

# Negative Regulation of Abscisic Acid Signaling by the *Fagus sylvatica* FsPP2C1 Plays A Role in Seed Dormancy Regulation and Promotion of Seed Germination<sup>1</sup>

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FsPP2C1 was previously isolated from beech (*Fagus sylvatica*) seeds as a functional protein phosphatase type-2C (PP2C) with all the conserved features of these enzymes and high homology to ABI1, ABI2, and PP2CA, PP2Cs identified as negative regulators of ABA signaling. The expression of FsPP2C1 was induced upon abscisic acid (ABA) treatment and was also up-regulated during early weeks of stratification. Furthermore, this gene was specifically expressed in ABA-treated seeds and was hardly detectable in vegetative tissues. In this report, to provide genetic evidence on FsPP2C1 function in seed dormancy and germination, we used an overexpression approach in *Arabidopsis* because transgenic work is not feasible in beech. Constitutive expression of FsPP2C1 under the cauliflower mosaic virus 35S promoter confers ABA insensitivity in *Arabidopsis* seeds and, consequently, a reduced degree of seed dormancy. Additionally, transgenic 35S:FsPP2C1 plants are able to germinate under unfavorable conditions, as inhibitory concentrations of mannitol, NaCl, or paclobutrazol. In vegetative tissues, *Arabidopsis* FsPP2C1 transgenic plants show ABA-resistant early root growth and diminished induction of the ABA-response genes *RAB18* and *KIN2*, but no effect on stomatal closure regulation. Seed and vegetative phenotypes of *Arabidopsis* 35S:FsPP2C1 plants suggest that FsPP2C1 negatively regulates ABA signaling. The ABA inducibility of FsPP2C1 expression, together with the transcript accumulation mainly in seeds, suggest that it could play an important role modulating ABA signaling in beechnuts through a negative feedback loop. Finally, we suggest that negative regulation of ABA signaling by FsPP2C1 is a factor contributing to promote the transition from seed dormancy to germination during early weeks of stratification.

The phytohormone abscisic acid (ABA) plays important regulatory roles in many plant stress and developmental responses throughout the plant life cycle, particularly in the ability to sense and respond to various unfavorable environmental conditions, including drought, salt, and cold stresses during vegetative growth (Marcotte et al., 1992; Koornneef et al., 1998; Leung and Giraudat, 1998). In seeds, ABA is involved in the acquisition of nutritive reserves, desiccation tolerance, maturation, development, and maintenance of dormancy and germination (Marcotte et al., 1992; Rock and Quatrano, 1995; Koornneef et al., 1998).

Genetic analysis has identified the crucial role of ABA in seed dormancy, as well as the requirement for gibberellins (GAs) in germination (Koornneef and Karssen, 1994), mainly using *Arabidopsis* because of its excellent suitability for genetic and molecular

studies (Koornneef et al., 1984), and because its germination responses are similar to those of many species used in seed physiology research (revised in Koornneef et al., 2002). However, beech (*Fagus sylvatica*) seeds represent a suitable model to study seed dormancy of woody plants exhibiting a specially deep degree of dormancy maintained by ABA and overcome by stratification or gibberellic acid treatment (Nicolás et al., 1996, 1997, 1998).

Most of the physiological responses regulated by ABA include changes in gene expression, and many genes and proteins have been identified as involved in ABA signaling, 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 signaling molecules, including second messengers such as cADPR and Ca<sup>2+</sup> (Wu et al., 1997; Pandey et al., 2000). In particular, ABA signaling appears to involve RNA-binding proteins HYL1, ABH1, or SAD1 (Lu and Fedoroff, 2000; Hugouvieux et al., 2001; Xiong et al., 2001), and a complex network of positive and negative regulators, including kinases, phosphatases, and transcriptional regulators (for review, see Finkelstein et al., 2002; Abe et al., 2003).

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A role for protein phosphorylation/dephosphorylation in these ABA-mediated processes has been assessed involving several specific protein kinases and phosphatases (Leung and Giraudat, 1998; Finkelshtein et al., 2002). For instance, the protein kinase PKABA1, which is induced by ABA and suppresses GA-inducible gene expression in barley (*Hordeum vulgare*) aleurone layers (Gómez-Cadenas et al., 1999, 2001), the guard cell-specific protein kinase AAPK essential for ABA-induced stomatal closing (Li et al., 2000), or OST1 as a key element mediating stomatal regulation in response to drought (Mustilli et al., 2002). In addition, genetic evidences have shown the involvement of three Arabidopsis Ser/Thr protein phosphatases 2C (ABI1, ABI2, and AtPP2CA) as negative regulators of the ABA signal transduction cascade (Gosti et al., 1999; Merlot et al., 2001; Tahtiharju and Palva, 2001). Whereas ABI1 and ABI2 are key regulators of ABA signaling in seeds and in vegetative tissues (Leung et al., 1994, 1997; Meyer et al., 1994; Rodríguez et al., 1998), AtPP2CA does not appear to regulate ABA signaling in dehydration responses (Tahtiharju and Palva, 2001). A pharmacological approach has been very useful in the identification of other protein phosphatases that may function in ABA signaling. For instance, cyclosporin A, an inhibitor of Ser/Thr protein phosphatases of the type 2B (PP2B), reduces the ABA response in pea (*Pisum sativum*) epidermal peels (Hey et al., 1997). The inhibitor of Ser/Thr protein phosphatases of the type 1 (PP1) and the type 2A (PP2A), okadaic acid, reduces ABA-induced stomatal closure in Arabidopsis (Pei et al., 1997), but in contrast, it enhances ABA-induced stomatal closure in fava bean (*Vicia faba*; Schmidt et al., 1995) and activates ABA-responsive promoters in tomato (*Lycopersicon esculentum*) hypocotyl cells (Wu et al., 1997). The only disruption mutant found is in a regulatory subunit of PP2A (RCN1) and confers ABA insensitivity to Arabidopsis (Kwak et al., 2002). Finally, the stomatal closure induced by ABA in *Commelina communis* is prevented by phenylarsine oxide, a specific inhibitor of protein Tyr phosphatases (MacRobbie, 2002).

Most knowledge of the genes and pathways involved in ABA signaling has been mainly based on loss-of-function experiments. However, gain-of-function mutants (or transgenic plants) are more significant from a biotechnological viewpoint because the character of interest can be easily transferred to crop plants by transformation (Wilkinson et al., 1997).

We previously reported the cloning of *FsPP2C1*, a functional PP2C from beechnuts, and showed that *FsPP2C1* is up-regulated upon addition of ABA to seeds and also during early weeks of stratification (Lorenzo et al., 2001). Therefore, as tools to transform beech are not available, we took advantage of the constitutive expression of *FsPP2C1* in Arabidopsis to investigate the role of this protein in the regulation of ABA responses in dormancy and germination. Here,

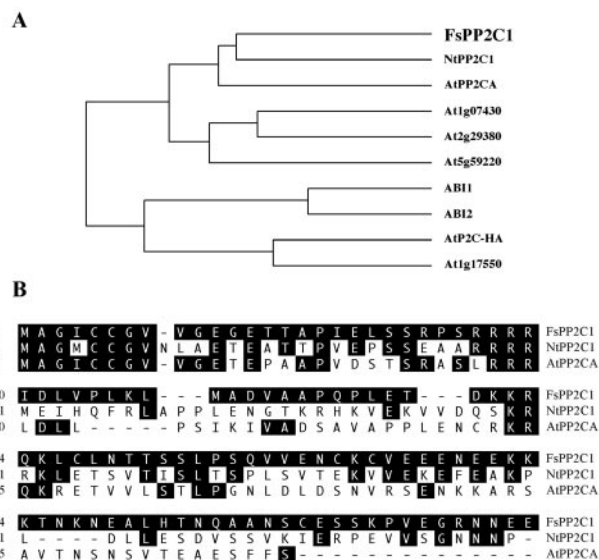
we show that gain-of-function of *FsPP2C1* is sufficient to confer ABA insensitivity in seed dormancy and germination under unfavorable conditions. Furthermore, *FsPP2C1* transgenic plants show ABA-resistant early root growth and diminished induction of the ABA-response genes *RAB18* and *KIN2*, but no effect on the stomatal closure regulation was observed. Taken together, these results are consistent with a role of *FsPP2C1* as a negative regulator of ABA signaling in beech seeds.

## RESULTS

### FsPP2C1 Is a Plant PP2C

We have previously reported the isolation and characterization of *FsPP2C1* as a functional plant PP2C, with all the conserved features of the catalytic domain of these proteins (Lorenzo et al., 2001). The phylogenetic relationship between *FsPP2C1* and the closely related plant PP2Cs is shown in Figure 1A. The cluster contains PP2Cs that have been physiologically characterized and proposed to participate in ABA signaling, in particular, Arabidopsis ABI1, ABI2 (Merlot et al., 2001), and PP2CA (Sheen, 1998; Tahtiharju and Palva, 2001). Additionally, two subgroups can be clearly distinguished, one including *FsPP2C1* and several PP2CA-like proteins, and the other one including ABI1 and ABI2.

Whereas the catalytic domain among these PP2Cs shows high identity, the N-terminal extension of



**Figure 1.** A, Phylogenetic tree of the plant PP2Cs reported to the updated databases with high similarity to beech *FsPP2C1* (AJ277743). Arabidopsis ABI1 (X77116), ABI2 (Y08965), AtPP2C-HA (AJ003119), AtPP2CA (D38109), AtPP2Cs (At1g17550, At1g07430, At2g29380, and At5g59220), and tobacco (*Nicotiana tabacum*) NtPP2C1 (Q9FEW0). Accession numbers are inside brackets. B, Alignment of the *FsPP2C1* N-terminal extension with AtPP2CA from Arabidopsis and NtPP2C1 from tobacco. Positions with identical amino acids residues are highlighted in black.

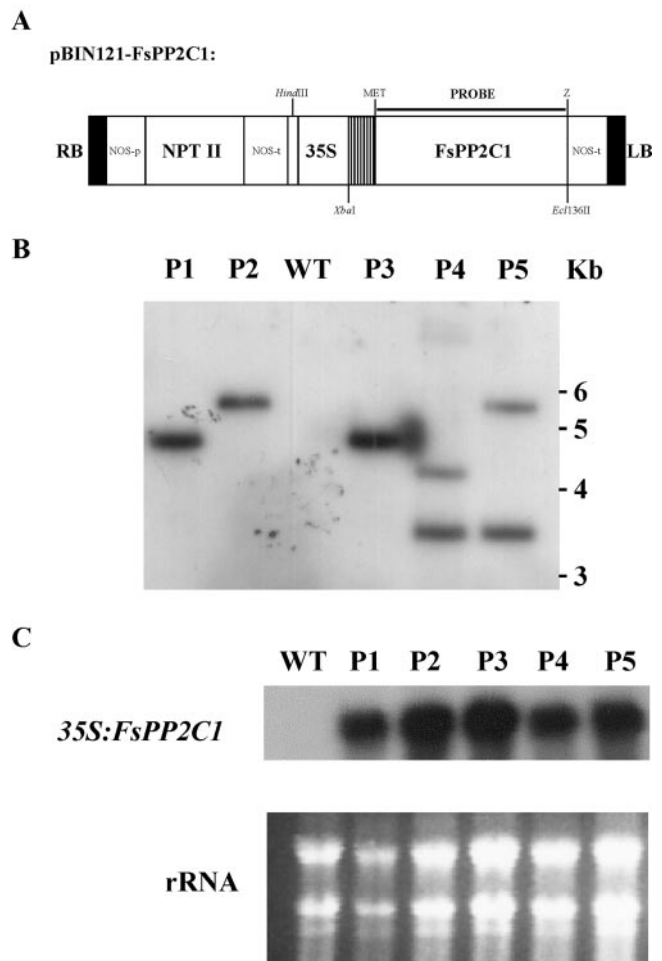
FsPP2C1 is only similar to that of AtPP2CA (Kuro-mori and Yamamoto, 1994) and NtPP2C1, showing some stretches of sequence identity (about 35%; Fig. 1B) and suggesting a functional similarity. AtPP2CA is a negative regulator of ABA responses during cold acclimation (Tahtiharju and Palva, 2001). *NtPP2C1* is up-regulated in response to drought stress, but its role in ABA signaling has not been investigated (Vranova et al., 2000).

#### Generation and Characterization of *35S:FsPP2C1* Transgenic Lines

Previously, we showed that *FsPP2C1* was specifically expressed in ABA-treated dormant seeds and this expression negatively correlated with germination (Lorenzo et al., 2001). To gain genetic evidence on the role of *FsPP2C1* in seed dormancy and germination, we used an overexpression approach in Arabidopsis because transgenic work is not feasible in beechnut. Transgenic plants were created as described in "Materials and Methods" (Fig. 2A). T<sub>1</sub> and T<sub>2</sub> kanamycin-resistant lines were recovered and five T<sub>3</sub> homozygous lines were finally selected. Southern-blot analysis displayed double and single insertions of the *35S:FsPP2C1* transgene (Fig. 2B). Expression levels of the *35S:FsPP2C1* transgene in the five different lines analyzed by northern blot are shown in Figure 2C. High expression was observed in all the transgenic lines, whereas no expression was detected in Arabidopsis wild-type plants, as expected. Out of the five T<sub>3</sub> homozygous lines obtained, the three lines with a single insertion were selected for further analysis.

#### Constitutive Expression of *FsPP2C1* in Arabidopsis Confers ABA Insensitivity in Seeds

The specific induction of *FsPP2C1* expression by ABA in beech seeds (Lorenzo et al., 2001) as well as the sequence homology of *FsPP2C1* to AtPP2CA, ABI1, and ABI2, prompted us to test whether constitutive expression of *FsPP2C1* in Arabidopsis would affect ABA sensitivity in seeds. Seed germination in media supplemented with ABA of *FsPP2C1*-overexpressing transgenic plants is shown in Figure 3A. After 10 d poststratification, radicle emergence and development of green and expanded cotyledons was partially inhibited in wild-type seeds at 1  $\mu$ M ABA and completely under 3  $\mu$ M ABA. In contrast, *35S:FsPP2C1* seeds were able to germinate and grow under these conditions, and, therefore, they showed reduced sensitivity to ABA. To further substantiate this result, we compared the germination of ABA-deficient (*aba2-11*), ABA-insensitive mutants (*abi1* and *abi2*), and wild-type seeds with that of *35S:FsPP2C1* transgenics in media supplemented with ABA (Fig. 3B). The addition of 0.5  $\mu$ M ABA decreased the percentages of germination in wild type and

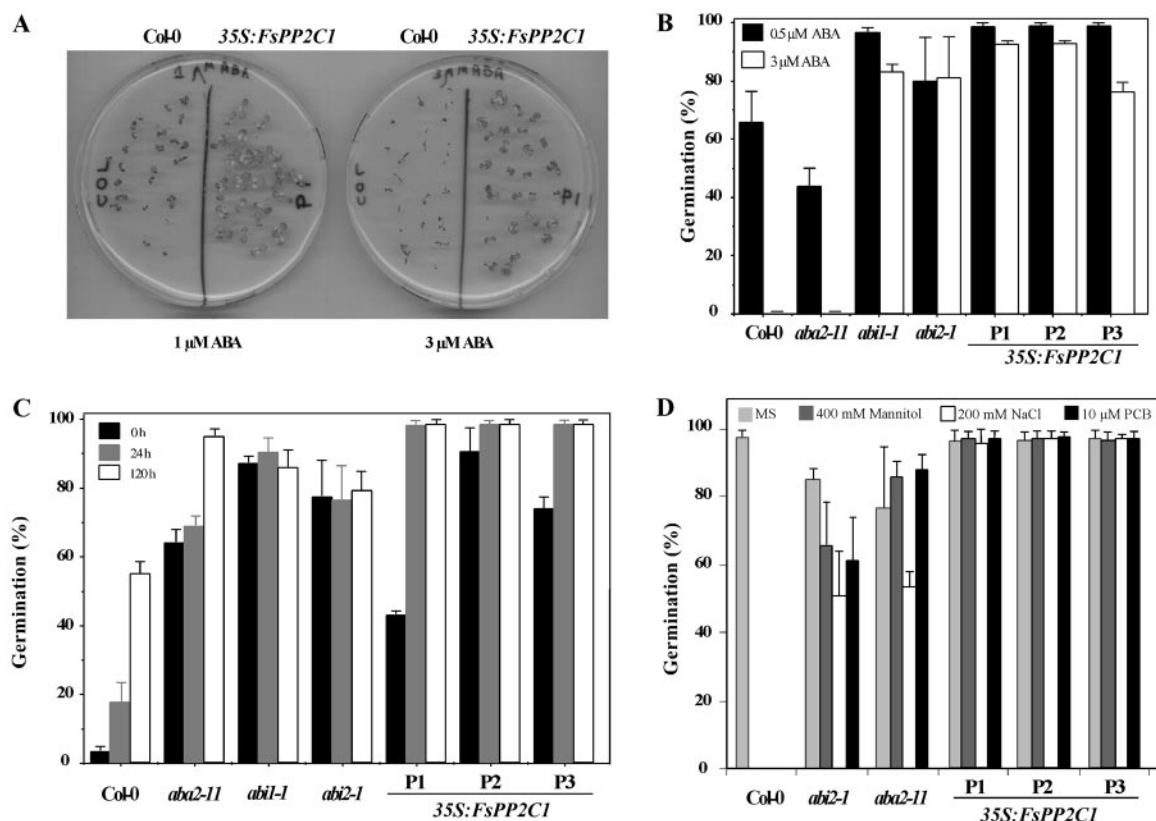


**Figure 2.** Generation and molecular analysis of *35S:FsPP2C1* transgenic lines. A, Construct used for plant transformation. RB, Right T-DNA border; LB, left T-DNA border; 35S, cauliflower mosaic virus 35S promoter; NPTII, neomycin phosphotransferase II; NOS-p, NOS promoter; NOS-t, NOS terminator. B, Southern-blot analysis of transgenic lines overexpressing *FsPP2C1*. Wild type (WT), Col-0 background; P1 to P5, transgenic lines harboring a *35S:FsPP2C1* transgene. Genomic DNA was digested with *Hind*III, blotted onto a nylon membrane, and hybridized with the *FsPP2C1* probe depicted in A. DNA ladder was used as molecular size markers. C, RNA-blot analysis of transgenic lines overexpressing *FsPP2C1*. Total RNA (10  $\mu$ g/line) from wild-type (Col-0) plants and P1 to P5 transgenic plants was isolated and hybridized with *FsPP2C1*. Bottom, ethidium bromide-stained gel showing rRNAs.

*aba2-11* to 65% and 45%, respectively, whereas no germination at all was observed under 3  $\mu$ M ABA. Clearly, under a concentration fully inhibitory for the wild-type (3  $\mu$ M ABA), *FsPP2C1*-overexpressing lines reached over 80% germination, which is very similar to the one observed for the ABA-insensitive *abi1-1* and *abi2-1* mutants.

Mature Arabidopsis seeds exhibit primary dormancy when freshly released from the mother plant, which means that seeds are unable to germinate under the appropriate environmental conditions without the help of dormancy-breaking agents such as





**Figure 3.** A, ABA germination assay of *35S:FSP2C1* seeds. Picture showing the differences in germination of *FsPP2C1*-overexpressing seeds and wild-type (Col-0) plants after 10 d in 1 and 3  $\mu\text{M}$  ABA. B, Percentage of seeds that germinated and developed green cotyledons in the presence of 0.5  $\mu\text{M}$  ABA (black bar) and 3  $\mu\text{M}$  ABA (white bar). Seeds were scored 7 d after sowing. C, Dormancy assay of *FsPP2C1*-overexpressing seeds. Germination percentage was determined at 5 d after 0 h (black bar), 24 h (gray bar), and 120 h (white bar) of stratification at 4°C. Three independent *FsPP2C1* transgenic lines in wild-type (Col-0) backgrounds were used, as well as the indicated ABA mutants and wild-type plants. D, Stress germination assays. Germination rating is represented as the percentage of seeds that germinated and developed green cotyledons in the presence of Murashige and Skoog medium, 400 mM mannitol, 200 mM NaCl, or 10  $\mu\text{M}$  PCB. Three independent *FsPP2C1* transgenic lines in wild-type (Col-0) backgrounds were used (P1, P2, and P3), as well as the indicated mutants and wild-type plants. Seeds were scored 7 d after sowing. Error bars represent  $\pm$  SD of three independent experiments with about 100 seeds plated per data point and carried out with similar results.

stratification or GAs (Koorneef and Karssen, 1994). To determine the degree of dormancy of *35S:FSP2C1* seeds, we compared the germination percentage of the seeds harvested at the same time after different cold treatment periods (0, 24, and 120 h) with that of wild-type plants and ABA-related mutants that produce nondormant seeds (*aba2-11*, *abi1-1*, and *abi2-1*). As a result, all of the *FsPP2C1* transgenic lines exhibited a reduced dormancy compared with the wild type, very similar to that of *aba* and *abi* mutants (Fig. 3C). In the absence of stratification at 4°C, *FsPP2C1* transgenic seeds were able to germinate, reaching 100% germination after 1 d of treatment.

An additional seed germination assay was carried out in the presence of paclobutrazol (PCB), a well-known inhibitor of GA biosynthesis. GAs are antagonistic to ABA, and, therefore, seeds with reduced sensitivity to ABA (or diminished ABA levels), show PCB-resistant germination (Koorneef et al., 1998). In contrast to wild-type seeds, *35S:FSP2C1* and *aba2-11*

and *abi2-1* seeds were able to germinate and develop green cotyledons in medium supplemented with 10  $\mu\text{M}$  PCB, indicating a reduced requirement for GAs at this developmental stage (Fig. 3D).

These results demonstrate that *35S:FSP2C1* seeds exhibit a reduced degree of seed dormancy and insensitivity to inhibition of germination by exogenous ABA and PCB, suggesting that *FsPP2C1* might be involved in ABA responsiveness in seeds.

#### *35S:FSP2C1* Seeds Exhibit Resistance to Salt and Osmotic Stresses

It has been previously suggested that ABA regulates many different stress responses. To test whether the ABA resistance induced by overexpression of *FsPP2C1* is also effective against other ABA-mediated stresses that increases ABA levels, we analyzed the seed germination response of *FsPP2C1*

transgenic lines in the presence of high concentrations of NaCl and mannitol (Fig. 3D) and compared it with that of wild-type plants.

Seed germination under 400 mM mannitol and 200 mM NaCl leads to a severe delay in radicle emergence and further growth arrest in wild-type individuals; in contrast, *35S:FSP2C1* seeds were able to germinate and develop green cotyledons under such conditions, even to a higher extent than *abi2* and *aba2* mutants do (Fig. 3D). These results indicate that *FsPP2C1*-expressing seeds are osmotolerant and resistant to inhibitory salt concentration in this germination assay.

#### Effect of *FsPP2C1* Overexpression in Vegetative Tissues

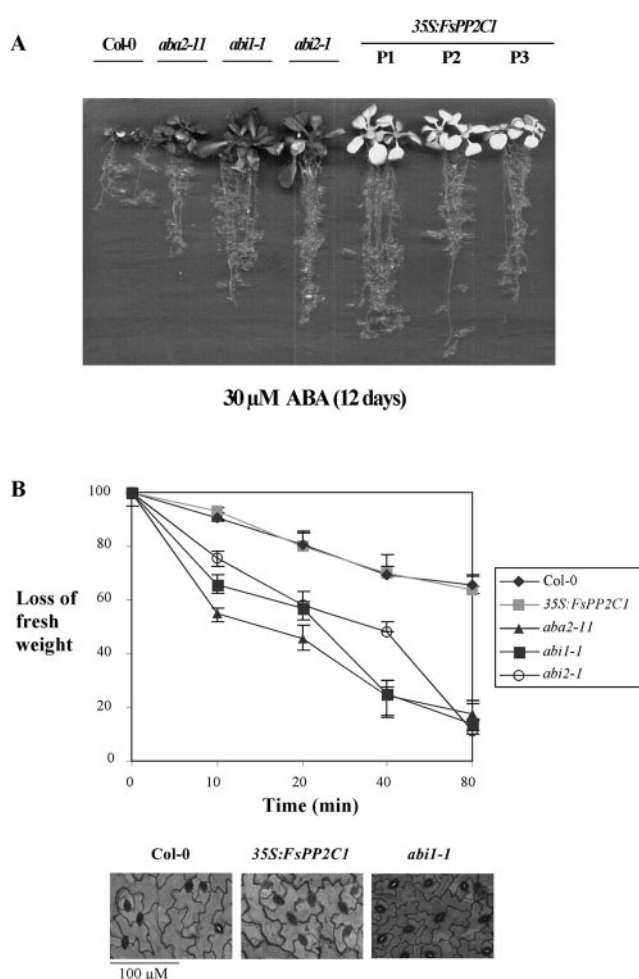
To determine whether *FsPP2C1* overexpression affected whole plant phenotypes, we analyzed ABA sensitivity in vegetative tissues of wild-type plants, ABA-related mutants (*aba2-11*, *abi1-1*, and *abi2-1*), and three *35S:FSP2C1* transgenic lines.

ABA has an inhibitory effect on root growth and consequently, ABA-insensitive mutants are resistant to this ABA-mediated process (Himmelbach et al., 1998). Twelve-day-old *35S:FSP2C1* seedlings grown in the presence of 30  $\mu\text{M}$  ABA showed a reduced inhibition of root growth compared with wild-type plants, and similar to that of *abi* mutants (Fig. 4A).

However, prolonged culture of *35S:FSP2C1* plants under 30  $\mu\text{M}$  ABA led to growth arrest of the aerial part of the plant and yellowing of the leaves, whereas *abi1-1* and *abi2-1* mutants remained green and grew under these conditions (Fig. 4A). When grown in the absence of ABA, the *FsPP2C1*-overexpressing plants did not display any visible phenotypic alteration (data not shown).

ABA triggers stomatal closure and consequent reduction in water loss under drought conditions. The Arabidopsis ABA-insensitive mutants *abi1-1* and *abi2-1* are impaired in the ABA-induced stomatal closure and, therefore, in their ability to limit transpiration upon drought. On the contrary, *FsPP2C1* overexpression did not affect stomatal regulation because detached leaves of *35S:FSP2C1* plants showed similar rates of transpiration than wild type under ambient conditions (35% relative humidity) and clearly different from *aba* and *abi* mutants, which lost approximately 40% to 50% fresh weight after 40 min (Fig. 4B). Furthermore, preopened stomata of *35S:FSP2C1* plants closed similarly to wild type (one representative line is shown in Fig. 4B, bottom) as compared with *abi1* mutant where stomata failed to close, consistent with the inability of *abi1* plants to reduce water loss upon drought.

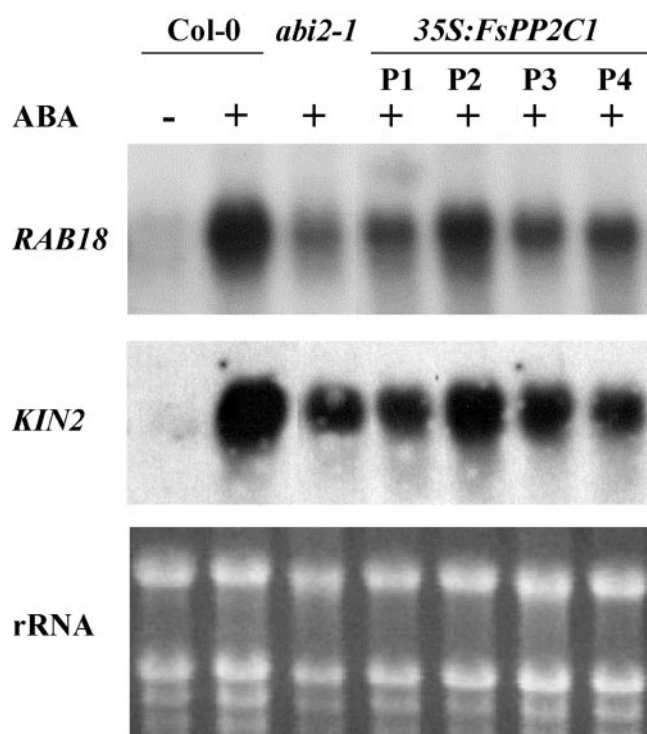
To examine whether the reduction in ABA sensitivity in transgenic plants was accompanied by altered expression of ABA-responsive genes, we compared the expression of *RAB18* and *KIN2* in *35S:FSP2C1* with that of Columbia (Col-0) and *abi2-1*



**Figure 4.** A, Root growth assay for scoring ABA sensitivity. Growth of Col-0, *aba2-11*, *abi1-1*, *abi2-1*, and *35S:FSP2C1* plants in medium supplemented with 30  $\mu\text{M}$  ABA. The picture was taken after 12 d of the transfer of 5-d-old seedlings from Murashige and Skoog medium to plates containing 30  $\mu\text{M}$  ABA. B, Micrographs showing the stomatal closure regulation of *35S:FSP2C1* plants compared with wild-type plants and *abi1-1*. All pictures were taken at the same scale (Bar = 100  $\mu\text{m}$ ). Loss of fresh weight was measured in detached rosette leaves of Col-0, *35S:FSP2C1*, or ABA mutant (*aba2-11*, *abi1-1*, and *abi2-1*) plants. Data values represent one of three independent experiments with similar results.

(Fig. 5). *RAB18* (Lang and Palva, 1992; Parcy et al., 1994) is an ABA-inducible gene whose expression is drastically inhibited in *abi1-1* and *abi2-1* mutants (Leung et al., 1997), and *KIN2* is a cold-regulated gene previously described as ABA inducible (Wang et al., 1995) but down-regulated in the *abh1* mutant (Hugouvieux et al., 2001). In that respect, *35S:FSP2C1* plants behave as *abi2-1* mutant as they showed a severe reduction (3- to 5-fold) in the expression of *RAB18* and *KIN2* upon ABA treatment.

Taken together, these results indicate that expression of *FsPP2C1* in vegetative tissues partially interferes with ABA signaling, affecting ABA-mediated inhibition of root growth and ABA-responsive



**Figure 5.** Expression of the ABA-regulated *RAB18* and *KIN2* genes in *35S:F<sub>s</sub>PP2C1* plants (P1 to P4) compared with wild-type (Col-0 transgenic plants with the kanamycin resistance gene) and *abi2-1* controls. mRNA levels of the indicated genes were determined by northern-blot analysis using total RNAs (10  $\mu$ g/line) isolated from mock-treated (-) or ABA-treated (+, 50  $\mu$ M ABA for 3 h) plants. Bottom, ethidium bromide-stained gel showing rRNAs.

gene expression but having no effect on stomatal regulation.

## DISCUSSION

In seeds, ABA has been shown to play an important role in the formation, maintenance of dormancy, and inhibition of germination (Koornneef and Karssen, 1994; Bewley, 1997), and later on in the early seedling growth arrest under unfavorable environmental conditions (López-Molina et al., 2001; González-Guzmán et al., 2002).

Our work is focused on beech seed physiology (Nicolás et al., 1996) and on the mechanisms involved in ABA action during seed dormancy and germination (Nicolás et al., 1997, 1998; Lorenzo et al., 2001, 2002). In our previous reports (Lorenzo et al., 2001, 2002), we described two PP2Cs, *F<sub>s</sub>PP2C1* and *F<sub>s</sub>PP2C2*, which represent the first PP2Cs described in woody plants (Kerk et al., 2002). *F<sub>s</sub>PP2C1* catalytic domain showed high identity to the ABA-related PP2Cs *ABI1*, *ABI2*, and *AtPP2CA*, widely involved in ABA signaling in Arabidopsis, but divergences in the N-terminal extension were found. The amino acid sequence identity to *AtPP2CA* (Kuromori and Yamamoto, 1994) and *NtPP2C1* (Vranova et al., 2000)

in the noncatalytic N-terminal region is nearly 35%. The N-terminal extension has been suggested to facilitate the interaction with different substrates (Rodríguez, 1998), and N-terminal deletions of *ABI1* and *AtPP2CA* led to enhanced PP2C activity, indicating a regulatory function for this domain (Sheen, 1998). Interestingly, evidence that *AtPP2CA* C-terminal (catalytic) domain is directly involved in the interaction with *ATK2* (Chérel et al., 2002) and *ATK3* (Vranova et al., 2001) has been provided.

In addition to their sequence similarity, *F<sub>s</sub>PP2C1* is also similar to *ABI1*, *ABI2*, and *AtPP2CA* in their inducibility by ABA. The specificity of their function has been suggested to depend on their differential expression patterns. Thus, *AtPP2CA* is highly expressed in leaves (Tahtiharju and Palva, 2001), whereas *ABI2* is mainly expressed in stems and roots, and *ABI1* strongly expressed in leaves, roots, and stems (Leung et al., 1997). In the case of *F<sub>s</sub>PP2C1*, transcript expression is also tissue specific and was found to accumulate in ABA-treated seeds rather than in other vegetative tissues (Lorenzo et al., 2001), suggesting that their function might be restricted to this tissue. Beside *F<sub>s</sub>PP2C1*, no other PP2C with a seed-specific expression pattern has been reported. In fact, most of the phenotypes observed in the transgenic plants are related to the seed.

The features of *F<sub>s</sub>PP2C1*, that is, sequence similarity to ABA-related PP2Cs and ABA up-regulation in seeds, made it a logical candidate as a regulator of ABA signaling in seeds and, consequently, as a regulator of seed dormancy. Genetic evidence was necessary to assess whether *F<sub>s</sub>PP2C1* functions as a positive or a negative regulator of seed dormancy and germination. In the present study, we used an over-expression approach in Arabidopsis to investigate *F<sub>s</sub>PP2C1* function, due to the lack of genetic tools in beechnuts (Fig. 2). Studies of orthologous genes and functional tests in heterologous systems have shown that the ABA signal transduction pathway is mostly conserved among evolutionary distant plant species (for review, see Finkelstein et al., 2002). In case *F<sub>s</sub>PP2C1* was a positive regulator of ABA signaling, constitutive expression in Arabidopsis could lead to enhanced seed responses to ABA and, consequently, to enhanced dormancy. Conversely, in case *F<sub>s</sub>PP2C1* was a negative regulator of ABA signaling, constitutive expression would lead to diminished ABA-response in seeds and, consequently, to a reduced dormancy.

Seeds of *35S:F<sub>s</sub>PP2C1* transgenic plants displayed reduced dormancy, similar to the *abi* mutants (Fig. 3C), which is indicative of diminished responsiveness to endogenous ABA in seeds (Gosti et al., 1999). Whereas the germination of wild-type Arabidopsis seeds was suppressed by 3  $\mu$ M ABA, *F<sub>s</sub>PP2C1*-overexpressing transgenic seeds emerged radicle and developed green and expanded cotyledons under these conditions (Fig. 3, A and B), as ABA-insensitive



mutants do (Koornneef et al., 1984; Finkelstein, 1994). Taken together, these data are consistent with a role of FsPP2C1 as a negative regulator of ABA signaling in seeds. In addition to up-regulation by ABA, *FsPP2C1* expression also increased after 2 weeks imbibition at 4°C, suggesting a role for this gene during the first weeks of stratification (Lorenzo et al., 2001). Taking into account that FsPP2C1 is a negative regulator of ABA signaling, blockade of the ABA response by FsPP2C1 might be a requisite to break dormancy during stratification. The reliability of the physiological role of FsPP2C1 is also reinforced by the germination assay in the presence of the GA biosynthesis inhibitor PCB (Fig. 3D). GAs and ABA play antagonistic roles in seed germination, and apparently, ABA-insensitive (or ABA-defective) individuals need less GAs during germination (Léon-Kloosterziel et al., 1996). Clearly, the GA requirement was reduced in *35S:FSP2C1* seeds compared with wild-type seeds (Fig. 3D), suggesting that expression of FsPP2C1, through blockade of ABA signaling, positively regulates seed germination.

Another important role of ABA is the prevention of seed germination under unfavorable water conditions (González-Guzmán et al., 2002). Compelling evidence has shown that osmotic stress delays seed germination and arrests early seedling development. Several studies show that ABA plays an inhibitory role in these processes because ABA-insensitive and ABA-deficient mutants are able to bypass them (Werner and Finkelstein, 1995; Léon-Kloosterziel et al., 1996; López-Molina et al., 2001; González-Guzmán et al., 2002). As previously observed with *aba2*, the three *FsPP2C1*-expressing lines tested were able to germinate and carry out early growth in medium with the organic solute mannitol as well as inhibitory salt concentration (200 mM NaCl) compared with the incapability of wild-type plants (Fig. 3D). These results indicate that FsPP2C1-induced resistance is not restricted to a nonionic osmotic imposed stress, and further suggests a general insensitivity to osmotic stress. Although *FsPP2C1* transcripts were not affected by water deficit in beechnuts (Lorenzo et al., 2001), *FsPP2C1* overexpression in Arabidopsis overcome the inhibition of seed germination under low water potential conditions.

Interestingly, constitutive and ectopic expression of *FsPP2C1* partially influences ABA responses in vegetative tissues of Arabidopsis. Thus, inhibition of early root growth by 30  $\mu$ M ABA was notably reduced in *35S:FSP2C1* seedlings compared with wild-type plants. However, *FsPP2C1*-overexpressing seedlings are more sensitive to ABA at further stages of development. After 12 d in 30  $\mu$ M ABA, *35S:FSP2C1* leaves showed wilting, whereas *abi1-1* and *abi2-1* mutant leaves remained green and turgid (Fig. 4A). Another well-characterized ABA-mediated response is stomatal closure regulation. ABA promotes

stomatal closure reducing water loss by transpiration during drought. Transpiration rate measured by the loss of fresh weight of detached rosette leaves in *FsPP2C1*-overexpressing plants, and the stomatal closure showed an ABA-response similar to wild-type plants, clearly different from *abi* mutants impaired in the ABA-induced stomatal closure in Arabidopsis and, therefore, in their ability to limit transpiration upon drought (Pei et al., 2000; Fig. 4B). In the same way, ABA-mediated drought responses were not affected by inhibition of *AtPP2CA* expression (Tahtiharju and Palva, 2001).

These results indicate that ABA responsiveness by *FsPP2C1* overexpression is mainly restricted to seeds and they also suggest lack of the corresponding signaling component (substrate, activator, or partner) or instability of *FsPP2C1* as a foreign protein in these tissues. In beech vegetative tissues, other PP2Cs may act in response to ABA, as the *FsPP2C2* previously described by Lorenzo et al. (2002) with a broad range of expression.

The attenuation of the ABA signal in *35S:FSP2C1* plants is further sustained by the diminished induction of the ABA-responsive genes *RAB18* and *KIN2*. *RAB18* is a dehydrin only found in ABA-treated plants and accumulates in Arabidopsis dry seeds (Nylander et al., 2001). *abi1-1* and *abi2-1* mutants show impaired induction of this gene (Leung et al., 1997) that is induced in antisense *AtPP2CA* plants (Tahtiharju and Palva, 2001). *KIN2* is a cold-regulated protein whose transcriptional regulation and gene expression are induced by low temperature, ABA, osmoticum, and dehydration (Wang et al., 1995; Abe et al., 2003). The induction of *KIN2* was severely reduced in *abh1* mutant (Hugouvieux et al., 2001).

ABA responses depend on coordinated interactions between positive and negative regulators required for the proper control of this complex signaling pathway that operate in a cell. Some genes have been identified as negative regulators of the pathway, including the homeodomain protein, *ATHB6* (Himmelbach et al., 2002), and the farnesyl transferase  $\beta$ -subunit, *ERA1* (Cutler et al., 1996; Pei et al., 1998). Protein farnesylation of certain signaling proteins appears crucial for negative regulation of ABA signaling, as recently suggested in the case of *ABI3* (Brady et al., 2003). In addition, genetics of *abi1* and *abi2* mutants, transient expression studies, as well as analysis of transgenic PP2CA antisense plants characterize PP2Cs as negative regulators of ABA signaling (Sheen, 1998; Gosti et al., 1999; Merlot et al., 2001; Tahtiharju and Palva, 2001). Therefore, a complex regulatory mechanism seems to have evolved to attenuate ABA signaling and avoid undesirable effects (i.e. inhibition of cell cycle) due to sustained activation of the ABA pathway.

Recent controversy on the role of *ABI1* has arisen from the work of Wu et al. (2003). On the basis that

ABI1 overexpression in *Arabidopsis* does not affect the ABA-signaling pathway and that microinjection of ABI1 protein had no apparent effect on ABA-induced *RD29A-β-glucuronidase* and *KIN2-β-glucuronidase* expression, the authors conclude that their results are not compatible with a negative regulatory role of ABI1 in ABA signaling. These results rise the question whether ABI1 catalyzes a rate-limiting step in ABA signaling more than questioning its role as a negative regulator of the pathway. However, the results of Wu et al. (2003) contrast with previous results reported by Sheen (1998), which showed that overexpression of ABI1 in maize (*Zea mays*) protoplasts led to a blockade of ABA-inducible gene expression. Nevertheless, additional overexpression experiments as well as loss-of-function studies with several PP2Cs are required to resolve the above controversy.

Results reported in this work with FsPP2C1 are in agreement with those reported by Sheen (1998) for ABI1 and PP2CA, and they show for the first time that overexpression of a PP2C in transgenic plants leads to a diminished response to ABA, therefore, we conclude that FsPP2C1 functions as a negative regulator of ABA signaling in seeds and early seedling growth. FsPP2C1 overexpression leads to a reduced seed dormancy, as compared with untransformed plants, and a promotion of germination under inhibitory concentrations of ABA or high-osmotic media. This ABA-insensitive phenotype, together with the ABA-induced expression of FsPP2C1 transcripts specifically in seeds, suggests that this PP2C, through modulation of ABA signaling, is a factor contributing to promote the transition from seed dormancy to germination during early weeks of stratification.

## MATERIALS AND METHODS

### Plant Material

The *Arabidopsis* wild-type and transgenic plants used throughout this work were the Col-0 ecotype. They were routinely grown in a growth chamber under 40% humidity, a temperature of 22°C, and with a 16-h light/8-h dark photoperiod at 80 to 100  $\mu\text{E m}^{-2} \text{s}^{-1}$  in pots containing a 1:3 vermiculite:soil mixture. For in vitro culture, seeds were surface sterilized in 70% (v/v) ethanol and 1% (v/v) Triton X-100 for 20 min, soaked for 10 min in 2.5% (v/v) bleach and 0.05% (v/v) Triton X-100, and finally, washed four times in sterile distilled water. Stratification of the seeds was conducted during 3 d at 4°C, otherwise as indicated. Afterward, seeds were sowed on Murashige and Skoog (1962) plates containing solid medium composed of Murashige and Skoog basal salts and 1% (w/v) Suc, solidified with 1% (w/v) agar and the pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber.

### Vector Construction and Plant Transformation

To generate the construction, the coding region of the FsPP2C1 cDNA was excised from the pSKFsPP2C1 (Lorenzo et al., 2001) using an *XhoI-XbaI* double digestion and was subcloned into *SmaI-Ecl136II* doubly digested pBIN121 vector. The T-DNA region of the pBIN121-FsPP2C1 construct was transferred to *Agrobacterium tumefaciens* C58C1 (pGV2260; Deblaere et al., 1985) by electroporation. *Arabidopsis* plants (Col-0 ecotype) were transformed by the floral dip method (Clough and Bent, 1998). Seeds were harvested and plated on kanamycin selection medium (50  $\mu\text{g mL}^{-1}$ ) to

identify T<sub>1</sub> transgenic plants. From 34 T<sub>1</sub> kanamycin-resistant lines recovered, approximately 60% showed an ABA-insensitive phenotype in seed germination as compared with untransformed plant or plants transformed with the empty vector. Approximately 100 of the T<sub>2</sub> seeds were plated on Murashige and Skoog kanamycin agar plates and transgenic lines with a 3:1 (resistant:sensitive) segregation ratio were selected. Southern-blot analysis was performed as described by Lorenzo et al. (2002) to select lines carrying a single T-DNA copy. T<sub>3</sub> progenies homozygous for kanamycin resistance were used for further studies.

### Germination Assays

To measure ABA sensitivity, seeds were plated on solid medium composed of Murashige and Skoog basal salts, 1% (w/v) Suc, and different concentrations of ABA (0.5, 1, or 3  $\mu\text{M}$ ). For the dormancy assay, seed lots to be compared were harvested on the same day from individual plants grown in identical environmental conditions and were stratified during 0, 1, and 5 d at 4°C. Each value represents the average germination percentage of 80 to 100 seeds with the SES of at least three replicates.

To determine sensitivity to inhibition of germination by high osmoticum, the medium was supplemented with 400 mM mannitol and by salt with 200 mM NaCl. To measure PCB sensitivity, seeds were plated on medium containing 10  $\mu\text{M}$  PCB. The percentage of seeds that had germinated and developed fully green expanded cotyledons was determined in all the assays after 7 d of sowing.

### Root Growth and Transpiration Assays

The root growth assay for scoring ABA sensitivity was done by measuring root growth after 5 d of the transfer of 7-d-old seedlings onto vertical Murashige and Skoog plates containing 30  $\mu\text{M}$  ABA. For the transpiration assays, the loss of fresh weight of excised leaves was measured at room temperature. To this end, four leaves at the same developmental stage and size from single 3-week-old plants were excised and fresh weight was determined at ambient conditions after the indicated periods of time.

In the stomatal study, leaves from 4-week-old plants with preopened stomata were detached, and paradermal sections of abaxial epidermis were fixed during 6 min in Romeis solution (44.8% alcohol, 10% formol, and 2% acetic acid, all v/v), washed with water during 15 min, stained for 5 min in Giemsa (Giemsa-Lösung; Merck, West Point, PA), and washed in running water during 15 min. Sections of the epidermis were dehydrated at 37°C until a color change was observed, and were then dehydrated with xilol for 3 min twice and Entellan (Merck). Observations and photographs were done on a light microscope (Ax70; Olympus, Melville, NY, with photographic digital system Apogee).

### Northern-Blot Analysis

Approximately 10 to 12 7-d-old seedlings were transferred from Murashige and Skoog plates to 125-mL flasks containing 25 mL of Murashige and Skoog solution and 1% (w/v) Suc. The flasks were shaken at 130 rpm under cool fluorescent light. After 10 d, seedlings were mock-treated or treated with 50  $\mu\text{M}$  ABA. Plant material was collected and frozen in liquid nitrogen. Total RNA was extracted as described (González-Guzmán et al., 2002), separated on formaldehyde-agarose gels, and blotted onto a nylon membrane. Blots were hybridized with random-priming <sup>32</sup>P-labeled probes (30  $\mu\text{Ci}$ ). Full-length cDNAs probe of FsPP2C1 was prepared as described previously (Lorenzo et al., 2001). The RAB18 probe was prepared by PCR amplification from genomic DNA of wild-type Col-0 plants with the primers described previously (González-Guzmán et al., 2002) and KIN2 probe was obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus; expressed sequence tag 224O21). Blots were exposed for 24 h in a Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA).

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