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Characterization of a dual plant protein kinase (FsPK1) up-regulated by abscisic acid and calcium and specifically expressed in dormant seeds of *Fagus sylvatica* L.

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Abstract

An abscisic acid (ABA)-induced cDNA fragment encoding a putative protein kinase (PK) was obtained by differential screening of a cDNA library from *Fagus sylvatica* seeds. The full-length clone, named FsPK1, was produced by 5′ rapid amplification of cDNA ends (RACE) extension. This clone contained the 11 catalytic domains present in all protein kinases, but displayed unusual characteristics found only in a few plant PKs. FsPK1 exhibits features of both serine/threonine and tyrosine protein kinases within the catalytic domain, a putative nuclear localization signal within the regulatory domain and the consensus sequence involved in binding of 14-3-3 proteins. The catalytic domain, expressed in *Escherichia coli* as a fusion protein, showed Ca^{2+}-dependent *in vitro* kinase activity and dual serine/threonine and tyrosine specificity. Transcription of the *FsPK1* gene was reduced by seed stratification at 4°C, and clearly increased when seeds were treated with 0.1 mM ABA, correlating with the inhibition of germination. Interestingly, *FsPK1* transcripts were enhanced when ABA (0.1 mM) and calcium (1 mM) were added together, while the addition of EGTA (calcium chelator) and 3,4,5,-trimethoxibenzoic acid 8-(diethylamino) octyl ester (TMB-8, a calcium antagonist) decreased its expression. Furthermore, *FsPK1* transcript expression was tissue specific and accumulated only in ABA-treated seeds, but not in any ABA-treated vegetative tissues examined. These results suggest that the expression of the corresponding protein could be related to the inhibition of germination mediated by ABA in a calcium-dependent pathway.

Keywords: abscisic acid, calcium, *Fagus sylvatica*, FsPK1, protein kinase, seed dormancy

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Introduction

Abscisic acid (ABA) plays a key role in the regulation of seed development, maturation and germination, by inducing reserve accumulation, desiccation tolerance, development of dormancy and adaptation to environmental stresses (Bewley, 1997). Development and maintenance of seed dormancy are adaptative processes, highly responsive to the environment, and the involvement of ABA has been implicated by a genetic approach using ABA-response mutants (*abi*) (Koornneef *et al*., 1984; Schwartz *et al*., 1997).

Most physiological responses regulated by ABA include changes in gene expression, and many genes and proteins have been identified as involved in ABA signalling, although the signal transduction cascades are not yet clearly established (Leung and Giraudat, 1998). However, substantial progress has been made in the characterization of several ABA signalling molecules, including second messengers such as cyclic-ADP-ribose and Ca^{2+} (Wu *et al*., 1997; Pandey *et al*., 2000), different enzymes regulating protein phosphorylation and dephosphorylation (Trewavas, 1988; Verhey and Walker-Simmons, 1997), and two putative transcriptional factors named ABI3 (Giraudat *et al*., 1992) and ABI4 (Finkelstein *et al*., 1998). In particular, ABA signalling appears to involve a complex network of both positively and negatively regulated kinases and phosphatases (Walker-Simmons, 1998; Campalans *et al*., 1999; Merlot *et al*., 2001).

Two major classes of protein kinases have been identified in plants, serine/threonine and tyrosine kinases (Hanks *et al*., 1988). However, homologues of bacterial histidine kinases (Bleecker and Schaller, 1996) and kinases with dual specificity for serine/threonine and tyrosine (Stone and Walker, 1995) have also been described in plants.

Protein phosphorylation controlled by kinases is a reversible process with the capability to respond to external and internal signals, and evidence suggests

its involvement in the hormonal regulation of seed dormancy (Trewavas, 1988; Walker-Simmons, 1998). While a specific protein kinase responsible for controlling dormancy has not yet been identified, several ABA and stress-responsive protein kinases have been described in seeds and other plant tissues, including MAPKs (mitogen-activated protein kinases) (Hirt, 2000), CDPKs (calcium-dependent protein kinases) (Urao *et al*., 1994) and PKABA1, whose expression is induced by ABA and various environmental stresses in dormant wheat embryos (Holappa and Walker-Simmons, 1995). The components of ABA signal transduction, their location in the signalling cascades and their interactions with second messengers are currently under study. Calcium is a second messenger in plants and plays a major role in the induction or activation of many ABA-regulated proteins (Campalans *et al*., 1999), although a calcium-independent pathway has also been proposed.

Our work focuses on the study of dormancy in *Fagus sylvatica* seeds and the role of ABA in the expression of specific genes involved. In previous reports we have shown that beechnut (*F. sylvatica*) seed dormancy is maintained by ABA and overcome by stratification or gibberellic acid treatment (Nicolás *et al*., 1996). Furthermore, ABA is able to regulate the expression of several dormancy-related genes (Nicolás *et al*., 1997, 1998; Lorenzo *et al*., 2001), some of them in a Ca^{2+}-dependent manner (Lorenzo *et al*., 2002). In this report we identify and characterize a cDNA clone (FsPK1) coding for a new dual specificity (Ser/Thr and Tyr) protein kinase, which is up-regulated by ABA and calcium, specifically expressed in ABA-treated seeds, and may participate in a signalling cascade related to ABA-induced dormancy.

Materials and methods

Plant material and germination conditions

Fagus sylvatica L. seeds (beechnuts) were obtained from the Danish State Forestry Tree Improvement Station, pretreated and imbibed as described previously (Lorenzo et al., 2001). The imbibition medium consisted of sterile water containing 100 µM ABA, 100 µM ABA + 1 mM $CaCl_2$, 100 µM ABA + 2 mM EGTA or 100 µM ABA + 200 µM TMB-8 [3,4,5,-trimethoxibenzoic acid 8-(diethylamino) octyl ester]. The concentrations used had been optimized by Nicolás *et al*. (1996) and Colorado *et al*. (1991). Seeds were maintained in the different media at 4°C (stratification temperature) from 1 to 6 weeks and separated into cotyledons and embryonic axes when needed.

Seedlings were obtained from 4-week-stratified seeds sown in moist vermiculite in a controlled-environment chamber under a 12-hour light and 12-hour dark cycle at 15°C, and harvested after 6 weeks. ABA-treated seedlings were watered every 2 d and moistened daily with a solution of 100 µM ABA, and the corresponding tissues were collected after 6 d. Both treated and untreated seedlings were separated into roots, leaves and stems. All collected tissues were frozen in liquid nitrogen and stored at –80°C.

Isolation of the full-length FsPK1 cDNA clone

A cDNA clone, of about 650 bp, was isolated by differential screening from a cDNA library constructed using poly(A^+) RNA from *F. sylvatica* seeds imbibed in 100 µM ABA for 2 weeks as a template (Nicolás *et al*., 1997). From the initial partial complementary DNA (cDNA) fragment, a full-length FsPK1 cDNA clone was produced by using 5′ rapid amplification of cDNA ends (RACE) to extend sequences. The 5′ RACE reactions were carried out by using the Rapid Amplification of cDNAs Ends system, version 2.0 (Invitrogen, Carlsbad, California, USA). The first strand was synthesized from 1 µg of poly(A^+) RNA from ABA-treated seeds, using the primer SP1-PK (for gene-specific primer one) [5′-CATCCCACATGAAAGCAAATGTACAAG-3′], and tailed with poly(G) following the vendor's procedure. The first round of polymerase chain reaction (PCR) amplification was carried out with the primer SP2-PK (for gene-specific primer two) [5′-CTCGAGCTGGTGAACTACAAAAGCCAGGTC-3′] and AAP (5′ RACE Abridged Anchor Primer, 5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′, vendor-provided) in the PCR mix. The PCR protocol was: one cycle of 94°C (5 min), 35 cycles of 94°C (1 min), 55°C (1 min), 72°C (2 min), and one cycle of 72°C (10 min). The first-round amplification product was diluted (1:100) and then used as a template for a second round of amplification with SP2-PK and AUAP (Abridged Universal Amplification Primer, 5′-GGCCACGCGTCGACTAGTAC-3′, vendor-provided). The reaction conditions and protocol followed for second-round PCR were the same as for the first one. The 1500 bp PCR product obtained was subcloned into pCR 2.1 vector (Invitrogen) and sequenced.

DNA sequencing

Plasmid DNA templates were isolated by the Wizard *Plus* Minipreps DNA Purification System (Promega, Madison, Wisconsin, USA). Determination of the nucleotide sequence of the cDNA clone was performed on an ABI 377 sequencer (Applied Biosystems, Inc., Foster City, California, USA) using

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the *Taq* DyeDeoxy™ Terminator Cycle Sequencing kit. The DNA and deduced protein sequences were compared to other sequences in the EMBL databases (GenBank and SwissProt, respectively), using the FASTA algorithm.

Construction of the family tree

A phylogenetic tree, illustrating the relationship among different plant protein kinases, was obtained from the final alignment using CLUSTAL W method (Thompson *et al*., 1994) with the megalign program from DNAstar 4.2 (DNAstar Inc., Madison, Wisconsin, USA)

Northern blot analysis

Total RNA from seeds and seedlings was extracted using the Qiagen Pack-500 cartridge (Qiagen, USA, Valencia, California, USA), following the manufacturer's protocol, and analysed by Northern blotting, following the same procedures described in Nicolás *et al*. (1997). Blotted membranes were hybridized with the FsPK1 ^{32}P-labelled probe and processed as described in Lorenzo *et al*. (2001). They were then exposed to X-Omat films (Kodak, Rochester, New York, USA) and the autoradiograms analysed on a Bioimage 60S Image Analyzer (Millipore Ibérica S.A., Madrid, Spain, Visage 4.6K Software).

Expression and purification of FsPK1 recombinant protein

The catalytic domain of FsPK1 was amplified by PCR with primers [5′-GGATCCAAATCGAAGTACCGG-3′, containing the *Bam*HI site (underlined) and the start of the catalytic domain, and 5′-CTCGAGATAAAGAT**TCA**TGTGA-3′, containing the *Xho*I site (underlined) and the stop codon (in bold)], subcloned in frame into the *Bam*HI and *Xho*I sites of the pMAL-c2 vector (New England Biolabs, Beverly, Massachusetts, USA) and verified by DNA sequencing. The FsPK1 catalytic domain was expressed in *E. coli* DH5α as a maltose-binding protein (MBP). Cells carrying the recombinant plasmid were grown at 30°C in Luria Bertani (LB) medium/0.2% glucose 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 2 h of induction, and recovered in 2 ml of MBP buffer [20 mM Tris–HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol] supplemented with 0.25% Tween 20 and lysed by sonication. The fusion protein was affinity purified using amylose resin (New England Biolabs), following the manufacturer's instruction. Proteins were analysed by SDS–PAGE and Coomassie Blue staining. Protein concentration was measured using a Bio-Rad protein assay kit (BioRad, Hercules, California, USA), based on the method of Bradford (1976), using bovine serum albumin as the standard.

Assay for protein kinase activity

For the PK activity assays, 500 ng of the fusion protein were used in a 20 µl reaction mixture containing 50 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 4 µCi [γ-^{32}P]dATP, and 10 µg of α-casein (Ali et al., 1994) or 2 µg of myelin basic protein (Wilson et al., 1995). The reactions were incubated for 15 min at 25°C. In some assays, protein kinase inhibitors such as staurosporine (10 µM) (Ser/Thr PK inhibitor) or genistein (10 µM) (Tyr PK inhibitor; Akiyama et al., 1987) (concentrations previously found effective by Conrath et al., 1997), were used. Aliquots (2 µl) were removed after the indicated time period and placed on Whatman GF/C filters (Whatman, Kent, UK). The reaction was stopped by washing the filters three times with 200 µl of 20% (w/v) trichloroacetic acid containing 10 mM sodium pyrophosphate and once with 95% ethanol (v/v). Filters were dried and mixed with 1 ml of Ready Safe™ Liquid Scintillation Cocktail (Beckman Instruments, Inc., California, USA), and radioactivity was determined in a Tri-Carb® 2100 TR Liquid Scintillation Analyzer (Packard Instrument Company Inc., Bedford, Massachusetts, USA).

Results

Isolation and characterization of a cDNA coding for a protein kinase from Fagus sylvatica

A partial clone of about 0.65 kb was initially identified by differential screening. The full-length cDNA clone, named FsPK1 (accession number AJ298992), was obtained using 5′ RACE and sequenced; the DNAstar analysis showed that it was 1610 bp long, containing the ATG start codon, the poly(A) and a complete open reading frame of 1440 nucleotides, encoding a putative polypeptide of 480 amino acids with a predicted molecular weight of 54.8 kDa and a pI of 9.34.

Comparison of the deduced amino acid sequence of FsPK1 with the databases (GENEMBL and SWISSPIRALL) showed the 11 catalytic subdomains described in all PKs (Hanks *et al*., 1988) (Fig. 1A) and displayed 72% identity with the PK GmPK6 from soybean (Feng *et al*., 1993) (Fig. 1B), and more than 60% identity with other protein kinases from *Arabidopsis*. The catalytic subdomain VIb

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(HRDLKPEN) of FsPK1 contained the conserved motifs for serine/threonine protein kinases, and subdomains VIII (GTYRWMAPE) and XI (CWX_6RPEF) showed the conserved residues of tyrosine kinases (Fig. 1C) (Hanks *et al*., 1988), suggesting that FsPK1 is a kinase with a possible dual specificity for both serine/threonine and tyrosine, as found previously by Feng *et al*. (1993) in a soybean protein kinase. As is usual in most protein kinases, less sequence identity was found in the regions spanning the catalytic subdomains. Additionally, it is worth noting the presence of a possible regulatory signal of nuclear location (Fig. 1B) (Feng *et al*., 1993), and the consensus sequence RSXSXXP in the N-terminal region (Fig. 1B), described by Muslin *et al*. (1996) as involved in binding of 14-3-3-type proteins.

The phylogenetic relationship between FsPK1 and other fully characterized plant protein kinases is rather limited (Fig. 2), and appears to be included in a separate group together with GmPK6, with which it shares the highest homology, dual specificity and similar unusual features.

Kinase activity of recombinant FsPK1 protein

To establish the possible dual kinase activity of the protein, the catalytic domain of FsPK1 was cloned into the pMAL-c2 vector (New England Biolabs) and expressed in *E. coli* as a maltose-binding protein (MBP). Cells carrying the recombinant plasmid were grown, and the production of recombinant proteins was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG). The fusion proteins were recovered from *E. coli* cells that had been grown at 30°C and purified by affinity chromatography on amylose columns (Fig. 3A). The purified recombinant protein had an apparent molecular mass of *c*. 80 kDa, consistent with that predicted from the amino acid sequence of MBP–FsPK1 (42 kDa for MBP and 38 kDa for FsPK1).

The *in vitro* kinase activity of the FsPK1 fusion protein was assayed in the presence of [γ-^{32}P]dATP using α-casein or myelin basic protein as substrates, and, thus, we obtained direct biochemical evidence that FsPK1 encoded a functional protein kinase, showing Ca^{2+}-dependent kinase activity (Fig. 3B). In the absence of the divalent cation Ca^{2+}, little detectable activity was present, and it was greatly reduced in the presence of 5 mM EGTA (calcium chelating agent). This kinase activity also decreased when staurosporine (Ser/Thr protein kinase inhibitor) or genistein (Tyr PK inhibitor) were added to the *in vitro* assay, and no activity was detected when both PK inhibitors were added together, indicating dual activities (Ser/Thr and Tyr PK) of FsPK1 (Fig. 3B)

Regulatory effects of ABA and calcium on the expression of FsPK1 *gene in dormant beechnuts*

The effect of ABA and calcium on the accumulation of *FsPK1* mRNA and its relation to dormancy in beechnuts were explored by Northern blot analysis (Fig. 4A). *FsPK1* transcript content was initially low in dormant seeds and during stratification at 4°C in water, a treatment efficient in breaking dormancy in these seeds; expression decreased as the stratification period proceeded and dormancy was dissipated (Fig. 4B). The addition of ABA, which maintains seed dormancy, increased the expression of *FsPK1* during imbibition and delayed seed germination (Fig. 4B). Seeds treated with $CaCl_2$, EGTA or TMB-8 showed similar germination percentages to those obtained during stratification at 4°C and remained viable after 6 weeks of treatment (data not shown), but none of them modified significantly the effects of ABA on seed germination (Figs. 4B, 5B). However, when ABA was added together with calcium, there was a greater increase in the *FsPK1* transcript level, while the addition of either EGTA or TMB-8, together with ABA (Fig. 5A), reduced *FsPK1* gene expression during the treatment period, suggesting that calcium is necessary for the effect of ABA on the regulation of this gene.

Transcript tissue specificity

The expression of *FsPK1* was analysed in different parts of beech seeds and seedlings (6 weeks old) for tissue specificity. The *FsPK1* transcript was found to accumulate in the cotyledons of ABA-treated seeds, whereas the level of expression in other ABA-treated or -untreated tissues (roots, stems and leaves) was undetectable (Fig. 6).

Discussion

Abscisic acid (ABA) is involved in the control of plant growth and development, including seed dormancy and germination (Bewley, 1997). The mechanism of ABA action is still unknown, but now it seems clear that phosphorylation/dephosphorylation events mediated by specific protein kinases (PKs) and protein phosphatases (PPs) play an important role in ABA-regulated processes (Leung and Giraudat, 1998; Campalans *et al*., 1999; Gómez-Cadenas *et al*., 1999, 2001; Shen *et al*., 2001).

Seed dormancy constitutes an intrinsic impediment to germination. We have been using *F. sylvatica* as a model to study seed dormancy of woody plants, and previously reported that these seeds display embryo dormancy, maintained by ABA and overcome by stratification at 4°C or gibberellic acid treatment. These treatments regulate the expression of a group of seed-

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Figure 1. (A) Schematic diagram of FsPK1, showing the regulatory domain containing the putative nuclear localization signals (white boxed) and the catalytic domain with the 11 PK subdomains (black boxed). (B) Alignment of the derived amino acid sequences from FsPK1 and GmPK6 from soybean. Identical amino acids are indicated with asterisks (*) and conservative amino acid substitution are indicated with dots (.). The consensus sequence of 14-3-3-type proteins is underlined. (C) Comparison of the catalytic subdomains VIb, VIII and XI of FsPK1 with those of Ser/Thr and Tyr PKs.

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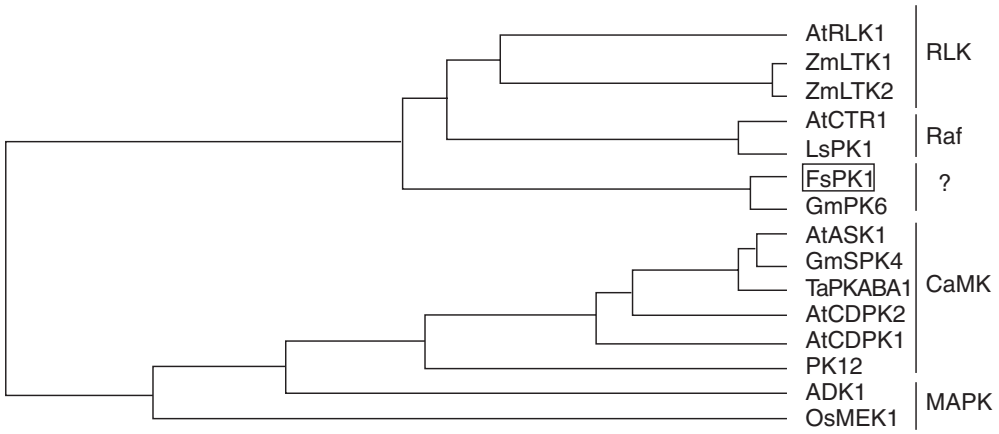

Figure 2. Phylogenetic tree of different plant protein kinases: *Fagus sylvatica* FsPK1 (AJ298992) (boxed); *Arabidopsis thaliana* AtASK1 (M91548), AtCDPK1 (D21805), AtCDPK2 (D21806), AtCTR1 (L08789), AtRLK1 (M84658), ADK1 (U48779), PK 12 (U73937); *Glycine max* GmPK6 (M67449), GmSPK4 (L38855); *Lycopersicon esculentum* LsPK1 (Y13273); *Oryza sativa* OsMEK1 (AF080436); *Triticum aestivum* TaPKABA1 (M94726); *Zea mays* ZmLTK1 (AF023164), ZmLTK2 (AF023165). Accession numbers are inside brackets. Proteins included in different PK families are indicated: RLK, receptor-like kinases; Raf, protein kinases of the Raf family; (?), possible new group of protein kinases; CaMK, calcium–calmodulin-dependent protein kinases; MAPK, mitogen-activated protein kinases.

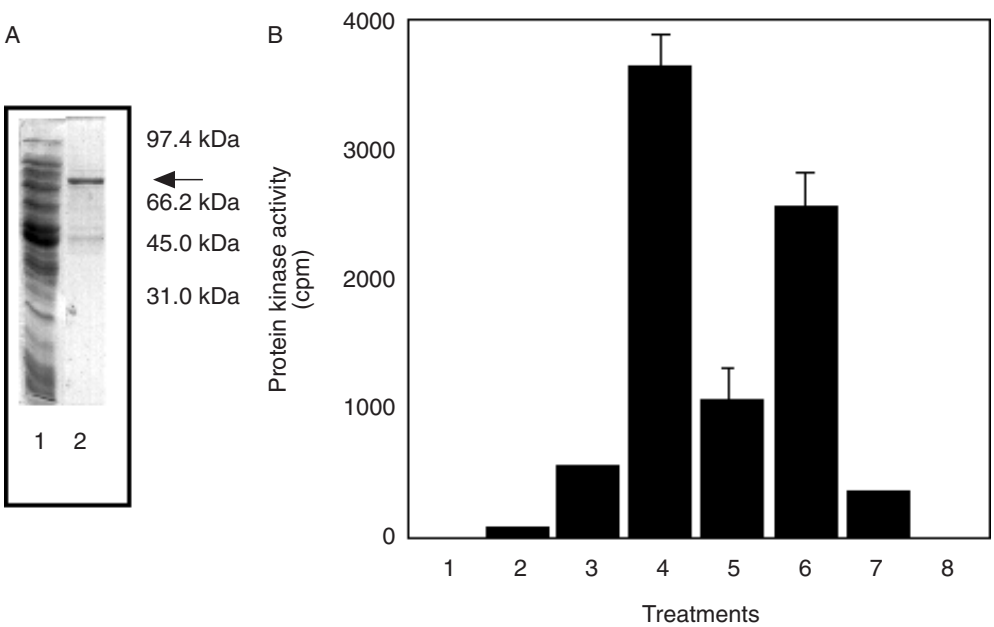

Figure 3. (A) Expression and purification of the fusion protein pMAL–FsPK1. Soluble extracts of *E. coli* (lane 1) and purified protein (lane 2) were resolved by 12% SDS–PAGE and stained with Coomassie Brilliant Blue. The position of the fusion protein is indicated by an arrow. (B) *In vitro* kinase activity of FsPK1: (1) denatured protein used as control; (2) maltose-binding protein (MBP) used as control; (3) FsPK1 activity without Ca^{2+} in the *in vitro* assay; (4) with Ca^{2+} (1 mM) added; (5) with EGTA (5 mM) + Ca^{2+} (1 mM); (6) with staurosporine (10 µM) + Ca^{2+} (1 mM); (7) with genistein (10 µM) + Ca^{2+} (1 mM) and (8) with staurosporine and genistein + Ca^{2+} (1 mM). Each value represents the average of duplicate assays, and kinase activity is expressed as cpm of ^{32}Pi incorporated per µg of protein kinase.

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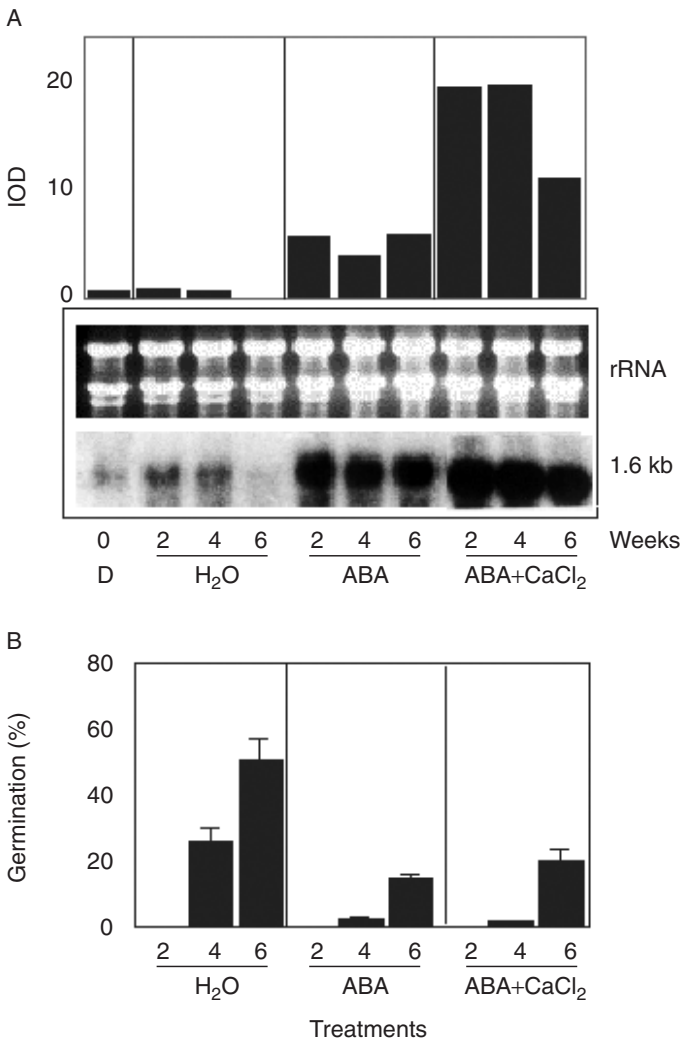

Figure 4. (A) Northern blot analysis of total RNA isolated from dormant *F. sylvatica* seeds (D) and dormant seeds imbibed for 2, 4 and 6 weeks at 4°C in water, 100 µM abscisic acid (ABA) or 100 µM ABA plus 1 mM $CaCl_2$. Ten µg RNA were used per lane and hybridized with the FsPK1 cDNA probe. Top panel: IOD (integrated optical density; arbitrary units). Lower panel: stained gel showing rRNAs. (B) Germination percentages of beechnut seeds obtained with the indicated treatments at 4°C.

expressed genes (Nicolás *et al*., 1996), including a glycine-rich protein (Nicolás *et al*., 1997), a GTP-binding protein (Nicolás *et al*., 1998) and two protein phosphatases (PP2C) (Lorenzo *et al*., 2001, 2002), which are up-regulated by ABA in hydrated, growth-arrested seeds, and seem to be related to dormancy.

In the present study, we have isolated and characterized a new gene, *FsPK1*, and provide evidence that it belongs to a new group within the protein kinase (PK) family. The *FsPK1*-predicted gene product contains the 11 catalytic subdomains present in all protein kinases (Fig. 1A) (Hanks *et al*., 1988), and shows high similarity to different plant protein kinases, primarily with GmPK6 from soybean (Feng *et al*., 1993) (Figs 1B, 2). The catalytic domain contains the conserved motifs for serine/threonine protein kinases in subdomain VIb (KPE) and also for tyrosine kinases within subdomains VIII (RWM) and XI (CWX_6RPEF) (Fig. 1C) (Stone and Walker, 1995). These results suggested that FsPK1 is a kinase with possible dual specificity for serine/threonine and tyrosine, as previously found by Feng *et al*. (1993).

The catalytic domain of FsPK1, expressed in *E. coli* as maltose-binding fusion protein (Fig. 3A), exhibited Ca^{2+}-dependent kinase activity (Fig. 3B) and a response to PK inhibitors consistent with the sequence-predicted dual specificity (Ser/Thr and Tyr PK). Further molecular confirmation of the kinase

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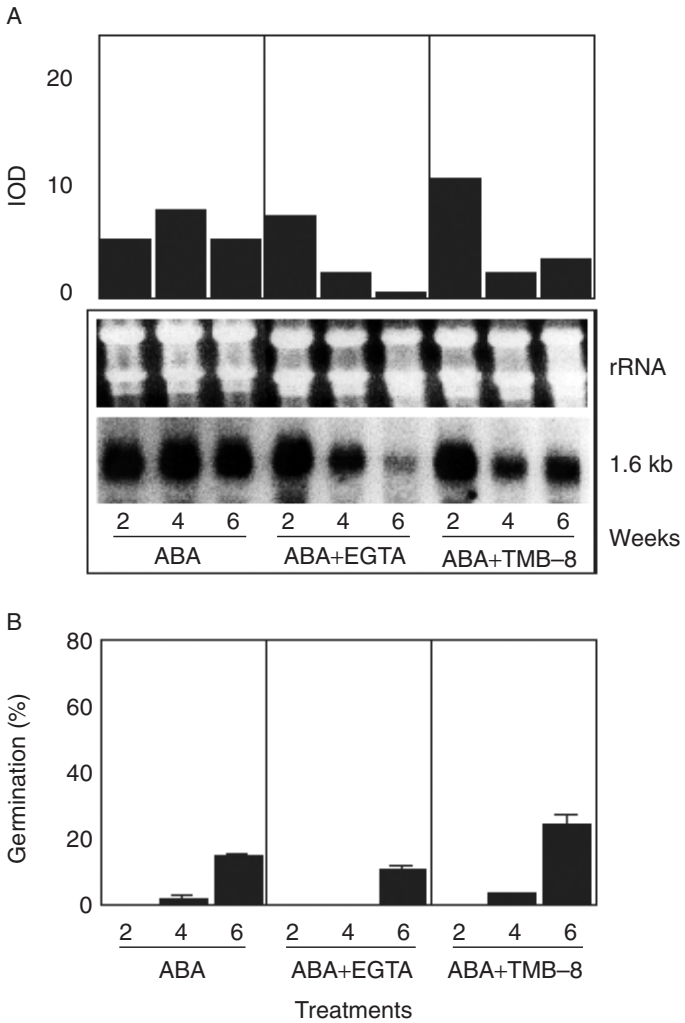

Figure 5. (A) Northern blot analysis of total RNA isolated from dormant *F. sylvatica* seeds imbibed at 4°C during 2, 4 and 6 weeks in 100 μM ABA, 100 μM ABA plus 2 mM EGTA or 100 μM ABA plus 200 μM TMB-8. Ten μg RNA were used per lane and hybridized with the FsPK1 cDNA probe. Top panel: IOD (integrated optical density; arbitrary units). Lower panel: stained gel showing rRNAs. (B) Germination percentages of beechnut seeds obtained with the indicated treatments at 4°C.

activity, using other substrates and performing autophosphorylation assays, is under investigation. There are not many plant protein kinases with both activities described so far, and just a few of them, such as ADK1 from *Arabidopsis thaliana* (Ali *et al*., 1994), or PK12 (Sessa *et al*., 1996) and NtDSK1 (Cho *et al*., 2001) from tobacco, have been confirmed to have dual specificity *in vitro*. FsPK1 would be the first dual plant PK induced by ABA.

FsPK1 contains some unusual characteristics for this kind of protein, such as a possible nuclear localization signal (Fig. 1B) (Feng *et al*., 1993), and the consensus sequence RSXSXXP in the N-terminal region (Fig. 1B), described by Muslin *et al*. (1996) as involved in binding of 14-3-3-type proteins. The 14-3-3 proteins have been reported to act as protein kinase regulators in many signal transduction cascades, or as transcription factors regulated by different hormonal and environmental signals, including ABA and some kinds of stress (Finnie *et al*., 1999).

Analysis of the expression of *FsPK1* by Northern blotting (Figs 4A, 6) shows that the transcript for this gene is present in cotyledons of dormant seeds and decreases after 4 weeks of stratification at 4°C, coincident with the release of seed dormancy (Nicolás *et al*., 1996) and reduction of ABA sensitivity

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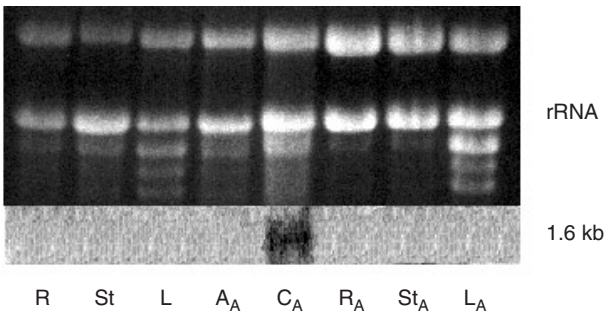

Figure 6. Expression pattern of FsPK1 transcripts in *F. sylvatica* seeds (2 weeks imbibed in ABA at 4°C), and untreated seedling (6-week-old) tissues [roots (R), stems (St), leaves (L)] and ABA-treated tissues [embryonic axes (A_A) cotyledons (C_A), roots (R_A), stems (St_A) and leaves (L_A)]. RNA (10 µg lane^{-1}) was used and hybridized with the FsPK1 cDNA probe. Top panel: ethidium bromide-stained gel showing rRNAs.

or content (Walker-Simmons, 1987; Steinbach *et al*., 1995). FsPK1 clearly increased after ABA treatment, which maintains dormancy and prevents germination of *F. sylvatica* seeds (Nicolás *et al*., 1996). This gene is only highly expressed in ABA-treated dormant seeds, particularly in cotyledons, but expression was not detected in any other ABA-treated or -untreated tissues (Fig. 6), suggesting that the *FsPK1* gene may have a role in beechnut dormancy or germination.

Since its expression is low in dormant and stratified seeds in the absence of ABA, and increases in the cotyledons only when seeds are incubated in ABA, FsPK1 seems to have little or no role in the induction of seed dormancy, nor in the control of embryo growth, but, rather, it may play a role in the maintenance of dormancy by inhibiting germination or controlling reserve mobilization.

A Ca^{2+}-dependent pathway has been proposed for the expression of some specific genes regulated by ABA, and the role of protein kinases in Ca^{2+}-mediated ABA signalling has been studied in various plants (Campalans *et al*., 1999; Pandey *et al*., 2000). In our study, the joint addition of ABA and calcium increased the transcript content as compared to the effect of ABA alone (Fig. 4A). The addition of high concentrations of EGTA or TMB-8, together with ABA (Fig. 5B), reduced the level of expression of *FsPK1*. These results showed the possible importance of calcium in the regulation of this ABA-induced gene. However, neither EGTA or TMB-8 modified the germination percentages of the seeds, suggesting that FsPK1 is not the unique ABA-regulated PK involved in the control of dormancy/germination of these seeds. While Botella *et al*. (1996) reported a calcium-dependent expression of a CDPK (calcium-dependent protein kinase) from mungbean, FsPK1 is not a CDPK, because it does not have any of its characteristics (e.g. FsPK1 does not contain a calmodulin-like domain in the C-terminus).

In summary, our results suggest that *FsPK1* gene expression is up-regulated by ABA in cotyledons, enhanced by calcium and associated with dormancy and/or inhibition of germination in *F. sylvatica* seeds. The unusual domains that may be present in this protein suggest that it could be involved in the final steps of the ABA signalling cascade inside the nucleus. Since genetic analysis is not feasible in beechnuts, we have initiated the construction of *Arabidopsis* plants overexpressing FsPK1, which will provide more information about the possible role of this protein kinase in ABA signalling and the control of germination.

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