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The Auxins IAA and 4-Cl-IAA Differentially Modify Gibberellin Action via Ethylene Response in Developing Pea Fruit

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Abstract

This study explores the unique growth-regulatory roles of two naturally occurring auxins, indole-3-acetic acid (IAA) and 4-chloroindole-3-acetic acid (4-Cl-IAA), and their interactions with gibberellin (GA) during early pea (*Pisum sativum* L.) fruit development. We have previously shown that 4-Cl-IAA can replace the seed requirement in pea pericarp growth (length and fresh weight), whereas IAA had no effect or was inhibitory. When applied simultaneously, gibberellin (GA_3 or GA_1) and 4-Cl-IAA had a synergistic effect on pericarp growth. In the present study, we found that simultaneous application of IAA and GA_3 to deseeded pericarps inhibited GA_3-stimulated growth. The inhibitory effect of IAA on GA-stimulated growth was mimicked by treatment with ethephon (ethylene releasing agent), and the inhibitory effects of IAA and ethylene on GA-mediated growth were reversed by silver thiosulfate (STS), an ethylene action inhibitor. Although pretreatment with STS could retard senescence of IAA-treated pericarps, STS pretreatment did not lead to IAA-induced pericarp growth. Although 4-Cl-IAA stimulated growth whereas IAA was ineffective, both auxins induced similar levels of ethylene evolution. However, only 4-Cl-IAA-stimulated growth was insensitive to the effects of ethylene. Gibberellin treatment did not influence the amount of ethylene released from pericarps in the presence or absence of either auxin. We propose a growth regulatory role for 4-Cl-IAA through induction of GA biosynthesis and inhibition of ethylene action. Additionally, ethylene (IAA-induced or IAA-independent) may inhibit GA responses under physiological conditions that limit fruit growth.

Key words: Hormonal interaction; Auxin; Gibberellin; Ethylene; *Pisum sativum*; Fruit development; Pericarp; Pea.

Introduction

Fruit development involves a complex interaction of molecular, biochemical, and structural changes that transform a fertilized ovary into a mature fruit. This

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coordination involves integration of hormonal and environmental signals to bring about a specific pattern of fruit (seed and pericarp) growth and development (Ozga and Reinecke 2003). How the different classes of plant hormones (specifically, gibberellins [GAs], auxins, and ethylene) interact with each other to bring about the coordination of seed and pericarp growth is not well understood. Moreover, it is generally assumed that all biologically active compounds within a hormone class elicit similar responses in fruit and other plant tissues, with differences only in the magnitude of the response (for example auxins, indole-3-acetic acid [IAA], 4-chloroindole-3-acetic acid [4-Cl-IAA], and indole-3-butyric acid [IBA]).

Gibberellins (bioactive GA_1 and GA_3; Garcia-Martinez and others 1991; Rodrigo and others 1997), auxins, IAA and 4-Cl-IAA (Magnus and others 1997), and ethylene (Orzáez and others 1999) are naturally occurring hormones in pea fruit. 4-Cl-IAA (1 to 100 μM), when applied to pollinated deseeded pea pericarps 2 days after anthesis (DAA), promotes elongation and fresh weight growth (Reinecke and others 1995). In combination with GA_1 or GA_3, 4-Cl-IAA stimulates growth of deseeded pea pericarps synergistically (Ozga and Reinecke 1999). However, IAA (0.1–1000 μM) is inactive or inhibitory to deseeded pericarp growth (Reinecke and others 1995).

Gibberellins have been implicated in many aspects of reproductive development (Pharis and King 1985). Pea plants metabolize GAs by the early 13-hydroxylation pathway: $GA_{12} \rightarrow GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20} \rightarrow GA_1$ (Sponsel 1995). Using the pea split-pericarp system, van Huizen and others (1995, 1997) found that 4-Cl-IAA-stimulated gene expression (*PsGA20ox1*) and enzyme activity of GA 20-oxidase (a multi-functional enzyme that converts GA_{53} to GA_{20}). Ozga and others (2003) showed that 4-Cl-IAA also stimulated message levels of *PsGA3ox1*, which codes for the enzyme GA 3β-hydroxylase, which converts GA_{20} to biologically active GA_1. However, IAA was ineffective in stimulating message levels of *PsGA20ox1* and *PsGA3ox1* (Ngo and others 2002; Ozga and others 2003). Silver thiosulfate (STS) pretreatment resulted in similar *PsGA20ox1* message levels for both IAA-treated and control deseeded pericarps (Ngo and others 2002). These data showed that two pea auxins had differing effects on the GA biosynthetic pathway, and that the lack of effect of IAA on pericarp *PsGA20ox1* expression was not due to a negative effect of IAA-induced ethylene.

To further understand how two naturally occurring auxins, 4-Cl-IAA and IAA, can elicit different fruit developmental processes in pea, we investigated the interaction between these auxins and GA in stimulating early pea fruit development, and the role of ethylene in modifying GA action. Although the involvement of ethylene in fruit ripening has been extensively studied, little is known about the role of ethylene in early fruit development. In pea fruit lacking fertilized ovules, and in the corolla, ethylene may act as a senescence hormone (Orzáez and others 1999). The capacity for IAA or 4-Cl-IAA to induce ethylene evolution in pea fruit has not been studied; however, both auxins can stimulate ethylene evolution in vegetative tissues of pea. IAA stimulated 1-aminocyclopropane-1-carboxylate synthase (ACS; the rate-limiting enzyme in the ethylene biosynthetic pathway) message levels (*PsACS1* and *PsACS2*), enzyme activity, and ethylene evolution in internodes of 5- to 6-day-old etiolated pea seedlings (Peck and Kende 1995, 1998). Both 4-Cl-IAA and IAA induced ethylene production in light-grown pea shoot cuttings, with 4-Cl-IAA giving a higher and more sustained ethylene release (Ahmad and others 1987).

In this study, we investigated the effect of IAA and 4-Cl-IAA on GA-induced pericarp growth, the role of ethylene in auxin-stimulated growth, and the profile of IAA- and of 4-Cl-IAA-induced ethylene production in pea pericarp tissue. The ethylene-releasing agent, ethephon, and an ethylene-action inhibitor, STS, were used to determine if ethylene could mimic the effects of IAA on pericarp growth. The data presented show that the difference in the growth response of the pericarp to these two endogenous auxins does not lie in their ability to stimulate ethylene biosynthesis. Instead, the data indicate that the two auxins induce differential developmental responses in pea fruit, in part, through different effects on the GA response pathway. IAA (at levels that induce ethylene synthesis) and ethylene inhibit signal transduction/response to GA, resulting in inhibition of pericarp growth. 4-Cl-IAA stimulates growth via auxin/GA signal transduction pathways, and it reduces tissue sensitivity to ethylene, which can interfere with GA growth promotion.

Materials and Methods

Plant Material

Pea seeds (*Pisum sativum* L.) line I_3 (Alaska-type) were grown at 19/17°C (day/night) under a 16-h photoperiod with 400 μE m^{-2} s^{-1} from cool white fluorescent and incandescent lights (van Huizen

and others 1995). For treatments, flowers from the first and second flowering nodes were removed and one fruit per plant between the third and fifth flowering nodes was used. Subsequent flowers and lateral buds were removed as they developed, the terminal apical meristem remained intact, and the pericarp stayed attached to the plant during all experiments unless noted.

Growth Studies

Two-DAA pea pericarps (ovaries) were either left intact, split (suture opposite the seeds; SP), or split and deseeded (SPNS and hormone treatments) as described by Ozga and others (1992). GA_3, 4-Cl-IAA, and/or IAA (5 or 50 μM in 0.1% [v/v] aqueous Tween 80) were applied immediately after deseeding to the inner pericarp wall (endocarp) and daily thereafter to 6 DAA for long-term growth experiments (30 μL, 2 and 3 DAA; and 40 μL, 4, 5, and 6 DAA). For the IAA plus STS dose–response experiment, IAA at 0, 0.1, 1, 10, and 100 μM was applied daily from 2 to 6 DAA following a pretreatment with STS at 2 DAA. Ethephon (30 μL; 1.7 or 6.9 mM) was applied once immediately after splitting and deseeding the pericarp. When ethephon was applied in combination with other hormone treatments, a 30-μL aliquot (1.7 or 6.9 mM) was applied once, 90 min after the initial auxin and/or GA_3 treatments. For treatments requiring STS (1 mM), a 20-μL aliquot was applied once as a pretreatment immediately after splitting and deseeding the fruit. Hormone solutions were applied 30 min after STS application. For long-term growth experiments, the pericarps attached to the plant were enclosed in a clear plastic bag to maintain moisture. Fruits were harvested at 9 DAA (7 days after initial treatment) and measured for growth. Increase in fruit length was determined by subtracting length at 2 DAA from the length at 9 DAA. In short-term experiments (0–24 h), pericarps received the same treatments but were not enclosed in a clear plastic bag while attached to the plant.

Ethylene Evolution Assay

To determine if induction of ethylene evolution by auxin requires pericarps to be attached to the plant, deseeded pericarps, detached or attached, were incubated with IAA (50 μM) and the ethylene evolution from the pericarps was compared. Two-DAA pericarps were split and deseeded and immediately treated with 30 μL of 50 μM IAA. The 12-h on-plant-incubated pericarps remained attached to the plant and open to the atmosphere. The 12-h off-plant-incubated pericarps were immediately detached from the plant after IAA-treatment and placed in an uncapped 7-mL glass vial in the growth chamber. After 12 h of incubation, the on-plant pericarps were harvested and the off-plant pericarps were collected to measure the rate of ethylene evolution. At 12 h after treatment of the pericarps on the plant with IAA, ethylene evolution was significantly more than control pericarps (3.3-fold higher than SPNS-treated plants). However, application of IAA (12 h) to detached pericarps did not stimulate ethylene evolution above the control detached SPNS pericarps. The absence of auxin-induction of ethylene evolution in the detached-treated pericarps is most likely related to dehydration of pericarp tissue after treatment (pericarp fresh weight: off-plant 48% lower than on-plant). Therefore, deseeded pericarps attached to the plant during the hormone incubation period were used in all other experiments.

For 24-h control treatments, 2-DAA SP, and SPNS pericarps were treated once with 30 μL 0.1% (v/v) aqueous Tween 80, and for hormone treatments 2-DAA deseeded pericarps were treated with auxins (IAA or 4-Cl-IAA) and/or GA_3 (30 μL, 50 μM hormone in 0.1% [v/v] aqueous Tween 80). All treatments were incubated on the plant for 4, 8, 12, or 24 h, except SPNS which was additionally incubated for 1 and 2 h. Two DAA deseeded pericarps were also treated with ethephon (30 μL; 1.7 or 6.9 mM); the pericarps were incubated on the plant for 1, 2, 4, 8, 12, and 24 h (ethephon 6.9 mM), or 4, 8, 12, and 24 h (ethephon 1.7 mM).

To determine if deseeded pericarps could respond to a second application of auxin, 2-DAA pericarps were split and deseeded and were either treated at 0 h (one treatment) with 30 μL of 50 μM IAA, 4-Cl-IAA, or 0.1% Tween 80, or they were treated at 0 h and 24 h (two treatments). Pericarps were harvested at 12, 24, and 36 h after the initial treatment, and assayed for ethylene production.

Ethylene Quantitation

In all cases, after the on-plant treatment periods, pericarps were harvested and immediately placed in 7-mL glass vials sealed with rubber septums (1 to 4 pericarps per vial). After 1 h, a 1-mL gas sample from the headspace of the sealed vial was withdrawn via a Hamilton gas-tight syringe. The headspace gas sample was injected onto a 2.9 m × 6.35 mm o.d. 80/100 mesh Porapak N column attached to a Hewlett Packard 5890 gas chromatograph (GC)

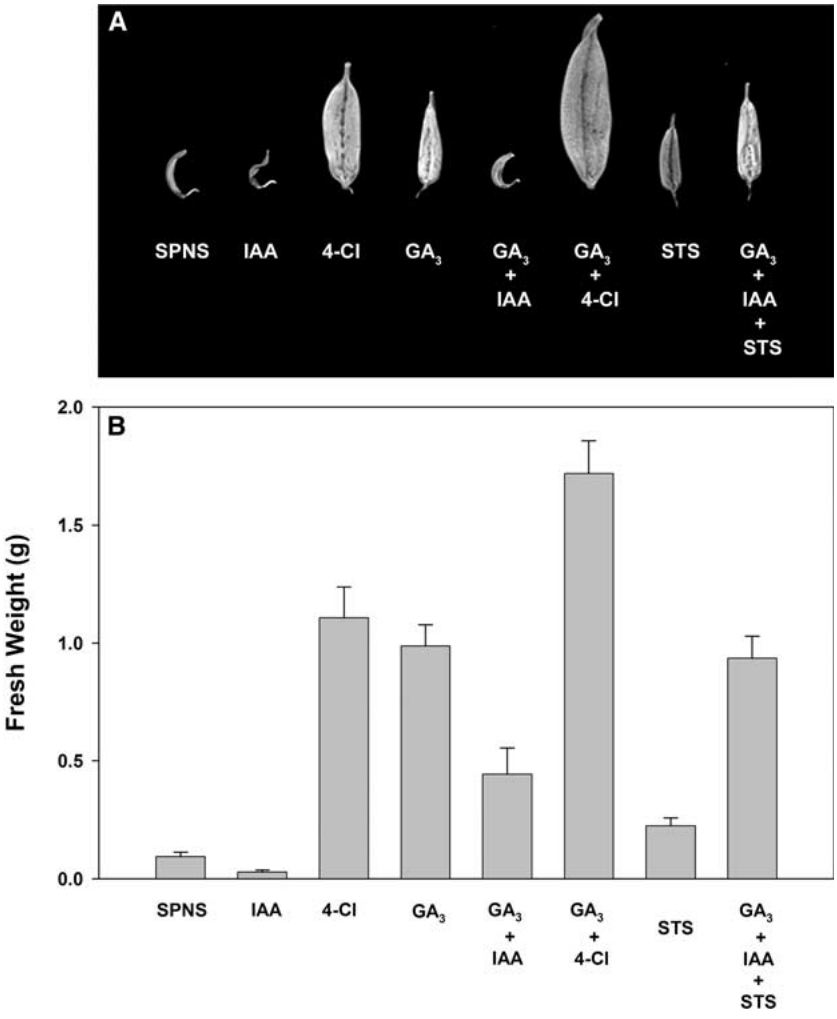

Figure 1. Effect of natural pea hormones (GA_3, IAA, 4-Cl-IAA) and ethylene-action inhibitor silver thiosulfate (STS) on pericarp growth. **A**. Representative pericarps harvested at 7 DAA (5 days after initial treatment). Deseeded pericarps were treated daily for 5 days with 50 μM of GA_3, IAA, GA_3 + IAA, 4-Cl-IAA (4-Cl), or GA_3 + 4-Cl-IAA (GA_3 + 4-Cl), or 0.1% (v/v) aqueous Tween 80 (SPNS). For STS-treated pericarps, STS (20 μl, 1 mM) was applied once (on the first day of the experiment) either immediately after pericarp splitting and deseeding (STS) or as a pretreatment 30 min prior to hormone treatment (GA_3 + IAA + STS). **B**. Growth (fresh weight) of hormone-treated pericarps. Deseeded pericarps were treated with hormone solutions as described in **A** and harvested 9 DAA (7 days after initial treatment). Data are means ± SE, n = 7 to 38.

fitted with a flame-ionization detector. The injection and detection ports were set at 250°C and the column oven was maintained at 120°C. Nitrogen was used as the carrier gas at a flow rate of 30 mL min^{-1}. Ethylene content was determined by comparing the sample response to an equal volume of 0.96 μl L^{-1} ethylene primary standard. The hormone-induced ethylene evolution rate (nL g fresh $weight^{-1}$ h^{-1}) was calculated by subtracting the rate of ethylene evolved from the SPNS control pericarps from that of the hormone-treated pericarps.

Results

Hormone-induced Pericarp Growth

When seeds were removed at 2 DAA, pericarp growth was arrested and pericarp tissues shriveled and senesced within 7 days (split pod no seeds [SPNS] treatment; Figure 1). 4-Cl-IAA stimulated deseeded-pericarp growth; however, pericarps treated with IAA did not grow (Figure 1). By 2 days after initial treatment (4 DAA), IAA-treated pericarps were 56% lower in fresh weight than the SPNS control pericarps (data not shown) and this trend continued through 7 days after the initial treatment (Figure 1B). When applied in combination, 4-Cl-IAA enhanced GA_3-induced pericarp growth, whereas IAA significantly inhibited GA_3-stimulated pericarp growth (Figure 1 A, B).

Application of STS (1 mM; an ethylene action inhibitor) to deseeded pericarps inhibited tissue senescence (7 days after the initial treatment), and STS-treated pericarps were greater in fresh weight than the SPNS controls (Figure 1 A, B). Treatment with STS (1 mM) also blocked the IAA-induced inhibition of pericarp growth but did not result in IAA-stimulation of pericarp growth when compared to the STS-treated control (Table 1). Silver thiosulfate pretreatment had a minimal effect on 4-Cl-IAA-induced pericarp growth (Table 1).

Table 1. Effects of IAA, 4-Cl-IAA and STS on Pea Pericarp Growth

	Treatments	Length[a]	Fresh weight[b]	Dry weight[b]	*n*
A[c]	IAA 100 μM + STS	5.6 ± 0.7	0.199 ± 0.039	0.022 ± 0.005	7
	IAA 10 μM + STS	7.8 ± 0.7	0.213 ± 0.052	0.022 ± 0.005	8
	IAA 1 μM + STS	7.5 ± 0.8	0.160 ± 0.020	0.017 ± 0.002	8
	IAA 0.1 μM + STS	5.4 ± 1.4	0.240 ± 0.038	0.043 ± 0.015	7
	SPNS + STS	9.6 ± 0.8	0.209 ± 0.039	0.030 ± 0.007	8
B	4-Cl-IAA 50 μM	26.3 ± 2.0	0.755 ± 0.122	0.084 ± 0.015	12
	4-Cl-IAA 5 μM	16.7 ± 0.8	0.280 ± 0.044	0.028 ± 0.005	12
	IAA 50 μM	3.4 ± 0.4	n.d.[d]	n.d.	8
	IAA 5 μM	6.0 ± 0.9	n.d.	n.d.	7
	4-Cl-IAA + IAA 50 μM	27.4 ± 1.4	0.764 ± 0.102	0.080 ± 0.013	11
	4-Cl-IAA + IAA 5 μM	15.8 ± 0.9	0.321 ± 0.054	0.033 ± 0.006	11
	SPNS	8.2 ± 1.0	n.d.	n.d.	11
C	4-Cl-IAA 50 μM	30.3 ± 1.6	0.908 ± 0.186[e]	0.095 ± 0.013	8
	4-Cl-IAA 50 μM + STS	28.6 ± 1.1	1.290 ± 0.118[e]	0.140 ± 0.016	8

[a] *Maximum increase in length (mm) from 2 to 9 DAA.*
[b] *Weight in g at 9 DAA.*
[c] *Results (**A**), (**B**), and (**C**) were from separate experimental dates.*
[d] *Not determined. Majority of pericarps had shriveled and abscised.*
[e] *Averaged from n = 4.*

When STS was applied as a pretreatment to GA_3 plus IAA-treated pericarps, the inhibitory effect of IAA on GA_3-treated fruit growth was eliminated (Figure 1 A, B). To test if ethylene could mimic the inhibitory effect of IAA on GA_3-induced pericarp growth, ethephon (the ethylene-releasing agent) was applied to deseeded pericarps. By day 2 after treatment, ethephon-treated (6.9 mM) pericarps were 56% lower in fresh weight than the SPNS control pericarps (data not shown). By 7 days after treatment, ethephon at 1.7 and 6.9 mM advanced pericarp-tissue senescence compared to the SPNS control (significantly lower fresh weight than SPNS control) and inhibited GA_3-induced pericarp growth (Figure 2).

Ethephon application mimicked the inhibitory effect of IAA on GA_3-stimulated growth, whereas pretreatment with STS blocked this inhibition (Figure 2 A, B). The growth of 4-Cl-IAA-treated (50 μM) deseeded pericarps was not reduced by ethephon at 1.7 mM (data not shown) and 6.9 mM (Figure 2 A, B). In addition, the growth of 4-Cl-IAA-treated deseeded pericarps was not affected by simultaneous application of IAA (Table 1).

Ethylene Evolution in Control and Ethephon-treated Fruit

Intact pericarps released relatively low amounts (7–27 nL g fresh weight^{-1} h^{-1}) of ethylene over the 24-h treatment (2–3 DAA) period (Figure 3A). Splitting of the pericarp (SP) resulted in a 5.6-fold increase in the rate of ethylene evolution 4 h after splitting. The rate of ethylene evolution from split pods decreased to 48 nL g fresh weight^{-1} h^{-1} by 8 h, three times the rate measured from 8-h intact fruit (Figure 3A). At 1 and 2 h after splitting and deseeding of the pericarp (SPNS), the rate of ethylene evolution increased 1.7- and 2.4-fold compared to the intact 0-h control. SPNS treatment triggered a maximum rate of ethylene evolution by 4 h, a 4.7-fold increase after the splitting and deseeding (Figure 3A). By 24 h after treatment, the ethylene evolution of SP- and SPNS-treated pericarps had dropped by 3.8 and 2.3 fold from their high points, respectively. During the 24-h period, intact fruit and SP pericarps grew 1.9- and 1.7-times greater in fresh weight, respectively, than the SPNS pericarps (Figure 3B).

Ethylene evolution increased rapidly to 589 nL g fresh weight $^{-1}$ h^{-1} by 1 h after ethephon (6.9 mM) application to 2-DAA deseeded pericarps (Figure 3C). Then, 8 h after ethephon treatment (6.9 mM), ethylene evolution decreased by 2.4-fold from the rate at 1 h, and it was maintained at this rate at least until 24 h (Figure 3C). Deseeded pericarps treated with 1.7 mM ethephon evolved ethylene at a lower rate (2.6-fold at 4 h after application) when compared to the 6.9 mM ethephon treatment (Figure 3C). By 12 h after application, both ethephon treatments evolved ethylene at similar rates. The average rates of ethylene evolution over the 24-h treatment period were 2 times and 5 times above the SPNS control for 1.7 mM and 6.9 mM ethephon, respectively (calculated from

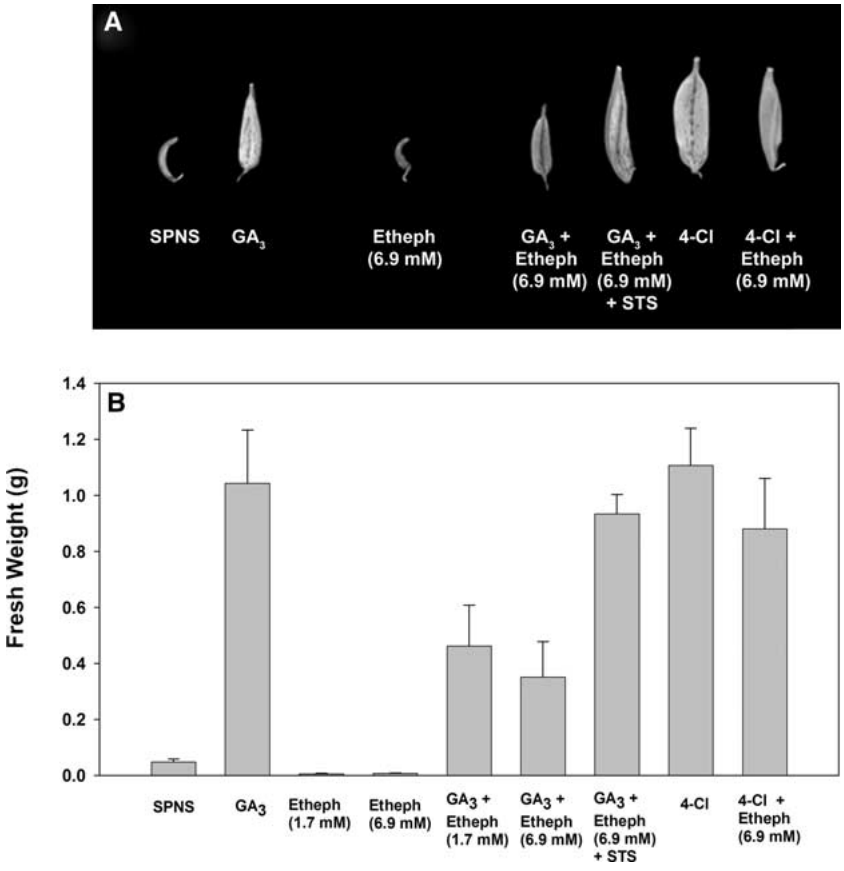

Figure 2. Ethephon inhibition of GA_3-induced pericarp growth. **A**. Representative pericarps harvested at 7 DAA (5 days after initial treatment). De-seeded pericarps were treated daily for 5 days with 50 μM of 4-Cl-IAA (4-Cl) or GA_3, or 0.1% (v/v) aqueous Tween 80 (SPNS). Ethephon (etheph) was applied only once, to the deseeded pericarps, either immediately (ethephon 1.7 or 6.9 mM) or 90 min after GA_3 or 4-Cl-IAA application (GA_3 + ethephon or 4-Cl-IAA + ethephon). STS (1 mM) was applied once on day 1 of the experiment as a pretreatment 30 min prior to hormone treatment (GA_3 + ethephon + STS). **B**. Growth (fresh weight) of hormone-treated pericarps. Deseeded pericarps were treated with hormone solutions as described in **A** and harvested at 9 DAA (7 days after initial treatment). Data are means ± SE, n = 4–38.

raw data). The fresh weight of ethephon-treated deseeded pericarps was less than that of the control (SPNS) after 24 h (Figure 3D). To see if ethephon had a negative impact on growth due to its acidity rather than ethylene release, we treated pericarps with aqueous 0.1% Tween 80 acidified with phosphoric acid (acidified to ca. pH 2.3), comparable to the acidity of 6.9 mM ethephon in aqueous 0.1% Tween 80. We observed no inhibitory effect of the acid treatment on pericarp growth (split pericarp plus seeds: 2.55 ± 0.16 g fresh weight) as compared to the unacidified water control treatment (2.22 ± 0.43 g fresh weight) when harvested 7 days after treatment (9 DAA, n = 3 to 4).

Hormone-induced Ethylene Evolution

IAA and 4-Cl-IAA at 50 μM stimulated similar rates of ethylene evolution in 2-DAA deseeded pericarps over a 24-h period (Figure 4 A, C). Application of GA_3 to the auxin-treated pericarps did not change the ethylene evolution profile compared to the auxin-alone treatments (Figure 4 A, C). Ethylene evolution in GA_3-treated pericarps was equal to that in untreated SPNS pericarps (Figure 4E). Significant changes in hormone-induced pericarp growth usually require more than 24 h to be observed. However, 24 h after treatment with 4-Cl-IAA and 4-Cl-IAA plus GA_3, pericarps were larger than the SPNS control (Figure 4D).

Deseeded pericarps treated with IAA or 4-Cl-IAA immediately after deseeding (0 h) were capable of renewed auxin-stimulated ethylene production upon reapplication of the auxin 24 h after the initial treatment (Figure 5). 4-Cl-IAA stimulated a higher rate of ethylene evolution 12 h after the reapplication (36-h time point) compared to IAA. De-seeded pericarps treated with IAA or 4-Cl-IAA only once, immediately after deseeding (0 h), evolved similar amounts of ethylene 36 h after hormone application as the SPNS-untreated control (Figure 5).

Discussion

IAA and 4-Cl-IAA are naturally occurring plant hormones in pea fruit (*Pisum sativum* L.; Magnus and others 1997). 4-Cl-IAA is also a natural component of seeds of other plants of the Vicieae tribe of the Fabaceae family, including *Vicia faba*, *Lens*

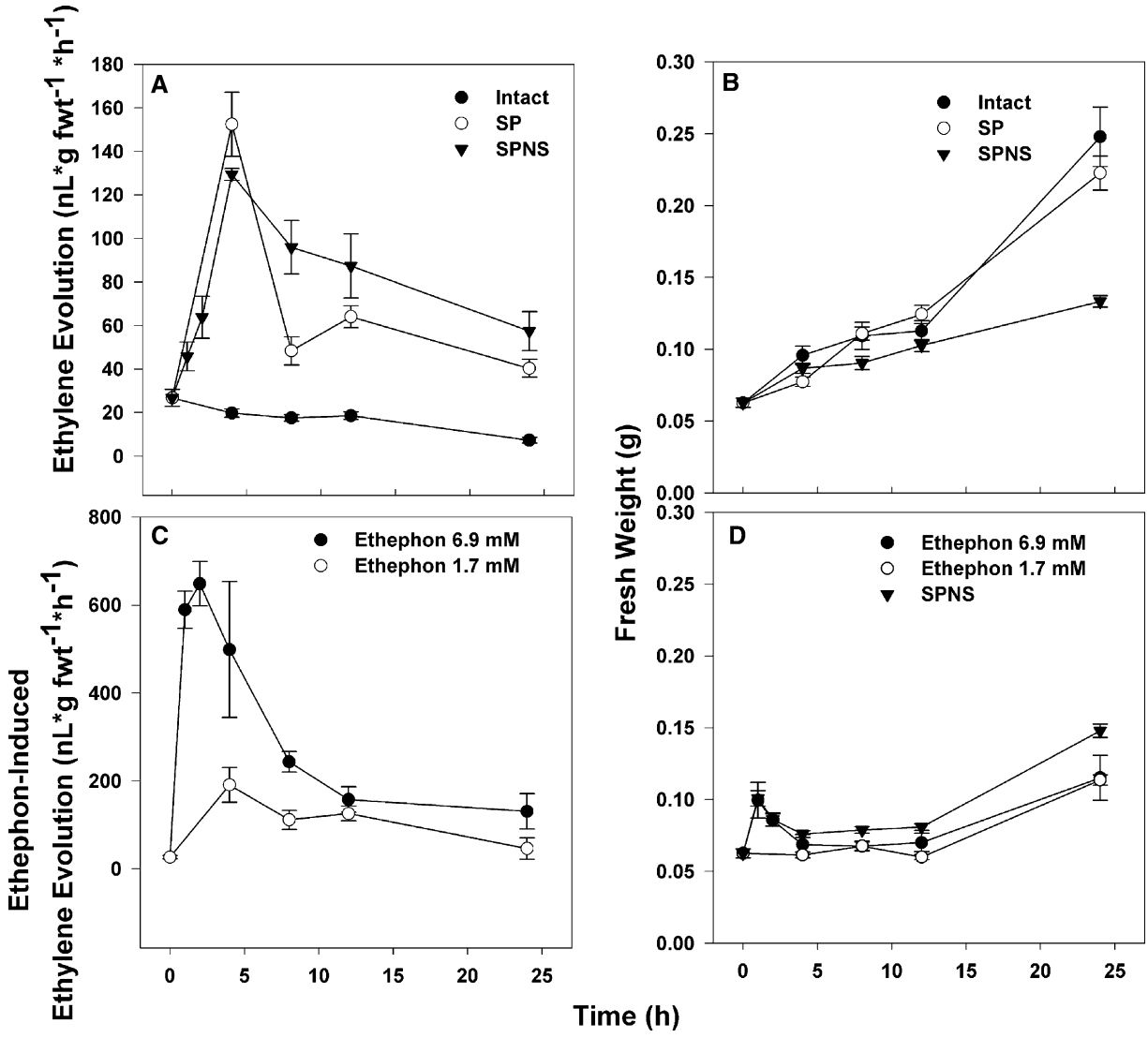

Figure 3. Rate of ethylene evolution and growth (fresh weight) of Intact, SP, SPNS pericarps (**A** and **B**), and ethephon-treated deseeded pericarps (**C** and **D**). Two DAA pericarps were left intact (Intact treatment), split (SP), or split and deseeded (SPNS). Ethylene evolution (**A**) and pericarp growth (fresh weight; **B**) were monitored over a 24-h period. Data are means ± SE, n = 4–19. Two-DAA pericarps were split and deseeded and immediately treated with ethephon (1.7 or 6.9 mM) or 0.1% (v/v) aqueous Tween-80 (SPNS). Ethylene evolution (**C**) and growth (fresh weight; **D**) from the pericarps were monitored over a 24-h period. Data from **C** are presented as the ethephon-stimulated ethylene evolution, where the rate of ethylene evolution from SPNS pericarps was subtracted from that of the ethephon-treated pericarps. Data are means ± SE, n = 4.

culinaris, and *Lathyrus* species (Engvild and others 1980, 1981). IAA and 4-Cl-IAA are likely involved in regulating fruit growth and development in pea (Ozga and others 2002), and in other plants of the Vicieae tribe (for example, *Vicia faba*; Pless and others 1984). In the present study we investigated the differential interactions of 4-Cl-IAA and IAA with ethylene and GA growth responses and extended our understanding of how both auxins affect fruit developmental processes.

Auxin-induced Ethylene Evolution and Biological Activity

4-Cl-IAA treatment promoted pea pericarp growth, whereas IAA at lower concentrations did not stimulate growth and at higher concentrations promoted pericarp senescence when compared to the non-hormone-treated control (Reinecke and others 1995). In the present study, 4-Cl-IAA treatment stimulated ethylene synthesis and gave ethylene

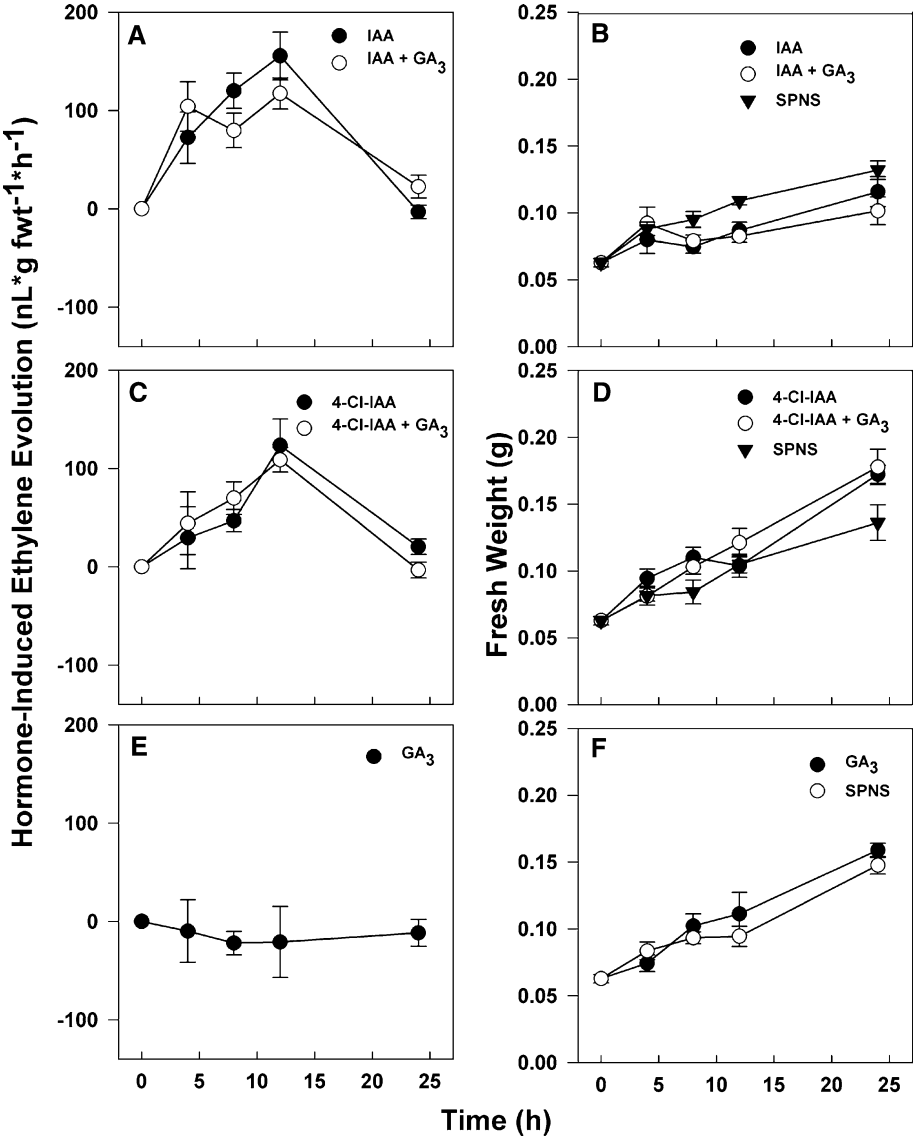

Figure 4. Ethylene evolution and pericarp growth in control and hormone-treated pericarps. Two-DAA pericarps were split, deseeded, and immediately treated with 50 μM IAA or IAA + GA_3 (**A** and **B**); 4-Cl-IAA or 4-Cl-IAA + GA_3 (**C** and **D**); GA_3 (**E** and **F**); or 0.1% (v/v) Tween 80 (SPNS). Subsequently, pericarps were harvested at 0, 4, 8, 12, and 24 h. Data are presented as the hormone-induced ethylene evolution, where the rate of ethylene evolution of the SPNS pericarps was subtracted from that of the hormone-treated pericarps. Data are means ± SE, n = 6–8.

developmental profiles similar to IAA-treated pericarps; thus, the difference in pericarp growth response between the two endogenous auxins was not due to a qualitative difference in ethylene biosynthesis. Ethylene levels peaked 12 h after treatment of deseeded pericarps with either auxin, producing an approximately 3-fold increase in ethylene levels above the SPNS control. In pea fruit, both auxins likely stimulate synthesis of the regulatory ethylene-biosynthetic enzyme 1-aminocyclopropane-1-carboxylate synthase (ACS), because ACS is stimulated by IAA in many plants including etiolated pea stems (IAA at 100 μM induced an 8-fold increase in ethylene level 6 h after treatment in etiolated pea seedlings; Peck and Kende 1995; Peck and Kende 1998).

Silver thiosulfate pretreatment reversed the IAA-induced reduction in pericarp growth (5–100 μM IAA), suggesting that auxin-induced ethylene production was responsible for growth inhibition of pericarp by IAA. Silver thiosulfate pretreatment had a minimal effect on 4-Cl-IAA-induced (50 μM) pericarp growth. Furthermore, when 4-Cl-IAA and IAA were applied simultaneously, the pericarp growth response was the same as with 4-Cl-IAA alone, and 4-Cl-IAA-treated pericarps did not respond negatively to ethylene (ethephon treatment). These data support the hypothesis that the negative effect of IAA on pericarp growth is due to stimulation of ethylene synthesis, and that the response of pericarp to ethylene is uniquely altered by 4-Cl-IAA.

Ethephon was used as an external source of ethylene (Warner and Leopold 1969) to determine if ethylene could mimic the effects of IAA on deseeded pericarps. The magnitude of ethylene pro-

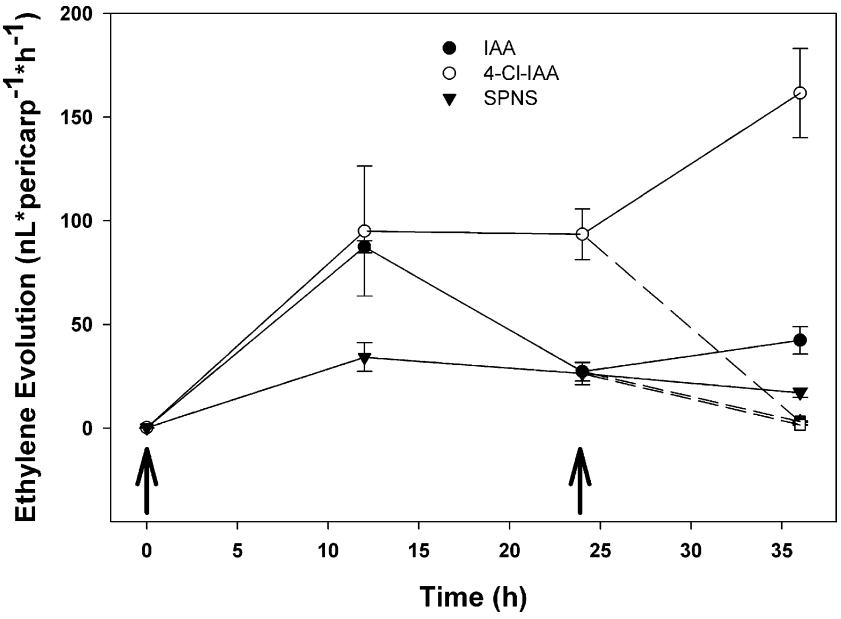

Figure 5. Sensitivity of pericarp tissue to reapplication of auxin (IAA or 4-Cl-IAA) 24 h after initial treatment. Two DAA pericarps were split, deseeded, and treated at 0 h (one application; broken lines at 24 to 36 h) or 0 h and 24 h (two applications; solid lines at 24 to 36 h) after splitting with 50 μM IAA or 4-Cl-IAA, or 0.1% (v/v) aqueous Tween 80 (SPNS). Pericarps were harvested at 12, 24, and 36 h after initial treatment and assayed for ethylene production. Arrows indicate time of hormone application. Data are means ± SE, n = 3–4.

duction by 1.7 mM ethephon was more like that produced by 50 μM IAA than when 6.9 mM ethephon was applied. However, for ethephon applied to the surface of the tissue, it is important to note that the amount of ethylene that permeates the tissue resulting in a physiological response would be only a fraction of the released ethylene because the pericarps were open to the atmosphere during treatment.

The profile of ethylene evolution after ethephon treatment (greatest within 1–4 h after application) was earlier than auxin-induced ethylene production, which peaked 12 h after application (Figures 3C, and 4 A, C). Nevertheless, ethephon mimicked the inhibitory effect of IAA on pericarp growth when applied alone or in combination with GA_3 (Figure 2). The inhibitory effect of ethephon (as well as IAA) on pericarp growth was also reversed by application of STS (Figures 1 and 2). These data support the hypothesis that the inhibition by IAA of pericarp growth occurs through IAA-induced ethylene. At levels below those required to stimulate significant ethylene biosynthesis and in actively growing pea fruit, IAA likely has developmental roles other than growth inhibition.

To further elucidate how ethylene modulates pea pericarp growth (fertilized ovaries), we studied ethylene evolution from intact and split pericarp tissues. Splitting of the pericarp (SP and SPNS) increased ethylene evolution beyond that observed in untreated pericarps, likely in part because of wounding at the cut suture (Orzáez and Granell 1997; Peck and Kende 1998; Orzáez and others 1999) and increased ethylene release from the inner pericarp. Senescence-induced ethylene was likely low during the reported ethylene measurements (2 to 3 DAA) because fruit deseeded at 2 DAA can be rescued at 3 DAA by treatment with GA plus 4-Cl-IAA and have not begun irreversible programmed senescence (van Huizen and others 1995).

GA, Auxin, and Ethylene Interaction in Pea Fruit Development

In pea fruit, seeds are required for pericarp elongation (Eeuwens and Schwabe 1975; Ozga and others 1992). GA_3 and 4-Cl-IAA can substitute for the seeds and stimulate pericarp growth (Ozga and Reinecke 1999). In contrast, IAA inhibits the stimulatory effect of GA_3 on pericarp tissue. Application of STS to deseeded pericarps treated with GA_3 plus IAA reversed the inhibitory effect of IAA on GA_3-stimulated growth. These data suggest that IAA-induced ethylene inhibits the growth-promoting effect of GA in pericarp tissue, whereas 4-Cl-IAA, the other naturally occurring auxin, differs from IAA in this respect. The differing biological activities of IAA and 4-Cl-IAA for pea fruit are unusual because the growth-promoting effect of IAA is the standard comparison for an auxin growth assay (Reinecke and others 1995). The pea pericarp assay differs from most auxin assays in three regards: the tested tissue remains attached to the plant, 4-Cl-IAA is tested in a tissue known to contain 4-Cl-IAA, and the tested tissue is fruit.

The level of IAA required to stimulate ethylene biosynthesis in plant tissues varies with type and developmental stage of the tissue (Burg and Burg 1968). Further, tissue receptivity to ethylene will determine the extent of the response to ethylene

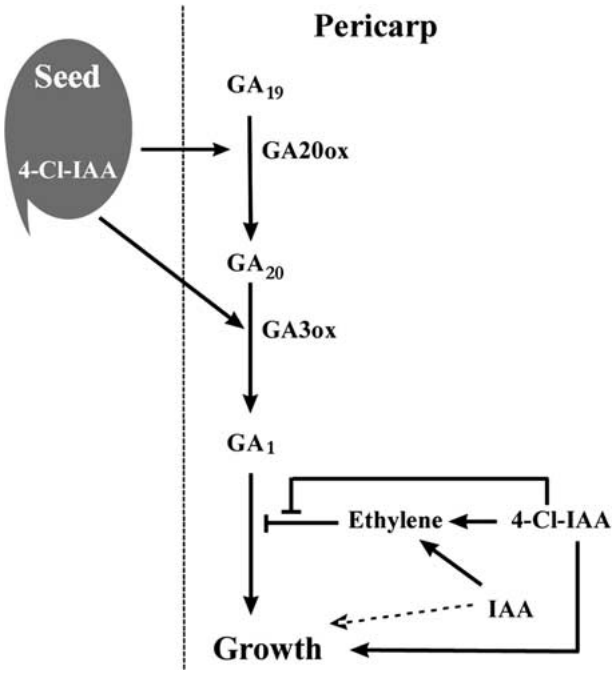

Figure 6. Working model of hormonal interactions in early pea fruit development. Seed-derived 4-Cl-IAA is transported to the pericarp, where it stimulates GA biosynthesis (via increased GA20ox and GA3ox gene expression) leading to increased levels of GA_1 for growth. 4-Cl-IAA also acts to inhibit ethylene action, which results in greater GA response, and 4-Cl-IAA acts to stimulate growth directly through auxin-response pathways. Under physiological conditions unfavorable for fruit growth, ethylene (IAA-induced or IAA-independent) can inhibit GA action, resulting in further growth reduction.

(Peck and Kende 1998). The level of endogenous auxins in non-pollinated or deseeded pericarps entering senescence (from 3 to 5 DAA) is not known. However, moderate amounts of IAA occur in young pericarps (Magnus and others 1997). Endogenous IAA in pericarp tissue may contribute to pericarp ethylene evolution, and during senescence the lack of sufficient growth-stimulatory factors increases the tissue sensitivity of pericarp to ethylene.

Non-pollinated pea fruits exhibit increased ethylene evolution as the tissue enters senescence (Orzáez and others 1999). Aminoethoxyvinylglycine (an ethylene biosynthesis inhibitor; Gomez-Gomez and Carrasco 1996) and STS (Orzáez and Granell 1997) can retard senescence, and exogenous ethylene can stimulate senescence (Orzáez and Granell 1997) in non-pollinated pea fruit. Ethylene (10 μL L^{-1} in air) was also observed to induce carpel senescence in non-pollinated GA_3-treated pea fruit (Orzáez and Granell 1997). The action of ethylene in non-pollinated fruit may be caused in part by ethylene inhibition of GA signal transduction/response, tipping the balance of hormone activity to favor senescence. This sequence of events could occur in fruit if developmental problems occurred within the ovary (including stress, seed abortion, and change in sink status resulting in fruit senescence).

Integration of Hormonal Signals

Recently, DELLA proteins, a subfamily of the GRAS family of putative transcriptional regulators (Richards and others 2001) have been proposed to play a key integrative role in a GA, auxin, and ethylene signal response network in *Arabidopsis*. DELLA proteins were initially identified as GA signaling components, where GA promotes growth of plants by opposing the effects of nuclear DELLA proteins by destabilizing them (Richards and others 2001). Achard and others (2003) found that ethylene alters the relationship between GA and the DELLA protein RGA in the roots of *Arabidopsis* seedlings, in this case, making RGA in the root nuclei more resistant to the opposing effects of GA. The ethylene-induced stabilization of DELLA proteins such as RGA is suggested to increase their growth-repressing effects, contributing to the ethylene-mediated inhibition of root growth. In addition, Fu and Harberd (2003) observed that reduction of auxin transport from the shoot apices (by shoot apex removal or naphthylphthalamic acid treatment) to the roots of *Arabidopsis* seedlings delayed the GA-induced disappearance of the growth repressor RGA from the root cell nuclei, and root growth was inhibited. Application of IAA to decapitated seedlings (grown in the presence of exogenous GA) resulted in destabilization of RGA from root nuclei at a similar rate as GA-treated intact seedlings.

The interactions of GA, auxins, and ethylene on pea pericarp growth observed in this study are consistent with those observed in *Arabidopsis* root growth, and they indicate that these hormones may interact similarly in different plant organs and species. Thus, the pericarp growth-stimulating auxin 4-Cl-IAA stimulates GA biosynthesis in the pericarp tissue and may also act to enhance the GA-induced destabilization of DELLA proteins to bring about pericarp growth. 4-Cl-IAA also alters the growth inhibitory effects of ethylene on pericarp tissue, possibly by blocking/reducing ethylene signal transduction and/or inhibiting ethylene stabilization of DELLA proteins. Plants that do not contain 4-Cl-IAA have alternative mechanisms of reducing ethylene action, as evidenced by differential sensitivities of tissues to ethylene. IAA-induced ethylene

could act to stabilize DELLA-like proteins, reducing GA-induced pericarp growth.

As it becomes evident that a significant amount of interaction occurs between different signaling pathways of different hormone classes, it is important to acknowledge that different auxins may have dramatically different effects on the network of signaling pathways in plants. This is important not only when considering the effects of naturally occurring auxins (IAA, IBA, and 4-Cl-IAA) on the signaling pathways, but also of the synthetic auxins, such as 1-naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid, which are frequently used in *Arabidopsis* mutant screening and characterization experiments (for example, auxin and ethylene interaction; Lincoln and others 1990; Luschnig and others 1998).

A working model of our proposed interactions of GA, auxins, and ethylene during early pea fruit growth is illustrated in Figure 6. We hypothesize that 4-Cl-IAA acts (1) as a seed-derived factor that promotes GA biosynthesis in the pericarp, thereby stimulating growth; (2) as a factor that inhibits ethylene action, resulting in a higher response to the GA produced; and (3) as a factor that stimulates growth directly through auxin-like activity. Under physiological conditions that limit pericarp growth, ethylene (IAA-induced or IAA-independent) can inhibit GA responses.

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