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Leucine-Rich Repeat Receptor-Like Kinase1 Is a Key Membrane-Bound Regulator of Abscisic Acid Early Signaling in Arabidopsis [W]

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Abscisic acid (ABA) is important in seed maturation, seed dormancy, stomatal closure, and stress response. Many genes that function in ABA signal transduction pathways have been identified. However, most important signaling molecules involved in the perception of the ABA signal or with ABA receptors have not been identified yet. Receptor-like kinase1 (RPK1), a Leu-rich repeat (LRR) receptor kinase in the plasma membrane, is upregulated by ABA in *Arabidopsis thaliana*. Here, we show the phenotypes of T-DNA insertion mutants and *RPK1*-antisense plants. Repression of *RPK1* expression in Arabidopsis decreased sensitivity to ABA during germination, growth, and stomatal closure; microarray and RNA gel analysis showed that many ABA-inducible genes are downregulated in these plants. Furthermore, overexpression of the RPK1 LRR domain alone or fused with the Brassinosteroid-insensitive1 kinase domain in plants resulted in phenotypes indicating ABA sensitivity. RPK1 is involved in the main ABA signaling pathway and in early ABA perception in Arabidopsis.

INTRODUCTION

The phytohormone abscisic acid (ABA) regulates many of the physiologically important events in the adaptation of vegetative tissues to abiotic stresses, such as drought and high salinity, as well as in seed maturation and dormancy and plant development. ABA synthesis and accumulation are promoted by water stress in plant cells, and ABA triggers stomatal closure in guard cells and regulates the expression of many genes whose products may function in dehydration tolerance of vegetative tissues and seeds (McCourt, 2001; Finkelstein et al., 2002). Many genes that play important roles in the ABA signaling pathway have been identified by molecular and genetic approaches. They include genes for transcription factors (*ABI3*, *ABI4*, and *ABI5*), protein phosphatases (*ABI1* and *ABI2*), protein kinases, a farnesyl transferase (*ERA1*), a phospholipase D (*PLD* α), an SnRK2 protein kinase (*SRK2E*/*OST1*), a G protein, and small GTPases (Li et al., 2000; Lemichez et al., 2001; McCourt, 2001; Sang et al., 2001; Finkelstein et al., 2002; Guo et al., 2002; Mustilli et al., 2002; Yoshida et al., 2002; Zheng et al., 2002; Coursol et al., 2003). Recently, proteins involved in posttranscriptional regulation and protein turnover were shown to modify ABA signaling pathways (Lu and Fedoroff, 2000; Hugouvieux et al., 2001; Xiong et al., 2001). However, the important signaling molecules or the principal proteins involved in the ABA perception machinery have not been identified genetically. Few ABA binding proteins have been identified biochemically.

Previous studies thus far suggest that protein phosphorylation/dephosphorylation is an important regulator of ABA signaling. The ABA signal is perceived at both cytoplasmic and extracellular sites (Finkelstein et al., 2002), and signaling events should occur at the plasma membrane for extracellular ABA perception (Yamazaki et al., 2003). ABA microinjected into the cytoplasm of guard cells was able to trigger stomatal closure and inhibit inward K^+ currents (Allan et al., 1994; Schwartz et al., 1994). Extracellularly applied ABA–BSA conjugate, which cannot enter the cell, was able to trigger *RAB18* or *Em* gene expression, stimulate membrane depolarization, and activate ion channels (Schultz and Quatrano, 1997; Jeannette et al., 1999). Furthermore, ABA binding proteins have been detected in the

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microsome fraction of Arabidopsis by the use of ABA–protein conjugates as affinity probes (Pedron et al., 1998). These findings show that the plasma membrane is one of the most important sites for the perception of ABA in Arabidopsis.

Leu-rich repeat receptor-like protein kinases (LRR-RLKs) that contain an extracellular LRR and a Ser/Thr kinase domain and are localized at the plasma membrane are important in the transduction of various plant environmental and developmental signals (Tichtinsky et al., 2003). Many LRR-RLKs are encoded in the plant genome (Shiu and Bleecker, 2001), but most of their functions are unknown, with the exception of several well-characterized proteins, such as CLAVATA1 (Clark et al., 1997), a brassinosteroid (BR) receptor, BR-insensitive1 (BRI1) (Wang et al., 2001a), a systemin receptor (Scheer and Ryan, 2002), a flagellin receptor, FLS2 (Gomez-Gomez et al., 2001), and a phytosulfokine receptor (Matsubayashi et al., 2002). These reports show that the LRR domain alone, or the protein complex, functions as a receptor of both peptide and nonpeptide hormones.

RPK1 is an LRR-RLK isolated from *Arabidopsis thaliana*, and expression of the gene is induced by ABA, dehydration, high salt, and low temperature (Hong et al., 1997). To determine the function of RPK1 in ABA perception in plants, we analyzed the expression pattern of RPK1 at the cellular level to show the localization of RPK1 on the plasma membrane. We also analyzed *RPK1* knockout mutants and antisense-*RPK1* transgenic lines. The loss of function of *RPK1* showed ABA insensitivity in seed germination, plant growth, stomatal closure, and gene expression in Arabidopsis. We present several lines of evidence for an important role of RPK1 in ABA signal transduction. On the basis of our results, we discuss the function of RPK1 in ABA perception and signal transduction.

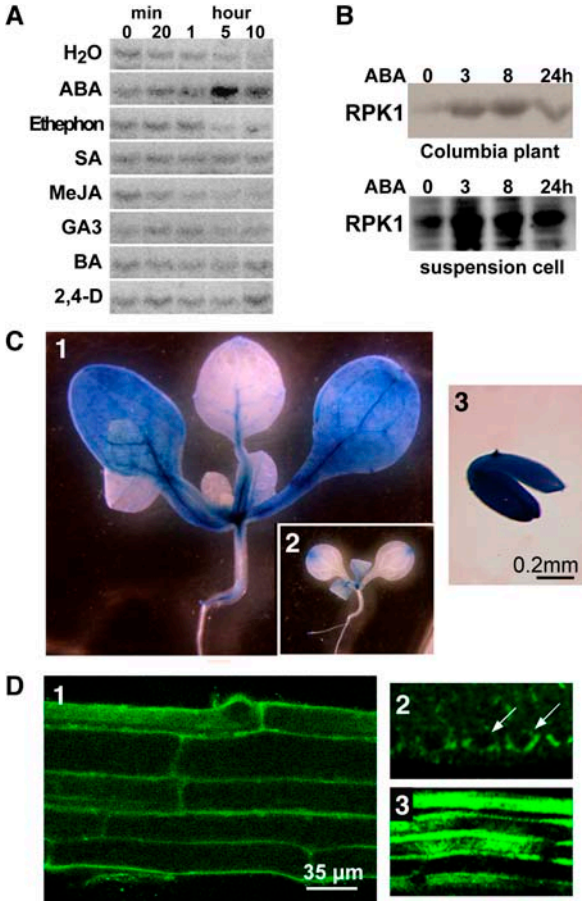

Figure 1. Expression Patterns of the *RPK1* Gene and RPK1 Protein.

(A) ABA-induced expression of *RPK1*. Total RNAs were extracted from wild-type Arabidopsis (ecotype Columbia) treated with distilled water or one of several plant hormones: 1 mM ethephon or 100 μM ABA, SA, MeJA, GA_3, BA, or 2,4-D. The blot was hybridized with a ^{32}P-labeled RPK1 probe.

(B) RPK1 protein expression is upregulated by ABA treatment. Crude proteins were extracted from wild-type plants or suspension cells treated with 100 μM ABA and blotted on phenylmethylsulfonyl fluoride membranes after SDS-PAGE. RPK1 protein was detected with anti-RPK1 antibody.

(C) Tissue-specific expression of the *RPK1* promoter–*GUS* fusion. Activity was higher in the plant treated with 100 μM ABA for 10 h (1) than in the untreated plant (2). High GUS activity was detected in the embryo of the mature seed (3).

(D) Detection of fluorescence of RPK1-GFP under a confocal microscope in the root of a transgenic plant expressing 35S:RPK1-GFP. RPK1-GFP was localized on the cell surface (1). The image was distorted and localized on the cell membrane when cells were plasmolyzed by treatment with 0.8 M mannitol (2). Arrows indicate individual cells. GFP was localized in whole cells of the 35S:GFP transgenic plant (3).

RESULTS

Expression Pattern of RPK1

RPK1 is an LRR-RLK isolated from Arabidopsis. Its expression is upregulated by ABA, dehydration, high salt levels, and low temperature (Hong et al., 1997). To determine the role of RPK1 in ABA signaling, we analyzed the effects of various plant hormone treatments, including ABA, on the expression of *RPK1* (Figure 1A). *RPK1* was expressed at low levels, even under control conditions, and its mRNA accumulation was specifically upregulated by ABA but not by other plant hormones, namely, ethephon, salicylic acid (SA), methyl jasmonate (MeJA), gibberellic acid (GA_3), cytokinin (6-benzylaminopurine [BA]), and auxin (2,4-D). RPK1 protein was detected in untreated plants by protein gel blot analysis using anti-RPK1 polyclonal antibody (Figure 1B). Specific upregulation of RPK1 by ABA was also shown at the protein level in mature Columbia ecotype plants and T87 suspension-cultured cells (Figure 1B). Figure 1C shows the expression pattern of the *RPK1* promoter–β-glucuronidase (*GUS*) fusion gene in transgenic Arabidopsis. GUS activity was increased by ABA treatment (Figure 1C, 1), especially in the cotyledons and vascular tissues; a low level of activity was found in untreated transgenic plants (Figure 1C, 2). Strong activity was also found in embryos of mature seeds (Figure 1C, 3). These expression patterns indicate that expression of the *RPK1* gene is activated specifically by endogenous as well as exogenous ABA.

We tested the cellular localization pattern of the RPK1 protein using an RPK1–green fluorescent protein (GFP) fusion construct,

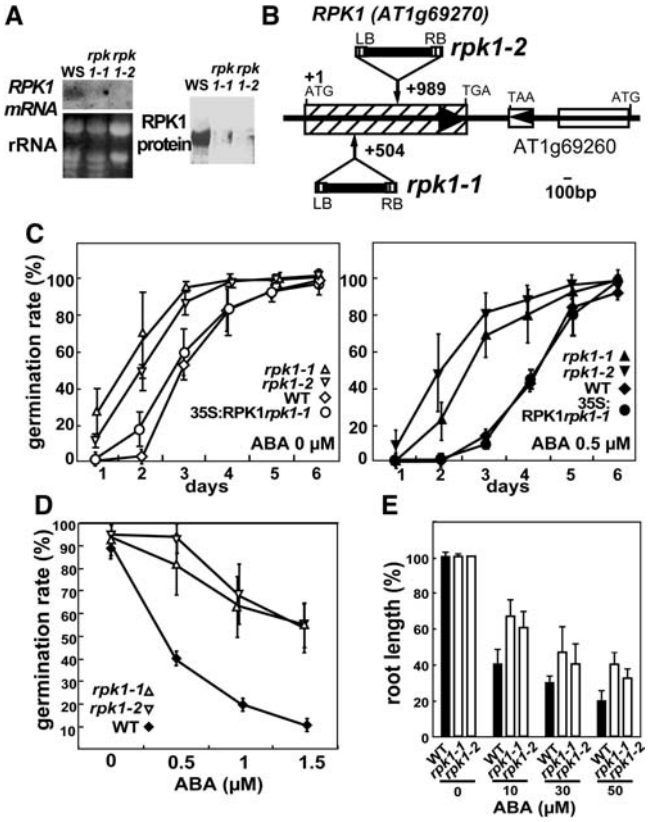

Figure 2. Phenotypes of *RPK1* Knockouts Plants.

(A) Expression levels of the *RPK1* gene and protein in knockout plants. Total RNAs extracted from *RPK1* knockout plants (*rpk1-1* and *rpk1-2*) and wild-type (Ws) plants were blotted and hybridized with a cDNA probe or a strand-specific probe of RPK1. Protein gel blot analysis of *RPK1* knockout (*rpk1-1* and *rpk1-2*) and wild-type (Ws) plants using anti-RPK1 antibody.

(B) T-DNA insertion site of *rpk1-1* and *rpk1-2*. T-DNA was inserted 504 and 989 bp, respectively, downstream of the ATG.

(C) and **(D)** Seed germination rates of *RPK1* knockouts.

(C) *rpk1-1*, *rpk1-2*, 35S:RPK1 *rpk1-1* (complemented), and wild-type plants were grown with (closed symbols) or without (open symbols) 0.5 μM ABA.

(D) Seed germination rates on day 4 at various concentrations of ABA. SD values were calculated from three individual experiments. $n = 30$ seeds per experiment.

(E) Root growth inhibition by ABA. Young seedlings of controls and *rpk1-1* were transferred to ABA-containing medium and incubated for 10 d. The relative root length was measured and is shown as a percentage of the root length grown without ABA. SD values were calculated from three individual experiments. $n = 10$ seedlings per experiment.

which is constitutively expressed in Arabidopsis roots. RPK1-GFP proteins were found on the cell surface under confocal microscopy (Figure 1D, 1). The GFP image was distorted when root cells were plasmolyzed by mannitol treatment (Figure 1D, 2), suggesting that RPK1 is localized on the plasma membrane. These results indicate that the RPK1 protein functions on the plasma membrane.

ABA Insensitivity of *RPK1* Knockouts and Antisense-*RPK1* Transgenic Plants

To analyze the function of RPK1 in ABA signaling in plant cells, we screened T-DNA–tagged Arabidopsis Wassilewskija (Ws) lines from the Arabidopsis Knockout Facility collection at Wisconsin University and obtained two *RPK1* knockout mutants, *rpk1-1* and *rpk1-2*, after PCR screening (Figure 2A). T-DNAs were inserted 504 and 989 bp downstream of the translational start

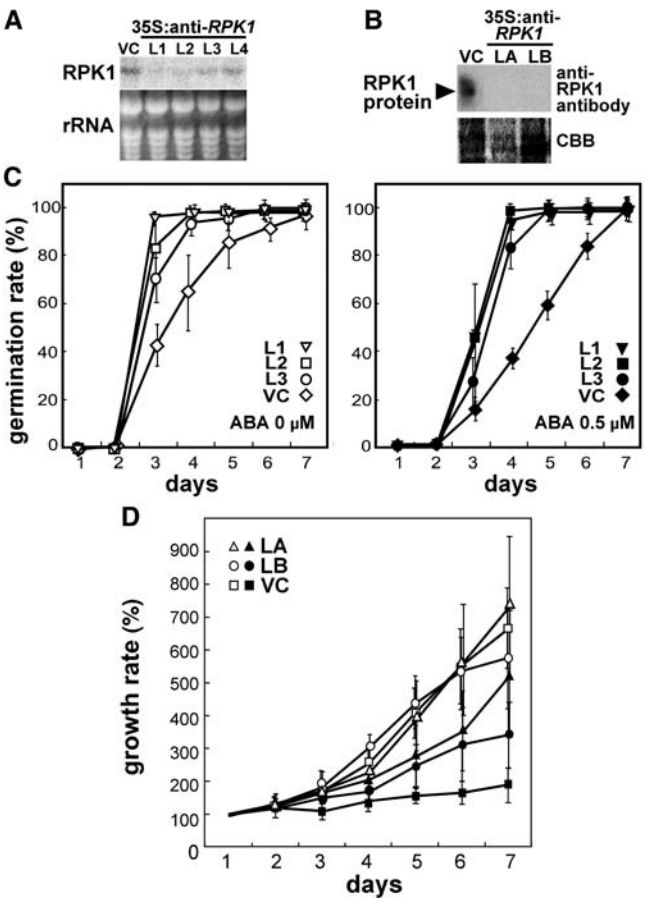

Figure 3. Phenotypes of Antisense-*RPK1* Transgenic Plants.

(A) Expression levels of *RPK1* in antisense transgenic plants. Total RNAs extracted from transgenic plants carrying the 35S vector (vector control [VC]) or from four transgenic lines carrying 35S:antisense-*RPK1* (L1 to L4) (Columbia) were blotted and hybridized with a cDNA probe or a strand-specific probe of RPK1.

(B) Protein gel blot analysis of the VC and antisense-*RPK1* transgenic suspension cells (LA and LB) using anti-RPK1 antibody.

(C) Seed germination of antisense-*RPK1* plants. VC and transgenic plants (L1 to L4) were grown with (closed symbols) or without (open symbols) 0.5 μM ABA. SD values were calculated from three individual experiments. $n = 30$ seeds per experiment.

(D) ABA sensitivity of cell growth rate of antisense-*RPK1* transgenic lines. Suspension cells were cultured in 100 μM ABA medium (open symbols) or in ABA-free medium (closed symbols). LA and LB, antisense-*RPK1* transgenic lines. SD values were calculated from three individual experiments.

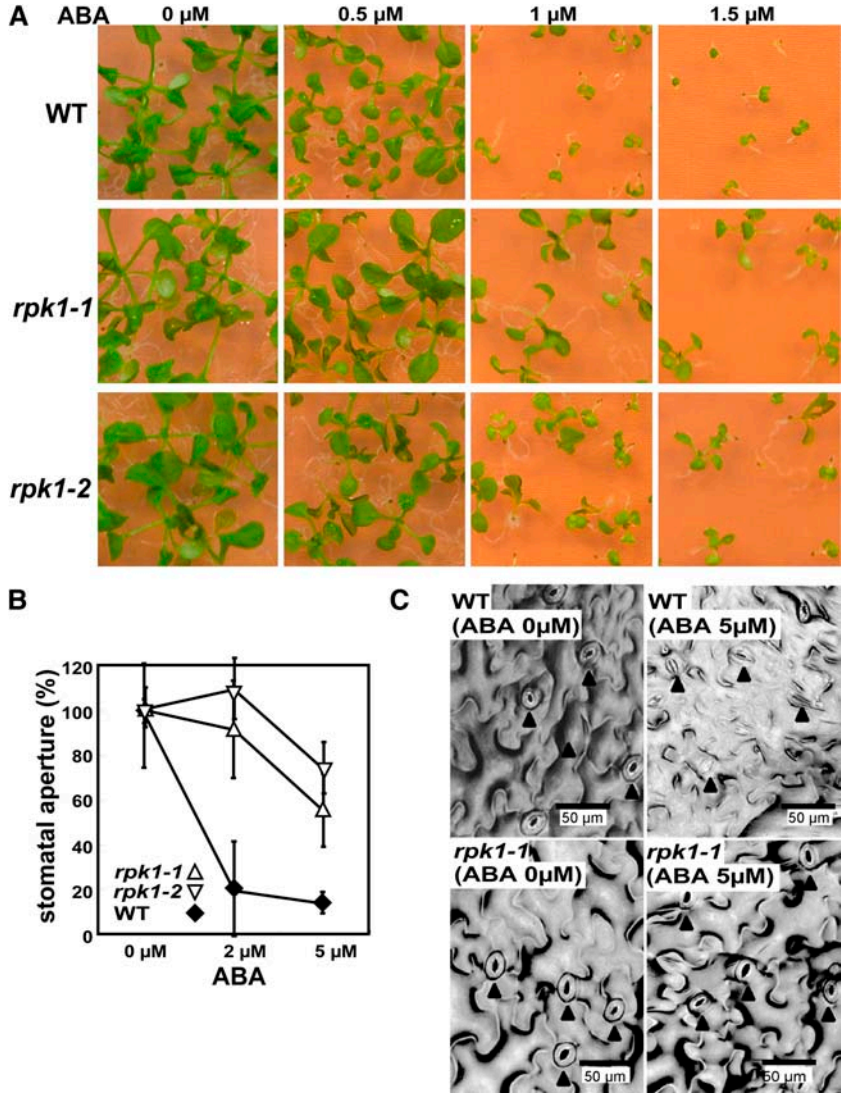

Figure 4. Plant Growth and Stomatal Closure of *RPK1* Knockout Plants in Response to ABA.

(A) Plants were grown for 2 weeks on medium containing ABA. WT, the wild type (Ws). On ABA-containing media, *RPK1* knockout plants grew and greened faster than did the wild type.

(B) Stomatal closure of guard cells as a result of ABA treatment. Leaves of wild-type and *RPK1* knockouts were treated with/without ABA for 2 h, and the stomatal aperture was measured. SD values were calculated from three individual experiments. $n = 10$ guard cells from two leaves of each plant per experiment.

(C) Guard cells of the wild type and *rpk1-1* treated with/without 5 μM ABA for 2 h.

site of the *RPK1* gene (Figure 2B) in *rpk1-1* and *rpk1-2*, respectively, and were confirmed by the sequences of the PCR products. *RPK1* expression was repressed in *rpk1-1* and *rpk1-2* homozygous lines at both transcription and translation levels (Figure 2A). These plants do not show any morphological alterations under normal growth conditions (data not shown). We examined the ABA sensitivities of the *RPK1* knockouts plants. The germination rates of the *RPK1* knockouts were higher than those of wild-type plants on both ABA-containing and ABA-free media (Figure 2C). Furthermore, overexpression of *RPK1* in *rpk1-1* (Figure 2C, 35S:RPK1*rpk1-1*) was able to complement the ABA insensitivity. Figure 2D shows the germination rates of the wild-type and *RPK1* knockout plants on ABA-containing media at various concentrations of ABA. The slightly higher germination rate without ABA might indicate insensitivity to internal ABA. Thus, the *RPK1* knockout plants showed an ABA-insensitive phenotype during germination. The negative effect of ABA on root growth was also reduced in the *RPK1* knockouts (Figure 2E).

We then generated antisense-*RPK1* transgenic plants in which *RPK1* mRNA accumulation was suppressed under the 35S promoter of *Cauliflower mosaic virus*. Antisense-*RPK1* plants also showed higher germination rates with or without ABA than

did controls (Figure 3C). *RPK1* suppression in the antisense-*RPK1* Arabidopsis lines was clearer in lines L1 and L2 than in L3 and vector control (VC) plants (Figure 3A). The antisense-*RPK1* L1 and L2 plants showed a stronger phenotype than the L3 plants (Figure 3C). These results indicate that RPK1 repression resulted in ABA insensitivity in Arabidopsis.

We also generated antisense-*RPK1* transgenic T87 suspension cell lines whose RPK1 protein levels were clearly decreased on protein gel blot analysis (Figure 3B). Suspension culture cells contain specific cell regulation mechanisms that maintain proliferation processes. We analyzed the negative effect of ABA on Arabidopsis cell growth (Figure 3D). In ABA-containing media, VC cells grew poorly and showed arrested cell reproduction; the antisense-*RPK1* cell lines continued growing. These antisense transgenic cells remained green (data not shown), whereas VC cells turned yellow in ABA-containing media. This result indicates that RPK1 has an elemental role in cell proliferation that is controlled by ABA. These ABA-insensitive phenotypes of the RPK1 knockouts and antisense transgenic plants suggest that RPK1 is an important positive regulator of the ABA signal transduction pathway of Arabidopsis.

ABA Insensitivity of the *rpk1* Mutants in Plant Growth and Stomatal Closure of Guard Cells

We further analyzed the ABA insensitivities of *RPK1* knockouts (Figure 4). The growth rate of wild-type Arabidopsis was severely inhibited by exogenously applied ABA. When knockout plants were grown on seed germination media (GM) agar plates containing ABA, growth retardation by ABA was suppressed markedly in the *rpk1* knockout plants compared with the wild-type plants (Figure 4A). ABA promotes stomatal closure in guard cells to avoid water loss during drought stress. When control plants were treated with 5 μM ABA for 2 h, most of the stomata were closed. In the *RPK1* knockout plants under the same condition, the stomata remained open (Figures 4B and 4C). These results indicate that loss of function of RPK1 causes ABA insensitivity also during plant growth and stomatal closure, suggesting that RPK1 is a positive regulator of the ABA response and regulates diverse ABA responses in development and stress responses.

RPK1 Regulates ABA-Inducible Gene Expression

To understand how RPK1 controls the ABA response at the gene expression level, we analyzed downregulated genes in antisense-*RPK1* transgenic plants and T87 cells. The 7000 full-length (RIKEN Arabidopsis full-length) cDNA microarray (Seki et al., 2002a) using mRNA prepared from ABA-treated antisense-*RPK1* plants and T87 cells was performed and results are shown in Supplemental Table 1 online. The expression patterns of several downregulated genes in antisense-*RPK1* transgenic plants were confirmed by RNA gel blot analysis (Figure 5A). Expression levels of other well-known genes regulated by ABA, such as *ADH1* (MacNicol and Jacobsen, 2001), *RD22*, *MYC2*, *AREB1*, and several *ERD* genes (Shinozaki and Yamaguchi-Shinozaki, 2000), also decreased in antisense-*RPK1* plants. RNA gel blot analysis revealed that the expression levels of most ABA-inducible genes were decreased in the *rpk1-1* mutant (Figure 5A).

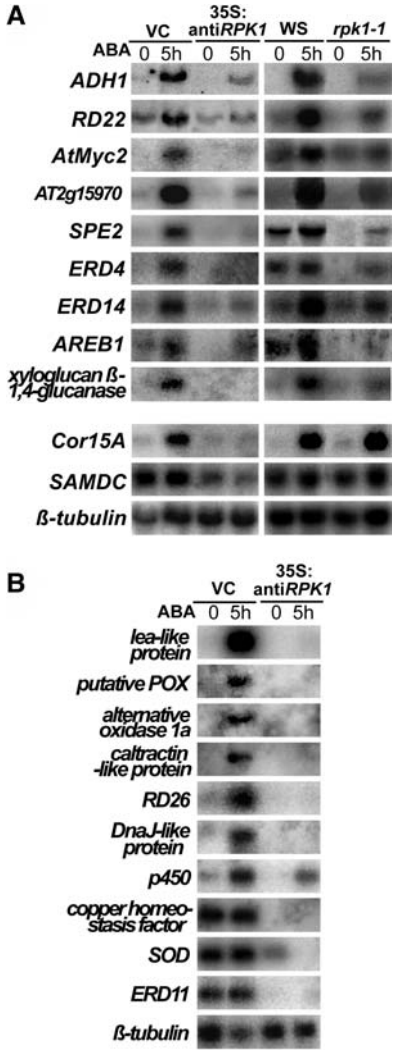

Figure 5. RNA Gel Blot Analysis of RPK1 Target Genes.

Total RNAs were extracted from wild-type (Ws), VC, *rpk1-1*, and antisense-*RPK1* transgenic suspension cells treated with or without 10 μM ABA for 5 h.

(A) RNA gel blot analysis of *rpk1-1* and antisense-*RPK1* transgenic plants. cDNA fragments of *ADH1* (alcohol dehydrogenase), *RD22*, *AtMYC2*, AT2g15970 (similar to cold acclimation protein), *SPE2* (Arg decarboxylase, AT4g34710), *ERD*s, *AREB1*, xyloglucan β1,4-glucanase (AT4g30270), *COR15A*, *SAMDC* (*S*-adenosylmethionine decarboxylase, AT3g02470), and β-tubulin genes were used as probes.

(B) RNA gel blot analysis of antisense-*RPK1* transgenic suspension culture cells. cDNA fragments of LEA-like protein (AT4g02380), putative *POX* (peroxidase, AT2g18950), alternative oxidase 1a (AT3g22370), caltractin-like protein (AT2g46600), *RD26*, DnaJ-like protein (AT4g36040), cytochrome p450 (AT4g19230), copper homeostasis factor (AT3g56240), *SOD* (superoxide dismutase, AT4g25100), *ERD11*, and β-tubulin genes were used as probes.

Table 1. Genes Downregulated in *rpk1-2* Seedlings as Identified in Microarray Analysis

Gene[c]	AGI Code	Experiment 1[a] Ratio (±SD)[d]	Experiment 1[a] P Value 1[e]	Experiment 1[a] P Value 2[e]	Experiment 2[b] Ratio (±SD)[d]	Experiment 2[b] P Value 1[e]	Experiment 2[b] P Value 2[e]	ABA Signal Transduction[f]	ABRE Position[g]
Glycosyl hydrolase family 19 (chitinase)	AT2G43620	0.12 (0.03)	2.9E-29	2.8E-26	0.15 (0.02)	1.1E-26	1.0E-24		
2S storage protein-like	AT5G54740	0.16 (0.01)	1.9E-25	7.2E-24	0.44 (0.02)	2.6E-10	1.8E-08	ABRE, Abi3	−64
Extensin4	AT1G76930	0.17 (0.02)	2.8E-25	7.9E-24	0.27 (0.04)	4.5E-20	9.2E-17		
Peroxidase, putative	AT5G64120	0.19 (0.02)	2.7E-24	2.1E-22	0.10 (0.01)	3.0E-28	2.5E-29	ABRE, induction	−61
Late embryogenesis abundant M17 protein	AT2G41260	0.22 (0.03)	1.7E-22	6.1E-20	0.58 (0.05)	1.6E-06	2.8E-04		
12S cruciferin seed storage protein	AT4G28520	0.24 (0.02)	5.2E-21	1.7E-19	0.44 (0.06)	1.8E-05	8.3E-04	ABRE, Abi3	−108
Unknown protein	AT1G14880	0.25 (0.14)	5.6E-21	6.1E-11				ABRE	−734
Peroxidase, putative	AT5G39580	0.26 (0.04)	1.2E-20	1.5E-17	0.14 (0.03)	3.9E-27	4.7E-25	Induction	−915
Sugar transporter-like protein	AT4G36670	0.26 (0.01)	1.6E-18	1.6E-17	0.34 (0.00)	9.0E-15	1.0E-14	Induction	−402
pEARLI 1-like protein	AT4G12470	0.32 (0.01)	3.9E-11	1.1E-09	0.56 (0.06)	7.0E-08	1.5E-05		
Expressed protein	AT2G25510	0.32 (0.03)	7.7E-17	1.3E-14	0.60 (0.06)	1.9E-01	6.1E-01		−935
Putative RLK	AT2G19190	0.33 (0.03)	1.7E-14	1.9E-12	0.10 (0.01)	1.4E-29	3.3E-28		
FAD-linked oxidoreductase family	AT1G30720	0.34 (0.01)	5.4E-15	1.4E-14	0.19 (0.01)	2.3E-23	6.6E-23	Induction	
MMK2	AT1G59580	0.34 (0.02)	3.3E-13	2.9E-11	0.18 (0.05)	7.6E-25	3.8E-20		−904
Legumin-like protein	AT5G44120	0.36 (0.08)	2.6E-15	1.8E-10	0.8 (0.03)	2.3E-02	9.2E-02		−588
Receptor protein kinase, putative	AT1G51800	0.36 (0.04)	1.2E-13	1.2E-06	0.21 (0.01)	1.7E-22	3.0E-21		
Oleosin	AT4G25140	0.37 (0.09)	3.4E-10	3.4E-10				ABRE, Abi3	−185
Light-repressible receptor protein kinase, putative	AT1G51850	0.41 (0.04)	5.5E-12	6.4E-09	1.28 (0.04)	2. 8E-19	3.7E-16		
Unknown protein	AT2g05580	0.41 (0.12)	9.0E-11	5.4E-13					
FAD-linked oxidoreductase family	AT1G30730	0.42 (0.01)	1.3E-08	7.2E-08	1.21 (0.04)	2. 0E-22	4.8E-19	Induction	
Integral membrane family protein	AT4G15620	0.42 (0.03)	9.9E-09	7.9E-08					−91
UTP-glucose glucosyltransferase-like protein	AT3G50740	0.42 (0.06)	4.5E-13	1.2E-09	0.65 (0.03)	2.6E-05	1.3E-03	Induction	
Expressed protein	AT3G22640	0.43 (0.00)	1.1E-10	1.7E-10	0.72 (0.07)	1.1E-03	1.7E-02		−411
Putative protease inhibitor	AT2G38870	0.43 (0.02)	2.6E-11	3.8E-10	0.21 (0.03)	3.0E-23	4.3E-21		−601
2-Oxoglutarate-dependent dioxygenase family	AT2G36690	0.44 (0.24)	5.4E-13	8.8E-04	0.17 (0.00)	1.6E-24	2.5E-24		−772
Nucleoid DNA binding protein cnd41-like protein	AT5G10760	0.44 (0.05)	1.2E-07	1.4E-07	0.96 (0.05)	9.7E-01	9.9E-01		−238
Expressed protein	AT1G61380	0.45 (0.04)	1.9E-05	2.6E-05	0.41 (0.08)	2.0E-13	4.3E-09	Induction	
Expressed protein	AT1G52200	0.45 (0.09)	1.1E-12	1.0E-07	0.39 (0.06)	2.0E-14	7.9E-11		
Peroxidase, putative	AT5G05340	0.45 (0.12)	9.8E-10	4.4E-05				Induction	−922
Type 2 peroxiredoxin, putative	AT1G65970	0.46 (0.02)	3.0E-10	5.8E-09	0.27 (0.05)	1.6E-20	1.8E-16	ABRE, Abi3	
Glucuronosyl transferase-like protein	AT3G46690	0.47 (0.06)	1.0E-09	8.9E-06	0.30 (0.05)	1.8E-18	3.9E-15		−89
Glutathione transferase, putative	AT1G69930	0.47 (0.12)	2.4E-09	4.8E-06				Induction	−480
Glycosyltransferase family	AT3G46700	0.48 (0.00)	2.2E-08	7.1E-07	0.34 (0.01)	6.4E-12	1.1E-10		

(Continued)

Table 1. (continued).

Gene[c]	AGI Code	Experiment 1[a] Ratio (±SD)[d]	Experiment 1[a] P Value 1[e]	Experiment 1[a] P Value 2[e]	Experiment 2[b] Ratio (±SD)[d]	Experiment 2[b] P Value 1[e]	Experiment 2[b] P Value 2[e]	ABA Signal Transduction[f]	ABRE Position[g]
Similar to Mlo proteins from *Hordeum vulgare*	AT2G39200	0.48 (0.13)	1.1E-11	1.1E-05	0.25 (0.06)	1.0E-21	3.5E-17		
Peroxidase, putative	AT4G36430	0.49 (0.15)	1.4E-10	1.2E-04	0.44 (0.03)	4.4E-11	1.4E-09		
FAD-linked oxidoreductase family	AT1G26420	0.49 (0.02)	1.8E-08	3.7E-08	0.20 (0.08)	2.1E-22	9.9E-15		
1-Aminocyclopropane-1-carboxylate synthase-like protein	AT4G26200	0.49 (0.17)	5.2E-12	1.7E-04	0.33 (0.08)	1.6E-17	7.1E-12		
Protein kinase-like protein	AT4G11890	0.49 (0.00)	5.9E-07	7.1E-07				Induction	−550
AP2 domain protein RAP2.3	AT3G16770	0.49 (0.02)	5.9E-09	3.7E-08	0.59 (0.00)	7.0E-06	2.0E-05		

[a] Experiment 1: downregulated genes in ABA-treated *rpk1* young plants (ABA-treated *rpk1-2*/ABA-treated Ws).
[b] Experiment 2: downregulated genes in untreated *rpk1-2* young plants (untreated *rpk1-2*/untreated Ws).
[c] Genes with ratio (ABA-treated *rpk1-2*/ABA-treated Ws) of <0.5 are listed.
[d] Values represent the means of two replicates. SD, standard deviations of two replicates.
[e] P values of two replicates. P values < 0.0005 were studied.
[f] ABA responses were detected in other experiments (Kroj et al., 2003) or by microarray analysis (K. Maruyama, unpublished data).
[g] ABA response *cis*-sequence; ABREs were detected in the promoter.

Expression of several genes that were downregulated in antisense-*RPK1* plants was not affected in the *rpk1-1* mutant. This different expression may be due to differences in the ecotypes of Arabidopsis and/or to the existence of RPK1 homologs in the Arabidopsis genome, whose expressions might be suppressed in antisense-*RPK1* transgenic plants.

Several downregulated genes in the antisense-*RPK1* transgenic T87 cells were confirmed by RNA gel blot analysis (Figure 5B). Expression levels of these ABA-inducible genes were clearly decreased in the antisense-RPK1 suspension cells. Interestingly, the expression levels of several genes that express constitutively, such as *ERD11* and *SOD*, which are involved in reactive oxygen species (Laloi et al., 2004), were also suppressed.

We then attempted to identify the RPK1-regulated genes in the *rpk1-2* knockout mutant during seedling development using an Agilent Arabidopsis 2 Oligo Microarray (Agilent Technologies, Palo Alto, CA), which covers >21,000 Arabidopsis genes. Total RNA samples were prepared from ABA-treated *rpk1-2* and wild-type plants and used for microarray analysis. We performed two experiments for each RNA sample using different labels, cy3 or cy5. The downregulated genes with ratios of <0.5 are summarized in Table 1 and in Supplemental Tables 2 and 3 online. Among these downregulated genes in *rpk1-2*, we found the genes for 2S storage protein, 12S storage protein, type 2 peroxiredoxin, and oleosin, which are regulated by ABI3 in the embryo (Kroj et al., 2003). Other seed storage proteins, such as pEARLI1 and legumin proteins, were also included in the downregulated genes. Approximately 44% of the downregulated genes were ABA inducible, as confirmed by the Agilent Arabidopsis 2 Oligo Microarray with 3-week-old Arabidopsis plants (K. Maruyama, unpublished data).

Function of the RPK1-LRR Domain in ABA Signaling

We tried to analyze the function of the RPK1-LRR domain in ABA signal transduction, especially ABA perception, in Arabidopsis. First, we constructed transformation vectors for overexpression of a truncated RPK1 protein containing the RPK1-LRR and transmembrane domains (Figure 6A, RPK1-LRR) and a chimeric protein of the RPK1 N-terminal region including the LRR, transmembrane, and juxtamembrane domains fused to the BRI1 kinase domain (BRI-KD) (Figure 6A, RL-BK). Transgenic plants overexpressing the truncated RPK1 showed ABA insensitivity in plant growth (Figure 6B) and seed germination (Figure 6C) on ABA-containing media. These results suggest that overexpression of the LRR domain has a dominant-negative effect on RPK1.

When the chimeric RL-BK protein was overexpressed in Arabidopsis, most of the transgenic plants in each line showed normal growth (Figure 7A, 1 and 2). However, several RL-BK plants showed strong dwarf phenotype (Figure 7A, 4), and a few showed weak dwarf phenotype, whose growth was repressed at the later stage, in each transgenic line (Figure 7A, 3). We named the transgenic lines in the order of the expression levels of RL-BK (L1 to L3; Figure 7B, left). Their dwarf phenotype became more apparent as the expression levels increased (Figures 7B, left, and 7C). We performed quantitative RT-PCR to detect *BRI1* expression levels in the RL-BK transgenic lines (Figure 7B, right). The expression levels of *BRI1* in both the dwarf and normal growth RL-BK overexpressors were slightly lower than that in the control plants and extremely higher than that in the *bri1* mutant, indicating that the dwarf phenotype of the RL-BK overexpressors is not caused by cosuppression of the endogenous *BRI1* gene.

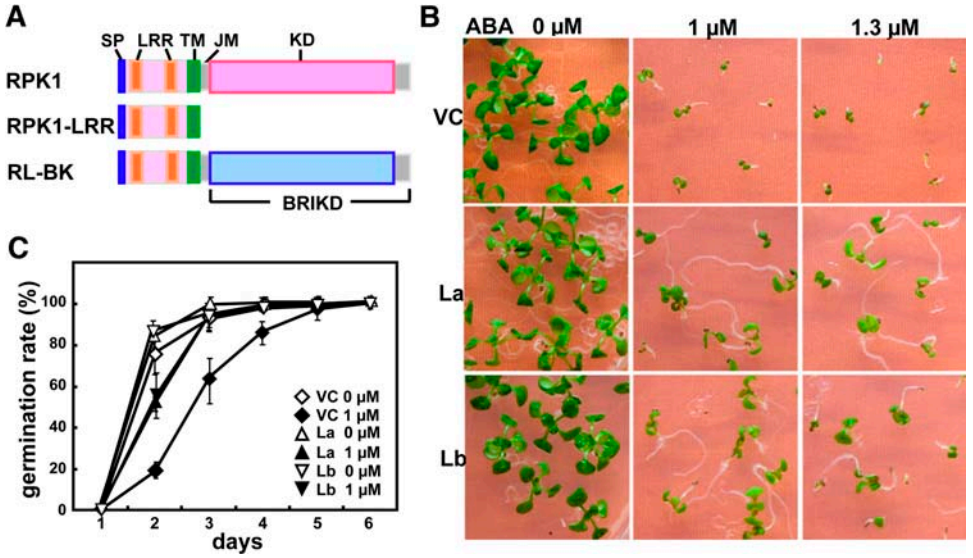

Figure 6. Protein Constructions and Dominant-Negative Phenotypes of Overexpressor of RPK1-LRR.

(A) Constructions of the RPK1 protein, the RPK1-LRR domain, and the BRI1-KD chimeric protein (RL-BK). RPK1-LRR contains a signal peptide (SP), LRR, and transmembrane domain (TM). RL-BK contains the RPK1 N-terminal region, including the juxtamembane domain (JM) and BRI1-KD.
(B) and **(C)** RPK1-LRR ox phenotypes.
(B) Plant growth on media with/without ABA for 2 weeks.
(C) Germination rates of the VC and the RPK1-LRR overexpressing lines (La and Lb) on media with/without ABA (0 or 1 μM).

These results suggest that overexpression of RL-BK has a negative effect on BR signal transduction in transgenic plants. Therefore, we analyzed expression of a BR-related gene in the RL-BK–overexpressing dwarf plants and found that the expression level of the BR-inducible *CYCD3* gene was decreased (Figure 7B, left). To define the effect of ABA application on the growth of the RL-BK overexpressors, we then tested the transgenic plants on ABA-containing media. Interestingly, the numbers of dwarf plants increased as the ABA concentration increased (Figure 7C).

Furthermore, we performed microarray analysis to detect regulated genes in the RL-BK dwarf plants using the Agilent Arabidopsis 2 Oligo Microarray. Total RNA samples prepared from untreated RL-BK dwarf plants of lines L1 and L3 and from the control plants carrying the transformation vector were used for microarray analysis (see Supplemental Tables 4 and 5 online). Table 2 shows the downregulated genes (ratio of <0.35) in the RL-BK dwarf plants. These genes include many that encode auxin-induced proteins and that are known to be BR responsive (Mussig et al., 2002; Goda et al., 2004). The results suggest that the BR signaling pathway is repressed in the RL-BK dwarf transgenic plants. On the other hand, we found that many upregulated genes in the RL-BK overexpressors with a ratio of >3.0 (Table 3) contain ABA-inducible genes and seed storage protein genes. The RL-BK transgenic plants showed delayed seed germination on ABA-containing media, suggesting increased sensitivity to ABA. This result suggested that the RL-BK physiological response to ABA is consistent with the microarray data (Figure 7D).

DISCUSSION

We isolated and analyzed two T-DNA insertion knockout mutants of *RPK1*, which encodes an ABA-inducible LRR-RLK. The *rpk1-1* and *rpk1-2* knockout mutants did not show any morphological alterations in normal growth conditions, but they showed insensitivity to ABA during germination, plant growth, and stomatal closure (Figures 2 and 4). The *rpk1* mutation was complemented by overexpression of the *RPK1* gene, indicating that the mutation allowed recessive ABA insensitivity in Arabidopsis. We generated antisense-*RPK1* transgenic plants and found that these plants were also ABA-insensitive during seed germination. Furthermore, reduction of ABA sensitivity was correlated with a decrease in the levels of *RPK1* mRNA accumulation in transgenic plants (Figure 3). These observations indicate that the ABA-insensitive phenotype of the antisense-*RPK1* transgenic plants is highly affected by the decrease in *RPK1* expression. Thus, RPK1 seems to function as an important positive regulator in ABA signal transduction.

To clarify the RPK1 function during the cell growth of cultured cells, we constructed antisense-RPK1 transgenic suspension culture cells and analyzed their ABA sensitivity during cell proliferation. The control T87 cells showed arrested cell reproduction in the presence of ABA, whereas the antisense-*RPK1* cell lines continued growing in the presence of ABA (Figure 3). ABA was found to prevent DNA replication, keeping the cells in the G1 stage, when it was applied just before the G1/S transition of tobacco (*Nicotiana tabacum*) BY-2 cells (Swiatek et al., 2002). This ABA-mediated inhibition of cell cycle progression may be

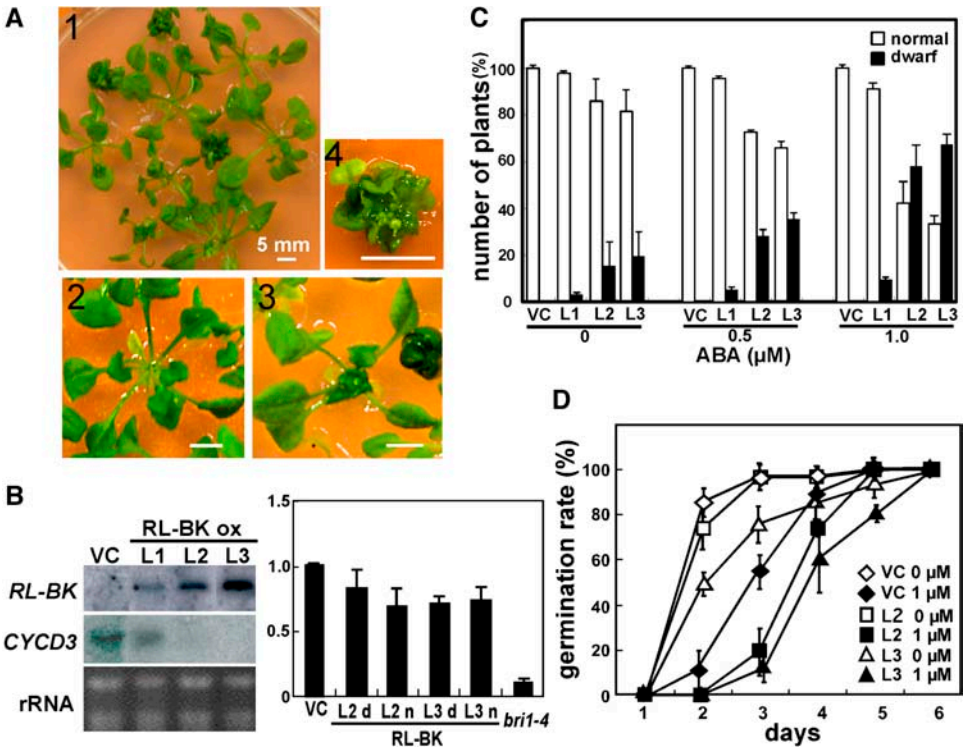

Figure 7. Phenotypes of the RL-BK Overexpressing Transgenic Plants.

(A) RL-BK overexpressor (line L3; 1) shows normal growth plants (2) and the BR-insensitive phenotype plants (3, weak phenotype; 4, strong phenotype). 2 to 4, RL-BK L3 plants in higher magnification.

(B) Left panel: RNA gel blot analysis of the RL-BK overexpressing (RL-BK ox) lines using a ^{32}P-labeled RL-BK chimeric gene or a BR-responsive *cyclinD3* gene as probes. Right panel: *BRI1* expression levels in the RL-BK transgenic lines, L2 and L3, and *bri1-4*. Total RNA from dwarf (d) and normal plants (n) of the same RL-BK line were isolated separately and used for quantitative RT-PCR. Experiments were repeated three times. Data represent means ± SD.

(C) Proportions of dwarf plants in the RL-BK overexpressing lines (L1 to L3), which were named in order of the strength of expression levels of RL-BK.

(D) Germination rates of the VC and the RL-BK overexpressing lines (L2 and L3) on media with/without ABA (0 or 1 μM).

repressed in the antisense-RPK1 transgenic culture cells, suggesting the key role of RPK1 in ABA signaling in cultured cells.

The microarray analysis of the *RPK1* mutant and antisense-RPK1 transgenic plants showed reduced expression levels of various ABA-inducible genes (Figure 5, Table 1; see Supplemental Table 1 online). Microarray analyses using ABA-treated whole plants (Seki et al., 2002c), the *abi1-1* mutant (Hoth et al., 2002), and guard cells (Leonhardt et al., 2004) have revealed a diversity of ABA-regulated genes. We found many such ABA-regulated genes in the results of our microarray analysis, indicating that RPK1 positively regulates many ABA-inducible genes that are controlled through various ABA-dependent signal transduction pathways. Previous genetic studies have shown that various negative regulators, such as ABI1, ABI2, ERA1, ROP10, AtRac1, and ABH1, regulate the important steps of the ABA signal transduction pathways (Finkelstein et al., 2002; Leonhardt et al., 2004). Several positive regulators in ABA signaling, such as SRK2E/OST1 and RCN1, have been identified (Kwak et al., 2002; Mustilli et al., 2002; Yoshida et al., 2002). These negative regulators are known to govern the ABA signaling cascades in normal growth conditions. On the other hand, when ABA is synthesized under stress conditions, positive regulators are activated and transmit the signal to downstream pathways (Finkelstein et al., 2002; Leonhardt et al., 2004). These findings and our results suggest that RPK1 functions at early steps of the ABA signal transduction pathway, so that it can regulate many ABA-responsive genes that are controlled through various ABA-dependent signal transduction pathways. Interestingly, the downregulated genes in the microarray analysis of the *RPK1* mutants and antisense transgenic plants also included several genes that were not inducible by ABA application, such as reactive oxygen species–related genes (Figure 5, Table 1). Crosstalk between ABA signaling and other signaling seems to exist and affect gene expression patterns in the RPK1 signal transduction pathway. The genes downregulated in the antisense-*RPK1* suspension cells were different from those downregulated in the antisense-*RPK1* transgenic plants; this difference may be due to cell specificity in plants and suspension cells.

Previous studies have shown that signaling events occur at the plasma membrane in extracellular ABA perception (Yamazaki et al., 2003). We showed the localization of RPK1-GFP at the plasma membrane in Arabidopsis cells (Figure 1). From the

Table 2. Genes Downregulated in RL-BK Overexpressing Plants as Identified by Microarray Analysis

		Experiment 1 (Transgenic Line L1)[a]			Experiment 2 (Transgenic Line L3)[b]		
Gene[c]	AGI Code	Ratio (±SD)[d]	P Value 1[e]	P Value 2[e]	Ratio (±SD)[d]	P Value 1[e]	P Value 2[e]
Unknown protein	AT5G64770	0.12 (0.04)	4.7E-26	1.3E-23	0.19 (0.00)	1.2E-21	1.5E-21
Auxin-induced protein-like	AT5G18020	0.16 (0.02)	2.5E-22	2.7E-21	0.22 (0.04)	1.3E-20	5.3E-17
Thionin, putative	AT1G66100	0.17 (0.00)	1.5E-21	1.2E-19	0.24 (0.00)	8.0E-18	3.7E-17
GAST1-like protein	AT1G74670	0.17 (0.02)	3.0E-25	2.5E-23	0.18 (0.02)	2.0E-24	5.1E-23
Fructose-bisphosphate aldolase-like protein	AT4G26530	0.18 (0.04)	2.5E-25	5.8E-22	0.23 (0.01)	1.3E-20	4.5E-20
Expansin, putative	AT2G40610	0.19 (0.03)	2.7E-24	1.1E-21	0.16 (0.01)	3.7E-25	3.3E-24
Auxin-induced protein, putative	AT1G29500	0.21 (0.07)	1.3E-23	1.6E-17	0.27 (0.05)	1.2E-18	1.4E-15
Expressed protein	AT4G18970	0.25 (0.03)	4.9E-21	9.9E-19	0.25 (0.02)	1.3E-20	5.0E-19
Early auxin-induced protein, IAA19	AT3G15540	0.26 (0.12)	1.3E-17	1.1E-11	0.41 (0.03)	7.0E-11	8.5E-09
Auxin-induced protein-like	AT5G18030	0.29 (0.03)	1.1E-17	1.7E-15	0.29 (0.04)	4.7E-18	1.4E-14
Expressed protein	AT5G52900	0.31 (0.05)	5.0E-18	1.4E-14	0.39 (0.03)	2.7E-13	1.3E-11
Expressed protein	AT1G32080	0.31 (0.00)	4.2E-16	8.3E-16	0.37 (0.00)	2.0E-13	2.7E-13
Auxin-induced protein (SAUR), putative	AT5G18010	0.32 (0.04)	1.1E-15	5.5E-13	0.38 (0.02)	1.9E-12	3.4E-11
myb-related transcription factor mixta, putative	AT1G18710	0.32 (0.02)	4.6E-15	5.9E-13	0.44 (0.08)	3.1E-11	1.6E-07
Putative auxin-induced protein AUX2-11	AT1G04240	0.33 (0.03)	3.8E-16	9.4E-14	0.42 (0.02)	1.8E-11	2.4E-10
Putative protein	AT5G11420	0.33 (0.05)	5.5E-17	9.4E-14	0.34 (0.04)	3.7E-16	2.1E-13
Auxin-induced protein-like	AT5G18060	0.33 (0.06)	3.0E-16	2.2E-13	0.37 (0.03)	8.2E-15	3.1E-14
Auxin-induced protein-like	AT5G18080	0.33 (0.15)	1.1E-19	2.2E-09	0.38 (0.10)	9.7E-15	3.0E-09
Methyladenine glycosylase family protein	AT5G44680	0.34 (0.16)	5.8E-17	4.5E-13	0.37 (0.03)	3.1E-14	2.7E-12

[a] Experiment 1: downregulated genes in untreated RL-BK line L1 (L1/control).
[b] Experiment 2: downregulated genes in untreated RL-BK line L3 (L3/control).
[c] Genes with ratio (untreated RL-BK transgenic plants/untreated VC plants) of <0.35 are listed.
[d] Values represent the means of two replicates. SD, standard deviations of two replicates.
[e] P values of two replicates. P values < 0.005 were studied.

localization of the RPK1 protein and global effects in ABA responses in loss-of-function mutants of RPK1, we can speculate that RPK1 binds ABA directly or indirectly and functions as a positive factor in ABA signaling, which governs many ABA-related cellular and molecular events. Another possibility is that RPK1 functions in the perception of unknown secondary messengers or peptide hormones involved in early ABA signaling.

RPK1 gene expression was specifically upregulated by ABA treatment, and phenotypes of *RPK1* loss-of-function mutants were observed in the presence of exogenous ABA, suggesting that RPK1 functions effectively at specific growth stages at which ABA operates, such as during seed dormancy and under water stress. Recently, ABA hypersensitivity of a T-DNA insertion mutant of a putative G protein–coupled receptor gene, *GCR1*, was reported (Chen et al., 2004; Pandey and Assmann, 2004). GCR1 was considered as a possible negative regulator of GPA1-mediated ABA responses (Wang et al., 2001b) via GA and BR signal transduction (Chen et al., 2004). The diverse responsiveness of heterotrimeric G proteins (Jones, 2002) and GCR1 to several phytohormones in Arabidopsis suggested that the GCR1-related ABA signaling system may not directly associate with the RPK1-mediated ABA signaling cascades under ABA-accumulated conditions in Arabidopsis. Possible interaction between GCR1 and sphingosine-1-phosphate or ABA receptors has been suggested (Pandey and Assmann, 2004). These results suggest that several kinds of ABA receptor complex might exist and be activated in various cell- or stage-specific responses to ABA in Arabidopsis cells.

Plant RLK proteins contain a ligand binding extracellular domain, a single membrane-spanning domain, and a cytoplasmic Ser/Thr kinase domain, and many classes of the genes are encoded in the plant genome (Shiu and Bleecker, 2001). More than 600 RLKs in the Arabidopsis genome contain various types of intracellular domains. The RLK extracellular domains are thought to function in their specific ligand bindings. The functional roles of several RLKs have been shown; they can perceive extracellular ligands, including hormones, and regulate downstream cascades and cellular events (Morris and Walker, 2003; Tichtinsky et al., 2003). The RPK1 protein contains two LRR amino acid sequences in its extracellular domain (Hong et al., 1997). The *RPK1* gene family in Arabidopsis genome contains

Table 3. Genes Upregulated in RL-BK Overexpressing Plants as Identified by Microarray Analysis

		Experiment 1 (Transgenic Line L1)[a]			Experiment 2 (Transgenic Line L3)[b]			
Gene[c]	AGI Code	Ratio (±SD)[d]	P Value 1[e]	P Value 2[e]	Ratio (±SD)[d]	P Value 1[e]	P Value 2[e]	ABA Response[f]
BR-6-oxidase (BR6ox2)	AT3G30180	9.0 (0.04)	6.3E-28	7.5E-28	8.0 (1.13)	7.6E-28	4.3E-26	BR feedback[g]
Xyloglucan endotransglycosylase (XTR7)	AT4G14130	6.4 (0.37)	1.0E-25	6.8E-25	5.1 (0.03)	7.5E-23	9.2E-23	Induction
Steroyl acyl carrier protein desaturase	AT1G43800	5.8 (2.34)	7.1E-22	4.1E-17	2.6 (0.20)	7.3E-11	2.9E-10	
Oleosin	AT4G251	4.7 (0.45)	1.1E-22	6.3E-21	4.2 (0.15)	2.0E-20	1.2E-19	Response
Expressed protein	AT1G75750	4.7 (1.45)	3.2E-24	1.9E-18	3.7 (1.00)	5.5E-21	4.5E-15	
Legumin-like protein	AT5G44120	4.3 (0.09)	5.3E-21	1.3E-20	3.4 (0.07)	2.1E-17	6.1E-17	Response
12S cruciferin seed storage protein	AT4G28520	4.2 (0.19)	7.1E-21	5.8E-20	3.7 (0.18)	5.0E-19	5.1E-18	Response
Expressed protein	AT2G19800	4.8 (0.89)	1.3E-20	9.3E-16	2.8 (0.51)	1.3E-15	3.3E-10	Guard cell[h]
2S storage protein-like	AT5G54740	3.8 (0.08)	4.6E-19	1.3E-18	3.2 (0.00)	1.9E-16	1.9E-16	Response
Unknown protein	AT2G05580	3.7 (1.05)	6.7E-20	7.5E-14	2.4 (0.07)	9.8E-11	3.2E-09	
Jasmonate-inducible protein, putative	AT1G52100	3.7 (0.48)	9.9E-20	6.6E-17	3.1 (0.17)	7.6E-16	1.3E-14	
Expressed protein	AT3G02480	3.7 (0.03)	1.0E-18	1.5E-18	2.2 (0.09)	1.7E-10	1.7E-09	Induction
Oleosin	AT5G40420	3.4 (0.34)	5.3E-18	4.7E-16	2.8 (0.54)	9.2E-16	3.1E-11	Response
Embryo-specific protein 1 (ATS1)	AT4G26740	3.4 (0.88)	2.4E-19	8.4E-14	2.8 (0.35)	4.5E-15	7.7E-12	
Putative protein	AT3G55240	3.4 (0.35)	2.9E-18	5.9E-16	2.3 (0.07)	4.3E-11	2.5E-10	
Expressed protein	AT2G39030	3.4 (0.52)	3.3E-18	2.5E-14	2.6 (0.27)	2.4E-13	1.2E-10	Induction
Embryo-specific protein 1 (ATS1)	AT5G55240	3.3 (0.03)	4.0E-16	7.3E-16	2.9 (0.13)	6.6E-14	4.7E-13	
Plant defensin protein, putative (PDF1.4)	AT1G19610	3.3 (0.31)	1.0E-17	1.4E-15	2.2 (0.29)	2.3E-11	3.4E-08	
Glycosyl hydrolase family 18	AT4G19810	3.2 (0.65)	6.5E-18	1.9E-13	2.8 (0.63)	4.4E-16	1.0E-10	Induction
Glycosyl hydrolase family 1	AT3G21370	3.2 (0.13)	1.1E-16	8.6E-16	3.0 (0.15)	7.0E-16	9.7E-15	
Late embryogenesis abundant protein LEA-like	AT5G06760	3.2 (1.12)	4.3E-19	3.0E-11	1.9 (0.47)	2.8E-09	4.7E-04	Induction
Similar to chloroplast nucleoid DNA binding protein-like	AT4G16563	3.2 (0.69)	2.3E-17	2.2E-12	3.1 (0.35)	1.6E-15	2.6E-13	
Putative seed storage protein (vicilin-like)	AT2G28490	3.1 (0.35)	3.2E-17	1.2E-14	2.6 (0.11)	7.1E-15	6.4E-11	
Expressed protein	AT1G72450	3.1 (0.20)	1.6E-16	5.3E-15	3.2 (0.13)	5.0E-17	3.7E-15	
Hypothetical protein	AT1G17710	3.1 (0.37)	5.1E-17	2.5E-14	2.4 (0.29)	4.3E-12	5.2E-11	
Putative protein	AT5G26260	3.1 (0.21)	1.9E-16	6.6E-15	2.1 (0.54)	1.0E-09	2.8E-08	
Hevein-like protein precursor (PR-4)	AT3G04720	3.1 (0.36)	6.4E-17	3.8E-14	2.2 (0.30)	9.5E-12	1.4E-08	

[a] Experiment 1: upregulated genes in untreated RL-BK line L1 (L1/control).
[b] Experiment 2: upregulated genes in untreated RL-BK line L3 (L3/control).
[c] Genes with ratio (untreated RL-BK transgenic plants/untreated VC plants) of >3 are listed.
[d] Values represent the means of two replicates. SD, standard deviations of two replicates.
[e] P values of two replicates. P values < 0.005 were studied.
[f] ABA responses are described in Table 1.
[g] See Shimada et al. (2003).
[h] See Leonhardt et al. (2004).

two related genes (*RPK1* and AT3g02130) that show the high sequence similarity in the kinase domain but not in the LRR domain (Shiu and Bleecker, 2001). To characterize the function of the RPK1 LRR domain, we generated transgenic plants overexpressing a truncated RPK1 protein containing the LRR domain and the membrane-spanning domain (RPK1-LRR, Figure 6). The transgenic plants with RPK1-LRR showed ABA insensitivity in seed germination and growth (Figure 6). The results suggest that the RPK1-LRR domain plays an important role in the perception of an ABA signal.

To further analyze the function of RPK1-LRR, we generated transgenic plants overexpressing the chimeric protein RL-BK, a fusion of RPK1-LRR and BRI1-KD (Figure 7). The function of the BRI1-LRR domain has been confirmed in the characterization of a chimeric protein formed from BRI1-LRR and the kinase domain of a rice (*Oryza sativa*) disease resistance LRR-RLK, Xa21 (He et al., 2000). The chimeric receptor initiated defense responses in rice cells upon treatment with BR, showing that BRI1-LRR is essential for BR perception (He et al., 2000). Further analysis has detected BR binding activity of the BRI1-LRR domain (Wang et al., 2001a). From these results, we first expected that RL-BK would initiate the BR signal transduction pathway upon treatment with ABA. However, the seed germination of these plants showed increased sensitivity to ABA (Figure 7D). Overexpression of RL-BK also caused a dominant-negative effect on BR signal transduction and strong dwarf phenotypes. Many BR-responsive genes were downregulated in the transgenic plants (Table 2). It is known that the repression of BR signal transduction causes ABA hypersensitivity (Steber and McCourt, 2001). However, the dominant-negative effect was controlled in an ABA-dependent manner in the transgenic plants overexpressing RL-BK (Figure 7). Furthermore, the microarray analysis showed that many ABA-responsive genes included in the RPK1 signal transduction pathway were upregulated in the transgenic plants overexpressing RL-BK (Table 3). These results suggest that the specific RPK1-mediated ABA signal transduction pathway was activated in the RL-BK transgenic plants. RL-BK overexpression in the transgenic plants showed modulated ABA and BR responses through the ABA signal perception of RPK1-LRR.

It has been shown that a BR receptor LRR-RLK, BRI1, requires heterodimerization with another LRR-RLK, BAK1, for its receptor function (Li et al., 2002; Nam and Li, 2002; Li, 2003). Another LRR-RLK, CLV1, which regulates meristem development, can form a heterodimer with an LRR-containing protein, CLV2 (Jeong et al., 1999). This suggests that plant RLKs form dimers or tetramers to become active and transduce the ligand binding signal to the downstream cascades. Isolation and characterization of the RPK1-specific partner in its ABA signaling complex will elucidate the mechanisms of ABA signal perception of RPK1. We are now performing yeast two-hybrid screening to identify RPK1-interacting proteins.

In conclusion, we showed that an RLK, RPK1, functions in ABA signal transduction pathways such as seed germination, stomatal closure, root growth, cell proliferation, and gene expression. RPK1 has a transmembrane domain and is localized on the plasma membrane. The LRR domain of RPK1 may function in early ABA signal perception and constitute an ABA receptor complex. We are now analyzing the ABA binding ability of the RPK1 protein to provide us with more information on ABA perception.

METHODS

Plant Materials and Isolation of *rpk1* Knockouts

Arabidopsis thaliana (Columbia [Col] ecotype) was grown on GM agar plates under continuous illumination of ~2500 lux at 22°C. Transgenic plants and control plants were grown on GM agar plates containing kanamycin (20 mg/L). Arabidopsis (Col) T87 cells were maintained in JPL medium (Takahashi et al., 2001) by gentle agitation under continuous illumination of 2500 lux at 22°C. The *rpk1* T-DNA knockout of Arabidopsis was isolated by screening the University of Wisconsin Biotechnology Center Arabidopsis Knockout Facility collection of 72,960 BASTA-resistant T-DNA mutant lines (ecotype Ws). A gene-specific primer, 5′-GATCTGCACAAGTACAACTGTGATTCCTT-3′, located 1029 bp upstream of the ATG codon of RPK1, and a T-DNA specific primer, 5′-CATTTTATAATAACGCTGCGGACATCTAC-3′, were used for screening. The position of the T-DNA insert was determined by sequencing of PCR products carrying the T-DNA and genome junctions. After establishing the presence of homozygotes, we confirmed by a backcross that *rpk1-1* was recessive and had a single insertion of T-DNA.

RNA and Protein Gel Blot Analyses

Total RNA was prepared by the method described previously (Osakabe et al., 2002) from Arabidopsis plants grown on GM agar plates for 2 weeks or T87 cells grown in JPL medium for 4 d and treated with or without 10 or 100 μM ABA. Total RNAs were also extracted from wild-type plants treated with distilled water or the plant hormones ABA (Sigma-Aldrich, St. Louis, MO), ethephon (2-chloroethyl-phosphonic acid; Sigma-Aldrich), MeJA (Wako, Osaka, Japan), 2,4-D (Sigma-Aldrich), BA (Sigma-Aldrich), GA_3 (Sigma-Aldrich), and SA (Sigma-Aldrich). Thirty micrograms of total RNA was fractionated in a 1% agarose gel containing formaldehyde and blotted onto a nylon membrane. The membrane was then hybridized with a ^{32}P-labeled *RPK1* cDNA fragment or full-length cDNA (Seki et al., 2002b) as a probe. Hybridization was performed in 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 25 mM sodium phosphate buffer, pH 6.5, 10× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), and 250 μg/mL denatured salmon sperm DNA at 42°C for overnight. The membranes were then washed three times with 0.1× SSC and 0.1% SDS at 60°C for 15 min and subjected to autoradiography.

Crude proteins were extracted from wild-type or knockout plants grown on GM agar plates for 3 weeks or from T87 transgenic and wild-type cells grown for 4 d in JPL medium treated with/without 100 μM ABA, then separated by SDS-PAGE and blotted onto a phenylmethylsulfonyl fluoride membrane. RPK1 protein was detected with anti-RPK1 peptide antibody with the ECL Plus protein gel blotting detection aystem (Amersham Biosciences, Piscataway, NJ).

ABA Sensitivity

ABA-sensitive assays were performed as follows. Seeds of antisense-*RPK1*, *rpk1-1*, *RPK1-LRR* ox, and 35S:RPK1*rpk1-1* that carried the 35S:RPK1 full-length cDNA in *rpk1-1* and seeds of control plants were sterilized and plated on GM medium containing 1% sucrose and 0 or 0.5 μM ABA. Seeds were grown under continuous light at 22°C. To test the ABA sensitivity of root growth, seeds of the mutants and wild-type plants were grown on GM medium for 6 d. After germination, the seedlings were transferred to fresh GM medium containing various concentrations of ABA. After 10 d of culture, the root lengths were measured. For stomatal movement assay, detached rosette leaves of 4-week-old *rpk1* knockouts and control plants grown on soil were incubated for 2 h in 0, 2, and 5 μM ABA solution containing 20 mM KCl, 1 mM $CaCl_2$, and 5 mM Mes-KOH, pH 6.15 (Pei et al., 1998). Guard cells were photographed under a color laser three-dimensional profile microscope (Keyence, Osaka, Japan).

Construction of Transgenic Plants

The sense or antisense full-length cDNA fragment of *RPK1* was cloned into the pBE2113 vector (Mitsuhara et al., 1996), which contains the

modified 35S promoter of *Cauliflower mosaic virus* and the Ω sequence of the *Tobacco mosaic virus*. For construction of the RPK1-GFP fusion, we used the subcloned *RPK1* cDNA as a template and 5′-TGTGAAGAAAAA-GATCTAGAATGAAACTTC-3′ as a vector primer to amplify the 1617-bp fragment (from ATG to an internal *Xba*I site). The fragment was then digested with *Xba*I and cloned into the *Xba*I site of the pBI121-sGFP (S65T) vector. To amplify the 815-bp promoter region of the *RPK1* gene, we used 5′-GAGGAATACACGTCAAGCTTAAATAAAAAT-3′ and 5′-GGA-TCCCAGAAGTTTCATTTTTCTTCTTTT-3′, and the amplified fragment was cloned into the pBI101 vector. The constructs were then introduced into *Agrobacterium tumefaciens* C58. Six-week-old wild-type Arabidopsis (Col) or *rpk1-1* plants were transformed by the vacuum infiltration method. Fifteen antibiotic-resistant transgenic lines carrying each construct were further analyzed. Arabidopsis T87 suspension cells cultured for 3 d were coincubated with Agrobacterium for 2 d to transform the constructions and then selected out on a callus induction medium plate containing kanamycin. The selected calluses were then incubated in JPL medium.

Reporter Gene Assays

The tissue-specific activity of GUS was detected by the overnight incubation of 2-week-old transgenic plants carrying the RPK1 promoter-GUS construct, in a GUS staining buffer (1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 0.1 M sodium phosphate buffer, pH 7, and 8 mM β-mercaptoethanol) at 37°C. Staining was stopped by transferring samples into 70% ethanol, and the chlorophyll was then destained. For GFP analysis, roots from 2-week-old transgenic plants containing 35S:RPK1-GFP or 35S:GFP were examined under a confocal microscope.

Agilent Oligo Microarray Analysis

Five-day-old seedlings of the *rpk1-2* and wild-type Ws plants were treated with 10 μM ABA for 5 h. Total RNAs were prepared from the ABA-treated *rpk1-2*, wild-type Ws plants, the 35S:RL-BK transgenic plants, and the control plants carrying the vector grown on GM agar plates for 3 weeks with Trizol reagent (Gibco BRL, Cleveland, OH) and used for the preparation of Cy5-labeled and Cy3-labeled cDNA probes. Samples were subjected to microarray experiments using the Agilent Arabidopsis 2 Oligo Microarray (Agilent Technologies). All microarray experiments, including the data analysis, were performed according to the supplier's manual (http://www.chem.agilent.com/scripts/generic.asp?lpage=11617andindcol=Nandprodcol=Y). The reproducibility of microarray analysis was assessed by a dye swap in each experiment. Genes showing a signal value <600 in both Cy3 and Cy5 channels of the control plants were not considered for the analysis. Feature extraction and image analysis software (version A.6.1.1; Agilent Technologies) was used to locate and delineate every spot in the array and to integrate each spot's intensity, filtering, and normalization using the Lowess method. Gene clustering analysis was performed with Genespring 6.1 (Silicon Genetics, San Carlos, CA).

RIKEN Arabidopsis Full-Length cDNA Microarray Analysis

Trizol reagent was used to extract total RNA for microarray analysis from VC or antisense-*RPK1* transgenic plants or T87 cells treated with 10 μM ABA for 5 h. mRNA was prepared with an MACS mRNA isolation kit (Miltenyi Biotec, Bergisch, Gladbach, Germany). mRNAs from the control and antisense plants (antisense T87 cells) were used for preparation of Cy5-labeled and Cy3-labeled cDNA probes, respectively. These cDNA probes were mixed and hybridized with 7K RIKEN Arabidopsis full-length cDNA microarray containing 7000 Arabidopsis cDNAs. The information on the 7K RIKEN Arabidopsis full-length cDNA microarray is available at http://pfgweb.gsc.riken.go.jp/projects/microarray.html. A λ control template DNA fragment (TX803; Takara, Tokyo, Japan) was used as an external control to equalize hybridization signals generated from different samples. To assess the reproducibility of microarray analysis, each experiment was repeated three times. Genes showing a signal value <1000 (which was typically twice the mean background value) in control were not considered for the analyses. The image analysis and signal quantification were performed with QuantArray version 2.0 (GSI Lumonics, Oxnard, CA). Gene clustering analysis was performed with Genespring (Silicon Genetics).

BRI1 Gene Expression Analysis

The *BRI1* gene expression levels in the RL-BK transgenic plants were detected by quantitative RT-PCR. Total RNAs were isolated from the 14-d-old dwarf and normal plants of the transgenic lines (L2 and L3) and control plants with Trizol reagent (Gibco BRL). Reverse transcription of all the RNA samples was performed with SuperScript III RNase H reverse transcriptase (Invitrogen, Carlsbad, CA). SYBR Premix Ex Taq (Takara) was used for real-time quantitative PCR. We used LightCycler3 (Roche Diagnostics, Indianapolis, IN) and repeated experiments three times. For detection of *Bri1*, PCR primers 5′-CATTCTTGATCTTTCAAG-CAATAAGCTCGATGGG-3′ and 5′-ATCCCATCGCCACACTACCAGCA-AGGGACG-3′ were used. Melting curves confirmed that only one product was amplified. Specific cDNA was quantified with a standard curve based on known amounts of amplified *BRI1* fragment. The 18S rRNA was also amplified and used for calibration.

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