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

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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, *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 wilty 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 planta*.

Guard cell protoplasts (4.8×10^7; 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 (protein kinase ABA1) subfamily (*9*) of protein kinases in subdomains I and VIb. *PKABA1* is transcriptionally upregulated by ABA (*9*) and PKABA1 may suppress gene induction by gibberellic acid during cereal grain germination (*10*).

Degenerate primers, whose design was based on conserved sequences in subdomain II of the PKABA1 subfamily and on the AAPK peptide sequence corresponding to protein kinase subdomain VIb, were used for reverse transcription–polymerase chain reaction (RT-PCR) with guard cell total RNA as template (*11*). The 310–base pair (bp) product that was generated encoded the previously determined AAPK peptide sequences as well as an amino acid sequence similar to that of the PKABA1 subfamily from subdomains II to VIb. This product was used to screen a *V. faba* guard cell cDNA library. A full-length cDNA of the appropriate size and sequence to encode AAPK was obtained.

The deduced AAPK sequence shows greatest homology to the PKABA1 subfamily (Fig. 2). However, the predicted protein also has unique regions, and none of the other PKABA1 family members has been implicated in stomatal function. Northern analysis (*12*) shows that *AAPK* is expressed in guard cell protoplasts, but not in mesophyll cell protoplasts, flowers, leaves, or seeds (Fig. 3), paralleling the guard cell specificity previously observed for AAPK activity (*4*). Southern analysis (*5*) implies that *AAPK* is a single copy gene.

Functional analysis of the *AAPK* gene product was complicated because ABA activation of AAPK does not occur in vitro. ABA activation is evident only when AAPK is extracted from intact guard cells previously treated with ABA (*4*), which presumably reflects a requirement for an intact cellular signaling cascade. Therefore, a green fluorescent protein (GFP)–tagged construct of AAPK (pAAPK-GFP) was made (*13*), expressed in guard cells (*14*), and shown to produce a kinase whose activity was upregulated by ABA treatment of the cells (*14*). The conserved lysine residue in subdomain II of protein kinases is critical for adenosine triphosphate (ATP) binding. Mutation of this residue yields kinases with reduced or absent catalytic activity (*15*). To create a comparable AAPK mutant, Lys^{43} in AAPK was mutagenized to an alanine and a pAAPK(K43A)-GFP construct was created (*13*, *16*). The kinase encoded by this construct had reduced activity (*14*), as predicted.

Next, *V. faba* leaves were biolistically transformed with pGFP, pAAPK-GFP, or pAAPK(K43A)-GFP (*17*–*19*). Abaxial epidermal peels were isolated, and transformed guard cells, indicated by their green fluorescence, were assayed for ABA-prevention of stomatal opening or for stomatal closure stimulated by ABA, CO_2, or darkness (*19*). The "half-aperture" 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

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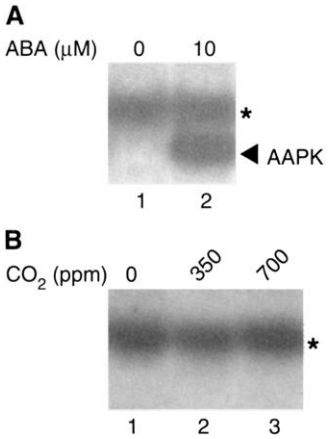

Fig. 1. ABA, but not other signals that cause stomatal closure, activate AAPK. AAPK autophosphorylation activity was assayed by the in-gel kinase method (*4*, *7*). Closing signals applied to the guard cell protoplasts were darkness [(**A**), lane 1], ABA (10 μM [±]-*cis,trans*-ABA) [(A), lane 2], and elevated CO_2 concentrations (700 ppm CO_2), versus the normal concentration of 350 ppm Co_2 [(**B**), lane 3]. The asterisk indicates a previously identified Ca^{2+}-dependent protein kinase (*28*). The arrowhead indicates AAPK.

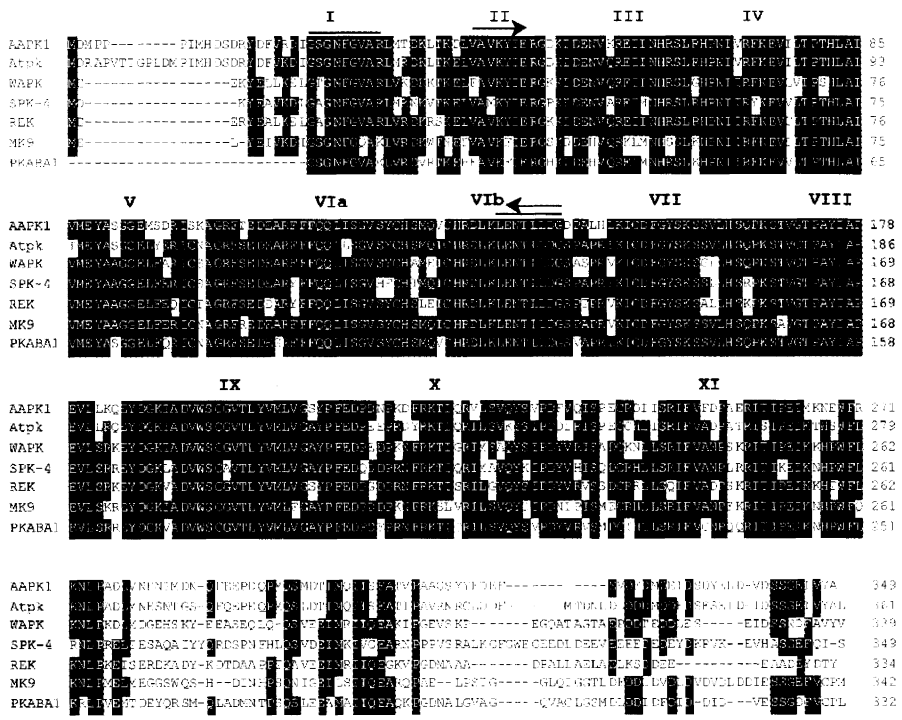

Fig. 2. Alignment of the deduced AAPK amino acid sequence with those of homologous protein kinases. GenBank accession numbers are: AAPK (AF186020), *Arabidopsis* Atpk (L05562), tobacco WAPK (AF032465), soybean SPK-4 (L38855), rice REK (AB002109), ice plant MK9 (Z26846), and wheat PKABA1 (M94726). Amino acids are highlighted when there are at least four identical residues among the seven sequences. Conserved subdomains of the protein kinase family are indicated by roman numerals. Peptide sequences obtained by tandem mass spectrometry are marked by lines. Peptide regions used for designing degenerate PCR primers are indicated by arrows. Sequences were aligned by the Clustal method in MegAlign (DNASTAR, Madison, WI). Numbers indicate amino acid positions (*16*).

1), but had no effect on stomatal closure induced by CO_2 or darkness. Transformation with wild-type AAPK (pAAPK-GFP) had no effect on any of the responses assayed (Table 1), suggesting either that native AAPK was not rate-limiting or that recombinant AAPK was not sufficiently overexpressed to alter function.

To determine the cellular basis for the mutant AAPK effect, guard cell protoplasts were isolated from biolistically transformed leaves and subjected to patch-clamp analyses (*20*). Slow anion channel activation is implicated in stomatal closure (*6*, *21*–*23*). Anion loss presumably depolarizes the membrane potential, thus driving an efflux of K^+, followed by H_2O efflux and stomatal closure. We show here that in *V. faba*, ABA activates slow anion channels (Fig. 5) (*24*), not only reversing the typical decay in anion current magnitude over time in the whole cell configuration (*25*) but also increasing anion current magnitude. Guard cells transformed with pGFP or pAAPK-GFP show normal ABA regulation of anion currents (Fig. 5). However, in guard cells transformed with pAAPK(K43A)-GFP, ABA activation of anion channels is eliminated (Fig. 5).

We suspect that the K43A mutant kinase competes with the activity of native AAPK in a dominant negative fashion. First, the kinase inhibitor K-252a inhibits (i) native AAPK activity (*4*, *5*), (ii) ABA-induced stomatal closure (*22*), and (iii) ABA regulation of anion channels in untransformed cells (Fig. 5B), implying that the channels are indeed normally regulated

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 CO_2 treatment was 700 ppm CO_2. 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.

	GFP		AAPK-GFP		AAPK (K43A)-GFP	
	Transformed	Untransformed	Transformed	Untransformed	Transformed	Untransformed
			Closure			
ABA	−2.52 ± 0.29 (36)	−2.54 ± 0.35 (36)	−2.59 ± 0.30 (36)	−2.58 ± 0.24 (36)	−0.36 ± 0.26 (56)*	−2.55 ± 0.21 (56)
Control	0.10 ± 0.09 (10)	0.09 ± 0.09 (10)	0.11 ± 0.10 (10)	0.12 ± 0.11 (10)	0.12 ± 0.10 (24)	0.13 ± 0.10 (24)
CO_2	ND	ND	ND	ND	−2.23 ± 0.44 (36)	−2.31 ± 0.46 (36)
Control					−0.09 ± 0.09 (10)	−0.11 ± 0.10 (10)
Darkness	ND	ND	ND	ND	−2.08 ± 0.40 (36)	−2.08 ± 0.46 (36)
Control					0.12 ± 0.11 (10)	0.13 ± 0.11 (10)
			Opening			
ABA	0.42 ± 0.22 (36)	0.45 ± 0.27 (36)	0.43 ± 0.22 (36)	0.41 ± 0.28 (36)	0.42 ± 0.17 (46)	0.44 ± 0.15 (46)

*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 (*21*), 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 CO_2 uptake, and thus accelerate growth or increase yield.

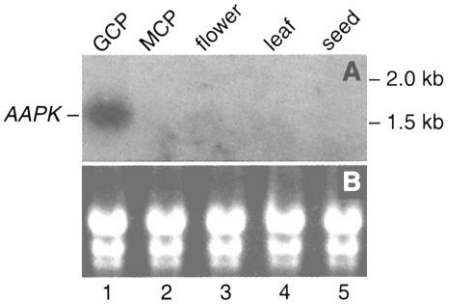

Fig. 3. Specific expression of *AAPK* in guard cells. (**A**) Northern blot analysis with 10 μg total RNA per lane (*12*). The *AAPK* transcript is approximately 1.6 kb. (**B**) Ethidium bromide staining of the gel used in (A), confirming equal sample loading. GCP, guard cell protoplasts; MCP, mesophyll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

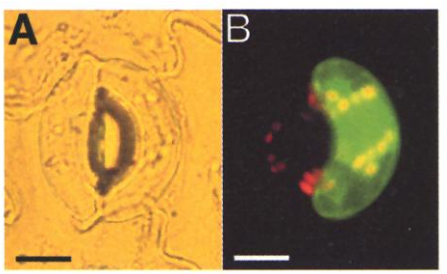

Fig. 4. Block of ABA-induced stomatal closure by AAPK(K43A) mutant kinase. (**A**) Brightfield image showing that treatment of *V. faba* epidermal peels with 25 μM [±]-*cis*,*trans*-ABA causes closure of "half-stomates" associated with untransformed guard cells (left cell), while half-stomates associated 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.

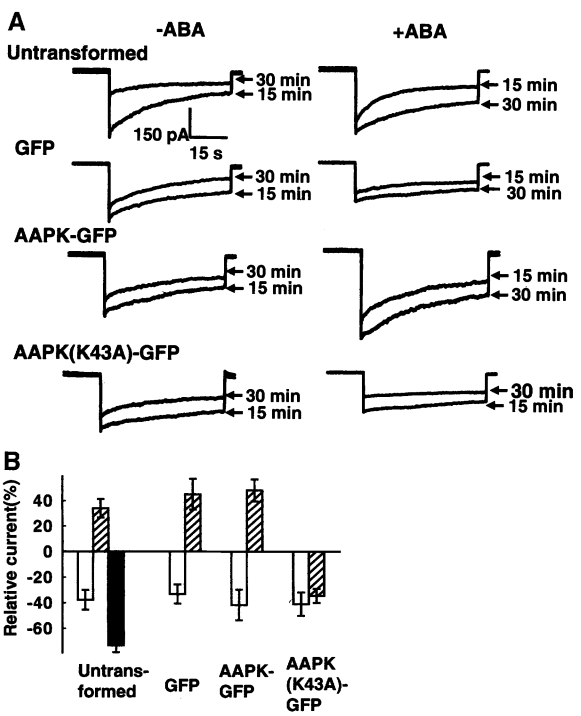

Fig. 5. Mutant AAPK blocks ABA-activation of slow anion channels in *V. faba* guard cell protoplasts. (**A**) In untransformed cells, or in cells transformed with GFP (pGFP) or AAPK (pAAPK-GFP) constructs, the typical decay in anion current magnitude over time in the whole cell configuration is prevented and anion current magnitude is enhanced by 50 μM [±]-*cis*,*trans*-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: $-[(I_{15min} - I_{30min})/I_{15min}] \times 100$. ABA was applied 15 min after attaining a stable whole-cell configuration. From left to right, bars represent mean ± SE of 9, 12, 5, 6, 5, 5, 9, 7, and 8 cells. Open bar, −ABA; striped bar, +ABA; solid bar, +ABA + K252a).

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11. Degenerate primers, 5′-TTGC(C/T)(A/G)T(G/C)AA(A/G) TACATCGAA-3′ (forward primer) and 5′-CCATC(C/T)A(A/G)NAGNGT(A/G)TTTTC-3′ (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* (TRIzol reagent, Gibco-BRL, Grand Island, NY) and used for RT-PCR. The PCR product was labeled with [^{32}P]dCTP (Rediprime II, Amersham 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 ^{32}P-labeled Bgl II–Csp45 I fragment of the AAPK cDNA clone, which includes the cDNA sequence corresponding to the relatively unique AAPK NH_2-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′-GAATCTCCACTACGACGCCGTTACTTCCG-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. Ho, 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 mutation.
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 protein was immunoprecipitated with GFP peptide antibodies (Clontech, Palo Alto, CA) and protein A–Sepharose CL-4B (Amersham Pharmacia Biotech, Picataway, NJ). Immunoprecipitated proteins were assayed for kinase activity using histone III-S (Sigma) as substrate (*4*). No histone phosphorylation by the pGFP control immunoprecipitate was observed. Analogous to native AAPK (*4*), histone phosphorylation by the pAAPK-GFP immunoprecipitate was enhanced when the immunoprecipitate was isolated from ABA-treated guard cells. When the same experiment was performed with pAAPK(K43A)-GFP, histone phosphorylation was reduced.
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16. 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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19. *V. faba* leaves were bombarded with DNA-coated [pGFP, pAAPK-GFP, or pAAPK(K43A)-GFP] gold particles (BioRad, Hercules, CA) with the use of a particle delivery system 1000/He (BioRad, Hercules, CA) as described [J. Marc *et al.*, *Plant Cell* **10**, 1927 (1998)]. Stomatal complexes were then assayed for ABA-prevented opening or closure stimulated by ABA, CO_2, or darkness. Conditions for opening experiments were as described [S. M. Assmann and T. I. Baskin, *J. Exp. Bot.* **49**, 163 (1998)] except that the incubation solution was 10 mM MES, 30 mM KCl, pH 6.1 with or without 50 μM [±]-*cis*,*trans*-ABA. For closure experiments, transformed leaves were illuminated with 0.20 mmol m^{-2} s^{-1} white light for 2.5 hours to open stomata. Epidermal peels were then taken and placed in incubation solution under darkness or treated with 25 μM [±]-*cis*,*trans*-ABA, or with 700 parts per million (ppm) CO_2 for 1 hour.
20. Abaxial guard cell protoplasts were isolated as described [X.-Q. Wang, W.-H. Wu, S. M. Assmann, *Plant Physiol.* **118**, 1421 (1998)]. Whole-cell patch-clamp experiments were performed as previously established (*22*). Pipette solution contained 100 mM KCl, 50 mM tetraethylammonium, 2 mM $MgCl_2$, 6.7 mM EGTA-$(Tris)_2$, 3.35 mM $CaCl_2$, 10 mM Hepes, pH 7.1 (Tris), and 5 mM Mg-ATP. Bath solution contained 40 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM MES-Tris pH 5.6. Osmolalities were adjusted with sorbitol to 500 mosmol/kg (in the pipette) or 470 mosmol/kg (in the bath). Protein kinase inhibitor K252a (Calbiochem, La Jolla, CA) was prepared as a 2 mM stock in dimethyl sulfoxide (DMSO); DMSO controls showed no effect.
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30. We thank the following for their advice: R. Cyr (provision of GFP expression vector), C. Granger (bombardment experiments), S. Gilroy and S. Ritchie (GFP photography), L. Haubrick (Northern blotting), and J. A. Snyder (CO_2 experiments). We also thank W. Lane and the Harvard Microchemistry Facility for mass spectrometric sequencing. Supported by NSF grant MCB-9874438.

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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.

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 β-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 (*crtI*) originating from *Erwinia uredovora* (GenBank accession number D90087) placed under control of the endosperm-specific glutelin (Gt1) and the constitutive CaMV (cauliflower mosaic virus) 35*S* promoter, respectively. The phytoene synthase cDNA contained a 5′-sequence coding for a functional transit peptide (*10*), and the *crtI* gene contained the transit peptide (*tp*) sequence of

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