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ACC Oxidase and ACC Synthase Expression Profiles after Leaning of Young Radiata (*P. radiata* D. Don) and Maritime Pine (*P. pinaster* Ait.) Seedlings

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Abstract Loss of verticality in conifers affects the normal wood development by inducing changes and chemical modifications in tree stems called compression wood. It is known that ethylene influences the response during this abnormal wood-forming process. The expression pattern of genes involved in the ethylene biosynthesis pathway during gravitropic response in gymnosperms has been identified in adult trees. Young seedlings of radiata pine were inclined to reveal the expression pattern of these genes by the quantitative real-time PCR (qRT-PCR) technique. The seedlings were exposed to gravitropic stimuli and harvested after 2.5 and 10 h (early responses) of inclination, and after 24 h (late response). Sampling includes transverse cuts at three heights of the whole stem of inclined seedlings. Our data revealed that genes encoding for 1-aminocyclopropane-1-carboxylate oxidase (ACO) and 1-aminocyclopropane-1-carboxylate synthase (ACS) were differentially expressed during the time of leaning, and, interestingly, at the basal portion of radiata pine stems. Additionally, transcriptional analysis in maritime pine showed a conserved profile of gene activation in conifers, and in mature compression wood, ACO gene transcription was strongly upregulated. These results indicate that the concerted activation of genes involved in ethylene biosynthesis could be responding to leaning signals in young radiata and maritime pine seedlings.

Keywords ACC oxidase · ACC synthase · Ethylene biosynthesis · Gravitropism · *Pinus radiata*

Introduction

Stem cells from trees respond to gravity through the induction of differential growth between upper and lower sides. Displacement of stems by slope or other mechanical stress leading to loss of verticality and tilting of the trees results in the formation of reaction wood. This response is unilateral and creates physical wood strains that force the stem back toward its original vertical orientation (Scurfield 1973; Wilson and Archer 1977; Timell 1986). The process of gravitropism can be divided into three sequential steps: (1) gravity perception, (2) signal transduction, and (3) differential growth in elongating organs with the consequent asymmetric radial growth in the responding parts (Fukaki and Tasaka 1999; Haswell 2003). The signal transduction pathway that controls the response to inclination in young seedlings is still unclear but seems to be a response related to intrinsic growth direction, phytohormone distribution, and interaction (Timell 1986; Sundberg and others 1994; Little and Eklund 1999).

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Ethylene has been proposed to be associated with the gravitropic response (Kang and Burg 1974; Wheeler and Salisbury 1980, 1981; Clifford and Oxlade 1989; Philosoph-Hadas and others 1996; Love and others 2009). Also, the involvement of auxins has been supported (Clifford and others 1983; Harrison and Pickard 1986; Kaufman and others 1985; Woltering 1991), but their contribution to the control of stem gravitropism and knowledge about their function are still uncertain.

Ethylene is a gaseous phytohormone involved in many aspects of plant growth and development such as seed germination, fruit ripening, response to biotic and abiotic stresses (Kende 1993; Bleecker and Kende 2000; Wang and others 2002), and determining certain aspects of tree form (Dolan 1997). The genes encoding its biosynthetic pathway are part of a multigenic family whose expression is strongly regulated by external and internal stimuli (Kende 1993; Johnson and Ecker 1998).

In higher plants ethylene is produced by a branch of the methionine cycle in which *S*-adenosyl methionine (SAM) is converted to 1-aminocyclopropane-1-carboxylate (ACC) and 5′-methylthioadenosine by ACC synthase (ACS, *S*-adenosyl-L-methionine methyl-thioadenosine-lyase, EC 4.4.1.14) (Kende 1993). ACC is subsequently oxidized by ACC oxidase (ACO, 1-aminocyclopropane-1-carboxylate oxidase, EC 1.14.17.4) (former ethylene-forming enzyme), to yield ethylene, HCN, and CO_2. Although it is established that ethylene is produced during wood formation and influences wood development when applied exogenously (Little and Savidge 1987; Little and Pharis 1995), genes encoding ACS and ACO in cambial tissues under the compression wood formation phenomenon have been characterized only in old adult trees. In a 21-year-old *Pinus contorta* tree, endogenous ACC was identified on the lower side in association with compression wood formation (Savidge and others 1983). In maritime pine, reports described that transcripts encoding for ACO and ACO proteins were upregulated during compression wood formation in 22- and 15-year-old maritime pine trees (*Pinus pinaster* Ait.), respectively (Plomion and others 2000; Le Provost and others 2003). Interestingly, Barnes and others (2008) suggested that the rapid increase in transcript levels of an ACS gene induced by bending could be involved in the early response to gravistimulation in loblolly pine (*Pinus taeda* L.).

In adult trees, ethylene synthesis was induced in pine stems that were mechanically bent (Leopold and others 1972) and the ethylene precursor ACC was detected in wood at the bottom of lodgepole pine (*P. contorta* Dougl. ex Loud.) branches, where compression wood was formed, but not at the top of the same branches, indicating a specific distribution and biosynthesis of ethylene at different heights of affected stems (Savidge and others 1983). Ethylene is known to increase radial growth in conifer stems if it is applied as Ethrel, an ethylene-forming compound (Barker 1979). In addition to increasing cambial activity, several studies showed that this hormone played a major role in the control of xylem differentiation, by inducing the activity of enzymes involved in lignification or by affecting polysaccharide deposition during cell wall formation (for review, see Eklund and Tiltu 1999).

In the present work the spatial and time-specific expression pattern of ACS and ACO genes were analyzed in stems in response to a gravitropic stimulus in 1-year-old *P. radiata* D. Don and *P. pinaster* Ait. seedlings. The questions to answer are: (1) Does the expression of ACC synthase and ACC oxidase change in response to leaning and (2) is the expression pattern of these genes similar in the two conifer species?

Materials and Methods

Sampling of Tissues and Differentiating Xylem

The experiments were performed in two conifer species: radiata pine (*Pinus radiata* D. Don.) and maritime pine (*Pinus pinaster* Ait.). The pots that held 1-year-old half-sib radiata pines, around 30 cm tall and which came from an open pollinated orchard, were tilted at 45°. The initial response to gravitropic stimulus of radiata pine seedlings was recorded after 2.5 h of inclination at the apical zone. Sampling of inclined seedlings' stems was performed after 2.5, 10, and 24 h of leaning. At each sampling time a group of nine inclined seedlings were collected, their stems divided in different zones, pooled, and immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. In addition, a group of nine noninclined seedlings were sampled at each sampling time as a control to evaluate changes in gene expression along the day. Control 0 and 24 h were the same. Sampling was initiated at 9:00 a.m. Seedling stems were divided into three parts to determine gene expression along the stem, corresponding to the apical zone (Stem 1), medial zone (Stem 2), and basal zone (Stem 3) (Fig. 1).

The pots that held 1-year-old half-sib seedlings of maritime pine, which came from the Research Unit of INRA-Pierroton (Cestas, France), were tilted at 45°. After 24 h of inclination, a group of ten inclined or noninclined seedlings were sampled, separating their stems in three parts: apical zone (Stem 1), medial zone (Stem 2), and basal zone (Stem 3) (Fig. 1). Additionally, as major transcriptional changes were previously reported in maritime pine seedlings after 10 h of inclination (Herrera and others 2010), to maximize information retrieval, the stem separating superior (S) and inferior (I) halves was cut longitudinally in the curvature zone. Control samples were the

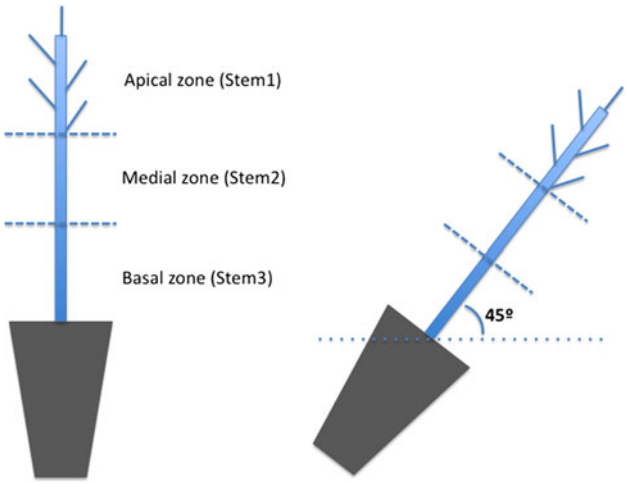

Fig. 1 Schematic drawing showing the inclination methodology and sampling at different heights of stems after gravistimulation. The pots containing the seedlings were tilted at a 45° angle and samples were collected after different bending times. Control and inclined stems were cut at three different heights

noninclined seedlings. Tissues from each sampling were pooled and immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction.

In addition, 22-year-old clonal maritime pine (*Pinus pinaster* Ait.) adult trees at the Forest Research Unit of INRA-Pierroton (Cestas, France) were artificially bent at 45° as described previously using a string (Le Provost and others 2003). Differentiating xylem associated with CW (compression wood) and with OW (opposite wood) corresponding to the superior and inferior halves of the bent trunk at the crown, respectively, was harvested. Samples were collected after 6, 24 h, and 8 days of bending by scraping after removal of bark, phloem, and cambium. Tissue samples were flash frozen in liquid nitrogen and stored at −80°C until use.

Total RNA Extraction

Total RNA was extracted from 200 mg of frozen tissue using the CTAB extraction procedure described previously (Le Provost and others 2007). Remaining traces of DNA were removed with RQ1 RNase-free DNase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Concentration was estimated by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). Three independent RNA extractions were taken from each frozen sample.

Quantitative Real-Time PCR

Primers for quantitative real-time PCR (qRT-PCR) (Higuchi and others 1993) were designed using Beacon Designer v 2.0 software (PREMIER Biosoft, Palo Alto, CA, USA). Housekeeping genes *PrUBC2* and *PrUBC7*, corresponding to ubiquitin carrier proteins TC81737 and TC82803, respectively, were selected from our local ESTs database generated from *Pinus radiata*. In addition, the 40S ribosomal protein S27 gene, called *Ge066D02* (accession BX252550), was used. The corresponding primer set sequences for housekeeping genes are listed in Supplementary Table 1. For each normalization gene, analyses of transcript accumulation in inclined and noninclined stem seedlings were performed and minimal variations in the expression level were obtained in stem samples, either over time or along the stem (data not shown).

For the ACS gene, primers were designed based on GenBank accession DQ871026 (listed in Supplementary Table 1). The amplicons obtained were sequenced at Macrogen (Seoul, Korea) to verify the unique correspondence to the specific gene. To evaluate ACO from *P. radiata*, different contigs [or Tentative Consensus sequences (TCs)] coding for ACOs found on the TIGR database of pine that were expressed on inclined wood were first amplified, cloned, and sequenced. From the sequences obtained, three radiata pine oligonucleotide-specific primers were designed (Supplementary Table 1): TC158318 was named ACO3, TC164905 was named ACO4, and TC186139 was named ACO5.

For qPCR analysis, template cDNA from each sample was synthesized using 1 µg of DNase-treated total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA), in accordance with the manufacturer's instructions. The first-strand RT reaction product was diluted tenfold, and 2 µl was used for each qPCR reaction. The cycle threshold (Ct) line was determined manually as the point where the R^2 value for the standard curve reached its highest point. iQ SYBR® Green Supermix (Bio-Rad Laboratories) was used on qPCR quantifications in a final volume of 20 µl, following the manufacturer's protocol. All experiments were run on a Chromo4™ Multicolor Real-Time PCR Detection System (Bio-Rad) with the following cycling conditions: initial denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. The instrument was set to measure dye fluorescence at the end of each cycle. The melting curve was analyzed for each primer pair to discard possible family gene member's amplification. Additionally, agarose gels were run to discard possible multiband products for each qPCR gene analyzed.

The standards consisted of a serial dilution of PCR amplicons prepared from each cDNA. The standard curves were determined from duplicate reactions of the dilution series of each amplicon. Data were analyzed using the Excel (Microsoft Corp., Redmond, WA, USA) macro GENEX v1.10 (gene expression analysis for iCycle iQ® real-time PCR detection system, v1.10, 2004; Bio-Rad Laboratories), using the methods derived from the

algorithm of Vandesompele and others (2002). Statistical analyses were performed using Student's *t* test.

Results

Selection of ACC Oxidase Encoding TCs

Based on the TIGR database for pine, a search for different TCs that encoded for proteins with ethylene-forming enzyme function was performed. Eight different TCs were found on the TIGR pine database in 2009 from libraries built from xylem of trees exposed to inclination and were named ACO1 to ACO8. TCs were rechecked in the TIGR database to verify the correct TC numbers after the new release of the database in 2011. Primers were designed to amplify the sequences in radiata pine, but only three of them provided amplification products. The amplification products were sequenced (Macrogen, Seoul, Korea) and specific primers for qRT-PCR assay were designed. These three TCs were identified as *Pinus radiata* (*Pr*) *PrACO3*, *PrACO4*, and *PrACO5*. The predicted amino acid sequences showed homology to ACO proteins described from *Arabidopsis*, apple, tomato, tobacco, poplar, loblolly pine, maritime pine, and white spruce, and shared the conserved motifs distributed within all members of the Fe II-dependent oxygenase/oxidase family, including the HXD…H motif (Supplementary Fig. 1). Additionally, multiple amino acids that are needed by Fe-ascorbate oxidases are present in the sequences analyzed, conserving all of the important residues highlighted in the characterized loblolly pine ACO1 (Yuan and others 2010).

Gene Expression Profile in Response to Inclination

To obtain a global expression pattern of genes involved in ethylene biosynthesis after the gravitropic response, transcript quantification was performed in whole-stem samples obtained from 45°-inclined young seedlings of radiata and maritime pine. Seedlings grown under vertical growth conditions (noninclined) were used as controls. qPCR analysis showed that the expression level of the *ACO3* gene was significantly induced about 20-fold after inclination in both species (Fig. 2). Higher induction was observed in radiata than in maritime pine (threefold). *ACO4* transcripts were induced only during inclination of maritime pine seedlings, and no differences were shown in radiata pine stems. In the case of *ACO5*, transcripts were detected only in radiata pine, and a clear induction in the expression was observed in inclined seedlings. As no ACO5 transcripts were detected in maritime pine, no further analyses were performed in this species for ACO5.

The expression of ACS, the first enzyme involved in the biosynthesis of ethylene was also analyzed. The expression pattern of *ACS* was similar to *ACO3* and *ACO5*, with a significant increase in the expression level observed in inclined stems compared to control (Fig. 2). A stronger induction of about tenfold was observed in radiata pine compared to maritime pine.

To better characterize the expression profile of these genes, a time-course experiment of 45°-inclined seedlings was performed in radiata pine seedlings (Fig. 3). In addition, transcript quantification was carried out in stems cut at three different heights to determine the time and place of transcript expression. Transcripts for *ACO3* and *ACO5* were induced (about 30- and 4-fold, respectively) after 10 h of inclination, mainly in the basal portion of the stem (Stem 3), with the expression level being reduced after that (Fig. 3). *ACO4* transcripts showed nonsignificant differences in expression levels over time (Fig. 3). On the other hand, *ACS* transcripts increased about twofold after 2.5 h of inclination, mainly in the medium portion of the stem, and remained high after 10 h but then decreased. In addition, ACS transcript induction was earlier than ACOs in radiata pine seedlings.

A similar experiment was set up in maritime pine seedlings. As minor global transcriptional changes were reported previously after 2.5 h of inclination and major changes after 10 h (Ramos and others, unpublished), simplified sampling was used in maritime pine for 10 and 24 h of inclination. As a way to get more information, the stem was cut longitudinally. In maritime pine, transcripts for *ACO3* were highly expressed, about eightfold, after 24 h in the basal zone of the stem (Fig. 4) compared to noninclined seedlings. Interestingly, the analysis performed in the superior and inferior halves of inclined stems indicated that after 10 h of inclination *ACO3* showed no differences between both sides (Fig. 4). A significant induction, about twofold, in *ACO4* transcripts was observed after 10 h in the inferior part of inclined stems of maritime pine seedlings (Fig. 4); however, nonsignificant changes were observed at different stem heights after 24 h of inclination. ACS transcripts remain almost constant after inclination as seen by comparing the different stems' portions (Fig. 4). Nevertheless, a significant asymmetric distribution of ACS transcripts was observed, with a high expression level in the superior half compared to the inferior half.

Ethylene Biosynthesis Genes Are Regulated in Wood Tissues During the Gravitropic Response of Adult Trees

To confirm the ACO expression pattern reported previously (Plomion and others 2000; Le Provost and others 2003), and also analyze the ACS gene, xylem samples were obtained from a 22-year-old maritime pine tree that was mechanically bent. Expression analyses by means of qPCR methodology were performed in xylem samples prepared

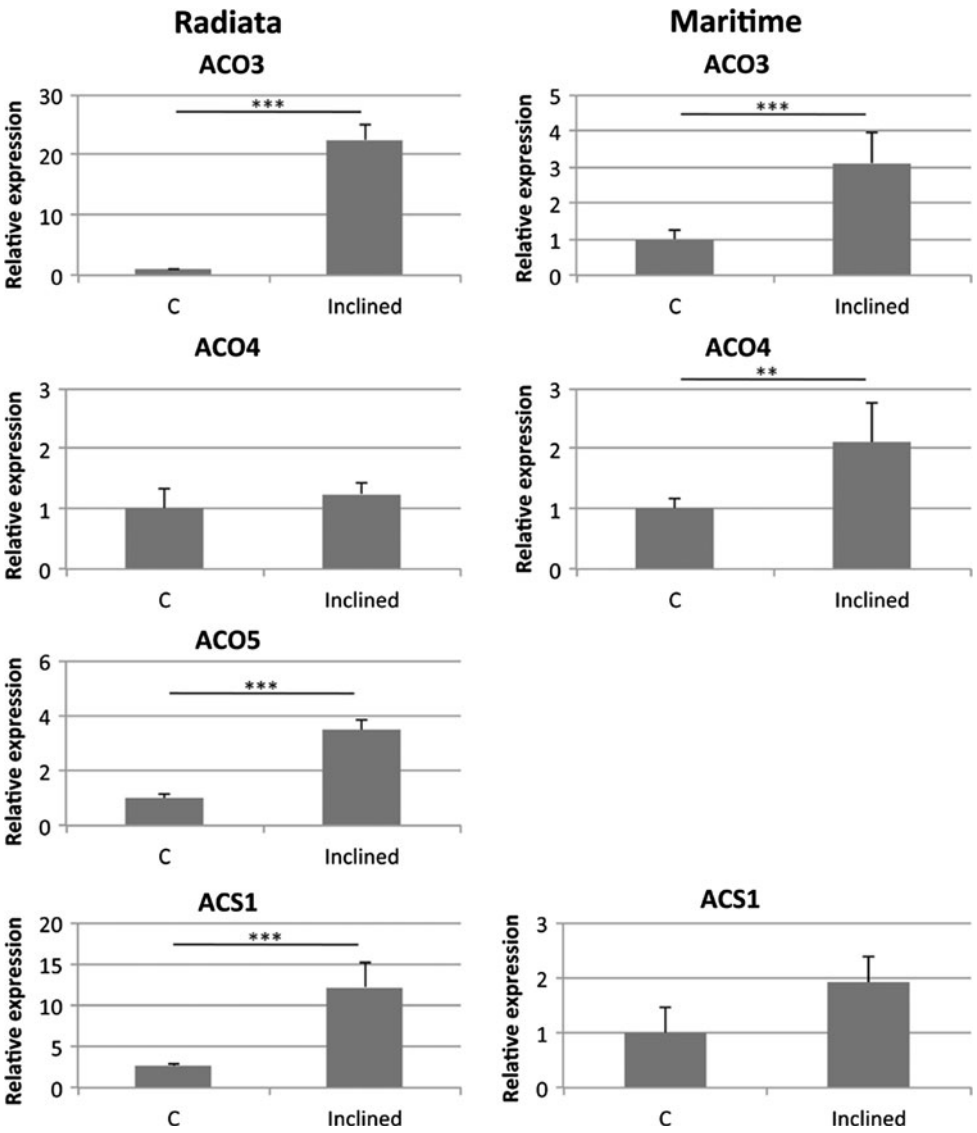

Fig. 2 Expression analysis by qPCR of ethylene biosynthesis genes in seedlings exposed to gravitropic stimuli. *ACOs* and *ACS* transcripts were analyzed in whole stems of inclined radiata and maritime pine seedlings compared to noninclined plants as controls. Samples correspond to equimolar mixture of cDNA samples prepared from stems collected during the first 24 h of leaning. The expression values represent the ratio between the expression level of the corresponding gene compared to housekeeping genes (*Ge066D02* and *UBC7*). Values are mean ± SD of at least three independent measurements. *Asterisks* indicate statistical significance (**$P < 0.01$, ***$P < 0.001$, Student's t test)

from compression and opposite wood tissues. Trees were bent at a 45° angle and differentiating xylem samples were collected at three different times. No differences in *ACO3* transcript levels between both sides of the trunk were found after 6 and 24 h of bending, but a significant decrease (threefold) in the expression level in opposite wood was observed after 8 days (Fig. 5). *ACO4* showed an important induction (threefold) in the transcript level in compression wood compared to opposite wood after 8 days of bending (Fig. 5). *ACS* transcripts showed no significant differences between compression and opposite wood in response to bending (Fig. 5).

Discussion

Ethylene modulates a wide variety of processes in plants, including growth and stress responses (Bleecker and Kende 2000). Although ethylene biosynthesis has been studied extensively in angiosperms due to its diverse roles in fruit ripening, senescence, and abscission (Liang and others 1992; Park and others 1992; Clark and others 1997), few studies have examined ethylene biosynthesis and its importance in gymnosperms (Klintborg and others 2002; Yuan and others 2010). Reaction wood forms in bent tree stems (Timell 1986), and *ACO* transcripts and ACO protein have been identified as expressed during compression wood development in gymnosperms (Plomion and others 2000; Le Provost and others 2003; Yuan and others 2010) and tension wood in angiosperm trees (Andersson-Gunneråas and others 2003). Moreover, the involvement of ethylene application and compression wood formation has been widely studied, but the expression of the genes encoding for enzymes responsible for its biosynthesis in the gravitropic response, which probably finally drives compression wood formation in gymnosperms, is still not clearly understood.

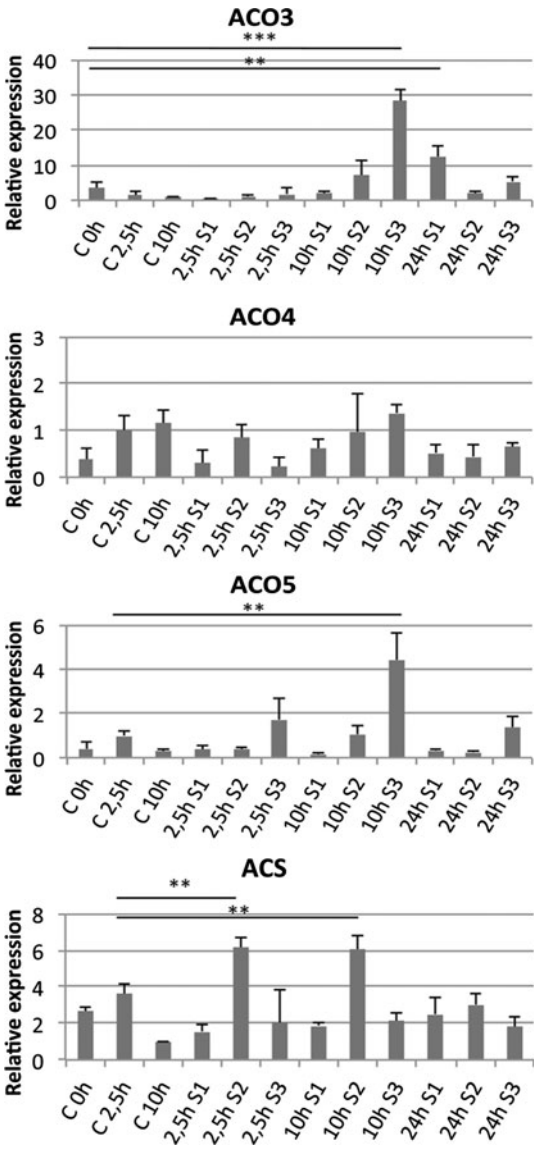

Fig. 3 Expression profiles of *ACOs* and *ACS* genes at three different stem heights during inclination of radiata pine seedlings. Expression analysis of *ACO3*, *ACO4*, *ACO5*, and *ACS* genes was performed by qRT-PCR. Sampling was performed after 2.5, 10, and 24 h of inclination, and stems were separated at three heights by transverse cuts (S1, apical zone; S2, medial; S3, basal). Control corresponds to a group of vertical plants harvested at the same time of sampling of inclined plants. The expression values represent the ratio between the expression levels of the corresponding gene compared to housekeeping genes (*Ge066D02* and *UBC7*). Data correspond to mean ± SD of three biological replicates. *Asterisks* indicate statistical significance (**$P < 0.01$, ***$P < 0.001$, Student's t test)

Previous studies of conifers suggested that ethylene biosynthesis genes could be implicated in the gravitropic response (Du and Yamamoto 2007). A comparative study of transcript accumulation for two genes of this pathway, *ACO* and *ACS*, has been done on young seedlings of radiata and maritime pine. Those genes showed differential expression profiles in response to being tilted at 45°. The intensity of the stem response is dependent on the angle of inclination, with the maximum effect observed at a tilt of 45° (Herrera and others 2010). Furthermore, differentially expressed proteins were identified and characterized after 22 h of inclination, but apex reorientation was observed to be as early as 2 h (Herrera and others 2010).

Three different TCs identified in the TIGR database and encoding as *ACO* transcripts and one *ACS* were found. A Blastp analysis was performed against sequences contained in GenBank for the *ACOs* sequences cloned. The predicted amino acid sequences showed *Picea glauca* ethylene-forming enzyme (AAA85365) as the best match. For the alignment ACC oxidases, sequences from gymnosperm as well as angiosperm species were considered, and the deduced protein sequence displayed all the conserved residues that confer the ACC oxidase activity (Supplementary Fig. 1).

The expression profile of these *ACOs* and *ACS* transcripts in a pool of inclined stem mix was evaluated. It was noted in the analysis that *ACO3*, *ACO5*, and *ACS* increased their expression level during reorientation in radiata pine (Fig. 2) and *ACO3*, *ACO4*, and *ACS* on maritime pine inclined stems (Fig. 2). At this point, it could be inferred that ethylene biosynthesis pathway genes are activated when seedlings from both species are inclined at 45°, but one cannot conclude that ethylene is important for the initiation of the response or that it is a modulator of other mechanisms involved in the gravitropic response of the stem. Recently, Lewis and others (2011) have shown that ethylene is involved in the control of flavonol accumulation. These observations supported the idea that ethylene could be related to the asymmetric distribution of auxins in tissues that respond to gravitropism, thus supporting the Cholodny-Went hypothesis (Went 1974).

A time-course experiment was performed to determine if there was a particular pattern of expression. The expression pattern of *ACO3* and *ACO5* was induced after 10 h of inclination at 45° (Fig. 3). On the other hand, *ACS* transcript accumulation was high at 2.5 h of inclination (Fig. 3), similar to the results reported by Barnes and others (2008). These observations are consistent with the sequential order for both enzymes in the ethylene biosynthesis pathway to provide the ACC substrate for ACO catalysis to produce ethylene (Yang and Hoffman 1984).

Distribution of ACC, the ethylene precursor, was studied in inclined branches of lodgepole pine. A specific localization at the bottom of the branches was found at the site where compression wood is formed (Savidge and others 1983). Based on that, qPCR analyses were performed at three different stem heights. For *ACO3* and *ACO5* transcripts a significant high expression level after 10 h of inclination was detected in radiata pine, being higher at the basal part of the stem. These results constitute

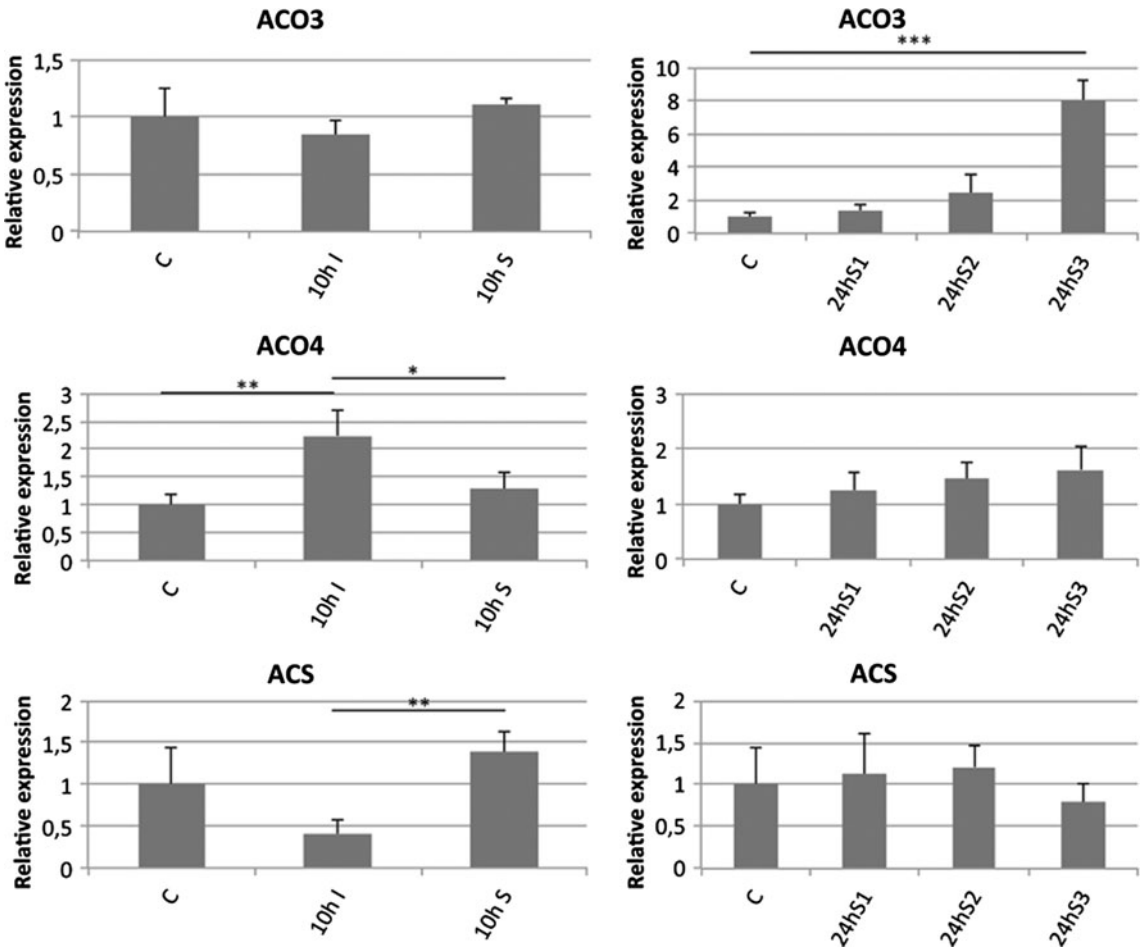

Fig. 4 Expression profiles of *ACOs* and *ACS* genes during inclination of maritime pine seedlings. Sampling was performed after 10 and 24 h of inclination. Longitudinal sampling of stems was performed after 10 h, separating superior (S) and inferior (I) halves. Transverse cuts of stem were employed at 24 h providing three stem heights. At each sampling time noninclined plants were sampled as controls. Expression values represent the ratio between the expression levels of the gene compared to housekeeping genes (*Ge066D02* and *UBC7*). Data correspond to mean ± SD of three biological replicates. *Asterisks* indicate statistical significance (**$P < 0.01$, ***$P < 0.001$, Student's *t* test)

the first report that explores the specific expression of ethylene biosynthesis genes in response to gravitropic stimuli along the stem. On the other hand, *ACO3* and *ACO5* are expressed only at the basal zone of inclined stems at 10 h, and *ACS* is expressed in the medial zone after 2.5 h of inclination, indicating spatial and temporal differential expression of the *ACS* gene, probably as an additional control point for the availability of free ACC, the substrate for ACO catalysis (Fig. 3). These observations agreed with the results of Andersson-Gunnerås and others (2003), who detected high levels of ACC in xylem sap during tension wood formation in poplar trees. The observation that ethylene biosynthesis gene expression increases in the basal portion of the stem agreed with the idea of modulation of accumulation of flavonols in segments of the stem that require an auxin differential distribution.

The expression patterns of *ACS* and *ACOs* genes were also determined in maritime pine seedlings. Analyses were done only at the time points where differences in expression were observed in inclined maritime pine seedlings. Similar expression profiles were observed in both species along the stem for *ACO3* (Fig. 2). Furthermore, *ACO4* transcripts were higher in the inferior half than in the superior half in maritime pine (Fig. 4). These results agreed with reports of gymnosperms showing the specific expression of these genes in the lower half of the stems (Le Provost and others 2003; Yuan and others 2010). Finally, the expression pattern of *ACO* in compression-associated xylem of mature maritime pine was confirmed (Fig. 5), supporting the results of previous reports on the expression of *ACO* transcripts and protein (Plomion and others 2000; Le Provost and others 2003) and validating our observations in young seedlings.

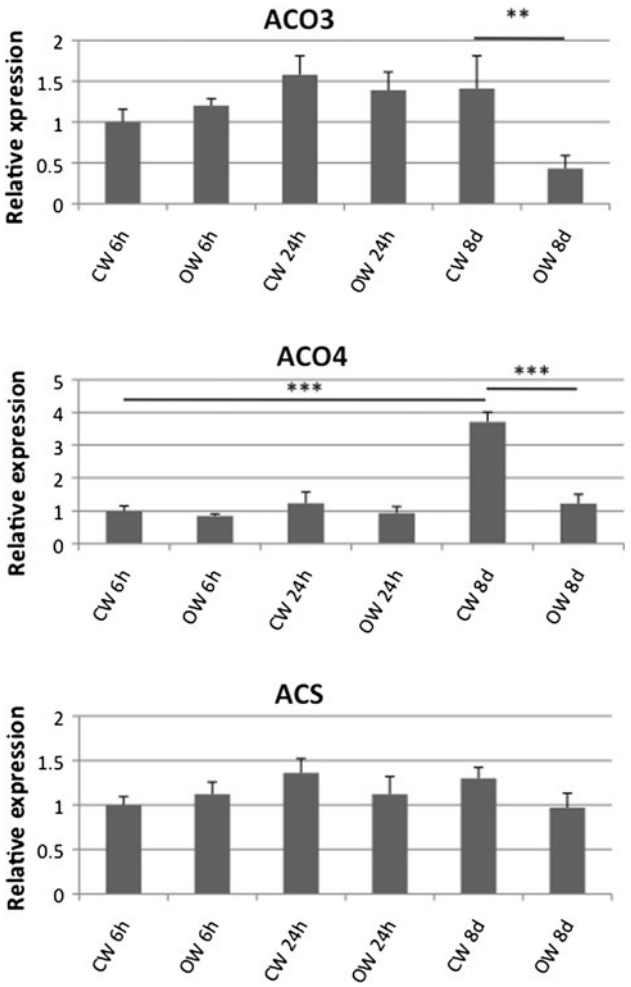

Fig. 5 Expression analysis of *ACO3*, *ACO4*, and *ACS* transcripts in xylem of compression wood (CW) and opposite wood (OW) of adult maritime pine trees. Differentiating xylem tissue was collected after 6, 24 h, and 8 days of bending. qRT-PCR was used for expression analysis of the genes. Values represent the ratio between the expression level of the corresponding gene compared to housekeeping genes (*Ge066D02* and *UBC7*). *Asterisks* indicate statistical significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t* test)

Yuan and others (2010) showed that two ACOs paralogs are present in loblolly pine, but only one was upregulated after seedling inclination. Deduced protein sequences of our partial ACOs from radiata pine showed 28% of identity with ACOs paralogs (Supplementary Fig. 1). Additionally, 50% identity to ACO protein from white spruce was found. Even with the lower percentage of identity, the sequences shared the responsible motif for the catalytic activity of ACO proteins, which was shown to be involved in metal ligation during the catalysis (Zhang and others 1997). Also, the amino acid residues that were previously described as conserved in all Fe-ascorbate oxidases are present (Tang and others 1993). In the case of PtACO1 and PpACO1, full-length protein sequences showed 47% of identity. When ACOs from radiata pine were compared to both PpACO1 and PtACO1, a 32% of identity was found. Furthermore, the transcriptional profile of *ACOs* genes showed the same expression pattern previously reported in maritime pine (Le Provost and others 2003). The low protein identity found may indicate that different ACOs are present within the pine species and could explain our findings in radiata pine where only ACO3 and ACO5 were regulated after inclination and the two different ACO3 and ACO4 were regulated in maritime pine. Moreover, only the *ACO4* transcript was upregulated in xylem from adult maritime pine, most interestingly in the trunk side where compression wood is formed.

In this report three different TCs that encoded ACO enzymes were analyzed, with two of them expressed in a temporal and spatial differential manner along the stem from both maritime and radiata pine seedlings. These observations agreed with previous reports on the promoter of two *ACOs* genes, where one was induced after inclination (Yuan and Dean 2010). Coincidently, both results suggest the existence of *ACO* isoforms, which are expressed specifically in response to inclination of stem tissue. On the other hand, *ACO* transcripts were downregulated in pine root tissue (data not shown), contrary to the upregulation observed in stems. In potato, transcripts homologous to *ACOs* were strongly expressed in leaves, contrary to the low level of expression detected in roots and tubers (Nie and others 2002). The emphasis of this report was on the differential expression of ACOs in stems, considering that the dynamic of ethylene in roots is totally different to stems.

Different studies have shown the relationship between compression wood formation and ethylene, but few have evaluated the expression pattern of the genes involved in the ethylene biosynthesis pathway during the development of the conifer gravitropic response in stems. The present work, done in young seedlings, showed a differential expression of ethylene biosynthesis genes along the stem during the gravitropic response. These results give clues about the specific space–time abundance of transcripts encoding for the ethylene biosynthetic pathway during the gravitropic response along the stem in conifer trees.

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