J Plant Growth Regul (2004) 23:44–53
DOI: 10.1007/s00344-004-0074-7

J P G R
Journal of Plant Growth Regulation

Evidence for Positive Regulation by Gibberellins and Ethylene of ACC Oxidase Expression and Activity During Transition From Dormancy to Germination in *Fagus sylvatica* L. Seeds

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Abstract

1-aminocyclopropane-1-carboxylic acid (ACC) oxidase is a key enzyme in the ethylene biosynthesis pathway. In the present report the changes in ACC content, ACC oxidase activity and ethylene production have been examined in *Fagus sylvatica* L. dormant seeds after stratification and different treatments that maintain or release dormancy. Additionally, a cDNA encoding an ACC oxidase (ACO) from *Fagus sylvatica* has been isolated and characterized. This clone, named *FsACO1*, exhibits high homology to ACC oxidases from several plant species and the corresponding enzyme, expressed in *Escherichia coli* as a fusion protein, is active in converting ACC into ethylene. The transcript levels of *FsACO1* are correlated with the ACC content, the ethylene production and the ACC oxidase activity measured in vitro as well as with the germination percentages observed in the seeds under the different treatments used in this study. There is a drastic increase in all these parameters when seeds are treated with GA_3 or ethephon (which releases ethylene in solution), hormones previously proven to be efficient in the breaking of dormancy of beech seeds. The stimulatory effect of ethephon is reverted by paclobutrazol, a well known GA biosynthesis inhibitor. These results indicate that ethylene biosynthesis is positively regulated by both gibberellins and ethylene and suggest a cross-talk regulation by these two hormones of the processes involved in the transition from seed dormancy to germination.

Key words: ACC oxidase; Ethylene; *Fagus sylvatica* L; Germination; Gibberellins; Hormone interactions; Seed dormancy

Received: 25 November 2003; accepted: 17 March 2004; Online publication: 1 June 2004
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The gaseous phytohormone ethylene acts as a signalling molecule in a large number of physiological processes accompanying plant responses to diverse stimuli throughout its life cycle, including senescence of plant organs such as fruits, leaves and flowers (Wang and others 2001), ripening of climateric fruits (Alexander and Grierson 2002), germination of some seeds (Matilla 2000), zygotic embryogenesis (Rodríguez-Gacio and Matilla 2001) or in response to mechanical wounding, pathogen invasion, drought and flooding or by auxin treatment (Voesenek and others 2003). Because ethylene regulates such diverse processes, its production in plants must be tightly regulated. In higher plants, ethylene is produced from methionine by a well - defined pathway (Bleecker and Kende 2000). Multiple genes encode key enzymes in the biosynthesis of ethylene and their transcripts are differentially regulated (Fluhr and Matoo 1996).

Formation of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), catalyzed by ACC synthase (ACS), is generally considered as the rate-limiting step of this biosynthetic pathway (Ge and others 2000; Wang and others 2002), although recent studies have shown that ACC oxidase (ACO), the enzyme that oxidizes ACC to ethylene, also plays an important role in the regulation of ethylene production in some developmental processes (Vriezen and others 1999). This enzyme is encoded by a multigene family and its expression has been proven to be differentially regulated by developmental and environmental factors (Vriezen and others 1999; Petruzelli and others 2000; Alexander and Grierson 2002). Ethylene also stimulates the ACO activity in a number of vegetative and reproductive tissues. This stimulation is correlated with the accumulation of ACO transcripts, suggesting a transcriptional regulation of these genes (Nadeau and others 1993; Drory and others 1993; Avni and others 1994; Barry and others 1996; Gómez-Jiménez and others 1998). Thus, different regulatory effects of ethylene on its own biosynthesis could occur in distinct processes.

The participation of ethylene on fruit ripening and senescence has been widely investigated, and there is also evidence for its involvement in the breaking of seed dormancy (Kepczynski and Kepczynska 1997). By using *etr1-1* and *ein2* mutants, Beaudoin and others (2000) have shown that ethylene negatively regulates dormancy in *Arabidopsis* seeds. However, the role of this hormone in the transition from seed dormancy to germination is still poorly understood.

Our research is focused on the role of ethylene and its relation to gibberellins during the breaking of dormancy and the onset of germination in *Fagus sylvatica* seeds. We have previously shown that stratification in water at 4°C and GA_3 treatment are able to break seed dormancy after 2 and 6 weeks of imbibition respectively (Nicolás and others 1996, 1997) and ethephon, a compound that releases ethylene in solution, accelerates the breaking of dormancy and enhances germination from the first week of treatment (Calvo and others 2003).

In this report, the regulation of ethylene biosynthesis has been studied during the transition from dormancy to germination in *Fagus sylvatica* L. seeds, measuring ACC content, in vitro ACO activity and ethylene production, showing evidence for the importance of gibberellins and ethylene itself, in the control of these processes. Additionally, we have isolated and characterized an ACC oxidase cDNA clone from *F. sylvatica* L. (*FsACO1*) encoding a functional ACO, and the effects of these hormonal treatments on the expression of the corresponding gene have also been examined.

Materials and Methods

Plant Material and Germination Conditions

Fagus sylvatica L. seeds (beechnuts) were obtained from the Danish State Forestry Tree Improvement Station. Seeds were dried to a moisture content of 10% and stored at −4°C in sealed jars. The pericarp was manually removed and seeds were sterilized in 1% sodium hypochlorite before imbibition in sterile water or solutions containing 700 μM ethephon (ETP), 100 μM GA_3, 10 μM paclobutrazol (PCB) or 700 μM ethephon +10 μM paclobutrazol (concentrations were optimized for these seeds in previous reports by Nicolás and others 1996 and Calvo and others 2003). Seeds were maintained in the different media at 4°C in the dark from 1 to 6 weeks.

Lawton and others (1994) reported that hydrochloric acid and phosphoric acid released during the breakdown of ethephon (Yang 1969) may result in the induction of some ethylene-induced genes in *Arabidopsis*. To check the specificity of the ethephon effects, some seeds were treated with 2 mM hydrochloric acid or 2 mM phosphoric acid in 50 mM phosphate buffer (pH 7.0) as a control (De Martins and Mariania 1999). It was confirmed that the germination percentages obtained and the transcript levels of *FsACO1* were not affected by this treatment (data not shown), inferring that the results obtained were due to the ethylene released from the ethephon.

Ethylene Determination and ACC Quantification

Ethylene production was measured in *Fagus sylvatica* seeds enclosed in an airtight chamber using the mercuric perchloride method (Abeles 1973; Akamine and Goo 1978). Briefly, 1 ml of the headspace gas was obtained with a plastic hypodermic syringe and injected into a Konik-3000-HRGC gas chromatographer (Konik, Somerset, NJ, USA) equipped with a flame ionization detector and 2 m × 4 mm glass column packed with 60–80 mesh Poropack-Q. The oven temperature was 60°C and the N_2, H_2 and O_2 flow rates were 35 ml/min, 30 ml/min and 300 ml/min, respectively. Ethylene identification was based on the retention time compared with standard C_2H_4 (purity 99.8%).

For ACC quantification, frozen seeds (1 g) were homogenized in 2.5 ml of 70% (v/v) ethanol at 4°C. The extracts were centrifuged at 12,000 × g for 10 min and the ethanol was fully removed from supernatants using a Speed Vac (Thermo Savant, Colin Drive Holbrook, NY, USA). Residues were taken up in distilled water and ACC contents were determined as described by Boller and others (1979) using the protocol of Lizada and Yang (1979). The ACC conversion efficiency of each sample was calibrated by adding a known amount of ACC as internal standard.

Extraction and Activity Assay of ACO

ACO was extracted using the method described by Ververidis and John (1991) with some modifications. Plant material (2 g) was ground in liquid nitrogen using a mortar and pestle and homogenized with 2 ml of extraction buffer (Vriezen and others 1999). The homogenate was centrifuged at 15,000 × g for 10 min at 4°C. The supernatant was used for the in vitro ACO assays using gas chromatography to measure the ethylene produced after incubation in the dark for 1 h at 30°C in capped 10-ml vials in a reaction mixture (Vriezen and others 1999). Reactions without enzyme or with boiled supernatant acted as controls, and in both cases, no ethylene production was detected. The protein concentration was determined following the Bradford's method (1976), using BSA as standard.

cDNA Synthesis, RT-PCR and Cloning of PCR Products

Poly(A^+) RNA was purified from total RNA by affinity chromatography in oligo(dT)-cellulose columns using the mRNA Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). cDNA was synthesized from 1 μg of poly(A^+) RNA obtained from ethephon-treated seeds using Superscript 1st Strand Synthesis kit for RT-PCR (Invitrogen, Carlsbad, CA, USA) and oligo-p(dT) as a primer, following the manufacturer's instructions.

This cDNA served as a template to isolate an ACC oxidase DNA fragment by PCR, using a pair of degenerate primers based on regions found to be highly conserved in other ACOs described in plants (ACO1: 5′-GA(C,T)GC(A,T,C,G)TG(C,T)(C,G)A(A,G)AA(C,T)TG GGG(A,T,C,G)TT-3′ and ACO2: 5′-CTTCAT(A,T,C,G)GC(C,T)TC(A,G)AA(A,T,C,G)C(G,T) (A,T,C,G)GGCTC-3′). The PCR reaction was performed as follows: 94°C for 5 min, 40 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for a min and a further 10 min at 72°C. The amplification products with the expected size (840 bp) were isolated from 1% agarose gel, cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced.

Isolation of the Full-length cDNA Clone

A cDNA library constructed in the Uni-ZAP XR vector (Stratagene, La Jolla, CA, USA) using poly(A^+) RNA from beech seeds as a template (Nicolás and others 1997), was screened with the PCR fragments as a probe. The recombinant cDNA clone was obtained and the full-length FsACO1 cDNA was excised from the phage in pBluescript SK(+) using the biological rescue recommended by the supplier (Stratagene).

DNA Sequencing

Plasmid DNA templates were isolated by the Wizard Plus Minipreps DNA Purification System (Promega Corporation, Madison, WI, USA). The nucleotide sequence of the cDNA clone was determined on an ABI 377 sequencer (Applied Biosystems Inc., Foster City, CA, USA) using the Taq DyeDeoxy™ Terminator Cycle Sequencing kit. The DNA and deduced protein sequences were compared to other sequences in the EMBL (GenBank and SwissProt databases, respectively), using the FASTA algorithm (Pearson and Lipman 1988).

Nucleic Acid Analysis

Total RNA from seeds was extracted using the Qiagen pack-500 cartridge (Qiagen, Valencia, CA, USA) following the manufacturer's protocol and analyzed by Northern blot following the same procedures described in Nicolás and others (1997). Blotted membranes were hybridized with a specific

^{32}P-labelled probe made from the 3′ noncoding region of the *FsACO1* cDNA clone to avoid a cross-hybridization with other ACC oxidases (Southern blot analysis suggested the existence of a multigene family in *F. sylvatica*, but when this *FsACO1* fragment was used as a probe the Southern displayed only one band; data not shown). Membranes were processed as described in Lorenzo and others (2001) and exposed to X-Omat films (Kodak), and the autoradiograms were quantified in an Image Analyzer Bioimage 60S (Millipore, Iberica SA, Madrid, Spain, Software Visage 4.6 K). Blots were repeated 3 to 4 times and the SD of quantified values was never higher than 5%.

Heterologous Expression of FsACO1 in *E. coli*

A cDNA fragment spanning the entire FsACO1 open reading frame was PCR-generated using the following primers: 5′-primer containing the *Nde* I site (5′-*CAT***ATG**GAGAACTTCCCAGTG-3′ the *Nde* I site is underlined and the ATG is in boldface) and 3′-primer containing the *Xho* I site (5′-*CTCGAG* **TTA**AGCGGTTGCAAT-3′; the *Xho* I site is underlined and the stop codon is in boldface). The PCR product was digested with *Nde* I and *Xho* I, and the fragment was inserted in frame into the *Nde* I and *Xho* I sites of the pET28a(+) vector (Novagen, Inc., Madison, WI, USA) and verified by DNA sequencing. *FsACO1* protein was expressed in *E. coli* BL21(DE3) as a histidine tag fusion protein, cells carrying the recombinant plasmid were grown at 30°C in 2x YT until A_{600} reached 0.6 units, and recombinant protein was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were harvested by centrifugation at 5000 × g for 5 min after 5 hours of induction and lysed using Bug Buster protein extraction reagent (Novagen). Proteins were analyzed by SDS-PAGE and Coomassie Blue staining and the ACO activity was assayed by measuring the ethylene production in the soluble phase of the heterologous protein expression using gas chromatography as described before.

Results

ACC Content, ACC Oxidase Activity and Ethylene Production During Stratification at 4°C of *F. sylvatica* Seeds Under Different Treatments

The ACC content (Figure 1A) as well as the ACC oxidase activity (Figure 1B) and the ethylene production (Figure 1C) were determined in stratified *Fagus sylvatica* seeds subjected to different treatments. The ACC content is high from the starting point of seed imbibition in water and progressively decreases along with the imbibition time. The ethylene production slightly increased after 2 weeks of stratification, although no significant changes were observed in ACC oxidase activity during the period studied. The addition of ethephon produced a drastic increase in ACC content, ACC oxidase activity and ethylene production compared with water as a control, pointing to a positive regulation of this biosynthetic pathway by ethylene. Additionally, exogenous application of GA_3 induced a clear increase in ACC content, ACC oxidase activity and ethylene biosynthesis, displaying a sustained increase in ethylene production through the first 4 weeks of imbibition (Figure 1C). Interestingly, the addition of paclobutrazol an inhibitor of GA biosynthesis, reverses the effect of ethephon in all measured parameters (Figure 1), suggesting an important role for gibberellins in the biosynthesis of ethylene.

Isolation and Characterization of a cDNA Clone from *Fagus sylvatica* L. Seeds Encoding an ACC Oxidase

Total RNA from ethephon-treated seeds was used for RT-PCR amplification using degenerated oligonucleotides corresponding to conserved sequences among ACC oxidases. A cDNA fragment of 840 bp, encoding a partial gene product with homology to ACC oxidases, was isolated and characterized. This cDNA fragment was used as a probe to screen a cDNA library constructed from mRNA of beech seeds (Nicolás and others 1997) and the corresponding full-length clone was obtained and registered in the EMBL and GeneBank Nucleotide Sequences Databases under the accession number AJ420189. The isolated cDNA clone was 1284 bp long and contained an ORF of 957 bp, which agrees with the mRNA size determined by Northern blot (1.3 kb approximately). The deduced protein had 319 amino acids (Figure 2) with a predicted molecular mass of 36 kDa. ACC oxidases are members of the family of 2-oxoglutarate-dependent dioxygenases. Accordingly, this protein contains 12 putative Fe^{2+}-binding amino acids (Roach and others 1995).

Comparison of the deduced amino acid sequence with EMBL databases revealed high identity with different proteins of the ACC oxidase family (Figure 2), hence, this clone was named *FsACO1*. The highest identity was found with ACC oxidases from

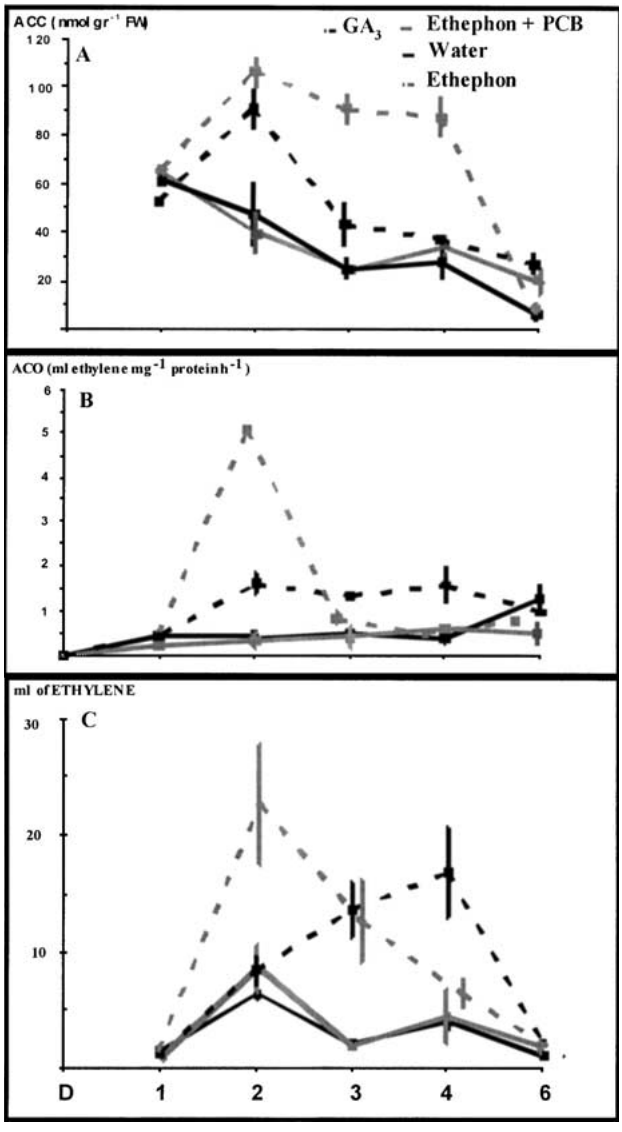

Figure 1. Changes in the final step of ethylene biosynthesis (A) ACC content, (B) ACC oxidase activity, (C) ethylene production during stratification at 4°C of *F. sylvatica* seeds treated with 700 μM ethephon, 100 μM GA_3, 700 μM ethephon plus 10 μM paclobutrazol and water. Data are mean of 3 different experiments ± SD.

Prunus persica (85.5%), *Prunus mume* (85.2%) and *Pelargonium hortorum* (84.6%).

Expression and Activity of *FsACO1* Recombinant Protein in *E. coli*

It has been previously shown that ACC oxidase cDNA clones from different species produce catalytically active enzymes when expressed as fusion proteins in *E. coli* (Zhang and others 1995; Brunhuber and others 2000; Rodríguez-Gacio and others 2004. The high degree of similarity between the amino acid sequences of the *FsACO1* protein and other ACC oxidases suggested that it may encode an enzyme of this type. To assess that *FsACO1* protein indeed had ACC oxidase activity, the coding fragment of this clone was expressed in an *E. coli* heterologous system using the pET28a(+) as the expression vector. After induction of the bacterial *E. coli* strain BL21(DE3) with IPTG, a protein band with an electrophoretic mobility in SDS/PAGE of 36 kDa (Figure 3A) was detected. Most of the expressed recombinant protein was found in the soluble phase, in which in vitro ACO activity was proved by assaying its ability to convert ACC into ethylene (Figure 3B).

Transcript Accumulation of *FsACO1* Gene

Northern blot analysis of total RNA hybridized with a probe from the *FsACO1* cDNA clone (Figure 4A), showed that these transcripts were almost undetectable in dry dormant seeds and slightly increased upon seed hydration at 4°C in water. After the addition of ethephon or GA_3, treatments proved to be efficient in the breaking of dormancy in beech seeds, the expression of this mRNA transcript clearly increased from the first week of treatment, specially in ethephon, correlating with the germination percentages obtained after these hormonal treatments (Figure 4B). Furthermore, when the seeds were imbibed in PCB, a well-known GA biosynthesis inhibitor, no expression was detected, and when PCB was added together with ethephon, treatment that produces germination percentages similar to those observed during stratification in water, the level of expression of *FsACO1* decreased to the same basal levels observed in the control in water (Figure 4), showing again evidence for a positive regulation of this gene by gibberellins and ethylene in *F. sylvatica* seeds.

Discussion

Fagus sylvatica L. seeds exhibit embryo dormancy that is eliminated by cold treatment at 4°C over a period longer than 6 weeks, and application of GA_3 or ethephon has proved to be efficient in releasing beechnuts from dormancy and in substituting for cold treatment, allowing seed germination (Nicolás and others 1996; Calvo and others 2003). In addition, the GA biosynthesis inhibitor, paclobutrazol, and the ethylene biosynthesis inhibitor, AOA, both maintain *Fagus sylvatica* L. seeds in the dormant state, but GA_3 is able to counteract the inhibitory effect of AOA on seed germination and ethephon

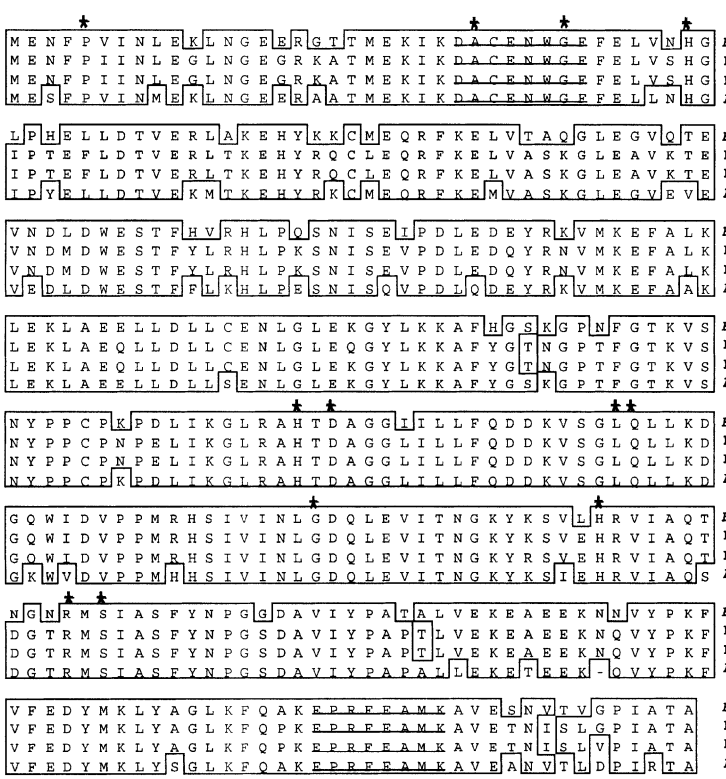

Figure 2. Alignment of the derived amino acid sequences from *FsACO1* with ACC oxidases from *Prunus persica* (accession number: X77232), *Prunus mume* (accession number: AB031027) and *Pelargonium hortorum* (accession number: U07953). The putative Fe^{2+}-binding residues are indicated with asterisks. The oligonucleotides used in the RT-PCR are underlined.

also reverses the effect of PCB (Nicolás and others 1997; Calvo and others 2003), which suggests some kind of interaction between GAs and ethylene in the regulation of seed dormancy/germination. In view of this premise, the aim of this work was to investigate the role of ethylene biosynthesis in breaking the dormancy as well as the possible involvement of GAs in the regulation of this process by ethylene.

Ethylene has been implicated in the breaking of dormancy in species such as peanut, apple or cocklebur (Kepczynski and Kepczynska 1997) and also some seeds have been shown to synthesize ethylene at the begining of seed germination (Matilla 2000). Ethylene can also fully rescue the germination defect of the *Arabidopsis* GA-deficient mutant *ga-1* (Karssen and others 1989; Koornneef and Karssen 1994), suggesting that some components of ethylene signalling are involved in the effects of GAs on this process.

The genetic mechanisms of ethylene action in breaking dormancy/onset of germination have not been elucidated and according to Brady and McCourt (2003), it would be interesting to determine if this hormone stimulates germination by altering GA biosynthesis or sensitivity, or if it acts through its own production or signalling components. Recently, Achard and others (2003) reported that at least part of the growth regulatory action of ethylene is mediated via its effects on the DELLA proteins, which act as repressors of growth in response to ethylene, auxin and GA signals.

In a previous report we showed that GA 20-oxidase gene expression is downregulated by GAs and ethylene, and suggested a cross-talk regulation by these two hormones during the transition from seed dormancy to germination (Calvo and others 2004).

Here we investigate the regulation of ACC oxidase and ethylene production by ethylene and GAs in relation to onset of germination in *F. sylvatica* dormant seeds. Our results show that in water-stratified seeds, ACO activity is low throughout imbibition, whereas ACC content is quite high and ethylene production slightly increases after 2 weeks of imbibition (Figure 1). These results correlate with the slow release from dormancy produced by stratification (Figure 4B) and suggest that ethylene has a role in the processes and that ACO activity is high enough to slowly increase ethylene levels during the first weeks of imbibition. The addition of ethephon (Figure 1) brings about a drastic increase in ACC content, ACC oxidase activity and ethylene production, pointing to a positive regulation of this biosynthetic pathway by ethylene and indicating again its involvement in the breaking of dormancy, especially considering the rapid increase in germination percentages under this treatment (Figure 4B). This positive regulation has also been

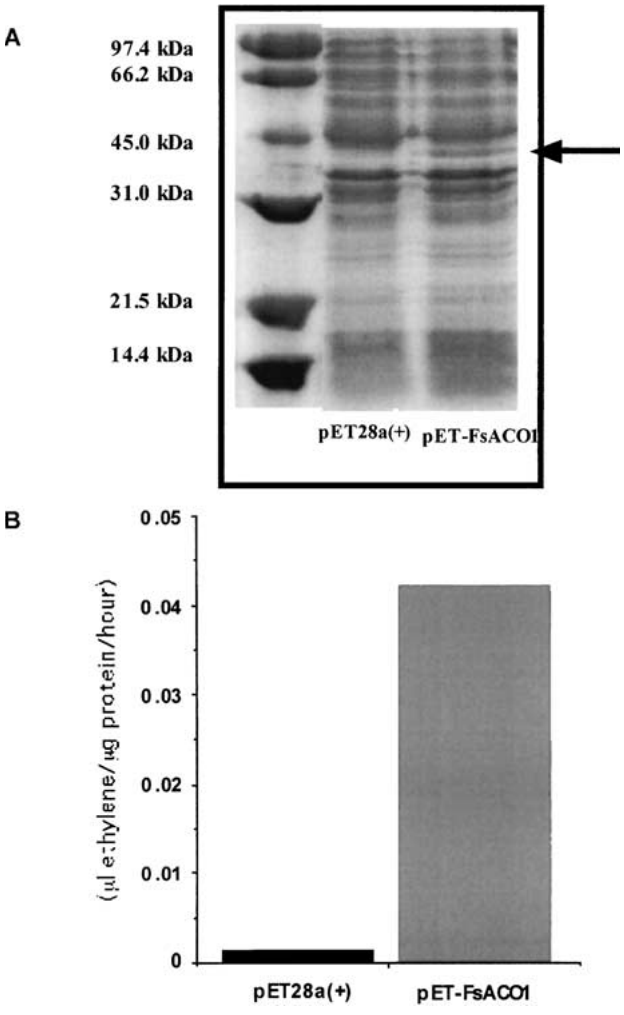

Figure 3. (A) Expression of the fusion protein pET-FsACO1. Soluble extracts of *E. coli* transformed with pET-FsACO1 or pET28a(+) were resolved by 12% (w/v) SDS-PAGE and stained with Coomassie Brilliant Blue. Position of the fusion protein is indicated with an arrow. (B) The in vitro ACO activity of recombinant FsACO1.

observed in pea seeds (Petruzelli and others 2000) as well as in seedlings, fruits and other vegetative tissues (Fluhr and Mattoo 1996; Johnson and Ecker 1998). Furthermore, exogenous application of GA_3 also produced a clear increase in ACC content, ACC oxidase activity and ethylene biosynthesis (Figure 1), although the levels were not as high as those obtained in treatment with ethephon, probably due to the wide and strong inductive effect of ethylene on its own synthesis. Moreover, we previously showed that AOA, an ethylene biosynthesis inhibitor, reduced the germination percentages in beech seeds during stratification, although its action was not as effective as PCB in the maintenance of seed dormancy (Calvo and others 2004), probably because AOA could not fully inhibit ethylene biosynthesis in the seed (Kepczynski and Kepczynska 1997).

In the present work, the addition of the GA biosynthesis inhibitor paclobutrazol (PCB) reverses the effect of ethephon in all measured parameters, indicating a relationship between GAs and ethylene biosynthesis. This was suggested for other species (Abeles and others 1992) and shows that endogenous GAs are necessary for ethylene production in beech seeds, although the mechanism involved in the interaction between these two hormones is still unknown.

To continue the study of this interesting subject on a more in depth level, we have attempted the molecular characterization and genetic regulation of ACO in beechnuts. Thus, we have isolated an ACO cDNA clone, *FsACO1*, and provide evidence that it encodes a functional ACC oxidase. *FsACO1* shows high identity with several proteins of the ACC oxidase family (Figure 2) and the coding fragment of this cDNA clone, expressed in *E. coli* as a fusion protein, shows ACC oxidase activity (Figure 3).

The expression analysis of this gene (Figure 4A) reveals that *FsACO1* transcripts are undetectable in dry dormant seeds and increase upon rehydration, although they remain low during the first 6 weeks of imbibition in water-stratified seeds, which indicates that low levels of ethylene production are required to slowly break dormancy (Figure 4B), just as observed in Figure 1. The addition of either GA_3 or ethephon, treatments that produce rapid dormancy release (Figure 4B), increased transcript levels of this gene from the first weeks of treatment, and gradually decreased to basal levels as the imbibition proceeded. *FsACO1* transcripts were also undetectable when GA biosynthesis was inhibited by the addition of PCB, a treatment that blocks seed germination. However, the joint addition of ethephon and PCB counteracted the stimulatory effect of ethephon in the expression of *FsACO1* transcripts, which supports a positive regulation of *FsACO1* expression by both hormones, although regulation at the post-transcriptional level cannot be discarded. This result additionally shows the importance of GA synthesis for the expression of this ethylene-regulated gene during the transition from dormancy to germination in beechnuts (Figure 4).

Although ACC synthase (ACS) is believed to be the key enzyme in regulating ethylene synthesis in different tissues (Yang and Hoffman 1984; Fluhr and Mattoo 1996), our results suggest that the transcriptional regulation of the *FsACO1* gene plays a major role in regulating ethylene production in beechnuts because the levels of ACS transcripts are

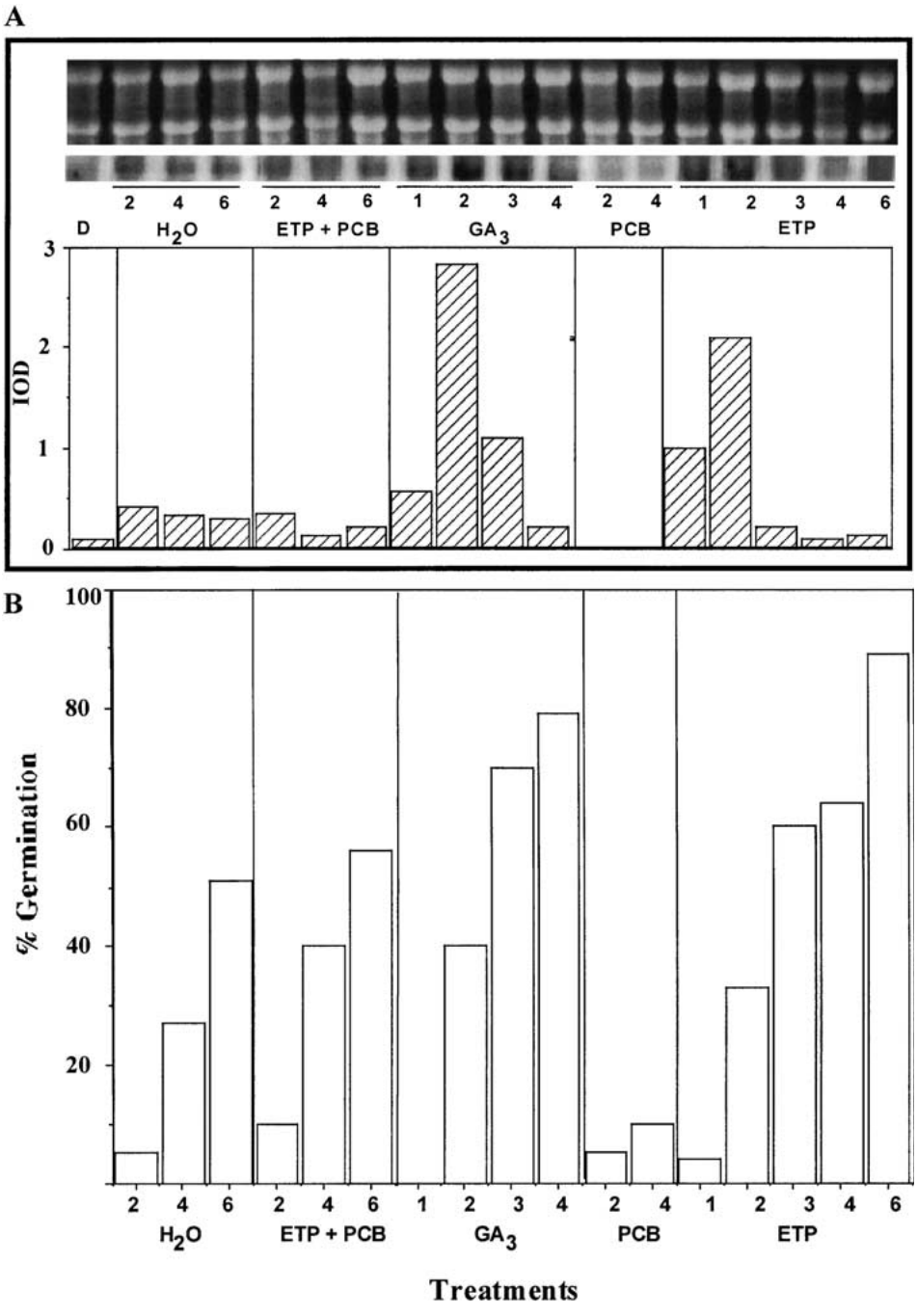

Figure 4. (A) Northern blot analysis of total RNA isolated from *F. sylvatica* dormant seeds (D) and seeds imbibed at 4°C in water, 700 μM ethephon plus 10 μM paclobutrazol, 100 μM GA_3, 10 μM Paclobutrazol and 700 μM ethephon. Ten μg RNA were used per lane and hybridized with *FsACO1* cDNA probe. Top panel: stained gel showing RNAs. Bottom panel: signals quantification (IOD: integrated optical density; arbitrary units). (B) Germination percentages of beechnut seeds obtained after the indicated treatments. The numbers indicate weeks of imbibition.

similar under all the treatments used (Calvo and others unpublished data).

Furthermore, the results of the *FsACO1* expression (Figure 4A) agree with the in vitro ACC oxidase activity, ACC content, and ethylene production measured under the different treatments used in this study (Figure 1) and also correlate with the seed germination percentages (Figure 4B).

In summary, all these results indicate the importance of ethylene and GAs in the regulation of ethylene biosynthesis during the breaking of dormancy/onset of germination in *F. sylvatica* L. seeds, and the existence of some type of cross-talk regulation of the *FsACO1* gene by these two hormones, as we previously proved in the regulation of a GA 20-oxidase gene, a key enzyme in the GAs biosynthetic pathway (Calvo and others 2004). However, further studies are required to elucidate the mechanisms of ethylene and GAs interaction during the transition from seed dormancy to germination.

ACKNOWLEDGMENTS

This work was supported by grants BFI2000-1361 from the Ministerio de Ciencia y Tecnología (Spain) and SA010/02 from Junta de Castilla y León.

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