AFP is a novel negative regulator of ABA signaling that promotes ABI5 protein degradation

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Abstract

Plants have evolved protective mechanisms to ensure their survival when threatened by adverse environmental conditions during their transition to autotrophic growth. During germination, there is a 2- to 3-d period during which a plant can execute growth arrest when challenged by water deficit. This postgermination developmental checkpoint is signaled by the stress hormone abscisic acid (ABA), which induces the expression of the bZIP transcription activator ABI5. The growth arrest efficiency depends on ABI5 levels, and abi5 mutants are ABA-insensitive and unable to execute the ABA-mediated growth arrest. Here we show that a novel ABI5-interacting protein, designated as AFP, can form high molecular weight (Mr) complexes with ABI5 in embryo-derived extracts. Like ABI5, ABI five binding protein (AFP) mRNA and protein levels are induced by ABA during seed germination. Two different afp mutant alleles (afp-1 and afp-2) are hypersensitive to ABA, whereas transgenic plants overexpressing AFP are resistant; in these plants, AFP and ABI5 protein levels are inversely correlated. Genetic analysis shows that abi5-4 is epistatic to [...]
AFP is a novel negative regulator of ABA signaling that promotes ABI5 protein degradation

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Plants have evolved protective mechanisms to ensure their survival when threatened by adverse environmental conditions during their transition to autotrophic growth. During germination, there is a 2- to 3-d period during which a plant can execute growth arrest when challenged by water deficit. This postgermination developmental checkpoint is signaled by the stress hormone abscisic acid (ABA), which induces the expression of the bZIP transcription activator ABI5. The growth arrest efficiency depends on ABI5 levels, and abi5 mutants are ABA-insensitive and unable to execute the ABA-mediated growth arrest. Here we show that a novel ABI5-interacting protein, designated as AFP, can form high molecular weight (Mr) complexes with ABI5 in embryo-derived extracts. Like ABI5, ABI five binding protein (AFP) mRNA and protein levels are induced by ABA during seed germination. Two different afp mutant alleles (afp-1 and afp-2) are hypersensitive to ABA, whereas transgenic plants overexpressing AFP are resistant; in these plants, AFP and ABI5 protein levels are inversely correlated. Genetic analysis shows that abi5-4 is epistatic to afp-1, indicating the ABA hypersensitivity of afp mutants requires ABI5. Proteasome inhibitor studies show that ABI5 stability is regulated by ABA through ubiquitin-related events. When expressed together, AFP and ABI5 are colocalized in nuclear bodies, which also contain COP1, a RING motif protein. Our results suggest that AFP attenuates ABA signals by targeting ABI5 for ubiquitin-mediated degradation in nuclear bodies.

[Keywords: Abscisic acid; ABI5; postgermination growth arrest; protein degradation]

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The transition from heterotrophic to autotrophic growth is one of the most fragile phases during the life cycle of a flowering plant. Plants have evolved protective mechanisms to ensure their survival under adverse environmental conditions during this transition. Recently, we have identified a developmental checkpoint that occurs during germination within ~2 d after stratification (Lopez-Molina et al. 2001). During this time interval, exposure to abscisic acid (ABA) or osmotic stress triggers a sustained but reversible growth arrest of germinated embryos (Lopez-Molina et al. 2001), which become osmo-tolerant (Lopez-Molina et al. 2001). Arabidopsis mutants deficient in ABA biosynthesis or ABA signaling are unable to execute this response (Koornneef et al. 1984; Finkelstein 1994; Laby et al. 2000; Lopez-Molina et al. 2002; Nambara et al. 2002). The latter category includes mutations in ERA1, which encodes a subunit of a farnesyl transferase (Cutler et al. 1996), and ABH1, which encodes a mRNA cap binding protein (Hugouvieux et al. 2001). Mutants bypassing the ABA-mediated growth inhibition were designated abl3, ab2, abl4, abl5, and abl8 (for ABA-insensitive), cho1, and cho2 (Koornneef et al. 1984; Finkelstein 1994; Lopez-Molina and Chua 2000; Quesada et al. 2000). These mutants have been isolated that either bypass this ABA-mediated growth arrest or show an enhanced response to ABA (for review, see Finkelstein et al. 2002). The latter category includes mutations in ERA1, which encodes a subunit of a farnesyl transferase (Cutler et al. 1996), and ABH1, which encodes mRNA cap binding protein (Hugouvieux et al. 2001). Mutants bypassing the ABA-mediated growth inhibition were designated abl1, abl2, abl3, abl4, abl5, and abl8 (for ABA-insensitive), cho1, and cho2 (Koornneef et al. 1984; Finkelstein 1994; Lopez-Molina and Chua 2000; Finkelstein et al. 2002, Nambara et al. 2002). The ABI8, CHO1, and CHO2 genes have not yet been cloned (Finkelstein et al. 2002, Nambara et al. 2002). ABI1 and ABI2 encode two homologous serine/threonine phosphatases of class 2C (Leung et al. 1994, 1997; Meyer et al. 1994, Rodriguez et al. 1998), and both abl1 and abl2 have identical Gly-
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to-Asp mutations in their phosphatase domains [Leung et al. 1994, 1997; Meyer et al. 1994, Rodriguez et al. 1998]. Although the targets of these phosphatases are not known [for review, see Finkelstein et al. 2002], the wild-type (WT) proteins, ABI1 and ABI2, have been proposed as negative regulators of ABA signaling that operates during germination, as well as in vegetative tissues [Gosti et al. 1999]. The ABI4 gene encodes an APETALA2 domain transcription factor [Finkelstein 1994, Finkelstein et al. 1998].

Two other ABA-SENSITIVE genes, ABI3 and ABI5, encode a putative acidic domain transcription factor [Giraudat et al. 1992] and a basic leucine zipper [bZIP] transcription factor [Finkelstein and Lynch 2000; Lopez-Molina and Chua 2000], respectively. Both abi3 and abi5 mutants were initially recovered by virtue of their ability to germinate in the presence of ABA [Koornneef et al. 1984; Finkelstein 1994; Lopez-Molina and Chua 2000]. Subsequently, it was demonstrated that both ABI5 and ABI3 mRNA and protein levels are stringently up-regulated by ABA or osmotic stress, but only during the time period when the postgermination growth arrest can occur [Lopez-Molina et al. 2001, 2002]. The identification of this molecular signature further supports the existence of a development checkpoint. As ABI3 plays a central role in the establishment of desiccation tolerance and dormancy during zygotic embryogenesis [Koornneef et al. 1984; Finkelstein 1994; Parcy et al. 1994, 1997], the osmo-tolerance of the postgermination arrested embryos is likely acquired through the recruitment by ABI3 of embryogenesis pathways implicated in desiccation tolerance [Lopez-Molina et al. 2002]. ABI3 has also been shown to be required for appropriate ABI5 expression [Finkelstein and Lynch 2000; Soderman et al. 2000; Lopez-Molina et al. 2002]. By using transgenic plants, we demonstrated that restoration of ABI5 levels in abi3 mutants could reestablish ABA-dependent growth arrest and expression of late embryogenesis genes [Lopez-Molina et al. 2002]. This result, together with those of others [Soderman et al. 2000], led to the notion that ABI5 acts downstream of ABI3.

The efficiency of the postgermination ABA-dependent growth arrest is directly dependent on ABI5 protein levels. [Lopez-Molina et al. 2001, Brocard et al. 2002]. We have demonstrated that ABA has two effects on the ABI5 protein: 1) it activates ABI5 as a growth repressor, including an increased promoter occupancy on its target promoters [Lopez-Molina et al. 2002], and 2) the hormone also signals the inhibition of ABI5 protein degradation via the 26S proteasome [Lopez-Molina et al. 2001]. The mechanism of ABI5 activation and the factors that regulate its ubiquitin-mediated proteolysis are unknown.

To gain further insight into the molecular mechanisms of ABA signaling that leads to postgermination growth arrest, we used yeast two-hybrid assays to isolate proteins that may interact with ABI5. Among the cDNA clones recovered, 15% encoded the same protein, which we designated as ABI five binding protein (AFP). Here, we present a molecular and genetic characterization of AFP and Arabidopsis mutants [afp] deficient in this protein. Our results indicate that AFP is a novel negative regulator of ABA signaling by facilitating the degradation of ABI5.

Results

Yeast two-hybrid screen identifies a novel ABI5 binding protein (AFP)

We used yeast two-hybrid assays to isolate and identify ABI5 interacting proteins by using a cDNA library made from mRNAs derived from a mixture of different Arabidopsis tissues [see Materials and Methods]. Fifteen percent of the cDNA clones recovered encode the same protein, which was designated AFP. AFP is located on chromosome 1 (accession no. AAF67775) and encodes a protein with a predicted molecular weight (Mr) of 36,383 [Fig. 1A], however, this protein migrates with an apparent mass of 42 kD on SDS gels [see below]. There are three genes (accession nos. NP_2189598, NP_2566163, AAA07175) encoding AFP-related proteins in the Arabidopsis genome, and this small AFP protein family appears to be plant-specific. By using yeast two hybrid assays, we found that the C-terminal region of AFP (amino acids 284–335) and domain III of ABI5 [amino acids 199–221] were necessary and sufficient for the interaction of the two proteins [Fig. 1B]. Each of these two domains is conserved within its own gene family [61%–77% and 81% amino acid identity for AFP and ABI5, respectively].

AFP and ABI5 have similar temporal expression patterns in response to ABA

Like ABI5, AFP mRNA and protein levels increased during seed development and desiccation reaching plateau values in mature seeds [Fig. 2A; data not shown]. After stratification and during germination, AFP mRNA and protein levels were induced by ABA within the developmental window when growth arrest can occur [Fig. 2A,B]. However, once seedling autotrophic growth was established, AFP was no longer detectable in all tissues examined [Fig. 2C]. These results, which are very similar to those obtained with ABI5 and ABI3 [Lopez-Molina et al. 2001, 2002], prompted us to examine whether ABI5 and AFP interact in vivo.

AFP and ABI5 interact in vivo

Coimmunoprecipitation experiments using extracts of germinating embryos treated with ABA showed that antibody to AFP was able to coimmunoprecipitate HA::ABI5 [Fig. 2D]. To explore the mode of interaction between the two proteins and to study the function of AFP, we identified two independent T-DNA insertions [Sussman et al. 2000] with lesions in the AFP locus. The mutant afp-1 has a T-DNA insertion in the coding sequence at codon 221, whereas afp-2 has an insertion in promoter sequences [Fig. 2E, upper panel]. Western blot
analysis showed that \textit{afp-1} is a null allele, whereas \textit{afp-2} is leaky [Fig. 2E, lower panel]. Glycerol gradient fractionation of extracts from ABA-treated WT embryos showed that AFP (42 kD) was distributed into two high Mr peaks in fractions 6 and 7 (∼150 kD) and 9 and 10 (∼60 kD; Fig. 2F). Similarly, ABI5 (50 kD) was detected predominantly in fractions 6 and 7, although not in the low-molecular-weight fractions. These results, which support the notion of high-molecular complexes of AFP/ABI5, are consistent with the coimmunoprecipitation data (Fig. 2D).

As expected, in \textit{abi5-4} extracts, which lack ABI5, most of the AFP protein was found in the lower-molecular-weight fractions. A similar shift in AFP distribution was found in \textit{afp-1} extracts [Fig. 2F].

\textbf{AFP is a negative regulator of ABA signaling}

The identification of an AFP/ABI5 complex suggests that AFP may regulate ABI5 functions. Therefore, we analyzed ABA responses of \textit{afp-1} and \textit{afp-2} mutant plants, as well as of \textit{355-AFP} transgenic plants. Western blot analyses indicated that AFP is not detectable in \textit{afp-1}, whereas \textit{afp-2} still contains low levels of AFP [Fig. 2E, lower panel]. In contrast, AFP levels in the \textit{355-AFP} plants were 5- to 10-fold higher compared with WT plants [see below]. In germination assays, \textit{afp-1} and \textit{afp-2} were hypersensitive to ABA compared with WT, whereas WT/\textit{355- AFP} transgenic plants were more resistant [Fig. 3A]. The ABA-hypersensitive phenotype of \textit{afp-1} could be complemented with \textit{355- AFP} in transgenic lines expressing AFP at WT levels [data not shown]. Because ABA-induced accumulation of AFP reached a maximum 5 d after stratification, we also examined the formation of growth-arrested germinated embryos after a prolonged exposure to the hormone. After incubation in the presence of 3 μM ABA for 8 d, >95% of the \textit{afp-1} embryos did not germinate, whereas >85% of WT embryos germinated and displayed arrested growth [Fig. 3B]. In contrast, WT/\textit{355- AFP} embryos fully germinated into green seedlings, even in the presence of 3 μM ABA. These results demonstrate that AFP is a negative regulator of ABA responses during germination.

\textbf{AFP acts upstream of ABI5 to regulate negatively ABI5 protein levels}

To see whether AFP function as a negative regulator depends on ABI5, we investigated the relationship between ABA responses and ABI5 levels. In WT, \textit{afp-1}, \textit{afp-2}, and a WT/\textit{355- AFP} line, ABI5 levels were inversely correlated with AFP levels at all ABA concentrations examined [Fig. 3C; data not shown]. These differences could not be directly attributed to differences in ABI5 mRNA levels, as the latter were very similar [Fig. 3C]. Because the \textit{afp-1/abi5-4} double mutant displayed ABA responses identical to those of \textit{abi5-4} [Fig. 3D], the ABA hypersensitive phenotype of \textit{afp} mutants must require ABI5. These results led us to conclude that the negative regulatory function of AFP is executed through its effects on ABI5 protein levels.

\textbf{ABI5 is ubiquitinated in vivo}

Previous work has shown that in extracts of germinating embryos, ABI5 protein stability is increased by ABA, but
the protein is rapidly degraded in vitro via 26S proteasomes on ABA removal (Lopez-Molina et al. 2001). To see whether ABI5 is also degraded by 26S proteasomes in vivo, we directly investigated the effects of proteasome inhibitors on ABI5 protein levels in living plants. Figure 4A shows that, after ABA removal, proteasome inhibitors blocked ABI5 degradation. Thus, ABI5 protein levels are subject to stringent regulation via the 26S proteasome during the establishment and maintenance of, and exit from, the ABA-dependent growth arrest. We next used a transgenic line constitutively expressing HA::ABI5 to study posttranslational regulation of ABI5 by ABA. As expected, proteasome inhibitors promoted the accumulation of poly-ubiquitinated ABI5 in vivo (Fig. 4B). This result confirms and extends previous in vitro data (Lopez-Molina et al. 2001).

**AFP enhances ABI5 proteolysis**

The inverse correlation between AFP and ABI5 protein levels and the degradation of ABI5 by 26S proteasomes suggests that one function of AFP could be to enhance ubiquitin-mediated proteolysis. To test this hypothesis, we first assayed the apparent ABI5 protein half-life by comparing the time course of decrease in ABI5 protein levels after ABA removal in WT, *afp-1*, and WT/35S-AFP.
embryos. Figure 4C shows that the apparent half-life of ABI5 was 21 h for \textit{afp-1} and 9 h for WT/35S-AFP compared with 14 h for WT.

We next examined whether these differences in half-lives reflected differences in 26S proteasome degradation rates. For this purpose, we used proteasome inhibitors to block ABI5 degradation in the three genetic backgrounds. In WT and a WT/35S-AFP transgenic line, treatment with proteasome inhibitors substantially increased ABI5 levels by 5- to 10-fold (Fig. 4D). A greater increase was observed in a WT/35S-AFP transgenic line than in WT plants primarily because, in the absence of the inhibitors, lower ABI5 levels were detected in the former. In contrast, ABI5 levels in \textit{afp-1} mutants were already as high as those in inhibitor-treated WT and a WT/35S-AFP transgenic line, and only a very small increase was seen on inhibitor treatment (Fig. 4D). Our results suggest that ubiquitin-mediated proteolysis of ABI5 is critically dependent on the level of AFP, which appears to facilitate ABI5-regulated proteolysis.

Our results thus far suggest that AFP could prevent ABI5 over-accumulation in the presence of ABA (Fig. 3C) and promote ABI5 degradation to terminate the stress signal on ABA removal so that growth can resume (Fig. 4C,D). Consistent with the latter, in WT plants, the apparent half-life of AFP is significantly longer than that of ABI5 such that AFP levels remain virtually unchanged during the period of ABI5 degradation (data not shown).

**AFP and ABI5 colocalize in nuclear bodies**

To determine the intracellular localization of AFP and ABI5, we transiently expressed 35S-ABI5::YFP (or ABI5::GFP) and 35S-AFP::CFP fusion genes, separately or together, in onion epidermal cells or \textit{Arabidopsis} \textit{afp-1} and \textit{abi5-4} mutant plants treated with 3 µM ABA for 8 d. Figure 5A shows that when individually expressed, both AFP::CFP and ABI5::YFP were distributed in the nucleus and the cytoplasm in \textit{abi5-4} and \textit{afp-1} mutants, respectively. However, when expressed together, the two fusion proteins became colocalized in the nucleus (Fig. 5A). Similar results were obtained when the same constructs were expressed in onion epidermal cells (Fig. 5B). Nuclear body formation was not detected after coexpression with an ABI5 mutant lacking the D3 domain (data not shown), which is needed for AFP interaction (Fig. 1B). As \textit{Arabidopsis} COP1, a RING motif protein, is known to be localized in nuclear bodies (von Arnim and Deng 1993; Ang et al. 1998), we investigated whether AFP/ABI5 might also reside in the same nuclear bodies when coexpressed with COP1 in onion epidermal cells. Figure 5C shows that this was indeed the case.
Discussion

So far, few mutations affecting negative regulators of the ABA-mediated growth arrest in Arabidopsis have been isolated. These regulators include ERA1, a subunit of a farnesyl transferase (Cutler et al. 1996), and ABH1, a mRNA Cap binding protein (Hugouvieux et al. 2001). However, the targets of these regulators have not yet been identified. Here, we show that AFP is a novel negative regulator of ABA responses and that it executes its function through the bZIP transcription activator ABI5. Two lines of genetic evidence support this conclusion: [1] mutants lacking {afp-1}, or deficient {afp-2} in, AFP are hypersensitive to ABA [Fig. 3A,B], and [2] the abis-5-4 mutation is epistatic to afp-1 [Fig. 3D]. This latter genetic interaction is confirmed by the observed physical interaction between ABI5 and AFP in coimmunoprecipitation experiments [Fig. 2D]. Moreover, glycerol fractionation experiments [Fig. 2F] indicate that ABI5 and AFP form a complex of ~200 kDa. We speculate that this complex likely contains an ABI5 leucine zipper dimer (~100 kDa), with one AFP molecule binding to each ABI5 monomer. The inverse correlation in ABI5 and AFP protein levels observed in afp mutants and 35S::AFP transgenic plants suggests that AFP can regulate negatively ABI5 protein levels, despite having similar ABI5 mRNA levels [Fig. 3C]. Therefore, this regulation must be posttranslational. In afp mutants, high ABI5 levels are little affected by 26S proteasome inhibitors [Fig. 4D], suggesting that ABI5 is more stable in an afp mutant background. Conversely, ABI5 appears to be less stable in 35S::AFP transgenic plants compared with WT, because in these transgenic plants, ABI5 levels show the highest increase on treatment with proteasome inhibitors [Fig. 4D]. Taken together, our results support the notion that AFP facilitates ubiquitin-mediated proteolysis of ABI5. The negative regulation of ABI5 serves to attenuate ABA signals, as well as to ensure rapid degradation of ABI5 after stress removal.

In contrast to ABI5, AFP levels appear to be only slightly elevated by proteasome inhibitors [Fig. 4D], suggesting that this protein is more stable and consistent with its role as a signal attenuator. We note that AFP continues to be induced by ABA in the abis-5-4 mutant [Fig. 3D]. This result indicates that ABA-induction of AFP can occur via an ABI5-independent pathway.

Subcellular localization studies showed that in the absence of AFP, ABI5 is diffusely distributed in the nucleo-
plasm, but AFP/ABI5 complexes are localized to discrete nuclear bodies. The ABI5 domain III is needed for interaction with AFP (Fig. 1B) and for colocalization in nuclear bodies (data not shown). The molecular mechanism that underlies this interaction-dependent colocalization remains to be elucidated. As both *afp* mutant alleles are hypersensitive to ABA, localization in nuclear bodies appears to not be required for ABI5 function. Indeed, these subnuclear structures may represent sites of ABI5 degradation, which is promoted by AFP. This conclusion is supported by the finding that the AFP/ABI5 nuclear bodies also contain COP1, a RING motif protein that is a putative E3 ligase (von Arnim and Deng 1993; Hardtke and Deng 2000). Nuclear bodies containing COP1 have been proposed to be sites of protein inactivation and degradation (Wang et al. 2001). Future experi-

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**Figure 5.** Colocalization of AFP, ABI5, and COP1 in nuclear bodies. (A) Subcellular localization of ABI5::YFP and AFP::CFP in different *Arabidopsis* genetic backgrounds. Cells were cobombarded with equal amounts of the DNA constructs indicated. Living cells were imaged through CFP and YFP filter channels. (B) Subcellular localization of ABI5::YFP (or ABI5::GFP) and AFP::CFP in onion epidermal cells. Onion peels were bombard with 3SS-ABI5::YFP (or 35S-ABI5::GFP), 3SS-AFP::CFP, or the two constructs together. Fluorescence images were collected through the YFP or the CFP filter channel. (C) Subcellular nuclear localization of ABI5, AFP::CFP and COP1::YFP in onion epidermal cells. Note that a few AFP/ABI5 nuclear bodies do not appear to contain COP1. Epidermal cells, co-bombarded with all three constructs, were imaged through CFP and YFP filter channels. Bars, 5 µm.
ments will determine whether ABI5 is a substrate of COP1 or other E3 ligases in nuclear bodies.

We have previously shown that ABA can inhibit ABI5 degradation in vegetative tissues of transgenic plants constitutively over-expressing ABI5. AFP and its family members share a highly homologous C-terminal domain, which interacts with domain III of ABI5 (Fig. 1B). It should be pointed out that domain III of ABI5 is highly conserved among ABI5 homologs. These include the AFB transcription factors in Arabidopsis, which have been shown to regulate the responses of a plant to osmotic stress (Choi et al. 2000; Kang et al. 2002; Kim et al. 2002). Therefore, our work opens the way to study the function of AFP homologs, which might play similar roles in ABA and stress signaling pathways operating in vegetative tissues.

Materials and methods

Plant material, plant transformation, growth conditions, and plasmid constructions

Plant material, growth conditions, and ABA-treatment were as described in Lopez-Molina et al. (2001). Germination frequencies were obtained by scoring radical emergence (n = 3, ~200 seeds per experiment). Plants were grown under identical conditions before seed harvesting. pBA002 (Kost et al. 1998) and pBin19 (Clontech) were used as binary vectors. T-DNA insertion mutants in AFP genes were obtained from the Arabidopsis Wisconsin-Madison knockout facility following the recommended procedure (Sussman et al. 2000). The PCR primers used in the screen were 5′-TGGATCGTGGCATAAAAATCTGAT TACTG-3′ and 5′-TGGATCGTGGCATAAAAATCTGAT TACTG-3′.

Yeast two-hybrid experiment

Yeast two-hybrid experiments were performed as described (Xie et al. 2002).

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

RNA extraction and Northern blot hybridizations were performed as described in Vicent and Delseny (1999) and Lopez-Molina and Chua (2001). Polyclonal anti-AFP antiserum was formed as described in Vicient and Delseny (1999) and Lopez-Molina and Chua (2001). AFP was immunoprecipitated with rabbit antibody to AFP for 2 h at 4°C. Protein A agarose beads were then added for 1 h. Beads were washed three times with the same buffer. An equal volume of SDS-loading buffer was added to stop the reaction. Proteins were analyzed by Western blots using mouse monoclonal antibody to HA as the first antibody. Glycerol gradient fractionation was performed by using extracts prepared from 6-day-old ABA-treated (3 µM) WT, afp-1, abi5-4 seedlings. Extraction buffer contained protease inhibitors as described above and 0.1% Triton X-100, 5% glycerol, 100 mM NaCl, and 50 mM Tris-HCl (pH 7.5). Extracts were loaded onto 5%–25% glycerol gradients and centrifuged for 16 h (250,000g). Fractions were collected, and proteins were precipitated with 10% TCA and resuspended in SDS gel sample buffer (Laemmli 1970) before Western blot analysis.

Subcellular localization experiments

Six-day-old Arabidopsis seedlings treated with 3 µM ABA or onion epidermal layers were bombarded according to standard procedures (Seki et al. 1998). ABI5:YFP, AFP:CFP, and YFP:COPI1 gene fusions were under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Ballesteros et al. 2001).

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References


