Journal of Experimental Botany, Vol. 61, No. 12, pp. 3475–3491, 2010
doi:10.1093/jxb/erq177 Advance Access publication 25 June, 2010
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

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-γ 1*, and *RbohD* and *F* genes is undoubtedly under the reciprocal cross-talk of the ethylene and PA_{PLD} 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 *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 sourrounding sites of Rboh activation (Torres *et al.*, 2005).

Rapidly diffusing across the cell membranes, H_2O_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_2O_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_2O_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_2O_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_2O_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; Schlagnhaufer *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 in the absence of ethylene. Binding of ethylene turns the receptor activity off, which results in removal of the downstream block on signal transduction (Fig. 2) (Gao *et al.*, 2008; Zhu and Guo, 2008; Lin *et al.*, 2009). A reduction in their overall number increases the 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_2O_2 (Desikan *et al.*, 2005). Thus, it is possible that ETR1 can act as a node mediating cross-talk between ethylene and H_2O_2.

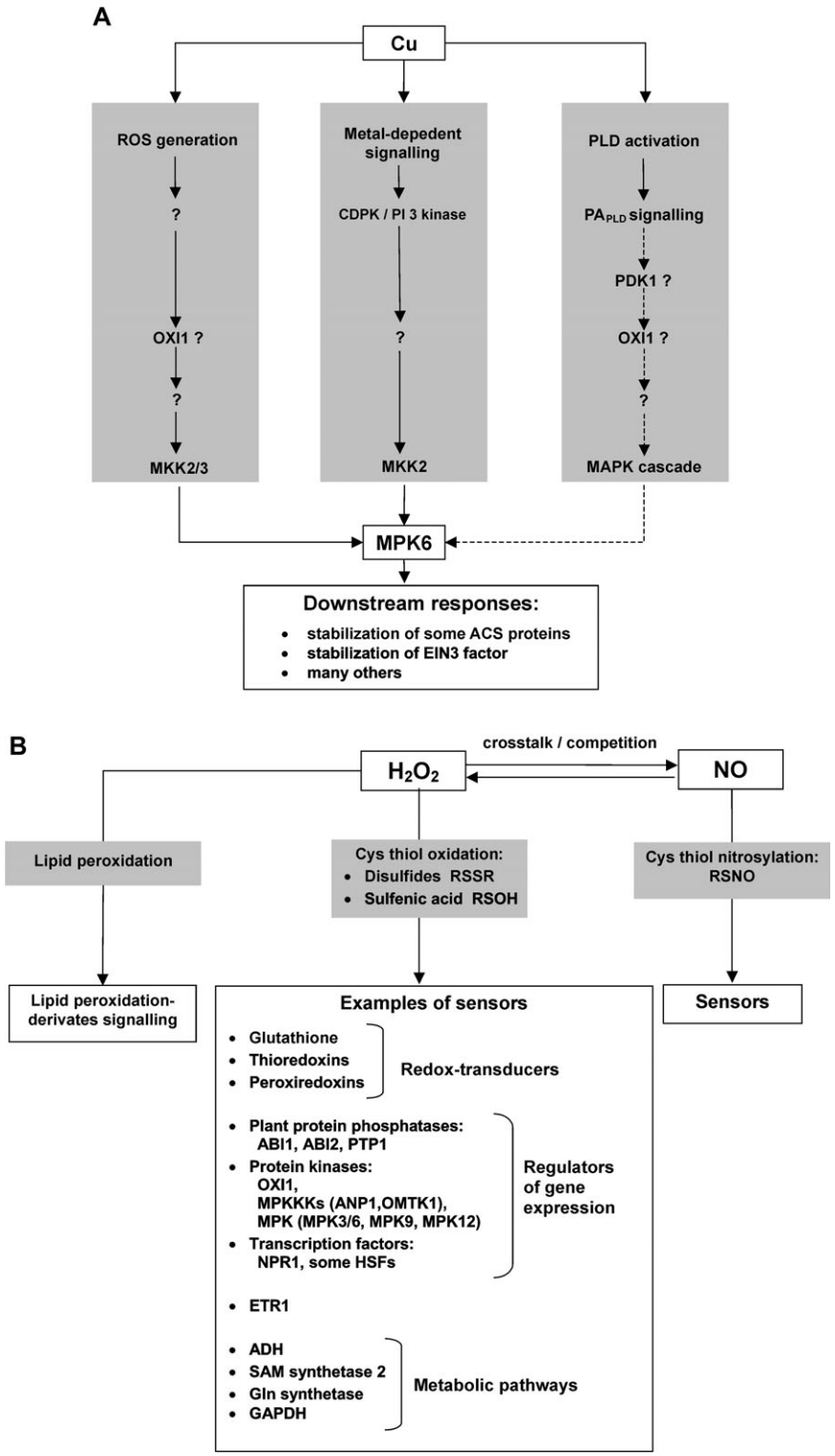

Fig. 1. A schematic representation of possible signalling routes of copper (A) and hydrogen peroxide (B) (the dotted line denotes a putative pathway; for details see the Introduction).

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 ($PA_{PLC/DGK}$) (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

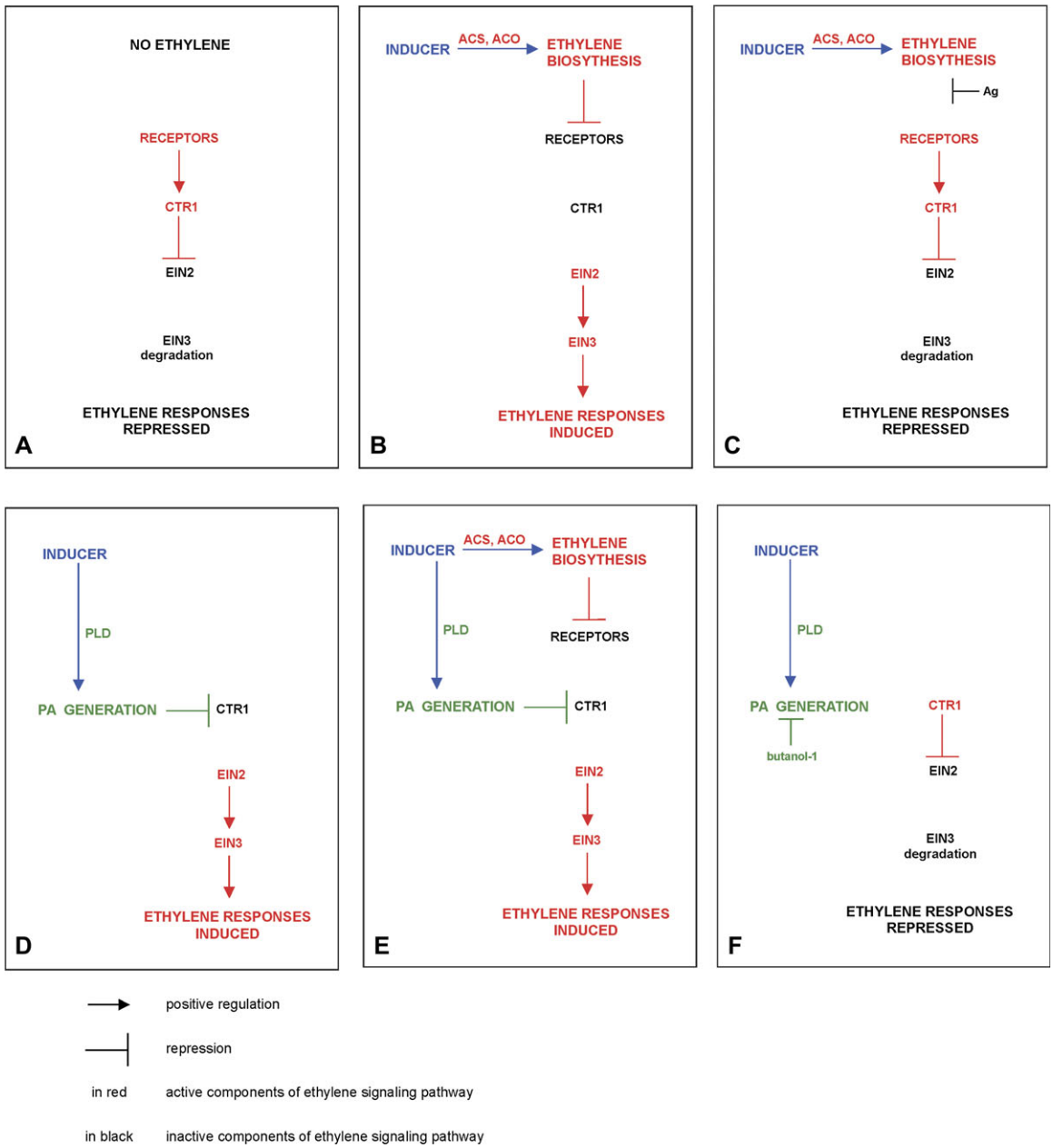

Fig. 2. A schematic presentation of relationships between the key components of the ethylene signal transduction pathway. (A and B) Ethylene action on downstream components of its signalling route. (C) Inhibitory effect of silver ions on ethylene signalling. (D) Inhibitory action of PA_{PLD} on CTR1 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_{PLP} signalling and its possible consequence.

determinants of the functioning of PA_{PLD} as a secondary messenger molecule which can act in opposite ways (Wang, 2000).

It has been assumed that PA_{PLD} can enhance the activity of Rboh-NADPH-oxidase (Yu *et al.*, 2008). A direct promotion of the catalytical activity of Rboh-oxidase by PLD-α1-derived PA species and the Cu^{2+}-induced elevation of both PA_{PLD} content and activation of Rboh-oxidase have been found in *Arabidopsis* (Zhang *et al.*, 2005) and wheat (Navari-Izzo *et al.*, 2006). Furthermore, Navari-Izzo *et al.* (2006) reported that the reduced PA_{PLD} level in wheat roots almost completely abolished the production of the superoxide anion. Nevertheless, the other isozyme of PLD, PLD-δ activated by H_2O_2, does not stimulate H_2O_2 formation; moreover, PA_{PLD} species generated by PLD-δ lead to a decrease in H_2O_2-promoted PCD (Zhang *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 *Arabidopsis* CTR1 that PA binds to CTR1, inhibits its kinase activity, and blocks interactions with the ethylene receptor ETR1 (Testerink *et al.*, 2007, 2008).

PLDs have the unique ability to transfer the phosphatidyl group of their substrates to a primary alcohol instead of water, which results in formation of phosphatidyl alcohols. The formation of the latter, especially that of inactive phospholipid such as phosphatidylbutanol, blocks PA_{PLD} production. Treatment with butanol-1 is used as a very effective method of inhibition of PA_{PLD} signalling in living cells (Morris *et al.*, 1997; Testerink and Munnik, 2005).

Ethylene receptors function as a dimer with a copper ion located in the hydrophobic pocket, but it has been shown that silver ions are able to replace copper. Despite the fact that the silver ion-occupied receptor binds ethylene, the binding site is apparently perturbed so that it is not able to induce the alterations that are necessary to elicit downstream signalling (Rodriguez *et al.*, 1999; Klee, 2002; Klee and Tieman, 2002; O'Malley *et al.*, 2005; Gao *et al.*, 2008). Thus, treatment of plants with silver represses the ethylene responses (see Fig. 2).

This observation has prompted us to check the effect of a decreased intracellular level of PA_{PLD} or the silver-induced ethylene insensitivity on the transcriptional activity of the ethylene biosynthesis enzymes, ethylene receptors, PLD, and Rboh-NADPH-oxidase genes. The aim of this study was to investigate the time course of expression of the above-mentioned genes at the early step of plant response to copper, exogenously applied H_2O_2, or CHX in *Brassica oleracea* etiolated seedlings. This investigation has provided evidence that the level of PA_{PLD} considerably affects the expression of the genes discussed and supports the putative regulatory involvement of PA_{PLD} in the ethylene signal transduction pathway.

Various plant species produce both the shorter and longer transcripts for the same ACS isozyme; moreover, the presence of the stress-induced incomplete spliced transcripts of some ACS isozymes has been reported (Nakagawa *et al.*, 1991; Rottmann *et al.*, 1991; Spanu *et al.*, 1993; Olson *et al.*, 1995; ten Have and Woltering, 1997; Peck and Kende, 1998). The role of these non-functional transcripts is still not recognized. Thus, it was decided to analyse the transcriptional pattern of the genes discussed considering the longer amplicons of their cDNA (462–718 bp) generated by a semi-quantitative RT-PCR method.

Materials and methods

Plant material and growth conditions

Brassica oleracea var. *alboglabra* A12 DH seeds used in the experiments were sterilized and sown onto MS medium containing 0.8% agar and 3% sucrose. They were grown for 6 d in the dark at 23 °C and then were finally transferred to fresh MS liquid medium and placed for an additional 20 h in darkness before the treatments. For treatment with different inducers of ethylene biosynthesis, appropriate amounts of CHX, $CuCl_2$, H_2O_2, $AgNO_3$, butanol-1, and isobutanol were added to liquid MS medium to a predicted final concentration. The sets of experiments were carried out after 0, 0.5, 1, 1.5, 2, 3, 4, and 6 h. After treatment, whole seedlings were collected, frozen in liquid nitrogen, and stored at –80 °C.

The final concentrations of agents were as follows: 0.005 mM CHX, 2.5 mM $CuCl_2$, 0.1 mM $AgNO_3$, 0.25% H_2O_2, 0.1% butanol-1, and 0.1% isobutanol.

The seedlings were treated at different time intervals with: (i) 2.5 mM $CuCl_2$; (ii) 0.1% butanol-1 added 16 h prior to addition of 2.5 mM $CuCl_2$; (iii) 0.1 mM $AgNO_3$ added 16 h prior to addition of 2.5 mM $CuCl_2$; (iv) 0.25% H_2O_2; (v) 0.1% butanol-1 added 16 h prior to addition of 0.25% H_2O_2; (vi) 0.005 mM CHX; (vii) 0.1% butanol-1 added 16 h prior to addition of 0.005 mM CHX; (viii) 0.1% isobutanol added 16 h prior to addition of 0.005 mM CHX; and (ix) 0.1% butanol alone.

RNA preparation

Total RNA was extracted from 7-d-old etiolated *B. oleracea* seedlings using the Trizol method (Invitrogen) according to the manufacturer's procedure. Total RNA was quantified by UV spectrophotometry and its integrity was assessed on a 1.2% ethidium bromide-stained formaldehyde agarose gel.

Primers

The following primers were used for specific amplification: *BO-ACS1*, F, 5′-TTCAGGATTATCATGGCTTGCCTG-3′ and R, 5′-CTTCTGCGACGCTGATAAACTCG-3′ (X82273); *BO-ACS3*, F, 5′-GAGTTCAGACAGGCAATTGCAC-3′ and R, 5′-AGTCCCATGTCTTTCGAGAGAC-3′ (AF338652); *BO-ACS4*, F, 5′-GAATCCTCACGGCATTATCCAG-3′ and R, 5′-AAGTTCGGTTTGGGTTGCTGTC-3′ (AB086353); *BO-ACS5*, F, 5′-CTAGTCTCAAGAGGAACGGTCAG-3′ and R, 5′-TTCTTGGAAGCGATGAAGTCAACG-3′ (AF074930); *BO-ACS7*, F, 5′-TGGGTTCAGAGAAAACGCACTG-3′ and R, 5′-CTGACACGTCATCGATGTTCTC-3′ (AF338651); *BO-ACS9*, F, 5′-AGCTAAGAATCCGGACGCAGCAG-3′ and R, 5′-CATGAACTCGTTTGGAGACTTCAC-3′ (AF074929); *BO-ACS11*, F, 5′-GTTCCAAGATTACTATGGCTTGCC-3′ and R, 5′-CACTTCTAGAACGCTGGTGAACTC-3’ (AF074928); *BO-ACO1*, F, 5′-GACAAGGTCAGTGGTCTCCAGCTTC-3′ and R, 5′-CCATTGACCAACAATTAACCACCAG-3′ (X81628); *BO-ETR1*, F, 5′-GCTCAAACACAGTCTTTAGCGAC-3′ and R, 5′-ATCACACTAAACCTCGCACCAG-3′ (AF047476); *BO-ERS1*, F, 5′-CTATAGGCGATGAGAAACGTCTG-3′ and R, 5′-GTGATTTGGCTGCAAGACGTAGC-3′ (AF047477); *BO-ETR2*, F, 5′-GGTTTCGGTTTACGGTTGATGC- 3′ and R, 5′-CTGTTCCATGGACTGATATGGAC-3′ (AB078598); *BO-PLDα1*, F, 5′-CAAGCTATATTGGATTGGCAGAG-3′ and R, 5′-AAATCCGGGAAACTCAGTGACG-3′ (AF090445); *BO-PLD α2*, F, 5′-CAAGCTATATTGGATTGGCAGAG-3′ and R, 5′-TGATATTACCTTCATTGTCCACAC-3′ (AF090444); *BO-PLDγ1*, F, 5′-TTTGCGTGTAGAGCTGTTGCAC-3′ and R, 5′-CAGCACTTCCCATGTCTATACTG-3′ (EU591736); *BO-PLDδ*, F, 5′-GGAAGGTGTTCGAGTTTTGCTAC-3′ and R, 5′-CACGGATCCTGAGTCGATAGAAC-3′ (AY113045); *BO-RbohD*, F, 5′-GGCACTGATAATGGAAGAGTTGG-3′ and R, 5′-CCATCCTTCCACTCCTTTCAC-3′ (AF424625); *BO-RbohF*, F, 5′-GCGGTAAAAGCGGACTTCTCAG-3′ and R, 5′-CGGTACTCCGCAATAAAACACTC-3′ (AB008111); and *BO-ACT1*, F, 5′-GCTATCCAAGCTGTCCTCTC-3′ and R, 5′-GAGAGCTTCTCCTTGATGTC-3′ (AF044573).

Primers for *BO-ACS5*, *9*, and *11* genes and for *BO-PLDδ*, *BO-RbohD*, and *BO-RbohF* genes were designed using the appropriate genes from *Sinapis arvensis* and *Arabidopsis thaliana*, respectively. The products of amplification were sequenced to confirm that these primers amplified fragments of the predicted genes from *B. oleracea* var. *alboglabra* A12 DH (see Supplementary data available at *JXB* online). The numbering of the broccoli ACS isozymes BO-ACS4, 5, 7, 9, and 11 was carried out according to their well-characterized counterparts from *Arabidopsis*, with the exception of BO-ACS1 and BO-ACS3 which correspond to AT-ACS6 and AT-ACS2, respectively.

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), $MgCl_2$, 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 PA_{PLD} 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, H_2O_2, 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, H_2O_2, and CHX is presented in Figs 3, 4, and 5. In contrast to the other *ACS* genes, the transcripts of the *BO-ACS9* 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 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/

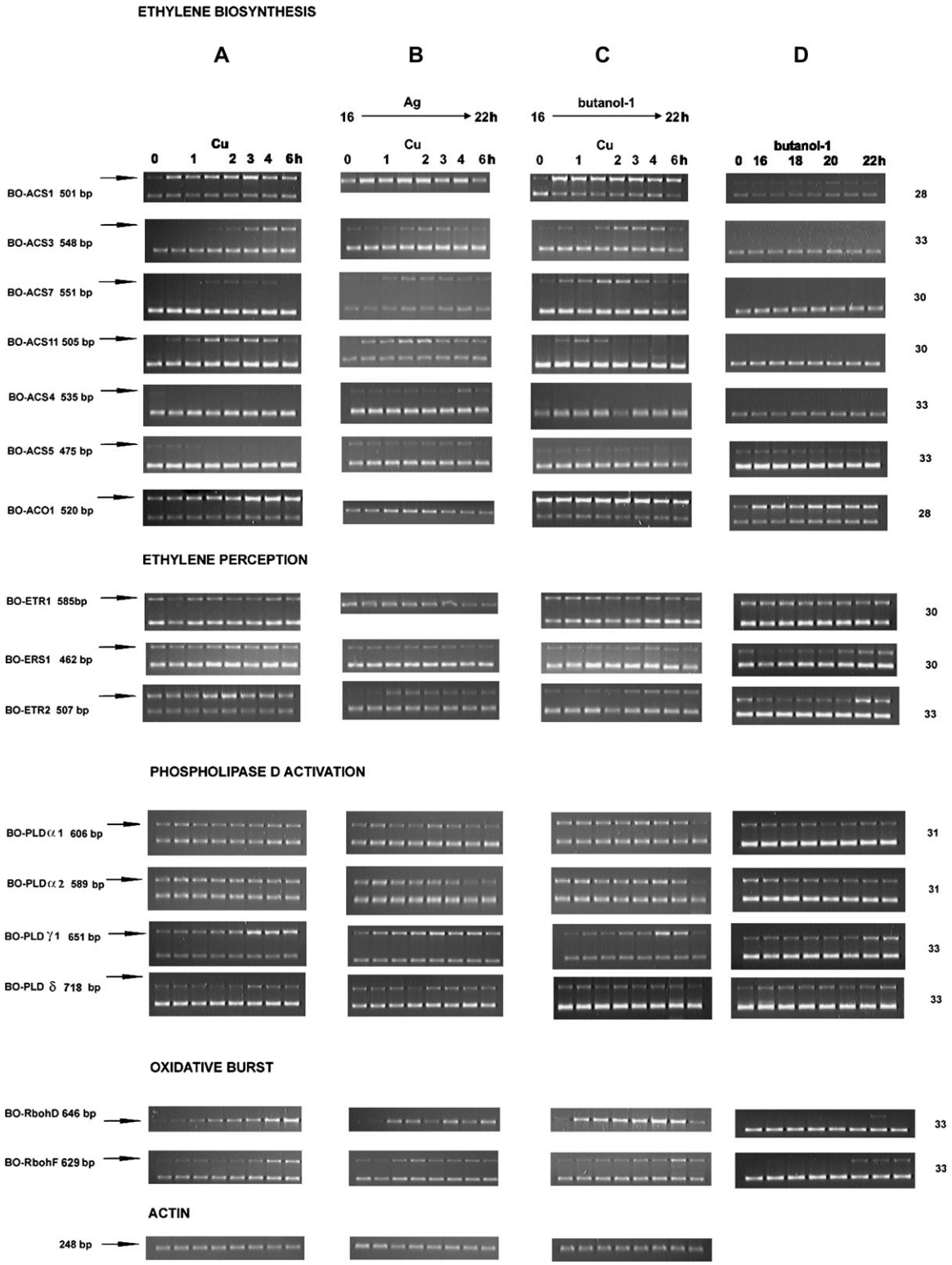

Fig. 3. The time course of transcriptional expression of genes encoding ethylene biosynthetic enzymes, ethylene receptors, phospholipases D, and Rboh-NADPH-oxidases in etiolated *B. oleracea* A12 DH seedlings treated with 2.5 mM $CuCl_2$ (A); 0.1 mM $AgNO_3$ and 2.5 mM $CuCl_2$ (B); 0.1% butanol-1 and 2.5 mM $CuCl_2$ (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.

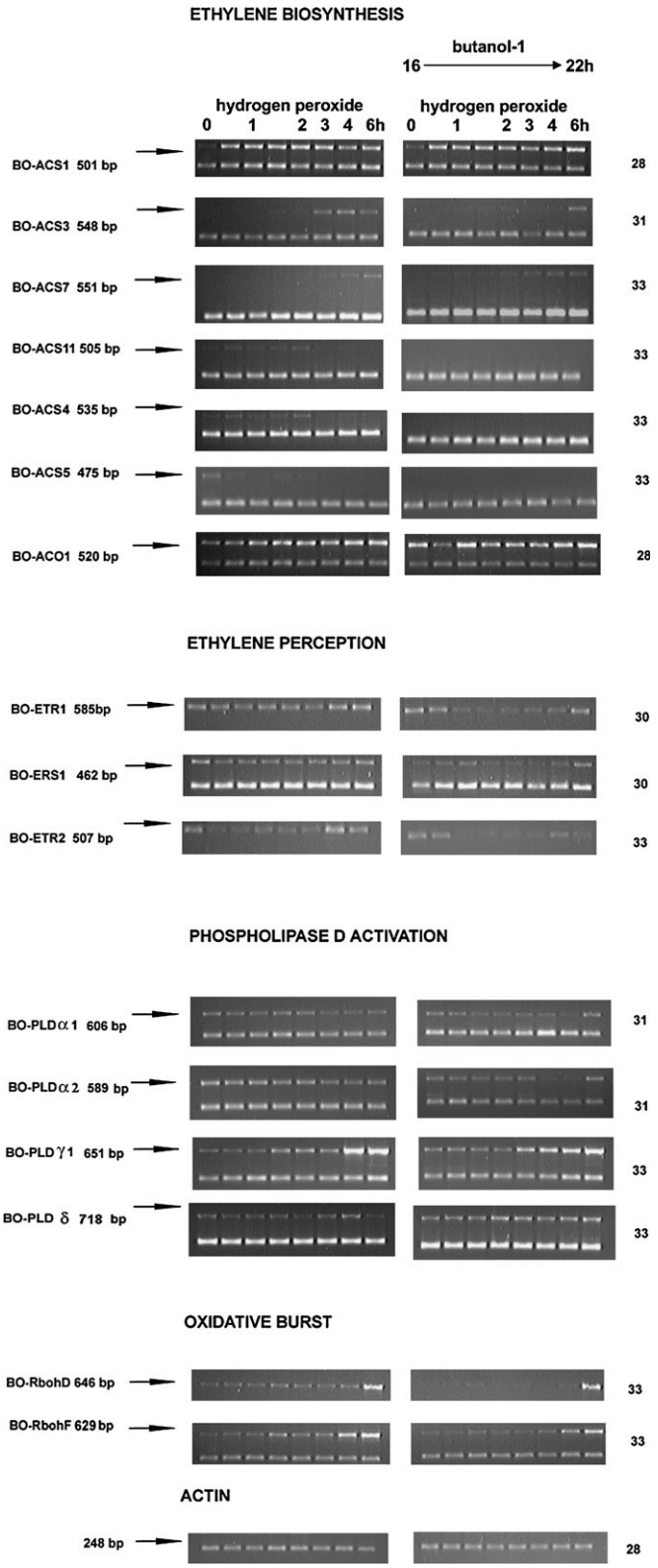

Fig. 4. The time course of transcriptional expression of genes encoding ethylene biosynthetic enzymes, ethylene receptors, phospholipases D, and Rboh-NADPH-oxidases in etiolated *B. oleracea* A12 DH seedlings treated with 0.25% H_2O_2 (left column) or 0.1% butanol-1 and 0.25% H_2O_2 (right column). Transcripts 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.

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_{PLD} signalling prior to the treatment of plants with copper, H_2O_2, 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_{PLD} 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 H_2O_2 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 H_2O_2. 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 H_2O_2 (Fig 4).

The pre-treatment with butanol-1 prior to addition of H_2O_2 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 H_2O_2 alone. In seedlings pre-treated with butanol-1 prior to addition of H_2O_2, the accumulation of transcripts of *ACS4*, *-5*, and *-11* was below the level of detection (detection at 33 cycles) (Fig. 4).

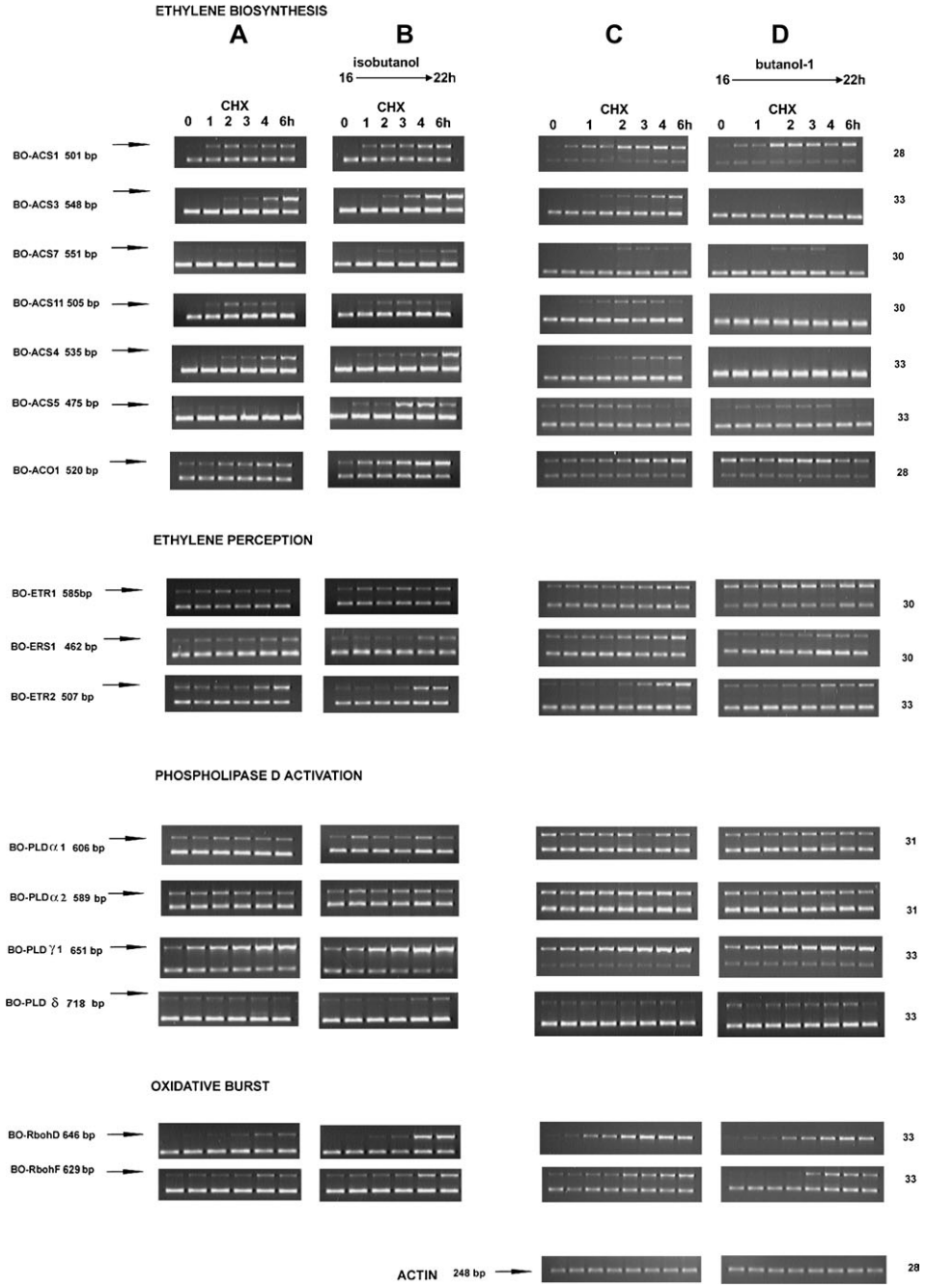

Fig. 5. The time course of transcriptional expression of genes encoding ethylene biosynthetic enzymes, ethylene receptors, phospholipases D, and Rboh-NADPH-oxidases in etiolated *B. oleracea* A12 DH seedlings treated with 5 μM CHX (A); 0.1% isobutanol and 5 μM CHX (B); 5 μM CHX (C); and 0.1% butanol-1 and 5 μM CHX (D). 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 each column. The expression of the actin housekeeping gene was used as an internal control. The results presented in column A and B are representative of two, those in C and D three 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 response to 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 cyles (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-treament 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 PA_{PLD} 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).

H_2O_2 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 H_2O_2 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 plants 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

ETR1 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 H_2O_2 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 H_2O_2 (Fig. 4). Moreover, the pre-treatment of seedlings with butanol-1 prior to addition of H_2O_2 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 isoforms 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_2O_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_2O_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_2O_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_2O_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_2O_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 up-regulated 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_2O_2 was very similar (Figs 3, 4). In contrast to *RbohF*, the expression of *RbohD* in response to H_2O_2 increased significantly later, after 6 h of treatment. Therefore, it should be considered whether the high concentration of H_2O_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_2O_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 isozymes (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 $PA_{PLC/DGK}$, while the second one involves a specific PA_{PLD} (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 PA_{PLD} can elevate the activity of Rboh-NADPH-oxidase (Yu *et al.*, 2008). In the present experiments, treatment with exogenously added H_2O_2 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 PA_{PLD} 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 PA_{PLD} signalling pathways occurring in the seedlings treated with copper (Fig. 6A), H_2O_2 (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 PA_{PLD}.

(ii) Ethylene up-regulates the expression of the *ACO1* gene encoding the main ACO isozyme, while PA_{PLD} 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 PA_{PLD}.

(iii) As the action of ethylene and PA_{PLD} 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 PA_{PLD} 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 PA_{PLD} whereas the *ETR1* gene is up-regulated by H_2O_2-induced PA_{PLD}, 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 H_2O_2 signalling (Desikan *et al.*, 2005).

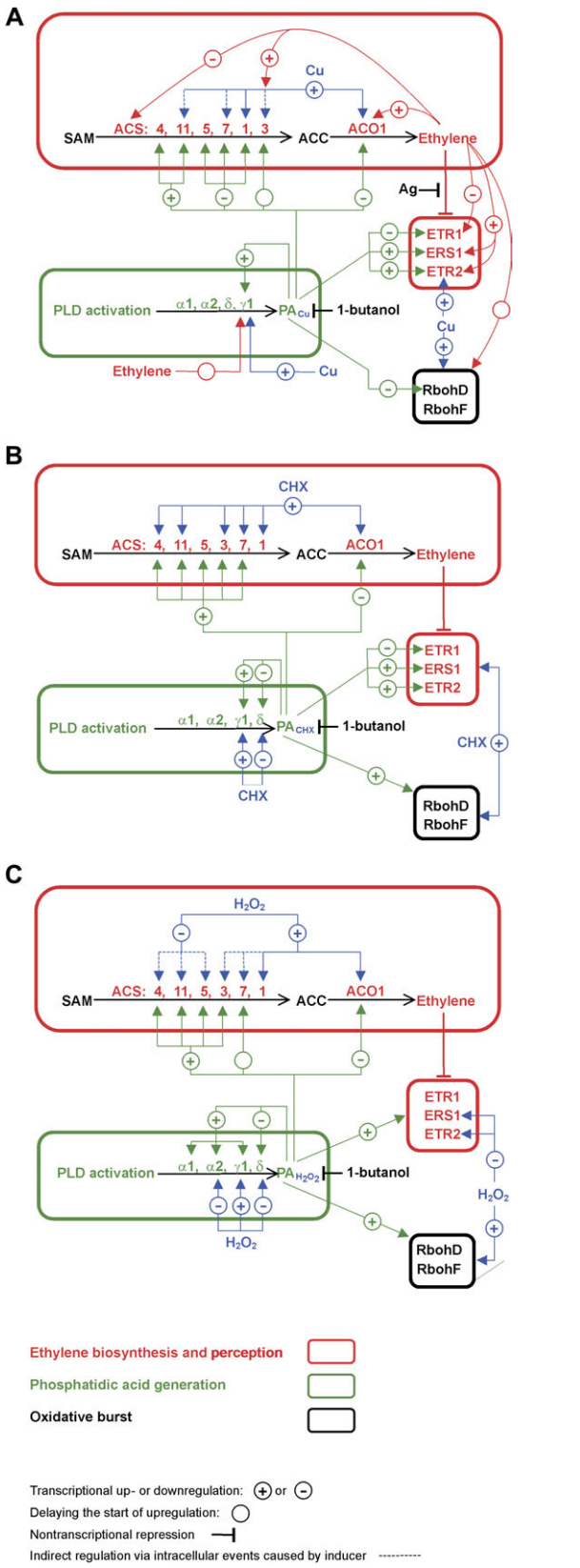

Fig. 6 Stimuli-induced transcriptional activity of the ethylene biosynthetic enzymes, ethylene perception, phospholipase D, and Rboh-NADPH oxidase genes by copper (A), CHX (B), and H_2O_2 (C) in 7-d-old etiolated seedlings of broccoli (for details see the Results).

(iv) The concerted short-term down-regulation of all the above discussed ethylene receptor genes in response to H_2O_2 may temporarily sensitize the plant tissues to the ethylene production that follows. In contrast to the other inducers, H_2O_2 somewhat decreases the high expression of the housekeeping *PLD-α1* and *-α2* genes, which probably results in a lowered concentration of the PLD-α-derived PA, a potential negative regulator of CTR1. Moreover, H_2O_2 decreases the low constitutive level of the *PLD-δ* transcripts. Therefore, during the response to H_2O_2 a concurrent regulation of genes encoding ethylene receptors and PLD-δ and -α isozymes always results in the opposite effects on the ethylene signalling pathway. On the other hand, H_2O_2 up-regulates the *PLD-γ1* gene. However, the expression of the latter gene is considerably lower than that of *PLD-α1* and *-α2*. A decrease in expression of the *PLD-δ*, *-α1*, and *-α2* genes correlates with the synchronous increase in the stress-induced *PLD-γ1* 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-β- and γ-derived PA species on the catalytical activity of PLD-α class isozymes in *B. oleracea* has been reported (Austin-Brown and Chapman, 2002).

(v) Generally, a very low constitutive level of PLD-δ 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-δ* gene. In protoplasts of *Arabidopsis*, PLD-δ-generated PA_{PLD} functions to decrease H_2O_2-promoted PCD, but the activation of a PLD-δ isozyme results from the activation of pre-existing PLD-δ rather than from the synthesis of the enzyme. Furthermore, in protoplasts of *Arabidopsis* after 3 h of treatment with H_2O_2 the level of PLD-δ 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-γ1*, *RbohD*, and *RbohF* genes. On the basis of the reports supporting the role of some PA_{PLD} in direct regulation of the catalytical activity of Rboh-NADPH oxidase, it can be speculated that PLD-γ1-derived PA_{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-γ1*, *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_{PLD} signalling molecules (PLD-γ1).

(viii) Ethylene delays the start of up-regulation of *ACS3*, *PLD-γ1*, *RbohD*, and *RhoF* genes and down-regulates the expression of *ACS4*, *5*, and *11* genes, in contrast to stress-induced PA_{PLD} which enhancse 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_{PLD}. Thus, the question is how the regulatory network of the above-mentioned genes recognizes distinct species of PA_{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_{PLD} signalling except the multiresponsive *ACS1*. In contrast to CHX-induced PA_{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 derivates and ethylene biosynthesis was suggested.

(xi) Considering the nature of the inducers such as H_2O_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 $PA_{PLC/DGK}$ 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_{PLD}, the short decline in earlier up-regulated expression of *ACO1* following addition of H_2O_2 or CHX could result from transitory stress-induced $PA_{PLC/DGK}$ (Fig. 4 right, 0.5 h after addition of H_2O_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_{PLD} and $PA_{PLC/DGK}$ 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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