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A protein phosphatase 2A from *Fagus sylvatica* is regulated by GA_3 and okadaic acid in seeds and related to the transition from dormancy to germination

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Several gibberellic acid (GA_3)-induced cDNA fragments encoding putative serine/threonine protein phosphatase (PP) 2A catalytic subunits were obtained by means of differential reverse transcriptase-PCR approach. The full-length clone, named FsPP2A1, isolated from a beechnut cDNA library, exhibited all the features of and homology to members of the PP2A family. By transient expression of FsPP2A1 in tobacco and Arabidopsis cells as a green fluorescent fusion protein, we have obtained evidence supporting the subcellular localization of this protein in both the cytosol and the nucleus. Analysis of FsPP2A1 expression during seed stratification shows that these transcripts increase in the presence of GA_3, a treatment proved to be efficient in breaking the dormancy of *Fagus sylvatica* seeds, but they are almost undetectable in dormant seeds or when dormancy is maintained after treatment with either abscisic acid or the gibberellin biosynthesis inhibitor paclobutrazol. The PP inhibitor okadaic acid (OKA) has a clear effect in decreasing both seed germination and FsPP2A1 expression. Furthermore, FsPP2A1 is specifically expressed in seed tissues, not being detected in other vegetative tissues examined. These results show the regulation of this PP by GA_3 and OKA in these seeds. Its relationship with the processes taking place during the transition from dormancy to germination is also discussed.

Introduction

Reversible protein phosphorylation is accepted as a major mechanism for the control of a variety of plant processes, including responses to hormones, pathogens and environmental stress (Smith and Walker 1996). This process is known to be mediated by protein kinases and protein phosphatases (PP), which constitute a significant proportion within the Arabidopsis genome. Several plant protein kinases have been isolated and functionally characterized; in contrast, information about PPs in higher plants is still limited (Chang et al. 1999, reviewed by Luan 2003). Using biochemical criteria, substrate specificity and dependence on divalent cations, the serine/threonine (ser/thr) PPs have been divided in four types: PP1, PP2A, PP2B and PP2C.

Research on PP2A is now emerging in plants, although the presence of phosphatase activities very similar to the mammalian PP type-2A had been previously reported (McKintosh and Cohen 1989, McKintosh et al. 1990, Jagiello et al. 1992). The PP2As are heterotrimeric proteins inhibited by okadaic acid (OKA), with a catalytic subunit (C) and two regulatory subunits (A and B) that modulate enzyme activity and mediate interactions with other proteins. PP2A catalytic

Abbreviations – GFP, green fluorescent protein; OKA, okadaic acid; ORF, open reading frame; PCB, paclobutrazol; PP, protein phosphatase; RT-PCR, reverse transcriptase-PCR.

subunit cDNAs have been isolated from various plant species, such as *Brassica napus* (McKintosh et al. 1990), *Arabidopsis thaliana* (Ariño et al. 1993, Casamayor et al. 1994), alfalfa (Pirck et al. 1993) or rice (Chang et al. 1999). Recently, the genes for PP2A regulatory A and B subunits have also been identified in Arabidopsis (Zhou et al. 2004, Camilleri et al. 2002) revealing important functions of PP2A regulation in all phases of plant growth and development.

By genetic analysis in Arabidopsis, a role for PP2A in hormone-mediated plant growth regulation has been demonstrated, including the control of plant morphology, developmental processes and hormonal signal transduction pathways (Rashotte et al. 2001, Camilleri et al. 2002, Kwak et al. 2002, Larsen and Cancel 2003). This is the case of roots curl in naphthylphthalamic acid-1 (RCN-1), encoding the subunit A of PP2As. The *RCN1* Arabidopsis mutant exhibits defects in root curling, hypocotyl hook formation, the polar transport of auxins and abscisic acid (ABA) responses (reviewed by Muday and DeLong 2001, Kwak et al. 2002). Additionally, after a systematic analysis in Arabidopsis, two A subunit isoforms (PP2AA2 and PP2AA3) have been identified, with overlapping functions but highlighting the pivotal significance of RCN1 (Zhou et al. 2004). Furthermore, the tonneau-2 (TON-2) protein shares homology with the B subunit of PP2A and is involved in cytoskeleton control in plants (Camilleri et al. 2002). The mutants *ton2/fass/gordo* show higher auxin content and ethylene production displaying significant alterations in morphogenesis (Torres-Ruiz and Jürgens 1994, Fisher et al. 1996).

Because the plant hormone gibberellin (GA) plays an essential role in plant growth and development, several genes and proteins regulated by this hormone have been identified. In the past years, Chang et al. (1999) have proved that ser/thr PP1 and PP2A may be involved in the germination of rice seeds. Kuo et al. (1996) proposed that GA binding to the receptor leads to activation of PP1 or PP2A, which alters the levels of cytosolic Ca^{2+} (Bush 1996), inducing the expression of GA-response genes.

Moreover, many responses regulated by GAs in wheat aleurone cells are suppressed by OKA, a specific inhibitor of PP1 and PP2A activity, that is changes in cytosolic Ca^{2+} levels, induction of hydrolitic enzymes, changes in gene expression and cell death (Jones and Jacobsen 1991, Kuo et al. 1996).

Our work is focused on the mechanism of gibberellic acid (GA_3) action during the transition from dormancy to germination in *Fagus sylvatica* seeds and in the expression of specific genes involved in this process. Although *Fagus* lacks the genetic tractability of Arabidopsis, it represents a suitable model to study seed dormancy of woody plants where little is known about the mechanisms controlling this process. We have shown that GA_3 produces a fast release from dormancy and increases the percentages of germination from the first week of treatment, while in the presence of ABA or the GA biosynthesis inhibitor paclobutrazol (PCB), the percentages of germination are scarce (Nicolás et al. 1996, 1997).

Here, we report the inhibitory effect of OKA treatment in beechnuts germination assays and the isolation and characterization of a full-length cDNA, FsPP2A1, encoding a ser/thr PP2A catalytic subunit. The corresponding transcripts are upregulated by GA_3 and downregulated by ABA, PCB and OKA treatments, which correlate with the release of dormancy/onset of germination in beechnuts. In addition, we provide evidence for FsPP2A1 representing a seed-specific PP localized both in the cytoplasm and in the nucleus within the cell.

Materials and methods

Plant material and germination conditions

F. 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 previously sterilized in 1% sodium hypoclorite before imbibition in sterile water containing 100 μM GA_3, 100 nM OKA, 100 nM OKA + 100 μM GA_3, 100 μM ABA or 10 μM PCB. Seeds were maintained in the different media at 4°C from 1 to 6 weeks.

F. sylvatica L. seedlings were obtained from 4-week-stratified seeds sown in a controlled environment chamber under a 12-h-light and 12-h-dark cycle at 15°C to 100 μE m^{-2} s^{-1} in moist vermiculite and harvested after 6 weeks. Then, they were separated into roots, leaves and stems. All collected tissues were frozen in liquid nitrogen and stored at −80°C.

Differential RT-PCR approach

The differential reverse transcriptase-PCR (RT-PCR) approach was basically performed as described in Lorenzo et al. (2001) with the following modifications. Briefly, total RNA from either GA_3-treated or ABA-treated seeds was extracted, and poly(A^+) RNA was purified from each preparation and used for cDNA synthesis. Each cDNA was used as a template for a PCR reaction with degenerate oligonucleotides corresponding to two subdomains conserved among the ser/thr PPs type 1 and

2A catalytic subunits (Ariño et al. 1993). The forward primer consisted of a 20-mer of the sequence 5′-GA(C,G)(C,A,T)T(G,C)(C,T)T(A,G,C)TGGTC(A,T,G)GA(T,C)CC-3′ encoding the DLLWSDP amino acid sequence, and the reverse primer was a 17-mer of the sequence 5′-(C,T)(C,A)(G,A)CA(G,A)TA(G,A)TT(G,T)GG(N)GC-3′ corresponding to the APNYCY amino acid sequence. PCR products were fractionated, and several DNA bands were amplified using as a template cDNA prepared from GA_3-treated seeds. In contrast, these DNA bands were absent when cDNA prepared from ABA-treated seeds was used as a template. A band of approximately 211 bp was excised from the gel, reamplified under the same PCR conditions, cloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced. As the predicted gene product encoded by this clone revealed homology to PP2A, it was named FsPP2A1.

Isolation of the FsPP2A1 cDNA clone

The full-length FsPP2A1 cDNA clone was isolated from a cDNA library constructed in the Uni-ZAP XR vector (Stratagene, La Jolla, CA) using poly(A^+) RNA from *F. sylvatica* seeds (Nicolás et al. 1997) with FsPP2A1 PCR fragment as probe. The recombinant cDNA of the clone selected for further analysis 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, Madison, WI). Determination of the nucleotide sequence of the cDNA clones was performed on an ABI 377 sequencer (Applied Biosystems, Inc., Foster City, CA) using the Taq DyeDeoxy™ Terminator Cycle Sequencing kit. The DNA and deduced protein sequences were compared with other sequences in the EMBL databases (GenBank and SwissProt, respectively), using the FASTA algorithm (Pearson and Lipman 1988).

GFP fusions and transient expression assays

A PCR fragment corresponding to the coding region of FsPP2A1 was obtained, and the green fluorescent protein (GFP) fused to the C-terminal end in the *Nco*I site of the vectors pMON30063 (Pang et al. 1996) and pPK100 using the following primers: forward primer, 5′-CTGAAAA*CCATGG*GGTCGCAC-3′; reverse primer, 5′-ATACGA*CCATGG*AAAAAATA-3′. Additionally, an N-terminal fusion construct was performed in the *Eco*RI site of the vector pMON30063 using the forward primer 5′-AGACTG*GAATTC*ATGCCGT-3′ and 5′-CAGGTAG*GAATTC*GAAAA-3′ as reverse primer.

Following sequence verification of the inserts by DNA sequencing, GFP-fusion and control constructs were transiently expressed by particle bombardment into tobacco BY2 (Bright Yellow 2) and Arabidopsis T87 cells. DNA absorption to gold particles and bombardment using a helium-driven particle accelerator (PDS-1000/He; BIO-RAD) were performed according to the manufacturer's recommendations. Five micrograms of plasmid were used per transformation, and all target materials were bombarded twice.

Fluorescence microscopy

The fluorescence photographs of cells expressing the GFP reporter gene under control of the 35S promoter were taken using a Zeiss Axiovert 200 confocal microscope and Bio-Rad Radiance 2100 laser scanning confocal imaging system with LaserSharp v.5 Image software acquisition. For GFP detection, the excitation source was an argon ion laser at 488 nm, and detection filters ranged from 515 to 530 nm.

Nucleic acid analysis

Genomic DNA was extracted using the Plant DNA Isolation kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's protocol. For Southern blot analysis, genomic DNA (10 μg) from *F. sylvatica* was digested with *Hin*dIII or *Eco*RI (both enzymes do not cut inside the FsPP2A1 genomic sequence), fractionated on 1% agarose gels and blotted onto Hybond-N nylon membranes (Amersham, Buckinghamshire, UK) according to the manufacturer's protocol. Blots were hybridized with the FsPP2A1 probe labelled with ^{32}P using the Random primed kit (Roche Diagnostics) by overnight incubation at 65°C in ×5 saline sodium citrate, SSC (×1 SSC is 0.15 M NaCl, 15 mM tri-sodium citrate), 1% sodium dodecyl sulfate (SDS) and ×5 Denhardt's solution [×1 Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll 400 and 0.02% polyvinylpyrrolidone]. Membranes were then washed at 65°C twice with ×2 SSC, 0.1% SDS for 5 min each and once with ×1 SSC, 0.1% SDS for 10 min and exposed to autoradiographic films (X-Omat, Kodak, Rochester, NY).

Total RNA was extracted from different tissues of seeds and seedlings using the Qiagen pack-500 cartridge (Qiagen, valencia, CA) following the manufacturer's protocol. In Northern blot analysis, 10 μg of total RNA were fractionated in denaturing formaldehyde agarose gels, transferred to nylon membranes (Hybond N, Amersham) and hybridized with a specific ^{32}P-labelled

probe obtained from the 3′ non-coding region of FsPP2A1 cDNA clone to avoid cross hybridization with other PP2As from *F. sylvatica*, as previously described (Lorenzo et al. 2001). Membranes were then exposed to X-Omat films (Kodak).

Results

Effect of OKA on seed dormancy and germination in *F. sylvatica*

In an effort to identify PPs type-2A among the components of the transduction pathways involved in seed dormancy and germination, we have investigated the effect of OKA, a ser/thr PP2A inhibitor, on the germination ability of *F. sylvatica* seeds. This study has also addressed the involvement of PP2As in the hormonal regulation of these processes.

Previously, Nicolás et al. (1997) reported that ABA maintains beechnuts dormancy while GA_3 is able to release it. As summarized in Table 1, using a different batch of seeds, our data corroborated those results, the germination percentages being 4% and 89% after 6 weeks of imbibition in the presence of ABA and GA_3, respectively.

The exogenous application of OKA (100 n*M*) clearly decreased the germination percentages of stratified seeds to approximately one-third from 3 weeks onwards (Table 1), pointing to an important role of these type of PPs in the processes controlling seed dormancy/germination. This effect observed after the addition of OKA resembled that obtained after the inhibition of GAs biosynthesis with PCB. The addition of OKA together with GA_3 produced a remarkable reduction in the germination percentage as compared with the treatment with GA_3 alone, but only during the first 4 weeks of imbibition, reaching similar values at the end of the studied period.

Isolation and identification of a cDNA clone from *F. sylvatica* seeds coding for type-1/2A PP

Serine/threonine PPs are key elements in ABA- and GA-signal transduction (Campalans et al. 1999, Chang et al. 1999, González-García et al. 2003, Sáez et al. 2004) and could play a crucial role as regulators of seed dormancy and germination. In this work, we attempted to identify PPs induced by GA_3, putatively involved in GA-signal transduction and related to the breaking of dormancy/onset of germination.

By means of differential RT-PCR approach using mRNA extracted from either GA_3-treated or ABA-treated seeds, and degenerate oligonucleotides corresponding to two motifs conserved among ser/thr PPs type 1 and 2A (DLLWSDP and APNYCY) (Ariño et al. 1993), some cDNA fragments encoding partial gene products with homology to PP2A were selected. These fragments were abundant in the cDNAs from GA_3-treated seeds but absent in cDNAs from ABA-treated seeds. Using one of these fragments as a probe to screen a cDNA library constructed from mRNA of *F. sylvatica* seeds (Nicolás et al. 1997), a full-length clone, named FsPP2A1, was isolated and submitted to the EMBL Nucleotide Sequence Database (accession number, AJ298829). This clone contained 1496 bp and an ORF of 921-bp-long. The deduced sequence of the corresponding protein had 306 amino acids in length, a predicted molecular mass of 35.15 kDa and contained the active site conserved in all the ser/thr PPs (in the position 53-115) (Fig. 1). FsPP2A1 shared high percent sequence similarity with the catalytic (C) subunit of plant type 2A PPs such as VfPP2A (97% identity) from *Vicia faba* (accession number AB039916), HbPP2A (96.4%) from *Hevea brasiliensis* (accession number, AF107464) and AtPP2A-1 (94.4% identity) from *A. thaliana* (At1g10430) (Fig. 1).

Table 1. Effect of okadaic acid (OKA) on the percentages of germination of *Fagus sylvatica* dormant seeds imbibed at 4°C under different treatments for the indicated periods of time (weeks). Data (±SD) are means of three experiments with approximately 100 seeds.

	Germination time (weeks)				
Treatments	1	2	3	4	6
H_2O	0%	5 ± 0.7%	17 ± 4%	27 ± 3.2%	51 ± 4.5%
ABA (100 μ*M*)	0%	0%	0%	1 ± 0.4%	4 ± 0.8%
GA_3 (100 μ*M*)	3 ± 0.6%	25 ± 3%	70 ± 7.0%	79 ± 3.0%	89 ± 1.9%
PCB (10 μ*M*)	0%	5 ± 0.5%	10 ± 0.9%	13 ± 0.6%	19 ± 2.3%
OKA (100 n*M*)	0%	3 ± 1.1%	5 ± 2.1%	7 ± 0.7%	19 ± 1.9%
OKA (100 n*M*) + GA_3 (100 μ*M*)	1 ± 0.2%	10 ± 2.8%	15 ± 1.1%	38 ± 1.6%	90 ± 2.9%

ABA, abscisic acid; GA_3, gibberellic acid; OKA, okadaic acid; PCB, paclobutrazol.

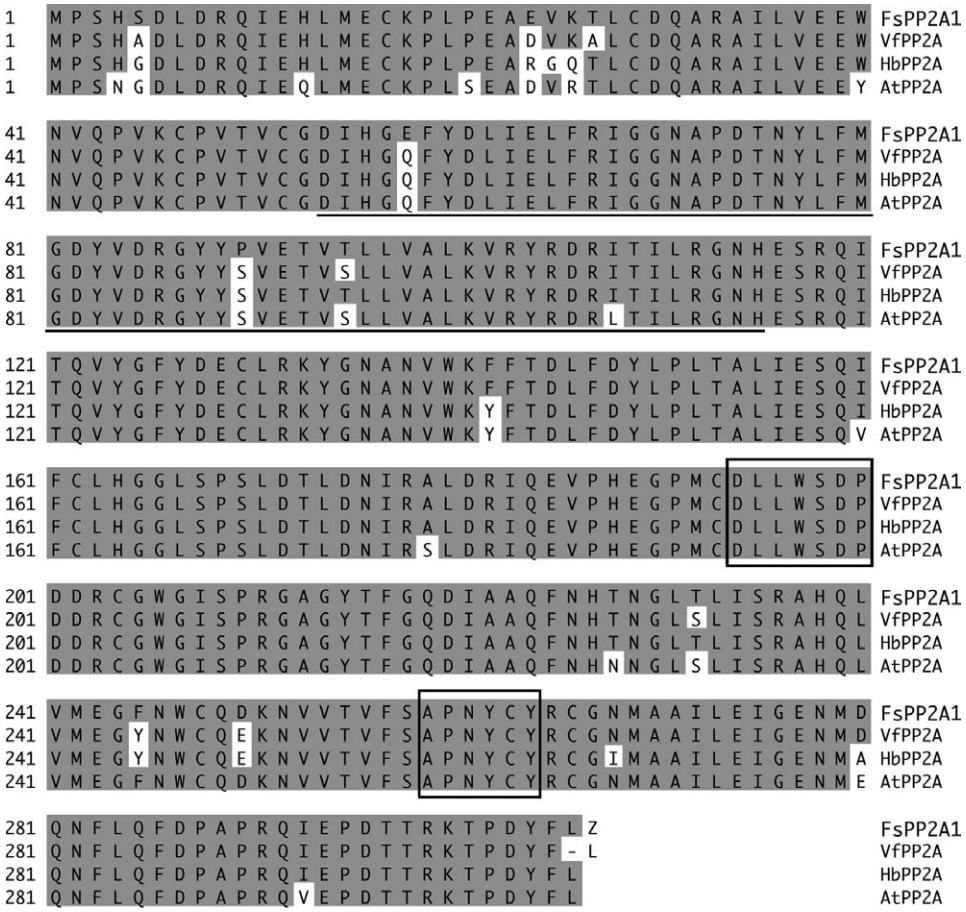

Fig. 1. Comparison of the deduced amino acid sequences of PP2As from *Fagus sylvatica*, *Vicia faba*, *Hevea brasiliensis* and *Arabidopsis thaliana*. Oligonucleotides used in the reverse transcriptase-PCR approach are boxed. The PP2A domain with the phosphorylase signature is underlined.

Subcellular localization of the FsPP2A1 protein

To ascertain the potential site of action of FsPP2A1, we investigated the subcellular localization of this protein using C-terminal and N-terminal GFP fusions of full-length FsPP2A1. As depicted in Fig. 2 (A), transient expression of these constructs in BY2 tobacco (*Nicotiana tabacum*) and T87 Arabidopsis cells showed a cellular expression pattern similar to that of the native GFP, demonstrating that the full-length protein appeared in both the cytoplasm and the nucleus. This exact localization of the protein was corroborated with confocal microscopy slides of cell sections through the nucleus, showing the expression of GFP-FsPP2A1 (Fig. 2B).

Southern blot analysis

In Southern blot analysis, hybridization of the full-length FsPP2A1 probe with genomic DNA revealed several bands in digests with restriction enzymes that do not cut inside the coding region of the gene (*Hin*dIII or *Eco*RI), suggesting the presence of a multigene family in *F. sylvatica* (Fig. 3A). When the genomic Southern blot was performed using the 3′-UTR of FsPP2A1 as the specific probe (Fig. 3B), a single band of hybridization was observed in both cases, as expected.

Regulatory effects of OKA and GA_3 on the expression of FsPP2A1 in beechnuts

To gain further insight into the role of FsPP2A1 on dormancy/germination of *F. sylvatica* seeds, the expression of FsPP2A1 was analysed by Northern blot of total RNA hybridized with a specific probe from the cDNA clone (Fig. 4).

FsPP2A1 transcript levels were almost undetectable in dry dormant seeds and slightly increased after stratification in water, being noticeable after 6 weeks of imbibition. The addition of GA_3, which produces a fast release from dormancy (Nicolás et al. 1996), clearly

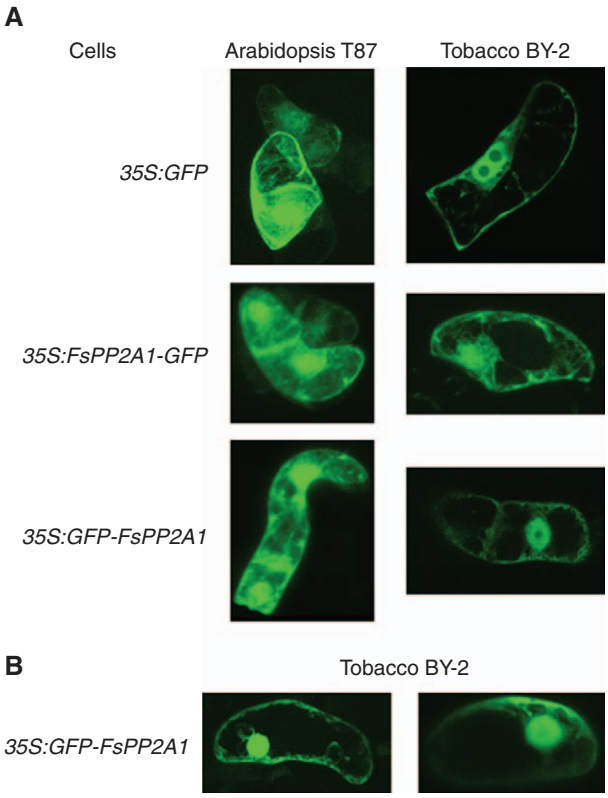

Fig. 2. Subcellular localization of FsPP2A1 in transiently transformed BY2 tobacco and T87 Arabidopsis cells. (A) Constructs delivered correspond to C- and N-terminal green fluorescent protein (GFP) fusions of full-length FsPP2A1 (*35S:FsPP2A1-GFP* and *35S:GFP-FsPP2A1*, respectively). Nuclear and cytosolic distribution of the GFP protein alone is shown as control (*35S:GFP*). The pictures show a merge of several slides. (B) Confocal microscopy slides of cell sections through the nucleus showing the exact localization of the GFP-FsPP2A1 protein in BY2 tobacco cells.

upregulated the expression of this gene, as the stratification period proceeds and germination percentages raise up to 70–80% (Table 1). Treatments which maintain seeds in dormant state and counteract the effect of GAs on dormancy breaking, such as ABA or the well-known GA biosynthesis inhibitor PCB (Nicolás et al. 1996), reduced the expression of this clone during the 6 weeks of stratification, reaching similar levels than those found in dry dormant seeds. These results indicated that the expression of this gene is rapidly activated by GA_3 mainly coinciding with the breaking of seed dormancy and the onset of germination.

Additionally, we analysed the expression of FsPP2A1 after the addition of the PP inhibitor OKA alone or together with GA_3 (Fig. 4) and found that it produced a drastic reduction in the transcript levels of this gene and completely inhibited the GA_3-induced FsPP2A1 expression.

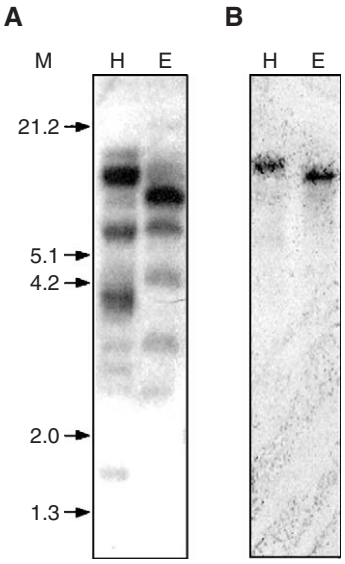

Fig. 3. Southern blot analysis of *FsPP2A1* gene. (A) Hybridization with the full-length FsPP2A1 probe. (B) Hybridization using the 3′-UTR of FsPP2A1 as specific probe. DNA (10 μg) from *Fagus sylvatica* was digested with *Hin*dIII (H) or *Eco*RI (E). λDNA cut with *Eco*RI and *Hin*dIII was used as molecular size markers (M).

Expression of FsPP2A1 in different tissues

FsPP2A1 tissue specificity was analysed in different parts of *F. sylvatica* seeds and seedlings (6-week-old) by Northern blot. The transcripts corresponding to FsPP2A1 clearly accumulated in the embryonic axes and cotyledons of the seeds, reaching the highest values in the embryonic axes of water and GA_3-treated seeds after 6 weeks of imbibition, precisely when dormancy has been alleviated and germination proceeds (Fig. 5). Moreover, its expression was undetectable in other seedling tissues examined, such as roots, stems and leaves, pointing to a seed-specific expression of the corresponding gene.

Discussion

F. sylvatica L. seeds display an endogenous dormancy that can be released by cold treatment (stratification) at 4°C over a period longer than 6 weeks. Exogenous addition of GA_3 has proved to be efficient in releasing beechnuts from dormancy and in substituting the cold treatment, allowing seed germination (Nicolás et al. 1996), as well as in antagonizing the effects of ABA on the maintenance of seed dormancy and on the expression of specific genes related to this process (Nicolás et al. 1996, 1997, 1998, Lorenzo et al. 2001, 2002b, 2003). Moreover, we have also reported the cloning and characterization of some genes, encoding an EIN-3-like

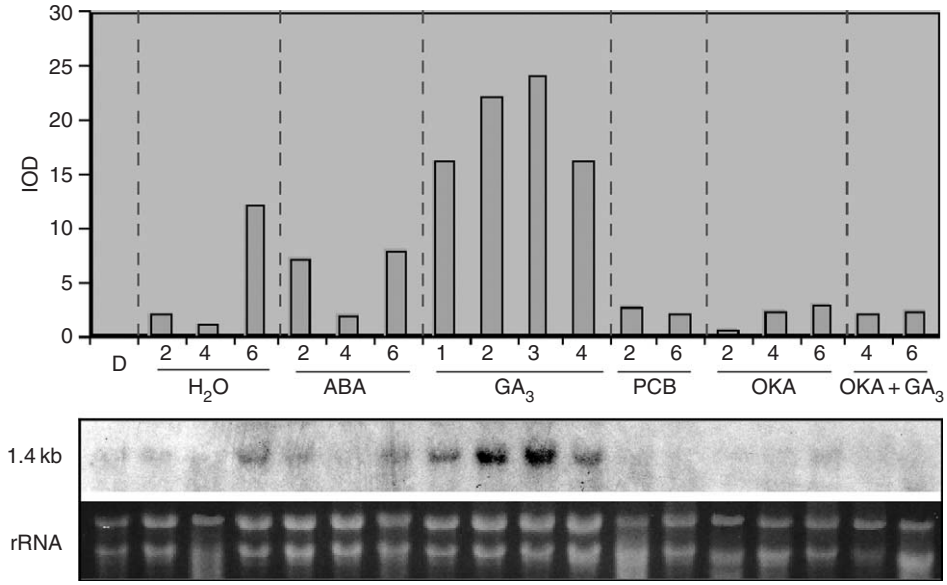

Fig. 4. Northern blot analysis of total RNA isolated from *Fagus sylvatica* dormant seeds (D) and dormant seeds imbibed from 1 to 6 weeks at 4°C in the presence of 100 μM abscisic acid (ABA), 100 μM gibberellic acid (GA_3), 10 μM paclobutrazol (PCB), 100 nM okadaic acid (OKA) or 100 nM OKA + 100 μM GA_3. Ten micrograms of RNA were used per lane and hybridized with FsPP2A1 probe. Top panel: IOD, integrated optical density; arbitrary units. Bottom panel: ethidium bromide-stained gel showing rRNAs.

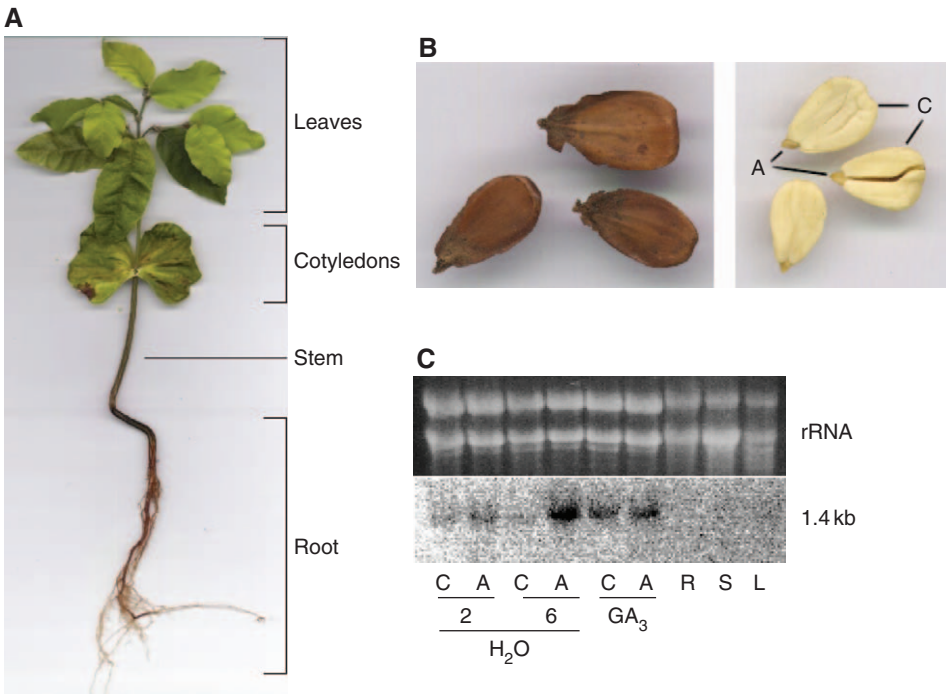

Fig. 5. Northern blot analysis of total RNA-isolated from: (A) *Fagus sylvatica* seedling tissues root (R), stem (S) and leaves (L). (B) *F. sylvatica* seeds tissues embryonic axes, A and cotyledons, C imbibed in water during 2 and 6 weeks or GA_3 during 3 weeks. (C) Ten micrograms of RNA were used per lane and hybridized with FsPP2A1 probe. Top panel: ethidium bromide-stained gel showing rRNAs.

protein (Lorenzo et al. 2000), and an AAA-ATPase (Lorenzo et al. 2002a) that are upregulated by GA_3 and seem to be related with the transition from dormancy to germination in beech seeds. These results indicate the significance of ABA and GA_3 in the control of expression of specific genes related to dormancy and

germination of these seeds and denote that this is a remarkable system to study the mechanisms involved in hormone action on the regulation of gene expression.

The fact that many responses regulated by GAs are restrained by the inhibitor OKA led to suggest a possible involvement of PP2A in the signal transduction pathway mediated by GAs (Kuo et al. 1996, Chang et al. 1999). Our present studies on germination of *F. sylvatica* dormant seeds show that OKA, a specific inhibitor of PP1/2A, has a clear effect in blocking seed germination and/or delaying the GA-induced breaking of dormancy (Table 1), suggesting that OKA-sensitive PPs (i.e. PP1/2A) might act early in the GA-signal transduction pathway that leads to the germination of the seeds.

PP2A activities have been shown to be involved in the ethylene-dependent induction of protein synthesis in tobacco (Raz and Fluhr 1993), or in the expression of light-inducible genes in maize (Sheen 1993). Remarkably, a role of these kinds of PPs has been shown in rice seed germination and GA-signal transduction (Chang et al. 1999). However, our understanding of GA-mediated processes involving phosphorylation/dephosphorylation events is just emerging.

The purpose of this study was to isolate PPs type-1/2A using a differential RT-PCR approach and identify those whose expression was increased after GA_3 treatment. Thus, we have isolated and further characterized FsPP2A1 and provide evidence that it belongs to the plant type 2A PPs family. The nucleotide and the deduced amino acid sequences of FsPP2A1 display the features described for these kinds of PPs, including the phosphorylase signature (Fig. 1). Furthermore, FsPP2A1 shows high similarity to different plant PP2As (Fig. 1). Additionally, transient expression studies using non-seed origin heterologous cells (tobacco and Arabidopsis) have been performed due to the lack of genetic tools in beechnuts. The expression of FsPP2A1 as a GFP fusion protein proves that FsPP2A1 is present in both the nucleus and the cytosol (Fig. 2), which is consistent with its ubiquity, broad substrate specificity and diverse cellular functions. Similar results have been recently obtained for other PP2As from Arabidopsis after a high-throughput protein localization (Koroleva et al. 2005). Within the complete sequence of the Arabidopsis genome, a great proportion of sequences encoding PP2A catalytic (5), regulatory A (3) and B (17) subunits have been found. All the PP2As reported belong to a multigene family with great similarity among the members, as we have also established in *Fagus* by Southern blot analysis (Fig. 3).

Expression of *FsPP2A1* is low in dormant beechnuts during the first weeks of stratification, increases after GA_3 treatment and correlates with the rise in the percentages of seed germination, as shown in Fig. 4. However, treatments with ABA, OKA, or the GA biosynthesis inhibitor PCB, which prevent the breaking of dormancy and inhibit germination, greatly reduce the expression of this clone. These results indicate that this *PP2A* gene is upregulated by GAs and show a correlation between FsPP2A1 expression and the processes involved in the transition from beechnuts dormancy to germination, because the corresponding protein is expressed precisely when dormancy is alleviated and germination starts. However, *FsPP2A1* does not seem to be so important later on during the germinative process, because the expression of this gene decays after 4 weeks in GA_3 (Fig. 2), and the germination percentages are high even in OKA + GA_3-treated seeds after this period (Table 1), being other components involved further for germination and seedling growth to proceed. The fact that GA_3 produces a fast release from dormancy (Nicolás et al. 1996) and increases the percentages of germination from the first weeks of treatment (Table 1) supports this proposal. Furthermore, the GAs synthesized upon stratification (Fernández et al. 1997) are responsible for the increase in the transcript levels observed during imbibition in water, because they disappear after the addition of PCB.

OKA is an inhibitor of PP2A activity, and in our study, we also show that it blocks the expression of FsPP2A1 at the level of transcript accumulation. Other authors have reported that OKA blocks gene expression in wheat aleurone cells (Kuo et al. 1996) and strongly inhibits the sucrose-inducible accumulation of mRNAs for sporamin, β-amylase and the small subunit of ADP-glucose pyrophosphorylase in petioles (Takeda et al. 1994) but, as far as we know, this is the first time that a transcriptional regulation of a PP type-2A by this compound has been established. Despite that the addition of OKA to beechnuts is able to block seed germination and to reduce the FsPP2A1 level of expression, the relation of this gene to the seed stratification process may be indirect. The effect of OKA in inhibiting an upstream PP1 or PP2A, that is required for the stability of the transcript or enhancement of transcription of the *FsPP2A1* gene is also assumed; being this upstream PP1/2A critical for the stratification process.

Another important consideration is the specificity of FsPP2A1 expression in the seed tissues, embryonic axis and cotyledons, rather than in other vegetative tissues examined (Fig. 5), which is in agreement with the involvement of this phosphatase in dormancy/germination processes and demonstrates that the differential RT-PCR developed in seed tissues is a good approach to isolate seed-specific genes and proteins, as previously described with FsPP2C1 (Lorenzo et al. 2001, and this work).

These results taken together lead us to think about an important function of FsPP2A1 in GA signalling, playing a key role in the onset of germination of *F. sylvatica* seeds, and it might be acting in the mechanisms responsible for the dephosphorylation of ABA-induced proteins involved in the maintenance of dormancy.

Finally, because genetic analysis is not feasible in beechnuts, we have initiated the isolation of T-DNA insertion mutants in Arabidopsis FsPP2A1 orthologue genes and the construction of Arabidopsis plants that overexpress this PP2A catalytic subunit. Both approaches will provide us more information about the role of this protein in GA signalling and in the transition from dormancy to germination.

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