ABSCISIC ACID SIGNAL TRANSDUCTION

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ABSTRACT

The plant hormone abscisic acid (ABA) plays a major role in seed maturation and germination, as well as in adaptation to abiotic environmental stresses. ABA promotes stomatal closure by rapidly altering ion fluxes in guard cells. Other ABA actions involve modifications of gene expression, and the analysis of ABA-responsive promoters has revealed a diversity of potential cis-acting regulatory elements. The nature of the ABA receptor(s) remains unknown. In contrast, combined biophysical, genetic, and molecular approaches have led to considerable progress in the characterization of more downstream signaling elements. In particular, substantial evidence points to the importance of reversible protein phosphorylation and modifications of cytosolic calcium levels and pH as intermediates in ABA signal transduction. Exciting advances are being made in reassembling individual components into minimal ABA signaling cascades at the single-cell level.

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INTRODUCTION

The scientific origins of abscisic acid (ABA) have been traced to several independent investigations in the late 1940s, but it was only in the 1960s that ABA was isolated and identified (1). Mutants affected in ABA biosynthesis are known in a variety of plant species (39, 146). The characterization of these mutants, together with physicochemical studies, has enabled the pathway of ABA biosynthesis to be elucidated in higher plants (134, 135, 146, 166). Two ABA biosynthetic genes have been recently cloned (88, 135) and should provide insights into the regulation and the sites of ABA biosynthesis.

A large body of evidence indicates that ABA plays a major role in adaptation to abiotic environmental stresses, seed development, and germination. The present review focuses on our current knowledge concerning how the ABA signal could be faithfully transduced to mediate these well-characterized physiological and developmental processes. Physicochemical and molecular genetic approaches have already provided fundamental insights into the diversity of ABA perception sites and other downstream components that could couple ABA stimuli to particular responses. It should be emphasized that many concepts about the relative importance of these components in the ABA signaling network are far from firmly established. Nonetheless, it is clear that the present development of single-cell systems should allow some of these components to be assembled into minimal signaling pathways within a cellular context.

ABA SIGNAL TRANSDUCTION IN SEEDS

Endogenous ABA content peaks during roughly the last two thirds of seed development before returning to lower levels in the dry seed (121). ABA is thus thought to regulate several essential processes occurring during the developmental stages that follow pattern formation of the embryo. These processes include the induction of seed dormancy, the accumulation of nutritive reserves, and the acquisition of desiccation tolerance.

Seed Dormancy and Germination

Exogenous ABA can inhibit the precocious germination of immature embryos in culture (121). Embryos of the ABA-biosynthetic mutants from maize exhibit precocious germination while still attached to the mother plant or vivipary (91). Seeds of the ABA biosynthetic mutants from Arabidopsis thaliana (71, 77) and Nicotiana plumbaginifolia (88) fail to become dormant. These observations support that endogenous ABA inhibits precocious germination and promotes seed dormancy.

Thus far, our knowledge on the signaling elements that mediate the regulation of seed dormancy and germination by ABA is primarily derived from genetic analysis. As summarized in Table 1, mutations that alter the sensitivity to ABA
Table 1  Main characteristics of the various mutations known to affect ABA sensitivity

<table>
<thead>
<tr>
<th>Species</th>
<th>Mutation</th>
<th>Dominance</th>
<th>Phenotype</th>
<th>Gene product</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hordeum vulgare</td>
<td>cool</td>
<td></td>
<td>ABA insensitivity in guard cells</td>
<td></td>
<td>118</td>
</tr>
<tr>
<td>Zea mays</td>
<td>vp1</td>
<td>R</td>
<td>ABA insensitivity in seeds</td>
<td>Seed-specific transcription factor</td>
<td>92, 120</td>
</tr>
<tr>
<td></td>
<td>rea</td>
<td>R</td>
<td>ABA insensitivity in seeds</td>
<td></td>
<td>144</td>
</tr>
<tr>
<td>Craterostigma plantagineum</td>
<td>cdt-1</td>
<td>D</td>
<td>Constitutive ABA response in callus</td>
<td>Regulatory RNA or short polypeptide</td>
<td>31</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>abi1</td>
<td>SD</td>
<td>ABA insensitivity</td>
<td>Protein phosphatase 2C</td>
<td>72, 78, 97</td>
</tr>
<tr>
<td></td>
<td>abi2</td>
<td>SD</td>
<td>ABA insensitivity</td>
<td>Protein phosphatase 2C</td>
<td>72, 79</td>
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<tr>
<td></td>
<td>abi3</td>
<td>R</td>
<td>ABA insensitivity in seeds</td>
<td>Seed-specific transcription factor</td>
<td>38, 72, 102, 108</td>
</tr>
<tr>
<td></td>
<td>abi4/5</td>
<td>R</td>
<td>ABA insensitivity in seeds</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>era1</td>
<td>R</td>
<td>ABA hypersensitivity&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;p&gt;β subunit of farnesyl transferase&lt;/p&gt;</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>era2/3</td>
<td>R</td>
<td>ABA hypersensitivity&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>gca1/8</td>
<td>D</td>
<td>Resistance to auxin, ethylene, and ABA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>axr2</td>
<td>D</td>
<td>Resistance to MeJa and hypersensitivity to ABA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>163</td>
<td></td>
</tr>
<tr>
<td></td>
<td>jar1</td>
<td>R</td>
<td>Resistance to MeJa and hyper-sensitivity to ABA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>143</td>
<td></td>
</tr>
<tr>
<td></td>
<td>jin4</td>
<td>R</td>
<td>Resistance to MeJa and hyper-sensitivity to ABA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bri1</td>
<td>R</td>
<td>Resistance to brass-inosteroids and hypersensitivity to ABA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sax</td>
<td>R</td>
<td>Hypersensitivity to auxin and ABA&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Nicotiana plumbaginifolia</td>
<td>iba1</td>
<td>R</td>
<td>Resistance to auxin, cytokinin, and ABA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Molybdenum cofactor biosynthesis</td>
<td>13, 80</td>
</tr>
</tbody>
</table>

<sup>a</sup>Dominance of the mutant alleles over wild-type; D, dominant; SD, semidominant; R, recessive.

<sup>b</sup>Molecular function of the product of the wild-type gene.

<sup>c</sup>Sensitivity of seed germination to exogenous ABA.

<sup>d</sup>Sensitivity of root growth to exogenous ABA.

<sup>e</sup>Sensitivities of root growth and stomatal closing to exogenous ABA.

<sup>f</sup>M. Felliher and G. Ephritikhine, personal communication.

<sup>g</sup>The *iba1* locus was renamed *ABA1* when it was found that *iba1* and other alleles are in fact ABA-deficient, as a result of a defect in the biosynthesis of a molybdenum cofactor that is required for multiple enzymatic activities including ABA aldehyde oxidase (the last step in ABA biosynthesis).
in seeds have been described in maize and in *A. thaliana*, and several of the corresponding genes have been cloned.

The maize *vp1* (*viviparous1*) (119) and *rea* (*red embryonic axis*) (144) mutations lead, like ABA biosynthetic mutations in this species, to vivipary. However, *vp1* (120) and *rea* (144) embryos do not have reduced ABA content but rather exhibit a reduced sensitivity to germination inhibition by exogenous ABA in culture. As is discussed below, the *VP1* gene encodes a seed-specific transcription factor (92).

The *A. thaliana* ABA-insensitive *ABI1* to *ABI5* loci were all identified by selecting for seeds capable of germinating in the presence of ABA concentrations (3–10 µM) that are inhibitory to the wild type (27, 72). The *abi1* (72), *abi2* (72), and *abi3* (72, 101, 108) mutants also exhibit, like *A. thaliana* ABA-deficient mutants, a marked reduction in seed dormancy. These three loci thus seem to mediate the inhibitory effects of endogenous ABA on seed germination. The *ABI1* (78, 97) and *ABI2* (79) genes encode homologous protein serine/threonine phosphatase 2C (PP2C) (12, 79). The *ABI3* gene is the ortholog of the maize *VP1* gene mentioned above (38). It is presently unclear whether the *ABI1*, *ABI2*, and *ABI3* loci act in the same or in partially distinct ABA signaling cascade in seeds (28, 110).

Mutations in the *ERA1* to *ERA3* (Enhanced Response to ABA) loci of *A. thaliana* were identified by a lack of seed germination in the presence of low concentrations of ABA (0.3 µM) that are not inhibitory to the wild type (24). The *era1* mutations also markedly increase seed dormancy. The *ERA1* gene encodes the β subunit of a farnesyl transferase, which may possibly function as a negative regulator of ABA signaling by modifying signal transduction proteins for membrane localization (24). The exact relationships between *ERA1* and the above-mentioned *ABI* loci are unknown.

**Reserve Accumulation and Acquisition of Desiccation Tolerance**

The accumulation of nutritive reserves and the acquisition of desiccation tolerance are associated with the expression of specific sets of mRNAs (60, 121). Transcripts encoding either storage proteins or late-embryogenesis-abundant (LEA) proteins thought to participate in desiccation tolerance can be precociously induced by exogenous ABA in cultured embryos (60, 121). The characterization of ABA-deficient mutants in *A. thaliana* (70, 77, 96, 101, 112) and in maize (91, 109) supports a contribution of endogenous ABA in the developmental expression of these genes in seeds.

The functional dissection of such ABA-responsive promoters, conducted primarily in transient expression systems, has identified several *cis*-acting elements involved in ABA-induced gene expression.
CIS-ACTING PROMOTER ELEMENTS A first category of elements is exemplified by the related motif I of the Rab16 LEA gene from rice (107, 141) and Em1a motif of the wheat Em LEA gene from wheat (44). These sequences share a G-box ACGT core motif (Table 2) and have been designated ABREs, for ABA Response Elements. ABRE-related sequence motifs are present in many other ABA-inducible genes, although their function in ABA signaling often remains speculative in the absence of experimental tests. For instance, only a subset of the multiple ABRE-like motifs present in certain promoters are indeed required for ABA regulation (20, 48, 117, 138, 139). A second type of cis-acting element has been identified in the maize C1 gene, a regulator of anthocyanin biosynthesis in seeds. The ABRE-like motifs present in C1 are not major determinants of the ABA responsiveness of this gene, whereas the distinct sequence motif designated as Sph element (Table 2) is essential for ABA induction of the C1 promoter (65).

Multimerized copies of ABREs (107, 141, 155) or of the Sph element (65) can confer ABA responsivity to minimal promoters. Single copies of these elements were, however, not sufficient for ABA response, suggesting that multimerization substituted for other aspects of the native promoter contexts of these elements. In fact, the smallest promoter units designated ABRCs shown to be both necessary and sufficient for ABA induction of gene expression appear to consist of (at least) two essential cis-elements. ABRCs can be composed of two G-boxes as in the case of the wheat Em promoter, where both the Em1a and

<table>
<thead>
<tr>
<th>Gene (Species)</th>
<th>Element</th>
<th>Sequence</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rab16 (Oryza sativa)</td>
<td>Motif I</td>
<td>GTACGTGGC</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>Motif III</td>
<td>GCCGCTTGGC</td>
<td>107</td>
</tr>
<tr>
<td>Em (Triticum aestivum)</td>
<td>Em1a</td>
<td>ACACGTGGC</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Em1b</td>
<td>ACACGTGCC</td>
<td>44</td>
</tr>
<tr>
<td>HVA22 (Hordeum vulgare)</td>
<td>ABRE3</td>
<td>GCCACGTACA</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>CE1</td>
<td>TGCCACCGG</td>
<td>138</td>
</tr>
<tr>
<td>HVA1 (Hordeum vulgare)</td>
<td>ABRE2</td>
<td>CCTACGTGCC</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>CE3</td>
<td>ACGCGTGCTTCC</td>
<td>139</td>
</tr>
<tr>
<td>C1 (Zea mays)</td>
<td>Sph</td>
<td>CGTGTGTCATGCAT</td>
<td>65</td>
</tr>
<tr>
<td>CDeT27-45 (Craterostigma plantagineum)</td>
<td>AAGCCCAATTTTCACA-GCCCGATAACC</td>
<td>104</td>
<td></td>
</tr>
</tbody>
</table>

The ACGT core in G-box-type ABREs is underlined.
Em1b G-box motifs (Table 2) contribute to the activity of the 75-bp Complex I (44, 155). In contrast, several other ABRCs consist of an ABRE and of a cis-element not related to G-boxes. The G-box-type motif I and the distinct motif III are essential for the ABA responsiveness of a 40-bp fragment of the rice *Rab16B* promoter (107). In the barley *HVA22* promoter, ABRC1 is a 49-bp fragment that comprises ABRE3 and the Coupling Element CE1 located 20-bp downstream (138). In the barley *HVA1* promoter, the 22-bp long ABRC3 consists of the coupling element CE3 directly upstream of ABRE2 (139). These results indicate that a diversity of ABRCs participate in ABA signaling in seeds.

**TRANS-ACTING FACTORS** The nature of the transcription factors that mediate ABA regulation of seed genes via the various above-mentioned cis-elements remains largely unknown. Most proteins that bind DNA sequences with ACGT core motifs contain a basic region adjacent to a leucine-zipper motif (bZIP-proteins). Several plant bZIP-proteins that bind to ABREs in vitro and/or are inducible (at the mRNA level) by ABA have been cloned (44, 68, 74, 100, 103, 105). However, few of these genes are known to be expressed in seeds, and thus far none has been unambiguously shown to be involved in ABA signaling in vivo (83).

In contrast, genetic and molecular evidence supports that the maize VP1 and *A. thaliana* ABI3 proteins are homologous transcription factors essential for ABA action in seeds. The *vp1* (91, 109) and *abi3* (28, 101, 108, 112) mutants are similarly altered in sensitivity to ABA, and in various other aspects of seed maturation including the developmental expression of storage proteins and LEA genes. The maize *VP1* (92) and *A. thaliana* ABI3 (38) genes, as well as the rice *OsVP1* (47) and *Phaseolus vulgaris* PvALF (19) genes, encode homologous proteins (Figure 1). These genes are all exclusively expressed in seeds (19, 47, 92, 112). However, when ectopically expressed in transgenic *A. thaliana* plants, ABI3 rendered vegetative tissues hypersensitive to ABA and

![Figure 1](attachment:image.png)

*Figure 1*  Schematic diagram of the architecture of the VP1/ABI3-like proteins. The maize VP1 (92), *A. thaliana* ABI3 (38), rice OsVP1 (47), and *Phaseolus vulgaris* PvALF (19) proteins display a similar and novel structural organization. They contain, in particular, four domains of high amino acid sequence identity: the A domain located in the large acidic (−) N-terminal region and three basic domains designated as B1, B2, and B3 in order from the N terminus. The sizes of these proteins range from 691 (VP1) to 752 (PvALF) amino acids.
activated expression of several otherwise seed-specific genes in leaves when exogenous ABA was supplied (110, 112). Similarly, in transient expression studies, VP1 (65, 92, 155), OsVP1 (47, 48), and PvALF (18, 19) could trans-activate various seed-specific promoters. When investigated, these trans-activations were found to require the acidic N-terminal domain of the relevant VP1/ABI3-like protein (48, 92), and domain-swapping experiments showed that the acidic N-terminal regions of VP1 (92) and PvALF (19) are indeed functional transcriptional activation domains. Thus, taken together, the above-mentioned results indicate that VP1/ABI3-like proteins act as transcription factors.

The maize \textit{vp1} (56, 91, 109) and \textit{A. thaliana abi3} (70, 77, 101, 108, 112) mutants exhibit a larger spectrum of seed phenotypes than the ABA-deficient mutants in the corresponding species. A possible explanation would be that the physiological roles of VP1 and ABI3 are not strictly confined to ABA signaling and rather that these proteins integrate ABA and other seed developmental factors. This model is also consistent with the observation that VP1/ABI3-like proteins can substantially trans-activate seed promoters in the absence of (added) ABA (19, 48, 92, 110), and that, within a given promoter, the cis-elements involved in VP1 responsiveness are partially separable from those involved in ABA responsiveness (65, 155).

In the wheat \textit{Em} promoter, the ABRE \textit{Em}1a (Table 2) that is essential for ABA induction also mediates transactivation by VP1 and synergism between ABA and VP1 (155). The conserved domain B2 of VP1 is required for \textit{Em} transactivation (54), but its exact role in this process remains unclear (54, 131). Furthermore, no specific binding of VP1 to G-box-like ABREs has been observed thus far (54, 92, 145), suggesting that VP1 is a coactivator protein that physically interacts with G-box-binding proteins (91, 155). Unlike for \textit{Em}, the combined response of the maize \textit{Cl} promoter to ABA and VP1 is less than additive, and transactivation of the \textit{Cl} promoter by VP1 is mediated exclusively by the sequence CGTCCATGCAT located within the Sph promoter element (Table 2) (65). The conserved basic domain B3 of VP1 is essential for \textit{Cl} transactivation, and the isolated B3 domain binds specifically to the above-mentioned sequence element in vitro (145). VP1 is, however, likely to interact with other DNA-binding proteins involved in ABA regulation of \textit{Cl}, because the entire Sph element is required for responsiveness of \textit{Cl} to both ABA and VP1 (65). The VP1/ABI3-like proteins thus appear to be multifunctional transcription factors that integrate ABA and other regulatory signals of seed maturation, most likely by interacting with distinct trans-acting factors that remain to be identified.

UPSTREAM SIGNALING ELEMENTS Genetic studies in \textit{A. thaliana} have identified a few other intermediates in the ABA regulation of gene expression in seeds. The already mentioned \textit{abi4} and \textit{abi5} mutants share with \textit{abi3} mutants...
a decreased ABA sensitivity in germinating seeds, as well as a reduced developmental expression of certain LEA genes. These three loci have thus been proposed to act in a common regulatory pathway of seed maturation (27). In addition, the recent analysis of abi3 fus3 and abi3 lec1 double mutants indicates that ABI3 acts synergistically with the FUSCA3 (FUS3) and LEAFY COTYLEDON1 (LEC1) genes to control multiple elementary processes during seed maturation, including sensitivity to ABA and accumulation of storage protein mRNAs (111). The ABI4, ABI5, FUS3, and LEC1 gene products are currently unknown.

Studies on barley aleurone protoplasts indicate that reversible protein phosphorylation is likely to be implicated in the regulation of gene expression by ABA in seeds. ABA rapidly stimulated the activity of a MAP kinase, and this stimulation appeared to be correlated with the induction of the Rab16 mRNA (69). Okadaic acid (an inhibitor of the PP1 and PP2A classes of serine/threonine protein phosphatases) inhibited the induction of the HVA1 (73) and Rab16 (51) mRNAs by ABA, and phenylarsine oxide (an inhibitor of tyrosine protein phosphatases) blocked the induction of Rab16 (51). The protein kinase and phosphatases revealed by these studies remain to be isolated.

Finally, suggestive evidence indicates that modifications of intracellular cytosolic free Ca\(^{2+}\) levels ([Ca\(^{2+}\)_i] and cytoplasmic pH (pH\(_i\)) may act as intracellular second messengers in the ABA regulation of gene expression in seeds. In barley aleurone protoplasts, ABA triggers an increase in pH\(_i\) (153) and a decrease in [Ca\(^{2+}\)_i] (35, 158). However, the exact contribution of these alterations in [Ca\(^{2+}\)_i] and pH\(_i\) to the regulation of gene expression by ABA could not be clearly established (152, 153).

ABA SIGNAL TRANSDUCTION IN STRESS RESPONSE

During vegetative growth, endogenous ABA levels increase upon conditions of water stress, and ABA is an essential mediator in triggering the plant responses to these adverse environmental stimuli (166). As is discussed below, substantial evidence supports that the increased ABA levels limit water loss through transpiration by reducing stomatal aperture. ABA is also involved in other aspects of stress adaptation. For instance, ABA-deficient mutants of A. thaliana are impaired in cold acclimation (87) and in a root morphogenetic response to drought (drought rhizogenesis) (154). The role of ABA in signaling stress conditions has also been extensively documented by molecular studies showing that ABA-deficient mutants are affected in the regulation of numerous genes by drought, salt, or cold (39, 60, 140). It should, however, be noted that the adaptation to these adverse environmental conditions also involves ABA-independent pathways (140). Finally, ABA is involved in the induction of gene expression by mechanical damage (116).
Regulation of Gene Expression in Response to Stress

**PROMOTER STUDIES** The regulation of gene expression by ABA in vegetative tissues appears to involve several signaling pathways. The ABA induction of distinct genes exhibit differential requirements for protein synthesis (164). In addition, several types of cis-acting elements are involved in ABA-induced gene expression in vegetative tissues. Many of the LEA genes that are abundantly expressed in desiccating seeds are also responsive to drought stress and ABA in vegetative tissues (60, 121). For several of these genes, the G-box-type ABREs already described (Table 2) are required for ABA induction both in seeds and in vegetative tissues (20, 44, 48, 107, 117).

In contrast, ABRE-like motifs are not involved in the ABA regulation of other stress-inducible genes such as the *A. thaliana RD22* (63) and the *Craterostigma plantagineum CDeT27-45* genes (104). The distinct sequence motif shown in Table 2 is essential for ABA responsiveness of *CDeT27-45* (104). In this case, as for ABREs (see section on ABA Signal Transduction in Seeds), the corresponding physiological trans-acting factors are presently unknown. Genes that are induced by ABA and encode other types of potential transcription factors include the *A. thaliana* homeobox gene *ATHB-7* (142) and several myb homologues from *A. thaliana* (151) and *Craterostigma* (62). The role of these genes in ABA signaling has, however, not been tested experimentally.

**POTENTIAL INTERMEDIATES** Studies performed on various model systems have identified several potential signaling intermediates in the ABA activation of gene expression in response to stress.

The resurrection plant *Craterostigma plantagineum* can tolerate extreme dehydration. However, in vitro propagated callus derived from this plant has a strict requirement for exogenously applied ABA in order to survive a severe dehydration (31). This property has been exploited for isolation of dominant mutants by activation tagging, in which high expression of resident genes activated by insertion of a foreign promoter would confer desiccation tolerance to the transformed cells without prior ABA treatments. One gene was identified (*CDT-1*), whose high expression did confer the expected phenotypes in calli and led to constitutive expression of several ABA- and dehydration-inducible genes (31). The function of the *CDT-1* gene is not immediately obvious, because it encodes a transcript with no large open reading frame. It is possible that the biologically active product of *CDT-1* is a regulatory RNA or a short polypeptide (31).

The ABA regulation of gene expression in vegetative tissues is likely to involve reversible protein phosphorylation events. Several stress- and ABA-inducible mRNAs that encode protein kinases have been identified (57–59). In epidermal peels of *Pisum sativum*, the ABA-induced accumulation of dehydrin mRNA was reduced by K-252a (an inhibitor of serine/threonine protein kinases).
and also by okadaic acid and cyclosporin A (an inhibitor of the PP2B class of serine/threonine protein phosphatases) (53).

The involvement of particular protein phosphatases 2C was revealed by the cloning of the \textit{ABI1} (78, 97) and \textit{ABI2} (79) genes of \textit{A. thaliana}. Their corresponding mutants show decreased sensitivities to the ABA inhibition of seedling growth, and defects in various morphological and molecular responses to applied ABA in vegetative tissues (28, 41, 72). Despite the highly similar architecture of the \textit{ABI1} and \textit{ABI2} proteins (79), their functions are unlikely to be completely redundant for all ABA actions. The \textit{abi1-1} and \textit{abi2-1} mutations lead to identical amino acid substitutions at equivalent positions in the \textit{ABI1} and \textit{ABI2} proteins, respectively (79), but have (at least quantitatively) different effects on some of the responses to exogenous ABA or water stress (25, 33, 41, 124, 142, 154).

The identification of stress- and ABA-inducible mRNAs that code for a Ca\textsuperscript{2+}-binding membrane protein in rice (30) and for a phosphatidylinositol-specific phospholipase C in \textit{A. thaliana} (55), respectively, provides circumstantial evidence for the possible involvement of Ca\textsuperscript{2+} in ABA signaling in vegetative tissues. In addition, ABA has been shown to induce increases in [Ca], and pH, in cells of corn coleoptiles and of parsley hypocotyls and roots (32).

**SINGLE-CELL SYSTEMS** Ambitious attempts are being made to reconstruct minimal stress and ABA signaling pathways by reassembling candidate components in single-cell systems.

In maize leaf protoplasts, the barley \textit{HVA1} promoter fused to reporter genes could be activated by applied ABA or by various stress conditions (136). In the absence of these stimuli, expression of the reporter gene could be induced by treating the protoplasts with 1 mM Ca\textsuperscript{2+} plus Ca\textsuperscript{2+} ionophores, or by overexpressing constitutively active forms of the normally Ca\textsuperscript{2+}-dependent ATCDPK1 and ATCDPK1a protein kinases from \textit{A. thaliana}. Overexpressing the catalytic domain of the wild-type \textit{A. thaliana} \textit{ABI1} PP2C inhibited the activation of \textit{HVA1} by ABA or by ATCDPK1 (136).

Microinjection experiments were conducted in hypocotyl cells of the tomato \textit{aurea} mutant to investigate the signaling cascade that mediates ABA activation of the \textit{A. thaliana} \textit{rd29A} (165) and \textit{kin2/or6.6} (157) promoters fused to the GUS reporter gene. This work supports the existence of a minimal linear cascade in which (a) ABA triggers a transient accumulation of cyclic ADP-ribose (cADPR) (for information on cADPR, see 3), (b) cADPR induces a release of Ca\textsuperscript{2+} from internal stores, and (c) a K-252a-sensitive kinase acting downstream of Ca\textsuperscript{2+} is required for activation of \textit{rd29A} and \textit{kin2} (Y Wu & N-H Chua, personal communication). Microinjection of the mutant \textit{abi1-1} protein inhibited ABA-, cADPR-, and Ca\textsuperscript{2+}-induced gene expression, and these
effects could be reversed by an excess of wild-type ABI1 protein (Y Wu, A Himmelbach, E Grill & N-H Chua, personal communication).

These two sets of results illustrate that combining genetics with single-cell analyses represents a promising approach for deciphering possible epistatic relationships between candidate components in ABA signaling cascades.

### Regulation of Stomatal Aperture

The stomatal pore is defined by a pair of surrounding guard cells. The closing and opening of the pore result from osmotic shrinking and swelling of these guard cells, respectively. In conditions of water stress, the increase in cellular ABA (45) or in apoplastic ABA at the surface of guard cells (161) is thought to provoke a reduction in turgor pressure of the guard cells, which in turn leads to stomatal closure to limit transpirational water loss. Applied ABA inhibits the opening and promotes the closure of stomatal pores (8, 16, 159). The enhanced transpiration of ABA-biosynthetic mutants (71, 77, 88) and transgenic plants expressing an anti-ABA antibody (7) provides evidence for such a role of endogenous ABA.

Unlike most other cells in higher-plant tissues, guard cells at maturity lack plasmodesmata (162). This property has afforded a single-cell system, directly accessible in planta, to investigate how ABA is perceived and transduced to trigger integrated physiological responses.

#### ABA PERCEPTION

Available evidence suggests that guard cells possess at least two sites of ABA perception involved in the regulation of stomatal aperture, one located at the plasma membrane and a second located intracellularly.

Stomatal closure can be triggered by extracellular application of ABA. However, the protonated form of the weak acid ABA (ABAH) readily permeates the lipid bilayer of the cell membrane (64). Guard cells of Commelina communis appear to have, in addition, a significant carrier-mediated uptake of ABA (86, 133). The requirement for an extracellular ABA receptor is indicated by the failure of ABA to inhibit the stomatal opening when microinjected directly into the cytosol of Commelina guard cells (5). In this species, extracellular ABA was, however, less effective in regulating stomatal aperture at high than at low external pH (that favors passive uptake of ABAH), providing indirect evidence for an intracellular ABA receptor (5, 106, 113, 133). This conclusion is also supported by the reports that stomatal closure could be triggered in Commelina by ABA released inside guard cells from caged ABA microinjected into the cytosol (2), and by ABA microinjected into guard cells in the presence of 1 μM extracellular ABA (133).

ABA closes stomatal pores by inducing net efflux of both K+ and Cl− from the vacuole to the cytoplasm, and from the cytoplasm to the outside of guard
cells (85, 86). Tracer flux studies, measuring rate of loss of $^{86}$Rb$^+$ from isolated Commelina guard cells, also indicate multiple actions of ABA, both inside and outside the cell. It has been proposed that internal receptors regulate tonoplast ion channels for release of vacuolar ions, whereas external receptors mediate the stimulation of ion efflux at the plasma membrane (85, 86).

ION CHANNELS REGULATED BY ABA  

Electrophysiological studies, either by whole cell impalement or by patch clamping of the plasma membrane of guard cell protoplasts or of isolated vacuoles, have identified a number of ionic channels present in these guard cell membranes. The nature of the tonoplast ion channels involved in the regulation of stomatal aperture by ABA is not yet clearly identified (159). Attention is thus focused here on ion transport mechanisms across the plasma membrane of guard cells.

ABA causes a depolarization of the plasma membrane (149). Two types of anion channels, which may reflect different states of a single channel protein (26, 132), have been identified in the plasma membrane of guard cells (50, 129). One of these types of anion channels (S-type) shows slow and sustained activation properties and is active over a wide range of voltage. Inhibitor studies (132) and the recent demonstration that ABA activates S-type anion channels (43, 114) indicate that these channels can account for the membrane depolarization and prolonged anion efflux required for ABA-mediated stomatal closure. Inactivation of the plasma membrane H$^+$-ATPase by ABA may, however, also contribute to the membrane depolarization during stomatal closure (40).

The guard cell plasma membrane contains two types of K$^+$ channels. The inward-rectifying K$^+$ channel is responsible for K$^+$ influx to the guard cell and is inhibited by ABA (76, 130, 149). The outward-rectifying K$^+$ channel is active only at membrane potentials positive to the equilibrium potential for K$^+$, and mediates K$^+$ efflux required for stomatal closure (14, 130, 149). The ABA-induced membrane depolarization mentioned above will thus activate outward-rectifying K$^+$ channels, and the magnitude of this outward K$^+$ current is further enhanced by ABA in a largely voltage-independent manner (14, 76).

SECOND MESSENGERS  

One of the earliest responses of guard cells to ABA is an increase in the concentration of cytosolic free Ca$^{2+}$ ([Ca$^2+$]) (2, 34, 61, 88a, 89, 128). The origin of the Ca$^{2+}$ required to elevate [Ca$^2+$] in response to ABA is unclear (90), but evidence favoring influx across the plasma membrane (128) or release from internal stores mediated by inositol 1,4,5-trisphosphate (17, 37, 75) and/or cyclic ADP-ribose (3, 90) has been presented. Experimental elevation of [Ca$^2+$] is sufficient to induce stomatal closure (37) and mimics several of the above-mentioned effects of ABA on ion channels in the plasma membrane of guard cells. Increased [Ca$^2+$] activates the S-type anion channel and inhibits
the inward-rectifying K\(^+\) channel (50, 127). These results thus provide strong evidence for the involvement of Ca\(^{2+}\)-coupled signal transduction cascade(s) in the regulation of guard cell turgor by ABA.

Several observations, however, indicate that Ca\(^{2+}\)-independent signaling pathways are also involved. The extent of ABA-induced increase in [Ca]\(_i\) is variable, and stomata could even close in response to ABA without any detectable change in [Ca]\(_i\) (2, 34, 89). It remains, however, unclear whether ABA always produces a local increase in [Ca]\(_i\) that does not systematically translate into global cytoplasmic change (89), or whether ABA can produce stomatal closure using only Ca\(^{2+}\)-independent signaling pathways (2). In any case, the fact that the outward-rectifying K\(^+\) channel is insensitive to increases in [Ca]\(_i\) (76, 127) indicates that other second messengers must be involved in the regulation of plasma membrane ion channels by ABA.

In particular, ABA increases the cytoplasmic pH (pH\(_i\)) of guard cells (15, 61). Studies in which guard cells were loaded with pH buffers or pH\(_i\) was experimentally modified support that the ABA-induced cytoplasmic alkalinization can account for ABA activation of the outward-rectifying K\(^+\) channel and also contribute to ABA inactivation of the inward-rectifying K\(^+\) channel (15, 42, 76, 98). The ABA regulation of ion channels in guard cells thus appears to involve, at least, both Ca\(^{2+}\)-dependent and pH\(_i\)-dependent signaling pathways.

DOWNSTREAM SIGNALING ELEMENTS How the ABA-induced Ca\(^{2+}\) and pH\(_i\) signals are then decoded and relayed by other cellular components in the transduction chain is not clear. The recent report that pH\(_i\) regulates the outward-rectifying K\(^+\) channel in isolated membrane patches from *Vicia faba* guard cells suggests that the signaling elements acting downstream of the pH\(_i\) signal are membrane associated (98). The Ca\(^{2+}\) signal is possibly relayed by particular protein kinases and phosphatases. Both Ca\(^{2+}\)-dependent protein kinases (115) and phosphatases (4, 23, 84) have been inferred to be potential candidates in modulating the activity of various ion channels in guard cells, but their precise roles in ABA signaling have not been clearly established at present.

There is nonetheless incontrovertible evidence that protein phosphorylation is a critical component in the ABA signal transduction controlling stomatal closure. In *Vicia faba* and *Commelina*, stomatal closure induced by ABA was abolished by kinase inhibitors while enhanced by inhibitors of the protein phosphatases PP1 and/or PP2A (125). These protein phosphatases, whose actions are Ca\(^{2+}\)-independent, have been implicated in the regulation of the inward-rectifying K\(^+\) channel (82, 148), outward-rectifying K\(^+\) channel (148), and S-type anion channel (114, 125) in *Vicia faba* and *Commelina* guard cells.

Recent reports described two protein kinases whose activities are rapidly stimulated by ABA in protoplasts from guard cells but not mesophyll cells in
*Vicia faba* (81, 99). Although they have a similar apparent molecular weight, the two protein kinases are probably distinct because they differ in their ability to autophosphorylate and in their substrate specificity in vitro (81, 99). The cellular targets of these kinases are not yet known, but because their activities are detectable minutes after induction by physiological concentrations of ABA, these kinases may be involved in the modulation of ion channel activities that are essential for rapid stomatal responses.

Genetic dissection of ABA signaling in guard cells has been rather limited despite an initial and promising report of the barley mutant *cool*, which displayed excessive transpiration and ABA-insensitive guard cells (118). Mutants with specific impairment in ABA-induced stomatal closure have not yet been identified in *A. thaliana*.

However, among the *gca1-gca8* (Growth Control by ABA) mutants that were isolated based on their reduced sensitivities to the inhibition of seedling growth by exogenous ABA, the *gca1* and *gca2* mutants display a disturbed stomatal regulation (10). The *abi1-1* and *abi2-1* mutants mentioned earlier also have ABA-insensitive guard cells as part of their pleiotropic phenotypes (114, 122).

What are the roles of the Ca\(^{2+}\)-independent (12) ABI1 and ABI2 protein phosphatases 2C (PP2Cs) in stomatal regulation by ABA? Taking advantage of the fact that the *abi1-1* mutation is dominant (72, 78), the mutant *A. thaliana* gene was introduced into the diploid tobacco *Nicotiana benthamiana* to facilitate electrophysiological measurements (6). Voltage clamp studies found that the mutant *abi1-1* transgene had no detectable effect on anion channels, but decreased ABA sensitivity of both the inward- and outward-rectifying K\(^{+}\) channels in the guard cell plasma membrane (6, 43). ABA sensitivity of these K\(^{+}\) channels and ABA-induced stomatal closure could be partially reestablished in the *abi1-1* transgenic plants by adding protein kinase inhibitors (6). Recent and important advances made in single-cell techniques have permitted measurements of ion channel activities in *A. thaliana* guard cells, which had previously posed technical difficulties because of their small sizes (114, 123). In patch-clamp studies of *A. thaliana* guard cell protoplasts, the ABA response of S-type anion channels was impaired in the *abi1-1* mutant. However, consistent with the reversibility of the effects of the *abi1-1* transgene in tobacco (6), treatment with the protein kinase inhibitor K-252a could partially restore ABA regulation of the anion channel and ABA-induced stomatal closure in the *A. thaliana abi1-1* mutant (114). The *abi2-1* mutation also suppressed the ABA activation of S-type anion channels, but curiously, this inhibition could not be counterbalanced by K-252a treatments (114). In addition, the *abi2-1* mutation also diminished the background activity of inward-rectifying K\(^{+}\) channels (114), an effect observed neither in the *abi1-1 A. thaliana* mutant (114) nor in the *abi1-1* transgenic tobacco plants (6). The differential effects of the *abi1* and *abi2* mutations on
the background activity of inward-rectifying $K^+$ channels, and the differential sensitivity of these mutants to K-252a, thus indicate that, although the ABI1 and ABI2 proteins are homologous PP2Cs, the functions of these proteins in stomatal regulation, as in other physiological responses, are nonredundant.

The apparent discrepancies between the above-mentioned results on $abi1-1$ transgenic tobacco and $abi1-1$ mutant A. thaliana plants may be indicative of the functional flexibility of ABI1 in maintaining an integrated ABA regulation of both $K^+$ and anion channels, depending on the imposed (environmental or experimental) conditions. This would be analogous to the relative importance of the ABA-induced increase in $[Ca]_i$, which is subjected to both experimental (89) and environmental (2) influences. A more serious problem in terms of formulating a unified model of stomatal regulation, however, is how many species-dependent differences exist in ABA signaling. For instance, various pharmacological studies indicate that PP1/PP2A protein phosphatases (distinct from the ABI1 and ABI2 PP2Cs) act as positive regulators of anion currents in A. thaliana guard cells (114), but as negative regulators in Vicia, Commelina, and N. benthamiana (43, 125). Therefore, it seems that detailed comparative studies with different species would be necessary before a fuller understanding of the signaling cascades that mediate stomatal regulation by ABA would emerge.

ABA SIGNALING TO GUARD CELL NUCLEUS As described above, stomatal guard cells have been used extensively as a cellular system to explore the transduction pathways that target the ABA signal to ion channels during stomatal closing. These responses are rapid and are thus thought to be operationally distinct from long-term ABA responses that require RNA and protein synthesis (166). However, hormone induction of gene expression at the transcriptional level can also take place within minutes, as has been observed in the case of auxin (93). The relevance of ABA in gene activation in guard cells has only been explored recently. Several promoters or mRNAs have been shown to be induced in guard cells by exogenous ABA treatment (53, 110, 137, 147, 157). These studies demonstrate that guard cells are competent to relay ABA signals to the nucleus. Initial attempts are being made to analyze whether identical or distinct signaling pathways mediate the ABA regulations of ion channels and gene expression in guard cells (53). It also remains to be established whether there are gene products directly implicated in controlling stomatal movements that are regulated by ABA transcriptionally.

CONCLUSION AND PERSPECTIVES

In recent years, the research into the molecular and physiological action of ABA has benefited tremendously from cross-fertilization of different expertise
and ideas. Studies with optically pure ABA analogues revealed that the stereo-
chemical requirements of ABA are not identical in all ABA responses (21, 156),
indicating that higher plants may contain several types of ABA receptors. Evi-
dence for ABA perception sites located both at the cell surface and intracel-
larly has come from analyses on the regulation of ion fluxes in guard cells.
Whether this model is applicable to other cell types, and to more long-term
responses involving changes in gene expression, are important questions to be
explored (for instance, see 36). In addition, even though locations of these
reception sites can be detected physiologically, we have no firm idea whether
they are functionally distinct, and if so, what physiological relevance they may
harbor. Cloning genes of ABA receptors and creating corresponding mutants
would thus constitute a major step toward dissecting these initial events of ABA
action.

Besides reception sites, we also need to improve further our understand-
ing of the more downstream events in the transduction cascades triggered by
ABA signals. Recent advances point to the central role of reversible protein
phosphorylation in mediating several of the physiological responses to ABA.
Much work, however, is clearly needed to identify the pertinent kinases and
phosphatases, and cellular targets of their action.

The ectopic expression of the seed-specific ABI3 gene in leaves resulted in
activation of several otherwise seed-specific genes. This observation and the
pleiotropic phenotypes of some ABA response mutants raise the question of
the degree of similarity between signaling cascades in the different tissues (or
cell types in the same tissue). It might be possible that many core components
of the ABA signaling network exist in different tissues. A simple model would
be that these central cascades are then regulated by cell-specific factors such
as the VP1/ABI3-like proteins. In this light, ABA-stimulated protein kinases
with apparent specificity to guard cells may play similar deterministic roles in
activating a branch of the ABA pathway in this cell type.

Isolation of additional mutants by judicious genetic screens (taking into ac-
count the potential for redundancy in signaling components) will no doubt
enrich our knowledge about regulatory points in ABA action, which might
include modulation in the activity of synthetic enzymes and transport of the
hormone (150). These two latter points have not been the subject of intense
investigations. Furthermore, mutations that simultaneously alter the respon-
siveness to ABA and other hormones or developmental clues, will permit a
fuller appreciation of cross-talk between ABA and other cellular signals. Sev-
eral mutants of this type have already been isolated in A. thaliana (Table 1).
The eventual cloning of the genes identified by mutational analyses will reveal
their molecular identity and, in some cases, suggest possible functions based on
homologies with known proteins. Genes with unexpected structural features
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(such as CDT-1 in C. plantagineum) will divulge novel signal transduction mechanisms, to the delight of researchers with iconoclastic tendencies.

The isolation of genes responsive to ABA, coupled with promoter analyses, are vital to our understanding of how combinatorial and synergistic actions of cis-acting elements and trans-acting factors are involved in the versatile control of the output response. Other approaches, such as biochemical purification and those based on interaction cloning, will undoubtedly complement the more established technologies in unraveling additional signaling elements or even signaling complexes.

Investigators with a pioneering spirit have already attempted to reconstruct particular signaling cascades by reassembling individual components suspected to play the relevant roles. These are much welcome advances, since these cellular systems will complement the more established guard cell model. It is certain that continuation of such a multidisciplinary approach will yield ever deeper insight not only into ABA signal transduction itself but also into how ABA and other signaling molecules jointly coordinate physiological responses.

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