

ABA SIGNALING NETWORKS IN ARABIDOPSIS

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ABSTRACT

Abcisic acid (ABA) regulates both seed maturation and germination, and several *ABA-insensitive (ABI)* loci encoding transcription factors are required for normal seed maturation and inhibition of germination and seedling growth by ABA. We are expanding our knowledge of this signaling network by determining which of these factors interact with each other, identifying additional factors that interact directly with the ABI gene products, and identifying genes that are directly regulated by the ABI factors. These studies have revealed extensive cross-regulation among these factors, and identified a novel plant-specific family of proteins that modifies ABA and abiotic stress sensitivity. Regulatory targets of the ABI factors constitute less than 10% of all ABA-regulated genes, including presumed desiccation protectants and additional regulators, but these may be core components of ABA response because ABI over-expression confers hypersensitivity to ABA and other stresses. Our results may lead to strategies for manipulation of seed nutritional, germination or storage qualities.

INTRODUCTION

The plant hormone abscisic acid (ABA) affects many agronomically important features of plant growth including embryo development, seed and bud dormancy, water movement and retention, tolerance of a variety of abiotic stresses (e.g. drought, salinity, and cold), and senescence. Many of these effects involve changes in gene expression, and transcriptional profiling studies in *Arabidopsis* have shown that as much as 5% of the genome is regulated by ABA (Hoth et al., 2002; Seki et al., 2002; Suzuki et al., 2003). However, it is likely that distinct subsets of these genes are ABA-responsive within any given organ or tissue type. The specificities of these responses depend on the local ABA concentration and the available signal transduction components. Consequently, one strategy to modify only subsets of ABA responses requires that we identify specific regulators with limited effects.

Attempts to understand ABA signaling have employed biochemical, pharmacological and genetic approaches to identify possible signal transduction components. To date, over 50 loci have been shown to affect some aspect of ABA signaling; many of these also regulate responses to other signals, such as other hormones or environmental conditions, providing a mechanism for integrating response to multiple signals (Finkelstein et al., 2002). Many of the mutants affecting these loci have no obvious phenotypic defects in the absence of ABA or stress treatments and, even under stress conditions, some phenotypes are detectable only by molecular analysis. The subtleties and complexities of these phenotypes are consistent with a high degree of genetic redundancy and many points of interaction or “cross-talk” among signaling pathways. The products of these loci include transcription factors, protein kinases and phosphatases, GTP-binding proteins, enzymes involved in phospholipid metabolism or RNA processing, and many unknowns. A current challenge is to discern which combinations of these components interact to form a signaling network and to identify regulators that can enhance stress tolerance without severely stunting growth.

Among these ABA effects, control of seed maturation and the subsequent commitment to seedling growth are major determinants of a plant’s reproductive success. Several loci affecting sensitivity to ABA at germination have been identified as the genes disrupted in the *ABA insensitive (abi)* mutants of *Arabidopsis* (Finkelstein et al., 2002). Three of these loci (*ABI3*, *ABI4* and *ABI5*) encode transcription factors. We are broadening our studies of the network by identifying additional genes that interact with or regulate these loci and investigating their function in ABA and/or stress signaling in seeds and seedlings.

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MATERIALS AND METHODS

Plant Genetic Materials

Single and double mutant lines with defects in *ABI4*, *ABI5*, *FUS3*, *LEC1*, *ABF3* and *ABF1* were isolated as described in (Brocard-Gifford et al., 2003; Finkelstein et al., 2005). T-DNA insertion lines for the *A5IPs* were obtained from the SIGnAL collection (Alonso et al., 2003), distributed by the ABRC.

Yeast Two-Hybrid Screen

A translational fusion of the DNA binding domain (BD) of GAL4 and all but the first eight amino acids of ABI5 was constructed as described in (Nakamura et al., 2001), and transformed into yeast (*Saccharomyces cerevisiae*) strain PJ69 (James et al., 1996) to be used as “bait” in a two-hybrid screen. An activation domain (AD) fusion cDNA library derived from 3-day-old etiolated tissue (Kim et al., 1997), distributed by the ABRC, was transformed into the yeast and cells containing potentially interacting fusions were selected by complementation of defects in histidine and adenine biosynthesis. Plasmids encoding the activation domain fusions were isolated and their fusion genes sequenced to identify the predicted interacting protein. Additional interactions were tested by transforming the AD fusion plasmids back into yeast, in combination with other BD fusions. Interactions were assayed qualitatively by complementation of the auxotrophies, and quantitatively by activation of a beta-galactosidase reporter gene, as described in (Nakamura et al., 2001).

Plant Material For Microarray Analyses

In order to emphasize the effects of each specific *ABI* regulator, we chose to compare ABA-responsive gene expression in *ABI* over-expression (*35S:ABI*) vs. mutant (*abi*) lines. Plants (11-12 days old) were treated with 50 μ M ABA and 20 μ M cycloheximide (CHX) for 4 hrs prior to harvest and RNA extraction; the short induction period and inclusion of CHX was intended to limit inductions to those that are primary effects of the constitutively over-expressed *ABI* factor. RNA was prepared by hot phenol extraction, followed by serial precipitations, as described in (Finkelstein et al., 1985). Prior to use in microarray experiments, effectiveness of the transgene expression and the ABA and CHX treatments were confirmed by Northern blot analyses of genes whose expression patterns were predictable based on previous studies.

Microarray Analyses

Affymetrix ATH1 Gene Chips were used to characterize transcriptomes associated with specific genomes and treatments. All procedures for generating labeled targets and hybridization of these targets to elements on the Gene Chips were carried out as recommended by Affymetrix. Briefly, cDNA was derived from total RNA and subsequently used to generate biotin labeled cRNA which was fragmented to lengths between 35 and 200 bp. Hybridization of the fragmented cRNA to the Gene Chip Array was carried out in a Affymetrix GeneChip Hybridization Oven 640. Appropriate washing and staining of the arrays was then conducted on an Affymetrix GeneChip Fluidics Station 400 under the control of Affymetrix Microarray Suite 5.0 software. The stained and washed array was finally scanned with an Agilent GeneArray Scanner.

The output information from the scanner, including intensity data and hybridization quality flags, and annotation information on the genes represented on the ATH1 Genechip were then used as input to GeneSpring 7 (Silicon Genetics/Agilent) for analysis and data mining. The program was used to normalize the intensity data. All values below 0.01 were set to 0.01. For purposes of comparison, the data from each chip were compared to the control chip which was the chip hybridized with targets derived from seedlings over expressing the *ABI4* gene in the absence of added ABA. Each measurement for each gene on each chip was divided by the median of that gene's measurements on the control chip. Only genes flagged as present or marginal in the experimental chips were considered for determining those that were up-regulated relative to the control. Similarly, only genes flagged as present or marginal in the control hybridization were considered for determining genes down-regulated with respect to the control.

RESULTS AND DISCUSSION

We have used three approaches to elucidating the network surrounding the *ABI* transcription factors: testing for potential interactions with known loci encoding additional

transcription factors, screening for proteins that can interact directly with the ABI proteins in a yeast two-hybrid system, and identifying regulatory targets of the ABI factors by microarray analyses.

Tests of Interactions Among Known Loci

ABI3, *ABI4*, and *ABI5* encode transcription factors that are members of three distinct families that play a major role in the seed/seedling transition and ABA response: the B3-, AP2-, and bZIP domain families (Finkelstein et al., 2002) (Figure 1). Other loci regulating embryonic identity were identified as defective in *leafy cotyledon (lec)* class mutants, including those encoding additional B3 family members (e.g. *FUSCA3* and *LEAFY COTYLEDON2*). Other bZIPs mediating ABA or stress-

responsive gene expression (ABFs, AREBs, and AtDPBFs) were identified based on binding to cis-acting ABA-response elements, but very little genetic information was available for the loci encoding these factors until recently (Bensmihen et al., 2005; Bensmihen et al., 2002; Kim et al., 2004).

Consequently, both the *LEAFY COTYLEDON (LEC)* class loci and the other members of the *ABI5/ABF/AREB/AtDPBF* clade of bZIPs were good candidates for interactors with the *ABI* transcription factor loci.

Furthermore, genetic interactions between *ABI3* and the *LEC* class loci had already been demonstrated (Parcy et al., 1997). To test for genetic interactions between the *LEC* class loci and *ABI4* or *ABI5*, or among potentially redundant *ABI5*-related bZIPs, we constructed and characterized double mutants. The potential for direct interactions was tested in a yeast two-hybrid assay, and by determining whether the various combinations of factors were co-expressed.

The genetic studies demonstrated both synergistic and antagonistic interactions among the *ABI* and *LEC* class factors, the nature of the interaction varying with the specific response (Brocard-Gifford et al., 2003). Although such interactions are most consistent with a complex combinatorial control network, no direct interactions were observed by two-hybrid assays (Brocard-Gifford et al., 2003). However, detailed expression analyses showed substantial cross-regulation among these loci, and distinct temporal and spatial regulation. Although all of these factors are expressed throughout embryo development, *LEC1* is most abundant earliest (Harada, 2001) and is required for a subsequent increase in *FUS3* expression (Brocard-Gifford et al., 2003). *FUS3* inhibits GA synthesis and promotes ABA synthesis, which in turn promotes *FUS3* stability, thereby enhancing *FUS3* activity in mid-embryogeny (Gazzarrini et al., 2004). *LEC1* and *FUS3* also regulate expression and/or stability of *ABI3* (Parcy et al., 1997), *ABI5*, and several related bZIPs (Brocard-Gifford et al., 2003). It is not known whether any of these reflect direct cross-regulation, but *ABI5* at least is auto-regulatory (Brocard et al., 2002).

In contrast to the dramatic defects of the *lec* class mutants, loss of function for most of the *ABI5* related clade of bZIPs have almost no discernible phenotype (Bensmihen et al., 2005; Bensmihen et al., 2002; Finkelstein et al., 2005; Kim et al., 2004). However, *ABI5* and *ABF3* appear to function redundantly, such that double mutants are more resistant to ABA than the monogenic parents (Finkelstein et al., 2005). Surprisingly, loci with similar effects antagonistically regulate each other's expression (Finkelstein et al., 2005), possibly providing a

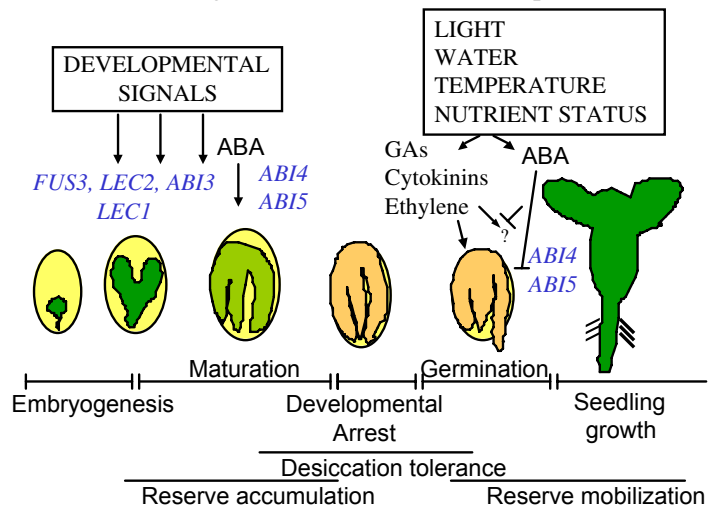


Figure 1. Factors regulating the seed/seedling transition

mechanism to emphasize regulation by distinct family members in different tissues or stages of development.

Screens for interactors

Relatively few direct interactions with the ABI factors were observed by two-hybrid assays. Therefore, we screened for additional factors that might participate in complexes affecting ABI-dependent signaling. The strongest interactions for ABI5 were observed with a family of four proteins containing 3 highly conserved (70-86% identical) domains, none of which have any known function. We have designated these A5IPs, for ABI5-interacting proteins. To determine whether these proteins participate in ABA signaling in plants, we obtained T-DNA insertion lines for all of the loci and constructed over-expression lines for most. Comparison of ABA sensitivity of *A5IP* loss- and gain-of-function lines confirms a role for all in ABA response (Figure 2). However, despite the high degree of sequence conservation, different family members have opposing effects on ABA sensitivity. Expression analyses and additional yeast two-hybrid assays indicate that the A5IPs may interact with multiple members of the ABI5-related bZIP clade in different tissues or developmental stages. Although the genetic data confirm the importance of the A5IPs in regulating ABA response, they do not address mechanism, which might involve effects on stability, activity or localization of ABI5 and related bZIPs (Figure 3).

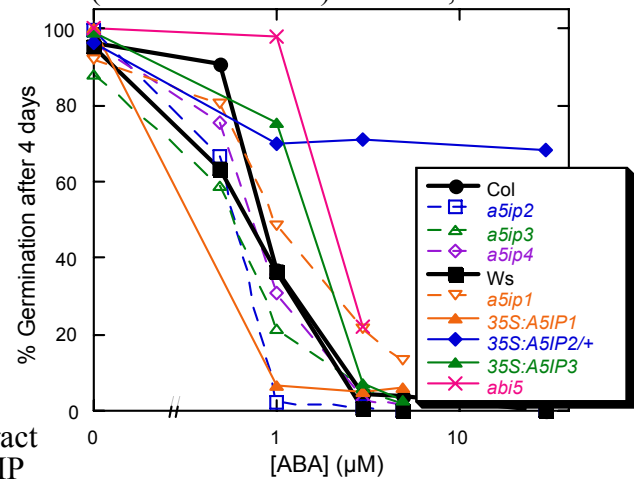


Figure 2. ABA sensitivity of *A5IP* mutant and over-expression lines, listed beneath their respective wild-type backgrounds.

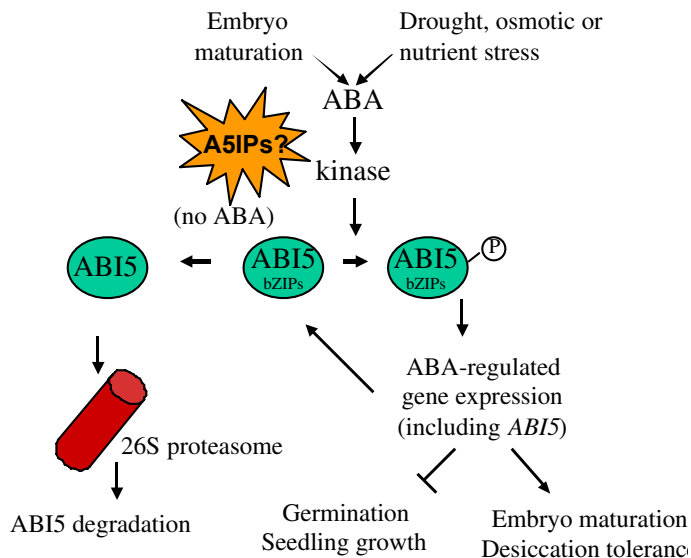


Figure 3. Hypothetical model of interactions among A5IPs, ABI5 and possibly related bZIPs affecting the commitment to seedling growth. The biochemical functions, and hence the mechanism of action, of the A5IPs are unknown.

Identifying regulatory targets of ABI transcription factors

Comparison of either loss- or gain-of-function lines for either ABI4 or ABI5 suggests that these factors have similar roles in ABA and stress response (Brocard et al., 2002; Finkelstein, 1994; Söderman et al., 2000). Furthermore, the ABA and stress hypersensitivity of the over-expression lines suggests that the subset of genes that are regulated by these loci are central components of ABA response. To identify specific regulatory targets of the ABI factors, we used microarrays to compare ABA-inducible expression in overexpression vs. mutant lines. Cycloheximide was included to limit transcript accumulation to those genes that do not require additional protein synthesis to promote their expression. These studies test the hypotheses that the ABIs have similar effects due to

the overlap in their targets, and that these targets include some regulators as well as genes encoding mediators of desiccation tolerance and other protective functions. In contrast to *abi1-1*, which perturbs regulation of greater than 90% of ABA-regulated genes (Hoth et al., 2002), each ABI appeared to regulate less than 100 ABA-inducible genes (~10% of the total), with only 20-30% of their targets in common. The largest class of shared targets is late embryogenesis abundant and related genes, which encode extremely hydrophilic proteins and are correlated with desiccation tolerance in many contexts (Wise and Tunnacliffe, 2004). Interestingly, additional targets include transcription factors, receptor-like kinases, protein phosphatases, and enzymes of phospholipid metabolism, suggesting that these ABIs may act in part by activating components of other signaling pathways. Reverse genetic studies are in progress to test the relevance of some of these other regulators to ABA or other stress responses.

CONCLUSIONS

These studies have shown that some of the *ABI* genes are members of gene families with overlapping functions that may be either synergistic or antagonistic, and that there is substantial cross-regulation of expression among some *ABI* genes and their homologs. We have also identified strong interactions with another novel family of proteins that affect ABI function in early seedling growth, the A5IPs. Finally, our microarray studies identified 15 potential ABI-regulated regulators that may contribute to ABI-dependent gene expression, as well as provide a mechanism to integrate responses to ABA and other stress signals. By integrating molecular, genetic and physiological data, we hope to develop a coherent model of ABA action that could have applications in modifying seed quality and yield or stress tolerance of plants.

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