Regulation of Abscisic Acid–Induced Stomatal Closure and Anion Channels by Guard Cell AAPK Kinase

Jiaxu Li, Xi-Qing Wang, Mark B. Watson, Sarah M. Assmann*

Abscisic acid (ABA) stimulates stomatal closure and thus supports water conservation by plants during drought. Mass spectrometry–generated peptide sequence information was used to clone a *Vicia faba* complementary DNA (cDNA), AAPK, encoding a guard cell–specific ABA-activated serine-threonine protein kinase (AAPK). Expression in transformed guard cells of AAPK altered by one amino acid (lysine 43 to alanine 43) renders stomata insensitive to ABA-induced closure by eliminating ABA activation of plasma membrane anion channels. This information should allow cell-specific, targeted biotechnological manipulation of crop water status.

The hormone ABA regulates various processes in plants including responses to stressors such as drought, cold, and salinity (1). During drought, ABA alteration of guard cell ion transport promotes stomatal closure and prevents stomatal opening, thus reducing transpirational water loss. That this is a fundamental component of plant desiccation tolerance is indicated by the wilt phenotype of some ABA-insensitive mutants of *Arabidopsis thaliana* [dominant mutations *abi1-1* and *abi2-1* (2)]. Conversely, the ABA supersensitive mutant, *era1*, shows enhanced drought tolerance (3). However, the *abi1-1, abi2-1*, and *era1* phenotypes are pleiotropic, showing altered seed dormancy (2, 3), for example, which indicates that these genes would not be ideal targets for biotechnological manipulations seeking specifically to regulate stomatal responses.

Guard cells express an AAPK, which has *Ca*^{2+}-independent and ABA-activated phosphorylation activities (4). AAPK activity is detected in guard cells but not in leaf epidermal or mesophyll cells (4) or in roots (5). AAPK is activated by ABA but not by darkness or elevated CO_{2} concentrations (Fig. 1), conditions that also engender stomatal closure (6). We thus hypothesized that AAPK could be a guard cell–specific ABA response regulator. Here we report cloning of the AAPK cDNA, AAPK function, and manipulation of that function in plants.

Guard cell protoplasts (4.8 × 10^{4}; 99.6% pure) were prepared (4) from *Vicia faba*. Protoplast proteins were extracted and subjected to two-dimensional (2D) gel electrophoresis. AAPK was identified as a 48-kD ABA-dependent and *Ca*^{2+}-independent autophosphorylation spot with the in-gel kinase assay (4, 7). The AAPK spot was excised and subjected to peptide sequencing by tandem mass spectrometry (8). Two sequenced AAPK peptides had similarity to the PKABA1 subfamily of protein kinases in subdomains I and VIb (9). The “half-aper- ture” of each transformed guard cell was compared with the half-aperture of the other, untransformed guard cell in the pair. Transformation with pAAPK(K43A)-GFP eliminated ABA-induced stomatal closure (Fig. 4) (Table 2).
A

ABA (μM) 0 10

1 2 AAPK

B

CO₂ (ppm) 0 350 700

Fig. 1. ABA, but not other signals that cause stomatal closure, activate AAPK. AAPK auto-
phosphorylation activity was assayed by the in-gel kinase method (4, 7). Closing signals ap-
tended to the guard cell protoplasts were darkness ([A], lane 1), ABA (10 μM [-cis-trans-
ABA] [A], lane 2), and elevated CO₂ concentrations (700 ppm CO₂) versus the normal con-
centration of 350 ppm CO₂ ([B], lane 3). The asterisk indicates a previously identified Ca²⁺-
dependent protein kinase (28). The arrowhead indicates AAPK.

Table 1. Overexpression of AAPK(K43A) in guard cells inhibits ABA-induced stomatal closure. V. faba leaves were transformed and stomatal responses measured as described in (19). ABA treatment was 25 μM (for closure) or 50 μM (for opening) [-cis-trans-ABA], elevated CO₂ treatment was 700 ppm CO₂. Except for the darkness treatment, peels were illuminated (0.20 mmol m⁻² s⁻¹ white light) for the duration of each treatment. All numbers represent the change in half aperture of stomata as measured in micrometers. ND, not determined. Numbers in parentheses indicate sample sizes.

<table>
<thead>
<tr>
<th></th>
<th>Transformed</th>
<th>Untransformed</th>
<th>ABA</th>
</tr>
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<tbody>
<tr>
<td>ABA Control</td>
<td>-2.52 ± 0.29(36)</td>
<td>-2.54 ± 0.35(36)</td>
<td>-2.59 ± 0.30(36)</td>
</tr>
<tr>
<td>CO₂ Control</td>
<td>ND</td>
<td>ND</td>
<td>-2.55 ± 0.21(36)</td>
</tr>
<tr>
<td>Darkness Control</td>
<td>ND</td>
<td>ND</td>
<td>-2.31 ± 0.46(36)</td>
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<tr>
<td>ABA (0.42 ± 0.22(36))</td>
<td>0.45 ± 0.27(36)</td>
<td>0.43 ± 0.22(36)</td>
<td>0.42 ± 0.17(46)</td>
</tr>
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*Significantly different from untransformed cells treated with ABA (P < 0.001, Student’s t test). Not significantly different from the AAPK(K43A)-GFP transformed ABA control (P > 0.05; Student’s t test).
by AAPK. Second, although dominant abi1-1 and abi2-1 mutations in ABI and ABI2 phosphatases confer ABA insensitivity to both anion channel activation and stomatal closure (2, 3), recently identified recessive loss-of-function mutations in ABI1 confer hypersensitivity to ABA (26). Thus, in wild-type plants an AAPK may mediate ABA-induced anion channel activation and stomatal closure through a phosphorylation event, while ABI1 opposes ABA action through a dephosphorylation event.

Neither wild-type nor mutant versions of recombinant AAPK affected ABA inhibition of stomatal opening (Table 1). ABA inhibition of stomatal opening and ABA promotion of stomatal closure may, therefore, employ different signaling cascades. Alternatively, and in contrast to current theory (27), ABA activation of anion channels may not be required for ABA inhibition of stomatal opening.

Agronomically, loss of ABA-stimulated stomatal closure in plants transformed with mutant AAPK under control of an inducible promoter might allow accelerated and controlled desiccation of crops that are dried before harvest or distribution. Basal levels of ABA remain even in irrigated crops (27); under these conditions, inhibition of AAPK activity might alleviate stomatal limitation of CO2 uptake, and thus accelerate growth or increase yield.

Fig. 3. Specific expression of AAPK in guard cells. (A) Northern blot analysis with 10 μg total RNA per lane (12). The AAPK transcript is approximately 1.6 kb. (B) Ethidium bromide staining of the gel used in (A), confirming equal sample loading. GCP, guard cell protoplasts; MCP, mesophyll cell protoplasts.

Fig. 4. Block of ABA-induced stomatal closure by AAPK(K43A) mutant kinase. (A) Brightfield image showing that treatment of V. faba epidermal peels with 25 μM [+]−cis,trans-ABA causes closure of “half-stomates” associated with untransformed guard cells (left cell), while half-stomates associated with transformed guard cells (right cell) remain open. Scale bar, 10 μm. (B) Fluorescence image corresponding to the brightfield image of (A), showing the green fluorescence resulting from expression of pAAPK(K43A)-GFP in the right guard cell. Scale bar, 10 μm.

Fig. 5. Mutant AAPK blocks ABA-activation of slow anion channels in V. faba guard cell protoplasts. (A) in untransformed cells, or in cells transformed with GFP (pGFP) or AAPK (pAAPK-GFP) constructs, the typical decay in anion current magnitude over time in the whole cell configuration is prevented and anion current magnitude is enhanced by 50 μM [+]−cis-ABA. By contrast, in cells transformed with mutant AAPK [pAAPK(K43A)-GFP], ABA has no effect, and current rundown proceeds. Currents at −85 mV are shown 15 and 30 min after achieving a stable whole-cell configuration. ABA was applied just after recording the 15-min traces. Note the “reversal” of the 15 and 30 min traces in the pAAPK(K43A)-GFP transformed guard cell treated with ABA. Currents were identified as slow anion currents by their time dependence, reversal potential, and sensitivity to the anion channel blocker 5-nitro-2-(3-phenylpropylamino)benzoic acid (29). (B) Summary of effects of AAPK and the kinase inhibitor K252A (1 μM) on ABA regulation of anion currents. Steady-state current magnitude at −85 mV (21, 22) at 15 min after ABA application relative to steady-state current magnitude just before ABA application is plotted using the formula: 

\[-[I_{\text{in}} - I_{\text{out}}]/I_{\text{in}}] \times 100.\]

ABA was applied 15 min after attaining a stable whole-cell configuration. From left to right, bars represent mean ± SE of 9, 12, 5, 5, 5, 5, 9, 7, and 8 cells. Open bar, −ABA; striped bar, +ABA; solid bar, +ABA + K252A.

References and Notes
5. J. J. and S. M. Assmann, data not shown.
7. Proteins from guard cell protoplasts treated with darkness, ABA, or elevated CO2 concentrations were separated with 12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Kinase autophosphorylation activities of subsequently renatured proteins were detected as described (I. Kameshita and H. Fujisawa, Anal. Biochem. 183, 22 (1989); Y.-H. Wang and R. Chollet, Arch. Biochem. Biophys. 304, 496 (1993)).
8. J. A. Loo, Bioconjugate Chem. 6, 644 (1995). The AAPK spot was excised from six 2D gels (first dimension, nondenaturing PAGE). AAPK peptides generated by in-gel digestion with trypsin were sequenced by tandem mass spectrometry on a Finnigan LCQ quadrupole ion trap mass spectrometer (Harvard Microchemistry Facility).
11. Degenerate primers, 5′-TGGC[T/R](A/G)(G/C)AAG(C/T)/GATCACTG(AG)(G)-3′ (forward primer) and 5′-CCATC(C/T) [A/G]GATCACTG(A/G)TTTC3′ (reverse primer), where N indicates A, T, G, or C were designed as indicated in Fig. 2. Total RNA was isolated from guard cell protoplasts of V. faba (Triloz reagent, Gibco-BRL, Grand Island, NY) and used for RT-PCR. The PCR product was labeled with [32P]dCTP (Rediprime II, Amer sham Pharmacia Biotech) and used to screen a V. faba guard cell cDNA library in λ-Zap II (Stratagene, La Jolla, CA). The resulting full-length AAPK cDNA was sequenced on both strands.
12. Total RNA was isolated from purified guard cells, mesophyll cells, flowers, leaves, and seeds of V. faba and Northern analysis was performed by standard methods. The probe was the 5′-labeled BglII–Csp45 fragment of the AAPK cDNA clone, which includes the cDNA sequence corresponding to the relatively unique AAPK NH2-terminal region (Fig. 2) and part of the 3′ untranslated region of the cDNA.
13. The AAPK coding sequence was amplified by PCR from the AAPK cDNA clone with the primers 5'-GAATTCCTAGAGCCCTCCTTT-3' and 5'-CCGTGCAACCATGGATATGGCATATACAAT-3'. The pAAPK-GFP construct was created by inserting (via Nco I digestion) the amplified AAPK coding sequence downstream of the 35S promoter and upstream of, and in frame with, the GFP coding sequence in the GFP expression vector (pGFP) described in (17). To create pAAPK(K43A)-GFP, the lysine at position 43 in the AAPK coding sequence was substituted with an alanine by site-directed mutagenesis (overlapping PCR method; [S. N. H., H. D. Hunt, R. M. Horton, J. K. Pullen, L. R. Pease, Gene 77, 51 (1989)]). Constructs were sequenced to confirm correct junction, orientation, and/or site mutational.

14. Fifteen million V. faba guard cell protoplasts were transfected with pGFP, pAAPK-GFP, or pAAPK(K43A)-GFP by polyethylene glycol-mediated DNA transfer (17). Protoplasts were lysed and recombinant AAPK (V. faba) was used to improve its nutritional value in this respect. A combination of transgenes enabled biosynthesis of provitamin A in the endosperm.

15. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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17. Further details of the nucleotide sequence coding for a bacte-

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29. X.-Q. Wang and S. M. Assmann, data not shown.

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Engineering the Provitamin A (β-Carotene) Biosynthetic Pathway into (Carotenoid-Free) Rice Endosperm

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Rice (Oryza sativa), a major staple food, is usually milled to remove the oil-rich aleurone layer that turns rancid upon storage, especially in tropical areas. The remaining edible part of rice grains, the endosperm, lacks several essential nutrients, such as provitamin A. Thus, predominant rice consumption promotes vitamin A deficiency, a serious public health problem in at least 26 countries, including highly populated areas of Asia, Africa, and Latin America. Recombinant DNA technology was used to improve its nutritional value in this respect. A combination of transgenes enabled biosynthesis of provitamin A in the endosperm.

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Vitamin A deficiency causes symptoms ranging from night blindness to those of xerophthalmia and keratomalacia, leading to total blindness. In Southeast Asia, it is estimated that a quarter of a million children go blind each year because of this nutritional deficiency (1). Furthermore, vitamin A deficiency exacerbates afflictions such as diarrhea, respiratory diseases, and childhood diseases such as measles (2, 3). It is estimated that 124 million children worldwide are deficient in vitamin A (4) and that improved nutrition could prevent 1 million to 2 million deaths annually among children (3). Oral delivery of vitamin A is problematic (5, 6), mainly due to the lack of infrastructure, so alternatives are urgently required. Success might be found in supplementation of a major staple food, rice, with provitamin A. Because no rice cultivars produce this provitamin in the endosperm, recombinant technologies rather than conventional breeding are required.

Immature rice endosperm is capable of synthesizing the early intermediate geranylgeranyl diphosphate, which can be used to produce the uncolored carotene phytoene by expressing the enzyme phytoene synthase in rice endosperm (7). The synthesis of β-carotene requires the complementation with three additional plant enzymes: phytoene desaturase and ζ-carotene desaturase, each catalyzing the introduction of two double bonds, and lycopene θ-cyclase, encoded by the lcy gene. To reduce the transformation effort, a bacterial carotene desaturase, capable of introducing all four double bonds required, can be used.

We used Agrobacterium-mediated transformation to introduce the entire β-carotene biosynthetic pathway into rice endosperm in a single transformation effort with three vectors (Fig. 1) (8). The vector pB19hpc combines the sequences for a plant phytoene synthase (psy) originating from daffodil (9) (Narcissus pseudonarcissus; GenBank accession number X78814) with the sequence coding for a bacterial phytoene desaturase (crf1) originating from Erwinia uredovora (GenBank accession number D90087) placed under control of the endosperm-specific gluulin (Gtl) and the constitutive CaMV (cauliflower mosaic virus) 35S promoter, respectively. The phytoene synthase cDNA contained a 5′-sequence coding for a functional transit peptide (10), and the crf1 gene contained the transit peptide (gp) sequence of

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