR reaction produced the 692-bp product from both the pericarp and seed samples. The more abundant seed product was purified using a GeneClean II kit (BIO 101, Vista, CA), taken up in 100 μL of water, and 1 μL was used as a substrate in a second round of PCR under the conditions described above. The amplified product was again isolated from an agarose gel, polished with *Pfu* DNA polymerase (Stratagene), and ligated into the pCR-Script SK(+) (Stratagene) cloning vector. The 692-bp cDNA cloned in pCR-Script SK(+) was transformed into *Escherichia coli* strain XL1 Blue.

DNA Sequence Analysis

Dye-terminator sequencing of the PCR-generated fragments cloned into pCR-Script SK(+) was performed using an automated sequencing system (373A, Perkin-Elmer/Applied Biosystems) at the DNA Services Laboratory, Department of Biochemistry, University of Alberta, using sequence-analysis software (version 2.1.1, Perkin-Elmer/Applied Biosystems).

Northern-Blot Analysis

For northern-blot analysis, total RNA (30 μg per sample) was denatured in 2.2 M formaldehyde/50% formamide and fractionated on a 1.2% agarose/2.2 M formaldehyde gel using a 20 mM Mops buffer (pH 7.0; Maniatis et al., 1982) and transferred to nylon membranes (Zeta-Probe GT, Bio-Rad) with 10× SSC. Equal loading and RNA integrity were ascertained by ethidium bromide staining of rRNA bands prior to membrane transfer. Membranes were baked for 2 h at 80°C under a vacuum.

RNA probes radiolabeled with [³²P]CTP were generated by *in vitro* transcription with T3 RNA polymerase according to the supplier's instructions (Riboprobe *in vitro* Transcription Systems, Promega) using the linearized plasmid as a template after digestion with *Sma*I. Prehybridization and hybridization of blots were performed at 50°C in a solution containing 60% formamide, 1× SSPE, 0.5% blotto (low-fat milk powder), 10% dextran sulfate, 1% SDS, and 0.25 mg/mL denatured salmon-sperm DNA.

Blots were hybridized for 18 h with the labeled probe, then washed twice at room temperature for 15 min in 2×

SSC, 0.1% SDS, and once at 70°C in 0.1× SSC, 1% SDS. As a developmental control, all blots were also probed with an *Arabidopsis thaliana* actin probe (clone pATC-4 from Dr. Robert J. Ferl, University of Florida, Gainesville). Blots were stripped in 0.1× SSC with 0.5% SDS at 95°C for 20 min. A [³²P]dATP random-primed actin cDNA probe was synthesized using the random primers DNA labeling system (GIBCO-BRL) according to the manufacturer's instructions.

Membranes were prehybridized and then hybridized with the actin probe at 65°C in 0.5 M Na₂HPO₄, pH 7.2, 7% SDS, and 1 mM EDTA for 18 h. Blots were washed twice for 30 min in 40 mM Na₂HPO₄, pH 7.2, 5% SDS, 1 mM EDTA, and twice for 30 min in 40 mM Na₂HPO₄, pH 7.2, 1% SDS, 1 mM EDTA at 65°C. For autoradiography the membranes were exposed to Kodak X-Omat AR film at -70°C. The amount of labeled antisense RNA hybridization to the RNA blot was quantitated by scanning the autoradiogram with an imaging densitometer (Bio-Rad). For quantitation of GA 20-oxidase gene expression during early pea fruit development (-2 to 6 DAA), the value for the GA 20-oxidase signal at 3 DAA was designated as 100% and all other signals were calculated relative to this sample.

For the 36-h time-course experiment, one extraction of 12 to 15 pericarps for the 2 DAA sample and one extraction for the 12 h after deseeding sample were performed, and these samples were run on all gels. The 2 DAA sample was used as a quantitative standard for all blots. The value for the GA 20-oxidase signal at 2 DAA (0-h treatment) on each autoradiogram was designated as 100% and all other signals were calculated relative to that sample.

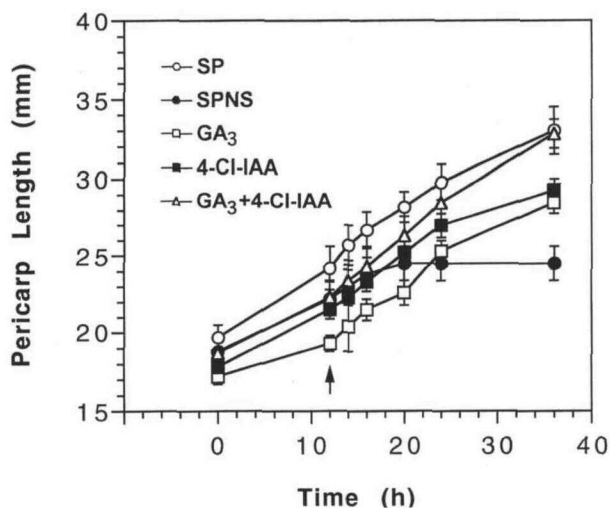


Figure 1. The effect of seeds (SP), seed removal (SPNS), and seed removal plus treatment with GA₃, 4-Cl-IAA, and GA₃ plus 4-Cl-IAA on pea pericarp growth. Two DAA pericarps were deseeded 12 h prior to GA₃ and/or 4-Cl-IAA (50 μM) or 0.1% Tween 80 application (SP and SPNS controls). The arrow indicates the time of hormone application. Data are means ± SE (n = 6).

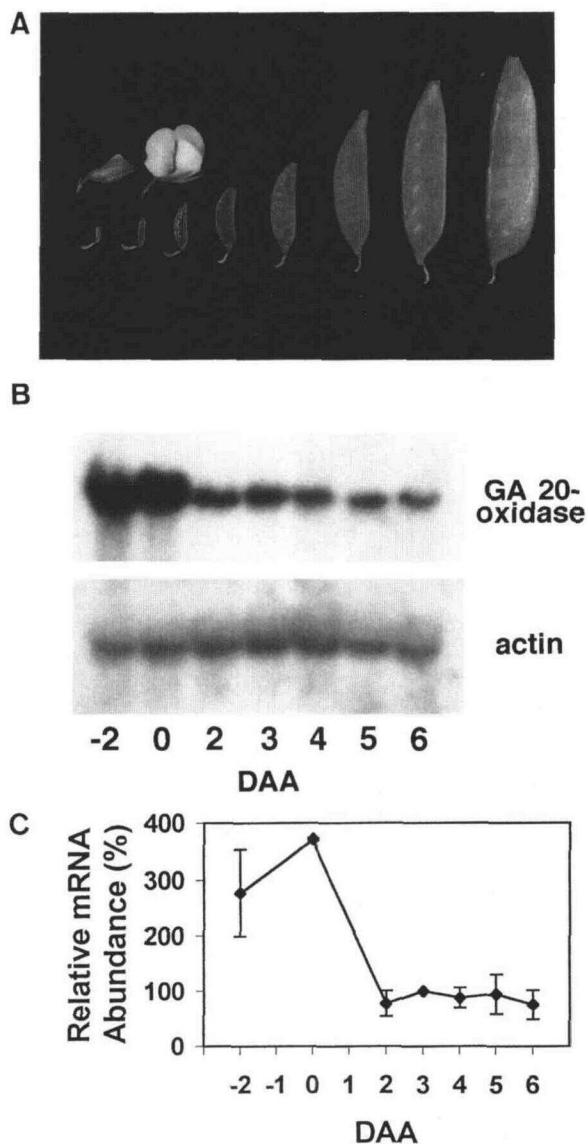


Figure 2. A, Early pea fruit development (-2, 0, and 1-6 DAA; flower bud and flower at anthesis are at -2 and 0 DAA, respectively). B, Corresponding northern-blot analysis of pea pericarp GA 20-oxidase mRNA expression (-2, 0, and 2-6 DAA; transcript size was approximately 1.4 kb). Reprobing of the blots with actin was performed as a developmental control. C, Hybridization signals to the GA 20-oxidase probe were analyzed by scanning autoradiograms with an imaging densitometer, and these values were normalized to the value for pericarps at 3 DAA (100%). The data represent the average of two replicates ± SE.

RESULTS

GA₃- and 4-Cl-IAA-Stimulated Pericarp Growth

Growth data (length) from pericarps used for RNA extractions for northern-blot analysis of GA 20-oxidase gene expression are presented in Figure 1. The length of pericarps with seeds (SP) and deseeded pericarps treated with 4-Cl-IAA, GA₃, and 4-Cl-IAA plus GA₃ increased during the 24-h period after hormone or 0.1% Tween 80 treatment (Fig. 1). The linear effect accounted for more than 95% of

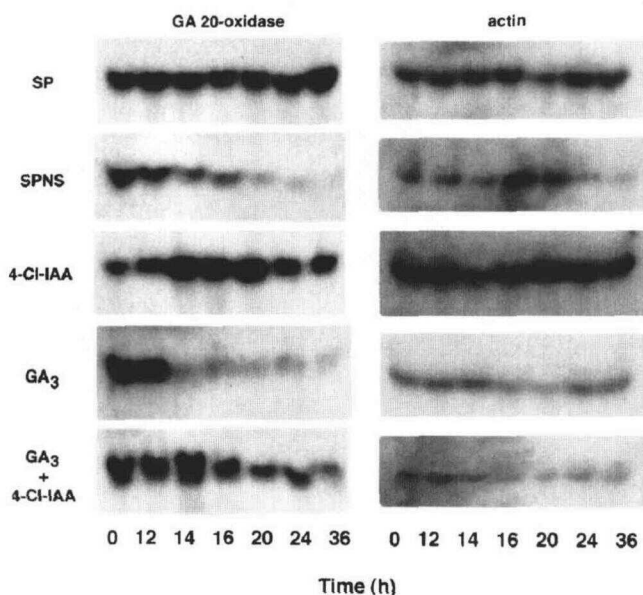


Figure 3. Time course of the levels of pea GA 20-oxidase mRNA (transcript size was approximately 1.4 kb) in pericarp with seeds (SP), deseeded pericarp (SPNS), and deseeded pericarp treated with GA₃, 4-Cl-IAA, and GA₃ plus 4-Cl-IAA. Pericarps at 2 DAA (0 h) were split (SP) or split and deseeded. GA₃ and/or 4-Cl-IAA (50 μM) or 0.1% Tween 80 (SPNS control) were applied 12 h after deseeding. An actin probe was used as a developmental control.

the variation during this period for these treatments and was highly significant (F test; $P < 0.0001$). When seeds were removed (SPNS) the rate of pericarp growth was significantly less (LSD; $P < 0.05$) than growth of pericarps with seeds (SP) and deseeded pericarp treated with 4-Cl-IAA, GA₃, or 4-Cl-IAA plus GA₃ (Fig. 1).

PCR Amplification of GA 20-Oxidase Fragment Using Degenerate Primers

The degenerate primers used for PCR to amplify the GA 20-oxidase sequence from pea seeds (6 DAA) were based on the amino acid sequence of the pea GA 20-oxidase (Garcia-Martinez et al., 1995; Martin et al., 1996). The primers yielded a product of the expected size (692 bp). The PCR-amplified product was cloned into pCR-Script SK(+) and sequenced. The PCR product was 98.3% identical at the nucleotide level to the pea GA 20-oxidase cDNA clone isolated by Martin et al. (1996).

GA 20-Oxidase Gene Expression

Pea pericarp GA 20-oxidase gene expression was investigated during flowering and early fruit development using northern-blot analysis. Pericarp GA 20-oxidase mRNA levels (transcripts approximately 1.4 kb in size) were highest from prepollination (−2 DAA) through anthesis (0 DAA; Fig. 2). By 2 DAA, GA 20-oxidase transcript levels had decreased 3-fold and remained at these levels through 6 DAA (Fig. 2).

The effect of seeds and hormones (4-Cl-IAA and GA₃) on the expression of GA 20-oxidase in pea pericarp was investigated over a 36-h treatment period (Figs. 3 and 4). GA 20-oxidase mRNA levels in pericarp with seeds remained relatively stable in the first 24 h after splitting the pericarp. After 36 h GA 20-oxidase transcript levels had increased to a level about twice that at 2 DAA. GA 20-oxidase mRNA levels in deseeded pericarp were similar to levels in pericarp with seeds during the first 12 h after seed removal; however, after 12 h GA 20-oxidase transcripts declined steadily to 5% of the original levels (2 DAA) after 36 h.

To allow sufficient time for the pericarp to become depleted of seed-produced factors that might affect the pericarp growth, hormones were applied to the pericarps 12 h after deseeding. When deseeded pericarps were treated with 4-Cl-IAA, a significant increase in GA 20-oxidase mRNA levels was detected within 2 h of hormone application (Figs. 3 and 4). Transcript levels in 4-Cl-IAA-treated deseeded pericarp remained elevated compared with all other treatments for up to 12 h after hormone application. The highest GA 20-oxidase transcript levels were observed between 2 and 8 h after 4-Cl-IAA application, with 4-Cl-IAA-treated deseeded pericarp exhibiting approximately 4- and 10-fold higher levels than that in SP and SPNS, respectively (Figs. 3 and 4).

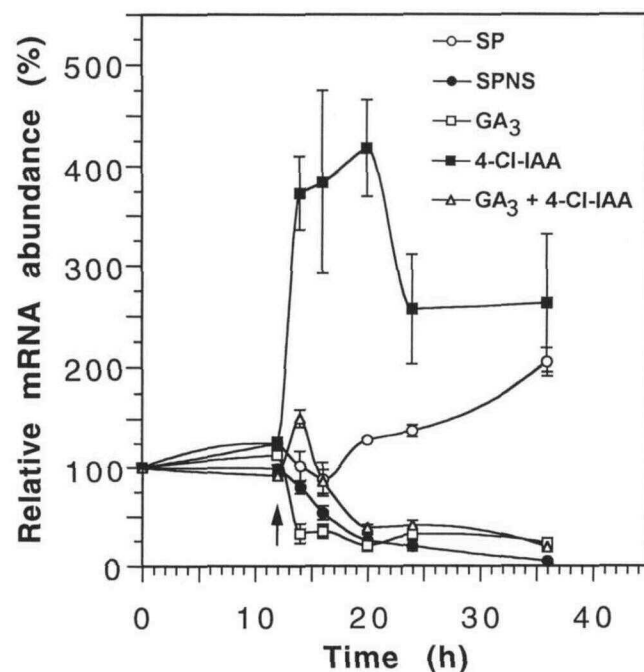


Figure 4. Relative abundance of GA 20-oxidase transcripts in pea pericarp treated as described in Figure 3. For the 2 DAA and the 12 h after deseeding samples, one extraction of 12 to 15 pericarps for each time period was performed and these samples were used as standards for all blots. Hybridization signals to the GA 20-oxidase probe were analyzed by scanning autoradiograms with an imaging densitometer and these values were normalized to the value for pericarps at 2 DAA (0 h; 100%). The arrow indicates the time of hormone application. The data represent the average of two replicates \pm SE, with the exception of the 4-Cl-IAA treatment, for which $n = 3$.

GA₃ significantly decreased GA 20-oxidase mRNA levels in deseeded pericarp within 2 h of application (Figs. 3 and 4). Subsequently, GA 20-oxidase transcript levels remained considerably lower than levels in SP. 4-Cl-IAA application to GA₃-treated deseeded pericarps (GA₃ plus 4-Cl-IAA treatment) delayed the decrease of GA 20-oxidase transcript levels from 2 to 8 h after hormone treatment (Figs. 3 and 4).

As a developmental control all blots were reprobbed with actin (used as a marker for the general mRNA population) to determine if treatment effects were specific to pericarp GA 20-oxidase mRNA or if they were due to a general trend in the total mRNA population. The GA 20-oxidase gene expression pattern for intact pericarp from -2 to 6 DAA (Fig. 2) and for deseeded pericarp with or without hormone application over the 36-h treatment period (Fig. 3) were not similar to the actin gene expression pattern.

DISCUSSION

GA 20-oxidase mRNA levels were 3-fold higher in pericarp from prepollination through anthesis than during the rapid phase of pericarp growth (2-6 DAA; Fig. 2). The high level of GA 20-oxidase mRNA detected prior to pollination (-2 DAA) and the previous report that emasculated and pollinated ovaries contain similar concentrations of GA₁₉ and GA₂₀ at anthesis (Garcia-Martinez et al., 1991) demonstrate that accumulation of pericarp GA 20-oxidase transcripts and GA₁₉ and GA₂₀ are not triggered by pollination events. Early (-2 DAA) high expression of pericarp GA 20-oxidase may indicate that pericarp-derived GAs are important for ovary development prior to anthesis. The lower but steady levels of pericarp GA 20-oxidase mRNA observed during the phase of rapid pericarp growth (2-6 DAA) in intact ovaries (Fig. 2) suggest that maintenance of pericarp GA biosynthesis is important for sustained pericarp growth.

We have also shown that GA 20-oxidase mRNA levels in pea pericarp are maintained when seeds are present and decreased after seed removal to 5% of the original levels (2 DAA) by 36 h (Figs. 3 and 4). These data demonstrate that seeds are required to maintain GA 20-oxidase mRNA levels for normal GA biosynthesis in the pericarp tissue. These findings are consistent with previous results from our laboratory that *in vivo* activity of the enzyme oxidizing GA₁₉ to GA₂₀ is maintained in pericarps with seeds (SP) and decreased to minimal levels in deseeded pericarps (SPNS) (Ozga et al., 1992; van Huizen et al., 1995). The pericarps are still viable after seed removal, as shown by their growth response to delayed hormone application (GA₃ plus 4-Cl-IAA; van Huizen et al., 1995).

4-Cl-IAA significantly increased levels of GA 20-oxidase transcripts in deseeded pericarp within 2 h of application compared with levels in SP or SPNS (Figs. 3 and 4). These data agree with our previous findings that the *in vivo* activity of the GA₁₉-oxidizing enzyme (conversion of [¹⁴C]GA₁₉ to [¹⁴C]GA₂₀ and [¹⁴C]GA₂₉) increased in deseeded pericarp after the 4-Cl-IAA treatment (van Huizen et al., 1995). These results suggest that 4-Cl-IAA can substitute for the seeds in maintaining GA biosynthesis in the

pericarp, at least in part by stimulating or maintaining GA 20-oxidase transcript levels.

GA 20-oxidase transcript levels (Figs. 3 and 4) and [¹⁴C]GA₁₉ metabolism (van Huizen et al., 1995) were significantly higher in deseeded pericarp treated with 4-Cl-IAA compared with pericarp with seeds, even though the rate of pericarp growth of 4-Cl-IAA-treated pericarp was similar to that of pericarp with seeds (Fig. 1; van Huizen et al., 1995). These data demonstrate that the effect of 4-Cl-IAA on GA 20-oxidase mRNA abundance and [¹⁴C]GA₁₉ metabolism is not simply an indirect effect of 4-Cl-IAA stimulating pericarp growth. In addition, reprobbed blots with actin (developmental control; Figs. 2 and 3) demonstrates that seed removal (SPNS) and hormonal effects on pericarp GA 20-oxidase mRNA levels were not simply a general trend in the total mRNA population.

Although the presence of 4-Cl-IAA in pea seeds has been known since the late 1960s (Marumo et al., 1968), the biological role of this endogenous halogenated auxin is not known. Reinecke et al. (1995) found that exogenous 4-Cl-IAA and, to a lesser extent, 5-Cl-IAA, promoted pericarp growth, whereas IAA, 6- and 7-chloro-, and 4-, 5-, 6-, and 7-fluoro-substituted IAA were inactive or inhibitory. In contrast, in pea stems and wheat coleoptile assays, IAA, 4-, 5-, 6-, and 7-Cl-IAA, and 5-F-IAA were all active, although maximum activity was observed at different concentrations (Hoffmann et al., 1952; Katekar and Geissler, 1983).

In pea fruit endogenous 4-Cl-IAA may have a specific role as a seed signal involved in the coordination of growth and development of the seeds and surrounding pericarp tissue. The presence of 4-Cl-IAA in pea pericarp tissue has recently been determined using unequivocal methods (Magnus et al., 1997). One possible seed-regulatory mechanism in young pea fruit would be the export of 4-Cl-IAA by seeds to the pericarp, where it stimulates GA biosynthesis (conversion of GA₁₉ to GA₂₀) at least in part by increasing the level and/or stability of GA 20-oxidase mRNA. However, experiments using the GA biosynthesis inhibitor paclobutrazol demonstrate that 4-Cl-IAA also has a direct auxin effect on growth (Ozga and Brenner, 1992).

In pea pericarp a decrease in GA 20-oxidase transcript levels was observed within 2 h of GA₃ application (Figs. 3 and 4). This is consistent with the observations that GA₃ applied to deseeded pericarp stimulated growth, but conversion of [¹⁴C]GA₁₂ to putative [¹⁴C]GA₂₀ was not detected (Ozga et al., 1992). These findings, along with studies on GA 20-oxidase expression in vegetative tissue (Phillips et al., 1995; Xu et al., 1995; Martin et al., 1996), support the view that bioactive GAs may control their own synthesis through end-product suppression of the GA biosynthetic pathway in vegetative as well as reproductive structures.

In our experiments the reduction in GA 20-oxidase expression by GA₃ was delayed by simultaneous application of 4-Cl-IAA (Figs. 3 and 4), demonstrating an interaction of these hormones in the regulation of GA 20-oxidase steady-state mRNA levels. In previous *in vivo* protein synthesis studies in our laboratory, the application of 4-Cl-IAA plus GA₃ mimicked the seed effect on protein synthesis in the pericarp (van Huizen et al., 1996). The GA 20-oxidase gene

expression and in vivo protein synthesis studies support our hypothesis that both hormones are involved in pea pericarp development. However, GA 20-oxidase mRNA and polypeptide patterns unique to GA₃ or 4-Cl-IAA treatment also indicate that their effects on these processes are not equivalent.

We now have evidence that seeds, GAs, and auxin (4-Cl-IAA) can regulate growth, in vivo activity of the enzyme oxidizing GA₁₉ to GA₂₀ (Ozga et al., 1992; van Huizen et al., 1995), in vivo protein synthesis (van Huizen et al., 1996), and GA 20-oxidase gene expression in pea pericarp. Furthermore, a comparison of the effects of 4-Cl-IAA on GA biosynthesis with those on GA 20-oxidase transcript levels indicate that the effects of 4-Cl-IAA on the synthesis of bioactive GA via GA₂₀ may be significantly mediated by the effects on gene expression.

These data provide critical information for testing the hypothesis that auxin (4-Cl-IAA) and GAs regulate synthesis of active GAs in the pea pericarp at the molecular level, as well as provide information that is fundamental to our understanding of fruit growth. These data also represent one of the few examples of one plant hormone specifically affecting the synthesis of another hormone at the gene level. Further research is planned to determine if seeds, 4-Cl-IAA, and GA regulate GA 20-oxidase expression at the transcriptional and/or posttranscriptional level and to investigate the mechanisms by which these factors control these processes.

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LITERATURE CITED

- Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**: 156–159
- Euwens CJ, Schwabe WW (1975) Seed and pod wall development in *Pisum sativum* L. in relation to extracted and applied hormones. *J Exp Bot* **26**: 1–14
- Garcia-Martinez JL, Lopez-Diaz I, Ward DA, Phillips AL, Gaskin P, Hedden P (1995) Molecular cloning of gibberellin 20-oxidases from legumes (abstract No. 99). 15th International Conference on Plant Growth Substances, International Plant Growth Substance Association, Minneapolis, MN
- Garcia-Martinez JL, Santes C, Croker SJ, Hedden P (1991) Identification, quantitation and distribution of gibberellins in fruits of *Pisum sativum* L. cv. Alaska during pod development. *Planta* **184**: 53–60
- Gillaspy G, Ben-David H, Gruissem W (1993) Fruits: a developmental perspective. *Plant Cell* **5**: 1439–1451
- Gilmour SJ, Zeevaart JAD, Schwenen L, Graebe JE (1986) Gibberellin metabolism in cell-free extracts from spinach leaves in relation to photoperiod. *Plant Physiol* **82**: 190–195
- Hoffmann OL, Fox SW, Bullock MW (1952) Auxin-like activity of systematically substituted indoleacetic acid. *J Biol Chem* **196**: 437–441
- Katekar GF, Geissler AE (1983) Structure-activity differences between indoleacetic acid auxins on pea and wheat. *Phytochemistry* **22**: 27–31
- Lange T, Hedden P, Graebe JE (1994) Expression cloning of a gibberellin 20-oxidase, a multifunctional enzyme involved in gibberellin biosynthesis. *Proc Natl Acad Sci USA* **91**: 8552–8556
- Magnus V, Ozga JA, Reinecke DM, Pierson GL, Larue TA, Cohen JD, Brenner ML (1997) 4-Chloroindole-3-acetic and indole-3-acetic acids in fruit of *Pisum sativum*. *Phytochemistry* (in press)
- Maniatis T, Fritsch EF, Sambrook J (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 202–203
- Martin DN, Proebsting WM, Parks TD, Dougherty WG, Lange T, Lewis MJ, Gaskin P, Hedden P (1996) Feed-back regulation of gibberellin biosynthesis and gene expression in *Pisum sativum* L. *Planta* **200**: 159–166
- Marumo S, Hattori H, Abe H, Munakata K (1968) Isolation of 4-chloroindolyl-3-acetic acid from immature seeds of *Pisum sativum*. *Nature* **219**: 959–960
- Ozga JA, Brenner ML (1992) The effect of 4-Cl-IAA on growth and GA metabolism in deseeded pea pericarp (abstract No. 12). *Plant Physiol* **99**: S-2
- Ozga JA, Brenner ML, Reinecke DM (1992) Seed effects on gibberellin metabolism in pea pericarp. *Plant Physiol* **100**: 88–94
- Ozga JA, Reinecke DM, Brenner ML (1993) Quantitation of 4-Cl-IAA and IAA in 6 DAA pea seeds and pericarp (abstract No. 28). *Plant Physiol* **102**: S-7
- Phillips AL, Ward DA, Uknes S, Appleford NEJ, Lange T, Huttly AK, Gaskin P, Graebe JE, Hedden P (1995) Isolation and expression of three gibberellin 20-oxidase cDNA clones from *Arabidopsis*. *Plant Physiol* **108**: 1049–1057
- Reinecke DM, Ozga JA, Magnus V (1995) Effect of halogen substitution of indole-3-acetic acid on biological activity in pea fruit. *Phytochemistry* **40**: 1361–1366
- Santes CM, Hedden P, Gaskin P, Garcia-Martinez JL (1995) Gibberellins and related compounds in young fruits of pea and their relationship to fruit-set. *Phytochemistry* **40**: 1347–1355
- van Huizen R, Ozga JA, Reinecke DM (1996) Influence of auxin and gibberellin on *in vivo* protein synthesis during early pea fruit growth. *Plant Physiol* **112**: 53–59
- van Huizen R, Ozga JA, Reinecke DM, Twitchin B, Mander LN (1995) Seed and 4-chloroindole-3-acetic acid regulation of gibberellin metabolism in pea pericarp. *Plant Physiol* **109**: 1213–1217
- Wu K, Li L, Gage DA, Zeevaart JAD (1996) Molecular cloning and photoperiod-regulated expression of gibberellin 20-oxidase from the long-day plant spinach. *Plant Physiol* **110**: 547–554
- Xu Y-L, Li L, Wu K, Peeters AJM, Gage DA, Zeevaart JAD (1995) The GA5 locus of *Arabidopsis thaliana* encodes a multifunctional gibberellin 20-oxidase: molecular cloning and functional expression. *Proc Natl Acad Sci USA* **92**: 6640–6644