Abscisic Acid Induces Rapid Subnuclear Reorganization in Guard Cells[1][w]

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While the phytohormone abscisic acid (ABA) is well established as a regulator of gene transcription and ion channel activity (Rock, 2000; Assmann and Wang 2001; Schroeder et al., 2001; Finkelstein et al., 2002), recent identification of RNA-binding proteins ABH1 (ABA hypersensitive), SAD1 (supersensitive to ABA and drought), and HYL1 (hyponastic leaves), whose mutation confers an ABA-hypersensitive phenotype (Lu and Fedoroff, 2000; Hugouvieux et al., 2001, 2002; Xiong et al., 2001), suggests that ABA may also play important roles in posttranscriptional RNA processing. Here we show that ABA dynamically regulates subnuclear architecture in guard cells, promoting the partitioning of a heterogeneous nuclear ribonucleoprotein (hnRNP)-type protein, AKIP1, into discrete subnuclear bodies or speckles via a process that has both Ca^{2+}-dependent and Ca^{2+}-independent steps and requires an active transcriptional machinery.

hnRNPs are mRNA-protein complex proteins (mRNP proteins) that bind RNA and affect its metabolism (Krecic and Swanson, 1999; Dreyfuss et al., 2002). Members of the hnRNP family are involved in all aspects of RNA metabolism, including pre-mRNA splicing, mRNA localization, mRNA stability, nuclear export of mRNA, and translational control (Dreyfuss et al., 1996, 2002; Krecic and Swanson, 1999; Mili et al., 2001; Reed and Magni, 2001). Binding of hnRNPs with nascent RNA transcripts results in the formation of ribonucleoprotein complexes (RNPs). RNPs are highly dynamic, and it has been demonstrated in mammalian cells that hnRNPs bind or dissociate from the target RNA at various stages of mRNA maturation until a distinct mRNP is formed and translocated from the nucleus to the cytoplasm for translation initiation (Dreyfuss et al., 2002). In plants, little is known about the function of hnRNPs (Albà and Pagès, 1998; Lorković et al., 2000; Lambermon et al., 2002; Lorković and Barta, 2002). We reported previously (Li et al., 2002) that AKIP1 is an hnRNP-like RNA binding protein from broad bean (*Vicia faba*). AKIP1 exhibits greatest sequence homology to mammalian hnRNP A/B and D proteins and has two RNA-recognition motifs. AKIP1 is phosphorylated by guard cell AAPK, an ABA-activated, Ca^{2+}-independent protein kinase involved in ABA-regulation of stomatal closure and plasma membrane anion channel activity (Li and Assmann, 1996; Li et al., 2000). Upon phosphorylation, AKIP1 becomes competent to bind dehydrin mRNA, which encodes a class of stress-protectant proteins (Close, 1996, 1997). ABA treatment of guard cells expressing AKIP1-green fluorescent protein (GFP) also results in rapid subnuclear clustering of AKIP1-GFP. In this report, we examine the signal transduction machinery involved in this phenomenon.

ABA PROMOTES REORGANIZATION OF AKIP1 INTO NUCLEAR SPECKLES

We previously reported that AKIP1-GFP is localized to the nucleus in guard cells of broad bean (Li et al., 2002). This phenomenon was confirmed by immunolocalization experiments using anti-AKIP1 polyclonal antibody (see supplemental figure, which can be viewed at www.plantphysiol.org). This is interesting because studies using mammalian cells showed that hnRNP D proteins associate with pre-mRNA, are present both in the cytoplasm and nucleus, and are important in mRNA stability and turnover (Dreyfuss et al., 1996, 2002; Krecic and Swanson, 1999; Mili et al., 2001; Reed and Magni, 2001; Xu et al., 2001). Arabidopsis UBA2a, an hnRNP-like RNA binding protein, has also been reported to function in stabilization of transcripts (Lambermon et al., 2002). Our observations to date suggest that AKIP1, despite sequence homology to hnRNP D proteins, is unlikely to fulfill any cytoplasmic functions due to its nuclear localization. Thus, at present, the functional classification of AKIP1 within the various hnRNP subclasses requires further experimental analysis.

To further characterize the effect of ABA on rapid relocalization of AKIP1 within the guard cell nucleus, we investigated the time-course of ABA-induced relocalization of AKIP1-GFP into nuclear speckles.

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We observed that ABA-induced relocalization of AKIP1-GFP into nuclear speckles increased with time, over the 30 min observation period (Fig. 1, A and B). The increase in number of speckles at 30 min is significantly greater in ABA-treated cells compared to control cells (Fig. 1C; $P \leq 0.05$, ANOVA). This rapid response is further supported by time-lapse photography of AKIP1-GFP relocalization into nuclear speckles, which indicates that redistribution occurs as early as 10 min following treatment with 50 μM ABA (see supplemental video). Such rapidity in the response is in good agreement with our previous observations in guard cell protoplasts that ABA can induce, within 15 min, phosphorylation of AKIP1 and activation of dehydrin mRNA accumulation (Li et al., 2002).

Ca^{2+}-DEPENDENT AND Ca^{2+}-INDEPENDENT SIGNALING STEPS COOPERATE TO MEDIATE ABA-INDUCED PARTITIONING OF AKIP1 INTO NUCLEAR SPECKLES

We next used AKIP1-GFP to further dissect the signaling pathway(s) associated with ABA-induction of AKIP1 relocalization into nuclear speckles. Pretreatment of epidermal peels with a transcriptional inhibitor cocktail consisting of actinomycin D and α-amanitin (TI) dramatically attenuated ABA-induced relocalization of AKIP1 into nuclear speckles (Fig. 1D). This result suggests that transcriptional activation and the accumulation of target mRNAs are required for relocalization of AKIP1 into nuclear speckles. An alternative hypothesis would be that the presence of these pharmacological agents interfered with AAPK phosphorylation of AKIP1. However, this latter hypothesis is not supported; in vivo phosphorylation assays illustrate that the same transcriptional inhibitor cocktail did not affect ABA-induced phosphorylation of AKIP1 in guard cell protoplasts (Fig. 1, F and G). Interestingly, these data suggest that ABA-induced AKIP1 phosphorylation alone is insufficient for relocalization of AKIP1 into nuclear speckles and suggest that relocalization is dependent on both ABA-induced phosphorylation of AKIP1 and ABA-stimulation of gene transcription.

Several studies have indicated that ABA-activation of stress-related transcription is Ca^{2+}-dependent (Sheen, 1996, 1998), and incubation of experimental material with buffers that chelate Ca^{2+} can reduce ABA-activation of gene expression (Wu et al., 1997; Webb et al., 2001). We decided to use a similar approach to examine the Ca^{2+} dependency of AKIP1 relocalization to nuclear speckles. We observed that pretreatment of epidermal peels with the Ca^{2+}-chelators, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), or EGTA inhibited ABA-induced relocalization of AKIP1 into nuclear speckles (Fig. 1E). This suggests that Ca^{2+}-chelation is interfering either with AAPK phosphorylation of AKIP1 and/or ABA-activation of gene transcription. To

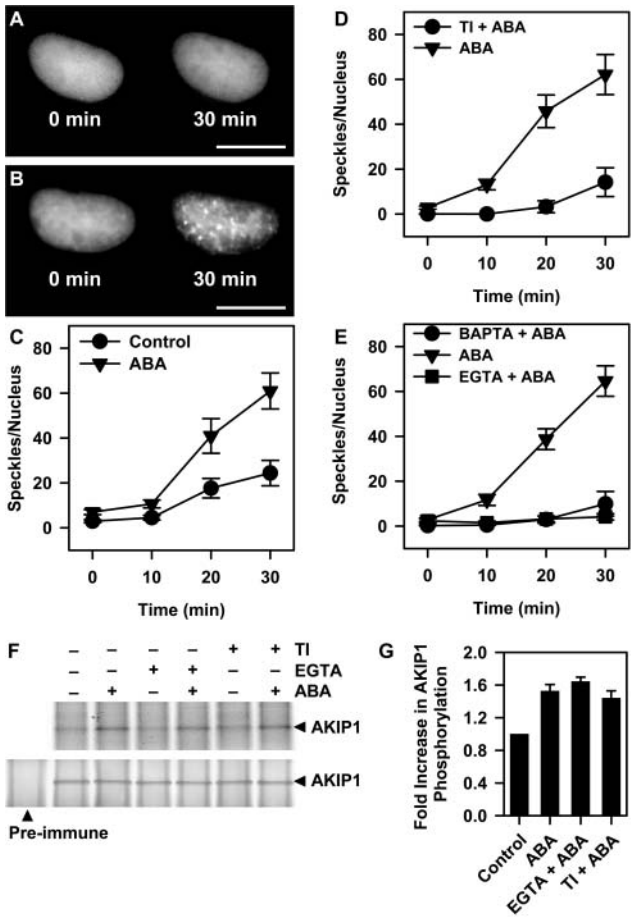

Figure 1. Effect of ABA on subnuclear relocalization of AKIP1-GFP. A, Representative images of the effect of a 30 min incubation with buffer only (10 mM MES, 50 mM KCl, pH 6.2) on the distribution of AKIP1-GFP in the nucleus ($n = 19$). B, Representative images of the effect of a 30 min treatment with buffer containing 50 μM ABA on the distribution of AKIP1-GFP in the nucleus ($n = 14$). C, Kinetics of AKIP1-GFP relocalization in the nucleus over a period of 30 min in guard cells incubated in buffer only ($n = 19$) or buffer containing 50 μM ABA ($n = 14$). D, Kinetics of 50 μM ABA-induced AKIP1-GFP relocalization in the nucleus over a period of 30 min in guard cells pretreated for 30 min with buffer only ($n = 15$) or buffer containing a transcriptional inhibitor cocktail consisting of 10 μg/mL actinomycin D and 50 μg/mL α-amanitin ($n = 16$). E, Kinetics of 50 μM ABA-induced AKIP1-GFP relocalization in the nucleus over a period of 30 min in guard cells pretreated for 30 min with buffer only ($n = 16$) or buffer containing 2 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid ($n = 17$) or 2 mM EGTA ($n = 7$). Values are mean ± SE. F, ABA-dependent phosphorylation of AKIP1 in vivo. Top portion: AKIP1 was immunoprecipitated from ^{32}P-labeled guard cell protoplasts using anti-AKIP1 polyclonal antibody. ABA was added at 50 μM with dimethyl sulphoxide (DMSO) as a vehicle. DMSO alone had no effect. Two millimolar EGTA or the transcriptional cocktail consisting of 10 μg/mL actinomycin D and 50 μg/mL α-amanitin did not significantly affect ABA-activation of AKIP1 phosphorylation in vivo. Bottom portion: Western blot showing that treatments did not alter the amount of AKIP1 protein available for phosphorylation. Data in both panels are representative of three separate experiments. G, Quantification of ABA-induced AKIP1 phosphorylation (as fold increase) in the absence or presence of 2 mM EGTA or a transcriptional cocktail consisting of 10 μg/mL actinomycin D and 50 μg/mL α-amanitin. Values are mean ± SE of three replicates. Bar = 10 μm.

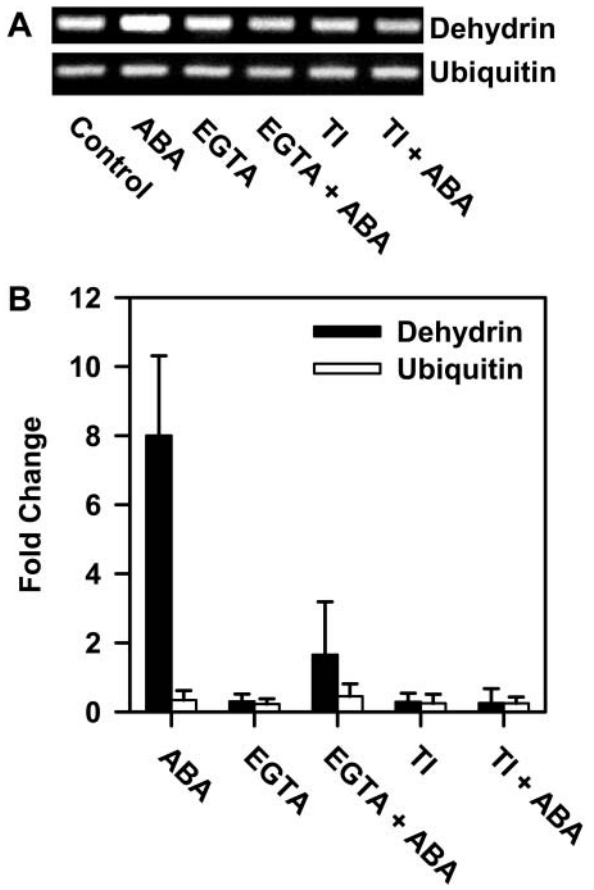

Figure 2. Effect of EGTA and transcriptional inhibitors on dehydrin gene expression in broad bean guard cells. Highly purified broad bean guard cell protoplasts were treated with DMSO for 30 min (control), 50 μM ABA for 30 min (ABA), preincubation with 2 mM EGTA for 30 min followed by DMSO for 30 min (EGTA control) or 50 μM ABA for 30 min (EGTA plus ABA), and preincubation with transcriptional inhibitor mix for 2 h followed by DMSO for 30 min (TI control) or 50 μM ABA for 30 min (TI plus ABA). cDNA was synthesized from total RNA isolated from these guard cell protoplasts. A, RT-PCR with dehydrin primers shows amplification of a specific product that is up-regulated by ABA. PCR with ubiquitin primers is used as control. B, Identical cDNA samples were subjected to quantitative real time RT-PCR, using the SYBR Green dsDNA binding dye method. Change in expression level is presented as fold change in comparison to control cDNA. Amplification with ubiquitin primers served as an internal control. Data shown are means $\pm$ SD of three experiments. $P < 0.03$ (ANOVA).

examine these hypotheses, we assayed for ABA-activation of AKIP1 phosphorylation in vivo using guard cell protoplasts and showed that EGTA did not significantly affect ABA-induced phosphorylation of AKIP1 (Fig. 1, F and G). This result is consistent with our previous observation that AAPK catalytic activity in vitro is Ca^{2+}-independent (Li and Assmann, 1996) and implicates Ca^{2+} participation in the speckling phenomenon at a step downstream or independent of AKIP1 phosphorylation.

To validate the use of the transcriptional inhibitors, and to evaluate the hypothesis that EGTA interference with ABA-induced gene expression might be responsible for its inhibitory effect on speckle formation, we analyzed the expression of the dehydrin gene in broad bean guard cells in response to ABA in the presence or absence of these pharmacological agents. Expression analysis by reverse transcription (RT)-PCR (Fig. 2A) was quantitatively confirmed using real-time PCR (Q-PCR; Fig. 2B). Q-PCR data are presented as fold change in expression level over and above the control level.

The PCR results show the expected attenuation of the ABA-induced increase in dehydrin level by the transcriptional inhibitors (Fig. 2, A and B), validating their efficacy in inhibiting production of the AKIP1 target mRNA, dehydrin. ABA up-regulation of dehydrin transcript levels was also blocked by EGTA. This result suggests that the Ca^{2+} chelator is interfering with ABA-activation of gene transcription. Because the transcriptional inhibitors and EGTA inhibited both dehydrin gene expression and AKIP1 relocalization, these data suggest that transcriptional events, plausibly regulated by cytosolic Ca^{2+}, are required for ABA-induced AKIP1 subnuclear partitioning.

DISCUSSION

Several functional categories of plant proteins have been shown to partition into nuclear speckles,

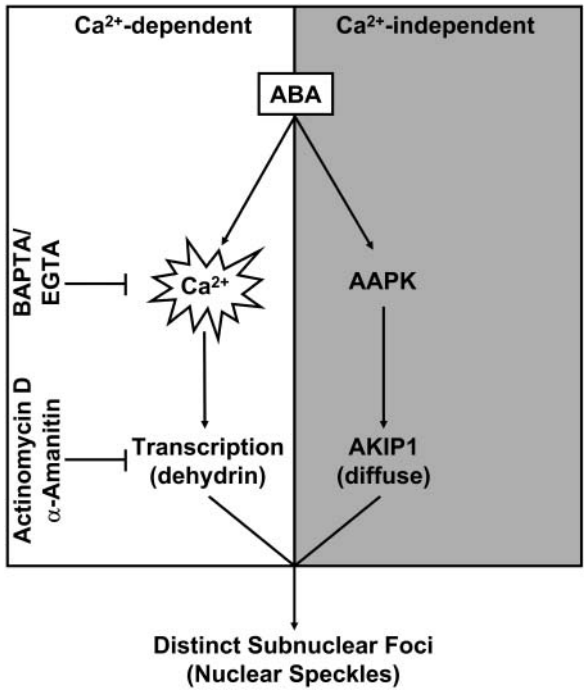

Figure 3. Working model for ABA-induced relocalization and retention of AKIP1 into nuclear speckles. ABA activates AAPK, leading to AKIP1 phosphorylation. At the same time, ABA activates transcription of dehydrin and/or other stress-related transcripts. Increased affinity of phosphorylated AKIP1 for dehydrin transcript facilitates relocalization into nuclear speckles.

suggesting the probable existence of different types of subnuclear bodies, as has been demonstrated for mammalian cells (Dreyfuss et al., 2002). Plant proteins known to exhibit a speckled localization include D polypeptide (a spliceosome protein; Glyn and Leitch, 1995) and atRSZ33, an Arg/Ser-rich RNA binding protein (Lopato et al., 2002), consistent with the speckled distribution of many mammalian splicing factors (Dreyfuss et al., 2002). The plant photoreceptors, phytochromes, and cryptochromes are also found in nuclear speckles (Yamaguchi et al., 1999; Más et al., 2000; Wang et al., 2001; Kircher et al., 2002; Yanovsky et al., 2002). Additionally, cryptochromes 1 and 2 as well as SPA1, a negative regulator of phytochrome A signaling, have been shown to colocalize with COP1, an E3 ubiquitin ligase (Osterlund et al., 2000; Wang et al., 2001; Seo et al., 2003). COP1 also colocalizes in speckles with AFP, which acts as a negative regulator of ABA signaling by promoting degradation of the colocalized ABI5 protein (Lopez-Molina et al., 2003). As such, the COP1-containing subset of nuclear speckles may function as sites for protein degradation (Osterlund et al., 2000; Lopez-Molina et al., 2003; Seo et al., 2003) analogous to clastosomes, which are nuclear bodies highly enriched in components of the ubiquitin-proteasome pathway, in mammalian cells (Lafarga et al., 2002). Future studies will be aimed at identifying other proteins residing in AKIP1-associated nuclear speckles, enabling determination of the exact category of subnuclear speckles in which AKIP1 resides, and elucidation of the role of AKIP1 protein in mRNA metabolism.

In conclusion, our data suggest that both Ca^{2+}-independent and Ca^{2+}-dependent processes cooperate to mediate relocalization of AKIP1 into nuclear speckles (Fig. 3). Our working model is that ABA acts via AAPK to modulate the phosphorylation status of AKIP1, priming it for subsequent binding of dehydrin and possibly other stress-related transcripts whose levels are up-regulated by ABA via cytosolic Ca^{2+} increases occurring during stress (Rock, 2000; Assmann and Wang, 2001; Evans et al., 2001; Webb et al., 2001). Our observations build on recent studies from the Fedoroff, Schroeder, and Zhu laboratories, suggesting that the double-stranded RNA binding protein HYL1, the RNA cap-binding protein ABH1, and the Sm-like snRNP SAD1 may have important functions in modulating ABA responses (Lu and Fedoroff, 2000; Hugouvieux et al., 2001, 2002; Xiong et al., 2001; Fedoroff, 2002; Kuhn and Schroeder, 2003). In particular, mutation of *ABH1* has been shown to result in ABA-hypersensitivity of seed germination and stomatal closure and altered ABA-regulation of guard cell ion channels (Hugouvieux et al., 2001, 2002). It will be of interest to determine if ABA also induces the partitioning of HYL1, ABH1, and SAD1 into nuclear speckles in a manner similar to that observed for AKIP1, and whether ABA affects the subnuclear distribution of the ABA-related proteins AFP and ABI5 (Lopez-Molina et al., 2003). Finally, our results reveal that the rapidity of ABA signaling to the nucleus to alter nuclear architecture in stomatal guard cells is comparable to the rate of ABA-induced turgor responses in these cells. Thus, we propose that rapid subnuclear reorganization may form an important component of acclimatory stress responses in plants.

MATERIALS AND METHODS

AKIP1-GFP Subcellular Localization

Seeds of broad bean (*Vicia faba*) cv Long Pod were sorted according to size and smoothness of seed coat. Seeds measuring about 2.5 cm in length and 1.5 to 1.8 cm in width were selected. The initial selection of seeds according to size ensures uniformity in terms of germination and plant growth. Plants were grown in Metromix 360 (Scotts-Sierra, Marysville, OH) under a 10-h day/14-h night regime (photosynthetic photon flux density: 200 μmol m^{-2} s^{-1}) at 22°C ± 2°C and watered every other day, alternating between one-half-strength Hoagland solution and water. Only the first fully-expanded leaves of 4- to 5-week-old plants were used for particle bombardment with AKIP1-GFP plasmids as described previously (Li et al., 2000, 2002). Expression was examined by fluorescence microscopy as described (Li et al., 2002). Fluorescence images of AKIP1-GFP distribution were acquired with a Sensys CCD camera (Photometrics, Trenton, NJ). The number of AKIP1-GFP labeled speckles was quantified from images taken at various times after incubation in 10 mM MES, 50 mM KCl, pH 6.2, with or without 50 μM ABA (A.G. Scientific, San Diego), 2 mM EGTA, or a transcriptional cocktail consisting of 10 μg/mL actinomycin D and 50 μg/mL α-amanitin. All chemicals were obtained from Sigma-Aldrich (St. Louis) unless otherwise stated.

In Vivo Phosphorylation Assay

Immunoprecipitation and in vivo phosphorylation assays were performed as previously described (Li et al., 2002).

RT-PCR and Real-Time Quantitative RT-PCR

Total RNA was isolated from guard cells of broad bean treated with Ca^{2+} chelators or transcriptional inhibitors using ISOGEN (Nippongene, Toyama, Japan). Five μg of total RNA was reverse transcribed using the Superscript II RT kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was diluted at a concentration of 1:100, aliquoted, and kept at 4°C throughout each experiment to avoid discrepancies in the data due to freeze-thaw cycles. The PCR amplification was performed with oligonucleotides specific for dehydrin and ubiquitin cDNAs using the following primers:

Deh forward: AACAAGGTACGGTGGAAGTG
Deh reverse: ATCCTCCAGTACCAGGAAGC
Ub forward: GCAGCTCGAGGATGGAAGGA
Ub reverse: CCAGCTGCTTACCCGCAAAG.

The position of these oligonucleotides was chosen so that the size of the PCR products is between 200 and 250 bp. The suitability of the oligonucleotide sequences in terms of efficiency of annealing was evaluated using the Primer 3 program. The identity of both dehydrin and ubiquitin RT-PCR products was confirmed by sequencing.

Q-PCR experiments were repeated three times independently, and the data were averaged. For Q-PCR, the cDNA was amplified in the presence of SYBR-Green I dye (Molecular Probes, Eugene, OR) at 0.125× final concentration using a DNA Engine Opticon 2 thermal cycler (MJ Research, Waltham, MA). Amplification of ubiquitin cDNA under identical conditions was used as an internal control to normalize the level of cDNA. The data obtained were analyzed with Opticon 2 software (MJ Research). Since SYBR Green I dye binds to the minor groove of any dsDNA, including specific products, nonspecific products, and primer-dimers, it is necessary to perform a melting curve analysis at the end of each Q-PCR experiment. Nonspecific products or primer-dimers can be identified as they melt at a lower temperature compared

to the specific amplicon. Specific melting temperatures obtained for ubiquitin (86°C) and dehydrin (84°C) validated the specific product formation.

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