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Ethylene promotes ethylene biosynthesis during pea seed germination by positive feedback regulation of 1-aminocyclo-propane-1-carboxylic acid oxidase

Luciana Petruzzelli[1], Immacolata Coraggio[1], Gerhard Leubner-Metzger[2]

[1]Istituto Biosintesi Vegetali, C.N.R., Via Bassini 15, 20133 Milano, Italy
[2]Friedrich Miescher-Institute, P.O. Box 2543, 4002 Basel, Switzerland

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Abstract. Increased ethylene evolution accompanies seed germination of many species including *Pisum sativum* L., but only a little is known about the regulation of the ethylene biosynthetic pathway in different seed tissues. Biosynthesis of the direct ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), the expression of ACC oxidase (ACO), and ethylene production were investigated in the cotyledons and embryonic axis of germinating pea seeds. An early onset and sequential induction of ACC biosynthesis, accumulation of *Ps-ACO1* mRNA and of ACO activity, and ethylene production were localized almost exclusively in the embryonic axis. Maximal levels of ACC, *Ps-ACO1* mRNA, ACO enzyme activity and ethylene evolution were found when radicle emergence was just complete. Treatment of germinating seeds with ethylene alone or in combination with the inhibitor of ethylene action 2,5-norbornadiene showed that endogenous ethylene regulates its own biosynthesis through a positive feedback loop that enhances ACO expression. Accumulation of *Ps-ACO1* mRNA and of ACO enzyme activity in the embryonic axis during the late phase of germination required ethylene, whereas *Ps-ACS1* mRNA levels and overall ACC contents were not induced by ethylene treatment. Ethylene did not induce ACO in the embryonic axis during the early phase of germination. Ethylene-independent signalling pathways regulate the spatial and temporal pattern of ethylene biosynthesis, whereas the ethylene signalling pathway regulates high-level ACO expression in the embryonic axis, and thereby enhances ethylene evolution during seed germination.

Key words: 1-Aminocyclopropane-1-carboxylic acid oxidase – Ethylene – Feedback regulation – *Pisum* (ethylene biosynthesis) – Seed germination – Signalling

Abbreviations: ACC = 1-aminocyclopropane-1-carboxylic acid; ACO = ACC oxidase; ACS = ACC synthase; MACC = malonyl-ACC; NBD = 2,5-norbornadiene

Correspondence to: G. Leubner-Metzger;
E-mail: leubner@fmi.ch; Fax: +41-61-6973976

Introduction

The gaseous plant hormone ethylene exerts profound effects in plants throughout their life cycle. Ethylene perception and biosynthesis are highly regulated, as has been demonstrated in a number of vegetative tissues, in seedlings, in ripening fruits and senescing flowers (reviews: Fluhr and Mattoo 1996; Johnson and Ecker 1998). These studies showed that ethylene can exert both a positive and a negative effect on its own biosynthesis. Formation and oxidation of 1-aminocyclopropane-1-carboxylic acid (ACC), the direct precursor of ethylene, are generally considered as the rate-limiting steps in the biosynthetic pathway. The ACC oxidases (ACO) and ACC synthases (ACS) are encoded by multigene families, and differential induction by developmental and environmental factors has been demonstrated (e.g. Barry et al. 1996; Bouquin et al. 1997; Oetiker et al. 1997). Positive and negative internal feedback regulation by ethylene of specific ACO and ACS genes respectively, has been demonstrated during tomato fruit ripening (Blume and Grierson 1997; Nakatsuka et al. 1998). Ethylene induced ACO but suppressed ACS in mung bean hypocotyls in a process that involves protein phosphorylation and dephosphorylation (Kim et al. 1997). Induction of specific ACO genes often involves endogenous ethylene, e.g. in leaves of tomato and melon (Blume and Grierson 1997; Bouquin et al. 1997), in tomato fruits during ripening (Blume and Grierson 1997; Nakatsuka et al. 1998), and in etiolated pea seedlings (Peck and Kende 1995; Peck et al. 1998). Thus, in several tissues ethylene enhances its own biosynthesis through a positive feedback loop that induces ACO.

Far less is known about the molecular basis of ethylene perception and biosynthesis in germinating seeds. Ethylene is implicated in the seed germination of many plant species (review: Kepczynski and Kepczynska 1997), e.g. poor germination is a feature of the *Arabidopsis* ethylene-insensitive mutant etr1 (Johnson and Ecker 1998). Increasing ethylene evolution accompanies germination of most seeds, e.g. in *Cicer arietinum*

(Gómez-Jiménez et al. 1998), *Lycopersicon esculentum* (Lashbrook et al. 1998), and *Pisum sativum* (Gorecki et al. 1991; Petruzzelli et al. 1995). Gómez-Jiménez et al. (1998) made the interesting observation that an ACO gene (*caaco1*) is transcriptionally induced in the embryonic axis of germinating *C. arietinum* seeds. The molecular mechanisms of gene regulation by ethylene have been thoroughly studied in vegetative tissues, senescing flowers and during fruit ripening (review: Johnson and Ecker 1998). In contrast, only a few reports present molecular data about ethylene-regulated gene induction during seed germination. These include genes encoding a cysteine proteinase in chickpea (Cervantes et al. 1994), a class-I β-1,3-glucanase in tobacco and pea (Leubner-Metzger et al. 1998; Petruzzelli et al. 1999), and three members of the *ETR* gene family encoding putative ethylene receptors in tomato (Lashbrook et al. 1998).

Here, the regulation of ethylene biosynthesis has been studied during pea seed germination by measuring ethylene evolution, ACC and malonyl-ACC (MACC) contents, in-vivo and in-vitro ACO activities, together with Northern analyses of *Ps-ACO1* and *Ps-ACS1* mRNA, and experiments utilizing 2,5-norbornadiene (NBD), which inhibits ethylene action (Sisler and Pian 1973).

Materials and methods

Plant material and germination experiments. Seeds of *Pisum sativum* L. cv. 'Espresso generoso' (Sais, Cesena, Italy) were surface-sterilized and incubated in the dark at 20 °C as described previously (Petruzzelli et al. 1999). In brief, seeds at different times after the start of imbibition were dissected into cotyledons and embryonic axis. Tissues were frozen immediately and stored at −70 °C for subsequent analyses. For experiments with a controlled ethylene-atmosphere, Petri dishes with seeds were incubated in gas-tight 10-l desiccators. Where indicated, ethylene (20 µl l^{-1}; Siad, Milano, Italy) or NBD (50 µl l^{-1}; Aldrich Chemical Co., Buchs, Switzerland) were introduced into the air phase for the 24-h period before harvest.

Ethylene evolution and in-vitro ACO activity. Embryonic axes (ca. 0.4 g) or cotyledons (ca. 3 g) aseptically dissected from seeds at the times indicated, were incubated in air-tight 20-ml flasks for 2 h at 25 °C in the light in order to measure ethylene evolution. Ethylene content in the gas phase was analysed using a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector and a stainless-steel column (183 cm long, 0.32 cm i.d.) packed with 80/100 Poropack Q. For measuring in-vitro ACO activity, protein extracts were prepared from frozen embryonic axes or cotyledon tissues. Tissues were pulverized in liquid nitrogen and homogenized in 2 ml g^{-1} of extraction buffer consisting of 100 mM Tris-HCl (pH 7.2), 10% (w/v) glycerol and 30 mM sodium ascorbate. The homogenate was centrifuged at 28 000 *g* for 15 min at 4 °C. The supernatant was used for the in-vitro ACO assays following the procedure of Malerba et al. (1995) at 30 °C for 15 min in 10-ml screwcap tubes fitted with a Teflon-coated septum. At the end of this time period, the quantity of ethylene released into the headspace was determined by gas chromatography.

Quantification of free and conjugated ACC. Frozen embryonic axes (0.5 g) or cotyledon tissues (1 g) were extracted in 5 ml of 70% (v/v) ethanol at 4 °C. Samples were centrifuged at 12 000 *g* for 10 min, the supernatants were evaporated under vacuum. Residues were taken up in distilled water and ACC contents were determined by the method of Boller et al. (1979) using the protocol of Lizada and Yang (1979). To determine the amount of conjugated ACC, the aqueous extracts were hydrolyzed in 6 M HCl to release ACC. After neutralization with saturated NaOH and centrifugation, the supernatant was assayed as indicated above. The amount of conjugated ACC is the difference between the contents in free and total ACC.

Analysis of RNA. Preparation of total RNA and RNA-blot hybridization were performed as previously described (Leubner-Metzger et al. 1998). The radiolabelled DNA probes were the 1.1-kb *Eco*RI cDNA of pPE8 for *Ps-ACO1* (Peck et al. 1993), the ca. 2-kb *Bam*HI-*Eco*RI and *Eco*RI cDNAs of the plasmids for *Ps-ACS1* and *Ps-ACS2* (Peck and Kende 1998), and the 1.8-kb *Eco*RI fragment of genomic DNA encoding tomato 18S ribosomal RNA (rRNA; Schmidt-Puchta et al. 1989). Hybridized membranes were washed at high stringency. Signals were detected and quantified with a PhosphoImager (Molecular Dynamics, Sunnyvalle, Calif., USA). The mRNA contents are expressed in arbitrary units corrected for RNA loading based on the 18S rRNA signals.

Results

Tissue-specificity of ethylene biosynthesis during pea seed germination. Under our standard conditions, i.e. imbibition in the dark at 20 °C, radicle protrusion through the testa started at ca. 30 h and was complete by ca. 50 h. Imbibed seeds were dissected into the embryonic axis and the fleshy storage cotyledons and both tissues were then analyzed separately. Figure 1A shows that accumulation of ACC, the direct precursor of ethylene, in the embryonic axis started at ca. 8 h, reached a ca. 10-fold maximal level at 36–48 h, and declined thereafter. While MACC and γ-L-glutamyl-ACC are conjugation products of ACC in plants (Fluhr and Mattoo 1996), MACC is the major conjugation product of ACC in various organs and tissues, including seeds (Amrhein et al. 1981; Hoffman et al. 1983). Conjugated ACC, assumed to be MACC, accumulated in the embryonic axis in parallel with ACC, but exhibited a slightly delayed onset (Fig. 1A). In contrast, ACC and MACC levels remained low and roughly constant in the cotyledons (Fig. 1A).

The enzyme activity of ACO, which converts ACC into ethylene, can be measured in vivo, i.e. ethylene production of intact tissues incubated with a saturating ACC concentration, and in vitro, i.e. ethylene production in assays with protein extracts (Fluhr and Mattoo 1996). In-vitro ACO activity increased in the embryonic axis after a lag of ca. 15–20 h, reached ≥350-fold maximal levels when radicle emergence was complete at ca. 48 h, and declined thereafter (Fig. 1B). In contrast, there was only a comparably small increase in in-vitro ACO activity in cotyledons at the very late time points (Fig. 1B). Measurements of in-vivo ACO activity yielded the same temporal and spatial expression pattern (data not shown). The ACO cDNA clone *Ps-ACO1* of pea (Peck et al. 1993) was used to hybridize RNA extracted from pea seed tissues by Northern analyses (Fig. 2). The *Ps-ACO1* mRNA was induced very early on in the embryonic axis during germination. While the transcript was not detected at the start of imbibition (Fig. 2A), a signal was already apparent at

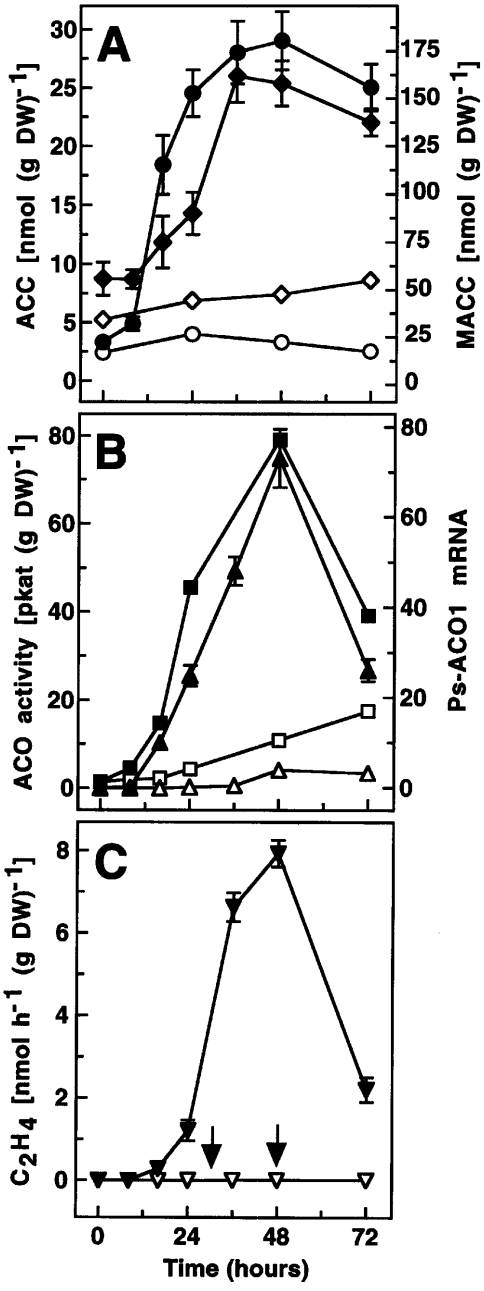

Fig. 1A–C. Tissue-specificity of ACC and MACC contents (**A**), in-vitro ACO enzyme activity and *Ps-ACO1* mRNA levels (**B**), and ethylene evolution (**C**) during pea seed germination. Seeds were incubated at 20 °C in the dark, harvested at the given time points, dissected into the embryonic axis (*closed symbols*) and the cotyledons (*open symbols*), and tissues were analysed separately. The ACC (●, ○) or MACC (◆, ◇) contents, in vitro ACO activities (△, ▲), and ethylene evolution (▼, ▽) are presented per g DW as nmol metabolite content, pkat enzyme activity, and nmol C_2H_4 h^{-1} release, respectively. The *Ps-ACO1* mRNA contents (□, ■) are expressed in arbitrary PhosphoImager units of the RNA-blot hybridization signals normalized for the signals obtained with 18S rRNA used as a loading standard. *Arrows* indicate the onset and the completion, respectively, of radicle emergence. With the exception of *Ps-ACO1* mRNA, mean values ± SE from at least three samples are presented

8 h. Quantification of the *Ps-ACO1* mRNA signals revealed a maximal increase of ca. 60-fold at 48 h, which was followed by a decline (Fig. 1B). Thus, the onset of

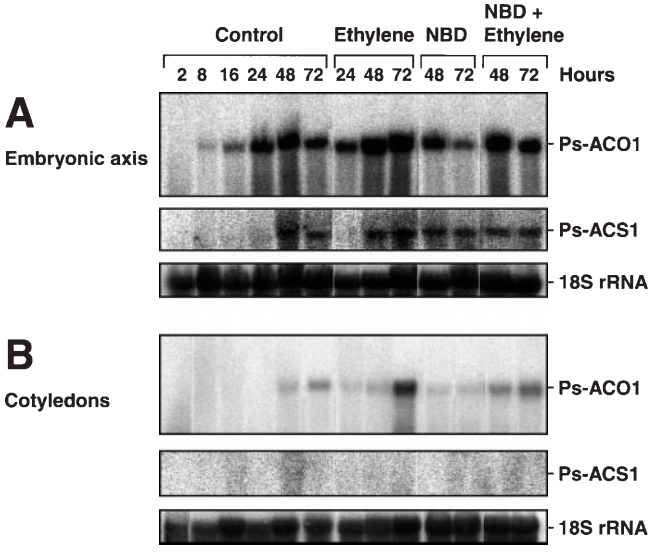

Fig. 2A,B. The effect of ethylene on the time course of *Ps-ACO1* and *Ps-ACS1* mRNA expression in the embryonic axis (**A**) and the cotyledons (**B**) during pea seed germination in the absence (*Control*) or presence of ethylene or NBD. Seeds were incubated at 20 °C in the dark and treated as indicated with 20 µl l^{-1} ethylene, 50 µl l^{-1} NBD, or 50 µl l^{-1} NBD plus 300 µl l^{-1} ethylene for the 24-h period before harvest. The pea ACO and ACS cDNA clones *Ps-ACO1* (Peck et al. 1993) and *Ps-ACS1* (Peck and Kende 1998), respectively, were used as probes to hybridize the RNA blots containing 20 µg total RNA per lane. The 18S rRNA signals were used as a loading standard and for the normalization of quantified *Ps-ACO1* and *Ps-ACS1* mRNA signals during PhosphoImager analyses

Ps-ACO1 mRNA accumulation preceded measurable ACO enzyme activity, and the further accumulation in the embryonic axis exhibited the same pattern as the ACO enzyme activity. No appreciable induction of *Ps-ACO1* mRNA was detected in the cotyledons, with the exception of the comparatively weak signals at the late time points (Figs. 1B,2B).

The time course presented in Fig. 1C shows that ethylene evolution was associated almost exclusively with the embryonic axis, whereas only background values were measured with the cotyledons. The embryonic axis exhibited an early onset of ethylene evolution at ca. 15–20 h, a ca. 30-fold maximal rate at ca. 48 h, and a decline thereafter. In summary, separate measurements in embryonic axes and cotyledon tissues, respectively, demonstrated that ethylene evolution is associated with the sequential and tissue-specific induction of ACC biosynthesis, *Ps-ACO1* mRNA, ACO enzyme activity, and ethylene production localized in the embryonic axis.

Regulation of embryonic-axis-specific ACO expression by endogenous ethylene. Ethylene induces ACO in the first internode of etiolated pea seedlings (Peck and Kende 1995). To determine if ACO is regulated by endogenous ethylene during the germination of pea, seeds were treated with combinations of ethylene and the inhibitor of ethylene action, NBD. Table 1 and Fig. 2A show that treatment with ethylene alone caused a 1.6- and 4.1-fold increase in *Ps-ACO1* mRNA, and a 1.2- and 2.5-fold increase in ACO

Table 1. Effect of ethylene and NBD treatment on the accumulation of ACO enzyme activity, *Ps-ACO1* and *Ps-ACS1* mRNA, ACC and MACC in the embryonic axis of germinating pea seeds 48 h and 72 h after the start of imbibition

Time	Treatment[a]	ACO activity[b]	mRNA content[c]		Metabolite content[d]	
			Ps-ACO1	*Ps-ACS1*	ACC	MACC
48 h						
	Control	74.7 ± 6.7	77	23	29 ± 3	158 ± 10
	Ethylene	90.3 ± 8.3	119	25	27 ± 2	127 ± 12
	NBD	39.7 ± 3.3	45	30	161 ± 12	242 ± 17
	Ethylene + NBD	91.4 ± 2.8	96	25	33 ± 2	101 ± 7
72 h						
	Control	26.4 ± 2.1	38	23	25 ± 2	137 ± 7
	Ethylene	65.3 ± 6.3	154	28	23 ± 2	107 ± 12
	NBD	6.6 ± 0.9	17	24	111 ± 10	201 ± 15
	Ethylene + NBD	24.3 ± 1.5	56	23	26 ± 3	138 ± 9

[a]Entire seeds were treated as indicated, with 20 μl l^{-1} ethylene or 50 μl l^{-1} NBD or 300 μl l^{-1} ethylene plus 50 μl l^{-1} NBD. Embryonic axes were dissected out from the imbibed seeds at 48 h and 72 h

[b]Mean in-vitro ACO activities [pkat (g DW)$^{-1}$] ± SE in at least three protein extracts

[c]mRNA contents are expressed as PhosphoImager units of RNA-blot hybridization signals normalized to the signals obtained with 18S rRNA used as a loading standard. The arbitrary values can not be compared between *Ps-ACO1* and *Ps-ACS1*. Equal amounts of RNA (20 μg) were loaded in each lane

[d]Mean ACC or MACC contents [nmol (g DW)$^{-1}$] ± SE in at least three extracts. The amount of conjugated ACC in the ethanol extracts was assumed to be MACC

activities in the embryonic axes at 48 h and 72 h, respectively. 2,5-Norbornadiene substantially inhibited this induction and its effect was completely reversed by simultaneous treatment with ethylene. Ethylene did not induce *Ps-ACO1* mRNA (Fig. 2B) and ACO activity (data not shown) in the cotyledons, with the exception of an appreciably minor effect at the 72-h time point. The pea cDNA clones *Ps-ACS1* and *Ps-ACS2* (Peck and Kende 1998) were used as probes during Northern blot analyses and detected *Ps-ACS1* mRNA (Fig. 2, Table 1), but not *Ps-ACS2* mRNA, in the embryonic axis at 48 h and 72 h. The *Ps-ACS1* mRNA signals were substantially weaker than the *Ps-ACO1* mRNA signals; not detected during the early phase in the embryonic axis; and not evident at any time in the cotyledons. Treatment with ethylene, NBD, or ethylene plus NBD did not appreciably affect the *Ps-ACS1* transcript levels. In contrast to *Ps-ACO1* mRNA and ACO enzyme activity, ethylene treatment did not affect the ACC contents of the embryonic axis at 48 h and 72 h (Table 1). Treatment with NBD caused a ca. 5-fold increase in the ACC content at these time points, and simultaneous treatment with ethylene completely reversed the NBD effect. 2,5-Norbornadiene caused a comparably smaller increase of the MACC contents, which was reversed by ethylene (Table 1). These findings indicate that overall ACC biosynthesis by ACS is not a target of up-regulation by endogenous ethylene during pea seed germination.

The inducing effect of ethylene on ACO was confined to the late phase of germination. Ethylene did not affect the levels of *Ps-ACO1* mRNA (Fig. 2A), ACO activity, and ACC at the early time point of 24 h. The 24-h ACO activities [pkat (g DW)$^{-1}$] in the embryonic axis were 24.5 ± 2.3 (control) and 23.7 ± 2.0 (ethylene), and the ACC contents [nmol (g DW)$^{-1}$] were 24 ± 2 (control) and 22 ± 2 (ethylene). Thus, endogenous ethylene does not regulate the temporal and spatial pattern of ACO expression during pea seed germination, but it enhances its own biosynthesis in the embryonic axis through a positive feedback loop on the *Ps-ACO1* gene.

Discussion

Embryonic-axis-specific localization of ACC formation and oxidation during pea seed germination. Seed germination of the pea cultivars 'Alaska' and 'Frijaune', i.e. radicle protrusion through the seed coat, is accompanied by an early increase in ethylene evolution (Gorecki et al. 1991; Petruzzelli et al. 1995). Earlier we found for the 'Frijaune' cultivar that the embryonic axis is the source of ethylene evolution (Petruzzelli et al. 1994). Our present results confirm this for the 'Espresso generoso' cultivar of pea and demonstrate that the sequential induction of the ethylene biosynthetic pathway is localized almost exclusively in the embryonic axis. The fleshy storage cotyledons, which make up most of the seed's volume and weight, do not, or only to a negligible degree, contribute to ethylene evolution during pea seed germination. We found that an early onset of ACC accumulation in the embryonic axis, was sequentially followed by the accumulation of *Ps-ACO1* mRNA, ACO activity, and ethylene evolution. An alternate fate of free ACC is its conjugation, which serves as a control mechanism for limiting ACC availability for ethylene formation (Amrhein et al. 1981; Fluhr and Mattoo 1996). The simultaneous accumulation of free and conjugated ACC in the embryonic axis of germinating pea seeds excludes the possibility that ACC is released from MACC stored in the dry seed, and strongly indicates that ACC biosynthesis in the embryonic axis is due to increased ACS enzyme activity. Very low, roughly constant amounts of free and conjugated ACC were observed in the cotyledons. These low amounts

exhibited a slight tendency towards a decrease and an increase of free and conjugated ACC, respectively, suggesting biosynthesis of MACC. Thus, the absence of free ACC due to conjugation could explain why there was no ethylene production associated with the cotyledons and supports the finding that malonylation of ACC can regulate ethylene production in seed tissues (Hoffman et al. 1983). The cDNA clones *Ps-ACS1* and *Ps-ACS2* of pea represent homologs of tomato class-III and class-II ACS, respectively (Peck and Kende 1995, 1998; Oetiker et al. 1997). We detected the transcripts of *Ps-ACS1*, but not of *Ps-ACS2* in the embryonic axis of germinating pea seeds. Using the ACO cDNA from etiolated pea seedlings (Peck et al. 1993) as a probe, we found accumulation of *Ps-ACO1* mRNA in the embryonic axis of germinating pea seeds. Accumulation of *Ps-ACO1* transcripts started at ca. 8 h, and was followed by increasing levels of ACO activity and ethylene evolution starting at 15–20 h. Maximal accumulation of *Ps-ACO1* mRNA, ACO activity, and ethylene evolution was detected when radicle emergence was complete at ca. 50 h. A similar temporal and spatial pattern of ethylene evolution and accumulation of ACO activity was found in germinating seeds of *C. arietinum* (Munoz De Rueda et al. 1995; Gómez-Jiménez et al. 1998). In *C. arietinum*, the embryonic axis was also the main producer of ethylene during germination and a cDNA clone (*caaco*) encoding ACO was isolated. Accumulation of *caaco* transcripts in the embryonic axis parallelled the increase in ACO enzyme activity, and the deduced amino acid sequence of this *C. arietinum* ACO is 86% identical to the sequence of *Ps-ACO1* (Gómez-Jiménez et al. 1998). Taken together, these findings suggest that (i) the induction of both rate-limiting steps of ethylene biosynthesis, namely ACC formation and oxidation, occur almost exclusively in the embryonic axis, and (ii) the induction of a *Ps-ACO1*-type of ACO accounts for most, if not all, of the ACO activity in the embryonic axis of germinating legume seeds.

Positive feedback regulation by ethylene of ACO expression during pea seed germination. Our most important finding is that endogenous ethylene promotes its own biosynthesis in the embryonic axis of germinating pea seeds through a positive feedback loop that enhances ACO. Treatment of germinating seeds with ethylene caused elevated levels of *Ps-ACO1* mRNA and ACO activity at the late time points of 48 and 72 h, but did not affect the amounts of ACC and the levels of *Ps-ACS1* mRNA. Treatment with NBD reduced levels of *Ps-ACO1* mRNA and ACO activity and caused dramatically increased ACC levels. Simultaneous treatment of seeds with NBD plus an excess of ethylene reversed the effects of NBD on *Ps-ACO1* mRNA, ACO activity, and ACC content. It is important to note that the ACC content did not decrease in response to treatment with ethylene, which strongly suggests that increasing ethylene evolution does not down-regulate overall ACS activity below the levels found during germination under control conditions. The increase in the ACC content upon inhibition of ethylene signalling is at least in part due to down-regulation of *Ps-ACO1* expression, but a negative feedback loop of ethylene on the expression of unknown ACS genes or posttranscriptional effects on ACS are likely to play an additional role (Kim et al. 1997; Oetiker et al. 1997; Nakatsuka et al. 1998). We conclude that endogenous ethylene enhances its own biosynthesis in the embryonic axis during the late phase of pea seed germination through a positive feedback loop that promotes *Ps-ACO1* gene expression. So far, such an autoregulatory mechanism for ethylene biosynthesis has not been described for germinating seeds, but has been demonstrated for a number of vegetative tissues, seedlings and ripening fruits (reviews: Fluhr and Mattoo 1996; Johnson and Ecker 1998). In agreement with the situation in pea seeds, endogenous ethylene induces *Ps-ACO1* mRNA and ACO activity in the first internode of 5- to 6-d-old etiolated pea seedlings (Peck and Kende 1995; Peck et al. 1998). Putative ethylene receptors and several components of the ethylene signalling pathway have been identified (Johnson and Ecker 1998). Far less is known about the developmental regulation of ethylene-responsiveness. Peck et al. (1998) demonstrated that the abundance of the *Ps-ACO1* transcript is highly localized to the inner, concave apical hook region of etiolated pea seedlings. Its abundance, but not its spatial distribution is regulated by ethylene. The *Ps-ACO1* transcript level can therefore serve as a molecular marker for both ethylene formation and ethylene responsiveness. In germinating pea seeds, the inducing effect of ethylene on ACO was confined to the late phase of germination. Treatment of imbibed pea seeds with ethylene did not affect the levels of *Ps-ACO1* mRNA, ACO activity, and ACC content at the early time point of 24 h. Thus, ethylene does not regulate the temporal and spatial pattern of ACO expression, but enhances its own biosynthesis in the embryonic axis through a positive feedback loop on ACO during the late phase of seed germination. This indicates that ethylene-independent signalling pathways are involved in the initial induction of ethylene biosynthesis in the early phase, and that the early phase of seed germination is characterized by low ethylene-responsiveness. Such a temporal pattern of ethylene-responsiveness during germination is supported by binding studies of ethylene with seeds of pea and other species (Sisler 1984), and by the transcript expression patterns in tomato seeds of three genes of the ethylene receptor family (Lashbrook et al. 1998). Furthermore, the accumulation of an ethylene-regulated class-I β-1,3-glucanase in the embryonic axis was confined to the late phase of pea seed germination (Petruzzelli et al. 1999). There is evidence that high-level induction of class-I β-1,3-glucanase during the germination of tobacco seeds depends on ethylene-induced transcription mediated by 'ethylene-responsive element binding proteins' (EREBPs), whereas ethylene-independent signalling pathways determine spatial and temporal patterns of expression (Leubner-Metzger et al. 1998). The EREBPs are transcription factors believed to be targets of ethylene-dependent signal transduction and bind to the AGCCGCC sequence (GCC box) present in the promoter regions of many pathogenesis-related proteins (Ohme-Takagi and Shinshi 1995). Interestingly,

sequences that are homologous to the GCC box are also present in the promoters of some ACO genes (Tang et al. 1993; Bouquin et al. 1997; Johnson and Ecker 1998). The elucidation of the up- and downstream components of the ethylene signal transduction pathway involved in the regulation of the positive feedback loop that enhances ethylene biosynthesis during seed germination will be the focus of future experiments.

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References

Amrhein N, Schneebeck D, Skorupka H, Tophof S, Stöckigt J (1981) Identification of a major metabolite of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid in higher plants. Naturwissenschaften 68: 619–620

Barry CS, Blume B, Bouzyen M, Cooper W, Hamilton AJ, Grierson D (1996) Differential expression of the 1-aminocyclopropane-ı-carboxylate oxidase gene family of tomato. Plant J 9: 525–535

Blume B, Grierson D (1997) Expression of ACC oxidase promoter-GUS fusions in tomato and *Nicotiana plumbaginifolia* regulated by developmental and environmental stimuli. Plant J 12: 731–746

Boller T, Herner RC, Kende H (1979) Assay for and enzymatic formation of an ethylene precursor, 1-aminocyclopropane-1-carboxylic acid. Planta 145: 193–303

Bouquin T, Lasserre E, Pradier J, Pech JC, Balague C (1997) Wound and ethylene induction of the ACC oxidase melon gene CM-ACO1 occurs via two direct and independent transduction pathways. Plant Mol Biol 35: 1029–1035

Cervantes E, Rodriques A, Nicolas G (1994) Ethylene regulates the expression of a cysteine proteinase gene duringh germination of chickpea (*Cicer arietinum* L.). Plant Mol Biol 25: 207–215

Fluhr R, Mattoo AK (1996) Ethylene – biosynthesis and perception. Critical Rev Plant Sci 15: 479–523

Gómez-Jiménez MC, Matilla AJ, Garrido D (1998) Isolation and characterization of a cDNA encoding an ACC oxidase from *Cicer arietinum* and its expression during embryogenesis and seed germination. Aust J Plant Physiol 25: 765–773

Gorecki RJ, Ashino H, Satoh S, Esahi Y (1991) Ethylene production in pea and cocklebur seeds of differing vigour. J Exp Bot 42: 407–414

Hoffman NE, Fu JR, Yang SF (1983) Identification and metabolism of 1-(malonylamino)-cyclopropane-1-carboxylic acid in germinating peanut seeds. Plant Physiol 71: 197–199

Johnson PR, Ecker JR (1998) The ethylene gas signal transduction pathway: a molecular perspective. Annu Rev Genet 32: 227–254

Kepczynski J, Kepczynska E (1997) Ethylene in seed dormancy and germination. Physiol Plant 101: 720–726

Kim JH, Kim WT, Kang BG, Yang SF (1997) Induction of 1-aminocyclopropane-1-carboxylate oxidase mRNA by ethylene in mung bean hypocotyls: involvement of both protein phosphorylation and dephosphorylation in ethylene signaling. Plant J 11: 399–405

Lashbrook CC, Tieman DM, Klee HJ (1998) Differential regulation of the tomato ETR gene family throughout plant development. Plant J 15: 243–252

Leubner-Metzger G, Petruzzelli L, Waldvogel R, Vögeli-Lange R, Meins F, Jr. (1998) Ethylene-responsive element binding protein (EREBP) expression and the transcriptional regulation of class I β-1,3-glucanase during tobacco seed germination. Plant Mol Biol 38: 785–795

Lizada MCC, Yang SF (1979) A simple and sensitive assay for 1-aminocyclopropane-1-carboxylic acid. Anal Biochem 100: 140–145

Malerba M, Crosti P, Armocida D, Bianchetti R (1995) Activation of ethylene production in *Acer pseudoplatanus* L. cultured cells by fusicoccin. J Plant Physiol 145: 93–100

Munoz De Rueda P, Gallardo M, Matilla AJ, Sánchez-Calle IM (1995) Preliminary characterization of 1-aminocyclopropane-1-carboxylate oxidase properties from embryonic axis of chickpea (*Cicer arietinum* L.) seeds. J Exp Bot 46: 695–700

Nakatsuka A, Murachi S, Okunishi H, Shiomi S, Nakano R, Kubo Y, Inaba A (1998) Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, 1-aminocyclopropane-1-carboxylate oxidase, and ethylene receptor genes in tomato fruit during development and ripening. Plant Physiol 118: 1295–1305

Oetiker JH, Olson DC, Shiu OY, Yang SF (1997) Differential induction of seven 1-aminocyclopropane-1-carboxylate synthase genes by elicitor in suspension cultures of tomato (*Lycopersicon esculentum*). Plant Mol Biol 34: 275–286

Ohme-Takagi M, Shinshi H (1995) Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. Plant Cell 7: 173–182

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

Peck SC, Kende H (1998) Differential regulation of genes encoding 1-aminocyclopropane-carboxylate (ACC) synthase in etiolated pea seedlings: effects of indole-3-acetic acid, wounding, and ethylene. Plant Mol Biol 38: 977–982

Peck SC, Olson DC, Kende H (1993) A cDNA sequence encoding 1-aminocyclopropane-1-carboxylate oxidase from pea. Plant Physiol 101: 689–690

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

Petruzzelli L, Harren F, Perrone C, Reuss J (1995) On the role of ethylene in seed germination and early growth of *Pisum sativum*. J Plant Physiol 145: 83–86

Petruzzelli L, Harren F, Reuss J (1994) Patterns of C_2H_4 production during germination and seedling growth of pea and wheat as indicated by a laser-driven photoacoustic system. Environ Exp Bot 34: 55–61

Petruzzelli L, Kunz C, Waldvogel R, Meins F, Jr., Leubner-Metzger G (1999) Distinct ethylene- and tissue-specific regulation of β-1,3-glucanases and chitinases during pea seed germination. Planta 209: 195–201

Schmidt-Puchta W, Kütemeier G, Günther I, Haas B, Sänger HL (1989) Cloning and sequence analysis of the 18S ribosomal RNA gene of tomato and a secondary structure model of the 18S rRNA of angiosperms. Mol Gen Genet 219: 17–25

Sisler EC (1984) Distribution and properties of ethylene-binding component from plant tissue. In: Fuchs Y, Chalutz E (eds) Ethylene: biochemical, physiological and applied aspects. Martinus Nijhoff/Dr. W. Junk Publishers, The Hague, The Netherlands, pp 45–54

Sisler EC, Pian A (1973) Effect of ethylene and cyclic olefins on tobacco leaves. Tob Sci 17: 68–72

Tang X, Wang H, Brandt AS, Woodson WR (1993) Organization and structure of the 1-aminocyclopropane-1-carboxylate oxidase gene family from *Petunia hybrida*. Plant Mol Biol 23: 1151–1164