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ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination

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Summary

The development of a germinating embryo into an autotrophic seedling is arrested under conditions of water deficit. This ABA-mediated developmental checkpoint requires the bZIP transcription factor ABI5. Here, we used *abi3-1*, which is also unable to execute this checkpoint, to investigate the relative role of ABI3 and ABI5 in this process. In wild-type *Arabidopsis* plants, ABI3 expression and activity parallel those described for ABI5 following stratification. During this process, transcript levels of late embryogenesis genes such as *AtEm1* and *AtEm6* are also re-induced, which might be responsible for the acquired osmotic tolerance in germinated embryos whose growth is arrested. ABI5 expression is greatly reduced in *abi3-1* mutants, which has low *AtEm1* or *AtEm6* expression. Cross complementation experiments showed that *35S-ABI5* could complement *abi3-1*, whereas *35S-ABI3* cannot complement *abi5-4*. These results indicate that ABI5 acts downstream of ABI3 to reactivate late embryogenesis programmes and to arrest growth of germinating embryos. Although ABI5 is consistently located in the nucleus, chromosomal immunoprecipitation (ChIP) experiments revealed that ABA increases ABI5 occupancy on the *AtEm6* promoter.

Keywords: ABI3, ABI5, ABA, checkpoint, ChIP, embryogenic program.

Introduction

The breaking of seed dormancy to establish seedling growth represents a fragile phase in the life cycle of a plant. During this developmental transition to autotrophic growth, plants must be able to monitor environmental water status and mount appropriate adaptive responses. In spite of the importance of these stress responses to a plant's survival, their underlying molecular mechanisms are poorly understood.

Because of the established role of ABA in water stress response, it is reasonable to assume that ABA signalling regulates the processes that break seed dormancy and establish seedling growth. Physiological studies on the inhibitory effect of ABA on radicle growth during germination have delineated three developmental stages (Bewley and Black, 1985). In *Chenopodium album*, ABA does not stop seeds from reaching stages 1 (outer testa splitting) and 2 (radicle extension) but arrests development in stage 3, that is, before radicle penetration of inner testa and endosperm layers. However, the molecular mechanisms underlying these processes and their physiological relevance are unknown. Recently, the *ABI5* gene encoding a basic leucine-zipper (bZIP) transcription factor has been cloned (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000). We showed that in wild-type (WT) *Arabidopsis* the ABI5 protein is essential to execute an ABA-dependent growth arrest, which sets in after breakage of seed dormancy but prior to autotrophic growth (Lopez-Molina *et al.*, 2001). The *abi5-4* mutant, which lacks ABI5, is unable to execute this arrest. ABI5 accumulation is induced by ABA only within a short interval of about 60 h following stratification, during which ABA and ABA-dependent ABI5 activity are essential to initiate growth arrest of germinated embryos. The arrested, germinated embryos remain viable but quiescent, and osmotolerant as long as ABA is present. We have shown that the formation of arrested, germinated embryos represents an adaptive mechanism to increase the survival rate of *Arabidopsis* under conditions of water deficit (Lopez-Molina *et al.*, 2001). Studying the determinants of ABA-induced *ABI5* expression and how ABI5 is activated by ABA are essential to understanding the

mechanisms underlying early growth arrest and the mechanism by which osmotolerance is developed in arrested, germinated embryos.

One approach to investigating these issues is to analyse the effects of other *Arabidopsis* mutations besides *abi5*, which also prevent growth arrest by ABA following the end of stratification (Finkelstein, 1994; Koornneef *et al.*, 1984). These include the dominant mutations in the PP2C phosphatases ABI1 and ABI2 (Gosti *et al.*, 1999; Leung *et al.*, 1994; Leung *et al.*, 1997; Rodriguez *et al.*, 1998), and recessive mutations in the AP2 factor ABI4 and the embryogenesis factor ABI3 (Finkelstein *et al.*, 1998; Giraudat *et al.*, 1992). The ABA-mediated growth arrest of germinated embryos and their consequent osmotolerance led us to hypothesize that in these embryos there may have been a recapitulation of signal transduction pathways that establish seed desiccation tolerance during late embryogenesis. For this reason, we chose to study in more detail the role of ABI3 in the establishment of arrested, germinated embryos. However, this task is rendered difficult by the fact that ABI3, unlike ABI5, is an essential embryogenesis factor (McCourt, 1999). Indeed, previous phenotypic studies of *abi3* showed that ABI3 is a factor whose activity is essential for late embryo development, which takes place only after embryonic cell division and morphogenesis is complete (McCourt, 1999; Nambara *et al.*, 1995; Parcy *et al.*, 1994). In particular, ABI3 is necessary for the expression of a large number of late embryogenesis genes thought to be essential for the acquisition of desiccation tolerance (Parcy and Giraudat, 1997; Parcy *et al.*, 1994). Given its importance in late embryo development, it is likely that ABI3 controls signalling events by influencing the expression and activity of other regulatory factors (McCourt, 1999). Here, we have used a weak *abi3* allele that still undergoes late embryo maturation but is not arrested by ABA during germination (Bies-Etheve *et al.*, 1999).

OSVP1, the ABI3 ortholog in rice, has been reported to interact with and modulate the transcriptional activity of TRAB1, a rice bZIP factor homologous to ABI5 (Hobo *et al.*, 1999; Lopez-Molina and Chua, 2000). Recently, ABI3 has been shown to interact with ABI5 in a yeast two-hybrid assay suggesting that ABI3 might modulate transcriptional activity mediated by ABI5 (Nakamura *et al.*, 2001). Seeds of both *abi3* and *abi5* mutants have low expression levels of late embryogenesis genes such as *AtEm1* and *AtEm6,* which encode hydrophilic proteins believed to be important for desiccation tolerance (Vicient *et al.*, 2000). This observation provides evidence for a genetic interaction between *ABI3* and *ABI5* during embryogenesis. ABI5 likely regulates the expression of *AtEm1* and *AtEm6* by binding to ABA-responsive elements (ABREs) located in their promoters (Carles *et al.*, 2002; Nakamura *et al.*, 2001). Like those of ABI5, ABI3 protein levels rapidly decrease following stratification under normal conditions (Lopez-Molina *et al.*, 2001; Parcy *et al.*, 1994). However, the inability of *abi3* mutant embryos to display growth arrest by ABA suggested that ABI3 might be required for this process; therefore, this protein might also be expressed *de novo* following stratification and on ABA exposure.

Here we show that during germination, ABA can recruit *de novo* late embryogenesis programmes to confer osmotic tolerance in arrested, germinated embryos. *ABI3*, *ABI5* and late embryogenesis genes are reactivated by ABA during a short development window. We show that ABI3 is required for the ABA-dependent growth arrest because it acts upstream of ABI5 and is essential for *ABI5* gene expression. Using chromosomal immunoprecipitations (ChIP), we show that ABA increases the ABI5 occupancy on the *AtEm6* promoter, although the transcription factor is consistently located in the nucleus. Finally, using mass spectrometry, we have identified phosphoamino acids in the three conserved domains of ABI5. Mutagenesis experiments indicate that these phosphoamino acids are individually not essential for ABA-dependent ABI5 activity.

Results

Expression of ABI3 defines three developmental time windows

We have previously identified a plant growth checkpoint triggered by ABA, which takes place during the first 60 h following stratification (Lopez-Molina *et al.*, 2001). Because the growth-arrested embryos were osmotolerant, we surmised that late embryogenesis pathways establishing desiccation tolerance in embryos might be reactivated during this ABA-mediated process. Previous reports have established the important role of ABI3 in late embryogenesis (McCourt, 1999; Nambara *et al.*, 1995; Parcy *et al.*, 1994). To investigate its role in the ABA-mediated growth arrest of germinated embryos, we generated a polyclonal antibody against recombinant ABI3 and confirmed its specificity (Figure 1a). This antibody was used to follow ABI3 expression levels following stratification as well as during embryo germination with or without ABA. Figure 1(b) presents the experimental procedure used to uncover three developmental time windows of different ABA responsiveness.

The first time window takes place during stratification. ABI3 levels declined significantly after 3 days of stratification, regardless of the presence or absence of the hormone (lane DS, A– and A+, Figure 1c). Figure 1(c) shows two consecutive developmental windows of different ABA responsiveness following the first time window. The second time window takes place between the end of stratification and 60–72 h thereafter. Indeed, in the absence of ABA, ABI3 levels decreased (Figure 1c, lane A–) eventually falling to below detectable levels 36 h following stratification (Parcy *et al.*, 1994). When ABA was added to the medium after stratification, ABI3 levels began to increase 1 day later,

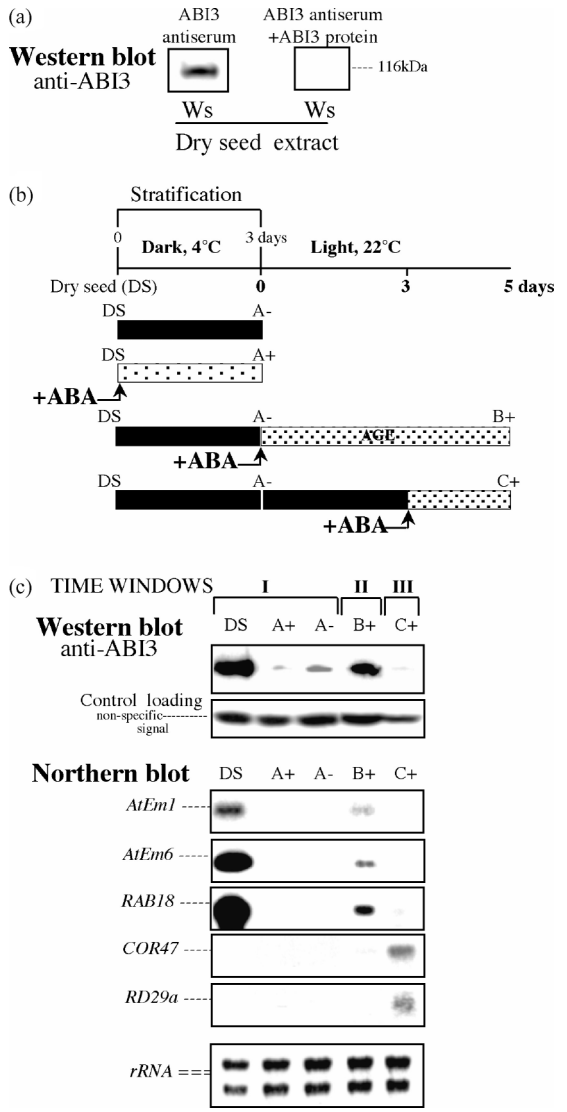

Figure 1. Northern and Western blot analyses reveal three developmental time windows of alternate ABA responsiveness following breakage of seed dormancy.
(a) Specificity of ABI3 antibody by competition experiments.
(b) WT Ws dry seeds (DS) were stratified and plant materials were harvested as following: sample A–, stratification without ABA; sample A+, stratification with 5 μM ABA; sample B+, seeds stratified without ABA were transferred to light in presence of 5 μM ABA for an additional 5 days; sample C+, seeds stratified without ABA were transferred to light in absence of ABA for 3 days and then transferred to 5 μM ABA for an additional 2 days.
(c) Western blot analyses of ABI3 protein levels and Northern blot analyses of *AtEm1*, *AtEm6*, *RAB18*, *COR47* and *RD29a* transcript levels performed on all samples. Protein and RNA were extracted from the same material. For Western blot analysis (10 μg lane^{-1}), a non-specific crossreacting signal (Mr = 30 kDa) was used as a loading control. For Northern blot analysis (3 μg lane^{-1}), rRNA levels were used as loading controls.

reaching plateau levels in growth-arrested, germinated embryos (Figure 1c, lane B+). However, ABI3 expression could not be induced when ABA was applied 3 days post-stratification (Figure 1c, lane C+) thereby defining the third developmental time window. These results of ABI3 paralleled those previously obtained with ABI5 (Lopez-Molina *et al.*, 2001). The data show that after stratification the embryogenesis factor ABI3 can be re-induced by ABA. Therefore, this factor may reactivate late embryogenesis pathways in germinated embryos on ABA exposure.

To test whether late embryogenesis genes are indeed re-induced in the growth-arrested germinated embryos, we analysed the expression of a set of ABA-responsive genes expressed in vegetative tissues and during late embryogenesis (Figure 1c). *AtEm1* and *AtEm6*, whose expression is regulated by ABI3 and ABI5 (Finkelstein and Lynch, 2000; Finkelstein, 1993; Lopez-Molina and Chua, 2000; Parcy and Giraudat, 1997; Parcy *et al.*, 1994), and *RAB18* (Lang and Palva, 1992; Parcy *et al.*, 1994) were chosen as representatives of late embryogenesis genes. After 3 days in the dark at 4°C, transcript levels of these late embryogenesis genes dropped from their peak levels in dry seeds (DS) to undetectable levels, and this reduction was not prevented by ABA (Figure 1c, lanes A– and A+). When ABA was present in the medium following stratification (Figure 1c, lane A+) transcript levels of *AtEm1*, *AtEm6* and *RAB18* began to increase 1 day thereafter, reaching plateau levels in arrested, germinated embryos (Figure 1c, lane B+). Early embryogenesis or vegetative ABA-responsive genes such as *COR47* and *RD29a* were not efficiently induced (Figure 1c, lane B+). This second time window coincides with the formation of growth arrested, germinated embryos (Lopez-Molina *et al.*, 2001). As expected, a 5- to 10-fold lower induction of *AtEm1* and *AtEm6* transcript levels was observed in *abi3-1* and *abi5-4* mutants (Bies-Etheve *et al.*, 1999; Lopez-Molina and Chua, 2000). Finally, in a third time window, only early embryogenesis or vegetative ABA-responsive genes (Parcy *et al.*, 1994) such as *COR47* and *RD29a* were efficiently induced if ABA was added 3 days post-stratification (Figure 1c, lane C+).

Taken together, our results demonstrate that transcript levels of late embryogenesis genes can be re-induced *de novo*, but only within a short developmental window (of 60–72 h) following the end of stratification. This developmental time window, during which the growth arrest of germinated embryos can occur, is preceded and followed by two time windows of distinct ABA responsiveness. The first one, triggered by stratification, is characterized by the decrease of late embryogenesis gene transcript levels in an ABA-independent manner, including *ABI3* (data not shown) and *ABI5* transcripts (Lopez-Molina and Chua, 2000; Lopez-Molina *et al.*, 2001). The third time window, taking place about 72 h post-stratification, is characterized by the onset of ABA responsiveness of early embryogenesis or vegetative ABA-responsive genes. The induction of *AtEm1* and *AtEm6* suggests that, in response to ABA, *Arabidopsis* can reactivate late embryogenesis pathways after embryo germination in order to acquire osmotolerance.

ABI3 and ABI5 protein levels in abi5-4 *and* abi3-1 *mutants*

Because ABA did not arrest growth of either *abi3-1* or *abi5-4* following stratification, we investigated whether these genetic lesions affect ABA signalling in a related or an independent manner. To this end, we monitored ABI3 and ABI5 protein levels in these two mutants after stratification, and in the presence or absence of ABA. Figure 2(a) shows that compared to WT plants, *abi5-4* plants displayed comparable ABI3 protein levels during the first 2 days in the presence of ABA. After 4 days, however, lower ABI3 levels were seen in *abi5-4* plants. This difference was particularly apparent at 6 days and likely reflects the developmental differences between WT and *abi5-4* at this stage rather than a downregulation of *ABI3* expression due to the ABI5 deficiency in the mutant. Whereas growth of the WT embryos was arrested by ABA, the *abi5-4* mutant embryos progressed to vegetative growth.

In contrast to *abi5-4*, ABI5 protein levels were strongly reduced (about 10-fold) at any time point in *abi3-1* plants in the presence of ABA (Figure 2b). This finding is consistent with a previous report in which *ABI5* transcript levels were shown to be downregulated in *abi3-1* seeds (Finkelstein and Lynch, 2000). These results indicate that ABI3 is essential for an ABA-dependent *ABI5* expression during the establishment of growth arrest of germinated embryos.

ABI3 overexpression cannot rescue the ABA-insensitivity of abi5-4

We investigated whether the slightly lower ABI3 levels in the *abi5-4* mutant could account for the ABA insensitivity of this mutant. To this end, we generated *abi5-4* transgenic lines carrying a *35S-ABI3* transgene. Transgenic lines of *abi5-4* expressing higher than WT ABI3 levels remained insensitive to ABA (Figure 2c). Neither *AtEm1* nor *AtEm6* transcripts were induced in *abi5-4*/*35S-ABI3* plant after 5 days in the presence of the hormone (Figure 2d). From these observations, we conclude that ABI3 is unable to complement the *abi5-4* mutant. These results are at variance with those of Soderman *et al*. (2000) using 13-day-old seedlings. These authors observed a hypersensitive response of *AtEm1* and *AtEm6* gene expression in 35S-ABI3 plants, irrespective of their genetic background, that is, C24, Ws, *abi5-1* (see also Parcy and Giraudat, 1997). However, discrepancies may be due to the fact that ectopic expression of ABI3 may recruit additional factors in seedlings which are not present in arrested and germinated embryos.

ABI3 overexpression confers ABA hypersensitivity to WT Ws transgenic plants

In contrast with the situation in *abi5-4* background, ABA hypersensitivity was observed when similar high ABI3 levels were expressed in WT Ws transgenic plants. Figure 2(e) shows that 0.5 μM ABA was able to arrest growth of germinated WT/35S-ABI3 transgenic embryos; however, this ABA concentration was insufficient to elicit a growth arrest in WT embryos. Whereas 0.5 μM ABA failed to cause the accumulation of detectable ABI5 levels in WT plants, the hypersensitivity observed in the WT/*35S-ABI3* transgenic lines was correlated with ABI5 accumulation (Figure 2e). These results are consistent with other observations showing that constitutive ABI3 expression in vegetative tissue leads to *ABI5* transcript over-accumulation (Finkelstein and Lynch, 2000). In the absence of ABA, ABI5 was undetectable in both WT (Lopez-Molina *et al*., 2001) and WT/35S-ABI3 lines (Figure 2f). Our results demonstrate that ABI3 is a positive regulator of *ABI5* expression during the formation of growth-arrested, germinated embryos, but its activity is dependent on the presence of ABA as it only induces ABI5 accumulation in the presence of the hormone. Taking advantage of the constitutive expression of ABI3 in vegetative tissues of the WT/*35S-ABI3* lines, we examined whether ABI3 protein levels would change on ABA exposure. Figure 2(g) shows that ABI3 levels were induced by ABA treatment suggesting that ABA regulates ABI3 accumulation post-transcriptionally.

ABI5 can rescue ABA-insensitivity of abi3-1

We next examined whether the 10-fold reduction in ABI5 protein levels observed in *abi3-1* (Figure 2b) could account for the ABA insensitivity of the mutant. We generated several *abi3-1* transgenic lines expressing different HA::ABI5 levels and compared them with *abi5-4* or WT Ler transgenic lines expressing comparable ABI5 protein levels (Figure 3a). Figure 3(a) shows that transgenic plants with similar ABI5 levels behaved similarly in ABA-triggered growth arrest of germinated embryos, irrespective of their genetic background. When ABI5 protein was expressed at steady-state levels comparable to those of WT (line #10, Figure 3a), *abi3-1/35S-ABI5* plants displayed ABA sensitivity similar to WT plants in seed germination assays (Figure 3b). Complementation was also observed at the level of *AtEm* gene expression (Figure 3b). In *abi3-1* plants, expression of the abi3-1 protein remained at low levels (Figure 3a,c).

Figure 3(a) also shows that the transgenic HA::ABI5 protein can induce the endogenous *ABI5* gene expression in *abi3-1* mutants as shown by the appearance of the endogenous ABI5 protein band, which migrated faster than the transgenic HA-tagged ABI5 band. Taken together, these results indicate that ABI3 is required for *ABI5* expression and that both ABI3 and ABI5 can induce *ABI5* expression in an ABA-dependent manner. In addition, our results suggest that ABI3 is not required for ABI5 activity.

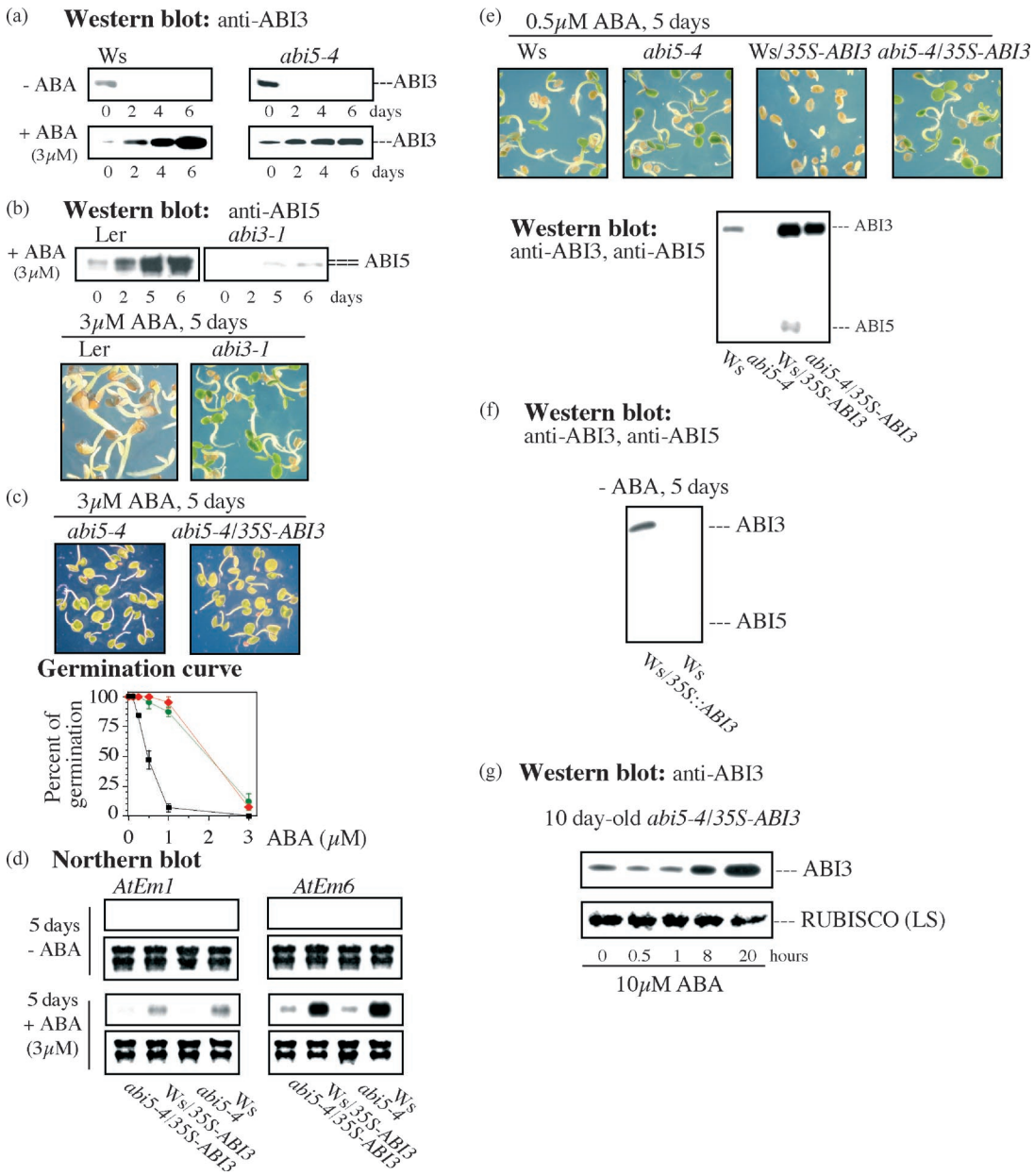

Figure 2. ABI3 expression is downregulated in the *abi5-4* mutant; ABI5 expression is strongly downregulated in the *abi3-1* mutant plant; ABI3 overexpression cannot rescue ABA insensitivity of *abi5-4* but causes ABA hypersensitivity in WT plants; ABA regulates ABI3 expression post-transcriptionally.
All pictures depict representative 5-day-old seedlings in the presence of ABA concentrations as indicated. Western and Northern blots were performed as described in Figure 1.
(a) Ws and *abi5-4* seeds were stratified and grown with or without 3 μM ABA. Proteins were extracted at the times indicated.
(b) WT Ler and *abi3-1* (Ler) seeds were stratified and grown on 3 μM ABA. Proteins were extracted at the times indicated.
(c) Germination frequencies of Ws (black curve), *abi5-4* (red) and *abi5-4/35S-ABI3* (green) seeds in the presence of ABA. Radical emergence was assayed 60 h after stratification.
(d) Ws, *abi5-4*, Ws/*35S-ABI3* and *abi5-4/35S-ABI3* seeds were stratified and grown in presence or absence of 3 μM ABA for 5 days before RNA extraction.
(e) Ws, *abi5-4*, Ws/*35S-ABI3* and *abi5-4/35S-ABI3* seeds were stratified and grown on 0.5 μM of ABA before protein extraction.
(f) Ws and Ws/*35S-ABI3* were stratified and grown without ABA for 5 days before protein extraction.
(g) 10-day-old *abi5-4/35S-ABI3* seedlings were transferred to 10 μM ABA plates. Proteins were extracted at the time points indicated. The Ponceau staining of the 55 kDa large unit of the ribulose 1,5-biphosphate carboxylase (RUBISCO) is used as a loading control.

ABI5 is constitutively localized in the nucleus

We previously reported that growth of germinating transgenic seeds constitutively expressing ABI5 were arrested only in the presence, but not in the absence, of ABA (Lopez-Molina *et al.*, 2001). Therefore, ABA regulates ABI5 activity.

One possible mechanism to regulate ABI5 activity might be to control its intracellular localization. To explore this

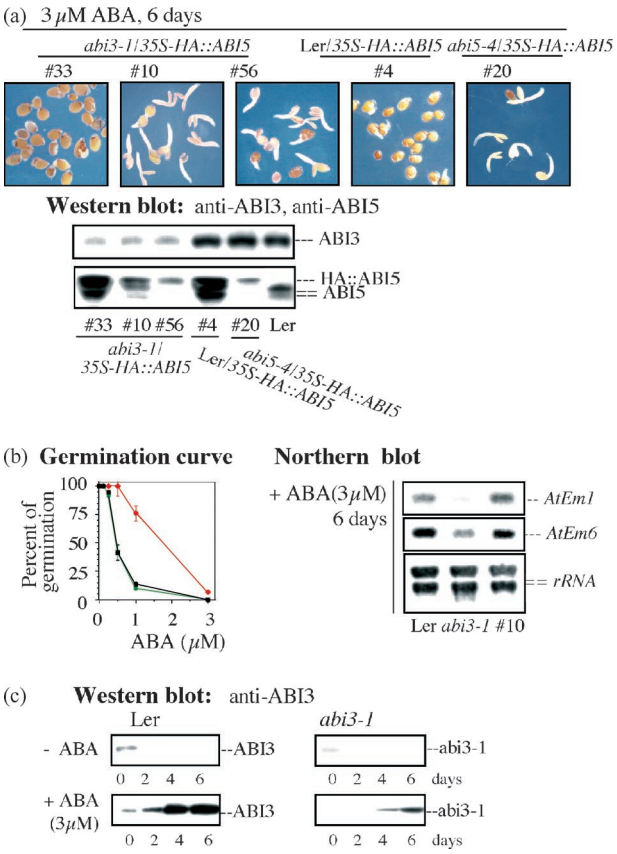

Figure 3. ABI5 can rescue *abi3-1* mutant ABA-insensitivity.
Western and Northern blots were performed as described in Figure 1.
(a) Transgenic lines carrying *35S-HA::ABI5* in *abi3-1* (3 different lines: #33, #10, #56), *abi5-4* and WT-Ler background were stratified and grown on 3 μM ABA for 6 days before protein and RNA extraction. Pictures depict representative 6-day-old seedlings.
(b) Germination frequencies of Ler (black curve), *abi3-1* (red) and *abi3-1/35S-HA::ABI5* (line #10, green) seeds in the presence of ABA. Radical emergence was assayed 60 h after stratification. Northern blots were performed on material treated as in Figure 3a.
(c) ABI3 expression is downregulated in *abi3-1* mutant plants. Experimental procedure is the same as in Figure 2a.

possibility *in planta*, we generated *abi5-4* transgenic plants carrying an *ABI5* promoter construct controlling the expression of the *ABI5* gene fused to the *GUS* marker gene (Figure 4). Three independent lines expressing similar amounts of the ABI5::GUS fusion protein were analysed. The ABI5::GUS protein fusion was functional as it was able to complement the *abi5-4* mutation (data not shown). Figure 4 shows that ABI5 was expressed in all parts of the dry seed embryo and was consistently localized in the nucleus. Following stratification and in the absence of ABA, the signal gradually disappeared from the cotyledons and the root tip (data not shown) but ABI5::GUS remained nuclear-localized 12 h following stratification (Figure 4). These results show that ABI5 is constitutively localized in the nucleus suggesting that nucleo-cytoplasmic shuttling is

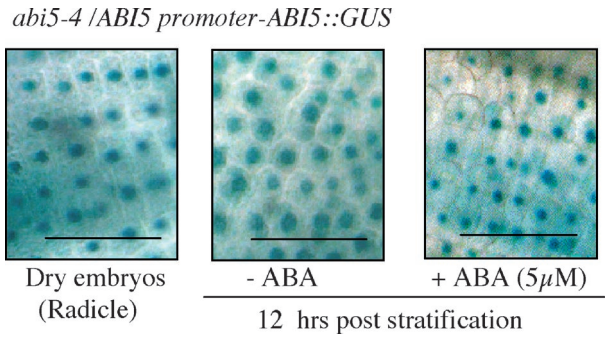

Figure 4. ABI5 is constitutively located in the nucleus.
Transgenic lines carrying a 2.6 kbp *ABI5* promoter controlling the expression of the *ABI5*::GUS fusion were stratified with or without 5 μM ABA and stained at the times indicated.
Pictures depict radicle tissue. Black bar: 50 μm.

not a major regulatory step of ABI5 following activation by ABA. This observation prompted us to assess ABI5 target promoter occupancy *in vivo* in the absence, versus the presence of ABA.

ABA promotes ABI5 binding to the AtEm6 promoter in vivo

ABI5 is normally not detectable in *Arabidopsis* cells in the absence of ABA and therefore its activity as a negative growth regulator requires its synthesis on ABA exposure. We again used *Arabidopsis* transgenic lines constitutively overexpressing HA::ABI5 to separate synthesis from activity. To investigate whether ABI5 promoter occupancy could be regulated by ABA *in vivo*, we used *in vivo* formaldehyde crosslinking followed by immunoprecipitation (chromosomal immunoprecipitations (ChIP)) and PCR amplification (Solomon and Varshavsky, 1985) of the ABI5 target gene *AtEm6*.

Four days post-stratification seeds treated with 0.5 μM ABA were harvested and incubated in formaldehyde for 10 min (see Experimental procedures). Extracts were prepared thereafter and chromatin was sheared to an average size of 500 bp. We next used PCR to measure the relative abundance of specific sequences bound to the immunoprecipitated HA-tagged proteins. Figure 5(a) depicts the relative location of the four sets of primers used for amplifying different genomic fragments located in the vicinity of *AtEm6*. One fragment was located 30 bp 5′ of the *AtEm6* transcription start site (Gaubier *et al.*, 1993) whereas the closest others where 4 kbp away. Figure 5(b) shows that in control experiments all four fragments were similarly amplified from sheared whole cell extracts (WCE). By contrast, in three independent experiments, the fragment containing *AtEm6* promoter sequences was preferentially amplified from immunoprecipitates performed on extracts of HA::ABI5 transgenic lines using an antibody specific to

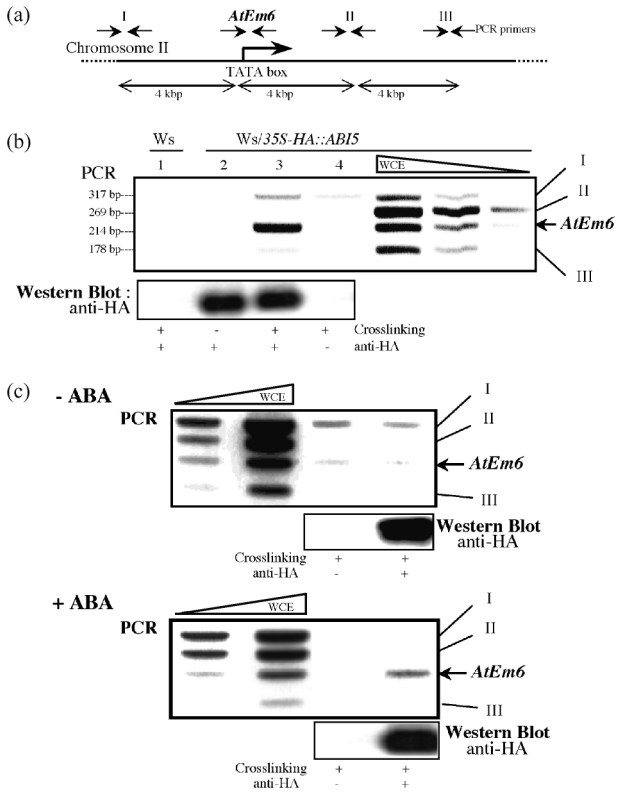

Figure 5. ABA increases the relative binding of ABI5 to *AtEm6* promoter *in vivo*.

(a) Location of four sets of primers used for amplifying different genomic fragments (I, *AtEm6*, II, III) located in the vicinity of *AtEm6* promoter and at 30 bp 5′ of the *AtEm6* transcription start site (*AtEm6*).

(b) *In vivo* formaldehyde crosslinking followed by immunoprecipitation and PCR amplification of the HA::ABI5 target gene *AtEm6* were performed on 4-day-old WT/*35S-HA::ABI5* seeds stratified and grown in the presence of 0.5 μM of ABA (Lane 3). WT Ws seeds treated with ABA (5 μM) were used as a negative control for HA antigen specificity (Lane 1). Seedlings not treated with formaldehyde were used as a negative control for crosslinking specificity (Lane 2). Agarose beads without antibody were used as a negative control for antibody specificity (Lane 4). PCRs on whole cell extract (WCE) dilutions showed that all four fragments from each locus were similarly amplified. This experiment was performed three times and similar results were obtained.

(c) HA::ABI5 binds preferentially to the *AtEm6* promoter in the presence of ABA. Four-day-old WT/*35S-HA::ABI5* seeds stratified and grown with or without 0.5 μM of ABA were used for ChIP analyses (see above). Western blot depicted signals are from the same exposure. This experiment was performed twice and similar results were obtained.

HA. This preferential amplification did not occur in immunoprecipitates obtained from extracts prepared from plants not expressing any HA-tagged protein (Figure 5b, lane 1). Amplification products other than the *AtEm6* promoter fragment could be observed from experiment to experiment but they were systematically observed in control beads (+ crosslinking, – antiHA, Figure 5b) and test beads (+ crosslink, + antiHA, Figure 5b) suggesting that their origin is not specific. Preferential amplification was also dependent on the presence of antibodies to HA in the immunoprecipitation procedure and the addition of formaldehyde (Figure 5b, lane 3). These results establish the feasibility of the ChIP technique to measure *in vivo AtEm6* promoter occupancy by ABI5 in transgenic plants constitutively expressing HA::ABI5.

To see whether the *AtEm6* promoter fragment is also preferentially amplified in the absence of ABA, transgenic seeds expressing HA::ABI5 were plated in the presence or absence of ABA. Samples were harvested and processed in parallel for ChIP and PCR analysis 4 days following stratification as described in Figure 5b. Given that transgenic tissue accumulates less ABI5 protein per cell in the absence of ABA (Lopez-Molina *et al.*, 2001), the amount of starting material was adjusted so as to recover equal amounts of ABI5 after immunoprecipitation (see Western blot in Figure 5c). Therefore, under these conditions, the plant material used without ABA treatment would contain relatively more plant cells, i.e. higher DNA amount than those treated with ABA, explaining perhaps the higher background obtained in the PCR reactions (Figure 5c). Figure 5(c) shows that in the absence of ABA two bands, including the *AtEm6* promoter fragment, could be detected after PCR in control beads obtained from crosslinked material and immunoprecipation without antibody to HA. When the antibody was used in the immunoprecipitation on the same plant material (see Western blot, Figure 5c), the same amplification pattern was observed suggesting that the signal obtained was non-specific (Figure 5c) and that no preferential promoter occupancy occurred. Similar results were obtained in two other independent experiments, leading to the same conclusion.

By contrast, when equal amounts of ABI5 were immunoprecipitated from plants treated with ABA, the *AtEm6* promoter PCR fragment was preferentially enriched only when the antibody to HA was added (Figure 5c). These results suggest that increasing occupancy of the *AtEm6* promoter represents an activation step of ABI5 by ABA.

Discussion

Three developmental windows of different ABA responsiveness

The molecular events surrounding the early life of a desiccated embryo once it is committed to break dormancy and germinate are not well understood. The importance of water availability and potential during this developmental process has prompted us to focus on the effects of ABA on these events. Using standardized stratification conditions and identical ABA concentrations our analyses uncover three developmental time windows starting from the initiation of stratification. Our molecular studies confirm previous physiological findings (Bewley and Black, 1985) and further clarify the nature of the molecular events taking

place in germinating seeds in the presence or absence of ABA.

A sharp drop in transcript and protein expression levels of late embryogenesis genes characterizes the initial period of 3 days from the time when seeds are imbibed to their transfer to normal growth conditions (stratification). These molecular events appear unaffected by exogenous ABA in the medium. This apparent ABA-insensitivity might be due to the physical barrier of the seed coat impeding penetration of exogenous ABA or reflect a developmental interval of reduced capacity for ABA signalling. Even though the degradation of the late embryogenesis proteins indicates the onset of active seed metabolism (Bove *et al.*, 2001), the physiological significance of an early ABA unresponsiveness is not clear.

Following the end of stratification, there is a time window of about 60 h during which ABA and osmotic stress, e.g. high salt or high mannitol, promote a secondary dormancy through the establishment of arrested, germinated embryos (Lopez-Molina *et al.*, 2001). This time period may correspond to stages 1 and 2 previously characterized in physiological studies (Bewley and Black, 1985). This time window is also characterized by the ABA-dependent induction of late embryogenesis gene expression (Figure 1c). The quiescent, germinated embryos are osmotolerant and can resume growth as soon as stress is removed (Lopez-Molina *et al.*, 2001).

Finally, when applied 60 h after stratification, ABA is unable to arrest growth. During this third time window the plants remain responsive to ABA as evidenced by the induction of vegetative ABA-dependent gene expression, but *ABI3* and late embryogenesis gene expression could no longer be activated (Figure 1c). This change in ABA-response gene expression profile is likely due to a developmental regulation.

De novo *recapitulation of late embryogenesis programmes following stratification in ABA*

We found that the re-induction of *ABI3* gene expression by ABA takes place only during the second time window. This observation is consistent with the notion that late embryogenesis pathways are recruited *de novo* to confer osmotolerance following stratification on ABA. As *abi3-1* is unable to execute this developmental checkpoint, we conclude that ABI3 regulates the expression of a number of genes important for the growth arrest of germinated embryos. These genes include *ABI5* whose expression is in turn essential for the expression of *AtEm1* and *AtEm6*. The latter two genes encode similar proteins containing repeats of a highly hydrophilic amino acid motif (Gaubier *et al.*, 1993). Related embryogenesis proteins from wheat have been shown to confer osmotic tolerance in yeast cells (Swire-Clark and Marcotte, 1999). Given the scarcity of our knowledge of the molecular events underlying late embryogenesis, it is difficult at this time to speculate on the actual physiological state of an arrested, germinated embryo and how it compares to its seed coat-surrounded counterpart. Additional work is needed to investigate in molecular terms the relationship between the secondary dormancy of growth-arrested, germinated embryos and the embryo dormancy in seed. Foley and Fennimore (1998) have generated inbred lines of wild oat (*Avena fatua*) that can develop secondary dormancy in seeds upon environmental treatment (Foley and Fennimore, 1998). Expression of *AfVP1*, the *A. fatua* homologue of ABI3, is strongly correlated with the level of embryo dormancy (Jones *et al.*, 1997).

Both the second and the third window display ABA responses, but different genes are induced by the hormone. Greening of seedlings marks the beginning of this time window; however, the absence of light does not affect the occurrence and length of the different windows (Lopez-Molina *et al.*, 2001). The mechanisms responsible for this precipitous change in ABA signalling outputs are unknown.

ABI3 acts upstream of ABI5

Although the mechanisms by which the expression of *ABI5* is regulated by ABI3 are unknown, a key observation of our work is that ABI5 can complement *abi3-1*, whereas ABI3 cannot complement *abi5-4*. This strongly suggests that ABI5 acts downstream of ABI3 to arrest growth of germinated embryos by ABA. We have also shown that ABI3 activity is controlled by ABA because ABI3 expression does not arrest growth in the absence of ABA (Parcy and Giraudat, 1997). When ABA is added, ABI3 is activated and triggers ABI5 accumulation leading to a hypersensitive response (Figure 2e). We have also used the same transgenic lines to show that ABA could increase ABI3 accumulation post-transcriptionally (Figure 2g). The observation that ABA can lead to a post-transcriptional accumulation and activation of ABI3 is reminiscent of results previously reported for ABI5 (Lopez-Molina *et al.*, 2001). Therefore, regulated proteolysis and activation are likely to be important aspects of ABA signalling, which requires future investigations.

ABI3/ABI5 interaction

Our results indicate that ABI5 activity, as a transcription factor, may not require ABI3. Two lines of evidence support this conclusion. Firstly, when WT ABI5 protein levels are restored in *abi3-1* transgenic plants, the ABA-insensitivity phenotype of *abi3-1* is rescued and transgenic *abi3-1* lines acquire WT ABA sensitivity (Figure 3a). This rescue also occurs at the molecular level as *AtEm1* and *AtEm6* transcript levels are restored to wild type levels (Figure 3b). In striking contrast, ABI3 cannot complement *abi5-4*

(Figure 2c). Second, lower than WT ABI5 levels, which are still able to confer ABA sensitivity in *abi5-4* control lines, can similarly complement *abi3-1* mutant lines (Figure 3a, line #56). Therefore, ABI5 functions with equal efficiency in an *ABI3* (WT) or in an *abi3-1* background.

This conclusion appears to be at variance with previous models describing the mode of action of ABI3 and ABI3-like proteins. Indeed, ABI3 and its orthologs can transactivate promoters containing ABA-responsive elements (ABREs) in transient transfection experiments in plant cells but their binding to ABA-responsive promoter DNA *in vitro* has remained elusive (Suzuki *et al*., 1997). This observation has complicated the interpretation of the mode of action of ABI3-like factors. By contrast, bZIP transcription factors, such as *Arabidopsis* ABI5 (Gampala *et al*., 2001), maize EmBP1 (Guiltinan *et al*., 1990) and rice TRAB1 (Hobo *et al*., 1999) are able to bind and transactivate ABRE-containing promoters *in vitro* (Carles *et al*., 2002), in transient experiments (Hill *et al*., 1996; Hobo *et al*., 1999; Razik and Quatrano, 1997) and *in vivo* (this study).

These and other observations have led to the proposal that not only ABI3 and ABI3-like transcription factors (Hill *et al*., 1996; Hobo *et al*., 1999; Razik and Quatrano, 1997) but also 14-3-3 proteins (Schultz *et al*., 1998) and histone H1 (Schultz *et al*., 1996) increase the transcriptional activity of target promoters by forming molecular complexes with ABI5 and ABI5-like factors. However, in all transient expression experiments reported so far (Gampala *et al*., 2001; Hobo *et al*., 1999) protein levels of transcription factors were not monitored. We have shown that both ABI5 and ABI3 overexpression can increase endogenous ABI5 protein levels *in vivo* in a concentration dependent manner (Figures 2e and 3a). Moreover, ABA also strongly regulates post-transcriptionally the accumulation of both ABI3 (Figure 2g) and ABI5 (Lopez-Molina *et al*., 2001). Therefore, one cannot exclude that the observed enhancements in target gene expression were due to a non-linear bZIP protein accumulation resulting from endogenous *ABI5* expression (and/or similar factors) and further enhanced by ABA-mediated ABI5 accumulation.

More recently, ABI3 was found to interact with ABI5 in a yeast two-hybrid assay (Nakamura *et al*., 2001). Furthermore VP1, the maize ABI3 ortholog, potentiates ABI5 activity in transient expression using rice protoplasts (Gampala *et al*., 2001). In spite of repeated attempts, we have been unable to detect any interaction between ABI3 and ABI5 neither in yeast two-hybrid experiments nor in *in vivo* co-immunoprecipitations even under very mild buffer conditions (data not shown). It should be pointed out that Hobo *et al*. detected an interaction between OSVP1 and TRAB1 in yeast-two hybrid assays but failed to reproduce it *in vitro* (Hobo *et al*., 1999). Nevertheless, our results do not rule out a possible *in vivo* interaction and co-operation between ABI3 and ABI5 because our co-immunoprecipitations experiments might not reveal weak and/or highly regulated interactions. In addition, it is possible that ABI3 may have two separate activities, one regulating *ABI5* transcript expression whilst the other regulating ABI5 protein activity by interacting with ABI5. Therefore, the ability of ABI5 to complement the *abi3-1* mutation does not exclude the possibility that the *abi3-1* mutation might affect the activity of ABI3 as a positive regulator of *ABI5* transcript expression without altering its activity as a regulator of ABI5 protein activity.

Investigating ABI5 activity in the absence of ABI3 *in planta* is difficult because of two considerations. First, during embryogenesis, ABI3 is normally expressed much earlier than ABI5 and severe *abi3* alleles alter embryonic cell fate suggesting that ABI3 may be a molecular switch gene in seed development, in addition to being a signalling component in ABA transduction (reviewed in McCourt, 1999; Rohde *et al*., 2000). For instance, the strong *abi3-4* allele produces characteristic green embryos, which fail to breakdown chloroplasts and have defects in cellular differentiation. Forcing ABI5 expression is of limited interest because of the different developmental context. Second, even for the strong *abi3-4* allele there is an accumulation of a large truncated version of ABI3 containing the acidic domain and the first basic domain (Parcy *et al*., 1994). This truncated ABI3 protein might still engage in a putative interaction with ABI5 (Nakamura *et al*., 2001).

Mechanisms of ABA-dependent ABI5 activation

Using an *ABI5::GUS* fusion gene under the control of *ABI5* promoter sequences, we have shown that ABI5 is constitutively localized in the nucleus in the presence or absence of ABA (Figure 4). This suggests that nucleo-cytoplasmic shuttling may not be a major regulatory step in ABI5 activity. Using ChIP on transgenic plants constitutively expressing HA::ABI5 we found that a PCR fragment encompassing the *AtEm6* promoter was preferentially amplified in plants treated with ABA. These data indicate that ABA increases the *in vivo* ABI5 occupancy of the target promoter suggesting that ABI5 binding could be part of its activation step. The molecular basis of ABI5 activation by ABA is presently unknown. One possibility is an ABA-dependent chromatin remodelling of the *AtEm6* locus to increase DNA accessibility of transcription factors.

We have shown that ABI5 is phosphorylated *in vivo* (Lopez-Molina *et al*., 2001). We and others (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000) have previously reported that ABI5 contained three conserved domains each containing the predicted phosphorylation sequence RQXS/T. Here, using mass spectrometry (Figure S1), we have shown that the three conserved domains located in the N-terminal portion of ABI5 are indeed phosphorylated *in vivo*. This prompted us to assess *in planta* the

role of the individual RQXS/T consensus phosphoaminoacids (i.e. S41, S42, S145, T201) in ABI5 activity. Our results indicate that none of the phosphoamino acids tested nor the conserved domains are individually essential for ABA-dependent ABI5 function in germination assays (Figure S1). Therefore, either ABI5 phosphorylation is not essential for its activity or else other phosphoamino acids not identified by mass spectrometry or untested by mutagenesis might be important. Another possibility could be that there is a functional redundancy between certain phosphoamino acids in each of the three conserved domains. Indeed, all the three domains contain the predicted phosphorylation sequence RQXS/T and elimination of only one of them may not have an impact on ABI5 activity. Clearly, additional mutagenesis experiments involving several phosphoamino acid combinations are required to address the role of phosphorylation in ABI5 activity.

The breaking of seed dormancy to establish seedling growth represents an essential developmental transition during which plants must be able to mount adaptive responses to environmental water stress. Continuing molecular characterization of this important event will shed light not only in basic plant biology but the results from these investigations are expected to have significant impact in plant biotechnology.

Experimental procedures

Plant material, plant transformation, growth conditions and plasmid constructions

Plant material was treated as described (Lopez-Molina *et al*., 2001). DNA manipulations were performed according to standard methods (Sambrook *et al*., 1989). Binary vectors vectors were pBA002 (Kost *et al*., 1998) and pBin19 (Clontech, Palo Alto, CA, USA). Inserts were generated by PCR amplification. For HA::ABI5 the HA tag (ATGTATCCATATGACGTGCCGGACTACGCCTCCCTC: MYPYDVPDYASL) was included in the primer and immediately precedes the ABI5 start codon. The final 35S-HA::ABI5 construct includes the entire *ABI5* ORF. The ABI5 promoter (2.6 kbp) driving the expression of ABI5::GUS fusion was constructed as follows: promoter DNA was amplified from WS genomic DNA with the following primers: CGAGTCGACGAGTGGACAACTCGGGTTCC, CGAGTCGACCATTTATCAAATCTAAGTCTCTATG, digested with *Sal*I and cloned into the *Sal*I site of pBI101 (Clontech). Subsequently, *ABI5* cDNA was amplified with CGAGATATCCAGTGGACAACTCGGGTTC and CGAGATATCATGGTAACTAGAGAAACGAAG. The resulting fragment was digested by *Eco*RV and cloned into the *Sma*I site of pBI101 containing *ABI5* promoter sequences. All constructs were verified by sequencing.

RNA extraction, ABI3 antibody production and Northern and Western blot analyses

RNA extraction and Northern blot hybridizations were performed as described (Sambrook *et al*., 1989; Vicient and Delseny, 1999). Northern blot probes were exactly as those described in Lopez-Molina and Chua (2000). ABI3 antibody was generated (Parcy *et al*., 1994) and Western blot analyses were performed as described (Lopez-Molina *et al*., 2001). ABI3 specificity was verified by competition experiments. 300 μl of rabbit serum containing antibody to ABI3 was incubated overnight by gentle rotation at 4°C with 100 μg of ABI3 peptide in 15 ml of TBS/5% milk. Western blot analyses were further performed on two equivalent membranes using antibody to ABI3 or with antibody pre-absorbed with peptide ABI3.

Chromosome Immunoprecipitation (ChIP)

Immunoprecipitation of *in vivo* fixed chromatin fragments was essentially performed according to (Orlando and Paro, 1993; Tanaka *et al*., 1997). Four hundred mg of WT/*35S-HA::ABI5* transgenic seeds were used for ABA treated material and 600 mg for untreated material. Seeds were stratified and grown for 5 days in the presence or absence of 0.5 μM ABA. Seedlings were further treated with or without 1% formaldehyde in 50 mM Tris-HCl pH 8, 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA for 10 min and rinsed twice (10 sec) with TBS. Formaldehyde-treated plants were blocked for 5 min in 125 mM glycine and tissues were ground in 13 ml of RIPA buffer supplemented with a cocktail of antiproteases and antiphosphatases (Lopez-Molina *et al*., 2001). Ground tissue was centrifuged for 15 min at 10 000 *g* and the supernatant passed through a 0.2-μm filter. This resulting material is called whole cell extract (WCE). WCE was sonicated 4 times for 30 sec using a Sonifier Cell disrupter 185, power 6 (Branson Sonic Power Co., Danbury, CA, USA); Chromatin was sheared into an average size of 500 bp. HA::ABI5 was immunoprecipitated as described (Lopez-Molina *et al*., 2001) using 3 μl (60 ng) of monoclonal antibody to HA coupled to agarose beads (Santa Cruz) or using as a control the same amount of agarose beads (Santa Cruz, CA, USA) without the coupled antibody. Beads were rinsed as described (Lopez-Molina *et al*., 2001). One tenth of the immunoprecipitate was analysed by Western blot using rabbit polyclonal antibody to HA (Santa Cruz) as the first antibody. The remaining sample (9/10) was used for DNA purification as described (Lopez-Molina *et al*., 2001). One tenth (5 μl) was used in PCR amplifications. Control PCRs of starting material were directly performed with 5 μl of a 100-fold dilution of the WCE obtained from the crosslinked material. Further 3-fold dilutions of WCE (see Figure 5) showed that under these conditions the PCR did not reach the plateau phase as the resulting PCR products diminished accordingly. Four pairs of primers (240 nM each) were used together in each PCR reaction. The primer sequences are available upon request. PCR cycles were done on a Stratagene Robocycler using EX-Taq polymerase (PanVera Corporation, Madison, WI, USA) according to the manufacturer's instructions. The PCR products were separated in a 2% agarose in presence of ethidium bromide (0.5 μg/ml) and photographed using an Eagle Eye still Video System (Stratagene, La Jolla, CA, USA).

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Supplementary Material

The following material is available from http://www.blackwell-science.com/products/journals/suppmat/TPJ/TPJ1430/TPJ1430sm.htm
ABI5 phosphorylation sites.
Immunoprecipitation and characterization of ABI5 phosphorylation.
Figure S1 Identification of ABI5 phosphorylation sites by mass spectrometry and phenotypes of lines expressing different ABI5 mutants in the *abi5-4* background.
(a) Mapping of ABI5 phosphorylation sites by MALDI-TOF mass spectrometry. Immunoprecipitated HA::ABI5 was subjected to SDS-PAGE followed by in-gel digestion with V8 protease or trypsin. The resulting peptides were extracted and analyzed by MALDI-TOF mass spectrometry. Pairs of peaks were observed that differed in mass by 80 Da (HPO_3), corresponding to unphosphorylated and phosphorylated peptides from ABI5: 31-50 (V8), 138-159 (V8) and 199-213 (trypsin). U designates the unphosphorylated peptide and P the corresponding phosphorylated peptide. LC-MS/MS was used to confirm the identity of each phosphorylated peptide.
(b) The phenotypes of transgenic lines expressing ABI5 phosphorylation site mutants (S41A, S42A, S145A and T201A) in the *abi5-4* background were analyzed in 3μM ABA at 5 days after stratification. Expression levels of the different ABI5 mutant derivatives were monitored by Western blot as described in Materials and Methods. Photographs depict representative seedlings 4 days post-stratification on 3μM ABA.

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