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RESEARCH PAPER

Immunolocalization of FsPK1 correlates this abscisic acid-induced protein kinase with germination arrest in *Fagus sylvatica* L. seeds

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Abstract

An enzymatically active recombinant protein kinase, previously isolated and characterized in *Fagus sylvatica* L. dormant seeds (FsPK1), was used to obtain a specific polyclonal antibody against this protein. Immunoblotting and immunohistochemical analysis of FsPK1 protein in beech seeds showed a strong immunostaining in the nucleus of the cells located in the vascular tissue of the embryonic axis corresponding to the future apical meristem of the root. This protein kinase was found to accumulate in the seeds only when embryo growth was arrested by application of ABA, while the protein amount decreased during stratification, previously proved to alleviate dormancy, and no protein was detected at all when seed germination was induced by addition of GA_3. These results indicate that FsPK1 may be involved in the control of the embryo growth mediated by ABA and GAs during the transition from dormancy to germination in *Fagus sylvatica* seeds.

Key words: ABA, *Fagus sylvatica*, germination, immunoblotting, protein kinase, seed dormancy.

Introduction

Dormancy is a complex phenomenon present in many seeds by which the embryo is unable to grow, and germination is blocked even under favourable environmental conditions (Bewley, 1997).

Fagus sylvatica seeds represent a suitable model with which to study this process in woody plants since they exibit a particularly deep embryo dormancy, which is expressed at temperatures higher than 5 °C (Muller and Bonnet-Masimbert, 1989; Derkx and Joustra, 1997) and is overcome by cold pretreatment (1–5 °C) (Muller and Bonnet-Masimbert, 1989), as this pretreatment has been shown to increase ABA degradation in the embryo axis (Le Page-Degivry *et al*., 1997). In addition, *Fagus* seed dormancy has been associated with the inability of the axis to synthesize ABA (Le Page-Degivry *et al*., 1997). Moreover, dormancy is maintained by exogenous application of ABA even at 4 °C (Nicolás *et al*., 1996) and released by GA_3 (Bonnet-Masimbert and Muller, 1976; Nicolás *et al*., 1996; Fernández *et al*., 1997) and ethylene (Falleri *et al*., 1997; Calvo *et al*., 2004).

ABA has been involved in different processes related to seed development, maturation, and dormancy induction, as well as in the inhibition of germination, where its capacity to induce the expression of specific genes is well established (Rock, 2000). While the mechanisms involved in the transition from dormancy to germination are still unknown, genetic analyses performed mainly in *Arabidopsis* have identified the crucial role of ABA in seed dormancy and the requirement of gibberellins for germination (Koornneef *et al*., 2000; Finkelstein *et al*., 2002),

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Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt; BSA, bovine serum albumin; DAPI, 4′-6′diamidino-2-phenylindol; NBT, 4-nitroblue tetrazolium chloride; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing Tween-20; PCB, paclobutrazol; PK, protein kinase; TBS, TRIS-buffered saline; TBST, TRIS-buffered saline containing Tween-20.

as well as the antagonistic interactions between ABA and gibberellins in controlling the germination ability (Koornneef and Karssen, 1994; Steber *et al*., 1998). Besides, physiological and genetic evidence indicates that ABA regulates seed dormancy/germination by phosphorylation/dephosphorylation events through the activation and/or expression of specific protein kinases (PKs) and phosphatases (PPs) (reviewed by Finkelstein *et al*., 2002).

Reversible protein phosphorylation plays an important role in processes controlled by ABA (Leung and Giraudat, 1998; Campalans *et al*., 1999; González-García *et al*., 2003; Sáez *et al*., 2004). There are many protein kinases already identified in seeds (Walker-Simmons, 1998) and some ABA-regulated PKs have been related with the mechanisms involved in seed dormancy/germination, such as PKABA1, a protein kinase with an acidic domain (Anderberg and Walker-Simmons, 1992), whose expression is induced by ABA and various environmental stresses in dormant embryos (Holappa and Walker-Simmons, 1995) and suppresses GA-inducible gene expression in barley aleurone layers (Gómez-Cadenas *et al*., 1999, 2001).

This research is a study of the role of ABA in the expression of specific genes involved in the transition from dormancy to germination in *Fagus sylvatica* (beechnuts). In this context, the isolation and characterization of FsPK1, a cDNA clone corresponding to a protein kinase from *Fagus*, whose expression was up-regulated by ABA specifically in the cotyledons and correlated with the maintenance of seed dormancy (Lorenzo *et al*., 2003) has previously been reported. Furthermore, the unusual domains present in this protein suggested that it could be involved in the final steps of the ABA signalling cascade inside the nucleus (Lorenzo *et al*., 2003).

To get a deeper insight into the role of FsPK1 from beechnuts and to determine whether the transcript levels correlate with the accumulation of the corresponding polypeptide, in this work, our previous expression studies are complemented with immunological analysis and immunohistochemical localization of the FsPK1 protein in *Fagus sylvatica* seeds subjected to different treatments, that maintain the dormant state (ABA) or are able to release it (cold stratification, GA_3), in order to confirm the proposed role of this protein in the germination arrest in beechnuts.

Materials and methods

Plant material

Fagus sylvatica L. seeds (beechnuts) were obtained from the Danish State Forestry Tree Improvement Station. Seeds were dried to a moisture content of 10% (fresh weight basis) 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 100 μM ABA, 100 μM GA_3 or 10 μM PCB (concentrations were optimized for these seeds in previous reports by Nicolás *et al*., 1996, 1997). Seeds were maintained from 2–6 weeks in the dark at 4 °C to study the accumulation of FsPK1 protein during the breaking of dormancy by cold treatment, as well as the effects of ABA and GAs on it.

Protein extraction

For protein isolation, seeds were frozen in liquid nitrogen and stored at –80 °C until required. Tissue was ground in liquid nitrogen and soluble proteins were extracted in 100 mM TRIS-HCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.1 mM ATP, 2 mM Asp, 20% glycerol, 0.5 mM DTT, and 67 mM β-mercaptoethanol (pH 7.8) as described by Romagni and Dayan (2000). Protein concentration was measured using a Bio-Rad protein assay kit (BioRad, Hercules, CA, USA) based on the method of Bradford (1976), using bovine serum albumin (BSA) as standard.

Purification of a specific polyclonal antibody for FsPK1

A partial clone of the previously characterized cDNA for FsPK1 was expressed in *Escherichia coli* as described by Lorenzo *et al*. (2003). The fusion protein was affinity-purified through amylose resin (New England Biolabs, Beverly, MA, USA) following the manufacturer's instruction. The pure truncated protein with a molecular weight of approximately 40 kDa was used for immunization of rabbits following the procedures established by Immunostep (Immunostep, Salamanca, Spain).

The serum used for immunoblots was obtained after five series of immunization of 16 d. Purification of the immunoglobulin fractions (IgGs) of the immune serum was carried out according to the instructions supplied by Immunostep. The resultant IgGs were concentrated by ultracentrifugation on a PM-10 membrane (Amicon, Beverly, Massachusets, USA). The antibody was purified by immunoaffinity using the methods described by Kelly *et al*. (1986) and Sakakibara *et al*. (1991). Approximately 100 μg of the affinity purified fusion protein FsPK1 were subjected to electrophoresis, transferred to nitrocellulose membranes and stained with Ponceau red. The protein band corresponding to 40 kDa was first blocked with 1% (w/v) BSA in phosphate-buffered saline (PBS) for 60 min at room temperature and incubated at 4 °C with 1.5 ml of IgG purified fraction diluted 1:10 in 1% PBST (PBS buffer containing 0.05% (v/v) Tween-20 and 1% (w/v) BSA). The blots were then washed with PBST four times over 6 min before the IgG was eluted by incubating the membranes for 1 min in the presence of 0.5 ml of 0.2 M glycine, pH 2.2. The eluted product was adjusted to pH 7.5 with TRIS-HCl, pH 8.8, extensively dialysed against PBS and stored at –20 °C.

Western blot analysis

For protein immunoblotting, 50 μg of soluble proteins from crude extracts of *F. sylvatica* seeds were analysed. Polypeptides were separated under denaturing conditions by SDS/PAGE (Laemmli, 1970) using a Bio-Rad Mini Protean II. The proteins on the gel were then electrophoretically transferred to nitrocellulose filters (0.45 μm, BA85, Schleicher and Schuell) and immunoprobed. Polyclonal FsPK1 antibody was used at 1:400 dilution in PBST with 0.5% (w/v) BSA. After three washes in PBST, the membranes were incubated for 1 h with the secondary antibody (goat anti-rabbit IgG peroxidase conjugate; Vector laboratories, Burlingame, CA, USA) diluted 1:1000 in PBST with 3% (w/v) BSA. After two washes in PBST, immunocomplexes were detected using peroxidase-conjugated antibodies. Peroxidase activity was revealed by incubation in 0.02% (v/v) H_2O_2 and 4 mM 4-chloro-1-naphthol in PBS.

Immunoblot and immunohistochemical localization

For immunolocalization of the protein, plant material was fixed and embedded in paraffin as described by Jackson (1992) and FsPK1 protein was immunolocalized essentially as described by Nylander *et al*. (2001). Plant material was fixed overnight at 4% (w/v)

paraformaldehyde in 0.1 M PBS (pH 7.2). Fixed material was dehydrated in a graded ethanol series (50–100%) and afterwards absolute ethanol was replaced by histological clearing agent (Histoclear, National Diagnostic, UK) and tissues were embedded in paraffin. Microtome 10 μm thick sections were affixed in poly-L-lysine coated glass slides, deparaffined in Histoclear, rehydrated in decreasing concentrations of ethanol (100–70%) and washed with TRIS-buffered saline (TBS). Endogenous alkaline phosphatase activity was blocked by boiling in 10 mM sodium citrate (pH 6.0) for 5 min at 750 W in a microwave oven. After the solution had cooled down, microscope slides were rinsed with water and TBS. After that, sections were blocked for 30 min in TBS containing 5% (w/v) BSA and incubated in primary antibody diluted in TBS with 3% (w/v) BSA for 2 h. Primary antibody was FsPK1 antisera used at 1:400 dilution. After several washes in TBST (TBS buffer containing 0.05% (v/v) Tween-20 and 1% (w/v) BSA), sections were incubated for 1 h with the secondary antibody (goat antirabbit IgG alkaline phosphatase conjugate; Sigma, St Louis, MO, USA) diluted 1:100 in TBS with 3% (w/v) BSA. Excess of antibody was washed off as described above. Development was performed in darkness in TBS containing 50 mM $MgCl_2$ (pH 9.4), 75 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt (BCIP; Roche, Mannheim, Germany) and 150 mg ml^{-1} 4-nitroblue tetrazolium chloride (NBT; Roche). Reactions were stopped by a 10 min rinse in water. All sections were dehydrated, mounted in rapid mounting media (Entellan, Merck, Germany), and observed under a light microscope.

Nuclei were counterstained with 4′-6′diamidino-2-phenylindol (DAPI, 1 μg ml^{-1}) fluorochrome. The samples were observed under a DAS Leica (Leica Inc., Wetzlar, Germany) DMRD epifluorescence microscope, equipped with a beam splitter (cut-off 400 nm), an excitation filter (340–380 nm), and a barrier filter (430 nm).

Results

Specificity of a polyclonal antibody against FsPK1 of beechnuts

Immunoblot analysis of FsPK1 revealed that the IgG affinity-purified from immune serum mainly recognized a 40 kDa polypeptide within a protein extract from *Escherichia coli* transformed with the partial FsPK1 cDNA (Fig. 1A, lane I). The polyclonal antibody purified by immunoaffinity cross-reacted only with the 40 kDa recombinant polypeptide in the same extract of proteins (Fig. 1A, lane II) and also specifically recognized a 55 kDa polypeptide, the translation product of FsPK1 mRNA, in protein extracts obtained from beechnuts (Fig. 1A, lane III), which coincides with the predicted size for this protein, as shown in the diagram of the whole protein including the catalytic domain and the N-terminal region (Fig. 1B).

Accumulation of FsPK1 during imbibition of beech seeds

In a previous report, a cDNA was isolated, corresponding to a functional protein kinase, named FsPK1, whose expression was up-regulated by ABA and decreased during stratification in water at 4 °C, which correlates with the maintenance or release from seed dormancy caused by these treatments (Lorenzo *et al*., 2003).

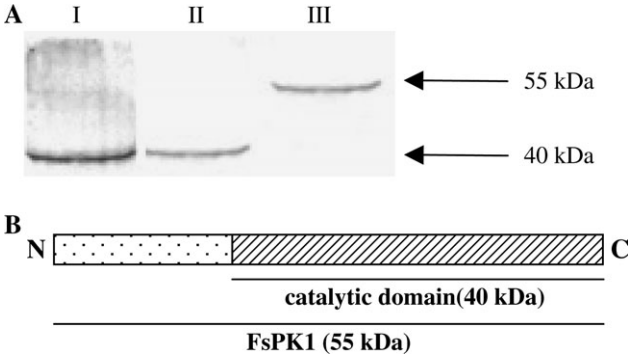

Fig. 1. Immunoblot analysis of FsPK1. (A) I, The IgG purified from immune serum mainly recognizes the truncated (40 kDa) FsPK1 polypeptide in protein extracts from transformed *Escherichia coli* cells. II, Specific cross-reaction of the immunopurified IgG to the 40 kDa polypeptide from the same protein extract. III, Specific identification of a 55 kDa polypeptide (full length FsPK1 protein) in protein extracts of beechnuts. The size (in kDa) of the major cross-reacting bands are indicated on the right. (B) Schematic diagram showing the N-terminus and the catalytic domain of FsPK1.

In the present study, the steady-state levels of FsPK1 protein have been examined by immunoblotting to determine whether the amount of FsPK1 mRNA detected in *Fagus* seeds (Lorenzo *et al*., 2003) subjected to the different treatments described in the Materials and methods was correlated with the accumulation of the corresponding polypeptide. Total soluble proteins were extracted from dormant, ABA-treated, GA_3-treated, PCB-treated, or stratified seeds and immunoprobed with the FsPK1 antibody (Fig. 2). The FsPK1 polypeptide was not detected in dormant seeds, clearly accumulated after ABA treatment, which inhibits seed germination, and was also present in the first weeks of imbibition in water, decreasing along with stratification at 4 °C, treatment that produces a slow release from dormancy. These results are coincident with the level of FsPK1 transcripts (Lorenzo *et al*., 2003). In addition, this protein was undetectable after GA_3 treatment, which quickly induces germination of dormant *Fagus* seeds and was present in seeds imbibed over 4 weeks in the presence of paclobutrazol (PCB), a GA biosynthesis inhibitor that maintains the dormant state in beechnuts (Nicolás *et al*., 1997), while a further addition of GA_3 to the PCB-treated seeds is able to inhibit the accumulation of FsPK1, since no signal was detected (Fig. 2B).

Tissue localization of FsPK1

Immunohistochemical analysis was performed to ascertain the spatial distribution of FsPK1 in *F. sylvatica* seeds and the effects on it of the different treatments that either maintain the dormant state or allow seed germination. Tissue sections from 2–6 weeks of imbibition subjected to the different treatments were incubated with the immunoaffinity-purified FsPK1 antibody and used in the immunolocalization experiments, as described in the Materials and methods.

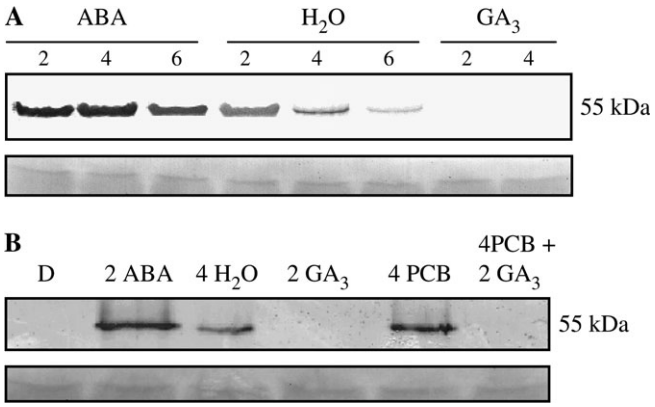

Fig. 2. Western blot analysis of FsPK1 polypeptide in a extract of proteins from *Fagus sylvatica* seeds. (A) Soluble proteins were extracted from seeds stratified at 4 °C from 2–6 weeks in 100 μM ABA, water, and 100 μM GA_3. (B) Soluble proteins extracted from dormant seeds (D) and seeds stratified 2 weeks in 100 μM ABA, 4 weeks in water, and 2 weeks in 100 μM GA_3 (as controls), 4 weeks in 10 μM paclobutrazol and 4 weeks in 10 μM paclobutrazol plus 2 weeks in 100 μM GA_3 in the dark. The M_r of the band cross-reacting to FsPK1 antiserum is indicated on the right. (A, B, bottom panels) Blots stained with Ponceau red as an equal loading indicator.

The signal for FsPK1 protein was clearly detected in ABA-treated seeds, both in the cotyledon and the embryo axis (Fig. 3), and specifically in the vascular cells (Fig. 4). In stratified seeds the staining was milder than in ABA-treated seeds, being mainly detected in the embryonic axis and decreasing along stratification. No signal was detected after 6 weeks of stratification nor upon GA_3 treatment (Fig. 3). In addition, immunohistochemical localization of FsPK1 in longitudinal sections of embryo axes of the ABA-treated seeds showed a precise staining in the nucleus of the cells within the vascular tissue of the apical meristem (Fig. 5A–D). The location of the nuclei was verified by a specific fluorescent DNA staining (DAPI) (Fig. 5E, F).

Discussion

There is strong biochemical and genetic evidence for a fundamental role of ABA in the induction and maintenance of seed dormancy and in the inhibition of the transition from embryonic to germinative growth in a variety of seeds (Le Page-Degivry and Garello, 1992; Leung and Giraudat, 1998; Grappin *et al*., 2000; Rock, 2000) including *Fagus sylvatica* (Le Page-Degivry *et al*., 1997), while GAs are usually required to overcome the germination arrest in ABA-modulated embryo dormancy (Steber *et al*., 1998; Debeaujon and Koornneef, 2000).

It has previously been reported that *Fagus sylvatica* seeds (beechnuts) display an embryo dormancy maintained by ABA and overcome by stratification at 4 °C or GA_3 treatment (Nicolás *et al*., 1996, 1997).

Moreover, protein phosphorylation controlled by kinases is involved in many hormonal signalling responses and particularly in ABA-mediated processes, including some PKs identified in seeds (Walker-Simmons, 1998).

The isolation of FsPK1 has been reported, a protein kinase from *Fagus sylvatica* induced by ABA in cotyledons that disappeared during stratification at 4° C (Lorenzo *et al*., 2003), coinciding with the release from seed dormancy and the onset of germination of these seeds (Nicolás *et al*., 1996).

To go further in the characterization of this protein, in the present work immunological analysis and immunohistochemical localization of FsPK1 have been performed. In order to do that, a specific polyclonal antibody was obtained against this protein kinase and its specificity was demonstrated using proteins from *E. coli* extracts expressing the corresponding fusion protein and total proteins obtained from *Fagus* seeds (Fig. 1).

Western blot assays show a concordance between the *FsPK1* expression (Lorenzo *et al*., 2003) and the amount of protein (Fig. 2), since it is hardly detected in dormant seeds, its accumulation increases after ABA treatment when seeds are unable to germinate and embryo growth is arrested (Nicolás *et al*., 1996; 1997; Lorenzo *et al*., 2003), and decreases during stratification in water where seeds are slowly being alleviated of dormancy, the embryo growth starts and the radicle emergence is evident after 6 weeks of imbibition (Nicolás *et al*., 1996).

Fagus dormant embryos contain high levels of ABA conjugates that are hydrolysed upon imbibition to constitute a potential source of active ABA, and cold stratification reduces the ABA levels in these seeds by both decreasing ABA synthesis and inducing ABA catabolism (Le Page-Degivry *et al*., 1997). These data correlate with the absence of FsPK1 in the dormant dry seed (Fig. 2B), its increase during the first weeks of imbibition in water and its disappearance along stratification (Fig. 2B), which together with its induction by ABA, indicate that this hormone regulates FsPK1 synthesis and accumulation. In addition, this protein is not immunodetected in GA_3 treatment (Fig. 2A), while it is present upon addition of paclobutrazol (PCB) and disappears after a further addition of GA_3, indicating that GAs are also involved in the control of FsPK1 accumulation. Therefore, FsPK1 accumulates in non-germinating seeds and disappears when seeds are able to germinate after the addition of GA_3 or upon stratification, which has been shown to increase the endogenous levels of GAs in beechnuts (Fernández *et al*., 1997), suggesting the involvement of FsPK1 in beechnut germination arrest.

Analysis of ABA- and GA-deficient mutants have established the important role of both hormones in the induction and breaking of seed dormancy (McCourt, 1999). However, experiments with double deficient mutants (Steber *et al*., 1998; Debeaujon and Koornneef, 2000) indicate that the level of dormancy or germination ability depend on the balance between ABA and GA, and hence, that seed dormancy is regulated by the interaction between both hormones (Brady and McCourt, 2003). This study's

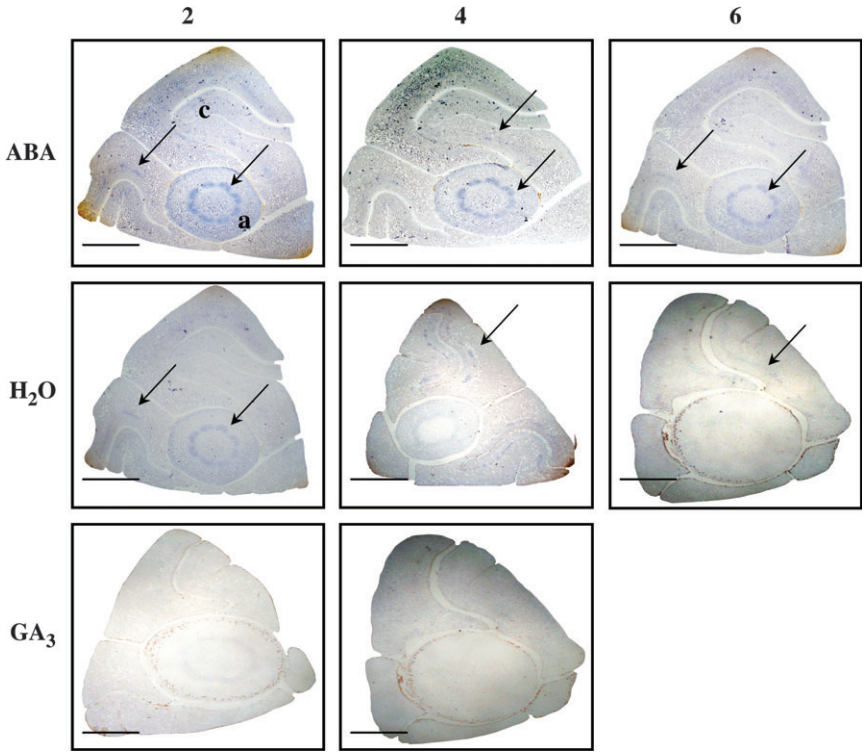

Fig. 3. Immunohistochemical localization of FsPK1 protein in transverse sections of ABA-treated, stratified or GA-treated seeds, respectively, from 2–6 weeks. Section of seeds were stained with primary FsPK1 antisera. The immunoreacted peptide was incubated with anti-rabbit IgG alkaline phosphatase conjugate antibody and was detected in PBS containing NBT/BCIP as substrates resulting in blue staining. Arrowheads indicate the immunostaining detected. Bars: 1 mm; c: cotyledons; a: embryo axis.

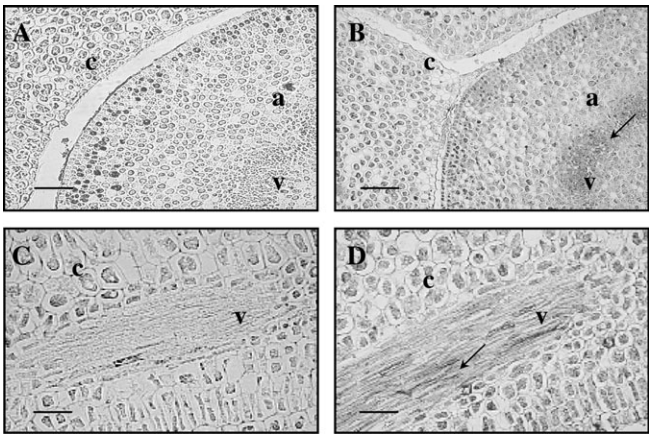

Fig. 4. (A, B) Magnification pictures of the transverse section of the ABA-treated seeds showing the vascular tissue of the embryo axis incubated without primary antibody as negative control (A) and with FsPK1 antisera (B). (C, D) Magnification pictures of the transverse section of the ABA-treated seeds showing the vascular tissue of the cotyledon incubated without primary antibody as negative control (C) and with FsPK1 antisera (D). Arrowheads indicate the immunostaining detected. Bars: 200 μm (A, B), 100 μm (C, D); c: cotyledons; a: embryo axis; v: vascular tissue.

results on FsPK1 agree with this proposal and suggest an antagonistic interaction between ABA and GAs in the regulation of the synthesis and accumulation of this protein, as shown for barley PKABA1 (Gómez-Cadenas *et al*., 2001) although a GA-modulated protein degradation cannot be discarded (Peng and Harberd, 2002).

To investigate further the possible role of this protein in the transition from dormancy to germination and/or in the embryo growth in beechnuts, the tissue localization of FsPK1 in seeds was undertaken by immunohistochemical analysis (Figs 3, 4, 5). This protein accumulates in ABA-treated seeds, mainly in the growth-arrested embryonic axes, but decreases and dissapears upon stratification and GA_3 treatment (Fig. 3). Therefore, these effects on the accumulation and decline of FsPK1 correlate with the germination ability of the seeds (Nicolás *et al*., 1996) and support a previous suggestion that this protein may be involved in the germination arrest (Lorenzo *et al*., 2003).

Furthermore, in the embryo axis of ABA-treated seeds FsPK1 protein is just located in the nucleus of the cells within the vascular tissue of the apical meristem (Figs 4, 5), which is in agreement with the nuclear localization signal found in the protein sequence (Lorenzo *et al*., 2003). In addition, this PK contains a consensus sequence involved in binding of 14-3-3 proteins (Lorenzo *et al*., 2003), and since these proteins have been reported to act in the nucleus as transcriptional complex regulators in response to different hormonal and environmental signals, including ABA (Finnie *et al*., 1999), it can be speculated that FsPK1 may participate as a component in this process.

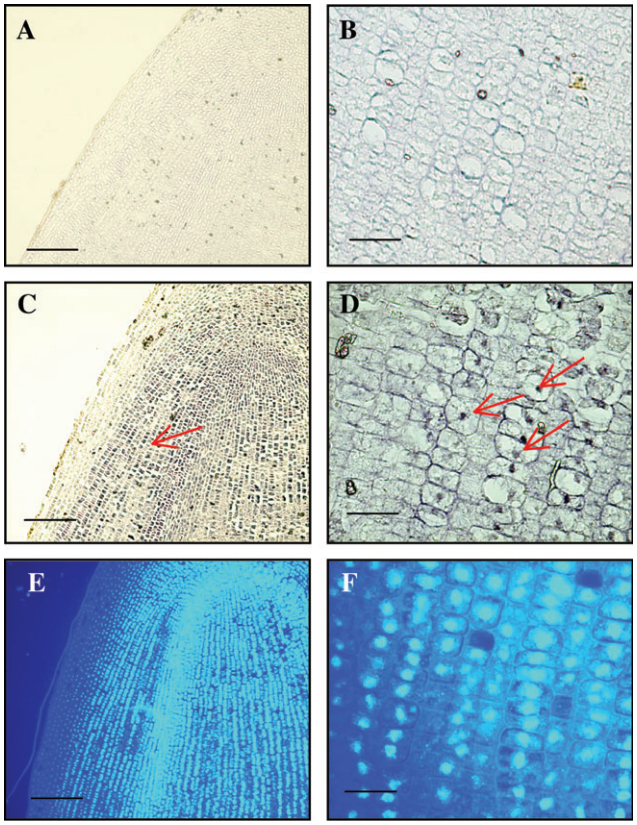

Fig. 5. Longitudinal sections of the cells located in the vascular tissue of the embryonic axis corresponding to the future apical meristem of the root in ABA-treated seeds incubated without primary antibody as negative control (A, B) and with FsPK1 antisera (C, D). (E, F) DAPI staining showing the induced fluorescence in the nuclei. Arrowheads indicate the immunostaining detected. Bars: 200 μm (A, C, E), 50 μm (B, D, F).

All these data suggest a regulatory role for this protein kinase in the mechanisms involved in the transition from dormancy to germination in *F. sylvatica* seeds, where it might participate in the final steps of the ABA signalling cascade inside the nucleus.

The specific location of FsPK1 in the vascular cells of the apical meristem of the embryonic axis (Fig. 5), the region of cell proliferation for root growth, together with protein accumulation when germination is arrested and its disappearance when germination starts, suggest that this protein may participate in the mechanisms regulating the onset of embryo growth in beechnuts, probably by interfering with the phloem function, which is critical to feed the growing cells near the root apex. A similar location has been reported for the transcript corresponding to a brassinosteroid up-regulated gene that promotes elongation in soybean epicotyls (Clouse, 1996) and for an mRNA encoding an ABA-induced and dormancy-related GTP-binding protein in *Fagus sylvatica* seeds (Nicolás *et al*., 1998).

Previous studies in apple proved the importance of cotyledons in embryo dormancy (Côme and Durand, 1971; Thévenot and Côme, 1973); the ABA present in the cotyledons was responsible for embryo dormancy and hormone leaching relieved dormancy allowing radicle growth. Interestingly, the FsPK1 transcript was only detected in beechnuts cotyledons (Lorenzo *et al*., 2003) while the protein is present both in cotyledons and embryo axes, indicating that the FsPK1 protein is being transported from the cotyledon to the embryo axis in ABA-treated seeds and, therefore, could participate in the germination arrest by blocking the embryo growth, while the cessation of this transport would result in the onset of seed germination.

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