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Calcium requirement for ethylene-dependent responses involving 1-aminocyclopropane-1-carboxylic acid oxidase in radicle tissues of germinated pea seeds*

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ABSTRACT

The Ca^{2+} requirements of ethylene-dependent responses were investigated in germinating seeds of *Pisum sativum* L. using 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (*Ps-ACO1*), ACC synthase (*Ps-ACS2*) and class I β-1,3-glucanase as molecular markers. Ethylene biosynthesis and responsiveness are localized to the elongation and differentiation zones of the pea radicle. Ethylene treatment induced ectopic root hair formation in the cell elongation zone and promoted root hair elongation growth in the radicles of germinated seeds. Characterized Ca^{2+} antagonists, including EGTA, lanthanum, verapamil, ruthenium red, W-7, lithium and neomycin, were used to test for the involvement of the apoplastic and the intracellular Ca^{2+}-pool, the Ca^{2+}/calmodulin complex and the phoshoinositide (PI) cycle in the ethylene responses. Ca^{2+} release from internal pools, but no appreciabe apoplastic Ca^{2+}, is involved in the transcriptional induction by ethylene of *Ps-ACO1* and in ectopic root hair formation in the radicle elongation zone of germinated pea seeds. Furthermore, the Ca^{2+}/calmodulin complex and the PI cycle seem to be involved in these ethylene responses. In contrast, both the intracellular and the apoplastic Ca^{2+}-pools are required for the negative and positive ethylene responses to the gene expression of *PS-ACS2* and class I β-1,3-glucanase, respectively; and, apoplastic Ca^{2+} also promotes root hair elongation growth. Tissues from adult plants and germinating seeds exhibit temporal and spatial differences in the signal/response coupling by Ca^{2+} of ethylene-regulated processes.

Key-words: *Pisum*; 1-aminocyclopropane-1-carboxylic acid oxidase; calcium signalling; embryonic root; ethylene signalling; root hair development.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; CaM, calmodulin; dz, differentiation zone; E, ethylene; EGTA, ethylene glycol-bis (β-aminoethyl ether)-*N*, *N*, *N′*, *N′*-tetra acetic acid; ez, elongation zone; NBD, 2,5-norbornadiene; Neo, neomycin; PI, phosphoinositide; RR, ruthenium red; rt, root tip; Ver, verapamil.

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**Dedicated to Nikolaus Amrhein, Zürich, on the occasion of his 60th birthday.*

INTRODUCTION

Neljubow (1901) discovered a reduction in elongation, a swelling of the shoot, and a change in the direction of growth as dramatic effects of ethylene treatment on etiolated pea seedlings. In addition to this so-called 'triple response', ethylene causes an intensified curvature of the apical hook and has pronounced effects on the root of etiolated dicot seedlings. Extensive progress has been made in elucidating the mechanisms of ethylene biosynthesis and signal transduction that constitute the molecular basis for the ethylene action on shoot development (Bleecker & Kende 2000). The gaseous plant hormone ethylene turned out to be an important part of a signalling network that regulates plant development and responses to environmental cues. This occurs in interactions with the transduction pathways for other plant hormones, for calcium (Ca^{2+}), and for tissue-specific factors. A major breakthrough was the identification of *ETR1* as an ethylene receptor of *Arabidopsis thaliana* (Bleecker *et al*. 1988). *ETR1* and other ethylene receptor genes turned out to be members of a multigene family with homology to bacterial two-component response regulators (Bleecker & Kende 2000; Lohrmann & Harter 2002). Downstream components of the ethylene-signalling pathway include *CTR1*, a negative regulator of the ethylene responses, which is closely related to Raf-like protein kinases. Such kinases are known to be the starting points for MAPK (mitogen-activated protein kinase) cascades, which provide signal amplification by protein phosphorylation. Protein phosphorylation and dephosphorylation events are important for the transduction of the ethylene signal (e.g. Raz & Fluhr 1993; Kim *et al*. 1997; Kwak & Lee 1997). The ethylene-inducible PK12 kinase is a member of the LAMMER class of protein kinases that phosporylate and physically interact with serine/arginine-rich splicing factors to alter their activity and the splicing of target mRNAs (Sessa *et al*. 1996; Savaldi-Goldstein,

Sessa & Fluhr 2000). Downstream of the signal transduction pathways are ethylene-regulated transcription activators or repressors, for example, the ethylene-responsive element binding proteins (Bleecker & Kende 2000; Ohta, Ohme-Takagi & Shinshi 2000). These transcription factors bind to the GCC box sequences present in ethylene-responsive promoter regions of many ethylene-regulated genes including class I β-1,3-glucanases (e.g. Leubner-Metzger *et al*. 1998) and 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO) (e.g. Tang *et al*. 1993; Bouquin *et al*. 1997). ACO and ACC synthase (ACS), which are the two key enzymes of ethylene biosynthesis, are encoded by multigene families, and exhibit differential and complex regulation by ethylene itself and by other developmental, hormonal and environmental factors (e.g. Barry *et al*. 1996; Oetiker *et al*. 1997; Peck & Kende 1998; Petruzzelli, Coraggio & Leubner-Metzger 2000). Ethylene induces the *Ps-ACO1* gene in the first internode of etiolated pea seedlings and its transcript is highly localized in the inner, concave apical hook (Peck & Kende 1995; Peck, Pawlowski & Kende 1998). Its abundance, but not its spatial distribution is regulated by ethylene and the *Ps-ACO1* transcript level can therefore serve as a molecular marker for both, ethylene formation and ethylene sensitivity. The primary factor in apical hook formation of dark-grown pea seedlings appears to be ethylene (Peck *et al*. 1998; Du & Kende 2001). Finally, Ca^{2+} interacts with ethylene signalling in shoots. Pharmacological experiments with Ca^{2+} antagonists suggest that apoplastic Ca^{2+} is required for the induction of ACO by ethylene in etiolated internodes of pea seedlings (Kwak & Lee 1997) and for the ethylene-induced shortening of pea epicotyls (Berry *et al*. 1996).

Far less is known on the molecular level about ethylene formation and ethylene sensitivity of roots and about the involvement of tissue-specific and Ca^{2+}-dependent factors with ethylene signalling in roots of etiolated seedlings (reviews: Bleecker & Kende 2000; Stevenson *et al*. 2000; Anil & Rao 2001). In *Arabidopsis thaliana*, Ca^{2+} mobilization via the phosphoinositide (PI) cycle seems to be involved in ethylene-regulation of ACS (Liang, Shen & Theologis 1996), but entire seedlings were used in this study and therefore it remains to be elucidated whether this regulation is localized in the shoot or the root tissues. Ethylene and Ca^{2+} are known to be positive regulators of root hair formation and growth in *A. thaliana* (e.g. Tanimoto, Roberts & Dolan 1995; Pitts, Cernac & Estelle 1998; Cao *et al*. 1999; Gilroy & Jones 2000). These studies utilized ethylene signal transduction mutants, but the effects of ethylene and Ca^{2+} on ACO and ACS gene expression were not investigated in root tissue of *A. thaliana.* The effects of ethylene and Ca^{2+} on ACO and ACS gene expression has been studied in root tissue of mung bean plants (Jung *et al*. 2000), but the physiological effects of ethylene and Ca^{2+} on root and root hair development were not investigated by these authors. Thus, tissue-specific factors and Ca^{2+} signalling appear to be involved in ethylene-mediated responses and regulate the key genes of ethylene-biosynthesis in etiolated seedlings of several species including pea. In contrast to shoot-tissue, little is known on the molecular level about the interaction of Ca^{2+} and ethylene in regulating ethylene biosynthesis in root-tissue and in germinating seeds.

In our earlier studies we found tissue-specific and ethylene-promoted gene expression of *Ps-ACO1* and class I β-1,3-glucanase to be confined to the embryonic axis and absent from the cotyledon tissue during seed germination of *Pisum sativum* (Petruzzelli *et al*. 1999, 2000). Such a spatial confinement to the embryonic axis is also found for the production of ethylene and its precursor ACC, as well as for malonyl-ACC (Petruzzelli *et al*. 2000), the major conjugation product of ACC (Amrhein *et al*. 1981). In the present study, we demonstrate that the temporal and spatial regulation of *Ps-ACO1* and class I β-1,3-glucanase gene expression is associated with the cell differentiation and elongation zones of the radicle. These root tissues have the highest ethylene-responsiveness among the different tissues of germinating pea seeds and are the locations of ethylene-induced root hair development. Results obtained by using various pharmacological agents known to affect the intracellular and apoplastic levels of Ca^{2+} by different mechanisms provide evidence that Ca^{2+} release from internal stores is involved in the induction of *Ps-ACO1* and the simultaneous suppression of *Ps-ACS2* gene expression by ethylene signalling. Furthermore, the effects of intracellular Ca^{2+} on ethylene-induced *Ps-ACO1* expression and root hair development in radicles of germinated pea seeds seem to involve calmodulin (CaM) and the PI cycle.

MATERIALS AND METHODS

Plant material, germination conditions and treatment 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). After 48 h of imbibition, seeds were pre-incubated in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.5) for 2 h without or with various inhibitors [ethylene glycol-bis (β-aminoethyl ether)-*N*, *N*, *N*′, *N*′-tetra acetic acid (EGTA), verapamil (Ver), ruthenium red (RR), W-7, neomycin (Neo), $LaCl_3$, LiCl; Sigma, Poole, Dorset, UK] added, as indicated. This pre-incubation was followed by further incubation for 8 h without (control) or with a defined concentration of ethylene (usually 30 μL L^{-1}; Siad, Milano, Italy). The ethylene concentration introduced in the air phase was verified by gas chromatography (GC). After the 8 h incubation in the presence of both the inhibitor and ethylene, the embryonic axes were dissected, frozen in liquid nitrogen and stored at –80 °C for subsequent analyses.

Analysis of RNA

Frozen axes were ground in liquid nitrogen and total RNA was prepared using TRIzol (Gibco/BRL, Gaithersburg, MD, USA). RNA (equal amounts per lane; usually 20 μg)

was fractionated in 1% agarose-formaldehyde gels and blotted onto nylon membranes (Hybond-N; Amersham Pharmacia Biotech AB, Uppsala, Sweden). RNA-blot hybridization was performed with radiolabelled DNA probes prepared from the 1.1-kb *Eco*RI cDNA of pPE8 for *Ps-ACO1* (Peck, Olson & Kende 1993) and the approximately 2 kb *Eco*RI cDNA of the plasmid for *Ps-ACS2* (Peck & Kende 1998) and the hybridized membranes were washed at high stringency as described earlier (Petruzzelli *et al*. 2000). To ensure equal RNA loading, the gels were stained with ethidium bromide after electrophoresis to visualize the rRNA bands.

Analysis of proteins

The *in vivo* ACO activity was measured by incubating approximately 0.5 g of embryonic axes in 60 mL vials with 2 mL buffer (50 mM MES, pH 6.5) with or without 5 mM ACC. Preliminary results showed that 1 h uptake yielded the maximal ACC-induced ethylene production. After this period the vials were closed and 1 h later the ethylene concentration in the headspace was measured by GC (Petruzzelli *et al*. 2000). The procedures used for extracting proteins, protein determination and immunoblot analyses were as described earlier (Petruzzelli *et al*. 1999, 2000). The anti-apple ACO antibody is known to cross-react with the chick-pea homologue of pea *Ps-ACO1* expressed in chick-pea embryonic axes (Gómez-Jiménez, Garcia-Olivares & Matilla 2001) and was used to detect the highly homologous *Ps-ACO1* in pea embryonic axes (Dong *et al*. 1992; Peck *et al*. 1993). A horseradish peroxidase-conjugated secondary antibody was used for detection with a chemiluminescence immunoblot detection kit (Amersham, Pharmacia Biotech AB).

RESULTS

Ethylene inducibility of ACO gene expression during pea seed germination is localized to specific zones of the emerging radicle

We have shown that increasing ethylene evolution of germinating pea seeds is due to ethylene-enhanced ACO gene expression (Petruzzelli *et al*. 2000). In this earlier study a concentration of 20 μL L^{-1} ethylene was used during a 24 h treatment period and induced *Ps-ACO1* mRNA and ACO enzyme activity exclusively in the embryonic axis, but not in the cotyledons. Immunoblot analysis of pea embryonic axis extracts presented in Fig. 1a shows that a polyclonal antibody directed against apple ACO cross-reacts with a 36 kDa immunoreactive band. The amino acid sequences of pea, chick-pea and apple ACO are highly homologous; for example, chick-pea ACO shares 86% of amino acid sequence identity with the Ps-ACO1 of pea (Dong *et al*. 1992; Peck *et al*. 1993; Gómez-Jiménez *et al*. 2001). The anti-apple ACO antibody is also known to cross-react with the ACO of the embryonic axis of germinating chick-pea seeds (Gómez-Jiménez *et al*.

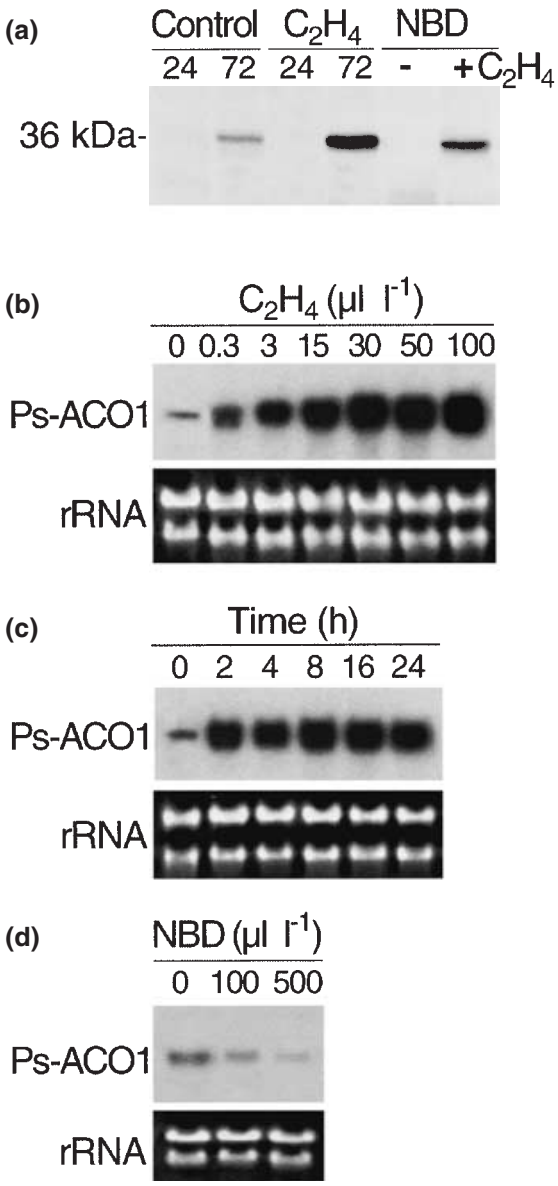

Figure 1. The effect of ethylene on the expression of the 36 kDa ACO antigen and of *Ps-ACO1* mRNA in the embryonic axis of pea seeds. (a) Immunoblot analysis of the 36 kDaA ACO antigen during the germination of pea seeds in the absence (Control) and presence of ethylene (C_2H_4) or NBD. Seeds were incubated 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} plus 300 μL L^{-1} ethylene for the 24 h period before harvest. The time in hours after the start of imbibiton is indicated. Equal amounts (10 μg protein per lane) of embryonic axes protein extracts was loaded. For corresponding protein samples, ACO enzyme activities and *Ps-ACO1* mRNA expression see Petruzzelli *et al*. (2000). (b–d) RNA blot analyses using the pea ACO cDNA clone *Ps-ACO1* (Peck *et al*. 1993) as a probe to hybridize with total RNA from pea embryonic axes (20 μg per lane; equal loading demonstrated by visualization of rRNA bands). The embryonic axes were dissected from pea seeds imbibed for 48 h followed subsequently by the indicated treatments. (b) Treatment for 8 h with the ethylene concentrations indicated. (c) Treatment with 30 μL L^{-1} ethylene for the times indicated. (d) Treatment for 8 h with the NBD concentrations indicated. A representative experiment from three independent experiments with the same result is presented.

2001). The spatial and temporal pattern of accumulation of the 36 kDa pea antigen during germination (Fig. 1a) correlated with *Ps-ACO1* mRNA and ACO enzyme activity (Petruzzelli *et al*. 2000). Treatment of germinating seeds with combinations of ethylene and the inhibitor of ethylene action, 2,5-norbornadiene (NBD) demonstrated that the 36 kDa pea antigen (Fig. 1a), as well as the *Ps-ACO1* mRNA and the ACO enzyme activity (Petruzzelli *et al*. 2000) of the embryonic axes, are regulated by endogenous ethylene. No other prominent bands were detected by the anti-ACO antibody and the apparent size of 36 kDa in the denaturing gel is in agreement with the calculated size from the sequence data for PS-ACO1 (Peck *et al*. 1993). Taken together, these findings are strong evidence that the 36 kDa pea antigen is the ethylene-inducible Ps-ACO1 protein that accumulates in the embryonic axis during germination.

Figures 1 and 2 show that the transcriptional induction of the *Ps-ACO1* gene in the embryonic axis of pea seeds is extremely ethylene-sensitive, fast and highly tissue-specific. Optimal responsiveness was observed in 48 h-imbibed seeds, which is just after radicle protrusion. Treatment with as little as 0.3 μL L^{-1} ethylene was sufficient for significant *Ps-ACO1* mRNA accumulation, and 15–30 μL L^{-1} ethylene caused a maximal response (Fig. 1b). A short treatment of only 2 h was sufficient for maximal *Ps-ACO1* transcript induction and accumulation in the embryonic axis (Fig. 1c). This induction was inhibited in a dose-dependent manner by exposing the seeds to NBD (Fig. 1d). Shoot- and root-tissue of the embryonic axis differed significantly with regard to the ethylene-responsiveness for *Ps-ACO1* gene induction. Ethylene treatment caused a massive accumulation of *Ps-ACO1* mRNA and 36 kDa ACO antigen in the radicle; but had only an appreciably weaker effect in the shoot of the embryonic axis (Fig. 2a). Within the radicle, ethylene-responsiveness for the *Ps-ACO1* gene induction was distributed as a gradient (Fig. 2b): highest in the cell differentiation zone (dz; above 6 mm) where the root hairs form, high in the cell elongation zone (ez; 2.5–6 mm); whereas the root tip (rt; 0–2.5 mm) exhibited low ethylene responsiveness. The ethylene-inducible class I β-1,3-glucanase of the pea embryonic axis (Petruzzelli *et al*. 1999) exhibited the same radicle-specific pattern of ethylene inducibility (data not shown). Thus, *Ps-ACO1* gene induction by ethylene during germination is a fast process, which is induced by minute amounts of ethylene and is mainly associated with the root-hair and elongation zones of the radicle.

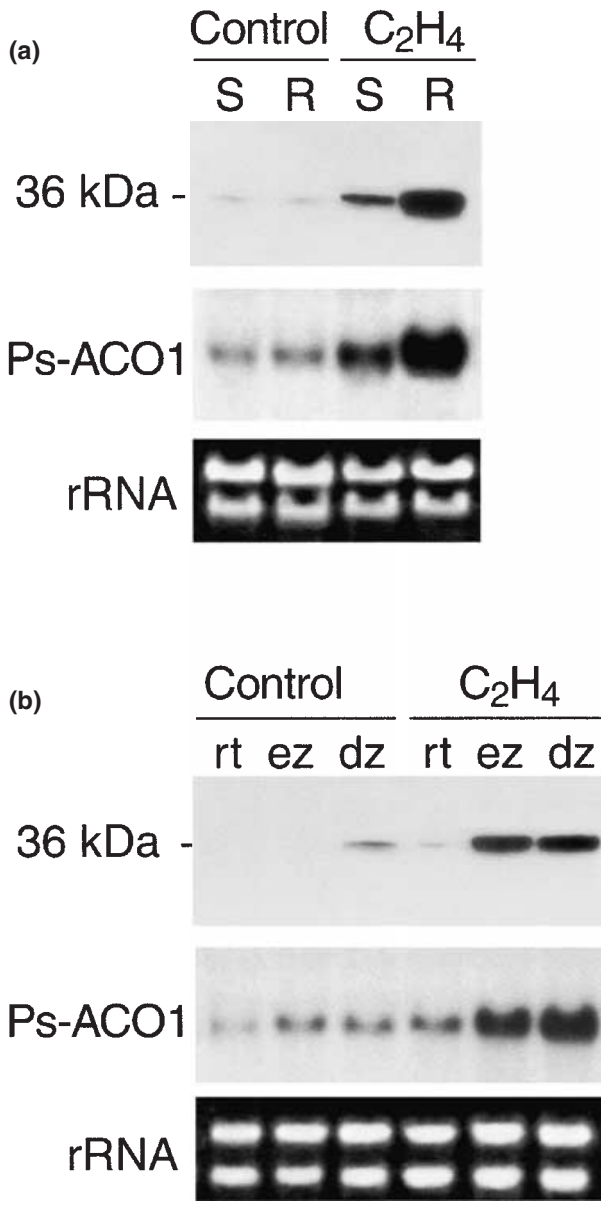

Figure 2. Tissue-specificity and ethylene responsiveness of the accumulation of the 36 kDa ACO antigen and of *Ps-ACO1* mRNA in the embryonic axes of pea seeds. The embryonic axes were dissected from pea seeds imbibed for 48 h followed subsequently by 8 h of treatment with 30 μL L^{-1} ethylene. Comparison of the ethylene-induction of *Ps-ACO1* in (a) shoot (S) and root (R) tissues and (b) the root tip (rt; 0–2.5 mm), cell elongation zone (ez; 2.5–6 mm), and cell differentiation zone (dz; > 6 mm) of the radicle. Immunoblot and RNA blot analyses were as described in Fig. 1.

Calcium requirement of ethylene-induced ACO gene expression in the radicle of germinated pea seeds

To investigate whether the ethylene-induced *Ps-ACO1* gene expression in the radicle of germinated pea seeds depends on Ca^{2+} we used characterized compounds, known to interfere with Ca^{2+} signalling at specific steps. These were added to the medium of 48 h-imbibed seeds and a 2 h pre-incubation with the compound alone was followed by a 8 h incubation in the presence of the compound plus treatment with 30 μL L^{-1} ethylene. The accumulation of transcripts, antigens and enzyme activities were subsequently analysed in the radicle tissue. To initially verify that Ca^{2+} by itself is not capable of mimicking the ethylene-effects we added up to 10 mM $CaCl_2$ to the seeds. The Ca^{2+} treatment (without

ethylene treatment) did not induce accumulation of *Ps-ACO1* mRNA (Fig. 3a), ACO 36 kDa protein and enzyme activity (Fig. 4) or class I β-1,3-glucanase (data not shown). Treatment with 5 mM EGTA, a membrane-impermeable Ca^{2+} chelator (Raz & Fluhr 1992), did not inhibit the ethylene-induced accumulation of *Ps-ACO1* mRNA (Fig. 3b) and of 36 kDa ACO protein and ACO enzyme activity (Fig. 4). Higher EGTA concentrations up to 10 mM also failed to inhibit the ethylene response on *Ps-ACO1* while causing toxic effects. The 5 mM concentration of EGTA interfered with three other ethylene responses in the radicle: (1) it inhibited the ethylene induction of class I β-1,3-glucanase accumulation (data not shown); (2) it induced *Ps-ACS2* transcript accumulation in the radicle of ethylene-treated seeds (Fig. 3b), whereas *Ps-ACS2* mRNA was almost undetectable in ethylene-treated and -untreated pea embryonic axes (Petruzzelli *et al*. 2000); and (3) it affected the root hairs, as described later. These results suggest that the apoplastic Ca^{2+} store is involved in the negative and positive ethylene response of *Ps-ACS2* and class I β-1,3-glucanase, respectively. In contrast, the apoplastic Ca^{2+} store appears not to be involved in the positive ethylene response of *Ps-ACO1* in the radicle of germinated pea seeds.

Lanthanum (La^{3+}) is known to compete externally with Ca^{2+} for channels located in the plasma membrane, but at high concentrations it also affects intracellular stores (e.g. Knight, Trewavas & Knight 1996; Leitner-Dagan & Weiss

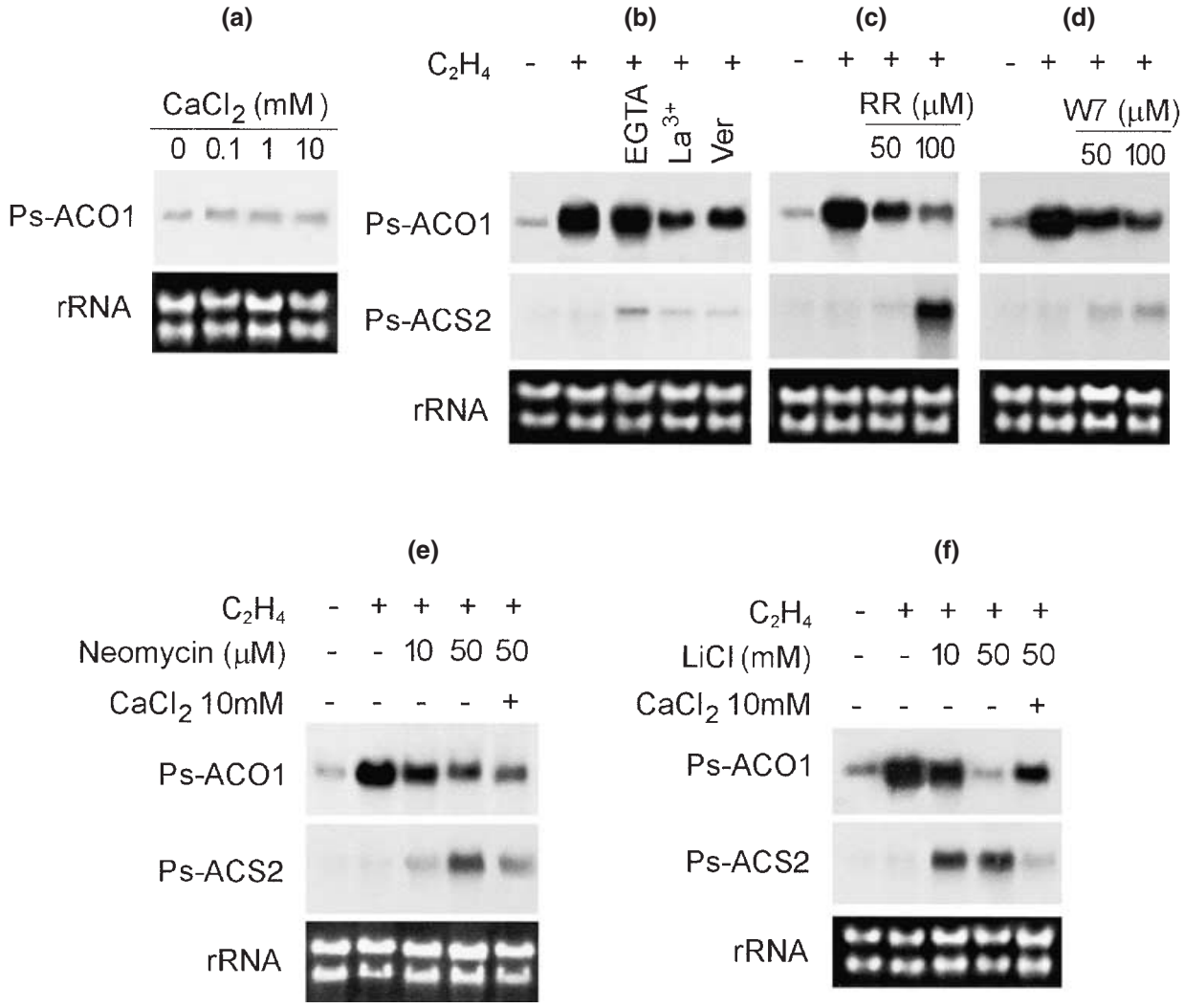

Figure 3. Effects of calcium antagonists on the regulation by ethylene of *Ps-ACO1* and *Ps-ACS2* transcript levels in the embryonic axes of germinated pea seeds. The 48 h-imbibed pea seeds were pre-incubated for 2 h in the presence or absence of the calcium antagonists followed by a further incubation for 8 h with 30 μL L^{-1} ethylene in presence or absence of the calcium antagonists. Subsequent RNA blot analyses was as described in Fig. 1. The effects of the following substances were investigated, as indicated: (a) different $CaCl_2$ concentrations without ethylene treatment. (b) Ethylene plus the membrane-impermeable Ca^{2+} chelator EGTA (5 mM) or the Ca^{2+} channel inhibitors $LaCl_3$ (1 mM) and verapamil (Ver, 100 μM). (c) Ethylene plus the Ca^{2+} channel inhibitor ruthenium red (RR). (d) Ethylene plus the CaM-binding inhibitor W-7. (e, f) Ethylene plus the inhibitors of the PI cycle neomycin and LiCl. A representative experiment from three independent experiments with the same result is presented.

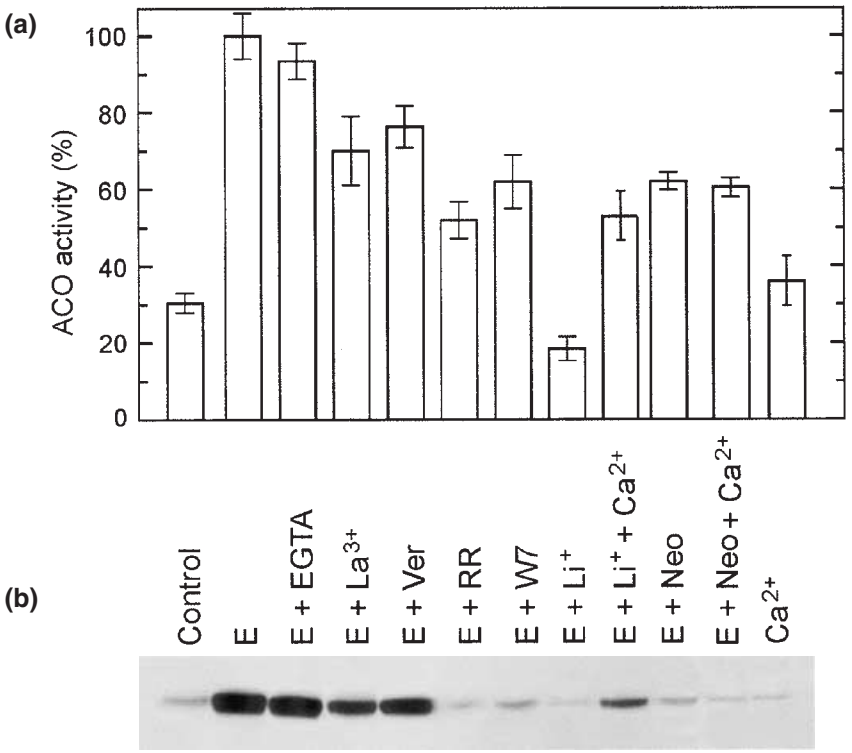

Figure 4. Effects of calcium antagonists on the ethylene-mediated induction in the radicle of germinated pea seeds of (a) *in vivo* ACO enzyme activity and (b) 36 kDa ACO antigen accumulation. The 48 h-imbibed pea seeds were pre-incubated for 2 h in the presence or absence of the calcium antagonists followed by a further incubation for 8 h exposure to 30 μL L^{-1} ethylene in the presence or absence of the calcium antagonists as described in Fig. 3; RR (100 μM), W-7 (100 μM), Li^+ (50 μM), Ca^{2+} (10 mM), Neo (50 μM). Subsequently the *in vivo* ACO of embryonic axes was measured and immunoblot analyses was performed with protein extracts from radicles as described in Fig. 1.

1999); verapamil (Ver) is known as a blocker of L-type Ca^{2+} channels in animal cells and also inhibits Ca^{2+} channels located in the tonoplast of plant root cells (Anil & Rao 2001); and ruthenium red (RR) is known as an irreversible inhibitor of plasma membrane Ca^{2+} channels, but also specifically blocks intracellular Ca^{2+} channels (e.g. Pineros & Tester 1997; Anil & Rao 2001). Treatment of imbibed pea seeds with 1 mM La^{3+}, 100 μM Ver or 100 μM RR reduced the ethylene-induced accumulation of *Ps-ACO1* mRNA (Fig. 3b & c). RR inhibited the accumulation of the 36 kDa ACO protein and of ACO enzyme activity in the radicle significantly, whereas the effects of La^{3+} and Ver were smaller (Fig. 4). As with EGTA, treatment with La^{3+}, Ver or RR induced *Ps-ACS2* transcript accumulation (Fig. 3b & c). These effects on *Ps-ACO1* and *Ps-ACS2* were strong for RR and weak for La^{3+} and Ver. The La^{3+} and RR, but not Ver, inhibited the ethylene-induction class I β-1,3-glucanase accumulation (data not shown). The CaM-binding inhibitor W-7 (Jung *et al*. 2000) was used to examine the involvement of the Ca^{2+}/CaM complex in ethylene signalling. Treatment with 100 μM W-7 inhibited ethylene-induced *Ps-ACO1* mRNA (Fig. 3d), 36 kDa ACO protein and ACO enzyme activity (Fig. 4) accumulation in the pea radicle. The W-7 slightly induced *Ps-ACS2* mRNA accumulation (Fig. 3d) and markedly inhibited class I β-1,3-glucanase accumulation (data not shown). Taken together with the effects of EGTA, these results suggest that intracellular Ca^{2+} interferes with ethylene signal transduction in the radicle of germinating pea seeds and can, at the same time, mediate induction of the *Ps-ACO1* gene and suppression of the *Ps-ACS2* gene.

Possible involvement of Ca^{2+} and the phosphoinositide cycle in the ethylene induction of *Ps-ACO1*

Lithium (Li^+) and neomycin are two inhibitors of the PI cycle and are known to interfere with Ca^{2+} effects on ethylene signalling (e.g. Liang *et al*. 1996; Jung *et al*. 2000; Stevenson *et al*. 2000). Neomycin or Li^+ treatment decreased the ethylene-induced accumulation of *Ps-ACO1* mRNA (Fig. 3e & f), 36 kDa ACO protein and ACO enzyme activity (Fig. 4) in the radicle of germinated pea seeds. As little as 10 μM neomycin was already effective and the inhibitory effect of neomycin was not suppressed by simultaneous addition of Ca^{2+}. Whereas 10 mM LiCl had only a weak effect, the ethylene response was almost completely abolished by 50 mM LiCl. In contrast to neomycin, the addition of 10 mM Ca^{2+} partially reversed the inhibitory effect of Li^+. Treatment with neomycin or Li^+ induced the accumulation of *Ps-ACS2* mRNA, and this effect was reversed by simultaneous addition of Ca^{2+} (Fig. 3e & f). Li^+ also inhibited the induction of class I β-1,3-glucanase and this effect was partially reversed by Ca^{2+} (data not shown). These findings suggest that the PI cycle is involved in Ca^{2+} effects on ethylene signalling.

Effects of Ca^{2+} antagonists on ethylene-induced root hair formation of pea radicles

The molecular effects of the Ca^{2+} antagonists and the spatial association of ethylene biosynthesis and responsiveness with the root hair and elongation zones prompted us to

investigate Ca^{2+} effects on ethylene-regulation of radicle morphology. The short ethylene treatment period of 8 h caused a slight reduction in radicle length of the 48 h-germinated seeds, which can be partially reversed by simultaneous treatment with NBD or Ca^{2+} antagonists (data not shown). However, pronounced effects of the 8 h treatment with ethylene were obvious on root hair formation and root hair elongation growth (Fig. 5). Root hair formation in untreated pea radicles is predominantly localized within an 6–13 mm zone from the apex. Root hairs are present in the cell differentiation zone (dz), but not the cell elongation zone (ez) of the control radicles presented in Fig. 5a, but are only visible in the 8 h-incubated radicles at higher magnification. Ethylene treatment caused enhanced root hair formation and enhanced root hair elongation growth and root hairs in ethylene-treated radicles were localized within a 3–13 mm zone (ez and dz) from the apex (Fig. 5b). These effects of ethylene treatment were reversed by simultaneous treatment with NBD (Fig. 5c). Thus, ethylene induces root hair formation also at ectopic places, for example, in the cell elongation zone (ez), and enhances root hair elongation growth. Ethylene-induced ectopic root hair formation in the elongation zone was not inhibited by EGTA, but EGTA inhibited root hair elongation growth. Treatment with Ca^{2+} alone did not induce ectopic root hair formation in the elongation zone (Fig. 5d), but promoted root hair elongation growth. Treatment with Ca^{2+} and ethylene resulted in ectopic root hair formation in the elongation

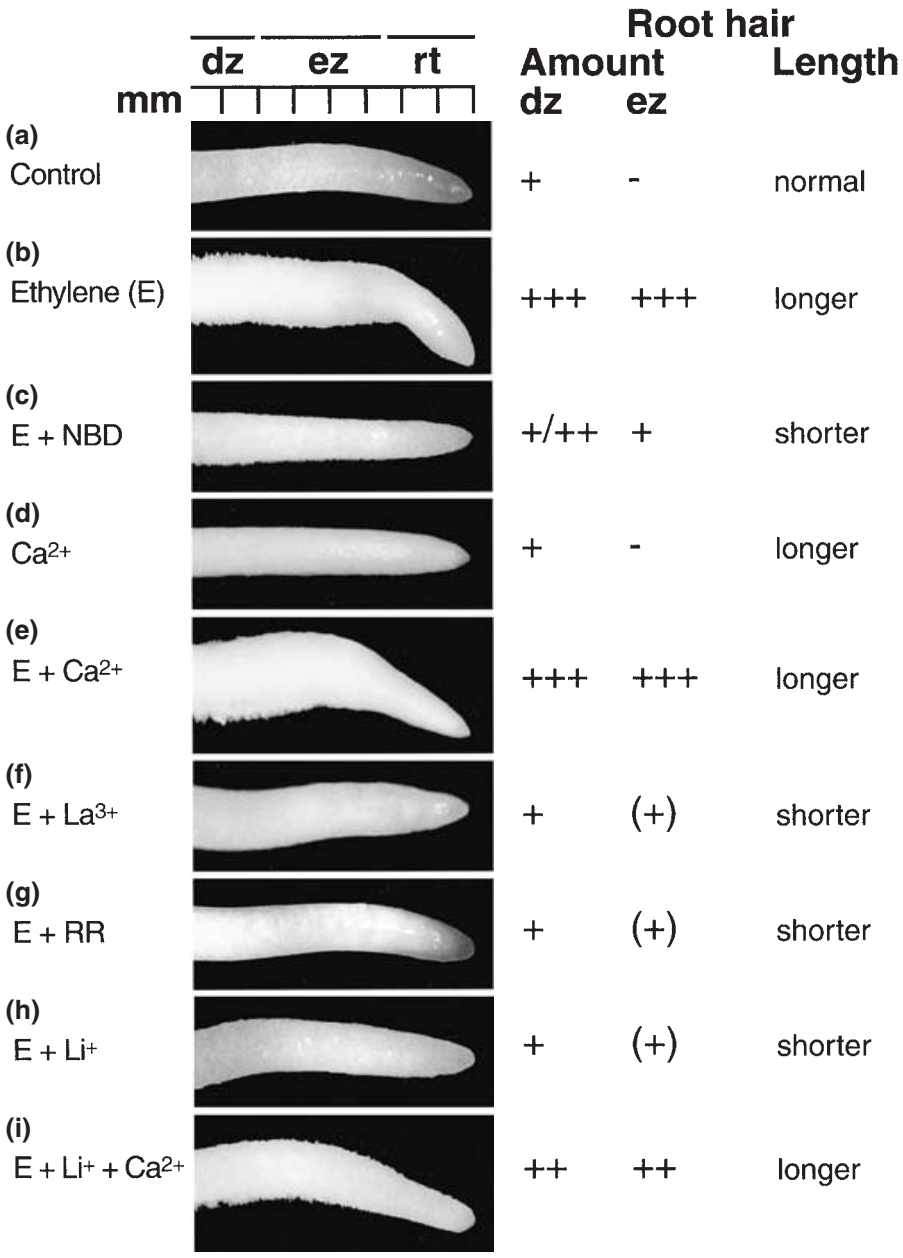

Figure 5. Effects of calcium antagonists on root hair formation and elongation growth of ethylene-treated germinated pea seeds. The 48 h-imbibed pea seeds were pre-incubated for 2 h in the presence or absence of the calcium antagonists followed by a further incubation for 8 h with exposure to 30 μL L^{-1} ethylene in the presence or absence of the calcium antagonists as described in Fig. 3. The cell differentiation zone (dz) where the root hairs normally form, the cell elongation zone (ez) where ethylene-induced ectopic root hairs form and the root tip (rt) are indicated. (a) Control radicle without ethylene treatment; note that root hairs are present in the dz, but not in ez, and are only visible in the 8 h-incubated radicles at higher magnification. (b) Ethylene-treated radicle with ectopic root hair formation. (C) Ethylene plus 300 μL L^{-1} NBD. (d) 10 mM $CaCl_2$ without ethylene treatment. (e) Ethylene plus 10 mM $CaCl_2$. (f) Ethylene plus 1 mM $LaCl_3$. (g) Ethylene plus 50 μM RR. (h) Ethylene plus 50 mM LiCl. (i) Ethylene plus 50 mM LiCl plus 10 mM $CaCl_2$. Estimations for the relative root hair amount ('+' = control radicle dz amount, '++' and '++ +' = increased amount, '(+)' = slightly decreased amount, '–' = absence of root hairs) and length are presented in the right part of the figure. Representative photographs of the bottom 8 mm radicle part.

zone and in promoted root hair elongation growth (Fig. 5e). All the Ca^{2+} antagonists tested interfered with the ethylene response and inhibited root hair formation, root hair elongation growth, and, at least partially, the formation of ectopic root hairs in the elongation zone. The La^{3+}, RR and Li^{+} were most effective and the ethylene-induced formation of ectopic root hairs in the elongation zone was almost completely inhibited (Fig. 5f–h). The Li^{+} also caused the strongest reduction in ethylene-induced ACO expression, which can be partially reversed by Ca^{2+} (Fig. 4). Simultaneous treatment with Ca^{2+} also reversed the inhibitory effect of Li^{+} on ethylene-induced ectopic root hair formation and on root hair elongation growth (Fig. 5i). Thus, ethylene, but not Ca^{2+} treatment alone, can induce ectopic root hair formation in the elongation zone of pea radicles. The release of Ca^{2+} from intracellular stores, but not apoplastic Ca^{2+}, seems to interfere with ethylene signalling of ectopic root hair formation. Ethylene-induced root hair elongation growth in pea radicles seems to involve both, intracellular and apoplastic Ca^{2+}.

DISCUSSION

The apical hook of dark-grown dicot seedlings is a protective structure for the apical shoot meristem. It pushes against the resistance during the growth through soil and it opens once the seedling emerges into the light. Physiological and genetic evidence shows that ethylene is involved in regulating closure and maintenance of the apical hook (e.g. Peck *et al*. 1998; Raz & Ecker 1999; Du & Kende 2001). *Ps-ACO1* gene induction in 5- to 6-day-old etiolated pea seedlings is highly localized to the inner, concave apical hook region and can serve as a molecular marker for both ethylene biosynthesis and ethylene responsiveness. Much earlier, before any appreciable shoot elongation growth, increasing ethylene evolution accompanies the germination of seeds of many plant species. Poor germination is a feature of the *A. thaliana* ethylene-insensitive mutant *etr1* (Bleecker *et al*. 1988) and the amount of ethylene evolution is positively correlated with seed vigour of pea (Gorecki *et al*. 1991). *Ps-ACO1* gene induction, ethylene biosynthesis and responsiveness are confined to the embryonic axis and are absent from the cotyledons of germinating pea seeds (Petruzzelli *et al*. 1999, 2000). Ethylene promotes ethylene biosynthesis during pea germination by positive feedback regulation of *Ps-ACO1* gene expression in the embryonic axis. Similar findings were made for *Cicer arietinum* seeds (Gömez-Jiménez, Matilla & Garrido 1998, Gömez-Jiménez *et al*. 2001). The adaptation of seedling roots to impeding soil conditions seems to involve ethylene and Ca^{2+}, but very little is known about the molecular basis of this process (Gilroy & Jones 2000).

In the present study with pea we demonstrate that induction by ethylene of *Ps-ACO1* mRNA, the 36 kDa Ps-ACO1 protein and ACO enzyme activity is mainly localized to the radicle and appreciably less to the shoot of the embryonic axis during seed germination. We observed the maximal ethylene responsiveness of *Ps-ACO1* gene induction in 2-day-old germinated pea seeds, which is directly after radicle emergence and is correlated with maximal ethylene evolution (Petruzzelli *et al*. 1999, 2000). Within the emerged embryonic axis, ethylene-inducibility of *Ps-ACO1* is distributed in a tissue-specific manner: strongest in the cell differentiation zone of the radicle, strong in the cell elongation zone of the radicle, and weak in the root tip and the shoot. Class I β-1,3-glucanase, our second molecular marker for ethylene responsiveness exhibits an identical spatial, temporal and ethylene-inducible expression pattern. Thus, not only is the radicle the first and main site of ethylene biosynthesis during pea germination, it also contains the tissues with the highest ethylene responsiveness. Ethylene treatment of germinated pea seeds resulted in reduced radicle elongation growth, and in enhanced root hair formation and root hair tip growth. In agreement with this, our finding that the cell elongation zone of the pea radicle is highly sensitive for ethylene perception is of particular interest. Ethylene has been shown to inhibit root elongation of many species (Bleecker & Kende 2000). In *A. thaliana* ethylene-mediated inhibition of root elongation is blocked in the *etr1* mutant (e.g. Bleecker *et al*. 1988), whereas the roots of the constitutive-response *ctr1* mutant are short and stunted. ACO induction by ethylene has been investigated in roots of 4-week-old mung bean plants (Jung *et al*. 2000), but so far not in the roots of the *A. thaliana* ethylene signal transduction mutants and not in the radicle of germinating seeds. The soil atmosphere can contain quite high concentrations of ethylene which appears to play an important role in the penetration of dense soil by roots (Bleecker & Kende 2000). A role for ethylene has also been proposed during programmed cell death important for root vascular tissue differentiation of germinated seeds (Cervantes *et al*. 2001). Ethylene also affected the root hairs of pea radicles and is known as a positive regulator of root hair development, which will be discussed later. Thus, regulation of pea radicle growth and root hair development appears to involve root-specific induction of ethylene biosynthesis and responsiveness.

Ca^{2+} plays a critical role as a second messenger in the signal/response coupling of many processes in higher plants that are regulated by environmental stimuli and by hormones (Anil & Rao 2001). Raz & Fluhr (1992) were the first to show that Ca^{2+} is involved in ethylene signalling leading to gene expression. The major Ca^{2+}-mediated signal cascades in plant cells function via elevation of $[Ca^{2+}]_{cyt}$, the cytosolic Ca^{2+} concentration (Anil & Rao 2001). To achieve $[Ca^{2+}]_{cyt}$ elevation, the cytosol has access to two types of Ca^{2+} stores/pools: the apoplast as an extracellular Ca^{2+} pool; and an intracellular Ca^{2+} pool including the vacuole, ER, mitochondria and possibly Golgi vesicles. A key question in investigating Ca^{2+} signalling is the determination of the relative contributions of these two types of Ca^{2+} pools. This can be experimentally achieved by using characterized Ca^{2+} channel blockers and Ca^{2+} chelators that inhibit $[Ca^{2+}]_{cyt}$ elevation and measure the inhibition of the downstream responses. Several of these substances can be used to discriminate between Ca^{2+} influxes from the apoplastic or the

intracellular Ca^{2+} pool. Our results with EGTA, La^{3+}, Ver, RR and W-7 strongly suggest that Ca^{2+} from intracellular pools, but not Ca^{2+} from the apoplast, is the main player that interacts with ethylene signal transduction in the radicle of germinated pea seeds. So far, such evidence has not been described for germinating seeds and for radicle tissues. Intracellular Ca^{2+}, but not apoplastic Ca^{2+}, enhances the gene induction by ethylene of *Ps-ACO1* in the pea radicle and intracellular Ca^{2+} is therefore a positive regulator of this response. This finding is in contrast to excised shoot internode segments from 5- to 6-day-old pea seedlings, in which the conclusion was drawn that Ca^{2+} influx from the apoplastic space is required for the ethylene response (Kwak & Lee 1997). Drawing this conclusion on the basis of experiments with EGTA is complicated by the fact that high EGTA concentrations have toxic effects and can inhibit ACO enzyme activity (Gallardo, Gömez-Jiménez & Matilla 1999). Our simultaneous investigation of different molecular markers also served as control in that the concentration of the Ca^{2+} antagonists used is effective and that the effects are not indiscriminately or generally inhibiting transcription and/or transcript stability. We used 5 mM EGTA, which is non-toxic for pea seeds, and found that it did not appreciably affect the levels of *Ps-ACO1* mRNA, Ps-ACO1 protein, and ACO enzyme activity. However, 5 mM EGTA is an effective concentration, it reduced class I β-1,3-glucanase and induced *Ps-ACS2* transcript accumulation in the ethylene-treated pea radicle. This finding is in agreement with the observation of an opposing regulation of ACO and ACS genes by pathways that transduce the ethylene signal (Kim *et al.* 1997; Oetiker *et al.* 1997; Bleecker & Kende 2000) and with the results from roots of 4-week-old mung bean plants (Jung *et al.* 2000). Thus, apoplastic Ca^{2+} appears to be involved as a negative regulator of specific ACS genes in ethylene-treated pea and mung bean roots. We found that La^{3+}, Ver, RR and W-7 inhibit *Ps-ACO1* gene expression and induce *Ps-ACS2* transcript accumulation in ethylene-treated pea radicles. In contrast to our findings for pea radicles, La^{3+} and Ver did not appreciably affect the mRNA levels of the *VR-ACO1* and *VR-ACS1* genes in the roots of 4-week-old mung bean plants (Jung *et al.* 2000). In agreement with our findings for pea radicles, RR and W-7 inhibited *VR-ACO1* gene induction in the mung bean roots, but only RR caused *VR-ACS1* gene induction. La^{3+}, Ver, and RR can affect both, the intracellular and the apoplastic Ca^{2+} stores (e.g. Knight *et al.* 1996; Pineros & Tester 1997; Leitner-Dagan & Weiss 1999; Anil & Rao 2001). Together with the effects of EGTA these observations suggest that the contributions of the intercellular and the apoplastic Ca^{2+} pools to the induction of ACO by ethylene differ between shoot and root, as well as between radicles of germinating seeds and roots of adult plants. Mobilization of Ca^{2+} from the intracellular stores appears to be the only requirement for the ethylene response of *Ps-ACO1* in radicles of germinating pea seeds, whereas intercellular and apoplastic stores are involved in this response in shoot and root tissues in older seedlings. The effects of W-7 are in agreement with this and are evidence for the involvement of the Ca^{2+}/CaM complex in ethylene signalling during pea seed germination. This is further supported by the finding that CaM gene expression in the embryonic axis of chick-pea seeds is correlated with ACO gene expression, ethylene biosynthesis and germination (Gómez-Jiménez *et al.* 1998; Nicolas *et al.* 1998). The Ca^{2+} is also involved in the response to abscisic acid (ABA), which regulates CaM expression during chick-pea seed germination. Taken together, this suggests that at least some of the interactions of ethylene and ABA signalling and at least some of the opposing effects of ethylene and ABA on seed germination could be mediated by the Ca^{2+}/CaM complex (Nicolas *et al.* 1998; Beaudoin *et al.* 2000).

Incomplete evidence suggests that most components of the PI cycle are present in plant cells and that the generation of second messengers such as inositol 1,4,5-triphosphate (IP3) can mediate Ca^{2+} release from intracellular stores (Stevenson *et al.* 2000). Li^{+} and neomycin are two inhibitors of the PI cycle and are known to interfere with Ca^{2+} effects on ethylene signalling in plants (e.g. Liang *et al.* 1996; Jung *et al.* 2000). Neomycin is a potent inhibitor of the binding of phosphatidylinositol-4,5-bisphosphate (PIP2) to phospholipase C (PLC); and Li^{+} is an inhibitor of the PI cycle by binding to inositol-phosphate phosphatases. Treatment of germinated pea seeds with neomycin or Li^{+} affected the ethylene-regulated expression of the *Ps-ACO1*, *Ps-ACS2* and the class I β-1,3-glucanase genes in the radicle: *Ps-ACO1* and class I β-1,3-glucanase were inhibited and *Ps-ACS2* was promoted. The same effect of neomycin and Li^{+} was also found for *VR-ACO1* and *VR-ACS1* in 4-week-old mung bean roots (Jung *et al.* 2000) and Li^{+} also regulated ACS gene expression in 5-day-old *Arabidopsis* seedlings (Liang *et al.* 1996). These findings support the proposal of Liang *et al.* (1996) that the PI cycle mediates Ca^{2+} mobilization from internal stores that, in turn, regulates the activity of Ca^{2+}-dependent protein kinase(s). Thus, elevation of $[Ca^{2+}]_{cyt}$, triggered by IP3, might represent a key step involved in positive and negative regulation by ethylene of *Ps-ACO1* and *Ps-ACS2* gene expression, respectively, in the pea radicle. As Ca^{2+} treatment alone was not capable of mimicking the ethylene responses, elevation of $[Ca^{2+}]_{cyt}$ alone does not represent a simple trigger. This is in agreement with the model that Ca^{2+} signalling is part of a network that integrates responses to a multitude of environmental, hormonal and developmental signals (Beaudoin *et al.* 2000; Gilroy & Jones 2000; Anil & Rao 2001).

Ethylene signal transduction mutants of *A. thaliana* were used to demonstrate that ethylene and Ca^{2+} are involved in the developmental regulation of root hair formation and root hair elongation growth (e.g. Tanimoto *et al.* 1995; Pitts *et al.* 1998; Cao *et al.* 1999; Gilroy & Jones 2000). Only some root epidermal cells, the trichoblasts, are destined to develop root hairs and these are more sensitive to ethylene. Ethylene treatment also induces root hair formation in the less ethylene-sensitive atrichoblasts, which normally do not develop into root hairs. Ca^{2+} is a positive regulator of root hair tip growth and thereby determines the elongation and

direction of root hair growth. We found that Ca^{2+} is not only a positive regulator of ethylene-induction of ACO in the elongation and differentiation zones, but also a positive regulator of ethylene-induced root hair formation and elongation growth in the pea radicle. According to Torrey (1965) three zones of the pea root can be distinguished: (1) the root tip zone (0–1.5 mm) including the cap, meristem and the region of maximal cell division; (2) the cell elongation zone characterized predominantly by elongation growth and the absence of root hairs (1.5–5.2 mm); and (3) the cell differentiation zone (above 5.2 mm) characterized by root hair formation. In agreement with this and using *Ps-ACO1* as a molecular marker, our findings demonstrate that the highest ethylene biosynthesis and responsiveness is localized in the elongation and differentiation zones of the pea radicle. Intracellular Ca^{2+}, but not apoplastic Ca^{2+}, appears to be involved in the ethylene-induction of *Ps-ACO1* and of ectopic root hair formation, for example, in the radicle elongation zone. Ethylene-insensitive mutants of pea are not available and ACO gene induction by ethylene has not been investigated in *A. thaliana* radicles. In contrast to root hair formation, ethylene-induced root hair elongation growth of pea seems to involve both intracellular and apoplastic Ca^{2+}. This is in agreement with similar studies with Ca^{2+} antagonists in *A. thaliana* (e.g. Wymer, Bibikova & Gilroy 1997; Gilroy & Jones 2000). Taken together this suggests that Ca^{2+} requirement for ethylene-dependent responses in the roots of *P. sativum* and *A. thaliana* are similar. We therefore propose that, as for the apical hook, ACO gene expression can be used as a molecular marker for ethylene biosynthesis and ethylene responsiveness in radicle tissues of germinated seedlings.

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