**MEETING REPORT**

# New Insights into ABA-Mediated Processes

The mechanisms by which phytohormones trigger such diverse tissue-specific and developmentally regulated responses have long eluded plant biologists. However, through the use of molecular and genetic approaches, new insights into the molecular basis of phytohormone action have emerged within the last decade. This is particularly true for responses elicited by the phytohormones ethylene, auxin, and abscisic acid (ABA; Davies, 1995).

The influence of ABA on the typical life cycle of a higher plant is critical during seed development, when ABA levels increase markedly (Rock and Quatrano, 1995). This increase is part of the signal that initiates regulatory pathways which, in turn, promote maturation of the seed and the acquisition of desiccation tolerance, and which also prevent precocious germination (i.e., vivipary; McCarty, 1995). ABA also plays an important role in activating responses to environmental stresses, such as osmotic and temperature extremes (Ingram and Bartels, 1996). Because activation of the ABA signal transduction cascade occurs very early in these responses, ABA acts as an internal signal that enables the immobile plant to survive such stresses.

Clearly, ABA triggers the expression of different sets of genes in different tissues. Hence, understanding the molecular and genetic bases of ABA biosynthesis and the differential responses of plant cells to the hormone has enormous implications for current agricultural practices and for the future engineering of crops with improved traits in seeds and/or increased tolerance of environmental stresses (Giraudat et al., 1994; Rock and Quatrano, 1994; Giraudat, 1995).

Recent progress in our understanding of the ABA response pathway was described during a highly focused workshop organized by Montserrat Pagés (CSIC, Barcelona, Spain) and Ralph Quatrano (University of North Carolina at Chapel Hill, USA) that was held in late October 1996 at the Juan March Foundation in Madrid, Spain. The workshop brought together a small group of scientists who are exploring ABA responses in a diverse set of experimental systems. The presenters discussed a combination of genetic, biochemical, and electrophysiological strategies that are revealing new insights into the biosynthesis and perception of the ABA signal and the molecular bases of ABA responses in embryos, seeds, hypocotyls, guard cells, and whole plants.

## ABA Biosynthesis

Advances in our understanding of the ABA biosynthetic pathway (Figure 1) represent a classic example of the effective application of combined genetic and biochemical approaches toward a difficult problem in plant biology. The majority of the enzyme activities have been defined through the biochemical analysis of mutant plants; the next step—cloning the corresponding genes—is in progress. Clearly, the identification and cloning of genes encoding the key enzymes in the pathway are essential prerequisites for the engineering of transgenic plants with altered ABA levels. Such plants would be very useful for the direct assessment of the roles of ABA in physiological processes.

Until very recently, none of these genes was available. However, Annie Marion-Poll (INRA, Versailles, France) and Don McCarty (University of Florida, USA) reported on the isolation of ABA biosynthetic genes from *Nicotiana plumbaginifolia*, Arabidopsis, and maize following the identification of transposon-tagged, ABA-deficient mutations in each species. Generally, such mutant plants germinate precociously and have a strong tendency to wilt but can be rescued by the application of exogenous ABA.

The first committed step in ABA biosynthesis, the epoxidation of zeaxanthin (Figure 1), is impaired in the *Activator* (*Ac*)-tagged *aba2* mutant of *N. plumbaginifolia*. The *ABA2* gene, which was isolated by using *Ac*-flanking sequences, encodes a chloroplast-imported, 72.5-kD protein with sequence similarities to bacterial monooxigenases and oxidases (Marin et al., 1996). When overexpressed in *Escherichia coli*, the ABA2 protein was shown to catalyze the con-

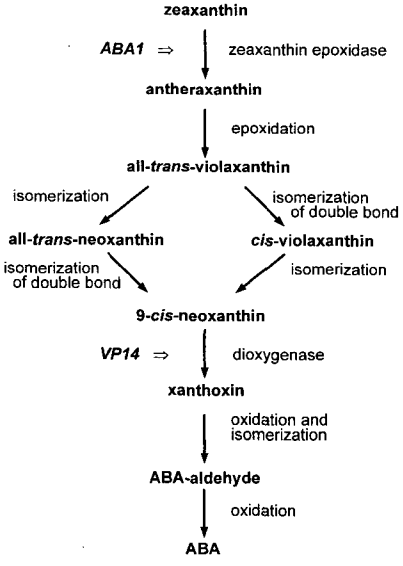


**Figure 1.** The ABA Biosynthetic Pathway in Higher Plants.

The scheme indicates the major steps in the biosynthesis of ABA, starting from zeaxanthin. *ABA1* and *VP14* indicate mutant loci from Arabidopsis and maize, respectively, for which the corresponding genes have been cloned.

version of zeaxanthin into antheraxanthin and violaxanthin in vitro (Figure 1). Moreover, the tobacco cDNA complemented both the tobacco *aba2* mutant and the corresponding Arabidopsis *aba1* mutant.

A similar approach was followed by McCarty, who reported the cloning of a novel maize viviparous mutation, *vp14*, which was identified in a population of *Mutator* (*Mu*)-tagged maize plants. McCarty showed that ABA levels are reduced in *vp14* embryos and that mutant embryos can be rescued by the application of exogenous ABA. However, no vegetative phenotype, including decreased sensitivity to ABA, was detected. Overexpression studies in *E. coli* with the corresponding Arabidopsis cDNA demonstrated that the *VP14* gene most likely encodes a member of a novel class of bacterial dioxygenases. Based on these findings and on biochemical analyses of the mutant plants, VP14 is thought to catalyze the oxidative cleavage of 9-*cis*-neoxanthin to xanthoxin (Figure 1).

Maarten Koornneef's (Wageningen Agricultural University, The Netherlands) presentation complemented the reports on the cloning of ABA biosynthetic genes. He described recessive mutations at two novel Arabidopsis loci, *aba2* and *aba3*. Both mutants have lower endogenous ABA levels than wild type, reduced seed dormancy, and suffer excessive water loss (Léon-Kloosterziel et al., 1996). Biochemical analyses suggest that these mutations affect the last two steps of ABA biosynthesis. Thus, the *ABA2* and *ABA3* genes, both of which should be cloned in the near future, are likely to encode enzymes that catalyze the oxidation and isomerization reactions that occur between xanthoxin, ABA-aldehyde, and ABA (Figure 1).

## ABA Perception

Despite numerous attempts, ABA receptors have yet to be been identified. However, Suzanne Abrams (Plant Biotechnology Institute, Saskatoon, Canada) described promising tools that she hopes to use to isolate and confirm the identity and function of potential receptor molecules, as well as to modify the bioactivity and stability of ABA.

To identify those parts of the ABA molecule that must be retained for activity, Abrams has constructed a series of ABA analogs in which portions of the structure have been altered. She has also coordinated a number of collaborations in which the ability of these analogs to trigger a variety of ABA responses, including inhibition of seed germination, activation of ABA-regulated gene expression, and induction of stress tolerance, is being tested. Abrams hopes to use the resulting information to identify carbon atoms in the ABA molecule to which photoaffinity ligands can be attached without affecting biological activity. Such analogs could potentially be used to identify the elusive ABA receptor(s).

Abrams also presented evidence that the bioactivity of one of the analogs, (+)-8′-methylene-ABA, is significantly higher than that of the natural ABA molecule. This is probably because of its increased in vivo stability, but it is also possible that the ABA receptor has a higher affinity for this analog. (+)-8′-methylene-ABA and other analogs still under development may be extremely useful for researchers and agriculturists seeking to increase the duration and level of specific ABA responses.

By analogy to the isolation of the serotonin 1c receptor (Julius et al., 1988), Mike Blatt and Barbara Leyman (Wye College, London, UK) presented a heterologous approach toward the functional identification of ABA receptors. Their approach hinges on the transient expression of *N. tabacum* leaf cDNA clones in Xenopus oocytes; the rationale is that ABA triggers an increase in $Ca^{2+}$ levels ($Ca^{2+}$ is a well known second messenger for ABA; Allan et al., 1994), which, in turn, activates endogenous $Ca^{2+}$-sensitive $Cl^-$ currents. Blatt and Leyman have been using patch clamping to monitor ABA-dependent changes in $Cl^-$ currents in Xenopus oocytes that are expressing pools of tobacco cDNAs. By successively diluting "positive" pools, they were able to identify a single cDNA clone that triggered an ABA-dependent activation of $Ca^{2+}$-sensitive $Cl^-$ currents. Blatt and Leyman are now characterizing this clone and investigating the influence of the putative receptor protein that it encodes on ABA responses in plants.

## Intermediates in the Response Pathway

Nam-Hai Chua (Rockefeller University, New York, USA) presented some very exciting results from his laboratory demonstrating that cyclic ADP-ribose (cADP-R) and nicotinic acid adenine dinucleotide phosphate (NAADP) are intermediates in ABA signaling pathways. Taking the same approach that previously led to the successful elucidation of intermediates in the light response pathway (Neuhaus et al., 1993; Bowler et al., 1994), Chua's group has been microinjecting subepidermal cells from the hypocotyl of the tomato mutant *aurea* with two ABA-responsive promoter–*GUS* fusions, *RD29a*::*GUS* and *KIN2*::*GUS*. *aurea* cells are optically ideal for the visualization of reporter gene products because they do not develop chloroplasts and anthocyanins.

No GUS activity was detected in injected cells unless ABA was present. However, in the absence of ABA, cADP-R was found to specifically induce expression of both gene fusions. Antagonists of cADP-R and NAADP blocked the induction of *GUS* expression, as did two kinase inhibitors, suggesting that phosphorylation is likely to be a key component of this signaling pathway. Jennifer Kuzma from the Chua laboratory also described her results showing that increases in cADP-R levels precede the induction of both *KIN2* mRNA accumulation and GUS activity.

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The Chua laboratory has taken advantage of the fact that *KIN2* expression increases in proportion to ABA concentration to devise a genetic approach to identify additional ABA signaling intermediates. Transgenic Arabidopsis plants expressing luciferase under the control of the *KIN2* promoter (*KIN2::LUC* plants) have been mutated, and Chua's group is screening the progeny for ABA hyper- and hyposensitive mutants on the basis of their luciferase activities in response to ABA. Any putative mutants isolated during this screen will be characterized with respect to the expression pattern of various ABA-, cold-, and desiccation-responsive genes.

Three presentations focused on ABA signal transduction in stomatal guard cells, a long-standing model system for examining ABA-mediated responses (Li and Assmann, 1996). Julian Schroeder (University of California, San Diego, USA) and Blatt both described their use of electrophysiological approaches to determine the position of the Arabidopsis *abi* lesions in the signaling pathway(s) that lead to reductions in guard cell turgor. Schroeder demonstrated for the first time the ability to patch clamp Arabidopsis guard cells, an advance that will allow genetics and electrophysiology to be brought together in the analysis of the ABA signaling pathway (see Pei et al., 1997, in the March issue of THE PLANT CELL).

Both laboratories produced further evidence that phosphorylation/dephosphorylation cascades are involved in ABA signal transduction (Armstrong et al., 1995; Schmidt et al., 1995; see also Leung et al., 1997, in press in the May issue of THE PLANT CELL). Schroeder showed that the *abi1* and *abi2* lesions both result in a disruption of the pathway that terminates at the slow activating anion channel (Pei et al., 1997), whereas Blatt demonstrated that the *abi1* lesion reduces the activity of the plasma membrane outwardly directed potassium channel. Schroeder also showed that the effects of the *abi1* and *abi2* mutations can be differentiated because the *abi1* mutant, but not the *abi2* mutant, can be rescued by a kinase inhibitor in the presence of ABA.

Alistair Hetherington (Lancaster University, UK) reported on the issue of ensuring specificity among $Ca^{2+}$-mediated signal transduction systems in guard cells and outlined a number of possible solutions to the problem. He presented data showing that guard cells are competent to respond to the novel intracellular calcium mobilizing second messenger cADP-R. These results and those from the Chua laboratory suggest that cADP-R functions as an intermediate in the ABA response pathway in two different cell types.

Also on the subject of second messengers, Blatt presented compelling evidence for the involvement of pH in guard cell signaling, and Schroeder described new tools for calcium imaging that will be useful in guard cells of Arabidopsis. Moreover, Hetherington discussed data showing that plants carrying the *abi1* and *abi2* mutations (either alone or in combination) were also affected in their ability to respond to signals that induce stomatal closure. Based on these results, he suggested that groups of several signaling components could be recruited to form a common signaling pathway—a "signaling cassette." Such a pathway could focus signals from different closure-inducing stimuli onto the specific ion channels that are responsible for stomatal closure.

### Transcriptional Regulation of ABA-Induced Genes

Several genes with potential functions in ABA responses were reported at the meeting to be induced by stress and/or ABA. These include genes encoding a protein kinase (Kay Walker-Simmons, Washington State University, Pullman, USA), a novel calcium binding protein (John Mundy, Copenhagen University, Denmark), a general DNA binding protein (M. Pagés), a bZIP protein (Tsukaho Hattori, Mie University, Tsu, Japan), and a homeodomain-containing leucine zipper protein (Dorothea Bartels, Max-Planck Institute, Köln, Germany; Peter Engstrom, Uppsala University, Sweden).

Based on this and previously published work, it has now become apparent that stress- and/or ABA-induced genes can be separated into three groups: (1) primary ABA-induced genes whose expression is independent of protein synthesis (i.e., the expression of another gene(s) is not required for ABA induction); (2) ABA-induced genes whose expression is dependent on the expression of other genes; and (3) stress-induced genes whose induction is independent of increases in ABA levels. Extensive promoter analyses are leading to a clearer picture of the DNA elements and protein factors that mediate the complex transcriptional regulation of genes involved in stress and/or ABA responses. Recent progress in these efforts was discussed in several talks.

Tuan-Hua David Ho (Washington University, St. Louis, USA) and his associates, who have taken both loss- and gain-of-function approaches to analyze the promoters of two ABA-induced barley genes, *HvA1* and *HvA22*, presented one example of this work. They have shown that in addition to sequence elements containing ACGT cores (such as the G-box motif), a novel promoter sequence termed the "coupling element" (CE) also appears to be necessary for ABA-mediated induction of these genes. Ho also showed that the combination of an ACGT-core element and a CE (e.g., CE1 [TGCCACCGG] or CE3 [ACGCGTGTCCTC]) defines the smallest promoter unit that is sufficient to confer ABA responsiveness in transient assays (Shen et al., 1996).

Ho speculated that combinatorial interactions between an ACGT-core element and a series of unique CEs could account for the specificity of the ABA

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response at the level of individual genes. Although ACGT-core elements are fairly widespread, even in the promoters of genes that do not respond to ABA, the lack of a CE element would render these promoters ABA insensitive. CE-like elements in the promoters of other ABA-induced genes, including the wheat *Em,* rice *Itp,* and maize *rab17* genes, were also described at the meeting.

Terry Thomas (Texas A&M University, College Station, USA) reported that a similar bipartite model may account for the developmental and ABA-mediated regulation of the carrot *Dc3* gene. The *Dc3* promoter can be split into distal and proximal regulatory regions. Seed-specific expression of *Dc3* is controlled by the proximal region only, whereas ABA-inducible expression in vegetative tissues requires the presence of both promoter regions. In particular, TTTCG-TGT motifs in the distal promoter region interact with consensus ACACNNG motifs in the proximal region to trigger vegetative expression of *Dc3.* Interestingly, ACGT-core elements are not necessary for ABA-induced transcriptional regulation of the *Dc3* gene.

Promoter sequences that are known to mediate ABA responses at the transcriptional level have been used as probes for the identification of the proteins that bind to them, either directly or via DNA binding proteins (Nantel and Quatrano, 1996). One model system makes use of the *Em* gene, which is a member of a set of genes that is expressed exclusively in maturing embryos in response to elevated levels of ABA.

*Em* expression during embryo maturation requires the regulatory locus *VP1* in maize and rice (McCarty et al., 1991; Hattori et al., 1995; Vasil et al., 1995) or its Arabidopsis homolog, *ABI3* (Parcy et al., 1994). McCarty's group has shown that the VP1 protein has different domains that appear to separately regulate processes involved in embryo maturation (i.e., its regulation of the *Em* gene) and the repression of germination (i.e., its inhibition of enzymes, such as $\alpha$-amylase, that are expressed during seed germination [McCarty et al., 1991; Vasil et al., 1995; Kao et al., 1996]). McCarty's group reported that the highly conserved, 140–amino acid, C-terminal domain of VP1 is required for germination-associated processes and that this portion of the protein interacts directly with the Sph sequence in the promoters of germination-specific genes (see Suzuki et al., 1997, in press in the May issue of THE PLANT CELL).

ABA induction, VP1 transactivation, and the synergistic increase in *Em* expression that is triggered by the combined presence of ABA and VP1 (Vasil et al., 1995) is supported by 76-bp region in the *Em* promoter that includes a G-box (CACGTG). Moreover, a highly conserved 18–amino acid region in VP1, BR2 (which is not in the C-terminal domain required for germination-associated responses), appears to be necessary for the transactivation of the *Em* gene in rice protoplasts (Hill et al., 1996).

How the interaction between VP1 and the Sph element is related to the G-box in the *Em* gene is not clear at this time. However, recent evidence from the Quatrano laboratory indicates that VP1 can interact with different promoter elements in a nonspecific manner, promoting the binding of a number of different sequence-specific factors. Hill et al. (1996) have proposed that these properties of the VP1 protein are related to those described for a new class of nuclear factors know as DNA chaperones (Travers et al., 1994). DNA chaperones modify DNA in a non-sequence-specific manner to enhance the association of sequence-specific factors with these regions. Thus, VP1, through its association with a range of different sequence-specific transcription factors, may mediate the regulation of a diverse set of genes and pathways associated with both the onset of dormancy in developing seeds (Dooner, 1985) and germination.

Quatrano's group has been using the yeast 2-hybrid assay to identify proteins that may interact specifically with VP1 on the *Em* promoter. One such protein is the rice protein GF-14, which is related to the class of human regulatory proteins known as 14-3-3s (Ferl, 1996). When recombinant GF-14 protein was incubated in nuclear extracts in the presence of a radiolabeled G-box probe, a novel G-box protein complex was formed, indicating that GF-14 interacts with a specific G-box binding factor. The interaction between GF-14 and VP1 and the formation of the novel G-box complex both require the N-terminal dimerization domain of the GF-14 protein. Quatrano proposed that the unidentified G-box binding factor, GF-14, and VP1 may all interact in vivo to stabilize and/or activate the regulatory complex responsible for the ABA-mediated induction of *Em* transcription.

Unlike *Em* induction, the induction of the Arabidopsis ABA-inducible gene *RD22* is dependent on protein synthesis. Kazuko Yamaguchi-Shinozaki (International Research Center for Agricultural Sciences, Tsukuba, Japan) reported that a 67-bp region of the *RD22* promoter, which is essential for the ABA-induced expression of this gene, contains several conserved motifs for DNA binding proteins, such as MYC and MYB. However, these ABA response elements do not contain any G-box motifs (Iwasaki et al., 1995). Yamaguchi-Shinozaki and her associates have cloned Arabidopsis MYC (*RD22BP1*) and MYB (*ATMYB2*) homologs that encode proteins capable of interacting with the 67-bp region in the *RD22* promoter. The expression of both of these genes is rapidly induced by dehydration, high salt, and treatment with ABA.

Yamaguchi-Shinozaki suggested that ABA may induce gene expression via two different modes of action. One mode, which is represented by the primary ABA-inducible genes typified by *Em*, involves the G-box and preexisting transcription factors of the basic leucine zipper (bZIP) type (Nantel and Quatrano, 1996). The other mode may require de novo synthesis of stress- and/or ABA-

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inducible transcription factors, as appears to be the case for the Arabidopsis *RD22* gene.

By analyzing the promoter of an Arabidopsis dehydration-induced gene, *RD29A*, the group headed by Yamaguchi-Shinozaki and Kazuo Shinozaki have identified a novel *cis*-acting element containing the 9-bp dehydration response element (DRE) TACCGACAT. This DRE is necessary for dehydration stress induction even in the absence of elevated levels of ABA. The same sequence has also been found in the promoters of ABA-responsive (Busk et al., 1997) and cold responsive (*COR*) genes (M. Thomashow, unpublished observations). However, Tapio Palva (University of Helsinki, Finland) discussed work with ABA-deficient Arabidopsis mutants showing that certain genes can still be induced by cold stress, even in the absence of elevated ABA levels.

It is not clear how dehydration and cold stress induce the expression of these genes without the participation of ABA. One possibility, which has been shown for the induction of *Em* expression in embryogenic rice suspension cultures undergoing salt stress (Bostock and Quatrano, 1992), is that these stress conditions increase the sensitivity of cells to ABA. In this way, minor increases in ABA levels would trigger more pronounced changes in gene expression.

### Genes Controlling ABA-Regulated Physiological Responses

Maarten Koornneef reported the isolation of several novel mutants that are able to germinate in the presence of inhibitors of gibberellin biosynthesis. In addition to new alleles at the *ABI3* locus and the mutations at the *ABA1* and *ABA2* loci described above, these include mutations at two reduced dormancy loci (*RDO1* and *RDO2*). Unlike other ABA-insensitive mutations, *rdo1* and *rdo2* have no effect on stomatal behavior. Together with previously described embryo-defective mutations (Meinke, 1995), these new hormonal and development mutants will undoubtedly prove to be very useful in furthering our understanding of the regulation of seed dormancy.

ABA is also an important component in processes that lead to the establishment of freezing tolerance. However, little is known about how ABA triggers this important response. As a first step toward the identification of genes that are involved in the cold acclimation process, Julio Salinas (CIT-INIA, Madrid, Spain) has isolated a freezing-sensitive mutant of Arabidopsis, named *frs1*. The reduced cold acclimation response of *frs1* can be partially alleviated by treatment with exogenous ABA. Surprisingly, however, endogenous ABA levels in the *frs1* mutant are higher than they are in wild-type Arabidopsis. This indicates that *frs1* is unlikely to be impaired in ABA synthesis and suggests instead that the *frs1* mutation may affect ABA perception or some other early step in the ABA signal transduction pathway. The identification of the corresponding gene will be most important in efforts to understand how freezing tolerance is acquired.

### Summary

It is clear from the studies presented at this workshop that there has been significant progress in our understanding of the ABA signaling pathway over the past several years. However, major gaps in our knowledge of the processes involved in this pathway still exist. For example, what is the nature of the ABA receptor(s)? What are the critical changes occurring in response to the ABA signal that result in ABA-dependent changes in gene expression? Finally, what is the link between the proteins that appear in response to ABA and tissue-specific physiological responses? Answers to these questions will continue to require analyses at many levels using systems that are amenable to a range of experimental approaches. Future workshops will ensure that these advances are discussed and that the appropriate questions are formulated in order that the dissection of the ABA response pathway can continue apace.

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