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ORIGINAL PAPER

The role of *PnACO1* in light- and IAA-regulated flower inhibition in *Pharbitis nil*

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Abstract In this study, the first ACC oxidase (*PnACO1*) cDNA from model short-day plant *Pharbitis nil* was isolated. The expression pattern of *PnACO1* was studied under different conditions (photoperiod and auxin), an adequate balance of which determines *P. nil* flowering. It was shown that the gene was transcribed in all the examined organs of the 5-day-old seedling and was strongly activated by auxin. Our results also revealed that *PnACO1* transcript accumulation in the cotyledons showed diurnal oscillations under both LD and SD conditions. On the basis of presented and previously obtained data, we suggest that flowering inhibition evoked by IAA in *P. nil* results from its stimulatory effect on both ACC synthase and oxidase gene expression and, consequently, enhances ethylene production.

Keywords ACC oxidase · Auxin · Ethylene · Flowering · *Pharbitis nil* (*Ipomoea nil*)

Introduction

Plant hormones are factors that integrate individual metabolic processes required for a functional transformation of the vegetative meristem into a generative one. A search for a substance responsible for flower induction caused by a relevant photoperiod allowed many chemical compounds to be identified, which have either a stimulating or inhibiting effect on the flowering of the model short-day plant (SDP) *Pharbitis nil*. The former group consists of gibberellins (Galoch et al. 2002; King et al. 1987; Wijayanti et al. 1996), cytokinins (Friedman et al. 1990; Galoch et al. 1996; Halevy et al. 1991) and prostaglandins (Groenewald and van der Westhuizen 2001). The latter group consists of auxins (Kulikowska-Gulewska et al. 1995; Wijayanti et al. 1997), ethylene (Amagasa and Suge 1987; Kęsy et al. 2008), jasmonic acid (Maciejewska and Kopcewicz 2003), abscisic acid (Wijayanti et al. 1997; Wilmowicz et al. 2008) and brassinosteroids (Kęsy et al. 2003). Among flowering inhibitors, the strongest effect is demonstrated by auxins and ethylene. Most data used for determining their role in regulating this process in *P. nil* come mainly from typically physiological studies. However, there is little information on auxin and ethylene interactions at the molecular level. The identification of genes encoding for IAA and ethylene metabolism enzymes in *P. nil*, as well as the determination of changes in the level of their expression in cotyledons and shoot apices of plants cultivated under different photoperiodic conditions, will promote a better understanding of photoperiodic flower induction.

In *P. nil*, the highest auxin effectiveness was observed following its application to the cotyledons directly before or during the first half of the inductive night (Amagasa and Suge 1987; Friedman et al. 1990; Kulikowska-Gulewska et al. 1995; Ogawa and Zeevaart 1967). In turn, the level of endogenous auxin in the cotyledons of *P. nil* seedlings before and during the first half of the inductive darkness remained low, only growing between hours 8 and 12 into the night (Bodson 1985). Application of auxin together with AVG (an ethylene biosynthesis inhibitor) restored the inductive effect of the long night (Kęsy et al. 2008). Therefore, it seems that auxin's inhibiting effect on *P. nil*

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flowering is achieved by an increased level of ethylene. It could be so, because it was proved that auxins induce the expression of some ACC synthases (Arteca and Arteca 1999; Frankowski et al. 2009; Kęsy et al. 2010; Tian et al. 2002; Tsuchisaka and Theologis 2004a), while the level of these genes' expression correlates with the level of ethylene production (Abel et al. 1995; Kęsy et al. 2008; Overmyer et al. 2000; Smith and Arteca 2000; Tian et al. 2002; Tsuchisaka and Theologis 2004b).

Depending on the plant species and the environmental conditions, ethylene production can be controlled both at the level of gene expression and the ACS and ACO enzyme activity (Finlayson et al. 1999; Kathiresan et al. 1996, 1998; Machackova et al. 1997). Individual genes encoding for enzymes that participate in this hormone's biosynthesis are expressed in diverse ways, with their products displaying different biochemical properties, which allows plants to respond to changing conditions in a time- and space-specific manner (Bleecker and Kende 2000; Kathiresan et al. 1996, 1998). Additionally, the accumulation level of transcripts of certain synthases (Frankowski et al. 2009; Kęsy et al. 2010), and perhaps also of ACC oxidases, is regulated by light and correlates with an increase in ethylene production (Kęsy et al. 2008).

In this study, the first ACO gene (designated *PnACO1*) from *P. nil* cotyledons was isolated and its expression levels were observed in the cotyledons of plants cultivated under both inductive and non-inductive photoperiodic conditions and following application of IAA. We also studied the *PnACO1* expression pattern in various vegetative organs of plants cultivated under continuous light (non-inductive control) and both under long inductive night and IAA treatment.

Due to the great commercial significance of ethylene, which controls the processes of development, maturation and senescence of flowers and fruits, most of the experimental studies published deal only with the analyses of the changes in the expression levels and activity of ACC synthases and oxidases in those organs. Hence, the research results presented herein are of a pioneering nature.

Materials and methods

Plant material

Seeds of *Pharbitis nil* Chois cv. Violet (Marutane Seed Co., Kyoto, Japan) were stirred with concentrated sulfuric acid for 45 min, washed under running tap water for 2 h and soaked for 24 h in water (26 ± 1 °C). The swollen seeds were sown in pots filled with vermiculite and sterile sand (1:1). The pots were transferred to a growth chamber at 26 ± 1 °C with continuous irradiance (130 μmol m^{-2} s^{-1} cool white fluorescent tubes Polam, Warsaw, Poland) for 5 days. IAA (0.5 mM) applications to both sides of the cotyledons were performed with the use of a small soft brush (about 50 μl per plant).

Plant material for *PnACO1* cDNA cloning

IAA was applied to the cotyledons of 5-day-old plants. After 2 h of IAA application, the cotyledons (without petioles) were harvested, immediately frozen in liquid nitrogen and stored at −80 °C.

Expression analysis under photoperiodic conditions

Plants were grown under long-day (LD) conditions (16 h of light and 8 h of darkness) for 5 days. A portion of 5-day-old plants was left under these conditions (variant I). The remainder of the plants grew in 16-h-long darkness (variant II), and some of these were treated with IAA (variant III). Cotyledons (without petioles) were harvested every 2 h after IAA application or transfer to the darkness and, additionally, at hours 1, 9 and 17. Subsequently, the cotyledons were frozen in liquid nitrogen and stored at −80 °C.

Organ expression

Plants were grown under continuous light for 5 days and subsequently divided into four portions. The first was left under the same conditions; the second grew under continuous light, but IAA was applied at the beginning of the 6th day; the third was exposed to a 16-h-long inductive night; and the fourth was transferred to a 16-h-long night period and treated with IAA at the beginning of the 6th day. The apices, petioles, cotyledons, hypocotyls and roots were picked at hours 2, 4, 8 and 16 after the beginning of the 6th day, immediately frozen in liquid nitrogen and stored at −80 °C. All manipulations during the dark period were performed under dim green safe light. Each experiment was repeated at least three times (biological replicates). All data are presented as mean ± standard error (SE).

Molecular cloning of *PnACO1*

Pharbitis nil tissue (1.0–1.5 g) was homogenized in a sterile chilled mortar with a pestle. Total RNA was isolated with Tri Reagent (Sigma) and genomic DNA was removed with Deoxyribonuclease I (Fermentas). All primers used were synthesized by the Laboratory of DNA Sequencing and Oligonucleotide Synthesis "oligo.pl" (Institute of Biochemistry and Biophysics, Polish Academy of Science, Warsaw, Poland). One microgram of total RNA primed with anchored oligo(dT)19 primers was used for first-

strand synthesis with RevertAid M-MuLV Reverse Transcriptase (Fermentas) according to the manufacturer's instructions. PCR, using degenerated primers 5′-CAAAGG GACATTACAAGAARTGCATGG-3′ (forward) and 5′-G CTARTGACATYCGWGTCCCGTC-3′ (reverse) constructed on the basis of conserved sequences encoding for *ACOs* in *Lycopersicon esculentum*, *Nicotiana tabacum*, *Nicotiana attenuata*, *Nicotiana glutinosa*, *Solanum tuberosum* (*Solanales* order) and *A. thaliana*, was performed in the T3 Thermocycler (Biometra). The total reaction volume contained 2 μL of first-strand cDNA, 2 μL of primer solution (10 μM), 5 μL of buffer B (containing 15 mM Mg^{2+}), 0.5 μL of 50 mM Mg^{2+}, 2 μL of 5 mM dNTP mix and 1.25 U of Blue Perpetual Taq DNA Polymerase$^{HOT\text{-}START}$ (EurX). The reaction mixtures were subjected to the following PCR conditions: 95 °C for 5 min, 1 cycle, 95 °C for 1 min, 55 °C for 45 s, 74 °C for 45 s for 35 cycles, followed by 1 cycle of incubation at 74 °C for 5 min. A 360-bp amplified cDNA fragment was isolated from an agarose gel with the GeneMATRIX Agarose Out DNA Purification Kit (EurX), cloned using the TOPO TA Cloning Kit (Invitrogen) and sequenced by "oligo.pl". A full-length cDNA coding for *PnACO1* was isolated using the BD SMART RACE cDNA Amplification Kit protocol from Clontech. mRNA used in 5′- and 3′-RACE-PCR was isolated with the Oligotex mRNA Mini Kit (Qiagen). 5′-RACE primer 5′-CAGCTTTCTCCACCA GAGCCGGTGCCGG-3′ and 3′-RACE primer 5′-GGTGG ACGTTCCTCCCATGCGCCACTCC-3′ were used, and were specific for the *PnACO1* cDNA fragment. PCR reactions were performed using the Advantage 2 PCR Enzyme System from Clontech. The reaction products were cloned using the TOPO TA Cloning Kit (Invitrogen). One Shot Mach1-T1 cells (Invitrogen) were plated onto Petri dishes containing S-Gal/LB Agar Blend (Sigma) and 50 μg mL^{-1} ampicillin (ICN). Ten white colonies were selected and cultured o/n in liquid 2× LB medium containing 50 μg ml^{-1} ampicillin. Plasmid DNA was isolated with the GeneMATRIX PLASMID MINIPREP DNA Purification Kit (EurX) and sequenced. Sequence data from this article have been deposited at GeneBank under accession number EF127817. Data analyses were performed in ClustalW (http://www.ebi.ac.uk/clustalw), BLAST 2.2 (http://www.ncbi.nlm.nih.gov/ BLAST) and DiAlign (http://www.genomatix.de/cgi-bin/dialign).

Expression analysis

Total RNA was isolated with the GeneMATRIX Universal RNA Purification Kit (EurX) according to the manufacturer's instructions. One microgram of total RNA primed with anchored oligo(dT)19 primers was used for first-strand synthesis with the RevertAid M-MuLV Reverse Transcriptase (Fermentas) according to the protocol. Semi-quantitative (SQ)RT-PCR conditions were optimized to a constitutively expressed actin gene (*InACT4*), which was used as an internal control. (SQ)RT-PCR analyses were performed with the gene-specific primers: *PnACO1* (550 bp) 5′-GAAAAGCTTAATGGCGCTGAGAGAGG-3′ (forward) and 5′-TCGTCTTGGAAGAGGAGGATTATG GCC-3′ (reverse); *InACT4* (240 bp) 5′-GAATTCGATATC CGAAAAGACTTGTATGG-3′ (forward) and 5′-GAATT CCATACTCTGCCTTGGCAATC-3′ (reverse). PCR mixtures were made as follows: 2 μL of first-strand cDNA, 2 μL of *PnACO1* primer solution (10 μM), 1.5 μL of *InACT4* primer solution (10 μM), 5 μL of buffer B (containing 15 mM Mg^{2+}), 0.5 μL $MgCl_2$ (50 mM), 2 μL of 5 mM dNTP mix and 1.25 U of Blue Perpetual Taq$^{HOT\text{-}START}$ DNA Polymerase (EurX). T3 Thermocycler parameters for the reaction were set up as follows: 95 °C for 5 min, 1 cycle, 95 °C for 1 min, 58 °C for 45 s, 74 °C for 1 min for 30 cycles, followed by 1 cycle of incubation at 74 °C for 5 min. The computer application used for the analysis was Quantity One (BioRad), and for the calculations and graphs we used MS Office Excel (Microsoft) and SigmaPlot 2001 v. 7.0 (SPSS Inc.).

Results

Isolation of cDNA for the ACC oxidase

For isolating the *PnACO1* gene we used degenerated PCR primers derived from conserved sequences of previously cloned *ACO* genes of other plant species. We performed RT-PCR amplification on total RNA from IAA-treated cotyledons. A DNA fragment of the expected size was recovered from the agarose gel and TA cloned into the plasmid vector. It was revealed through sequencing of the cDNA that the obtained fragment (designated as *PnACO1*) corresponded to other plant species' *ACOs*. Using RACE-PCR, the complete coding sequence of *PnACO1* (Gene Bank accession number EF127817) was obtained, which contains start and stop codons. The full-length *PnACO1* cDNA is composed of 1,259 bp and encodes for 309 amino acids (Fig. 1a). Based on a comparison between the deduced *PnACO1* amino acid sequence and ACC oxidase sequences from other plant species, it was found that the *PnACO1* protein, too, contains characteristic motifs: a cofactor binding one (Hsp177-X-Asp179-X(54)-Hsp234) and a co-substrate binding one (Arg244-X-Ser246) (Yoo et al. 2006). However, at the C-terminus of the *PnACO1* protein, an amino acid sequence was identified that was evolutionarily conserved and characteristic of ACC oxidases, containing the Lys and Arg residues essential for enzymatic activity (positions 294–301).

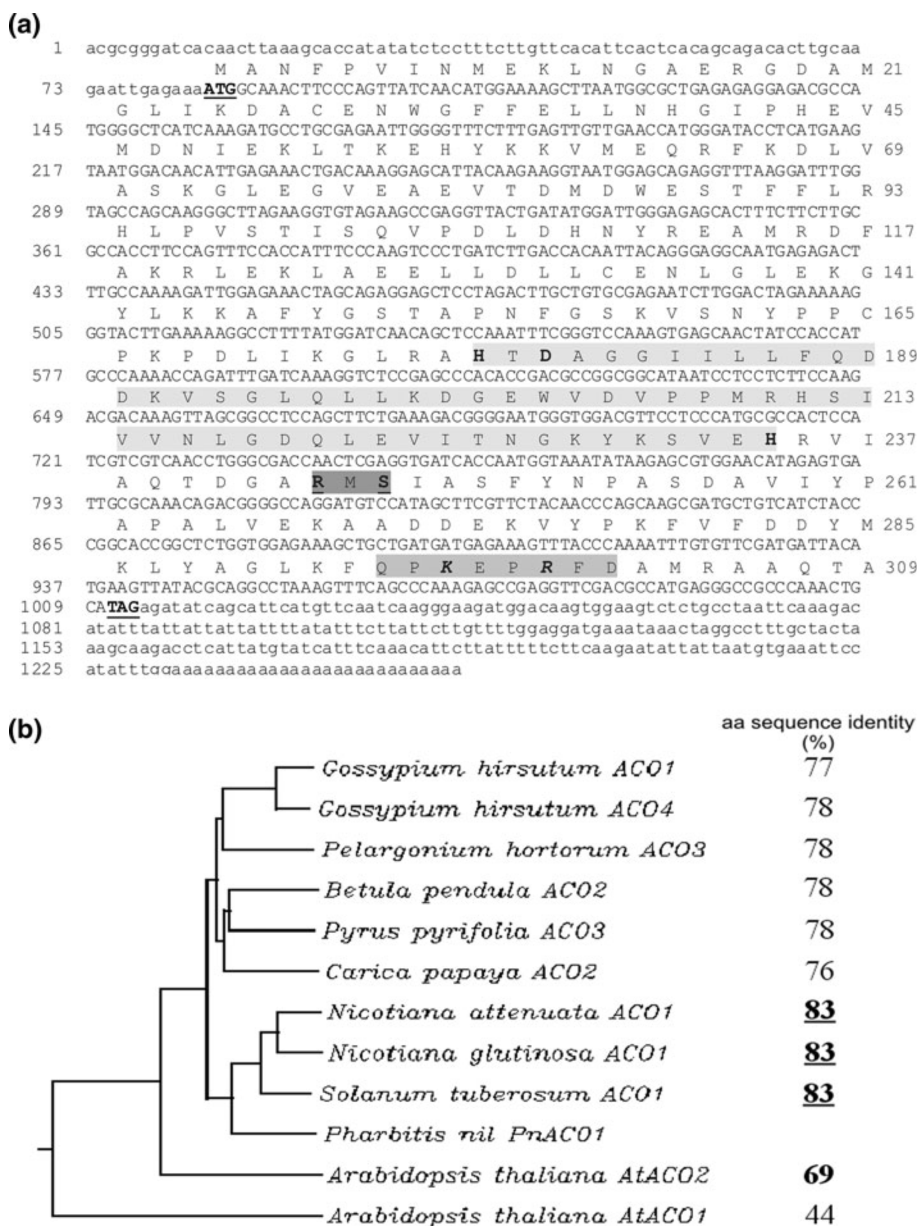

Fig. 1 **a** Coding sequence of *PnACO1* cDNA and the deduced amino acid sequence. Subsequent nucleotide positions are marked on the *left side* of the figure, and amino acid positions on the *right*. The translation initiation point (the start codon) and termination point (the stop codon) are *underlined* and in *boldface type*. *Small letters* are used for regions not subject to translation. *Light gray* denotes the motif forming the Fe(II) binding pocket comprising three characteristic amino acid residues (in *boldface type*) (Yoo et al. 2006). The ascorbic acid-binding motif comprising two characteristic amino acid residues (*underlined* and in *boldface type*) is marked *dark gray*. The evolutionarily conserved ACC oxidase sequence is marked *gray*, while the Lys and Arg residues essential for oxidase enzymatic activity are in *italics* and *boldface type*. **b** The phylogenetic relationship of *PnACO1* compared with the ACC oxidases from *A. thaliana* and members of the Solanales order. A phylogram tree was generated using ClustalW. *Percentages* placed in a column were generated in DiAlign and denote *PnACO1* amino acid sequence identity when compared with *A. thaliana* and other species ACOs. GeneBank accession numbers from *top* to *bottom*: ABC02397.1; ABA01476.1; AAB70884.1; AAN86821.1; BAD61000.1; AAS16934.1; AAR99394.1; AAA99792.1; AAK68075.1; EF127817; NP_176428.1; NP_179549.1. Details are described in the text

A comparison of ACO sequences from *A. thaliana* (*AtACO1* and *AtACO2*—the only fully described ones) and other plant species in the DiAlign with *PnACO1* shows that *PnACO1* has 69 % amino acid sequence identity to *A. thaliana* ACO2 (GB acc. no. NP_176428.1). Based on amino acid sequences, phylogenetic analysis revealed that *PnACO1* was very closely related to ACO1 (83 %) from *N. attenuata* (GB acc. no. AAR99394.1), *N. glutinosa* (GB acc. no. AAA99792.1) and *S. tuberosum* (GB acc. no. AAK68075.1) (Fig. b).

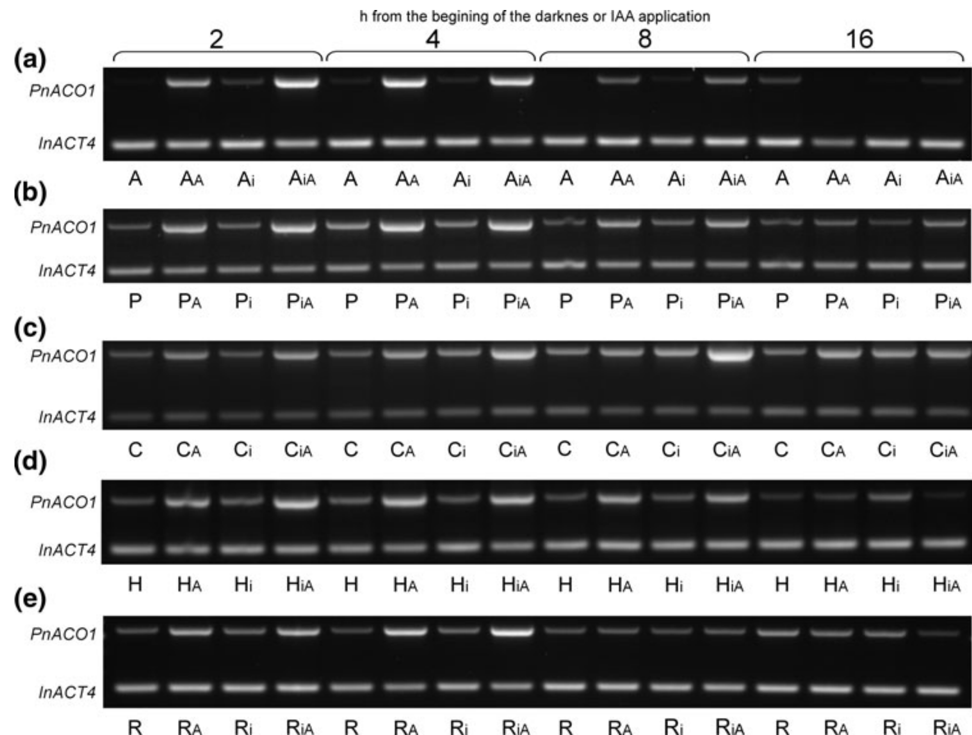

Fig. 2 (SQ)RT-PCR analysis of *PnACO1* mRNA in the vegetative organs of plants grown under diverse light conditions and IAA treatment. The *symbols below* gel pictures are: *A* apices, *P* petioles, *C* cotyledons, *H* hypocotyls, *R* roots; capitals with *no index* plants grown under constant light, *A-index* grown under constant light and treated with IAA, *i-index* transferred to 2, 4, 8 or 16 h of darkness (inductive night), *iA-index* transferred to darkness and treated with IAA. As an internal control, we used a constitutively expressed actin gene

PnACO1 expression in vegetative organs of *Pharbitis nil* seedlings

PnACO1 transcript accumulation occurs in all of the *P. nil* organs studied (Figs. 2, 3). The highest mRNA level of the gene in IAA-untreated seedlings was found in the cotyledons, while the lowest in the apices. However, addition of auxin to the cotyledons of *P. nil* seedlings causes the *PnACO1* expression level to rise in all of the organs studied, with that gene's mRNA level being the highest in the cotyledons (Figs. 2, 3).

The highest (four- or fivefold) increase in the *PnACO1* transcript level in IAA-treated plants was observed in shoot apices, petioles and hypocotyls, while the lowest (two- or threefold) in cotyledons. Apart from that, in all of the organs studied the level of *PnACO1* expression kept dropping following hour 8, to reach a level identified in plants not treated with the hormone at hour 16 (Figs. 2, 3).

PnACO1 expression under LD and SD conditions regulated by light and IAA

The highest *PnACO1* expression level in the cotyledons of *P. nil* seedlings cultivated under the long-day conditions is observed at hour 10 into the diurnal cycle (Fig. 4). It needs to be noted that a threefold increase in the level of mRNA of the gene studied at hour 10 is connected with the end of the dark period (the light is turned on at hour 8). However, in plants cultivated under the short-day conditions, a sixfold increase in the *PnACO1* transcript level is observed 1 h after the light is turned on (hour 17). In plants cultivated under these conditions, a clear increase in *PnACO1* expression was also observed at hours 9 and 10, with the increased level of the gene's mRNA maintained until hour 16. After hour 18 of the diurnal cycle, the level of *PnACO1* mRNA drops to the value observed in plants grown under non-inductive conditions. Application of IAA at the beginning of the inductive night results in a twofold increase (hour 2) of the level of *PnACO1* mRNA, which during the night grows to reach the maximum value at hours 14–16. Its further increase at hour 17, similar to plants not treated with auxin, is probably a consequence of the light having been turned on at hour 16. From hour 20 to 24 into the diurnal cycle, the transcriptional activity of *PnACO1* slows down to the initial value.

Discussion

In this study, using degenerated PCR primers, and then 5′- and 3′-RCE-PCR techniques, we isolated for the first time ACO cDNA from cotyledons of *P. nil*. (*PnACO1*) and determined its sequence. The *PnACO1* gene encodes for a protein whose expected molecular mass (34.82 kDa) is similar to that of ACC oxidases identified in other plant species (Hudgins et al. 2006; Hunter et al. 1999; Nie et al. 2001; Vriezen et al. 1999; Zanetti et al. 2002). The largest similarity with respect to the *PnACO1* amino acid sequence

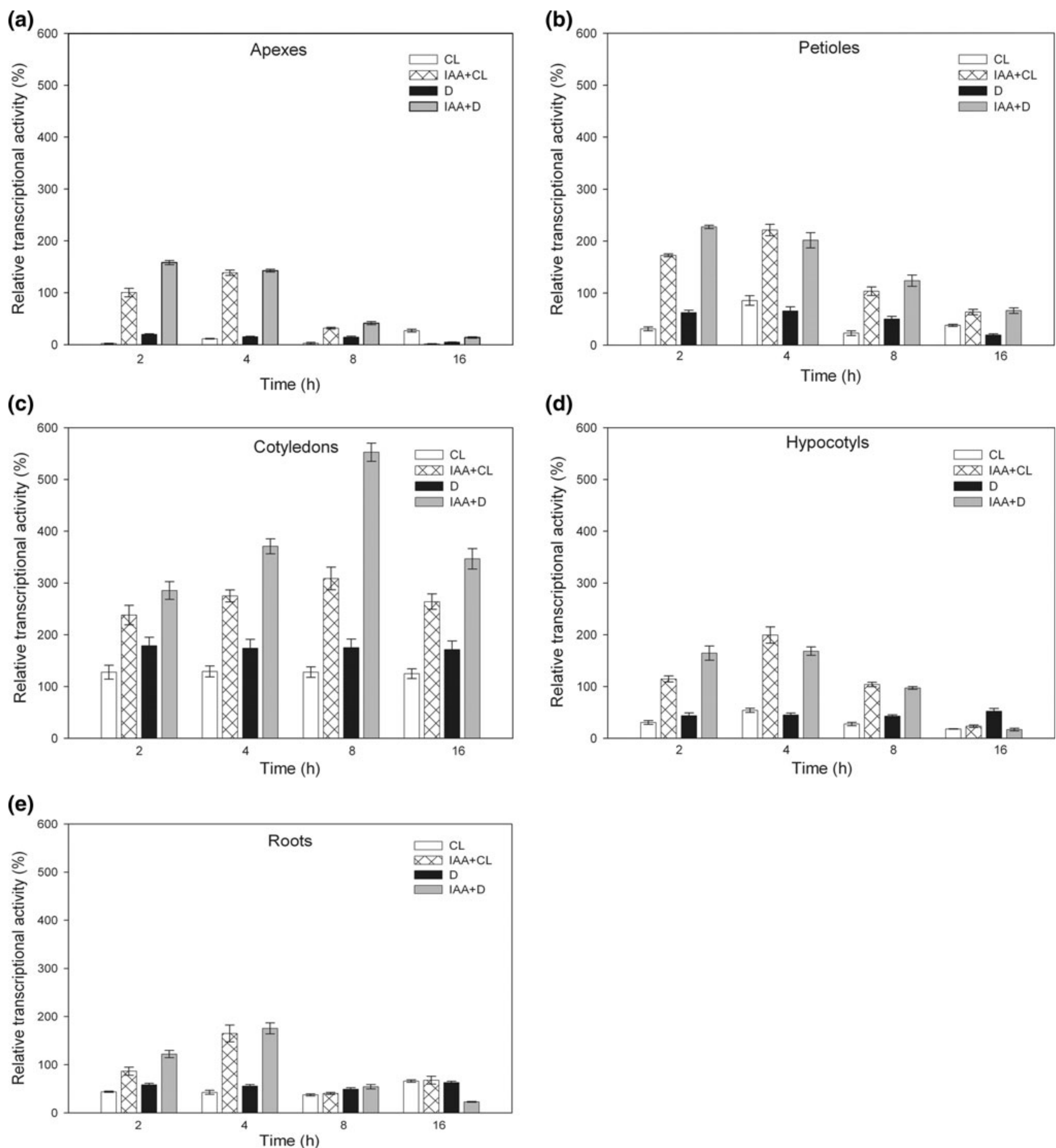

Fig. 3 The level of *PnACO1* transcript expression (related to *InACT4*) (three replicates) in the vegetative organs of plants grown under diverse light conditions and IAA treatment. The expression activity of *PnACO1* in the apices (**a**), petioles (**b**), cotyledons (**c**), hypocotyls (**d**) and roots (**e**) is shown in separate *bar charts*. *Empty bars* constant light, *checked bars* IAA + constant light, *black bars* inductive darkness, *checked gray bars* IAA applied before transfer to inductive darkness. SE is marked on the *bars*

(83 %) is shown in ACO1 proteins from *N. attenuata*, *N. glutinosa*, *S. tuberosum*, as well as *A. thaliana* ACO2—69 % (Fig. 1b). Because *PnACO1* contains motifs that are evolutionarily conserved and characteristic of ACC oxidases and these motifs are located within the Lys and Arg residues essential for protein activity (Yoo et al. 2006) (Fig. 1a), it can be suspected that this gene encodes for a functional enzymatic protein.

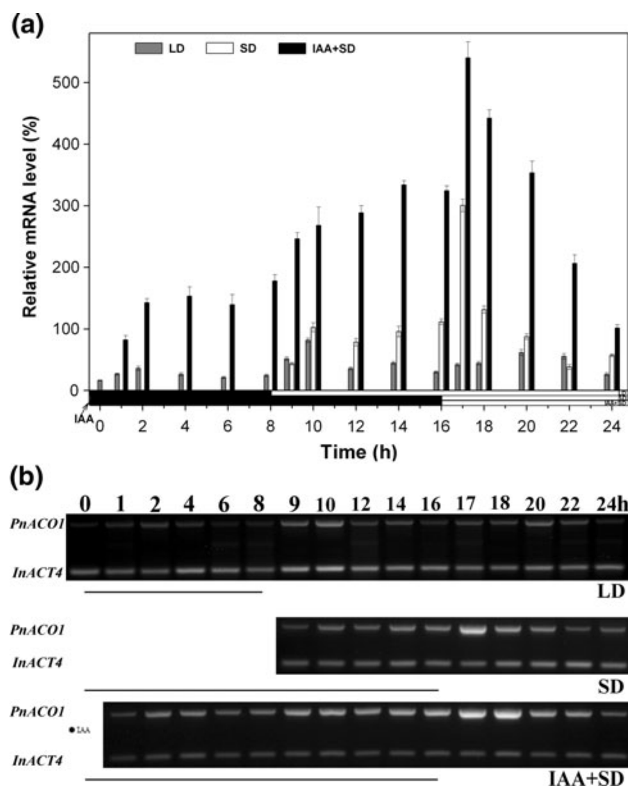

Fig. 4 **a** The level of *PnACO1* transcript expression (related to *InACT4*) (three replicates) in the cotyledons of plants grown under diverse light conditions and IAA-treated. *Gray bars* LD conditions, *empty bars* SD conditions, *black bars* IAA applied before transfer to SD conditions. SE is marked at the *bars*. **b** (SQ)RT-PCR analysis of *PnACO1* mRNA during a 24-h-long photoperiod and after IAA treatment. As an internal control we used a constitutively expressed actin gene. *Black lines* dark period; *black dot* IAA application to cotyledons

Due to the fact that the ethylene production level in various organs and tissues at different stages of the plant's development is regulated differently (Tsuchisaka and Theologis 2004b; Yamagami et al. 2003), we studied the expression pattern of *PnACO1* in the roots, hypocotyls, petioles, cotyledons and shoot apices of *P. nil* seedlings cultivated under continuous light, 16-h inductive darkness and after auxin treatment.

The results obtained show that *PnACO1* expression occurs in all the vegetative organs studied of *P. nil* seedlings cultivated under various lighting conditions and after the application of exogenous IAA. The highest level of this gene's transcript in plants not treated with auxin was observed in cotyledons, whereas the lowest accumulation of its mRNA was found in shoot apices (Figs. 2, 3).

Similar results were obtained from studies on the transcriptional activity of ACC oxidase genes in sunflower seedlings, with the highest *ACCO2* expression seen in cotyledons and the lowest in roots (Liu et al. 1997). *ACCO1* expression in these organs demonstrated the opposite pattern. At the same time, the level of *ACCO3* mRNA observed in cotyledons, roots and hypocotyls was equal, although low. In turn, in potato the highest *ACO1* mRNA concentration was found in leaves (Nie et al. 2001). An increased accumulation of *NG-ACO1* and *NG-ACO3* transcripts was observed in aging leaves of *N. glutinosa*, while the level of *NG-ACO2* mRNA was stable in these organs (Kim et al. 1998). A high level of *NG-ACO1* and *NG-ACO3* expression was also found in roots and that of *NG-ACO2* in stems.

An increased expression of the ACC oxidase gene in *P. nil* cotyledons (Figs. 2, 3), correlated with a substantial transcriptional activity of *PnACS1* and *PnACS2* (Frankowski et al. 2009; Kęsy et al. 2010), could be connected with the cotyledons reaching full maturity, or could be the first symptom of their aging. A similar effect was proved in the case of the cotyledons and leaves of *Morus alba*, in which an increased accumulation of the *MaACO1* transcript preceded the occurrence of aging symptoms. Additionally, many papers showed that ethylene can, on its own and as a result of positive feedback, regulate its biosynthesis by increasing the level of expression of genes encoding for ACC oxidases. The existence of this mechanism was proved in the leaves of tomato and melon (Blume and Grierson 1997; Bouquin et al. 1997), etiolated pea seedlings (Peck and Kende 1995; Peck and Kende 1998) or sprouting pea seeds (Petruzzelli et al. 2000).

Application of auxin to *P. nil* seedlings cultivated both under light and dark conditions causes an increase in the transcriptional activity of the *PnACO1* gene in all of the organs studied (Figs. 2, 3). The highest concentration of this gene's mRNA is observed at hours 4 or 8 (depending on the organ), after which the accumulation of the transcript reaches a level observed in plants not treated with IAA (hour 16). It must also be noted that the maximum level of *PnACO1* expression is shifted in time by 2–6 h in relation to the occurrence of *PnACS1* and *PnACS2* transcripts (Frankowski et al. 2009; Kęsy et al. 2010).

An increased expression of ACC-encoding genes after application of auxins was also observed in other plant species. In potato tubers, auxin has a significant stimulating effect on the accumulation of *ST-ACO3* mRNA (Zanetti et al. 2002). In turn, in etiolated hypocotyls of *Vigna radiata*, IAA increases the expression of *VR-ACO2*, while having no effect on the level of accumulation of the *VR-ACO1* transcript (Yu et al. 1998). Nevertheless, application of another concentration of IAA in further studies resulted in an increased transcriptional activity of both the genes studied (Song et al. 2005).

Our results show that, similarly to *ACSs*, the expression of genes encoding for ACC oxidases is controlled at various stages of plant development by internal (auxin) and environmental (light) factors and differs in time and space.

A high level of *PnACO1* expression in *P. nil* cotyledons treated with auxin shows what role ethylene and its interactions with auxin play in regulating the plant's growth and development also at early stages of its vegetation. Ethylene produced by shoot apices in dicotyledons limits cell enlargement in young leaves (Kieber et al. 1993; Lee and Reid 1996; Osborne 1991), and—by way of analogy—it could play the same role in other tissues, as well.

Auxin induces the expression of some of the ACC synthase genes (Arteca and Arteca 1999; Frankowski et al. 2009; Tian et al. 2002; Tsuchisaka and Theologis 2004b); therefore, its inhibitory effect on flowering is most probably the result of increased ethylene production (Frankowski et al. 2009; Kęsy et al. 2008). Additionally, the level of accumulation of some ACC synthase transcripts is also regulated by light (Frankowski et al. 2009) and correlates with increased ethylene production (Kęsy et al. 2008). We therefore studied whether the inhibitory effect of auxins on *P. nil* flowering is correlated with an increase in the transcriptional activity of the ACC oxidase gene, and also determined the level of this gene's expression in the cotyledons of seedlings cultivated under different photoperiodic conditions.

Based on the results obtained, it can be concluded that both light and auxin control the level of *PnACO1* transcript accumulation (Fig. 4). Significant changes in the level of this gene's mRNA occurred in plants cultivated both under the long- and the short-day conditions. While an increase in the transcriptional activity of *PnACO1* at hour 10 in the cotyledons of seedlings cultivated under the LD condition results from the light having been turned on at hour 8, the increase in the gene's expression observed at the same hour of the diurnal cycle in the cotyledons of seedlings cultivated under the SD conditions seems to be the consequence of an endogenous rhythm. The level of the gene's expression in plants cultivated under the SD conditions is also increased after the light is turned on (hour 17). Oscillations in the level of *PnACO1* mRNA are also observed in the case of IAA-treated plants, whereas an increase in its expression is additionally seen after the light is turned on (hour 17). Nevertheless, the increased level of the gene's transcriptional activity in IAA-treated plants that is maintained at hours 12–16 seems to be the result of positive feedback, as it correlates with an increased accumulation of *PnACS1* and *PnACS2* mRNAs (Frankowski et al. 2009; Kęsy et al. 2010). The data presented show clearly that *PnACO1* expression is regulated by light and displays diurnal oscillations. Additionally, the increase in *PnACO1* concentration after the light is turned on both under the SD and LD conditions is correlated with an increase in *PnACS1* transcript accumulation (Frankowski et al. 2009) and an increase in ethylene production (Kęsy et al. 2008).

ACC oxidase genes, whose expression is regulated by light, were also identified in the seedlings of *Phaseolus vulgaris* (Pidgeon et al. 1997), *Stellaria longipes* (Kathiresan et al. 1996), *Sorghum bicolor* (Finlayson et al. 1999) and *Vigna radiata* (Song et al. 2003). In *S. longipes* cultivated under the LD conditions, cyclic changes were found in the level of mRNA of the gene encoding for ACC oxidase, which were correlated with the enzymatic activity of the protein (Kathiresan et al. 1996). Cyclic accumulation of *SbACO2*, as well as cyclic fluctuations in the level of ethylene, was also observed in *S. bicolor* cultivated under conditions imitating shading (Finlayson et al. 1999). However, changes in the gene's transcriptional activity did not correlate with the enzymatic activity of its product. In turn, a slight drop in the level of the *VR-ACO2* transcript and the constant transcriptional activity of *VR-ACO1* were observed in the hypocotyls of etiolated *V. radiata* seedlings after they were exposed to light (Song et al. 2003).

Application of auxin to the cotyledons of *P. nil* seedlings at the beginning of the inductive night causes a clear increase in the accumulation of *PnACO1* mRNA from the first hours in the dark period (Fig. 4); that increase follows a rise in the accumulation of ACC synthase gene (*PnACS1* and *PnACS2*) transcripts (Frankowski et al. 2009; Kęsy et al. 2010). These data correlate with an increase in ethylene production after application of 1 mM IAA to the cotyledons of *P. nil* seedlings at the beginning of the inductive night (Kęsy et al. 2008; Wijayanti et al. 1997).

The data presented here and in our previous papers (Frankowski et al. 2009; Kęsy et al. 2008, 2010) clearly show that the inhibitory effect of auxins on *P. nil* flowering is the result of their stimulating effect on the expression of genes encoding for enzymes involved in ethylene biosynthesis (ACSs and ACOs), which leads to an increase in the concentration of ethylene that inhibits flowering directly.

Future perspectives

Although auxin plays a superior role in regulating many plant growth and development processes, still its participation in the photoperiodic induction of flowering occurs through cooperation with other phytohormones, including ethylene. Nevertheless, it cannot be excluded that ethylene has an influence on some other significant factor regulating flowering in this species, e.g., abscisic acid (Wilmowicz et al. 2008). It was shown that auxin-induced increase in ethylene production leads, in turn, to a drop in the accumulation of abscisic acid, whose increased level in the second half of the inductive night is a factor that inhibits *P. nil*'s transition from the vegetative development stage to generative differentiation.

Author contribution Emilia Wilmowicz and Kamil Frankowski designed and carried out the experiments, analyzed the data and wrote the manuscript. Agata Kućko carried out the experiments. Paulina Glazińska and Waldemar Wojciechowski were responsible for the preparation of figures and charts. Jan Kopcewicz and Jacek Kęsy helped in preparing the manuscript.

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