

The Arabidopsis A4 Subfamily of Lectin Receptor Kinases Negatively Regulates Absciscic Acid Response in Seed Germination^{1[W][OA]}

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Abscisic acid (ABA) is an important plant hormone for a wide array of growth and developmental processes and stress responses, but the mechanism of ABA signal perception on the plasma membrane remains to be dissected. A previous GeneChip analysis revealed that a member of the A4 subfamily of lectin receptor kinases (LecRKs) of Arabidopsis (*Arabidopsis thaliana*), At5g01540 (designated *LecRKA4.1*), is up-regulated in response to a low dose of ABA in the *rop10-1* background. Here, we present functional evidence to support its role in ABA response. *LecRKA4.1* is expressed in seeds and leaves but not in roots, and the protein is localized to the plasma membrane. A T-DNA knockout mutant, *lecrka4.1-1*, slightly enhanced ABA inhibition of seed germination. Interestingly, *LecRKA4.1* is adjacent to two other members of the A4 subfamily of LecRK genes, At5g01550 (*LecRKA4.2*) and At5g01560 (*LecRKA4.3*). We found that loss-of-function mutants of *LecRKA4.2* and *LecRKA4.3* exhibited similarly weak enhancement of ABA response in seed germination inhibition. Furthermore, *LecRKA4.2* suppression by RNA interference in *lecrka4.1-1* showed stronger ABA inhibition of seed germination than *lecrka4.1-1*, while the response to gibberellic acid was not affected in *lecrka4.1-1* and *lecrka4.1-1; LecRKA4.2* (RNAi) lines. Expression studies, together with network-based analysis, suggest that *LecRKA4.1* and *LecRKA4.2* regulate some of the ABA-responsive genes. Taken together, our results demonstrate that the A4 subfamily of LecRKs has a redundant function in the negative regulation of ABA response in seed germination.

Abscisic acid (ABA) is a hormone that modulates a wide spectrum of growth and developmental processes (such as seed maturation, dormancy, and germination) and responses to biotic and abiotic stresses (Finkelstein et al., 2002; Himmelbach et al., 2003; Xie et al., 2005). Although ABA signal perception and transduction pathways have not been clearly demonstrated yet, nearly 50 genes have been shown to affect ABA responses through forward and reverse genetics (Finkelstein et al., 2002). Among them, the ABA signaling proteins that are associated with the plasma membrane (PM) are of particular interest (Wang and Zhang, 2008; Wasilewska et al., 2008; Zheng and Xin, 2008). It is well recognized that PM is an important site for ABA perception (Finkelstein et al., 2002; Yamazaki et al., 2003; Wang and Zhang, 2008; Zheng and Xin,

2008), although direct biochemical evidence has been reported that ABA can also be sensed in the plastid (Shen et al., 2006) or the nucleus (Razem et al., 2006).

Two major types of PM-associated proteins have been increasingly recognized as important ABA perception or signaling components. The first one is the PM-integral receptor-like kinases. Among more than 600 receptor-like kinases in Arabidopsis (*Arabidopsis thaliana*), RPK1 was shown to be induced starting 1 h after the ABA treatment (Hong et al., 1997). Recently, the RPK1 mutants and antisense transgenic plants were shown to reduce seed dormancy and the sensitivity to ABA inhibition of seed germination (Osakabe et al., 2005). Although RPK1 controls the expression of many ABA-inducible genes, it remains to be determined whether RPK1 is a PM-localized ABA receptor.

The second type of PM-associated ABA signaling proteins includes heterotrimeric GTP-binding proteins such as GPA1 and AGB1 (Wang et al., 2001; Ullah et al., 2002; Pandey et al., 2006) and ROP monomeric GTPases such as ROP2, ROP6, and ROP10 (Lemichiez et al., 2001; Li et al., 2001; Zheng et al., 2002; Xin et al., 2005). Both of these groups of GTP-binding proteins turn on/off the signaling switch by cycling between GTP-bound and GDP-bound forms. Furthermore, these GTP-binding proteins likely act through their associations with the PM-localized receptors. For example, G-proteins can be activated by the G-protein-coupled receptors (GPCRs),

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based on the animal signaling paradigm. A putative GPCR called GCR1 has been reported to modulate ABA responses in seed dormancy, root growth, and stomatal behavior (Colucci et al., 2002; Pandey and Assmann, 2004). More recently, GCR2 was claimed to be another putative GPCR and to act as an ABA receptor (Liu et al., 2007), with contrasting arguments reported by other groups regarding the mutant phenotypes in ABA responses and the biochemical identity of the gene (Gao et al., 2007; Illingworth et al., 2008). Therefore, the PM-localized ABA receptors remain cryptic.

In an effort to understand the transcriptional control of ABA response by ROP10 small GTPase, a negative regulator of ABA signaling (Zheng et al., 2002), we recently performed an Arabidopsis GeneChip analysis (Xin et al., 2005). Our results led to the identification of a group of genes, including a lectin receptor kinase encoded by At5g01540, which are only up-regulated by 1 μ M ABA in *rop10-1* (Xin et al., 2005). For this particular group of genes, higher ABA concentrations (10 and 100 μ M) did not affect their expression in both the wild type and *rop10-1*, indicating that ROP10 is likely involved in the gating of low-dose ABA-regulated gene expression (Xin et al., 2005). The At5g01540 gene product has been grouped into the A4 subfamily of lectin receptor kinases (LecRKs; Barre et al., 2002); therefore, it is designated *LecRKA4.1* here. In this report, we characterize *LecRKA4.1* expression and protein subcellular localization patterns. More importantly, we show that *LecRKA4.1* and the adjacent two other members, *LecRKA4.2* and *LecRKA4.3*, have redundant functions in the negative regulation of ABA response in seed germination. Therefore, these results increase our understanding of the PM-associated ABA signaling events.

RESULTS

LecRKA4.1 Expression and Protein Subcellular Localization Patterns

To determine where *LecRKA4.1* is expressed, both reverse transcription (RT)-PCR and promoter:GUS reporter analyses were performed. RT-PCR analysis showed that *LecRKA4.1* is preferentially expressed in the shoots of young seedlings, while its transcript is barely detectable in the roots (Fig. 1A). *LecRKA4.1* is also weakly expressed in the stems and flowers but strongly expressed in the leaves (Fig. 1A). Consistent with the RT-PCR results, a 1.2-kb fragment of *LecRKA4.1* promoter directed GUS reporter expression in the shoots but not in the roots of young seedlings (Fig. 1, B and C). This indicates that the 1.2-kb promoter fragment likely contains all of necessary cis elements for proper expression of the *LecRKA4.1* gene. Using three homozygous *P_{LecRKA4.1}::GUS* lines, all with one T-DNA insertion, we found that the *LecRKA4.1* promoter is very active in the vascular system and trichomes of the leaf (Fig. 1D), and it is also expressed in

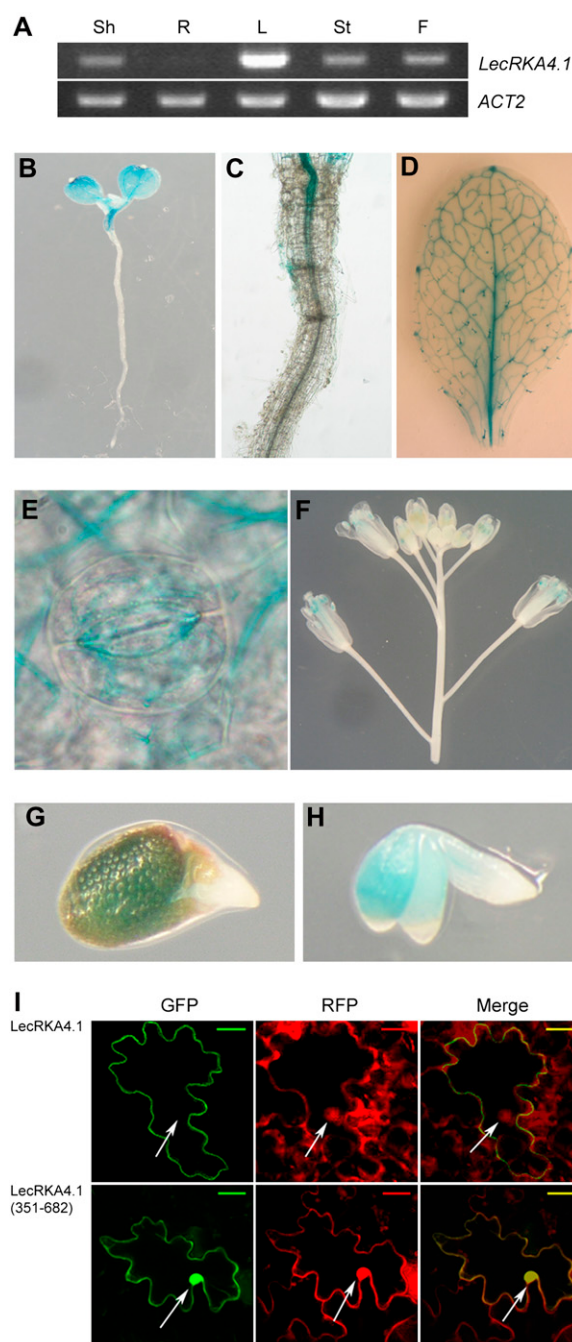


Figure 1. Expression and protein subcellular localization patterns of *LecRKA4.1*. A, RT-PCR analysis of *LecRKA4.1* expression in different organs. Shown are shoots (Sh) and roots (R) of 7-d-old seedlings, mature leaves (L), stems (St), and flowers (F). *ACT2* was used as an internal control. RT was prepared as described elsewhere (Gao et al., 2008). B to H, Expression patterns of *LecRKA4.1* as revealed by *P_{LecRKA4.1}::GUS*. Shown are a 5-d-old seedling (B), an enlarged view of a root segment immediately from the root-hypocotyl junction (C), a mature leaf (D), an enlarged view of guard cells (E), an inflorescence with several flowers (F), and a germinating seed with and without seed coat (G and H). I, Transient expression of *LecRKA4.1-GFP* and *LecRKA4.1³⁵¹⁻⁶⁸²-GFP* in leaf pavement cells. GFP images are shown at left. An mCherry version of RFP was used as a control (middle). The merged GFP and RFP images are shown at right. The arrows indicate the position of the nucleus. Bars = 20 μ m.

the guard cells (Fig. 1E). In addition, this promoter is active in anthers and stigmas but not in petals (Fig. 1F). We also found that the *LecRKA4.1* promoter is active in germinating seeds, although its activity is absent in the protruding radicles (Fig. 1, G and H).

The intronless *LecRKA4.1* gene has a translated region of 2,049 bp, encoding a protein of 682 amino acids. The predicted protein has an N-terminal signal peptide (amino acids 1–27), legume lectin α and β domains (amino acids 29–278), and a transmembrane domain (amino acids 310–332) that is followed by a protein kinase domain (amino acids 367–641) and the C terminus. To confirm that *LecRKA4.1* is a PM-localized protein, we transiently expressed *LecRKA4.1*-GFP fusion protein in leaf epidermal cells of a transgenic line that expresses an mCherry version of red fluorescent protein (RFP). Compared with RFP, which is localized to the cytoplasm and the nucleus, *LecRKA4.1*-GFP is exclusively localized to the PM (Fig. 1I). Because the N-terminal half of *LecRKA4.1* contains the signal peptide, lectin domains, and a transmembrane domain, we constructed a GFP fusion with the C-terminal half of *LecRKA4.1*, *LecRKA4.1*^{351–682}, which contains the predicted protein kinase domain. Transient expression in leaf epidermal cells showed that *LecRKA4.1*^{351–682}-GFP was expressed in the nucleus and the cytoplasm (Fig. 1I). This pattern is similar to that of the RFP control, as shown in the merged RFP and GFP images. Therefore, we conclude that the transmembrane domain-containing N-terminal half of *LecRKA4.1* is responsible for targeting *LecRKA4.1* specifically to the PM.

A T-DNA Knockout Mutant of *LecRKA4.1* Slightly Enhanced ABA Response in Seed Germination Inhibition

To demonstrate that the PM-localized *LecRKA4.1* has a function in ABA response, we isolated and characterized a T-DNA line of *LecRKA4.1* (SALK_070801), which is designated *lecrka4.1-1*. RT-PCR analysis showed that *LecRKA4.1* transcript was absent in *lecrka4.1-1* (Fig. 2A). Given that the T-DNA is located in the exon (1,434 bp from ATG), we believe that *lecrka4.1-1* likely represents a null allele of *LecRKA4.1*. *LecRKA4.1* is not expressed in the roots (Fig. 1A), and indeed, root growth inhibition response to ABA was not significantly altered in *lecrka4.1-1* (data not shown). In addition, we did not observe any difference in leaf water loss in *lecrka4.1-1* (data not shown), although the *LecRKA4.1* promoter is active in the guard cells (Fig. 1E). Therefore, we focused our ABA phenotypic characterization on seed germination inhibition. After 3 d on the medium containing 0.6 μ M ABA, the Columbia (Col) wild type had a germination percentage of 60%, but the germination percentage of *lecrka4.1-1* was only 27% (Fig. 2B). This 55% reduction of seed germination percentage in *lecrka4.1-1* indicates that the *LecRKA4.1* mutant slightly increased the ABA sensitivity of seed germination inhibition. To substantiate that this enhancement is due to the loss of *LecRKA4.1* function, a

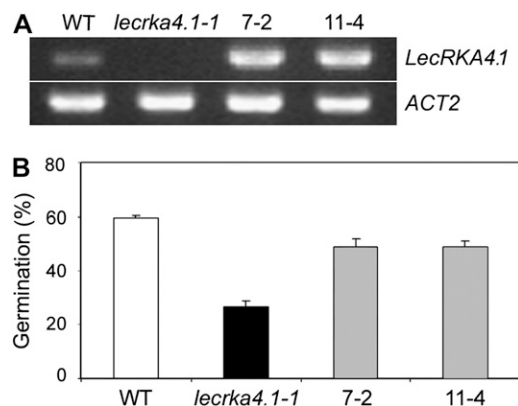


Figure 2. The *lecrka4.1-1* knockout mutant slightly enhances the ABA response in seed germination inhibition. A, RT-PCR analysis of *LecRKA4.1* mRNA expression in seedlings of the wild type (WT), *lecrka4.1-1*, and two transgenic lines (7-2 and 11-4) expressing the 2X35S:*LecRKA4.1* transgene in *lecrka4.1-1*. *ACT2* was used as an internal control. B, Functional complementation of *lecrka4.1-1* by 2X35S:*LecRKA4.1*. The transgenic lines 7-2 and 11-4 showed similar seed germination percentages as the wild type in the presence of 0.6 μ M ABA. Germination was scored at 3 d after the cold treatment. Data represent averages and SD of germination percentages for three replicates, each with about 40 to 50 seeds.

functional complementation test was performed. The full-length *LecRKA4.1* cDNA under the control of a 2X35S cauliflower mosaic virus (CaMV) promoter was transformed into *lecrka4.1-1*, and two independently transformed lines, 7-2 and 11-4, had high levels of *LecRKA4.1* transcript (Fig. 2A). In the absence of ABA, these two lines had almost 100% germination, similar to the wild type and *lecrka4.1-1*, and under 0.6 μ M ABA, they had seed germination percentages close to that for the wild type compared with reduced germination for *lecrka4.1-1* (Fig. 2B). These results suggest that *LecRKA4.1* is a negative regulator of ABA response in seed germination.

Mutants of *LecRKA4.2* and *LecRKA4.3* Slightly Enhanced ABA Response in Seed Germination Inhibition

The weak phenotype of *lecrka4.1-1* indicates that other members of the A4 subfamily of LecRKs could have redundant functions. Interestingly, two other members, At5g01550 (designated *LecRKA4.2*) and At5g01560 (*LecRKA4.3*), are immediately adjacent to *LecRKA4.1* (At5g01540), and they share very high amino acid sequence identity (65%–74%). Therefore, we isolated and characterized their T-DNA insertional lines. In the Salk T-DNA database, there was one line for *LecRKA4.2* (SALK_108000, designated *lecrka4.2-1*) and two lines for *LecRKA4.3*, SALK_003084 (designated *lecrka4.3-1*) and SALK_122197 (designated *lecrka4.3-2*). RT-PCR analysis showed that *LecRKA4.2* transcript was lower than that in the wild type and that *LecRKA4.3* was absent in the *lecrka4.3-1* and *lecrka4.3-2* alleles (Fig. 3A). As in the case of *lecrka4.1-1*, the T-DNA insertion in

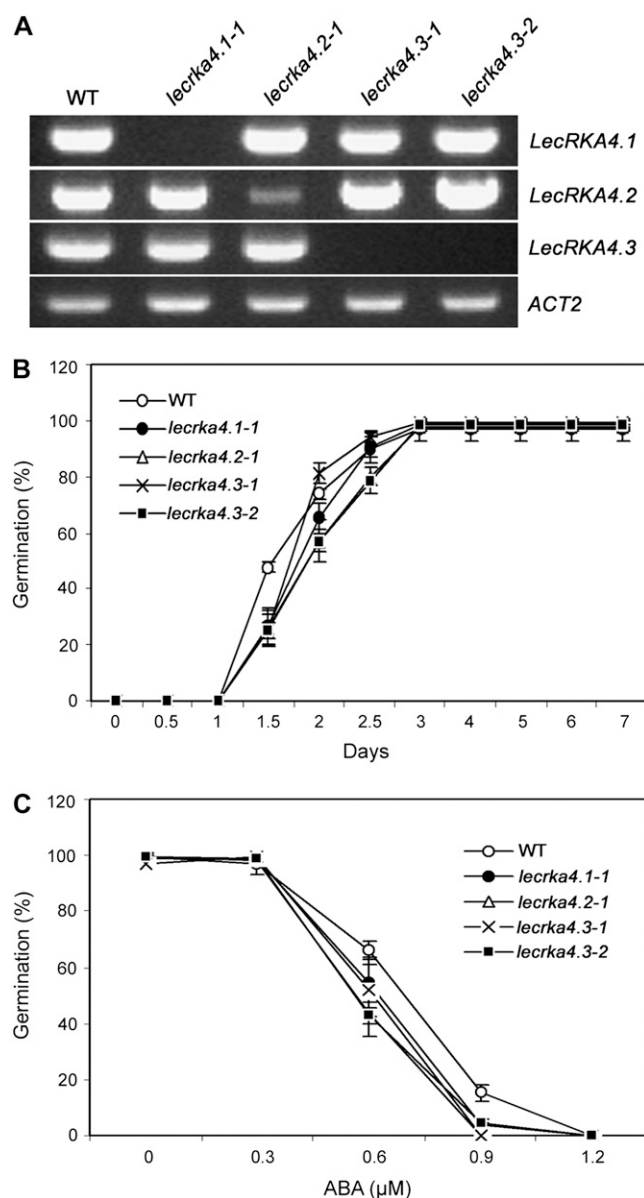


Figure 3. Mutants of *LecRKA4.2* and *LecRKA4.3* slightly enhance ABA response in seed germination inhibition. A, RT-PCR analysis of *LecRKA4.1*, *LecRKA4.2*, and *LecRKA4.3* transcript levels in seedlings of the wild type (WT) and various mutants. *ACT2* was used as an internal control. B and C, *lecrka4.2* and *lecrka4.3* mutants showed similar seed germination kinetic profiles with 0.3 μM ABA (B) and ABA dose-response patterns (C) as *lecrka4.1-1*. Germination for dose response was scored at 4 d after cold treatment. Data represent averages and SD of germination percentage for three replicates, each with about 40 to 50 seeds.

lecrka4.2-1, *lecrka4.3-1*, and *lecrka4.3-2* did not interfere with the expression of other A4 subfamily genes.

We then subjected the mutants to 0.3 μM ABA treatment. The germination kinetic profile showed that, similar to *lecrka4.1-1*, fewer *lecrka4.2-1*, *lecrka4.3-1*, and *lecrka4.3-2* seeds germinated than wild-type seeds at 1.5 d after cold treatment, and after 3 d, they all

germinated like wild-type seeds (Fig. 3B). At day 2, *lecrka4.3-2* still germinated less, while *lecrka4.3-1* had a similar germination percentage as the wild type. The reason for this allelic difference is unknown. A dose-response curve showed that after 4 d at both 0.6 and 0.9 μM ABA, the mutants for all three genes had lower germination rates than the wild type (Fig. 3C). These experiments indicate that the A4 subfamily of LecRKs encoded by the three genes clustered on chromosome 5 probably has a redundant function in ABA-mediated seed germination inhibition response.

lecrka4.1-1; *LecRKA4.2* (RNAi) Lines Exhibited a Stronger ABA Response in Seed Germination Inhibition

If *LecRKA4.1*, *LecRKA4.2*, and *LecRKA4.3* have redundant functions as negative regulators of ABA response, we would expect to see a stronger phenotype in their double and triple mutants. However, their extremely tight genetic linkage prevented us from obtaining double or triple mutants by crossing with each other. Therefore, we decided to use RNA interference (RNAi) to suppress *LecRKA4.2* and *LecRKA4.3* gene expression in the *lecrka4.1-1* background. Given their high sequence homology (77% identical for full-length cDNAs), we initially predicted that the *LecRKA4.2* RNAi using either the partial fragment of *LecRKA4.2* cDNA (1,130–1,760 bp) or the longer cDNA fragment (1–1,760 bp) would suppress both *LecRKA4.2* and *LecRKA4.3* expression. Two representative RNAi lines, S2 and F9, were chosen for further characterization. *LecRKA4.2* mRNA expression was greatly suppressed in S2 and F9, but *LecRKA4.3* expression was not altered (Fig. 4A).

Seed germination kinetic profiles for the wild type, *lecrka4.1-1*, and the two RNAi lines, S2 and F9, at 0.3 μM ABA showed that after 1.5 d, while *lecrka4.1-1* had a germination percentage about 50% lower than the wild type, S2 and F9 lines were even 50% to 80% lower than *lecrka4.1-1* (Fig. 4B). After 2 d, although *lecrka4.1-1* had a similar germination percentage as the wild type (approximately 82%), only 48% to 64% of seeds in S2 and F9 lines germinated. Furthermore, after 7 d, almost all of the wild-type and *lecrka4.1-1* seeds germinated, but 14% to 19% of the seeds in S2 and F9 still did not germinate. On the other hand, an ABA dose-response curve showed that after 4 d, S2 and F9 lines had lower seed germination percentages than *lecrka4.1-1* (Fig. 4C). We also examined the postgerminative seedling growth by scoring the cotyledon-greening phenotype, and the results showed that S2 and F9 had 50% fewer seedlings showing green cotyledons than *lecrka4.1-1* after 6 d in the presence of 0.6 μM ABA (Fig. 4D) or after 9 d in the presence of 0.9 μM ABA (Fig. 4E). Taken together, these results show that suppression of *LecRKA4.2* expression further enhances the ABA sensitivity observed in *lecrka4.1-1*.

To exclude the possibility that the enhanced response to ABA in *lecrka4.1-1* and *lecrka4.1-1*; *LecRKA4.2* (RNAi) lines is due to differences in seed germination

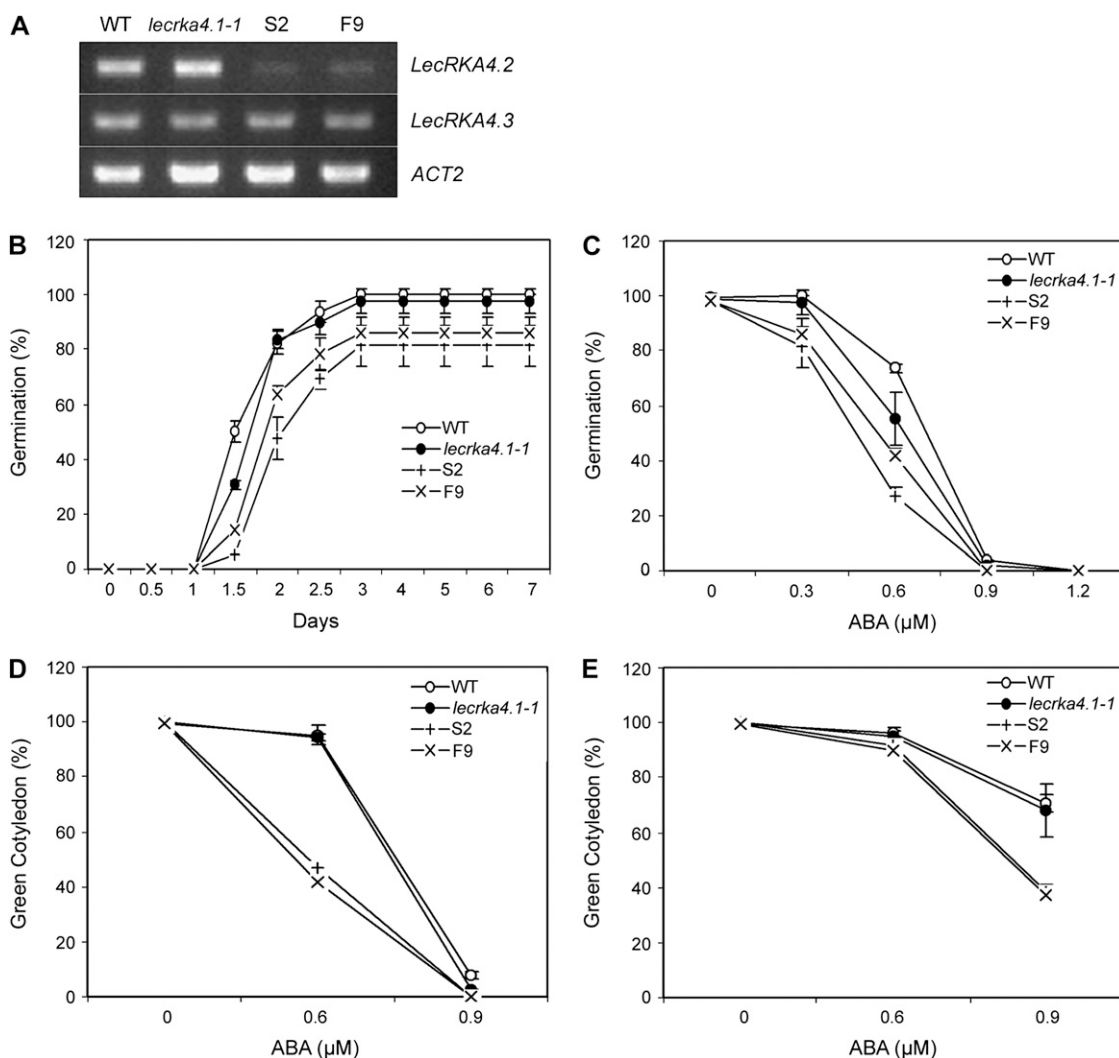


Figure 4. The *lecrka4.1-1; LecRKA4.2* (RNAi) lines enhance the ABA response in seed germination inhibition. A, RT-PCR analysis of *LecRKA4.2* and *LecRKA4.3* mRNA expression in seedlings of the wild type (WT), *lecrka4.1-1*, and two *LecRKA4.2* RNAi lines (S2 and F9) in the *lecrka4.1-1* background. *ACT2* was used as an internal control. B and C, S2 and F9 lines showed a stronger ABA seed germination inhibition response than *lecrka4.1-1*. Seed germination kinetic profiles (B) were determined using 0.3 μ M ABA, and ABA dose response (C) was measured at 4 d after cold treatment. Each data point represents the average and SD of three replicates, each with about 40 to 50 seeds. D and E, S2 and F9 lines showed smaller green cotyledon percentages at 6 d (D) and 9 d (E) under ABA stress. Each point represents the average and SD of three replicates, each with about 40 to 50 seeds.

or postgerminative seedling growth that are not related to ABA signaling, freshly harvested seeds of these mutant lines were allowed to germinate in the absence of ABA. The results showed that *lecrka4.1-1* and the S2 and F9 lines exhibited a very similar germination kinetic profile (Supplemental Fig. S1). Furthermore, treatments with 1 and 10 μ M GA₃, which antagonizes the ABA inhibitory effect in seed germination, did not result in lower sensitivity to GA₃ in *lecrka4.1-1* and the S2 and F9 lines (Supplemental Fig. S2). These results support the idea that the observed seed germination response in the *LecRKA4* subfamily gene knockout or RNAi lines is likely specific to ABA.

Expression of ABA-Responsive Genes Was Altered in *lecrka4.1-1; LecRKA4.2* (RNAi) Lines

To test whether ABA-responsive genes are negatively regulated by the A4 subfamily *LecRK*, expression of *ABI1* and *ABI2* genes was quantified by real-time RT-PCR. Consistent with a previous report (Leung et al., 1997), transcripts of *ABI1* and *ABI2* were dramatically up-regulated by 0.3 and 0.9 μ M ABA in the wild type (Fig. 5, A and B). In the presence of 0.3 μ M ABA, the expression levels for *ABI1* and *ABI2* in both *lecrka4.1-1* and the F9 RNAi line were slightly (60%–100%) higher than that in the wild type. However, there was no

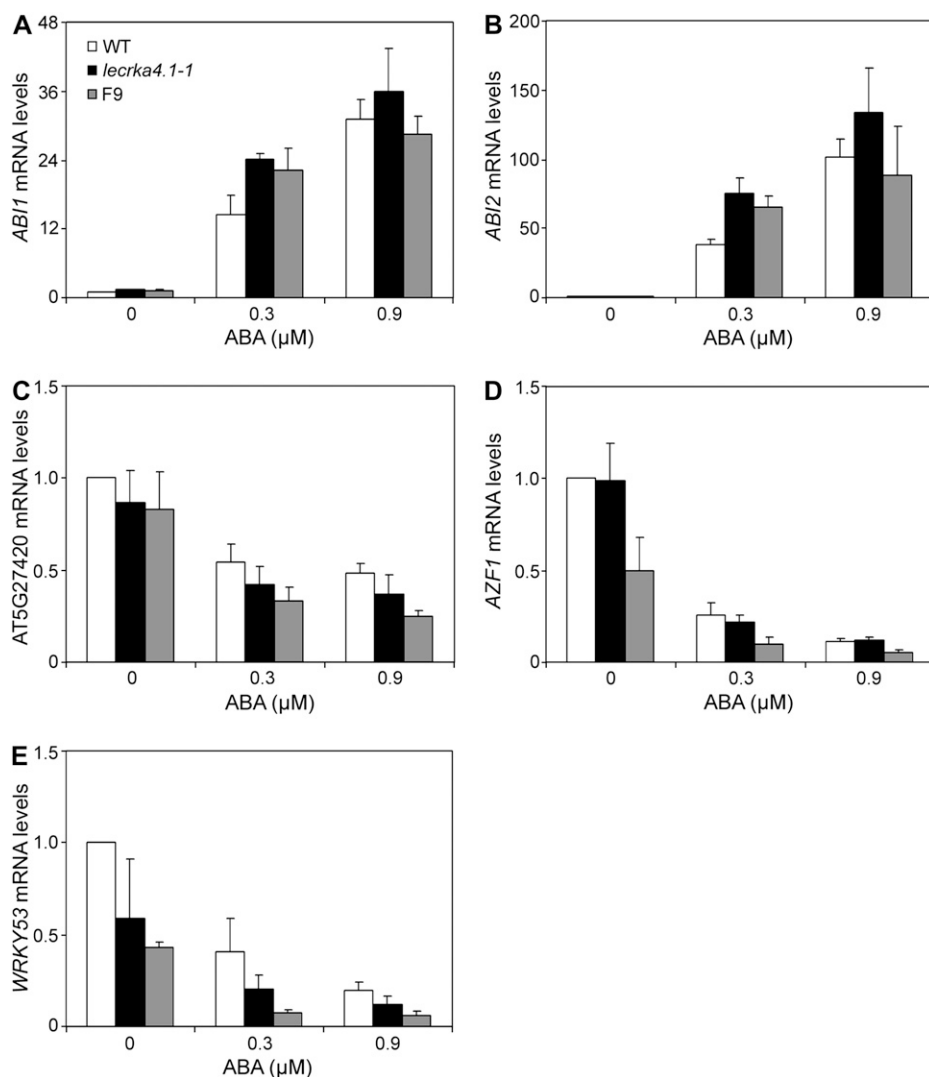


Figure 5. Real-time PCR analysis of gene expression in response to ABA. Four-day-old wild-type (WT), *lecrka4.1-1*, and F9 seedlings grown in liquid MS medium were treated for 12 h with 0, 0.3, and 0.9 μM ABA prepared in fresh MS medium. Expression of *ABI1* (A), *ABI2* (B), *At5g27420* (C), and two genes, *AZF1* (D) and *WRKY53* (E), which were selected from the assembled protein-protein interaction network (Fig. 6), was quantified using real-time PCR analysis. The mRNA levels for each of these genes were normalized with *ACT2*, and relative mRNA levels in nontreated wild-type seedlings for each gene were set at 1. Average and se values for three biological repeats are shown. The wild type, *lecrka4.1-1*, and F9 are designated in all panels as shown in A.

difference between *lecrka4.1-1* and the F9 line, and at 0.9 μM ABA, the difference between the wild type and the mutant/RNAi line disappeared. Interestingly, a C3HC4-type zinc finger family protein gene (*At5g27420*), whose expression was not altered in the wild type by 1, 10, and 100 μM ABA (Xin et al., 2005), was slightly down-regulated by 0.3 and 0.9 μM ABA (Fig. 5C). Importantly, in the presence of 0.3 μM ABA, its expression level was slightly lower in *lecrka4.1-1* than in the wild type, and F9 had an even lower expression level than *lecrka4.1-1* (Fig. 5C). Although the treatment with 0.9 μM ABA did not further reduce the expression of *At5g27420* in the wild type and *lecrka4.1-1*, the F9 line still displayed a similar difference compared with the wild type.

To further explore which genes are possibly regulated by the three members of A4 subfamily LecRK or their pathway, a protein-protein interaction network was assembled. The protein-protein interactions were based on both experimentally demonstrated or predicted physical interactions (Geisler-Lee et al., 2007)

and functional interactions implicated by a large number of gene expression analyses (Ma et al., 2007). We found that LecRK4.1 and LecRK4.2, but not LecRK4.3, were present in the network database, resulting in a total of 70 unique genes (nodes; Supplemental Table S1) that exhibit 149 physical or functional interactions (edges). Therefore, a network for LecRK4.1 and LecRK4.2 was assembled (Fig. 6). Functional categorical analysis of these 70 genes showed that genes involved in metabolism (in particular phosphate metabolism), protein fate (folding, modification, and destination), cellular transport, interaction with environments, and cell rescue, defense, and virulence were overly represented compared with the whole genome representation (Supplemental Table S1).

We then selected two transcription factor genes from the assembled network for quantitative analysis of their expression in *lecrka4.1-1* and the F9 line. *AZF1*, although not yet updated in the database as ABA suppressed, was recently shown to be down-regulated by ABA (Sakamoto et al., 2004), and its close homolog,

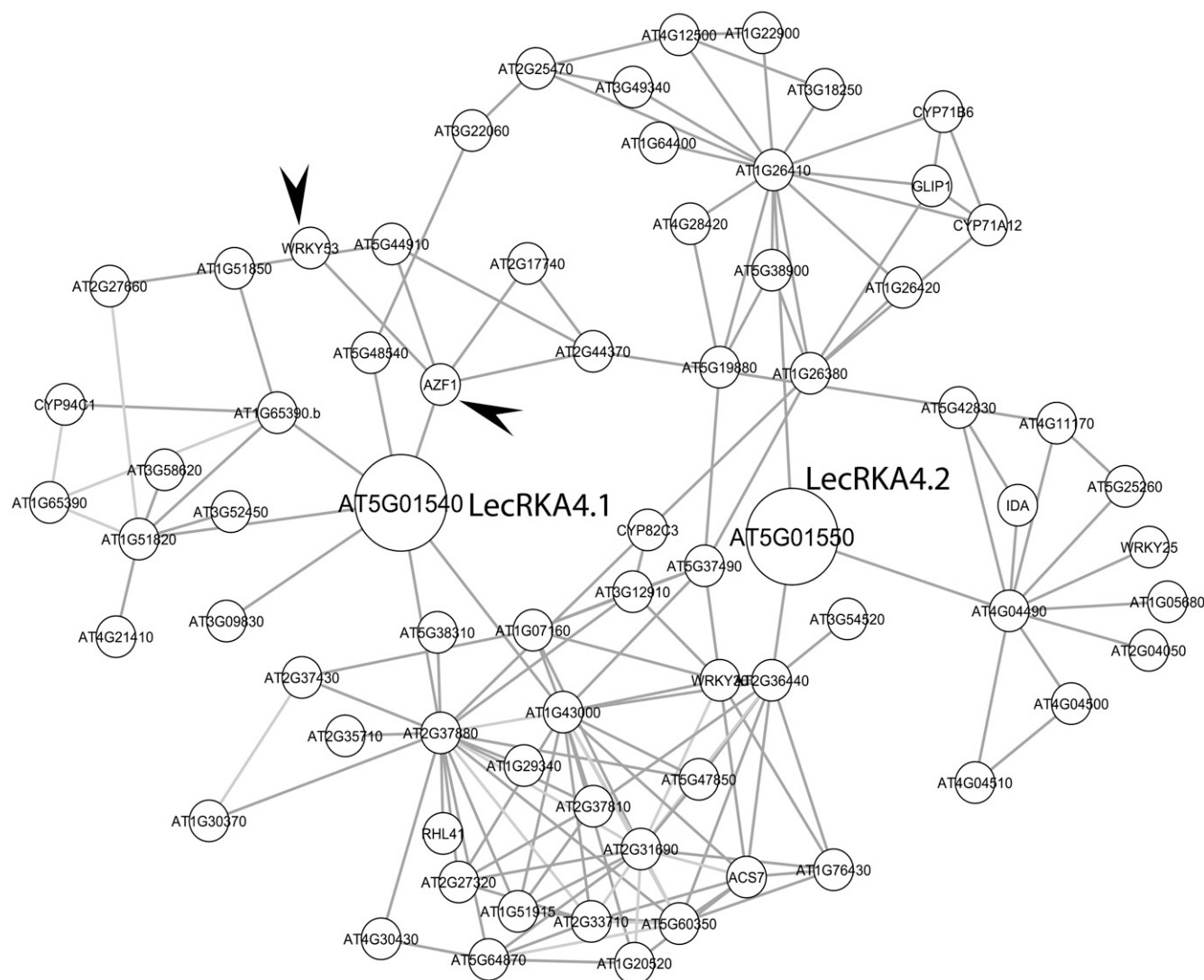


Figure 6. A LecRKA4.1 and LecRKA4.2 gene interaction network. Three members of LecRKA4 genes were mapped to the protein-protein interaction database, with only LecRKA4.1 and LecRKA4.2 present in the database. This analysis revealed a total of 70 unique genes (Supplemental Table S1) that showed 149 interactions, and a network was then assembled based on these interactions. Arrowheads indicate the two genes (*WRKY33* and *AZF1*) whose transcript levels were analyzed using real-time PCR (Fig. 5, D and E).

AZF2, was demonstrated to act as a transcription factor negatively regulating ABA response (Sakamoto et al., 2004). Consistently, we found that *AZF1* mRNA level was reduced by about 75% and 90% by 0.3 and 0.9 μM ABA, respectively (Fig. 5D). Surprisingly, although *AZF1* showed a direct functional interaction with LecRKA4.1 based on the assembled network (Fig. 6), there was no difference in its transcript levels between the wild type and *lecrka4.1-1* at 0, 0.3, and 0.9 μM ABA (Fig. 5D). However, F9 had a 50% lower expression level than the wild type in the absence of ABA and a 65% lower level at 0.3 μM ABA. At 0.9 μM ABA, a similar trend was observed. In contrast to *AZF1*, *WRKY53*, another transcription factor gene that

was down-regulated by ABA, as reported by the Genevestigator Web site (<https://www.genevestigator.ethz.ch/gv/index.jsp>) and in our experimental conditions (Fig. 5E), showed differential expression levels between the wild type, *lecrka4.1-1*, and F9 (Fig. 5E). Although there was no statistical difference between *lecrka4.1-1* and the wild type in the no-ABA control, the 50% lower level difference in *lecrka4.1-1* compared with the wild type at 0.3 μM ABA was statistically significant. Interestingly, as in the case of *At5g27420* under the 0.3 μM ABA treatment (Fig. 5C), F9 showed a further reduction in *WRKY53* expression compared with *lecrka4.1-1* (Fig. 5E). In the control, there was an approximately 2-fold difference between F9 and the wild type, but at

0.3 μM ABA, they showed a 5-fold difference. At 0.9 μM ABA, there was still a 3-fold difference between F9 and the wild type.

DISCUSSION

In this work, we provide genetic evidence that LecRKA4.1 and the other two most closely related LecRKs (LecRKA4.2 and LecRKA4.3) act as negative regulators of ABA response in seed germination. First, we show that single knockout mutants for each of these three genes exhibit subtle enhancements in ABA-inhibited seed germination. Second, down-regulation of *LecRKA4.2* by RNAi further enhanced the response in *lecrka4.1-1*. Third, this enhancement is not due to the developmental delay in *lecrka4.1-1* and *lecrka4.1-1; LecRKA4.2 (RNAi)* lines or to their altered sensitivity to GA_3 . Fourth, gene expression studies, coupled with gene network assembly, suggest that LecRKA4.1 and LecRKA4.2 control the expression of some ABA-responsive genes. Therefore, our results demonstrate that the A4 subfamily of LecRKs acts redundantly in the negative regulation of ABA response.

The identification of the PM-associated ABA signaling components, including ABA receptors, has been a significant challenge. Some of the components, such as RPK1 (Hong et al., 1997; Osakabe et al., 2005), are targeted for their possible functions in ABA response because of earlier observations that they are transcriptionally regulated by ABA in the wild type. However, the genes that are not transcriptionally affected by ABA in the wild type may not give investigators a clue to their possible function in ABA response and thus may potentially be overlooked. For example, *LecRKA4.1*, reported in this paper, was not regulated by 1, 10, and 100 μM ABA at the transcriptional level in the wild type in several DNA microarray experiments (Xin et al., 2005), nor was its promoter activity differentially regulated by low concentrations (0.3 and 0.9 μM) of ABA (Supplemental Fig. S3). Therefore, it would be difficult to assume its role in the control of ABA response. However, using the *rop10-1* background and only in the presence of a low dose of ABA, this gene was revealed to be slightly up-regulated in *rop10-1* (Xin et al., 2005). Clearly, transcriptome analysis involving various genetic backgrounds and different ABA doses will lead to the identification of novel components, in particular those associated with the PM, in the ABA signaling network.

However, it is difficult to elucidate how the A4 subfamily of LecRKs links to ROP10 small GTPase in ABA signaling. The observation that *LecRKA4.1* was up-regulated by a low dose (1 μM) but not at higher doses (10 and 100 μM) of ABA in *rop10-1* seedlings indicates that LecRKA4.1 might participate in the ROP10-gated, low-dose ABA response pathway (Xin et al., 2005). In this report, we show that the *lecrka4.1-1; LecRKA4.2 (RNAi)* lines enhanced the sensitivity to low doses (0.3–0.9 μM) of ABA in the seed germination

response and the cotyledon-greening process, but we were unable to test this hypothesis in the true leaves because we failed to observe any difference in leaf water loss between the *lecrka4.1-1; LecRKA4.2 (RNAi)* lines and the wild type. It is possible that LecRKA4.3 and/or the residual *LecRKA4.2* mRNA in these lines might still be functional to mask the low-dose ABA response in the true leaves. A further complication is that if *LecRKA4.1* transcript is higher in *rop10-1* (Xin et al., 2005), one would expect that increased *LecRKA4.1* expression would be necessary for the enhanced ABA response in *rop10-1* (Zheng et al., 2002). However, loss of *LecRKA4.1* function (and that of two other LecRK genes) resulted in a similar phenotype as *rop10-1*. The facts that *rop10-1* and *lecrka4.1-1* are in different ecotypic backgrounds and they have weak phenotypes prevented us from testing whether they act in the same or different pathways. Although we did not find any apparent difference in *ROP10* transcript level in the wild type and a *lecrka4.1-1; LecRKA4.2 (RNAi)* line treated by 0.3 and 0.9 μM ABA (data not shown), it is still possible that LecRKA4.1, LecRKA4.2, and LecRKA4.3 might function to regulate the ROP10 GTPase activity. If this is the case, this type of regulation is possibly subjected to the transcriptional control of the *LecRK* genes by ROP10 signaling. Given the complexity of the ABA response, in particular a need for plants to distinguish low versus high ABA concentrations and/or transient versus sustained ABA action, this kind of regulatory loop, if demonstrated, will be an important mechanism for plants to cope with their dynamic environments during growth and development.

Nevertheless, the identification of three members of the A4 subfamily of LecRKs as redundant, negative regulators of ABA response will provide novel insights into the complex ABA signaling networks. Recently, plastidic and nuclear ABA receptors have been found (Razem et al., 2006; Shen et al., 2006), together with the PM-localized RPK1, as an important ABA signaling component (Osakabe et al., 2005). One common feature of these components is that their mutants exhibit only weak or limited phenotypes (Osakabe et al., 2005; Razem et al., 2006; Shen et al., 2006). Similarly, we only observed the weak seed germination phenotype for various *LecRKA4* single mutants and the *lecrka4.1-1; LecRKA4.2 (RNAi)* lines. This indicates that ABA perception and response likely involve distinct receptors and/or signaling branches. Therefore, the identification of various components involved in ABA perception and signaling remains an important step before we have a full picture of ABA sensing and signaling.

The role of the legume lectin-like extracellular domain in ABA signaling is intriguing. Lectins are known to bind to carbohydrates, and some of them have been shown to act as defense proteins or in plant development (Herve et al., 1996; Navarro-Gochicoa et al., 2003; Andre et al., 2005; Chen et al., 2006; Wan et al., 2008). In Arabidopsis, there are a total of 42 LecRKs, and most of them have not been functionally

characterized yet (Barre et al., 2002). If oligosaccharides turn out to be the ligands for the A4 subfamily of LecRKs, how does that link to ABA response? Given that alterations in the biosynthesis of some polysaccharides, such as pectin and hemicellulose, lead to altered sugar responses (Li et al., 2007; Mouille et al., 2007; Gao et al., 2008) and that the cross talk of sugar and ABA responses exists in plants (Finkelstein and Gibson, 2002), one might speculate that oligosaccharides might exert an effect in sugar signaling that then indirectly affects the ABA response. However, the observed ABA-inhibited seed germination phenotype in the *LecRKA4* mutants or transgenic lines is less likely due to sugar signaling, as we did not observe any altered sugar response (data not shown). Another possibility is the ABA signaling-defense response link. The network analysis indicates that *LecRKA4.1* and *LecRKA4.2* have interactions with some genes, such as *WRKY53* (Murray et al., 2007) and *WRKY25* (Zheng et al., 2007), which are involved in defense response. The involvement of ABA signaling in the defense response has been reported (de Torres-Zabala et al., 2007), and a function for LecRK in disease resistance was reported in rice (*Oryza sativa*; Chen et al., 2006). Therefore, it is also possible that the A4 subfamily of LecRKs may act through ABA signaling to modulate disease response, or that in response to oligosaccharides that are associated with disease response, *LecRKA4* members indirectly exert their effect in the ABA response. However, the possibility that these LecRKs might directly bind ABA cannot be excluded. Clearly, testing the binding of the A4 subfamily of LecRKs by ABA at the physiologically relevant concentrations will be one future direction.

Another interesting question that needs to be addressed concerns the pathways controlled by these LecRKs. Consistent with their weak ABA response phenotypes, the mutants and transgenic lines exhibit slight differences in *ABI1/ABI2* expression from their wild types, indicating that these LecRKs may not control a major ABA response pathway but only modulate a minor branch of the pathway. Although more dramatic differences might be observed if three members of the A4 subfamily of LecRK genes are knocked out, currently this possibility cannot be tested because they physically cluster on the same chromosomal location. Furthermore, the assembled network and gene expression studies indicate that *LecRKA4.1* and *LecRKA4.2* may have overlapping and distinct response pathways. For example, while the mutation in *LecRKA4.1* increased *ABI1* and *ABI2* expression, *LecRKA4.2* (*RNAi*) did not further enhance their expression (Fig. 5, A and B). Similarly, it is less likely that *AZF1* was predominantly affected by *LecRKA4.1*, but a reduction in *LecRKA4.2* expression could enhance the ABA suppression of *AZF1* expression (Fig. 5D). Nevertheless, down-regulation of the genes *At5g27420* (encoding a zinc finger family protein) and *WRKY53* (a transcription factor) was enhanced by the loss of *LecRKA4.1* and *LecRKA4.2* function (Fig. 5, C and E).

Regardless of whether or not these LecRKs modulate a minor ABA signaling branch through overlapping or distinct pathways, it is important to first identify their downstream factors in ABA signaling in the future. Therefore, genetic and biochemical analyses of those components on the assembled network will provide further insights into the complex ABA signaling networks.

MATERIALS AND METHODS

Plant Materials, T-DNA Mutant Isolation, and Growth Conditions

The *lecrka4.1-1* (SALK_070801), *lecrka4.2-1* (SALK_108000), *lecrka4.3-1* (SALK_003084), and *lecrka4.3-2* (SALK_122197) mutants of *Arabidopsis* (*Arabidopsis thaliana*) were isolated from T-DNA insertion lines maintained in the *Arabidopsis* Biological Resource Center at The Ohio State University. For screening homozygous mutants, the following primers were used: XZP136 (sense, 5'-CATCCTCGCAGATTGAGATACAG-3') and XZP60 (antisense, 5'-TCGACGAAACATATCCTACCAACT-3') for *lecrka4.1-1*; XZP211 (sense, 5'-GTTTCAAAGGGAAGAACATACG-3') and XZP212 (antisense, 5'-GGGTTGGAGATAGCGTGAAGGTG-3') for *lecrka4.2-1*; and XZP213 (sense, 5'-CTCTGTCTTCTTTGCTTCTTCATCTCG-3') and XZP214 (antisense, 5'-CGGCTATAAAGAAGGTCCAGAG-3') for *lecrka4.3-1* and *lecrka4.3-2*. Seeds of the *Arabidopsis* wild type (Col), T-DNA insertion mutants, and transgenic plants were cold treated at 4°C for 3 to 4 d and then germinated and grown in the greenhouse for harvesting seeds, or in an incubator at 22°C with a 16-h-light/8-h-dark cycle for seed germination assay.

ABA and GA Response Assays

Seed germination was performed on half-strength Murashige and Skoog (MS) medium supplemented with various ABA concentrations. Petri plates were incubated at 22°C in the light after cold treatment (4°C for 3–4 d). Germination was scored every 12 h after incubation in the light, with the criterion being set as the complete protrusion of the radicle. For cotyledon-greening characterization, seedlings were observed from 5 to 9 d after cold treatment. For the seed germination assay with GA₃, seeds were plated on half-strength MS medium supplemented with various GA₃ concentrations. After 3 d of cold treatment, germination was scored every 2 h after incubation in the light, with the same germination criterion used above.

Regular and Real-Time RT-PCR Analyses

For regular PCR analysis of *LecRK* gene expression, 7-d-old young seedlings were used for total RNA using TRIzol (Invitrogen). A total of 5 µg of RNA was reverse transcribed in a 20-µL reaction using SuperScript III reverse transcriptase and oligo(dT)_{12–18} primer (Invitrogen) according to the instructions provided by the vendor. PCR was performed using Taq DNA polymerase, with *ACT2* as the internal control, using the primers *ACT2S* and *ACT2A* described previously (Xin et al., 2005). Gene-specific primer pairs XZP196 (sense, 5'-CTCTGCAGATGCATCCTCGCAGATTGAGATAC-3') and XZP197 (antisense, 5'-GTGCTACTGACTGATACGAGAAGTCG-3'); XZP206 (sense, 5'-TTCTTAATTAACCATGGATGCTCGTGCTCTTCTTGCT-3') and XZP205 (antisense, 5'-AAGGATCCAITTAAATGAATCTCTCTCGCGTGCAAT-3'); and XZP213 (sense, 5'-CTCTGTCTTCTTTGCTTCTTCATCTCG-3') and XZP232 (antisense, 5'-CTCCGCTTCTCTCTCGGTTTACTG-3') were used for the amplification of *LecRKA4.1*, *LecRKA4.2*, and *LecRKA4.3*, respectively. These experiments were repeated once.

For real-time PCR analysis of gene expression, seeds of *lecrka4.1-1*, its wide type (Col), and a *lecrka4.1-1*; *LecRKA4.2* (*RNAi*) line (F9) were incubated in 50 mL of liquid MS medium, pH 5.7, at 4°C for 3 d and then allowed to germinate and grow at 22°C for 4 d under a 16-h-day/8-h-night regime with continuous shaking at 150 rpm. After three rinses with freshly prepared MS liquid medium, young seedlings were treated with 0, 0.3, and 0.9 µM ABA that was dissolved with dimethyl sulfoxide (with the final concentration in the medium of 0.1%, v/v) and prepared in the MS medium. After 12 h of treatment, the seedlings were immediately frozen in liquid nitrogen and stored at -80°C until RNA isolation. Three biological replicates were performed at different

times for all genotype/treatment combinations. RNA RT was described above, and real-time quantitative PCR analysis was performed using the QuantiTect SYBR Green PCR kit (Qiagen). Real-time PCR was carried out in the Master-Cycler II (Cypheid) according to the manufacturer's protocol. The primers for *ACT2* were designed previously (Zheng et al., 2002). Gene-specific primer pairs were as follows: for *ABI1* (At4g26080), XZP331 (sense, 5'-AGAGTG-TGCCTTTGTATGGTTTAA-3') and XZP332 (antisense, 5'-CATCCTCTCTC-TACAATAGTTCGCT-3'); for *ABI2* (At5g57050), XZP333 (sense, 5'-GATGGA-AGATCTGTCTCAACGATT-3') and XZP334 (antisense, 5'-GTTTCTCCT-TCACTATCTCCTCCG-3'); for At5g27420, XZP177 (sense, 5'-CAGAGACAT-GACCCGTATGC-3') and XZP178 (antisense, 5'-GCGCCAGTACAGTGACG-TAT-3'); for *AZF1*, XZP281 (sense, 5'-AGCGGTCCGTTGTATTAA-3') and XZP282 (antisense, 5'-ACGCAAACGACTTGAAACAG-3'); and for *WRKY53*, XZP286 (sense, 5'-AATCTCCGGCATCGATAAAC-3') and XZP288 (antisense, 5'-GCCTCTCTCTGGGCTTATTC-3').

Construction of 2X35S:*LecRKA4.1*

The *LecRKA4.1* full-length cDNA was amplified using primers XZP147 (sense, 5'-ACTGCAGATGGGCACACAAAGATCCATG-3') and XZP148 (antisense, 5'-GCAGATCTCTACTGACTGATACGAGAAGTC-3'), with the underlined bases indicating the introduced restriction enzyme sites for cloning. The PCR product was digested by *Pst*I and *Bgl*II and then cloned into GZ42, which is based on pCambia1301 (B4), containing a hygromycin resistance selection marker and a 2X35S promoter of CaMV.

Construction of *P_{LecRKA4.1}*:GUS

The *LecRKA4.1* promoter fragment containing a 1.2-kb region upstream of the ATG codon and 20 bp of coding DNA downstream of ATG was amplified with primers XZP199 (sense, 5'-GGCGTCGACCTCAGTCCCATCTTTCTC-3') and XZP200 (antisense, 5'-CATGGATCCTTGTGTGCCCATGGGTG-3') using TaKaRa ExTaq (TaKaRa Bio). Underlined bases show the restriction enzyme sites for cloning. The amplified DNA fragment was digested by *Sal*I and *Nco*I and then cloned into *Sal*I and *Nco*I sites of the binary vector pCambia1301 (B4) that contains GUS and the CaMV 35S terminator. The resulting plasmid, XZ22, was then transformed into *Col* using the floral dip method (Clough and Bent, 1998). Independently transformed lines were used for GUS activity assay as described elsewhere (Dan et al., 2007).

Construction and Expression of *LecRKA4.1*-GFP, *LecRKA4.1³⁵¹⁻⁶⁸²*-GFP, and RFP

LecRKA4.1 full-length cDNA was amplified with XZP147 (sense, as described above) and XZP198 (antisense, 5'-CATCTAGACTGACTGATACGA-GAAGTCGAAGAAAC-3'), then digested by *Pst*I and *Xba*I and cloned into the pCambia3301-based binary vector GZ6 that contains GFP and the CaMV 35S promoter and terminator, resulting in the 35S:*LecRKA4.1*-GFP plasmid, designated XZ16. A cDNA fragment (1,051–1,339 bp) of *LecRKA4.1* was amplified with XZP196 (sense, as described above) and XZP193 (antisense, 5'-CTAAGCTTCGGTTGGGGATGTAATCG-3') and then digested by *Pst*I and *Hind*III. This fragment was cloned into XZ16 (digested by *Pst*I and *Hind*III), giving rise to the *LecRKA4.1³⁵¹⁻⁶⁸²*-GFP plasmid, designated XZ25. XZ16 and XZ25 were bombarded into young leaves of 35S:mCherry (RFP) lines, according to the instructions with the Bio-Rad Biolistic PDS-1000/He Particle Delivery System. The 35S:mCherry plasmid was constructed by replacing GFP in GZ6 (described above) with a cDNA fragment encoding the mCherry version of RFP (Shaner et al., 2004), which was PCR amplified from the pRSET-B mCherry plasmid kindly provided by Dr. Bo Liu (University of California, Davis). Two primers were used: the sense primer ZPP63 (5'-TATCTA-GAATGGTGAGCAAGGGCGAGGA-3') and the antisense primer ZPP64 (5'-GGGTTACTTGTACAGCTCGTCCATG-3'). Fluorescence was visualized under a Bio-Rad Radiance 2000 confocal laser scanning device (Carl Zeiss Microimaging) and analyzed using ImageJ 1.37 software (<http://rsb.info.nih.gov/ij/>), as described elsewhere (Yang et al., 2007).

Production of *lecrka4.1-1*; *LecRKA4.2* (RNAi) Transgenic Plants

The *LecRKA4.2* cDNA fragment (1–1,760 bp) was amplified with primers XZP206 (sense, 5'-TTCTTAATTAACCATGGATGCTCGTCTTCTTGCT-3') and XZP205 (antisense, 5'-AAGGATCCATTAAATGAATCTCTCTCTC-

GCGTGCAAT-3') using TaKaRa ExTaq (TaKaRa Bio). The amplified fragment was digested by *Sma*I and *Nco*I and then cloned into the pFGC5941 double-stranded RNA vector. Subsequently, this amplified fragment was digested by *Bam*HI and *Pac*I and cloned into the above resulting plasmid, giving rise to the RNAi plasmid, designated XZ20. Another RNAi plasmid, XZ21, was similarly obtained by amplifying the *LecRKA4.2* cDNA fragment (1,130–1,760 bp) with primers XZP204 (sense, 5'-AATCTAGACCATGGTCCGAGGAACTTA-TCGTCACC-3') and XZP205 (antisense, as described above). This fragment was then digested by *Sma*I-*Nco*I and *Bam*HI-*Xba*I and sequentially cloned into pFGC5941. XZ20 and XZ21 were then transformed into the *lecrka4.1-1* knock-out mutant using the floral dip method (Clough and Bent, 1998). Two representative lines, S2 and F9, respectively, from XZ21- and XZ20-transformed populations, were chosen for ABA response assays.

Network Assembly

We assembled a protein-protein interaction network using the network assembly methods as adopted previously (Chuang et al., 2007; Bromberg et al., 2008). Briefly, our network database includes the known protein interaction databases, which contain the data from yeast two-hybrid experiments, predicted interactions via orthology and cocitation (Geisler-Lee et al., 2007; http://bar.utoronto.ca/interactions/cgi-bin/arabidopsis_interactions_viewer.cgi), curation of the literature, and the recently reported interaction database derived from analysis of large-scale DNA microarray analyses (Ma et al., 2007). Three *LecRKA4* genes were mapped to their corresponding proteins in the network database. *LecRKA4.3* was not present in the assembled network database; thus, the resulting interactions were used to build the *LecRKA4.1* and *LecRKA4.2* interaction network.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At5g01540 (*LecRKA4.1*), At5g01550 (*LecRKA4.2*), and At5g01560 (*LecRKA4.3*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Seed dormancy behaviors of *lecrka4.1-1*, S2, and F9.

Supplemental Figure S2. Seed germination patterns of *lecrka4.1-1*, S2, and F9 are similar to those of the wild type in response to GA₃.

Supplemental Figure S3. Promoter activity of *LecRKA4.1* in response to ABA doses.

Supplemental Table S1. List of 70 genes in the network and their functional categories.

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