RESEARCH PAPER

Exogenously induced expression of ethylene biosynthesis, ethylene perception, phospholipase D, and Rboh-oxidase genes in broccoli seedlings

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Received 6 November 2009; Revised 21 May 2010; Accepted 25 May 2010

Abstract

In higher plants, copper ions, hydrogen peroxide, and cycloheximide have been recognized as very effective inducers of the transcriptional activity of genes encoding the enzymes of the ethylene biosynthesis pathway. In this report, the transcriptional patterns of genes encoding the 1-aminocyclopropane-1-carboxylate synthases (ACSs), 1-aminocyclopropane-1-carboxylate oxidases (ACOs), ETR1, ETR2, and ERS1 ethylene receptors, phospholipase D (PLD)-α1, -α2, -γ1, and -δ, and respiratory burst oxidase homologue (Rboh)-NADPH oxidase-D and -F in response to these inducers in Brassica oleracea etiolated seedlings are shown. ACS1, ACO1, ETR2, PLD-γ1, and RbohD represent genes whose expression was considerably affected by all of the inducers used. The investigations were performed on the seedlings with (i) ethylene insensitivity and (ii) a reduced level of the PLD-derived phosphatidic acid (PA). The general conclusion is that the expression of ACS1, -3, -4, -5, -7, and -11, ACO1, ETR1, ERS1, and ETR2, PLD-γ1, and RbohD and F genes is undoubtedly under the reciprocal cross-talk of the ethylene and PAPLD signalling routes; both signals affect it in concerted or opposite ways depending on the gene or the type of stimuli. The results of these studies on broccoli seedlings are in agreement with the hypothesis that PA may directly affect the ethylene signal transduction pathway via an inhibitory effect on CTR1 (constitutive triple response 1) activity.

Key words: ACC oxidase, ACC synthase, Brassica oleracea, ethylene, ethylene receptors, phosphatidic acid, phospholipase D.

Introduction

Ethylene production and perception regulate plant responses to a broad spectrum of various biotic and abiotic stimuli. The immediate precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC), is a product of the reaction controlled by 1-aminocyclopropane-1-carboxylate synthase activity (S-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14; ACS). The next step, conversion of ACC to ethylene, is catalysed by 1-aminocyclopropane-1-carboxylate oxidase (ACO).

S-Adenosyl-L-methionine → 1-aminocyclopropane-1-carboxylic acid → ethylene

Different expression of ACS and ACO isozymes encoded by multigene families in response to external and internal stimuli is controlled at the transcriptional and post-transcriptional level (Rottmann et al., 1991; Liang et al., 1992; Barry et al., 1996; Oetiker et al., 1997; Vogel et al., 1998; Kim et al., 2001; Tatsuki and Mori, 2001; Gallie and Young, 2004; Hernandez-Sebastia et al., 2004; Tsuchisaka and Theologis, 2004; Yoshida and Theologis, 2004; Yoshida et al., 2006; Ralph et al., 2007; El-Sharkawy et al., 2008; McClellan and Chang, 2008; Xue et al., 2008; Christians et al., 2009; Gallie et al., 2009; Lin et al., 2009; Tsuchisaka et al., 2009). In plants, ethylene biosynthesis is controlled by two systems: the ethylene autoinhibitory system 1, which generally operates during
normal vegetative growth of plant; and system 2, regulated by a positive feedback mechanism, usually responsible for the rapid increase in ethylene production in senescing ethylene-sensitive plant organs, and in ripening climacteric fruits (Nakatsuka et al., 1998; Barry et al., 2000; Kim et al., 2001).

Ethylene production and formation of reactive oxygen species (ROS) are the first biochemical alterations which participate in the signal transduction events involved in programmed cell death (PCD), playing an essential role in response to different abiotic stressors and in a plant defence reaction against various pathogens (Moeder et al., 2002; Woltering et al., 2003; Iakimova et al., 2008; Steffens and Sauter, 2009). In plants, in response to stress, one of the generators of extracellular ROS is the membrane-bound respiratory burst oxidase homologue (Rboh)-NADPH oxidase, catalysing the stimuli-induced extracellular production of superoxide anion which dismutates to hydrogen peroxide (Frahry and Schopfer, 2001; Beckett et al., 2004; Sgherri et al., 2007). However, Rboh function can be activated by exogenous ROS, and the subsequent oxidative burst can suppress death in cells surrounding sites of Rboh activation (Torres et al., 2005).

Rapidly diffusing across the cell membranes, H$_2$O$_2$ at low concentrations acts as a messenger molecule triggering tolerance against various stresses, but at high concentrations it orchestrates PCD. It has been thought that selective enzymatic or non-enzymatic oxidation of cysteine residues in sensor proteins is a general H$_2$O$_2$ signalling route which can directly cross-talk or compete with nitric oxide (NO) action (Hancock et al., 2005, 2006; Miller and Mittler, 2006; Forman et al., 2008; Neill et al., 2008; Wang and Song, 2008; Jammes et al., 2009; Forman et al., 2010; Paulsen and Carroll, 2010); for details see Fig. 1B. Nonetheless, in general, a close interplay of H$_2$O$_2$ with the other signalling molecules is realised at the transcriptional level (Dat et al., 2001, 2003; Vandenabeele et al., 2003).

All organisms have to maintain a balance between essential and toxic levels of copper. The enhanced activity of Rboh-NADPH oxidase and also an essential accumulation of H$_2$O$_2$ in the plasma membrane and cell walls in response to copper have been shown in different species (Quartacci et al., 2001; Yu et al., 2008; Zhang et al., 2008; Wu et al., 2009). Copper-induced oxidative stress results, first, from the directly catalysed formation of ROS via a Fenton-like reaction and, secondly, from a significantly decreased glutathione (GSH) level by copper ions. Depletion of intracellular GSH increases the cytotoxic effect of ROS (Mattie and Freedman, 2004). Copper can activate transcription through either oxidative stress- or metal-mediated mechanisms, leading to activation of mitogen-activated protein kinase (MAPK) signalling pathways (Ostrakhovitch et al., 2002; Jonak et al., 2004; Mattie and Freeman, 2004; Gaitanaki et al., 2007; Yeh et al., 2007; Chen et al., 2008; Mattie et al., 2008) (see Fig. 1A for details). Although copper and H$_2$O$_2$ effectively stimulate ethylene biosynthesis in higher plants, the most universal and potent inducer of expression of ACS genes is cycloheximide (CHX) (Yamagami et al., 2003). Its role in this induction has not been elucidated to date. CHX has mostly been considered as a eukaryotic protein synthesis inhibitor, but this seems to be only one aspect of its action in living cells. According to some suggestions (Li et al., 2001), CHX can (i) lead to transcriptional activation via loss of labile negative regulators; (ii) inhibit translation of protein products of autorepressive genes, and thus superinduce their transcription; (iii) prevent the synthesis of labile mRNA-degrading enzymes; (iv) cause RNAs to be trapped on polysomes, thus shielding them from cytoplasmic RNases; (v) induce phosphorylation of proteins usually involved in abscisic acid (ABA)-mediated activation; (vi) lead to direct transcriptional activation via phosphorylation of H3 histones; and (vii) uncouple DNA replication and chromatin assembly preventing the formation of a repressive chromatin structure.

Despite a general agreement that plants produce ethylene in response to exposure to copper ions, the copper-induced transcriptional activity of the ethylene biosynthesis genes has only been studied in a limited number of plants. In Arabidopsis, the only ACS6 gene characterised as multi-responsive responds to copper (Arteca and Arteca, 1999). The greatest stimulation has been observed in inflorescences and the youngest leaves, whereas light had an inhibitory effect (Arteca and Arteca, 2007). The copper-inducible expression of two different ACS genes in potato, two distinct ACS genes in Pelargonium hortorum, and an accumulation of ACS transcripts in different cultivars of tobacco have been reported (Avni et al., 1994; Wang and Arteca, 1995; Schlagnhauffer et al., 1997).

At least three, but more often more than three, members of the ACO family regulated in a gene-specific manner occur in the genomes of all plants investigated to date. Some of them are copper inducible, but there are only scarce data from studies performed on Pelargonium hortorum, Nicotiana tabacum, and Nicotiana glutinosa (Avni et al., 1994; Wang and Arteca, 1995; Clark et al., 1997).

In Arabidopsis, the N-terminal fragments of five ethylene receptors (subfamily 1, ETR1 and ERS1; subfamily 2, ETR2, ERS2, and EIN4) are involved in copper-mediated ethylene binding. Ethylene receptors act as negative regulators which actively repress expression of the ethylene-responsive genes; whereas an enhancement of ethylene responsiveness of tissues, whereas an enhancement decreases the ethylene sensitivity. Ethylene binding triggers ubiquitin-dependent receptor degradation, therefore synthesis of new receptors is the only way to turn off the ethylene response (Klee, 2002; Kevany et al., 2007). Moreover, it has been shown that ETR1 mediates stomatal closure in response to H$_2$O$_2$ (Desikan et al., 2005). Thus, it is possible that ETR1 can act as a node mediating cross-talk between ethylene and H$_2$O$_2$. 
Generally, phosphatidic acid (PA) represents ~1–2% of total phospholipids and is generated by two routes, either directly by phospholipase D (PLD; EC 3.1.4.4) activity or by the sequential action of phospholipase C and diacylglycerol kinase (PAPLC/DGK) (Arisz et al., 2009; Munnik and Testerink, 2009). The up-regulated activity of both routes occurs at the early step of response to stress (Wang, 2005; Bargmann and Munnik, 2006). Multiple types of PLDs reveal different catalytic and regulatory properties, and generate a distinct PA_{PLD} species (Li et al., 2009). Putatively the PA_{PLD} species, the location and timing of PA_{PLD} formation, and its intracellular level are essential
determinants of the functioning of PA\textsubscript{PLD} as a secondary messenger molecule which can act in opposite ways (Wang, 2000).

It has been assumed that PA\textsubscript{PLD} can enhance the activity of Rboh-NADPH-oxidase (Yu \textit{et al.}, 2008). A direct promotion of the catalytical activity of Rboh-oxidase by PLD-\textalpha{1}-derived PA species and the Cu\textsuperscript{2+}-induced elevation of both PA\textsubscript{PLD} content and activation of Rboh-oxidase have been found in \textit{Arabidopsis} (Zhang \textit{et al.}, 2005) and wheat (Navari-Izzo \textit{et al.}, 2006). Furthermore, Navari-Izzo \textit{et al.} (2006) reported that the reduced PA\textsubscript{PLD} level in wheat roots almost completely abolished the production of the superoxide anion. Nevertheless, the other isozyme of PLD, PLD-\delta activated by H\textsubscript{2}O\textsubscript{2}, does not stimulate H\textsubscript{2}O\textsubscript{2} formation; moreover, PA\textsubscript{PLD} species generated by PLD-\delta lead to a decrease in H\textsubscript{2}O\textsubscript{2}-promoted PCD (Zhang \textit{et al.}, 2003).

In mammals, the MAPK kinase kinase Raf-1 represents the best known molecular target of PA thus far. One of the plant homologues of Raf-1, CTR1 (constitutive triple response 1), functions as a negative regulator of the ethylene signalling pathway (Fig. 2). It has been shown for \textit{Arabidopsis} CTR1 that PA binds to CTR1, inhibits its kinase activity, and blocks interactions with the ethylene receptor ETR1 (Testerink \textit{et al.}, 2007, 2008).

\textbf{Fig. 2.} A schematic presentation of relationships between the key components of the ethylene signal transduction pathway. (A and B) Ethylene action on downstream components of its signalling route. (C) Inhibitory effect of silver ions on ethylene signalling. (D) Inhibitory action of PA\textsubscript{PLD} on CTR1 and its expected result. (E) Putative concerted action of both messenger molecules on ethylene signal transduction pathway. (F) Inhibitory effect of butanol-1 on PA\textsubscript{PLD} signalling and its possible consequence.
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RT-PCR analysis

First-strand cDNA synthesis was performed in a 20 µl reaction mixture containing 1 µg of total RNA, 0.2 µg of random hexamers, four dNTPs, RNase inhibitor, buffer, and M-MLV reverse transcriptase according to the manufacturer’s instructions (Promega).

To determine the temporal expression patterns of ACS, ACO, ethylene receptors, PLD, and Rboh-NADPH oxidase D and F genes during the various stresses, a semi-quantitative analysis of steady-state transcript levels using an RT-PCR with gene-specific primers was performed. Reaction mixtures contained 2.5 µl of 10× Mg-free buffer (Fermentas), MgCl₂, dNTPs, and forward and reverse primers to the final concentration 1.5 mM, 0.25 mM, and 250 nM, respectively. Each reaction mixture contained 1.5 µl of appropriate 4-fold diluted cDNA mixture and 1 U of Taq polymerase in a final volume of 25 µl, which was overlaid with 30 µl of mineral oil.

The reaction mixture was maintained at 95 °C for 5 min and then cycled 28, 30, 31, or 33 times at 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 90 s, with a final extension of 5 min at 72 °C. The numbers of cycles were determined experimentally for each analysed gene to detect the RT-PCR products in the linear range.

To ensure internal control of the reaction, the actin housekeeping gene was amplified simultaneously in one tube with the gene of interest with actin-specific primers. The concentrations of primers were selected to obtain sufficient amounts of both amplicons and to ensure that the primers would not limit the reactions. The number of cycles was chosen to ensure that both products were clearly visible on the agarose gel but stayed in the exponential phase of amplification (28–33 cycles).

The primers were added in such a manner that the final concentration of gene-specific primers was 250 nM, in contrast to the concentration of the actin-specific primers which was lower and most often equal to 200 nM for 28 cycles, 160 nM for 30 and 31 cycles, and 125 nM for 33 cycles. Moreover, the RT-PCRs for each gene of interest and the control actin gene have been carried out in independent thermocycler runs from the same cDNA stocks. RT-PCR products were analysed by 1.7% agarose gel electrophoresis and stained with ethidium bromide. Gels were visualized under UV light; images were taken using a gel documentation system.

Results

The steady-state transcript levels of genes of the ethylene synthesis pathway and perception, PLDD-α1, -α2, -γ1, -δ, and NADPH oxidase RbohD and F in 7-d-old etiolated seedlings of B. oleracea var. alboglabra ADH12 in time course experiments using semi-quantitative RT-PCR have been investigated.

To determine the possible influence of the PAPLD signalling on the expression of the above-mentioned genes, the study was performed on seedlings pre-treated or not with butanol-1 before addition of copper, H₂O₂, or CHX. The suggested effect of treatment with butanol-1 on the ethylene signal transduction pathway is shown in Fig. 2.

Among primary alcohols functioning as transphosphatidylation substrates, butanol-1 has been known to be the most potent; however, it has been shown that plant PLD isozymes differ in their transphosphatidylation potentials (Morris et al., 1997; Mansfeld et al., 2009). In algae, isobutanol functions as a transphosphatidylation substrate but it is significantly less effective than butanol-1, while animal PLDs do not use it (Munnik et al., 1995; Shen et al., 2001). Supplementary Fig. S1 at JXB online shows the effect of treatment of the dark- and light-grown broccoli seedlings with butanol-1 and isobutanol. In contrast to butanol-1, treatment of seedlings with isobutanol did not seem to visibly affect their growth and development. Treatment of seedlings with butanol-3 (a tertiary alcohol, which is neither a transphosphatidylation substrate nor an activator of PLD and is often used as a control in experiments involving butanol-1 action) caused abnormalities in root development (not presented). Thus, pre-treatment with isobutanol was preferred in control experiments of the effect of butanol-1 on the expression of the genes studied. The control experiment involved pre-treatment of seedlings with isobutanol prior to their treatment with the most potent inducer used, CHX (Fig. 5).

Ethylene regulates its own synthesis in a positive or negative feedback manner. To investigate the functioning of such a regulatory system, the response to copper was studied on seedlings pre-treated or not with silver ions. The silver treatment reduced the ethylene sensitivity in these seedlings. The effect of silver on ethylene signalling is illustrated in Fig. 2.

Expression of the ethylene biosynthesis enzymes genes

In broccoli after harvest, expression of one ACS and three ACO genes was characterised in florets, sepals, and yellowing leaves, and there are no data regarding expression of ACS and ACO genes in response to other stimuli (Pogson et al., 1995; Yang et al., 2003).

The detailed comparative analysis of the transcriptional pattern of ACS1, -3, -4, -5, -7, and -11 genes in response to copper, H₂O₂, and CHX is presented in Figs 3, 4, and 5. In contrast to the other ACS genes, the transcripts of the BO-AC9 gene were not detected under any type of stimulation. Among all the investigated ACS genes, ACS1 showed the highest accumulation of expressed mRNAs (detection of its amplicons at 28 cycles) in response to the earlier mentioned stimuli. The accumulation of transcripts of the other ACS genes was lower and thus they were amplified in a linear range using 31 or 33 cycles.

The ACS1 gene was the only expressed in the same manner rapidly and explicitly in response to different concentrations of copper, in contrast to the other genes whose expression was strongly dependent on the concentration of the stressor (the results of treatment with 0.5 mM copper are not presented). Therefore, the conclusion is that the transcriptional induction of these genes observed in seedlings treated with 2.5 mM copper may result from the transcriptional induction of the stressor (the results of treatment with 0.5 mM copper are not presented). Therefore, the conclusion is that the transcriptional induction of these genes observed in seedlings treated with 2.5 mM copper may result from the following events occurring in plants at its higher concentration (Fig. 3).

The pre-treatment of seedlings with silver prior to addition of copper hastened the beginning of up-regulation of the ACS1, -3, -7, and -11 genes and/or affected the level of transcripts, relative to their expression in seedlings treated with copper alone. Thus, the suggestion has been made that ethylene controls the start of up-regulation and/
Fig. 3. The time course of transcriptional expression of genes encoding ethylene biosynthetic enzymes, ethylene receptors, phospholipases D, and Rboh-NADPH-oxidases in etiolated B. oleracea A12 DH seedlings treated with 2.5 mM CuCl₂ (A); 0.1 mM AgNO₃ and 2.5 mM CuCl₂ (B); 0.1% butanol-1 and 2.5 mM CuCl₂ (C); or 0.1% butanol-1 alone (D). Transcripts were assayed by RT-PCR. The number of cycles used to detect mRNAs of certain genes is marked on the right-hand side of the columns. The expression of the actin housekeeping gene was used as an internal control. The results shown in columns A and B are representative of three, and those in C and D of two separate experiments. Experimental details are described in the Materials and methods; for other details, see the Results.
or the level of mRNA of these genes via negative feedback. The ACS3 was the only one of the ACS genes investigated whose copper-enhanced expression was slightly down-regulated in the absence of ethylene signalling (Fig. 3). In seedlings treated with copper alone, the low constitutive expression of the ACS4 and ACS5 genes was down-regulated after 30 min and 90 min of the stressor action, whereas in seedlings pre-treated with silver such a down-regulation of ACS5 appeared later after 6 h of treatment with copper, but in the case of ACS4 it did not occur by this time. It seems that the constitutive expression of both genes is negatively controlled by the copper-enhanced ethylene production.

In broccoli, during the time course of the experiment, a low level of transcripts of constitutively expressed ACS1 and 5 genes was not essentially affected by treatment with butanol-1 alone, whereas the expression of other ACS genes was not detected (Fig. 3D). Nonetheless, the interruption of the PA preceding prior to the treatment of plants with copper, H2O2, and CHX visibly affected the expression of all ACS genes studied to a different degree and in different manners in response to them.

In seedlings pre-treated with butanol-1 prior to the addition of copper the pattern of expression of the ACS1, -3, -5, and -7 genes resembled that observed in seedlings pre-treated with silver (Fig. 3B, C). In contrast, the ACS4 and ACS11 genes did not respond in a similar way to both pre-treating agents and the observed differences can suggest that they require the presence of PA or of an appropriately high level of intracellular PA to be effectively transcribed.

The ACS1 gene seems to be the only gene whose expression was rapidly, significantly, and directly up-regulated by H2O2 itself. An increase in the expression of the ACS3 and ACS7 genes followed much later and could be generated by other events in seedlings concomitant with the action of the inducer rather than directly by H2O2. In contrast, the very labile low expression of ACS4, -5, and -11 genes was down-regulated and hardly perceptible after 2 h of treatment with H2O2 (Fig 4).

The pre-treatment with butanol-1 prior to addition of H2O2 did not considerably affect the expression of the ACS1 gene, essentially delayed the beginning of up-regulation of the ACS3 gene, enhanced the constitutive level of ACS7, and hastened the start of its up-regulation in comparison with the response of these genes to H2O2 alone. In seedlings pre-treated with butanol-1 prior to addition of H2O2, the accumulation of transcripts of ACS4, -5, and -11 was below the level of detection (detection at 33 cycles) (Fig. 4).

**Fig. 4.** The time course of transcriptional expression of genes encoding ethylene biosynthetic enzymes, ethylene receptors, phospholipases D, and Rboh-NADPH-oxidases in etiolated B. oleracea A12 DH seedlings treated with 0.25% H2O2 (left column) or 0.1% butanol-1 and 0.25% H2O2 (right column). Transcripts were assayed by RT-PCR. The number of cycles used for detection of mRNAs of certain genes is marked on the right-hand side of the columns. The expression of the actin housekeeping gene was used as an internal control. The results presented are representative of two separate experiments. Experimental details are described in the Materials and methods; for other details, see the Results.
Considerably increased expression of all the ACS genes discussed, except ACS5, in response to the treatment with CHX was observed (Fig. 5). In comparison with the earlier described inducers, the detection of transcripts of the ACS1, -4, and -5 genes occurred at the same number of cycles (28, 33, and 33, respectively), ACS7 and ACS11 at a lower number of cycles (30 cycles), and ACS3 at a higher number (33 cycles). In seedlings pre-treated with butanol-1 and subsequently treated with CHX, the transcripts of ACS3, -4, and -11 genes were below the limit of detection, whereas those of ACS1, -7, and -5 only slightly altered the pattern of expression in comparison with that in seedlings treated with CHX alone (Fig. 5). The level of constitutive expression of ACS5 was labile and dependent on the set of seedling used; however, it always declined in response to CHX.
In seedlings pre-treated with isobutanol and subsequently treated with CHX, the transcriptional patterns of all ACS genes were very similar to those observed in the seedlings treated with CHX alone, except ACS5. Surprisingly, pre-treatment with isobutanol prior to the addition of CHX results in the increased expression of the ACS5 gene in response to this latter stimulus (Fig. 5).

The presence of transcripts of ACO2 and ACO3 genes in stimulated and non-stimulated seedlings was controversial (data not presented). In contrast to these genes, ACO1 revealed a rather high constitutive level of expression (detection at 28 cycles) and was up-regulated in response to the inducers used (Figs 3, 4, and 5). The ACO1 gene was highly expressed in response to butanol-1 alone, which implies a significant role for PAPLD signalling in negative regulation of this gene (Fig. 3). The addition of copper to the seedlings previously pre-treated with butanol-1 for the next 3 h only slightly increased the enhanced earlier expression of the ACO1 gene, but after 4 h of the treatment it led to a decrease (Fig. 3).

The pre-treatment with silver did not considerably affect the constitutive expression of the ACO1 gene but in seedlings pre-treated with silver its up-regulation in response to copper was lowered and remained shorter in comparison with that in response to copper alone. This implies that ethylene controls the level of transcripts of the ACO1 gene by positive feedback (Fig. 3).

H2O2 or CHX alone enhanced the level of transcripts of ACO1 after 1 h or 3 h of treatments, respectively (Figs 4, 5). An increased expression of the ACO1 gene caused by pre-treatment with butanol-1 was temporarily down-regulated after the subsequent addition of H2O2 or CHX, but in both cases it rapidly returned to its up-regulated level. Nevertheless, for the seedlings treated with butanol-1 and subsequently with CHX after 4 h of treatment with this latter stimulus, the enhanced expression of ACO1 decreased and stabilized at a constitutive level.

In seedlings pre-treated with isobutanol and subsequently treated with CHX, the timing of expression of the ACO1 gene was similar to that observed in the seedlings treated with CHX alone, but the level of its transcripts was visibly higher (Fig. 5).

**Expression of the ethylene receptor genes**

ETRI and ERS1 genes displayed moderate constitutive expression which allowed the detection of amplicons of their transcripts at 30 cycles. In contrast, the transcripts of ETR2 were detected at 33 cycles and this gene was the only one whose expression was significantly transitorily up-regulated in response to copper, whereas the accumulation of ETR1 and ERS1 mRNAs fluctuated about the level of the control, with a temporary increase in the latter.

In the plants treated with butanol-1 alone, the ETR1 gene was expressed at similar levels to untreated plants. In contrast, the expression of ERS1 and ETR2 decreased; however, after 21 h of treatment with butanol-1 it returned to the initial levels (Fig. 3).

The pre-treatment of seedlings with butanol-1 prior to the addition of copper resulted in a lowered expression of ETR2 and ERS1, and in a slight increase in the expression of ETR1, which nevertheless was down-regulated later, remaining at a constitutive level after 4 h of copper action (Fig. 3). The pre-treatment of seedlings with silver prior to addition of copper affected the expression of ETR1, ERS1, and ETR2 in a similar manner to that described for the seedlings pre-treated with butanol-1 (Fig. 3).

In response to H2O2 the expression of all of the ethylene receptor genes investigated was transiently down-regulated; nevertheless, it returned to control levels or was even slightly up-regulated after 4–6 h of treatment with H2O2 (Fig. 4). Moreover, the pre-treatment of seedlings with butanol-1 prior to addition of H2O2 reduced the expression of all these genes much more (Fig. 4).

The treatment of seedlings with CHX only slightly enhanced the expression of ETR1, in contrast to ERS1 and ETR2 which were up-regulated to a greater degree. In the seedlings pre-treated with butanol-1, the constitutive expression of ETR1 increased and only slightly fluctuated throughout the whole time course of the subsequent action of CHX. In contrast, the expression of ERS1 was decreased and remained at a low level during the whole treatment with CHX. The abundance of the ETR2 transcripts distinctly decreased in comparison with that in the seedlings treated with CHX alone (Fig. 5).

In seedlings pre-treated with isobutanol and subsequently treated with CHX, the patterns of expression of ETR1, ERS1, and ETR2 genes did not differ significantly from those observed in the seedlings treated with CHX alone (Fig. 5).

**Expression of the PLD and Rboh-NADPH oxidase genes**

It has been established that in B. oleracea the PLD-α2 isozyme is somewhat more active than -α1, and they both slightly differ in their preference for substrates of hydrolysis (Dippe and Ulbrich-Hofmann, 2009); and at least two PLD-γ isforms occur (Novotna et al., 2003).

In the present study, the expression of the PLD-α1, -α2, -γ1, and -δ genes has been investigated. The abundance of the PLD-α1 and -α2 transcripts in control and stressed seedlings allowed detection of amplicons of their transcripts at 31 cycles, while these of the PLD-γ1 and -δ genes were detected at 33 cycles. PLD-α1, -α2, and PLD-δ did not respond significantly to copper throughout the time of treatment, whereas the accumulation of the PLD-γ1 mRNAs was essentially increased (Fig. 3).

In seedlings treated with butanol-1 alone, the constitutive levels of PLD-α2 declined somewhat, in contrast to PLD-γ1 and -δ which showed barely perceptible up-regulation (Fig. 3).

The pre-treatment of seedlings with butanol-1 prior to the subsequent addition of copper did not considerably affect the level of PLD-α1 and -α2 transcripts during 4 h of treatment with this latter stimulus, but strongly
down-regulated the expression of the genes after this time. The same treatment slightly lowered the constitutive expression and the copper-induced up-regulation of the PLD-γ1 gene; however, after 4 h it was essentially down-regulated in a manner similar to the case of expression of PLD-α1 and α2 genes. The pre-treatment of seedlings with silver did not significantly influence the expression of PLD-α1 and α2 genes but, after the addition of copper, a visible decline in the abundance of the PLD-α1 transcripts occurred, and after 3 h the accumulation of PLD-α2 mRNA decreased somewhat. In seedlings pre-treated with silver, the copper-induced up-regulation of the PLD-γ1 gene started earlier but the level of transcripts was lower in comparison with that was observed in response to copper alone. The expression of PLD-δ was rather unaffected by the treatments discussed above (Fig. 3).

The treatment with H$_2$O$_2$ transiently lowered the transcriptional expression of the PLD-α1 and α2 genes. In contrast, in response to this stimulus the accumulation of transcripts of the PLD-γ1 gene were greatly increased. In seedlings pre-treated with butanol-1, the addition of H$_2$O$_2$ essentially temporarily down-regulated the expression of both α1 and α2, whereas the timing of expression of PLD-γ1 only was somewhat changed in comparison with that observed in the seedlings treated with H$_2$O$_2$ alone. Constitutive expression of the PLD-α1 and α2 genes did not alter throughout the 6 h of treatment of the seedlings with CHX, whereas the expression of PLD-γ1 clearly increased. In plants pre-treated with butanol-1, the timing of expression of PLD-α1, PLD-α2, and PLD-γ1 genes did not change considerably in comparison with that in the seedlings treated with CHX alone (Fig. 5).

H$_2$O$_2$ and CHX, when used alone, down-regulated the low expression of the PLD-δ gene after 6 h of treatment of seedlings. In both cases, the pre-treatment with butanol-1 increased the level of PLD-δ mRNA but only when followed by treatment with H$_2$O$_2$ did it prevent the down-regulation of expression of the PLD-δ gene (Figs 4, 5).

In seedlings pre-treated with isobutanol and subsequently treated with CHX, the expression of PLD-α1, PLD-α2, and PLD-γ1 genes did not differ significantly from that observed in the seedlings treated with CHX alone; however, the level of transcripts of the PLD-γ1 gene was somewhat elevated (Fig. 5).

After 6 h of treatment with CHX alone, the expression of the PLD-δ gene was decreased, but when pre-treated with isobutanol prior to addition of CHX it was even slightly upregulated after this time (Fig. 5).

The respiratory burst oxidase homologue (Rboh) gene family encodes the key enzymatic subunit of the plant Rboh-NADPH oxidase. The PA elevated activity of Rboh-NADPH oxidase can cause collapse of antioxidant systems that scavenge ROS (Yu et al., 2008). In Arabidopsis, there are 10 different Rboh genes whose expression is mainly transcriptionally controlled in a tissue-specific manner, but RbohD and F genes belong to the group expressed throughout the whole plant (Torres et al., 2002; Kwak et al., 2003; Torres and Dangl, 2005).

In broccoli, in response to the earlier discussed stimuli, the expression of both genes increased, but in different ways. Generally, the abundance of transcripts of the RbohF gene was lower than that of the RbohD gene (both detected at 33 cycles). In response to copper, an increase in the expression of RbohD significantly preceded the expression of RbohF. The up-regulation of expression of RbohD occurred earlier in the seedlings pre-treated with silver in comparison with those treated only with copper. In seedlings pre-treated with butanol-1 the hastened and enhanced up-regulation of the RbohD gene stopped after 6 h of treatment with copper. Neither pre-treatment affected the abundance of transcripts of the RbohF gene but only hastened its slight up-regulation (Fig. 3).

The treatment of plants with butanol-1 alone did not considerably affect the expression of RbohD and F genes, causing low transitory expression of RbohD after 21 h and similarly low expression of RbohF after 20 h of stimulation (Fig. 3).

The expression of RbohF in response to copper and in response to H$_2$O$_2$ was very similar (Figs 3, 4). In contrast to RbohF, the expression of RbohD in response to H$_2$O$_2$ increased significantly later, after 6 h of treatment. Therefore, it should be considered whether the high concentration of H$_2$O$_2$ could maintain the expression of RbohD at a constitutive level blocking its up-regulation for at least 4 h. The pre-treatment with butanol-1 diminished the level of transcripts of RbohD and F but generally did not alter the time course of their response to H$_2$O$_2$.

Both genes discussed were up-regulated in a similar manner in response to the treatment with CHX and in both of them the pre-treatment of seedlings with butanol-1 only somewhat decreased the accumulation of their transcripts (Fig. 4).

In seedlings pre-treated with isobutanol and subsequently treated with CHX, the timing of expression of RbohD and F genes resembled that observed in the seedlings treated with CHX alone; however, the expression of RbohD and F genes was visibly higher after 4-6 h of treatment with CHX in plants pre-treated with isobutanol (Fig. 5).

Discussion

It has been assumed that two systems, the ethylene autoinhibitory system 1 and system 2, regulated by a positive feedback mechanism control the transcriptional activity of genes encoding ACS and ACO isoforms (Nakatsuka et al., 1998; Barry et al., 2000; Kim et al., 2001). In the absence of ethylene, its receptors operate via the Raf-1-like kinase, CTR1, whose activity suppresses the ethylene responses. In the presence of ethylene, receptors do not stimulate CTR1, which results in its inactivation and induction of a response to ethylene (Zhu and Guo, 2008) (Fig. 2). Testerink et al. (2007, 2008) reported that PA can be a negative regulator of CTR1 via its binding to CTR1’s kinase domain and through reduction of the binding of CTR1’s kinase domain to the ethylene receptor,
ETR1. A direct effect of PA on the activity of CTR1 suggests the possibility of turning on of ethylene signalling in the absence of ethylene (Fig. 2D). PA, present in the endoplasmic reticulum all the time, may be involved in a complicated mechanism of CTR1 regulation.

In plants, PA signalling is associated with a broad spectrum of biotic and abiotic stimuli. The host–pathogen interactions stimulate a biphasic PA response. The first, rapid phase following a few minutes of stress generally involves PAPlcDgk, while the second one involves a specific PApld (Wang, 2000; Laxalt and Muunik, 2002; den Hartog et al., 2003; Meijer and Munnik, 2003; Bargmann et al., 2006; Navari-Izzo et al., 2006). Downstream from PA formation, the concomitant biphasic accumulation of ROS takes place (van der Luit et al., 2000; de Jong et al., 2004; Andersson et al., 2006; Arisz et al., 2009). It has been believed that the stress-induced PApld can elevate the activity of Rboh-NADPH-oxidase (Yu et al., 2008). In the present experiments, treatment with exogenously added H2O2 can mimic the first initial phase of the oxidative burst.

A comparative analysis of expression of the genes under discussion in (i) ethylene-sensitive seedlings; (ii) seedlings whose sensitivity to ethylene was reduced at the level of receptors; and (iii) seedlings with a lowered level of PApld has been made in a previous section (Figs. 3, 4 and 5). Below, the inter-relationships concluded to occur between the action of inducers and the ethylene and PApld signalling pathways occurring in the seedlings treated with copper (Fig. 6A), H2O2 (Fig. 6C), and CHX (Fig. 6B) are summarized.

In conclusion, the following phenomena were observed:

(i) Expression of the ACS1 gene encoding the key ACS isozyme is regulated in an autoinhibitory manner by ethylene and not affected or negatively affected by PApld.

(ii) Ethylene up-regulates the expression of the ACO1 gene encoding the main ACO isozyme, while PApld seems to be its negative regulator. These two signalling molecules affect the expression of ACO1 in opposite ways. Putatively, the relatively high constitutive expression of ACO1 is under the permanent control of the cross-talk between ethylene and PApld.

(iii) As the action of ethylene and PApld is synergic in transcriptional regulation of ACS1, ACS7, and ETR1 genes (down-regulation), and ERS1 and ETR2 genes (up-regulation), it seems likely that the expression of the above-mentioned genes may be controlled via the ethylene signalling pathway in which one PApld mimics ethylene action through direct repression of CTR1 activity. The only deviation from this rule is that the ACS7 gene is up-regulated by CHX-induced PApld whereas the ETR1 gene is up-regulated by H2O2-induced PApld, but both genes are down-regulated by the others. At this point it is worth noting the potentially dual role of ETR1 which has also been reported to be a mediator in H2O2 signalling (Desikan et al., 2005).
(iv) The concerted short-term down-regulation of all the above discussed ethylene receptor genes in response to H$_2$O$_2$ may temporarily sensitize the plant tissues to the ethylene production that follows. In contrast to the other inducers, H$_2$O$_2$ somewhat decreases the high expression of the housekeeping PLD-$\alpha 1$ and -$\alpha 2$ genes, which probably results in a lowered concentration of the PLD-$\alpha$-derived PA, a potential negative regulator of CTR1. Moreover, H$_2$O$_2$ decreases the low constitutive level of the PLD-$\delta$ transcripts. Therefore, during the response to H$_2$O$_2$ a concurrent regulation of genes encoding ethylene receptors and PLD-$\delta$ and -$\alpha$ isozymes always results in the opposite effects on the ethylene signalling pathway. On the other hand, H$_2$O$_2$ up-regulates the PLD-$\gamma 1$ gene. However, the expression of the latter gene is considerably lower than that of PLD-$\alpha 1$ and -$\alpha 2$. A decrease in expression of the PLD-$\delta$, -$\alpha 1$, and -$\alpha 2$ genes correlates with the synchronous increase in the stress-induced PLD-$\gamma 1$ gene, implying the regulation of these distinct classes of PLD genes in an opposite manner. At this point it is worth noting that the inhibitory effect of PLD-$\beta$- and -$\gamma$-derived PA species on the catalytical activity of PLD-$\alpha$ class isozymes in *B. oleracea* has been reported (*Austin-Brown and Chapman*, 2002).

(v) Generally, a very low constitutive level of PLD-$\delta$ transcripts was detected throughout the experiment, and the only stimulus able to modify its level was the treatment with butanol-1. Thus it could be speculated that the intracellular level of PA may be involved in a permanent control of the transcriptional activity of the PLD-$\delta$ gene. In protoplasts of *Arabidopsis*, PLD-$\delta$-generated PA$\text{PLD}$ functions to decrease H$_2$O$_2$-promoted PCD, but the activation of a PLD-$\delta$ isozyme results from the activation of pre-existing PLD-$\delta$ rather than from the synthesis of the enzyme. Furthermore, in protoplasts of *Arabidopsis* after 3 h of treatment with H$_2$O$_2$ the level of PLD-$\delta$ protein essentially decreased (*Zhang et al.*, 2003).

(vi) There is an extremely good correlation of the level of transcripts and the time course of expression for PLD-$\gamma 1$, RbohD, and RbohF genes. On the basis of the reports supporting the role of some PA$\text{PLD}$ in direct regulation of the catalytical activity of Rboh-NADPH oxidase, it can be speculated that PLD-$\gamma 1$-derived PA$\text{PLD}$ species and RbohD and F oxidase may have a close functional relationship and it can be reasonably presumed that they are involved in the second phase of the oxidative burst (*Sang et al.*, 2001; *Zhang et al.*, 2005; *Yu et al.*, 2008).

(vii) The increased expression of the ACS1 gene encoding the most potent ACS isozyme precedes the enhanced expression of the PLD-$\gamma 1$, RbohD, and RhoF genes whose up-regulation is delayed by ethylene. Therefore, it can be concluded that the earliest ethylene production, mainly controlled by ACS1, can orchestrate or temporarily repress the expression of genes encoding the key catalytic subunits of enzymes generating superoxide anions (Rboh-NADPH oxidase D and F) or stress-specific PA$\text{PLD}$ signalling molecules (PLD-$\gamma 1$).

(viii) Ethylene delays the start of up-regulation of ACS3, PLD-$\gamma 1$, RbohD, and RhoF genes and down-regulates the expression of ACS4, 5, and 11 genes, in contrast to stress-induced PA$\text{PLD}$ which enhance the expression of the above genes. There are two exceptions to this rule, ACS5 and RbohD, whose expression is down-regulated by the copper-induced PA$\text{PLD}$. Thus, the question is how the regulatory network of the above-mentioned genes recognizes distinct species of PA$\text{PLD}$ and responds to them in a different way.

(ix) CHX significantly induces the expression of all ACS genes discussed except ACS5. All of them are under the positive control of CHX-induced PA$\text{PLD}$ signalling except the multiresponsive ACS1. In contrast to CHX-induced PA$\text{PLD}$ signalling which does not significantly affect the level of the ACS1 transcripts, its expression is efficiently stimulated by CHX.

(x) The results of pre-treatment with isobutanol (Fig. 5) allowed a distinction to be made between ACS5, ACO1, and RbohD whose expression was essentially affected by isobutanol action, and the remaining genes whose expression did not alter significantly. It has been reported that volatile isobutyl derivatives are abundantly synthesized in broccoli seedlings when only a few days old (*Fernandes et al.*, 2009); thus the possibility of reciprocal relationships between metabolism of isobutyl derivate and ethylene biosynthesis was suggested.

(xi) Considering the nature of the inducers such as H$_2$O$_2$ (generated by plants during their response to pathogen attack) and CHX (found as an antifungal antibiotic of some soil-borne *Streptomyces* species), it could be reasonably expected that both of them can affect the PA$\text{PLC/DGK}$ signalling route. PLC and PLD are affected in opposite ways by copper ions. Copper transiently enhances the catalytical activity of PLD but considerably inhibits that of PLC (*Pina-Chable et al.*, 1998; *Navari-Izzo et al.*, 2006). Our speculation is such that in the seedlings characterized with a lowered concentration of PA$\text{PLD}$, the short decline in earlier up-regulated expression of ACO1 following addition of H$_2$O$_2$ or CHX could result from transitory stress-induced PA$\text{PLC/DGK}$ (Fig. 4 right, 0.5 h after addition of H$_2$O$_2$; Fig. 5D, 0.5 h and 1.0 h after addition of CHX). Such a decline in the accumulation of the ACO1 transcripts did not occur in the seedlings treated with butanol-1 and subsequently treated with copper (Fig. 3B). This could support the view that both PA$\text{PLD}$ and PA$\text{PLC/DGK}$ are the negative regulators of ACO1 expression.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Figure S1.** Illustration of the effect of the long-term treatment of broccoli seedlings with 0.1% butanol-1 and 0.1% isobutanol. Seedlings were germinated in the dark on MS medium with addition of 3% sucrose, and 1 d after germination (denoted as time 0) 10 randomly chosen
seedlings were transferred to the same medium without supplements (control seedlings); to medium with 0.1% butanol-1; and to medium with 0.1% isobutanol, and were kept in dark (A) or light (16 h light/8 h dark, B) conditions throughout the next 7 d.

Figure S2. Sequence alignment of partial cDNAs of BO-ACS5, 9, and 11, BO-PLDδ, and BO-RbohD and F (GU942464, GU942465, GU942466, GU942467, GU942468, and GU942463, respectively) with their counterparts from Arabidopsis thaliana and Sinapis arvensis (SA-ACS2, 3 and 4, AT-ACS5, 9 and 11; AF074928, AF074929, and AF074930, NM_125977, NM_114830, and NM_116873, respectively; AT-PLDδ, AT-RbohD and F; NM_179170, NM_124165 and NM_105079, respectively)

Acknowledgements

We are grateful to Hanna Korcz-Szatkowska for help in preparing plant materials.

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