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INVOLVEMENT OF THE IAA-REGULATED ACC OXIDASE GENE *PNACO3* IN *PHARBITIS NIL* FLOWER INHIBITION

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The study examined the influence of light and auxin on the transcription level of *PnACO3*, a gene involved in ethylene production, in relation to the inhibitory effect of ethylene on flower induction in the short-day plant *Pharbitis nil* (=*Ipomoea nil*). Exogenous auxin was shown to increase the level of *PnACO3* mRNA, with the effect depending on the experimental conditions. Light did not affect the level of *PnACO3* mRNA. Applying auxin to seedling cotyledons at the beginning of inductive night boosted *PnACO3* transcriptional activity even threefold during the next few hours, supporting our previous suggestion that the inhibitory effect of auxin on *P. nil* flowering results from its stimulatory effect on ethylene production.

Key words: ACC oxidase, auxin, ethylene, flowering, *Pharbitis nil*.

INTRODUCTION

The plant hormone ethylene is involved in controlling a number of physiological processes throughout the whole course of ontogenesis, including seed sprouting, responses to environmental stimuli, vegetative and generative development, and aging (Ecker, 1995). Due to its structure and biosynthetic pathway, simpler than other plant hormones, the mechanisms regulating its concentration within the cell are relatively well recognized. A significant role in this regulation is played by genes encoding enzymes involved in ethylene biosynthesis (ACC synthases and oxidases), whose expression is variously controlled and which varies in time and space (Kathiresan et al., 1996; Kathiresan et al., 1998; Bleecker and Kende, 2000; Frankowski et al., 2009; Kęsy et al., 2010). Ethylene's role in regulating plant growth and development involves its direct effect on the expression of the relevant effector genes, but also on the biosynthesis, distribution and signal transduction pathways of other hormones and signal particles (Stepanova and Alonso, 2005). The many interactions between ethylene and other plant hormones regulate a number of physiological processes, including those of flowering.

Ethylene and auxins have various effects on flowering. They can inhibit or stimulate it. Flower stimulation was observed in long-day plants (LDPs) cultivated under noninductive conditions, whereas inhibition often takes place in short-day plants (SDPs) cultivated under an inductive photoperiod (Bernier et al., 1988). Ethylene and auxins are among the compounds that show the strongest inhibitory effect on flowering in the model SDP *Pharbitis nil*. Ethylene only inhibits generative induction of *P. nil* completely when applied in the second half of 16 h inductive night (Suge, 1972; Friedman et al., 1990; Kulikowska-Gulewska et al., 1995; Kęsy et al., 2008; Wilmowicz et al., 2008). Auxin applied to cotyledons of *P. nil* seedlings just prior or during the first half of the inductive dark period has a similar effect (Ogawa and Zeevaart, 1967; Amagasa and Suge, 1987; Kulikowska-Gulewska et al., 1995). Both IAA application at the beginning of inductive darkness and disruption of long night by red illumination cause a multiple increase of ethylene production and finally inhibit flowering completely (Kęsy et al., 2008). IAA application leads to increased accumulation of the

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Abbreviations: ACC – 1-aminocyclopropane-1-carboxylic acid; ACO – 1-aminocyclopropane-1-carboxylate oxidase; ACS – 1-aminocyclopropane-1-carboxylate synthase; IAA – indolilo-3-acetic acid; SDP – short-day plant; LDP – long-day plant.

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transcripts of genes encoding ACC synthases (Frankowski et al., 2009; Kęsy et al., 2010). In this work we described the gene encoding ACC oxidase in *P. nil* and analyzed its expression in cotyledons of seedlings cultivated under inductive and noninductive conditions and after IAA treatment. We assessed the transcriptional activity of that gene in different vegetative organs (shoot apices, petioles, cotyledons, hypocotyls, roots) of plants cultivated under various light conditions and treated with IAA.

MATERIALS AND METHODS

All procedures were performed as described by Frankowski et al. (2009) but using different PCR primers and PCR conditions. Degenerated primers 5'-AAGGTRRSCAAYTACCCWCCWTGTCC-3' (forward) and 5'-CAATYACSCKGTGYTCCACRCTCTTG-3' (reverse) (10 μM), 2 μl first-strand cDNA, 5 μl buffer B (containing 15 mM Mg^{2+}), 0.5 μl 50 mM Mg^{2+}, 2 μl 5 mM dNTP mix and 1.25 U Blue Perpetual Taq DNA Polymerase HOT START (EurX) were subjected to the following PCR conditions: 95°C for 5 min, 1 cycle, 95°C for 1 min, 55°C for 1 min, 74°C for 1 min for 35 cycles, followed by 1 cycle of incubation at 74°C for 5 min. A 390 bp amplified cDNA fragment was cloned and sequenced. A full-length cDNA encoding *PnACO3* was obtained using 5'-RACE primer 5'-CCAGACCTCATCAAGGGCCTCCGGGC-3' and 3'-RACE primer 5'-GCGGTGCTCCACGCTCTT GTATTTCCCG-3'. PCR reactions were run as described by Frankowski et al. (2009). Sequence data from this work have been deposited at GenBank ID: EF127818.

(SQ)RT-PCR primers: *PnACO3* (460 bp) 5'-GTTTGCAATGGAGCTGGAGAAGC-3' (forward) and 5'-CTTTCTCGACTAATGCAGGTGCTG-3' (reverse); *InACT4* (240 bp) 5'-GAATTCGATATCCGAAAA-GACTTGTATGG-3' (forward) and 5'-GAATTCCAT-ACTCTGCCTTGGCAATC-3' (reverse). PCR mixtures were composed of 2 μl first-strand cDNA, 2×1 μl *PnACO3* primer solution (10 μM), 2×0.75 μl *InACT4* primer solution (10 μM), 5 μl buffer B (containing 15 mM Mg^{2+}), 2 μl 5 mM dNTP mix and 1.25 U Blue Perpetual Taq DNA Polymerase HOT START (EurX) and subjected to the following conditions: 95°C for 5 min, 1 cycle, 95°C for 1 min, 58°C for 1 min, 74°C for 1 min for 30 cycles, followed by 1 cycle of incubation at 74°C for 5 min. All data are presented as means ±SE.

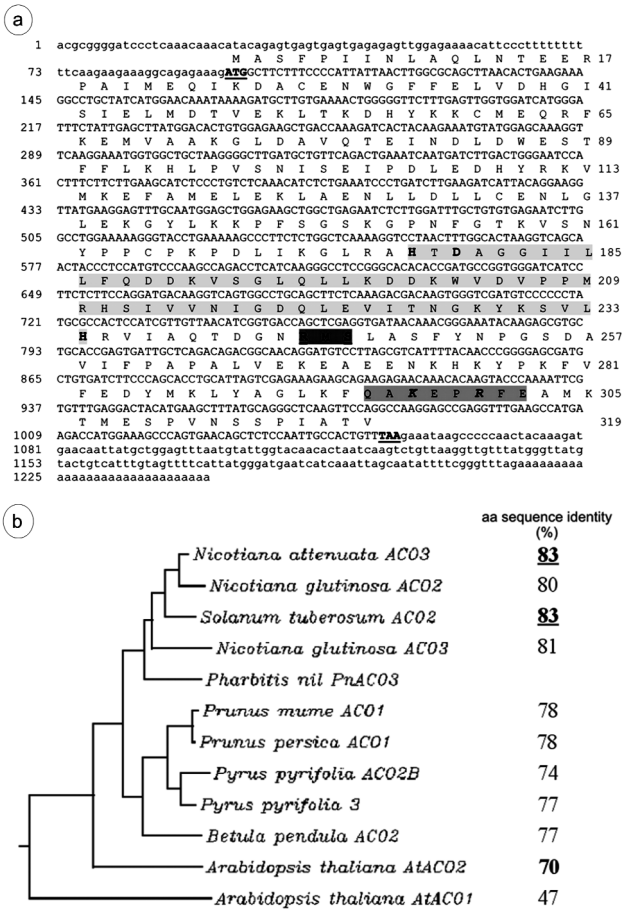

Fig. 1. (**a**) Coding sequence of *PnACO3* and the deduced amino acid sequence. Nucleotide positions marked on left side and amino acid positions on right side of figure. Start and stop codons are underlined and in boldface. Small letters are used for UTRs. Motif forming the Fe(II) binding pocket comprising three characteristic aa residues (boldface) is light gray (Yoo et al., 2006). Ascorbic acid-binding motif comprising two characteristic aa residues (underlined and in boldface) is dark gray. Evolutionarily conserved ACO sequence is gray. Lys and Arg residues essential for oxidase enzymatic activity are in italics and boldface, (**b**) Phylogenetic relationship of PnACO3 compared with ACC oxidases from *A. thaliana* and members of the order Solanales. Phylogram tree generated with ClustalW. Percentages within column were generated in DiAlign and denote PnACO3 amino acid sequence identity compared with *A. thaliana*'s and other species' ACOs. GenBank IDs from top to bottom: ABO32691.1; AAA99793.1; AAK68076.1; AAB05171.1; BAA90550.1; CAA54449.1; BAD60999.1; BAD61000.1; AAN86821.1; NP_176428.1; NP_179549.1.

RESULTS AND DISCUSSION

We obtained *PnACO3*, the sequence encoding ACC oxidase in *P. nil*, by RT-PCR with degenerated primers designed for highly conserved ACC oxidase sequence fragments from different plant species, and 5'-RACE- and 3'-RACE-PCR technique (Fig. 1a). The identified cDNA encodes a 319 aa protein with estimated 36.14 kDa molecular mass, similar to the molecular mass of ACOs found in other plant

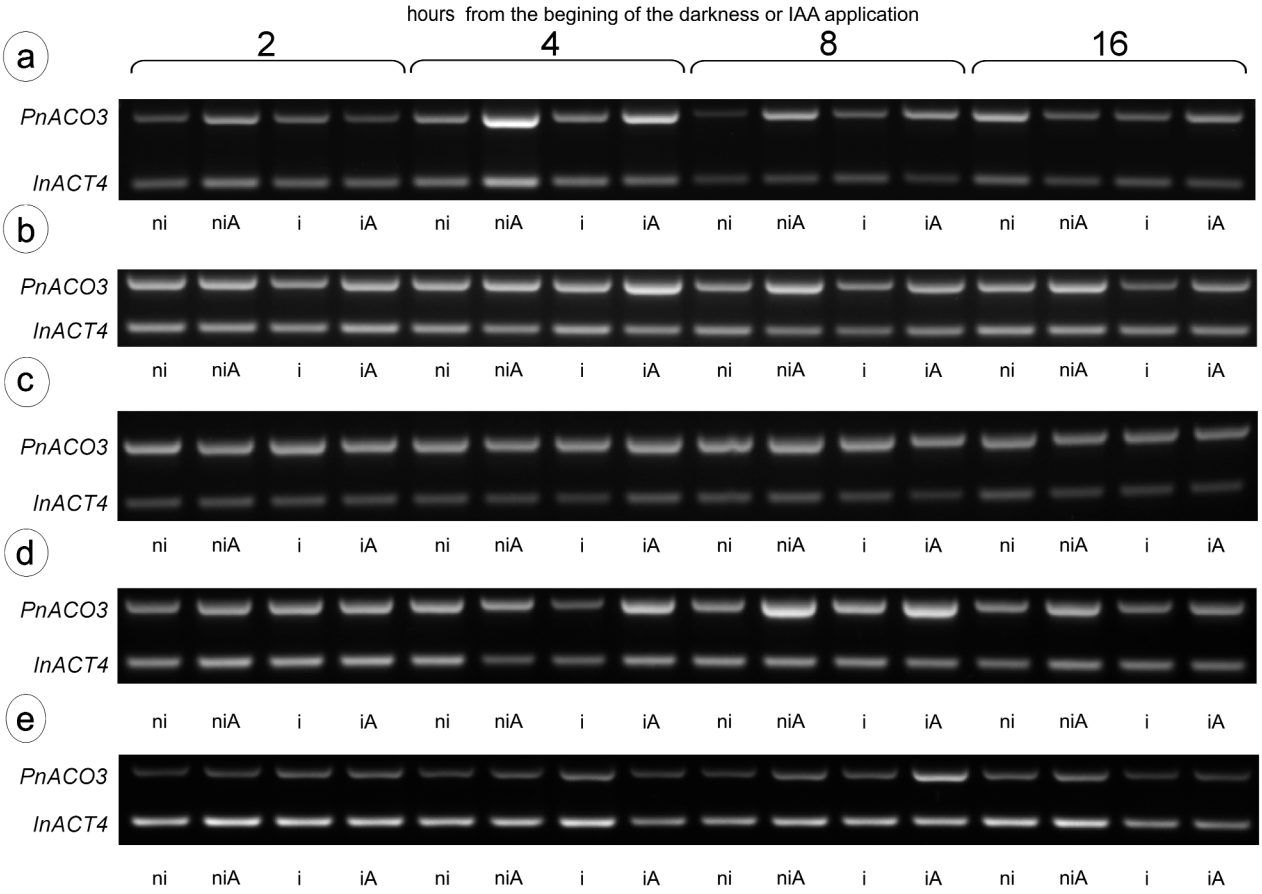

Fig. 2. Semi-quantitative RT-PCR analysis of *PnACO3* transcript accumulation in apices (**a**), petioles (**b**), cotyledons (**c**), hypocotyls (**d**) and roots (**e**) of plants grown under different light conditions and treated with IAA; ni – plants grown in constant light (not induced); niA – plants grown in constant light and treated with IAA; i – plants transferred to 2, 4, 8 or 16 h darkness (inductive night); iA – plants transferred to darkness and treated with IAA. A constitutively expressed actin gene was the internal control.

species (Zanetti et al., 2002; Hudgins et al., 2006). Phylogenetic analysis showed that the amino acid sequence of *PnACO3* protein is close to that of ACO3 from *Nicotiana attenuata* and ACO2 from *Solanum tuberosum* (83% identity), as well as ACO2 from *Arabidopsis thaliana* (70%) (Fig. 1b).

ACC oxidases are classified as oxogluterate-dependent dioxigenases, which – unlike other enzymes of this group – use ascorbic acid instead of 2-oxoglutarate as the reducer (Prescott and John 1996). A comparison of the *PnACO3* amino acid sequence to those of ACC oxidases identified in other plant species revealed that the *PnACO3* sequence contains a characteristic motif forming a cofactor (Fe(II)) binding pocket (Hsp177-X-Asp179-X(54)-Hsp234) and a cosubstrate (ascorbic acid) binding motif (Arg244-X-Ser246) (Yoo et al., 2006). Also, an evolutionarily conserved amino acid sequence characteristic of ACC oxidases was identified at positions 295 to 302. This sequence contains Lys and Arg residues essential for protein activity (Yoo et al., 2006). The presence of sequences encoding those regions, and the amino acid residues, suggests that the identified *PnACO3* cDNA encodes a functional enzymatic protein.

A significant role in ethylene biosynthesis regulation is played by the expression of genes encoding ACC synthases and oxidases; this expression is variously controlled and varies in time and space (Kathiresan et al., 1996; Kathiresan et al., 1998; Bleecker and Kende, 2000; Frankowski et al., 2009; Kęsy et al., 2010). Individual genes are expressed differently, and their products show different biochemical properties which allow plants to respond to changing conditions in a time- and space-specific manner.

PnACO3 was expressed in all of the vegetative organs of *P. nil* seedlings cultivated both under light

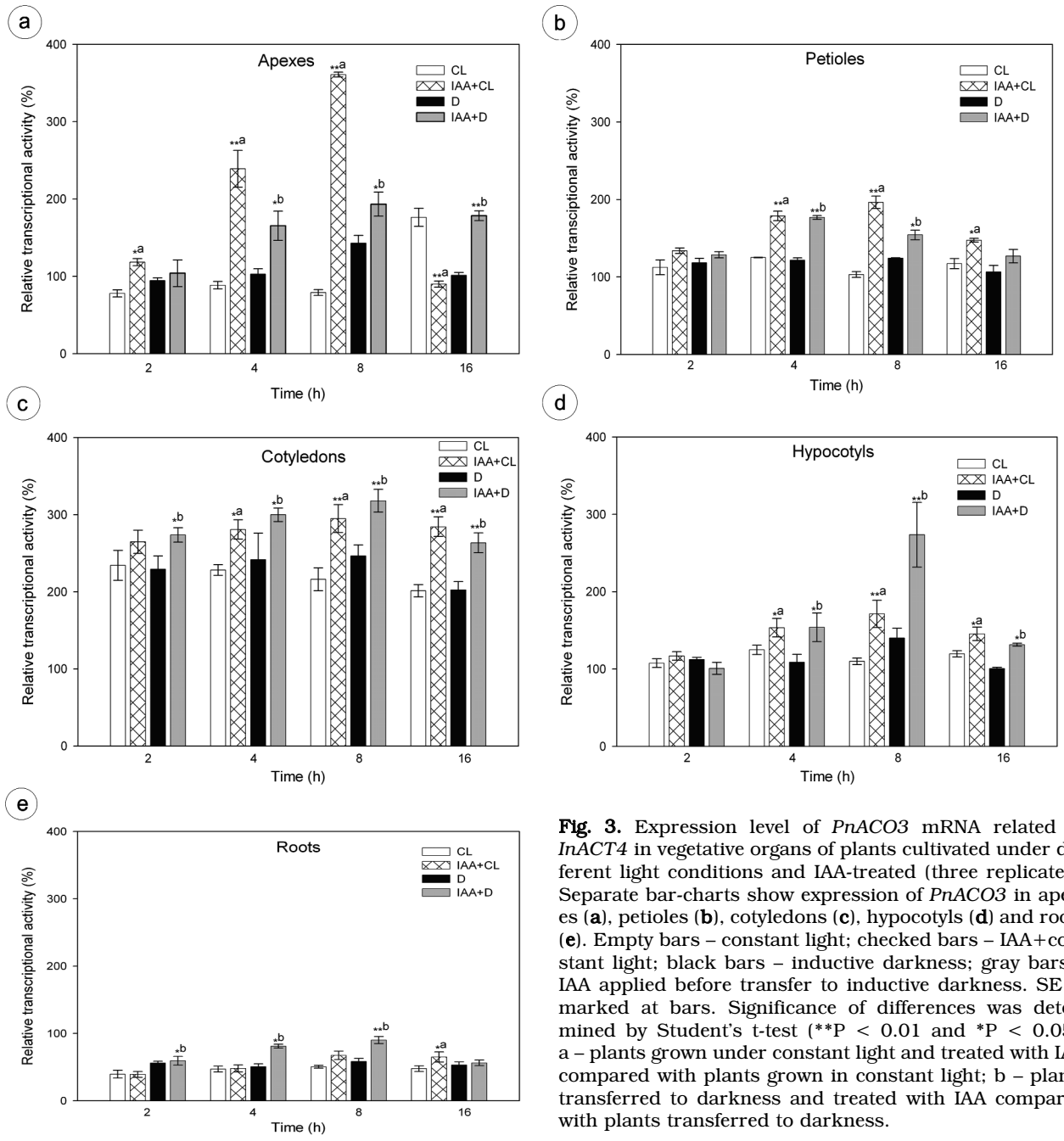

Fig. 3. Expression level of *PnACO3* mRNA related to *InACT4* in vegetative organs of plants cultivated under different light conditions and IAA-treated (three replicates). Separate bar-charts show expression of *PnACO3* in apexes (**a**), petioles (**b**), cotyledons (**c**), hypocotyls (**d**) and roots (**e**). Empty bars – constant light; checked bars – IAA+constant light; black bars – inductive darkness; gray bars – IAA applied before transfer to inductive darkness. SE is marked at bars. Significance of differences was determined by Student's t-test (**P < 0.01 and *P < 0.05); a – plants grown under constant light and treated with IAA compared with plants grown in constant light; b – plants transferred to darkness and treated with IAA compared with plants transferred to darkness.

and in darkness, as well as after auxin application. *PnACO3* mRNA accumulation in IAA-untreated plants was highest in cotyledons and lowest in roots (Figs. 2, 3). Liu et al. (1997) reported a similar expression pattern of ACC oxidase 2 in 4-day-old sunflower seedlings. In tulip, of the five ACO genes identified, only *TgACO3* was expressed in leaves and only *TgACO5* was expressed in stems; transcripts of the other three genes were identified in flowers (Momonoi et al., 2007). The higher *PnACO3* transcript level in cotyledons supports suggestions by Yang and Hoffman (1984) and Nie et al. (2002) that ACC is synthesized mainly in roots and then transported to higher parts of the plant, where it is converted to ethylene. High transcriptional activity of *PnACS1* has also been found in roots (Frankowski et al., 2009). As yet there is no clear evidence of ACC transport from roots. Alternatively, higher ethylene production could also be a sign of cotyledon ageing (Pan and Lou, 2008). Ethylene can regulate its own

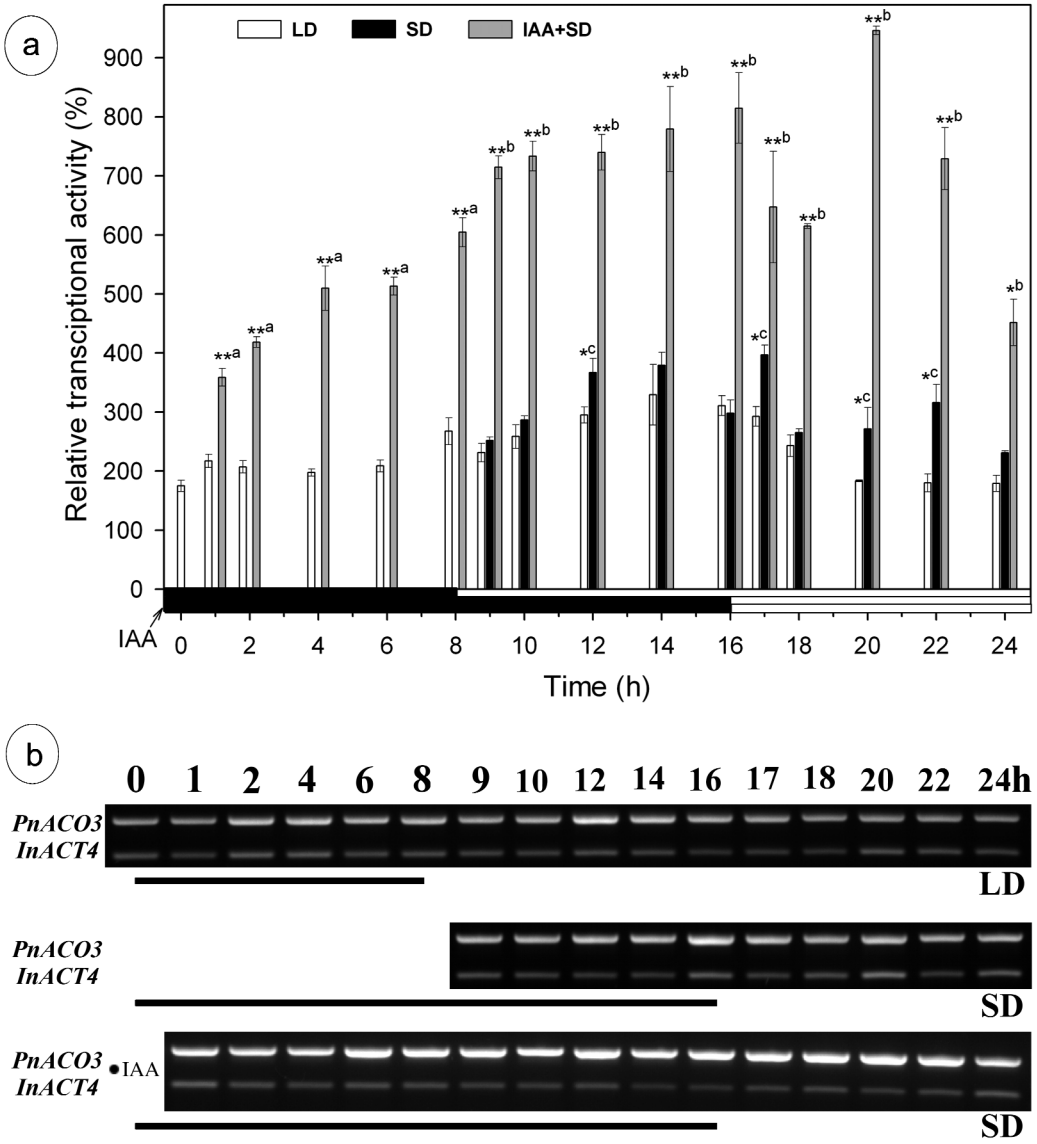

Fig. 4. (**a**) Level of *PnACO3* transcript accumulation related to *InACT4* (3 biological replicates) in cotyledons of plants cultivated under different light conditions and treated with IAA. Empty bars – LD conditions (8 h noninductive night), black bars – SD conditions (16 h inductive night), gray bars – IAA applied before transfer to SD conditions. SE is marked at bars. Significance of differences was determined by Student's t-test (**P < 0.01 and *P < 0.05); a – plants grown under SD conditions and treated with IAA compared with plants grown under LD conditions; b – plants grown under SD conditions and treated with IAA compared with plants grown under SD conditions; c – plants grown under SD conditions compared with plants grown under LD conditions, (**b**) (SQ)RT-PCR analysis of *PnACO3* transcript during 24 h photoperiod and after IAA treatment. A constitutively expressed actin gene was the internal control. Black lines – dark period; black dot – application of IAA to cotyledons.

biosynthesis by increasing the level of expression of genes encoding ACC oxidases (Peck and Kende, 1995; Blume and Grierson, 1997; Bouquin et al., 1997; Peck et al., 1998; Petruzzelli et al., 2000).

PnACO3 transcriptional activity increased after application of exogenous auxin in all of the organs studied in plants cultivated both under light and in darkness (Figs. 2, 3), reaching maximum at hours 4 or 8 depending on the organ. The highest *PnACO3* expression level lags behind the occurrence of *PnACS1* and *PnACS2* mRNA (Frankowski et al., 2009; Kęsy et al., 2010). Similar results after auxin application have been reported in other plant species such as *Vigna radiata* and *Solanum tubero-*

sum (Yu et al., 1998; Zanetti et al., 2002; Song et al., 2005). Peck and Kende (1995) studied the effect of IAA on the level of mRNA of genes encoding enzymes involved in ethylene biosynthesis. They suggested that the increase of ACO expression they observed in etiolated pea seedlings after applying auxin is in fact caused by an increase in ethylene production, with auxin playing only an intermediary role. Probably IAA causes an increase in the expression level and activity of ACC synthases, leading to an increased ACC concentration. This compound is then transformed into ethylene by ACC oxidase(s), a low concentration of which is maintained constantly in the cell, and the resulting ethylene – via positive feedback – induces the expression of genes encoding ACC oxidases. In our work, accumulation of the transcript and the enzymatic activity of ACC oxidase only increased 2 hours after application of the hormone. In *Vigna radiata*, ACC oxidase mRNA accumulation increased 7 hours after IAA application (Kim and Yang, 1994) and in *Petunia hybrida* 24 hours after IAA application (Tang et al., 1994).

Like auxins, ethylene is considered to be one of the most effective flower induction inhibitors in the model short-day plant *P. nil* (Suge, 1972; Friedman et al., 1990; Kulikowska-Gulewska et al., 1995; Kęsy et al., 2008). Since auxin's flower inhibition effect most probably results from increased ethylene production preceded by intensive transcriptional activity of ACC synthases (Kęsy et al., 2008; Frankowski et al., 2009; Kęsy et al., 2010), we studied the effect of IAA on *PnACO3* expression in cotyledons of seedlings cultivated under different photoperiods.

Exogenous auxin significantly increased the level of *PnACO3* mRNA, with the effect depending on experimental conditions. Light conditions had no effect on the level of *PnACO3* mRNA. Applying the hormone to *P. nil* cotyledons at the beginning of inductive night raised the *PnACO3* transcript concentration, which reached maximum at hour 20 (Fig. 4). Frankowski et al. (2009) and Kęsy (2010) indicated that the influence of exogenous IAA the transcriptional activity of ACC synthases increased as well, reaching maximum 1–2 hours after auxin application. These data are in accord with ethylene production measurements made after cotyledons of *P. nil* seedlings were treated with auxin (Kęsy et al., 2008), and they support the suggestion that the inhibitory effect of auxins on *P. nil* flowering results from their stimulating effect on ethylene production. There is an abundance of literature data on the effect of IAA and ethylene on generative induction, but their role in this process has not been fully explained (Kęsy et al., 2008). Our studies clearly show that the application of auxin to *P. nil* cotyledons is accompanied by an increase in the expression level of the genes encoding ACC synthase (*PnACS1* and *PnACS2*) (Frankowski et al., 2009; Kęsy et al., 2010) and then ACC oxidase (*PnACO3*), which as a consequence causes an increase of ethylene production (Kęsy et al., 2008). This suggests that auxin inhibits *P. nil* flowering by acting as an intermediary, via an increase of ethylene production. There is a network of mutual relations between hormones at the levels of their biosynthesis, functional mechanisms and signal transduction pathways. The specific balance among the phytohormones achieved in successive stages of plant development ensures the proper course of physiological processes, including the transition to flowering.

AUTHORS' CONTRIBUTION

EW carried out the experiments, analyzed the data and wrote the manuscript; KF, AK carried out part of the experiments; JKe, JKo helped in preparing the manuscript. The authors declare that there are no conflicts of interest.

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