Journal of Experimental Botany, Vol. 58, No. 6, pp. 1497–1503, 2007
doi:10.1093/jxb/erm011 Advance Access publication 21 February, 2007

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Indole-3-acetic acid and auxin herbicides up-regulate 9-*cis*-epoxycarotenoid dioxygenase gene expression and abscisic acid accumulation in cleavers (*Galium aparine*): interaction with ethylene

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Received 15 October 2006; Revised 5 January 2007; Accepted 9 January 2007

Abstract

Interaction between auxin and auxin-induced ethylene was suggested in previous work to up-regulate abscisic acid (ABA) biosynthesis in cleavers (*Galium aparine*) through stimulated cleavage of xanthophylls to xanthoxin, catalysed by 9-*cis*-epoxycarotenoid dioxygenase (NCED). Here, the effects of auxin on *NCED* gene expression were studied in relation to changes in ethylene synthesis and ABA levels. A gene from *G. aparine* shoot tissue was cloned based on sequence similarity to cloned *NCED* genes from tomato (*LeNCED1*), potato, *Phaseolus*, and *Arabidopsis*. When the roots of *G. aparine* plants were treated with 0.5 mM indole-3-acetic acid (IAA), IAA concentrations increased from 0.2 μM to 65 μM IAA in the shoot tissue after 3 h. Transient increases in *GaNCED1* mRNA levels were detectable as early as 1 h after treatment and reached maximum values of 40-fold, relative to the control, after 3 h. Increases in *GaNCED1* mRNA preceded increases in 1-aminocyclopropane-1-carboxylic acid and ethylene. Levels of ABA began to increase more slowly and, significantly, with a lag phase of 2 h, and reached levels 24-fold higher than those in controls after 24 h. *GaNCED1* gene expression was also stimulated by auxin herbicides. The ethylene-releasing compound ethephon induced *GaNCED1* transcript levels only moderately. In accordance with this, aminoethoxyvinylglycine and cobalt ions, which inhibit ethylene synthesis, only slightly affected the increase in *GaNCED1* transcript levels by IAA. However, both ethylene inhibitors decreased IAA-induced ABA accumulation by up to 70%. This suggests that auxin and auxin-induced ethylene are involved in ABA accumulation. While auxin is the primary trigger for *NCED* gene expression, ethylene appears to enhance ABA biosynthesis, possibly by up-regulation of NCED activity post-transcriptionally.

Key words: Abscisic acid, auxin herbicides, 9-*cis*-epoxycarotenoid dioxygenase, ethylene, *Galium aparine*, gene expression, indole-3-acetic acid.

Introduction

Indole-3-acetic acid (IAA), the principal natural auxin in higher plants, usually stimulates a variety of growth and developmental processes (Woodward and Bartel, 2005). However, with increasing concentrations, growth abnormalities are induced, which include leaf epinasty and growth inhibition of root and, to a greater extent, of shoot, followed by accelerated foliar senescence with chloroplast damage, and by the destruction of membrane and vascular system integrity, leading to desiccation, tissue necrosis, and decay (reviewed by Sterling and Hall, 1997; Grossmann, 2003). The effects of IAA on other hormones, such as ethylene and gibberellins, contribute to its ability to regulate this diverse range of developmental phenomena (Ross *et al.*, 2002; Grossmann, 2003). Auxin herbicides basically act as synthetic mimics of IAA at high

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Abbreviations: ABA, (+)-abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinyl-glycine IAA, indole-3-acetic acid; NCED, 9-*cis*-epoxycarotenoid dioxygenase.

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concentrations (Sterling and Hall, 1997; Grossmann, 2003). In sensitive dicot species, the herbicide syndrome is mediated by a newly discovered hormone interaction between sequentially induced ethylene and abscisic acid (ABA) biosynthesis (Hansen and Grossmann, 2000; Grossmann and Hansen, 2001; Grossmann, 2003). The induction of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase in ethylene biosynthesis appears to be the primary target process, following auxin herbicide signalling (Sterling and Hall, 1997; Grossmann, 2003). Isoforms of ACC synthase genes were shown to be differentially expressed or post-transcriptionally or post-translationally regulated by auxins within a few minutes of treatment (Wang *et al*., 2002; Tsuchisaka and Theologis, 2004; Chae and Kieber, 2005). Subsequently, massive accumulations of ABA in root and even more in shoot tissue were found (Scheltrup and Grossmann, 1995). This was exemplified first for the dicot weed cleavers (*Galium aparine*) after root treatment with the auxin herbicide quinmerac (Scheltrup and Grossmann, 1995). ABA concentrations were 70 times greater than those in controls 48 h after treatment. Induction of ABA accumulation by both auxin herbicides from the different chemical classes and IAA at high concentrations in a variety of dicot species was confirmed (Grossmann, 2003). Experiments with isolated roots and shoots revealed that auxin-induced increases in ethylene and ABA occur exclusively in the shoot tissue. From there, ABA appears to be translocated within the plant. Accumulated ABA mediates stomatal closure, which limits photosynthetic activity and biomass production, and is accompanied by the overproduction of reactive oxygen species such as hydrogen peroxide (Scheltrup and Grossmann, 1995; Grossmann *et al*., 2001). Growth inhibition, senescence and tissue decay are the consequences of this cascading process (Grossmann, 2000, 2003).

Recently, this causality was established by molecular dissection of the target pathways of ethylene and ABA (Hansen and Grossmann, 2000). This approach used different inhibitors of biosynthesis, tomato mutants defective in perception or synthesis of ethylene or ABA, and quantification of tissue levels of xanthophylls, xanthoxin, ABA, and its degradation and conjugation products (Hansen and Grossmann, 2000). The results suggested that auxin-stimulated ethylene triggers ABA biosynthesis by increasing xanthophyll cleavage, leading to increased production of the ABA precursor xanthoxin (Hansen and Grossmann, 2000). This key regulated step in ABA biosynthesis is catalysed by the plastid enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED), which is encoded by a family of *NCED* genes (reviewed by Schwartz *et al*., 2003; Tan *et al*., 2003; Xiong and Zhu, 2003; Taylor *et al*., 2005). The different isoforms of NCED appear to be expressed in different tissues, at different developmental stages, and in response to drought and osmotic stress (Schwartz *et al*., 2003; Tan *et al*., 2003; Xiong and Zhu, 2003; Taylor *et al*., 2005; Rodrigo *et al*., 2006). In leaves, stress-induced increases in *NCED* transcript levels were detected within 15–30 min after treatment (Qin and Zeevaart, 1999; Thompson *et al*., 2006). Therefore, it was hypothesized that auxin-induced ethylene induces NCED activity which, in turn, up-regulates ABA biosynthesis, possibly by inducing *NCED* gene expression (Grossmann and Hansen, 2000; Grossmann, 2003). In accordance with this, analysis of the promoter of tomato *LeNCED1* revealed five ethylene-responsive elements upstream of the gene, one of which was in the 0 to −595 bp region (Thompson *et al*., 2004). In addition, the signal transduction chains of ethylene and ABA were found to be partly overlapping and interfering (Beaudoin *et al*., 2000; Ghassemian *et al*., 2000). Moreover, recent gene expression studies in *Arabidopsis* showed that the auxin herbicide 2,4-dichlorophenoxyacetic acid induced the expression of *NCED1* and genes involved in ethylene signalling and biosynthesis (Raghavan *et al*., 2006).

The present study was undertaken to evaluate auxin effects on *NCED* gene expression in relation to ethylene and ABA biosynthesis. The investigations were particularly focused on the action of IAA, at high concentrations, and synthetic auxin herbicides. The effects were determined primarily in the shoot of *G. aparine* plants because (i) this dicot weed is very sensitive to auxins and (ii) the shoot tissue was found to be the principal site of biochemical auxin action (Grossmann, 2003). Since *NCED* genes have not been isolated from *Galium* species, a gene was cloned from *G. aparine* shoot tissue based on sequence similarity to cloned *NCED* genes from tomato (*LeNCED1*), potato, *Phaseolus*, and *Arabidopsis*. Following root treatment, the time-course of auxin-induced changes in transcript levels of NCED, through quantitative real-time RT-PCR, and immunoreactive ABA levels were analysed. Concomitantly, ACC synthase activity, and ACC and ethylene production were measured. For testing causality, the ethylene-releasing compound ethephon and various inhibitors of ethylene biosynthesis were used. The results suggest that a cross-talk between auxin and auxin-induced ethylene up-regulates *NCED* gene expression and downstream signalling in *Galium* shoot tissue, leading to ABA accumulation. To our knowledge, this is the first report of this phenomenon.

Materials and methods

Chemicals

The following compounds were used: the synthetic auxins 2-methoxy-3,6-dichlorobenzoic acid (dicamba), 7-chloro-3-methyl-8-quinolinecarboxylic acid (quinmerac), and 4-amino-3,6,6-trichloropicolinic acid (picloram) from Riedel-de Haen (Seelze, Germany) and BASF AG, Ludwigshafen (Germany). ACC,

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aminoethoxyvinyl-glycine (AVG), (+)-ABA, and IAA were obtained from Calbiochem (Bad Soden, Germany) or from Sigma-Aldrich (Steinheim, Germany).

Cultivation of plants in hydroponics

Seedlings of *G. aparine* L. at the first whorl stage (15 d after sowing) were raised to the third whorl stage, as described (Grossmann *et al*., 2001). Uniformly developed plants were transferred into 320 ml glass vessels containing 310 ml of half-strength Linsmaier–Skoog (1964) medium and maintained at 16/8 h light/dark cycles, 25/20 °C and 75% relative humidity (three plants per vessel, five replications). Light (530 μmol m^{-2} s^{-1}, 400–750 nm) was provided by Osram Powerstar HQI-R 250 W/NDL and Osram Krypton 100 W lamps. The solution was aerated throughout the experiment. After 1 d of adaptation, IAA or a synthetic auxin was added, alone or in combination with AVG or cobalt chloride, to the medium in acetone solution [0.1% (w/v) final concentration of acetone]. Controls received corresponding amounts of acetone, with no adverse effect on the growth of the plants. AVG and cobalt chloride were added to the medium from aqueous stock solutions. At various times after treatment, ethylene was measured. Shoots from parallel vessels were harvested, immediately frozen in solid CO_2, and stored at −80 °C. Plant material was powdered under liquid nitrogen. All experiments were repeated at least twice and proved to be reproducible. The results of one representative experiment with mean values and standard errors of data are shown.

Determination of ethylene production

After treatment in hydroponic vessels, detached shoots of plants were transferred to 100 ml glass cylinders containing 10 ml of half-strength Linsmaier–Skoog medium (one shoot per cylinder; six replications). The cylinders were sealed with rubber caps. After incubation for a further 3 h under light, a 1 ml gas sample of the head space was withdrawn and ethylene was measured by gas chromatography (Grossmann *et al*., 2001).

Determination of ACC

Samples of powdered plant material (100 mg; three replications) were extracted with 70% (v/v) aqueous ethanol. Following oxidative conversion, the ACC content was assayed as ethylene by gas chromatography (Lizada and Yang, 1979; Grossmann *et al*., 2001).

Determination of ACC synthase activity

A sample of powdered plant material (2 g, two replicates) was extracted in 3 ml of 100 mM EPPS (*N*-[2-hydroxyethyl]piperazine-*N'*-3-propanesulphonic acid)/potassium hydroxide buffer (pH 8.5) containing dithiothreitol (5 mM), pyridoxal phosphate (6 μM), leupeptin (10 μM), and Pefabloc SC (10 μM), and assayed as described previously (Hansen and Grossmann, 2000). The extract was centrifuged at 25 000 *g* for 10 min (4 °C) and the supernatant was passed through a Sephadex G25 column (volume 6 ml; Amersham Biotech, Uppsala, Sweden), which bad been equilibrated with 5 mM EPPS buffer (pH 8.5) containing dithiothreitol (1 mM), pyridoxal phosphate (6 μM), and Pefabloc SC (10 μM). The column eluate was used as enzyme preparation. The ACC synthase assay mixture, with a total volume of 0.6 ml, contained 0.3 ml of enzyme preparation in EPPS buffer (80 mM) with pyridoxal phosphate (20 μM) and *S*-adenosylmethionine (100 μM). After an incubation period of 2 h at 37 °C, the reaction was stopped by adding 20 μmol mercury (II) chloride. The ACC produced was determined subsequently by chemical conversion to ethylene (Lizada and Yang, 1979). All assays were replicated four times.

Determination of ABA and IAA

For ABA determination, powdered plant material (1 g) was extracted twice with 80% (v/v) aqueous methanol containing 10 mg l^{-1} butylated hydroxytoluene (three replicate extractions). The extracts were passed through a C_{18}-reverse phase prepacked column (Sep-Pak; Waters, Königstein, Germany) under dim light conditions, as described elsewhere (Weiler *et al*., 1986; Hansen and Grossmann, 2000). The effluent was concentrated under vacuum, dissolved in 3 ml of double-distilled water, acidified to pH 2.5 with 1 M HCI, and partitioned three times into ethyl acetate (3 ml). The organic solvent was evaporated to dryness under a N_2 stream and samples were redissolved in 2 ml of 5% (v/v) methanol in 0.1 M acetic acid. Separation of ABA in a 1 ml aliquot of the extract was performed by HPLC on a reverse-phase Nucleosil 120 5 μm C_{18} column (250×10 mm; Machery-Nagel, Düren, Germany) using a linear gradient from 5% (v/v) methanol in 0.1 M acetic acid to 95% (v/v) methanol. The sweep time was 45 min at a flow rate of 1 ml min^{-1}. The fractions containing ABA (26.0–27.0 min) were collected, concentrated *in vacuo* to dryness, and dissolved in a solution of 50 μl of 100% methanol and 950 μl of TRIS-HCl (50 mM, pH 7.8) for analysis using enzyme-linked immunosorbent assay (ELISA). The monoclonal antibody against ABA, 100% reactive against its respective antigen, was used for analyses according to a standard procedure described by Weiler *et al*. (1986).

For IAA determination, plant material was extracted as described above. Separation of IAA in a 500 μl aliquot of the extract in 30% (v/v) acetonitrile in 0.1 M acetic acid was performed by HPLC on an ODS-Hypersil column (250×4.0 mm, 5 μm particle size; Bischoff, Leonberg, Germany) using a linear gradient from 30% (v/v) acetonitrile in 0.17 M acetic acid to 95% (v/v) acetonitrile. The sweep time was 25 min at a flow rate of 1 ml min^{-1}. The fractions containing IAA (4.2–6.2 min) were collected, concentrated to the aqueous phase under a N_2 stream, acidified with 200 μl of 1 M acetic acid, and partitioned twice into ethyl acetate. After evaporation of the organic solvent, the samples were redissolved in 200 μl of 100% methanol. The extract was methylated with ethereal diazomethane, concentrated to dryness, and dissolved in a solution of 50 μl of 100% methanol and 450 μl of double-distilled water. IAA was analysed by ELISA using a monoclonal antibody against IAA methylester, 58% cross-reactive with indole-3-acetylglycine (Weiler *et al*., 1986).

The detection limit is ~1 pmol for IAA and 0.1 pmol for ABA, as estimated from standard curves. All samples were assayed at least in triplicate. Internal performance controls of assay accuracy and reliability were carried out according to Weiler *et al*. (1986). Recovery, as checked with internal standards added to the methanolic extracts of the ground tissues, was >70% for both phytohormones. Mean values with standard errors from three replicate extractions of plant material and hormone determinations are shown.

Molecular cloning of the GaNCED1 fragment

The *GaNCED1* gene fragment was cloned based on homology to GenBank entries for NCEDs from tomato (*LeNCED1*; accession no. AJ439079), *Arabidopsis thaliana* (accession no. NM 112304), potato (accession no. AJ276244), and *Phaseolus vulgaris* (accession no. AF190462). A pair of primers: forward CGCAATTACTGA-GAACTTCGTC, and reverse CGAGTTTGTTTCGGTTCAC-CATTC were designed based on the conserved sequences between the various cDNA clones. PCR conditions were 95 °C for 5 min and 35 cycles of 94 °C for 1 min, 64 °C for 1 min, and 72 °C for 3 min. The resulting fragment was cloned into pCR4®-TOPO (Invitrogen, Carlsbad, CA, USA) and confirmed for homology with the *NCED* genes from tomato, *Arabidopsis*, potato, and *Phaseolus* by DNA sequencing.

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Isolation of total RNA and real-time quantitative RT-PCR

Total RNA was isolated from shoot tissue of *G. aparine* using an Rneasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA samples were treated with RNase-free DNase and purified using the Qiagen mini-column. The concentration of RNA was photometrically determined at A_{260}/A_{280}. Real-time RT quantitative PCR was performed employing ABI PRISM 7000 (Applied Biosystems, Perkin-Elmer, Foster City, CA, USA) under conditions recommended by the manufacturer. The PCR profile was 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 95 °C for 15 s/60 °C for 1 min. All reactions contained 1×RT PCR buffer, 5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, and dUTP, 100 nM random hexamer, 0.625 U of Ampli-Taq Gold polymerase, 0.25 U of MultiScribe RNA reverse transcriptase and RNase inhibitor in a 20 μl volume, 100–300 ng of total RNA, and 800 nM of forward (GTTCCCGACTGCTTCTGCTT) and reverse primer (TCATGCAGGAGCCGATCAC). Three independent measurements were made. Data analysis was carried out using the ABI PRISM 7000 SDS Software, version 1.0 (Applied Biosystems). A plasmid containing *GaNCED1* was used in a standard curve assay, and *GaNCED1* transcripts were normalized as copy number per nanogram of total RNA. The relative standard deviation of the mean in these qPCR experiments was <10%.

Results and discussion

In order to measure gene expression levels of *NCED* in *G. aparine*, a fragment of the *GaNCED1* gene was cloned to carry out RT qPCR experiments. Potential cross-hybridization with other potential *NCED* gene family members in *G. aparine* was tested by Southern blot analysis. Since only one band was detected, it was concluded that a single-copy gene for *NCED* existed in *G. aparine* (data not shown).

In time-course experiments, roots of *Galium* plants at the third whorl stage were treated with 0.5 mM IAA by adding it to the hydroponic solution. Within 3 h, total immunoreactive IAA in the shoot tissue increased from 0.16±0.01 nmol g^{-1} FW to 59±2 nmol g^{-1} FW (data not shown). Assuming water constitutes 90% of tissue fresh weight, this represents shoot tissue IAA concentrations of ~65 μM. In the shoot tissue, transient increases in ACC synthase activity, as well as ACC and ethylene production were detectable within 2 h of treatment and peaked at 5 h, with values 12-fold (ACC synthase, ACC) and 6-fold (ethylene) higher than in control plants (Fig. 1). Levels of ACC synthase activity, ACC, and ethylene formation then declined. Compared with the stimulation of ethylene synthesis over time, immunoreactive levels of ABA began to increase more slowly and, significantly, with a lag phase of 2 h (Fig. 1), after which it continued to accumulate to levels up to 24-fold higher than those in control plants 24 h after treatment. ABA accumulation was preceded by large increases in *GaNCED1* gene expression (Fig. 1). At the transcriptional level, *GaNCED1* mRNA was transiently induced within 1 h of IAA treatment and increased to maximum values 40-fold greater than in controls 3 h after treatment; thereafter the level declined. The induction of *GaNCED1* gene expression coincided with the initial stimulation of ACC synthase activity and preceded increases in ACC and ethylene. In accordance with previous results showing that the shoot tissue is the principal site of auxin-induced ethylene and ABA accumulation (Grossmann, 2003), no increase in *GaNCED1* gene expression was found in the root tissue during the first hour after IAA treatment (data not shown). Comparing the effects of auxin herbicides from different chemical classes, such as quinmerac, dicamba, and picloram, it was demonstrated in *Galium* that the increase in *GaNCED1* gene expression is an effect common to all auxin herbicides (Fig. 2).

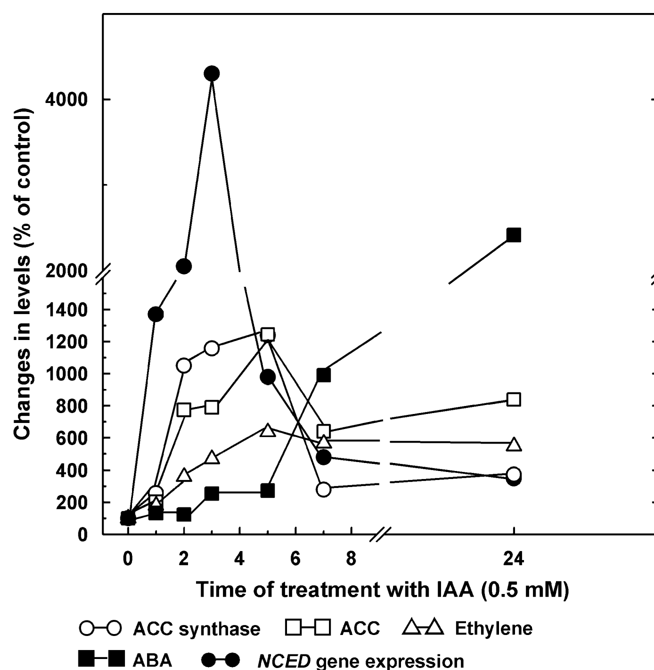

Fig. 1. Time-course of the effect of IAA on ACC synthase activity, ethylene formation, and levels of ACC, ABA, and *GaNCED1* transcript in shoot tissue of root-treated *G. aparine* plants at the third whorl stage grown in hydroponic solution. Vertical bars represent the SEM. Where bars are not shown, they are smaller than the symbols. In gene expression experiments, the relative standard deviation of the mean was <10%. Control values ±SE (100%), given at 3 h after treatment, were: ACC synthase activity 19±4 pmol g^{-1} FW×h; ACC 2140±620 pmol g^{-1} FW; ethylene formation 100±10 pmol g^{-1} FW×h; ABA 173±25 pmol g^{-1} FW.

Overall, the time-course of auxin-induced *GaNCED1* gene expression and ABA synthesis was similar to that observed in bean leaves after water stress (Qin and Zeevaart, 1999). ABA accumulation was preceded by a strong induction of *NCED* mRNA within 0.5 h of dehydration (Qin and Zeevaart, 1999). In *Galium* shoot tissue, the time course of IAA effects showed that increases in *GaNCED1* gene expression slightly preceded increases in ACC and ethylene, which preceded ABA accumulation. This suggests that auxin activates *NCED*

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gene transcription, followed by *de novo* NCED and ABA synthesis. In previous studies, a causal role of ethylene in auxin-induced ABA biosynthesis was shown (Hansen and Grossmann, 2000; Grossmann, 2003). Accordingly, application of ethephon increased *GaNCED1* gene expression 3 h after treatment, but only by 5-fold relative to the control (Fig. 3). Conversely, IAA-induced ethylene production was decreased by the application of AVG, an inhibitor of ACC synthase (Abeles *et al.*, 1992), and cobalt ions, which inhibit the conversion of ACC to ethylene by ACC oxidase (Abeles *et al.*, 1992; Fig. 4). Although AVG blocked both ACC and ethylene production, whereas cobalt ions only decreased ethylene formation, both inhibitors decreased IAA-induced ABA accumulation in *Galium* shoot tissue (Fig. 4). These results suggest that ethylene and not ACC triggers ABA accumulation. However, the inhibitory effects of AVG and cobalt ions on IAA-induced ABA accumulation were not accompanied by corresponding declines in IAA-induced *GaNCED1* transcript levels (Fig. 4). While the ethylene inhibitors decreased IAA-induced ABA accumulation by up to 70%, the increase in *GaNCED1* transcript levels was reduced by no more than 30%, indicating that auxin-induced ethylene is required for the auxin-induced ABA accumulation, but plays only a minor role in *NCED* gene expression (Fig. 4). Thus, auxin appears to be the primary trigger for gene activation of *NCED* which in turn is required for up-regulation of ABA biosynthesis. In addition, auxin-induced ethylene enhances ABA production, possibly by stimulating a signalling process downstream of *NCED* gene expression. Post-transcriptional mechanisms underlying an ethylene-mediated up-regulation of NCED activity may include increasing synthesis, activity and/or stability of the enzyme protein (Fig. 5). NCED is suggested to be regulated by ethylene because previous work has shown that enhanced NCED precursor supply, steps in the pathway after NCED, or reduced ABA degradation and conjugation did not contribute to stimulated ABA accumulation (Hansen and Grossmann, 2000).

Based on these results, previous work, and a reappraisal of the literature, it is speculated that ethylene-triggered ABA biosynthesis plays a principal role in plant growth regulation (Grossmann and Hansen, 2001). In accordance, ethylene and ethephon treatments have been shown to elicit processes such as growth inhibition, senescence, and delay of bud growth and bloom development, accompanied by increased ABA levels (Abeles *et al.*, 1992; Grossmann and Hansen, 2001). A rise in ethylene during fruit ripening in avocado has also been shown to be followed by an increase in ABA biosynthesis, which is regulated at the level of carotenoid cleavage (Chernys and Zeevaart, 2000). By contrast, experiments using mutants defective in ethylene signalling suggested that ABA biosynthesis is stimulated by the absence of a functional ethylene signal transduction pathway (Pierik *et al.*, 2006). For example, in *Arabidopsis etr1* and *ein2* ethylene-insensitive mutants and in ethylene-insensitive tobacco, ABA levels were found to be higher than in wild-type plants (Ghassemian *et al.*, 2000; Pierik *et al.* 2006). Furthermore, in mature seeds of ethylene-insensitive *Arabidopsis etr1-2* mutants, ABA was detected at higher levels than in wild-type seeds but had higher auxin levels than wild-type plants (Chiwocha *et al.*, 2005). Therefore, in mutants defective in ethylene signalling, increased auxin levels may be the trigger for ABA accumulation without an additional stimulatory effect mediated by

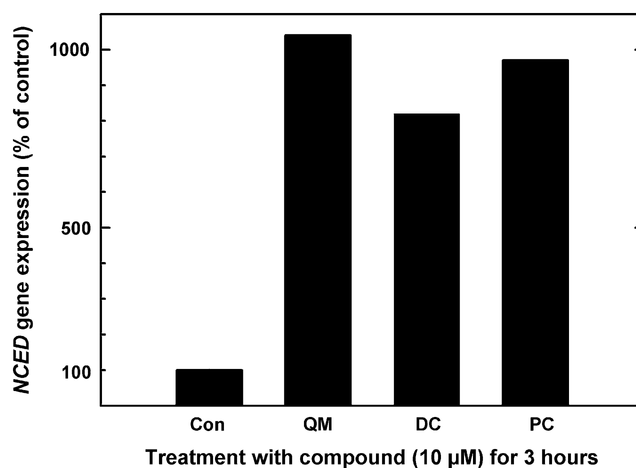

Fig. 2. Levels of *GaNCED1* transcript in shoot tissue of *Galium* plants 3 h after treatment of the hydroponic solution with 10 μM quinmerac (QM), dicamba (DC), or picloram (PC). The relative standard deviation of the mean was <10%.

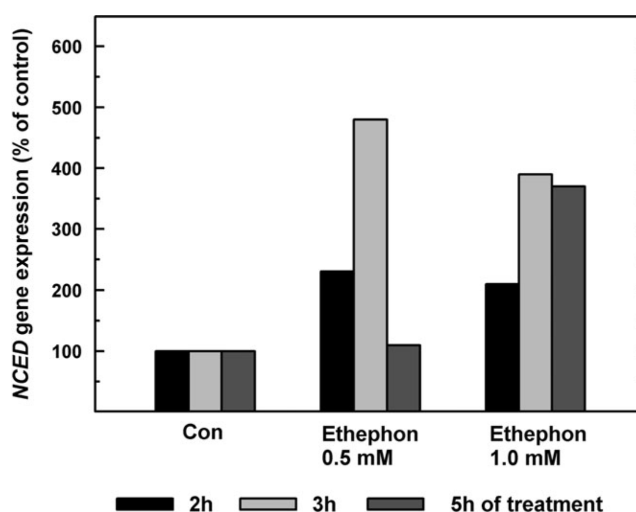

Fig. 3. *GaNCED1* transcript levels in shoot tissue of *Galium* plants 2, 3, and 5 h after treatment of the hydroponic solution with ethephon. The relative standard deviation of the mean was <10%.

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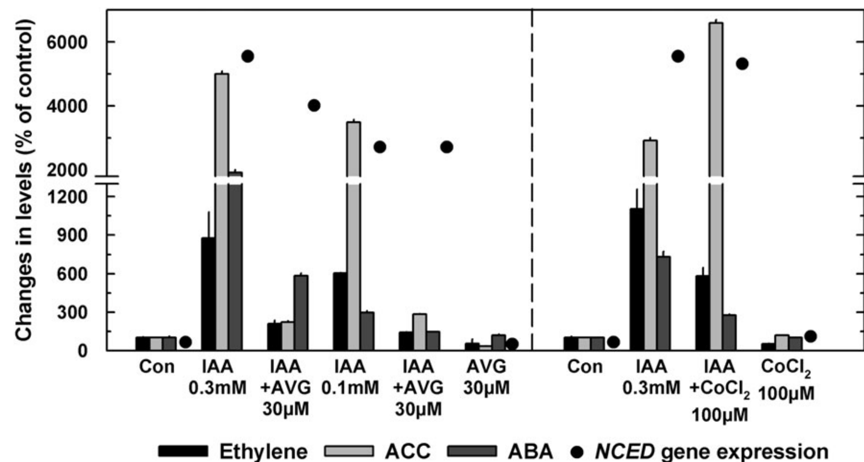

Fig. 4. Effects of IAA, AVG, and cobalt chloride, and combinations of the compounds on ethylene formation, and levels of ACC, ABA, and *GaNCED* transcript in shoots of *G. aparine*. For determination of changes in ethylene formation, and levels of ACC and ABA, the roots of *G. aparine* plants at the third whorl stage were treated with compounds and combinations in hydroponics for 24 h. For determination of *GaNCED1* transcript levels, plants were treated with AVG or cobalt chloride for 1 h before treatment with IAA for 1 h. Vertical bars represent the SEM. In gene expression experiments, the relative standard deviation of the mean was <10%. Control values ±SE (100%) were: ethylene formation 68±5 pmol g^{-1} FW; ACC 1880±100 pmol g^{-1} FW; and ABA 83±4 pmol g^{-1} FW.

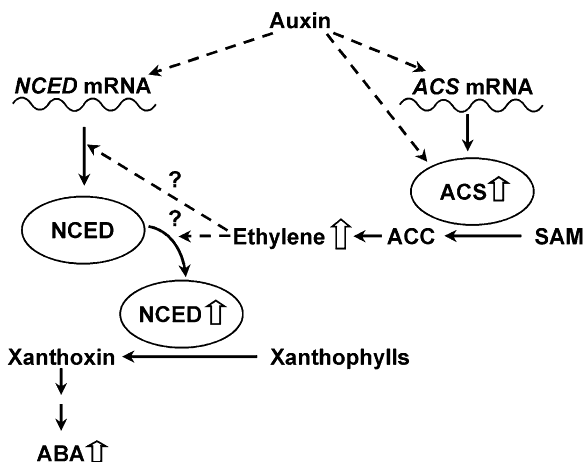

Fig. 5. Hypothetical model for interaction between auxin and auxin-induced ethylene in up-regulation of ABA biosynthesis. Auxin stimulates ACC synthase activity in ethylene formation at the transcriptional and post-transcriptional level (Wang and Ecker, 2002; Chae and Kieber, 2005). Auxin induces *NCED* gene expression as a prerequisite for the stimulation of ABA biosynthesis. Ethylene up-regulates NCED activity post-transcriptionally, leading to lasting ABA biosynthesis. Stimulatory effects are represented by dotted lines. ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC synthase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; SAM, *S*-adenosylmethionine.

ethylene signalling. Another explanation for enhanced ABA levels in ethylene-insensitive mutants could result from compensatory effects of ABA biosynthesis. In the tomato ethylene receptor mutant *never ripe*, the concentrations of xanthoxin in the shoot tissue were lower than in the wild type, whereas ABA concentrations were higher (Hansen and Grossmann, 2000). These results indicate a higher metabolic conversion of xanthoxin to ABA in this ethylene-insensitive mutant (Hansen and Grossmann, 2000). In addition, tomato mutants of *never ripe* also resisted, at least in part, the xanthoxin and ABA increase induced by auxins in wild-type plants (Hansen and Grossmann, 2000).

In conclusion, it is suggested that besides their stimulatory effects on gene expression in ethylene and gibberellin biosynthesis (Ross *et al.*, 2002; Grossmann, 2003), auxins are able to activate *NCED* gene expression, leading to enhanced ABA biosynthesis. Gene expression of *NCED* has been shown to be up-regulated when plant cells lose turgor in response to environmental stress or during the development of seeds and buds (Xiong and Zhu, 2003; Taylor *et al.*, 2005). Within a few hours of treatment of *Galium* plants, root-applied IAA led to enhanced *GaNCED1* transcript levels exclusively in the shoot tissue. During this time, the water content and the osmotic potential in the shoot tissue were not changed (Hansen and Grossmann, 2000). Consequently, IAA functions as a direct trigger of *NCED* gene expression in *Galium* shoot tissue. In addition, auxin-induced ethylene is speculated to up-regulate NCED activity post-transcriptionally, leading to lasting ABA biosynthesis (Fig. 5). To test this hypothesis, future research should explore the situation at the enzymatic level of NCED.

Acknowledgement

We thank Professor EW Weiler (University of Bochum, Germany) for a generous gift of antibodies for phytohormone analysis.

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