Abscisic Acid Signaling Through Cyclic ADP-Ribose in Plants

Yan Wu, *et al.*

Science **278**, 2126 (1997);
DOI: 10.1126/science.278.5346.2126

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.

identity of the proteins was confirmed by specific antibodies to Sec61α (Sec61p) and Sec61β (Sbh1p) (*17*).
14. S. Panzner, L. Dreier, E. Hartmann, S. Kostka, T. A. Rapoport, *Cell* **81**, 561 (1995)
15. For the purification of ribosomes, the yeast strain DF5 was grown in 3.5 liters of yeast extract, peptone, and dextrose medium. At an optical density of 600 nm (OD_{600}) of 1.0, the cells were washed with water and incubated for 15 min at 25°C in 100 mM tris-SO_4 (pH 9.4) and 10 mM DTT. After homogenization by French press in buffer A [50 mM triethanolamine-OAc (pH 7.5), 50 mM KOAc, 5 mM $MgCl_2$, 1 mM DTT, and 0.5 mM PMSF], the homogenate was centrifuged for 30 min at 100,000*g* at 4°C. The supernatant was layered over a continuous 10 to 40% sucrose gradient in buffer A. After centrifugation for 4.5 hours at 200,000*g* at 4°C, the monomeric ribosomes were pooled according to the A_{254} profile. The ribosomes were pelleted by centrifugation for 4.5 hours at 145,000*g* at 4°C, resuspended in water, and frozen in liquid N_2.
16. Purified Sec61 complex and ribosomes were incubated for 30 min on ice in a buffer containing 0.5% Triton X-100, 100 mM KOAc, 5 mM triethanolamine-OAc (pH 7.5), 5% glycerol, 1.5 mM $Mg(OAc)_2$, 0.5 mM DTT, and 0.5 mM $CaCl_2$. To separate unbound Sec61 complex from ribosome-bound Sec61 complex, we carried out gradient centrifugation using a 10 to 50% sucrose step gradient in 0.5% Triton X-100, 100 mM KOAc, 10 mM triethanolamine-OAc, 1 mM DTT, and 3 mM $Mg(OAc)_2$. After centrifugation for 60 min at 240,000*g* at 4°C, six fractions were collected manually. For saturation assays, the first two fractions were pooled as the unbound fraction and the following two as the bound fraction. Fractions were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and stained with SYPRO Red. The amount of protein was quantitated with the STORM system (red fluorescence) and NIH image. For immunoblotting, proteins were precipitated, separated on 10 to 20% SDS-PAGE gradient gels, transferred to a nitrocellulose membrane, incubated consecutively with anti-Sec61α or anti-Sec61β and horseradish peroxidase–conjugated donkey antibodies to rabbit, and detected by ECL as described (Amersham).
17. R. Beckmann *et al*., data not shown.
18. Incubation to form the ribosome-Sec61 complex was performed as described (*16*), and the mixture was diluted with 4 volumes of water immediately before it was applied to the grid. Grids for cryo–electron microscopy were prepared as described [T. Wagenknecht, R. Grassucci, J. Frank, *J. Mol. Biol*. **199**, 137 (1988); J. Dubochet *et al.*, *Q. Rev. Biophys*. **21**, 129 (1988)]. Micrographs were recorded under low-dose conditions on a Philips EM 420, with 1.5-μm defocus and magnification of 52,200 ± 2% as checked by a tobacco mosaic virus standard.
19. P. A. Penczek, R. Grassucci, J. Frank, *Ultramicroscopy* **53**, 251 (1994).
20. C. Bernabeu, E. M. Tobin, A. Fowler, I. Jabin, J. A. Lake, *J. Cell Biol*. **96**, 1471 (1983).
21. Micrographs were checked for drift, astigmatism, and presence of Thon rings by optical diffraction. Scanning was done with a step size of 25 μm corresponding to 4.78 Å on the object scale, on a Perkin-Elmer PDS 1010 A microdensitometer. Particles were selected by an automated selection procedure that differed from the one previously described [K. R. Lata, P. Penczek, J. Frank, *Ultramicroscopy* **58**, 381 (1995)] in that the particle candidates were compared directly with the reference set of 87 quasi–evenly spaced projections (*19*) of an existing reconstruction of the ribosome from yeast (*12*). A total of 13,178 particles were picked. The reconstruction was done with two independent approaches to obtain the orientations of the projections. In the first approach, an existing reconstruction of the ribosome from yeast (*12*) was used as a reference in the 3D projection alignment procedure (*19*). In the second approach, an initial reconstruction was obtained with the simultaneous minimization technique [P. Penczek, J. Zhu, J. Frank, *Ultramicroscopy* **63**, 205 (1996)]. In both cases, four steps of the 3D projection alignment procedure (*19*) were applied with a 2° angular interval. In each step, the refined 3D structure was calculated with 70% of the best matching particles (on the basis of the value of the cross-correlation coefficient). Both reconstructions proved to be indistinguishable within the measured resolution range. The final resolution, estimated with the Fourier shell correlation with a cutoff value at 0.5 [B. Böttcher, S. A. Wynne, R. A. Crowther, *Nature* **386**, 88 (1997)], was 26 Å.
22. R. Gilmore and G. Blobel, *Cell* **42**, 497 (1985).
23. In trying to gauge the correct threshold value, we were led by two criteria: (i) We observed the structure as the threshold was increased. There is normally a "plateau," a range of threshold values within which the appearance (or the volume encompassed) varies only slightly. (ii) Three-dimensional connectivity must not be violated, which means that, in this case, we could not choose a threshold, within the plateau defined above, that makes the connecting rod disappear. Thus, the plateau was further narrowed. These criteria were applied separately in the preparation of the 3D representations of the ribosome and the channel. Because of the residual uncertainty in the molecular boundaries, the measurements for pore size and the distance between channel and ribosome have an uncertainty of 25%.
24. We thank A. Fischer for the purification of the Sec61 complex; members of the Blobel lab and R. Agrawal for discussions; S. Darst and A. Malhotra for discussions, support with the electron microscopy, and assistance with the image processing; A. Heagle for help with the illustrations; and the National Center for Supercomputer Applications, University of Illinois at Urbana-Champaign, for computing support. Supported by grants from NIH (1R01 GM29169) and NSF (BIR 9219043) (to J.F.) and a fellowship of the Deutsche Foschungsgemeinschaft (to R.B.).

26 September 1997; accepted 17 November 1997

Abscisic Acid Signaling Through Cyclic ADP–Ribose in Plants

Yan Wu, Jennifer Kuzma, Eric Maréchal,* Richard Graeff, Hon Cheung Lee, Randy Foster, Nam-Hai Chua†

Abscisic acid (ABA) is the primary hormone that mediates plant responses to stresses such as cold, drought, and salinity. Single-cell microinjection experiments in tomato were used to identify possible intermediates involved in ABA signal transduction. Cyclic ADP–ribose (cADPR) was identified as a signaling molecule in the ABA response and was shown to exert its effects by way of calcium. Bioassay experiments showed that the amounts of cADPR in *Arabidopsis thaliana* plants increased in response to ABA treatment and before ABA-induced gene expression.

Plants endure environmental challenges such as drought, salinity, or cold by adjusting rather than escaping. These responses are mediated by ABA (*1*), which through unknown signals affects the regulation of many genes (*2*–*7*). One signaling intermediary is calcium (Ca^{2+}) (*8*). ABA-mediated increases in guard cell Ca^{2+} levels lead to stomatal closure (*9*). Ca^{2+} can also induce the expression of an ABA-responsive gene in maize protoplasts (*10*).

Three regulators of Ca^{2+} levels are inositol (1,4,5)-trisphosphate (IP_3) (*11*), cyclic adenosine 5′-diphosphate ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate ($NAADP^+$) (*12*–*15*). The receptor for IP_3 is known (*11*), whereas those for cADPR and $NAADP^+$ are not (*15*). A putative receptor for cADPR is the ryanodine receptor (RyR) (*15*). $NAADP^+$, a metabolite of nicotinamide adenine dinucleotide phosphate ($NADP^+$) identified in vitro, regulates a third Ca^{2+} channel that appears to be distinct from the IP_3 and cADPR receptors (*14*). cADPR can be produced by ADP–ribosyl cyclase or by CD38, a lymphocyte protein, both of which use nicotinamide adenine dinucleotide (NAD^+) as a precursor (*16*).

Both IP_3 and cADPR elicit Ca^{2+} release from beet storage root vacuoles (*17*), and a RyR-like activity, sensitive to cADPR, has been detected in beet microsomes (*18*). Here we demonstrate that cADPR is a likely in vivo intermediate of ABA signal transduction that exerts its effects by way of intracellular Ca^{2+} release.

We used microinjection to screen for compounds that may be involved in ABA responses. We studied the *Arabidopsis* genes *rd29A* (also termed *Iti78* and *cor78*) (*5*), a desiccation-responsive gene (*3*), and *kin2* (also termed *cor6.6*) (*5*), a cold-inducible gene (*2*). Both genes are rapidly induced by ABA, without requiring new protein synthesis.

We microinjected 7- to 10-day-old etiolated hypocotyls of the phytochrome-deficient tomato mutant *aurea* (*19*, *20*) with *rd29A-GUS*, *kin2-GUS* (*21*), and potential agonists and antagonists of ABA signal

Y. Wu, J. Kuzma, E. Maréchal, R. Foster, N.-H. Chua, Laboratory of Plant Molecular Biology, Rockefeller University, 1230 York Avenue, New York, NY 10021–6399, USA.
R. Graeff and H. C. Lee, Department of Physiology, University of Minnesota, Minneapolis, MN 55455, USA.

*Present address: Laboratoire de Physiologie Cellulaire Végétale, Departement de Biologie Moleculaire et Cellulaire, Universite Joseph Fourier et CEA-Grenoble, 17 rue des Martyrs, F-38054 Grenoble Cedex 9, France.
†To whom correspondence should be addressed. E-mail: chua@rockvax.rockefeller.edu

transduction. The *aurea* mutant was chosen because it has been used in previous investigations on phytochrome signaling pathways and therefore provided convenient controls. ABA was sufficient to induce gene expression from both constructs (Table 1). Neither *rd29A-GUS* nor *kin2-GUS* was activated in the absence of ABA; both were activated in response to ABA, but not to phytochrome A (phyA). By contrast, *cab-GUS*, a light-responsive gene, was activated by phyA but not ABA. Moreover, phyA induction of *cab-GUS* was negatively regulated by ABA, as reported previously (*22*). These results recapitulated specific activation of the reporter genes by their appropriate physiological signals in our system.

rd29A-GUS and *kin2-GUS* can be activated by coinjected Ca^{2+}. ABA induction of these two genes is blocked by EGTA (3 mM), a Ca^{2+} chelator (*9*) (Table 2). cADPR, an endogenous regulator of Ca^{2+}, triggers Ca^{2+} release from sea urchin microsomes (*15*) and from beet cell vacuoles in beet storage roots (*17*). To determine if cADPR could substitute for ABA or Ca^{2+}, we coinjected cADPR with *rd29A-GUS* or *kin2-GUS* (Table 2). At 1 μM (estimated intracellular concentration) (*19*), cADPR replaced ABA or Ca^{2+} in stimulating the two ABA-regulated genes. Lower (0.2 μM) or higher (2 μM) concentrations of cADPR were not as effective (*23*). Similar concentration dependence is often observed for ion channels, where low concentrations of agonists are below the threshold needed for activity and high concentrations lead to desensitization.

EGTA blocked cADPR induction of *rd29A* and *kin2* expression, consistent with the role of cADPR in releasing Ca^{2+} from internal stores (Table 2). A structural analog of cADPR and specific inhibitor of cADPR activity, 8-amino-cADPR (8-NH_2-cADPR) (*24*), blocked the stimulation of *rd29A-GUS* and *kin2-GUS* by ABA (Table 2). Heated cADPR or heated 8-NH_2-cADPR (100°C, 10 min) was inactive (Table 2). Upon heat treatment, cADPR is hydrolyzed to ADP-ribose which is ineffective as a Ca^{2+}-releasing agent (*25*).

Because *cab* gene is activated by phyA by way of Ca^{2+} (*19*), we examined whether cADPR could substitute for these two inducers. In contrast to *rd29A* and *kin2*, *cab-GUS* expression was induced by Ca^{2+} but not cADPR (Table 2). These results suggest that cADPR may elicit a unique cytoplasmic Ca^{2+} wave leading to a specific activation of ABA-responsive but not phyA-responsive genes. The expression of *cab-GUS* and CaMV*35S-GUS* (*26*) was not inhibited by 8-NH_2-cADPR, its heated derivative, or EGTA, thus ruling out a general cytotoxic effect (Table 2).

Microinjected *Aplysia* ADP–ribosyl cyclase, which synthesizes cADPR (*16*), activates reporter gene expression in the absence of ABA (Table 3). The effect was inhibited by coinjection of 8-NH_2-cADPR. *Neurospora* NADase, an enzyme that de-

Table 1. Specificity of phyA and ABA signaling pathways. Subepidermal cells of *aurea* hypocotyls were injected with either *rd29A-GUS*, *kin2-GUS*, or *cab-GUS* (*19*), with or without oat phytochrome A (phyA). Pipette concentrations were ~10,000 molecules/pl for phyA and 1 mg/ml for plasmid DNA (except for CaMV*35S-GUS*, 0.5 mg/ml). The injected volume was ~ 1 pl and the cell volume was estimated to be 160 pl (*19*). After injections, seedlings were incubated in KNOP salt medium (*19*) with or without ABA (50 μM) (*cis-*, *trans*-abscisic acid; Sigma) for 2 days. The results are given as the number of cells expressing GUS as a percentage of the total number of cells injected. Because the plant vacuolar volume is about 90% of the cell volume, the frequency of a micropipette hitting the cytoplasm rather than the vacuole is low. Using the constitutive 35*S* promoter (CaMV*35S-GUS*) (*26*) as a control, we found that 5 to 10% of the injected cells expressed GUS activity. Therefore, we regard values that fall within 5 to 10% as the maximum that can be obtained in our microinjection system. No quantitative significance is attached to numbers within this range.

GUS reporter	Coinjected phyA	Externally added ABA	GUS efficiency (%) (*n*)
rd29A	−	−	0 (208)
rd29A	−	+	9.2 (596)
rd29A	+	−	0 (234)
rd29A	+	+	6.3 (221)
kin2	−	−	0 (213)
kin2	−	+	6.3 (639)
kin2	+	−	0 (242)
kin2	+	+	7.4 (230)
cab	−	−	0 (202)
cab	+	−	6.8 (266)
cab	−	+	0 (218)
cab	+	+	0.4 (231)
35S	−	−	7.6 (236)

Table 2. cADPR can substitute for ABA or Ca^{2+} in the activation of *rd29A-GUS* and *kin2-GUS*. *Aurea* hypocotyls were injected with combinations of cADPR, 8-NH_2-cADPR (Molecular Probes), Ca^{2+}, *rd29A-GUS*, *kin2-GUS*, *cab-GUS*, and CaMV*35S-GUS* as indicated. Pipette concentrations of cADPR, 8-NH_2-cADPR, and Ca^{2+} were all 160 μM. Injected seedlings were subsequently incubated in KNOP medium in the presence or absence of ABA (50 μM) or in the presence or absence of EGTA (EGTA/AM, 3 mM) for 2 days. Δ: Heated cADPR or heated 8-NH_2-cADPR (*25*). EGTA/AM: Membrane-permeable version (Calbiochem-Novabiochem, La Jolla, California).

GUS reporter	Coinjected			Externally added		GUS efficiency (%) (*n*)
	cADPR	Ca^{2+}	8-NH_2-cADPR	ABA	EGTA	
rd29A	−	−	−	−	−	0 (205)
rd29A	−	−	−	+	−	9.7 (423)
rd29A	−	−	−	+	+	0 (434)
rd29A	−	+	−	−	−	8.1 (209)
rd29A	+	−	−	−	−	9.2 (436)
rd29A	+	−	−	−	+	0 (222)
rd29A	+Δ	−	−	−	−	0 (215)
rd29A	−	−	+	−	−	0 (220)
rd29A	−	−	+	+	−	0 (219)
rd29A	−	−	+Δ	−	−	0 (246)
rd29A	−	−	+Δ	+	−	7.5 (228)
kin2	−	−	−	−	−	0 (214)
kin2	−	−	−	+	−	8.6 (441)
kin2	−	−	−	+	+	0 (458)
kin2	−	+	−	−	−	8.2 (219)
kin2	+	−	−	−	−	9.0 (423)
kin2	+	−	−	−	+	0 (234)
kin2	+Δ	−	−	−	−	0 (229)
kin2	−	−	+	−	−	0 (236)
kin2	−	−	+	+	−	0 (217)
kin2	−	−	+Δ	−	−	0 (257)
kin2	−	−	+Δ	+	−	7.6 (211)
cab	−	−	−	−	−	0 (216)
cab	−	−	−	+	−	0 (221)
cab	−	+	−	−	−	5.5 (254)
cab	+	−	−	−	−	0 (218)
cab	+	+	−	−	−	7.7 (233)
cab	+Δ	+	−	−	−	9.7 (226)
cab	−	+	+	−	−	6.1 (212)
cab	−	+	+Δ	−	−	7.0 (242)
35S	−	−	−	−	−	10.3 (203)
35S	−	−	−	+	−	8.5 (246)
35S	−	−	−	+	+	9.0 (210)

grades NAD^+, the precursor of cADPR (*27*), inhibited ABA induction of the two reporter genes (Table 3). Coinjection of cADPR (intracellular concentration 1 μM), which is insensitive to NADase, overcame the inhibition. Injection of bovine serum albumin (BSA) had no effect (Table 3).

To examine the effect of protein phosphorylation on ABA signal transduction, we injected seedlings and treated them with protein kinase inhibitors K252a (2 μM) (*28*) and staurosporine (*29*) (100 nM). The response of reporter genes to ABA, cADPR, and Ca^{2+} was abolished by K252a and diminished by staurosporine (Table 4). The phosphatase inhibitor okadaic acid (OA) (*28*) (100 nM) stimulated reporter gene expression in the absence of ABA (Table 5). This activation was inhibited by 8-NH_2-cADPR, EGTA (3 mM), and K252a (2 μM), indicating that an OA-sensitive phosphatase may be upstream of cADPR, Ca^{2+} pools, and kinases in ABA signaling.

Table 3. ADP–ribosyl cyclase activates, NADase inactivates, *rd29A-GUS* and *kin2-GUS*. *Aplysia* ADP–ribosyl cyclase (ADPR cyclase) (7000 molecules; Sigma), *Neurospora* NADase (3000 molecules; Sigma), and BSA (7000 molecules; Boehringer Mannheim) were coinjected with either *rd29A-GUS* or *kin2-GUS*. Pipette concentrations of cADPR and 8-NH_2-cADPR were 160 μM. After injections, some of the seedlings were incubated in KNOP medium with ABA (50 μM).

GUS reporter	Coinjected					Externally added ABA	GUS efficiency (%) (*n*)
	ADPR cyclase	NADase	cADPR	8-NH_2-cADPR	BSA		
rd29A	+	−	−	−	−	−	6.6 (213)
rd29A	+	−	−	+	−	−	1.4 (211)
rd29A	−	+	−	−	−	−	0 (223)
rd29A	−	+	−	−	−	+	0 (425)
rd29A	−	+	+	−	−	+	6.9 (233)
rd29A	−	−	−	−	+	−	0 (218)
kin2	+	−	−	−	−	−	7.8 (218)
kin2	+	−	−	+	−	−	2.2 (226)
kin2	−	+	−	−	−	−	0 (225)
kin2	−	+	−	−	−	+	0 (443)
kin2	−	+	+	−	−	+	5.6 (231)
kin2	−	−	−	−	+	−	0 (214)

Table 4. Effects of kinase inhibitors on cADPR- and ABA-induced gene induction. Pipette concentrations of Ca^{2+} and cADPR were 160 μM. Injected seedlings were incubated in KNOP medium with either staurosporine (St.) (100 nM, Calbiochem-Novabiochem) or K252a (2 μM; BIOMOL Research Laboratories) for 2 days in the presence or absence of ABA (50 μM).

GUS reporter	Coninjected		Externally added			GUS efficiency (%) (*n*)
	cADPR	Ca^{2+}	ABA	K252a	St.	
rd29A	−	−	+	−	−	9.1 (241)
rd29A	−	−	−	+	−	0.4 (216)
rd29A	−	−	+	+	−	0 (229)
rd29A	−	−	+	−	+	0.4 (235)
rd29A	+	−	−	−	−	8.2 (219)
rd29A	+	−	−	+	−	0 (223)
rd29A	+	−	−	−	+	0 (228)
rd29A	−	+	−	−	−	5.9 (256)
rd29A	−	+	−	+	−	0 (266)
rd29A	−	+	−	−	+	0 (213)
kin2	−	−	+	−	−	8.8 (215)
kin2	−	−	−	+	−	0 (227)
kin2	−	−	+	+	−	0 (219)
kin2	−	−	+	−	+	0 (205)
kin2	+	−	−	−	−	6.4 (203)
kin2	+	−	−	+	−	0 (210)
kin2	+	−	−	−	+	0 (229)
kin2	−	+	−	−	−	7.1 (226)
kin2	−	+	−	+	−	0 (242)
kin2	−	+	−	−	+	0 (212)
35S	−	−	−	−	−	9.5 (241)
35S	−	−	+	−	−	11.1 (207)
35S	−	−	+	+	−	8.9 (203)
35S	−	−	+	−	+	8.6 (232)

Thus, cADPR-mediated induction of ABA-responsive gene expression may be positively regulated by protein kinases and negatively regulated by protein phosphatases. ABA-mediated guard cell closure in *Vicia faba* shows a similar response to OA and K252a, although *Arabidopsis* guard cells responded differently to the same inhibitors (*7*, *28*).

IP_3-triggered release of Ca^{2+} has been implicated in guard cell closure (*30*). We examined whether IP_3 was able to induce expression of ABA-responsive genes. Indeed, *rd29A* and *kin2* were activated by IP_3, and the activation was blocked by heparin (Table 6), a specific inhibitor of IP_3 receptor (*11*). By contrast, ABA activation of the two reporter genes was not affected by heparin, and the same inhibitor had no effect on cADPR-induced gene expression (Table 6). Because phospholipase C mRNA itself is induced by ABA treatment (*31*), the results suggest that IP_3 may be involved in the secondary rather than primary ABA response. Control experiments demonstrated that IP_3 was unable to activate *cab-GUS* (Table 6), thus

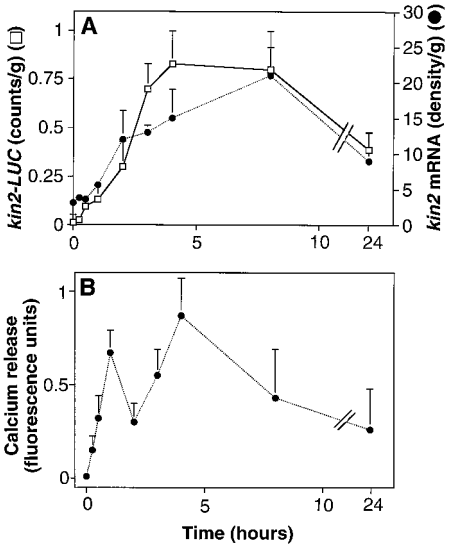

Fig. 1. cADPR levels during ABA induction. (**A**) Gene induction of *kin2* after ABA treatment (50 μM) was monitored by Northern blotting (*kin2* mRNA) and by in vivo imaging of plants harboring the *kin2-LUC* transgene (*kin2-LUC* activity). Results are expressed in normalized units per gram of fresh weight, either density for mRNA or counts for reporter gene activity, and are averages of two experiments. For clarity, only the positive standard deviation is shown. (**B**) Ca^{2+}-release activity of the extracts as measured during ABA induction. Ca^{2+}-release activities are expressed in fluo3 fluorescence units induced by cADPR extracted for 1 g of fresh tissue and are the averages of three experiments. For clarity, only the positive standard deviation is shown. The double bars on graphs (A) and (B) indicate an interruption in the *x* axis.

ruling out its role in phyA responses.

We examined whether GTP-binding proteins (G proteins) also participate in ABA signal transduction. GDP-β-S, an antagonist of heterotrimeric G proteins and inhibitor of *chs* and *cab* gene expression (*19*), had no effect on *rd29A-GUS* or *kin2-GUS* expression (*23*) over 2 days. Likewise, a G-protein activator, GTP-γ-S, was unable to induce reporter gene expression in the absence of ABA. These results demonstrate that G proteins are not directly involved in ABA signal transduction.

To assess cADPR levels in vivo, we used transgenic *Arabidopsis thaliana* plants carrying a *kin2*-luciferase transgene (*32*) that were treated with ABA. After assessing reporter gene activity (*33*), we harvested plants and measured amounts of endogenous *kin2* mRNA (*34*) (Fig. 1A) and cADPR-triggered Ca^{2+}-release activity (*12*, *34*) (Fig. 1B). Gene expression peaked after 4 to 8 hours of exposure to ABA (Fig. 1A). Levels of *LUC* reporter gene activity and endogenous *kin2* mRNA were similar throughout the time course. cADPR levels, as measured by the Ca^{2+}-release assays, increased after ABA treatment and before changes in transcription of ABA-responsive genes.

Using microinjection into tomato *aurea* (*20*) hypocotyls, we identified potential intermediates in ABA signal transduction. Agonists that could replace ABA in the microinjection experiments included OA, cADPR, and Ca^{2+}. cADPR has been described in the chloroplast-containing unicellular protist *Euglena gracilis* (*35*). cADPR levels in *E. gracilis* oscillated during the cell cycle, with maximal levels detected immediately before cell division. Recently, two serine-threonine phosphatases, ABI1 and ABI2, have been reported to be directly or indirectly involved in ABA signal transduction in *A. thaliana* (*6*). These phosphatases are insensitive to OA, and their relation to the ABA signaling pathway described here remains to be elucidated. In barley aleurone protoplasts, ABA is thought to activate mitogen-activated protein kinase by way of a tyrosine phosphatase (*36*). This step appears to be a prerequisite for ABA induction of *rab16* gene expression.

We propose that cADPR is a second messenger responsible for initiating the cascade of Ca^{2+} increases and subsequent Ca^{2+}-dependent phosphorylation and dephosphorylation during ABA signal transduction.

Table 5. Okadaic acid (OA) mimics ABA responsiveness. Hypocotyls were injected with reporter genes with or without 8-NH_2-cADPR (pipette concentration, 160 μM). After injections, seedlings were incubated in KNOP medium containing okadaic acid (OA, 100 nM, BIOMOL Research Laboratories) for 2 days. K252a (2 μM) and EGTA (EGTA/AM, 3 mM) were added to the incubation medium.

GUS reporter	Coinjected	Externally added			GUS efficiency (%) (*n*)
	8-NH_2-cADPR	OA	EGTA	K252a	
rd29A	−	−	−	−	0 (228)
rd29A	−	+	−	−	10.1 (423)
rd29A	+	+	−	−	0.2 (414)
rd29A	−	+	+	−	0 (250)
rd29A	−	+	−	+	0 (229)
kin2	−	−	−	−	0 (224)
kin2	−	+	−	−	9.5 (476)
kin2	+	+	−	−	0.5 (425)
kin2	−	+	+	−	0 (234)
kin2	−	+	−	+	0 (225)

Table 6. IP_3 may not be involved in the primary response of ABA. Pipette concentrations were as follows: IP_3, 320 μM; cADPR, 160 μM; 8-NH_2-cADPR, 160 μM; and heparin (Sigma), 4.8 mM. Injected seedlings were subsequently incubated in KNOP medium with or without ABA (50 μM) or with or without EGTA (EGTA/AM, 3 mM) for 2 days.

GUS reporter	Coinjected					Externally added		GUS efficiency (%) (*n*)
	IP_3	cADPR	Ca^{2+}	8-NH_2-cADPR	Heparin	ABA	EGTA	
rd29A	−	−	−	−	−	−	−	0 (211)
rd29A	+	−	−	−	−	−	−	8.7 (218)
rd29A	−	−	−	−	+	−	−	0 (212)
rd29A	+	−	−	−	+	−	−	0 (216)
rd29A	−	−	−	−	−	+	−	8.8 (445)
rd29A	−	−	−	−	+	+	−	6.9 (451)
rd29A	+	−	−	−	−	−	+	0 (229)
rd29A	+	+	−	−	−	−	−	9.6 (219)
rd29A	+	−	−	+	−	−	−	8.4 (225)
rd29A	−	+	−	−	+	−	−	10.5 (229)
kin2	−	−	−	−	−	−	−	0 (207)
kin2	+	−	−	−	−	−	−	7.4 (255)
kin2	−	−	−	−	+	−	−	0 (210)
kin2	+	−	−	−	+	−	−	0 (208)
kin2	−	−	−	−	−	+	−	6.7 (435)
kin2	−	−	−	−	+	+	−	6.7 (415)
kin2	+	−	−	−	−	−	+	0 (224)
kin2	+	+	−	−	−	−	−	8.8 (217)
kin2	+	−	−	+	−	−	−	9.1 (230)
kin2	−	+	−	−	+	−	−	8.9 (213)
cab	−	−	+	−	−	−	−	6.1 (214)
cab	+	−	−	−	−	−	−	0 (235)
cab	+	−	+	−	−	−	−	8.6 (220)
cab	−	−	+	−	+	−	−	8.8 (240)

REFERENCES AND NOTES

1. J. Giraudat *et al.*, *Plant Mol. Biol.* **26**, 1557 (1994).
2. S. Kurkela and M. Borg-Franck, *ibid.* **19**, 689 (1992).
3. K. Yamaguchi-Shinozaki and K. Shinozaki, *Mol. Gen. Genet.* **236**, 331 (1993).
4. S. J. Hey, A. Bacon, E. Burnett, S. J. Neill, *Planta* **202**, 85 (1997); G. L. de Bruxelles, W. J. Peacock, E. S. Dennis, R. Dolferus, *Plant Physiol.* **111**, 381 (1996); J. Ingram and D. Bartels, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 377 (1996); A. Kuo, S. Cappelluti, M. Cervantes-Cervantes, M. Rodriguez, D. S. Bush, *Plant Cell*, **8**, 259 (1996); V. Vasil *et al.*, *ibid.* **7**, 1511 (1995); F. Parcy *et al.*, *ibid.* **6**, 1567 (1994); P. M. Chandler and M. Robertson, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45**, 113 (1994).
5. M. F. Thomashow, in Arabidopsis, E. M. Meyerowitz and C. R. Somerville, Eds. (Cold Spring Harbor Laboratory, Plainview, NY, 1994), pp. 807–834.
6. S. Merlot and J. Giraudat, *Plant Physiol.* **114**, 751 (1997); J. Leung, S. Merlot, J. Giraudat, *Plant Cell* **9**, 759 (1997); J. Leung *et al.*, *Science* **264**, 1448 (1994); K. Meyer, M. P. Leube, E. Grill, *ibid.*, p. 1452.
7. Z.-M. Pei, K. Kuchitsu, J. M. Ward, M. Schwarz, J. I. Schroeder, *Plant Cell* **9**, 409 (1997).
8. D. S. Bush, *Annu. Rev. Plant Physiol. Mol. Biol.* **46**, 95 (1995).
9. M. R. McAinsh, C. Brownlee, A. M. Hetherington, *Nature* **343**, 186 (1990); J. I. Schroeder and S. Hagiwara, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9305 (1990).
10. J. Sheen, *Science* **274**, 1900 (1996).
11. M. J. Berridge, *Nature* **361**, 315 (1993).
12. H. C. Lee, T. F. Walseth, G. T. Bratt, R. N. Hayes, D. L. Clapper, *J. Biol. Chem.* **264**, 1608 (1989).
13. H. C. Lee, *Biol. Signals* **5**, 101 (1996); A. Galione, H. C. Lee, W. B. Busa, *Science* **253**, 1143 (1991).
14. E. N. Chini, K. W. Beers, T. P. Dousa, *J. Biol. Chem.*

270, 3216 (1995); H. C. Lee and R. Aarhus, *ibid.*, p. 2152.
15. H. C. Lee, *ibid.* **266**, 2276 (1991); H. C. Lee, in *Ryanodine Receptors*, V. Sorrentino, Ed. (CRC Pharmacology and Toxicology Series, Boca Raton, FL, 1995), pp. 31–50; L. G. Mészáros, J. Bak, A. Chu, *Nature* **364**, 76 (1993).
16. G. S. Prasad *et al*., *Nature Struct. Biol.* **3**, 957 (1996); H. C. Lee and R. Aarhus, *Cell Regul.* **2**, 203 (1991).
17. G. J. Allen, S. R. Muir, D. Sanders, *Science* **268**, 735 (1995).
18. S. R. Muir and D. Sanders, *FEBS Lett.* **395**, 39 (1996).
19. Y. Wu, K. Hiratsuka, G. Neuhaus, N.-H. Chua, *Plant J.* **10**, 1149 (1996); C. Bowler, G. Neuhaus, H. Yamagata, N.-H. Chua, *Cell* **77**, 73 (1994); Erratum: *ibid.* **79**, 743 (1994); G. Neuhaus, C. Bowler, R. Kern, N.-H. Chua, *ibid.* **73**, 937 (1993); Erratum: *ibid.* **79**, 743 (1994).
20. M. J. Terry and R. E. Kendrick, *J. Biol. Chem.* **271**, 21681 (1996).
21. We constructed *kin2-GUS* by ligating 1358 base pairs (bp) (−1358 to +8) of *A. thaliana kin2* flanking 5′ sequence to a GUS reporter gene cassette [R. A. Jefferson *et al*., *EMBO J.* **6**, 3901 (1987)]. For *rd29A-GUS*, 1291 bp of *A. thaliana rd29A* 5′ flanking sequence (−1291 to +8) were used. The *rbcS-3C* polyadenylate addition sequence was placed at the 3′ end in both cases.
22. Y. C. Chang and L. L. Walling, *Plant Physiol.* **97**, 1260 (1991).
23. Y. Wu, R. Foster, N.-H. Chua, unpublished data.
24. T. F. Walseth and H. C. Lee, *Biochim. Biophys. Acta* **1178**, 235 (1993).
25. H. C. Lee and R. Aarhus, *ibid.* **1164**, 68 (1993).
26. P. N. Benfey, L. Ren, N.-H. Chua, *EMBO J.* **8**, 2195 (1989).
27. H. Kim, E. L. Jacobson, M. K. Jacobson, *Science* **261**, 1330 (1993).
28. C. Schmidt, I. Schelle, Y.-J. Liao, J. I. Schroeder, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9535 (1995).
29. C. Bowler, H. Yamagata, G. Neuhaus, N.-H. Chua, *Genes Dev.* **8**, 2188 (1994).
30. Y. Lee *et al*., *Plant Physiol.* **110**, 987 (1996); E. V. Kearns and S. M. Assmann, *ibid.* **102**, 711 (1993).
31. T. Hirayama, C. Ohto, T. Mizoguchi, K. Shinozaki, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3903 (1995).
32. We constructed the *kin2-LUC* transgene by fusing *Arabidopsis kin2* 5′ upstream sequence (−1358 to −1) to a firefly *LUC* cassette that includes 67 bp of the tobacco mosaic virus 5′ untranslated sequence and the pea *rbcS-E9* poly(A) addition sequence.
33. A. J. Millar, S. R. Short, N.-H. Chua, S. A. Kay, *Plant Cell* **4**, 1075 (1992).
34. We measured Ca^{2+} release using a sea urchin microsomal assay. Microsomes were loaded with Ca^{2+}, and Ca^{2+} release from the purified extracts and standard compounds was measured by fluorimetry (*12*). Two peaks of Ca^{2+}-release activity from the last purification step were detected; one was primarily desensitized to cADPR, and the other to $NAADP^+$ (*14*). Neither peak exhibited desensitization to IP_3. Desensitization of channel activity has previously been used as a method to determine the qualitative presence of molecules [T. F. Walseth, R. Aarhus, R. J. Zeleznikar, Jr., H. C. Lee, *Biochim. Biophys. Acta* **1094**, 113 (1991)]. In addition, release activity from the first peak (that is, "cADPR" desensitized) was potentiated by caffeine, an agonist of cADPR activity, and inhibited (50 to 90%) by 8-NH_2-cADPR (0.2 μM in assay), an antagonist. Ca^{2+} release from the peak that was desensitized to cADPR was monitored over the time course and in three separate experiments (Fig. 1B). Ca^{2+} release consistently exhibited two maxima: one at 1 hour and the other at 4 hours after ABA treatment. Northern (RNA) blotting experiments were conducted essentially as described [S. A. Barnes *et al.*, *Plant Cell* **8**, 601 (1996)] with a *kin2* probe specific for the 3′ untranslated region (*2*).
35. W. Masuda *et al.*, *FEBS Lett.* **405**, 104 (1997).
36. M. L. W. Knetsch, M. Wang, B. E. Snaar-Jagalska, S. Heimovaara-Dijkstra, *Plant Cell* **8**, 1061 (1996).
37. We thank S. Cahill for advice on Ca^{2+} fluorimetry, D. Cowburn for use of his facilities, and R. F. Irvine for IP_3. Supported by NIH grant HD17484 (to H.C.L.) and U.S. Department of Energy (DOE) grant DE-FG02-84 ER20142 (to N.-H.C.). J.K. was supported by a Life Science Research Foundation–DOE Biosciences postdoctoral fellowship (5-29329), and E.M. was supported by a Human Frontier Science Program fellowship.

5 September 1997; accepted 14 November 1997

Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts

Angelika E. Schnieke,* Alexander J. Kind, William A. Ritchie, Karen Mycock, Angela R. Scott, Marjorie Ritchie, Ian Wilmut, Alan Colman, Keith H. S. Campbell†

Ovine primary fetal fibroblasts were cotransfected with a neomycin resistance marker gene (*neo*) and a human coagulation factor IX genomic construct designed for expression of the encoded protein in sheep milk. Two cloned transfectants and a population of neomycin (G418)–resistant cells were used as donors for nuclear transfer to enucleated oocytes. Six transgenic lambs were liveborn: Three produced from cloned cells contained factor IX and *neo* transgenes, whereas three produced from the uncloned population contained the marker gene only. Somatic cells can therefore be subjected to genetic manipulation in vitro and produce viable animals by nuclear transfer. Production of transgenic sheep by nuclear transfer requires fewer than half the animals needed for pronuclear microinjection.

Microinjection of DNA into the pronuclei of fertilized oocytes has been the only practical means of producing transgenic livestock since the method was established in 1985 (*1*). However, only a small proportion (~5%) of animals integrate the transgene DNA into their genome (*2*, *3*). In addition, because the timing and site of integration are random, many transgenic lines do not provide sufficiently high levels of transgene expression or germline transmission. The consequent inefficient use of animals and associated high costs are a major drawback to pronuclear microinjection.

In mice, embryonic stem cells provide an alternative to pronuclear microinjection as a means of transferring exogenous DNA to the germline of an animal and allow precise genetic modifications by gene targeting (*4*, *5*). However, despite considerable efforts, embryonic stem cells capable of contributing to the germline of any livestock species have not been isolated (*6*–*11*).

Recently, viable sheep have been produced by transfer of nuclei from a variety of somatic cell types cultured in vitro (*12*–*14*). We now demonstrate that nuclear transfer from stably transfected somatic cells provides a cell-mediated method for producing transgenic livestock.

We have used a transgene designed to express human clotting factor IX (FIX) protein in the milk of sheep. FIX plays an essential role in blood coagulation, and its deficiency results in hemophilia B (*15*). This disease is currently treated with FIX derived mainly from human plasma. Recombinant FIX produced in milk would provide an alternative source at lower cost and free of the potential infectious risks associated with products derived from human blood.

The transgene construct, pMIX1 (*16*), comprises the human FIX gene, containing the entire coding region (*17*), linked to the ovine β-lactoglobulin (BLG) gene promoter, which has been previously shown to provide a high level of transgene expression in ovine mammary glands (*18*). Analysis of pMIX1 expression in transgenic mice showed that seven of seven female founders expressed FIX in their milk (*19*). The level of expression in two animals (125 μg/ml) exceeded that achieved in previous studies (*20*, *21*), indicating that pMIX1 is functional and suitable for introduction into sheep.

Primary strains of ovine cells, termed PDFF (Poll Dorset fetal fibroblast) 1 to 7, were derived from seven day-35 fetuses from the specific pathogen-free flock at PPL Therapeutics (*22*). Sex analysis of each cell strain by the polymerase chain reaction (PCR) (*23*) revealed PDFF5 to be male and

A. E. Schnieke, A. J. Kind, K. Mycock, A. R. Scott, A. Colman, PPL Therapeutics, Roslin, Midlothian, EH25 9PP, Scotland, UK.
W. A. Ritchie, M. Ritchie, I. Wilmut, K. H. S. Campbell, Roslin Institute, Roslin, Midlothian, EH25 9PS, Scotland, UK.

*To whom correspondence should be addressed.
†Present address: PPL Therapeutics, Roslin, Midlothian, EH25 9PP, Scotland, UK.