

Phenotypic Analyses of *Arabidopsis* T-DNA Insertion Lines and Expression Profiling Reveal That Multiple L-Type Lectin Receptor Kinases Are Involved in Plant Immunity

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L-type lectin receptor kinases (LecRK) are membrane-spanning receptor-like kinases with putative roles in biotic and abiotic stress responses and in plant development. In *Arabidopsis*, 45 LecRK were identified but their functions are largely unknown. Here, a systematic functional analysis was carried out by evaluating phenotypic changes of *Arabidopsis* LecRK T-DNA insertion lines in plant development and upon exposure to various external stimuli. None of the LecRK T-DNA insertion lines showed clear developmental changes, either under normal conditions or upon abiotic stress treatment. However, many of the T-DNA insertion lines showed altered resistance to *Phytophthora brassicae*, *Phytophthora capsici*, *Pseudomonas syringae*, or *Alternaria brassicicola*. One mutant defective in *LecRK-V.5* expression was compromised in resistance to two *Phytophthora* spp. but showed enhanced resistance to *Pseudomonas syringae*. *LecRK-V.5* overexpression confirmed its dual role in resistance and susceptibility depending on the pathogen. Combined analysis of these phenotypic data and *LecRK* expression profiles retrieved from public datasets revealed that *LecRK* which are hardly induced upon infection or even suppressed are also involved in pathogen resistance. Computed coexpression analysis revealed that *LecRK* with similar function displayed diverse expression patterns. Because *LecRK* are widespread in plants, the results presented here provide invaluable information for exploring the potential of *LecRK* as novel sources of resistance in crops.

Plasma membrane-localized receptor-like kinases (RLK) are critical for plants to perceive and process environmental stimuli. In *Arabidopsis*, there are over 400 plasma membrane-localized RLK with a conserved cytosolic kinase domain and divergent extracellular domains (Shiu and Bleecker 2003). Several of these RLK have been analyzed in detail and demonstrated to play essential roles in plant development or plant immunity. For example, the RLK CLAVATA1 controls meristem cell proliferation and differentiation (Dievart et al. 2003), and RLK

such as FLS2, EFR, and CERK1 recognize microbe-associated molecular patterns (MAMPs) to initiate MAMP-triggered immunity (Boller and Felix 2009). Other RLK, such as BAK1/SERK3 and SOBIR1, function as central components in regulation of various receptor-mediated signaling processes (Chinchilla et al. 2009; Liebrand et al. 2013). More insight into the function of a wider and more diverse range of RLK would help to better understand how plants interact with their environment.

Based on the divergent extracellular domains, RLK can be divided into different classes. One of the largest RLK classes in plants comprises the L-type lectin receptor kinases (LecRK). LecRK are characterized by an extracellular lectin domain structurally resembling soluble lectins of leguminous plants (Hervé et al. 1996). In *Arabidopsis*, 45 LecRK have been identified that were divided into nine clades (i.e., clade I to IX) and seven singletons (Bouwmeester and Govers 2009). Given this large number, LecRK are expected to play diverse roles in plant development and defense. Thus far, functional characterization has been limited to a few *Arabidopsis* LecRK. For example, LecRK-IV.2 (alias SGC lectin RLK) was suggested to be essential for proper pollen development because a T-DNA insertion mutant showed deformed and collapsed pollen grains (Wan et al. 2008). LecRK-V.2 (alias LecRK-b2) and three LecRK from clade VI (previously named LecRK4.1, LecRK4.2, and LecRK4.3) were shown to be involved in regulating abscisic acid (ABA) response during seed germination (Deng et al. 2009; Xin et al. 2009). Later studies also demonstrated a role for LecRK in plant immunity. LecRK-I.9, a mediator of plant cell wall-plasma membrane integrity (Gouget et al. 2006), was shown to be crucial for *Phytophthora* resistance in *Arabidopsis* (Bouwmeester et al. 2011). More recently, the same LecRK was identified as the first ATP receptor in plants and shown to be required for ATP-mediated responses (Choi et al. 2014). As yet, it is not clear if the function of LecRK-I.9 as a *Phytophthora* resistance component is linked to its function as ATP receptor. Two other LecRK, LecRK-V.5 and LecRK-VI.2, were shown to be involved in bacterial resistance by regulating stomatal immunity (Desclos-Theveniau et al. 2012; Singh et al. 2012). The latter was found to associate with the bacterial flagellin-receptor FLS2 and to be a positive regulator of the flagellin-mediated defense responses (Huang et al. 2014). LecRK-IV.3 (alias AtLPK1) might have a role in resistance to fungal pathogens because *LecRK-IV.3* overexpression in *Arabidopsis* resulted in increased resistance to *Botrytis cinerea* (Huang et al. 2013). For several other *LecRK*, a func-

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tion in plant growth or stress response was anticipated based on in silico expression analysis (Bouwmeester and Govers 2009) but, as yet, experimental evidence for this is lacking.

The objective of the current work was to elucidate the biological function of individual *LecRK* in *Arabidopsis*. *LecRK* T-DNA insertion lines were obtained and analyzed for phenotypic changes i) in plant development under normal conditions or abiotic stress and ii) upon exposure to pathogens. The phenotypic data were then integrated with in silico expression data with the aim to uncover potential associations between *LecRK* gene expression and biological function. In addition, *LecRK-V.5*, of which a mutant showed altered disease phenotypes, was selected for more detailed study to validate its function.

RESULTS

Assembling the collection

of *Arabidopsis LecRK* T-DNA insertion lines.

The T-DNA Express database of the SALK Institute Genome Analysis Laboratory was used to search for T-DNA insertion lines for all 45 *LecRK* (Table 1). Preferably, homozygous lines bearing T-DNA insertions within the coding regions were selected to enhance the possibility of complete disruption of gene function. If not available, homozygous lines with insertions in the promoter (five lines), 5' untranslated region (UTR) (14 lines), 3'UTR (two lines), or segregating lines were obtained. In total, we obtained 62 T-DNA insertion lines covering 41 *LecRK*, all in a Col-0 background. The seed of the only T-DNA insertion line available for *LecRK-IV.3* (i.e., SAIL_529_D07) did not germinate, thereby reducing the number of lines to 61, covering 40 *LecRK*. Of these 61, 52 were indicated as homozygous lines and nine as segregating lines. To confirm this, we performed polymerase chain reaction (PCR) analysis and found that 42 of the 52 lines were indeed homozygous with correct T-DNA insertion sites. Of the remaining 10 lines, eight were found to be segregating at the site of the T-DNA insertion and two (i.e., SALK_053703C and SALK_019496C) did not show a T-DNA insertion in the anticipated target gene (i.e., *LecRK-IV.1*), indicating that these lines are either untransformed Col-0 or contain a T-DNA insertion elsewhere in the genome. Therefore, in total, we ended up with 17 segregating lines. These were propagated and the offspring was genotyped to select the homozygous ones. For five lines, however, we were not able to detect any homozygous offspring among over 60 plants tested for each individual line. This was the case for the single T-DNA insertion line that was available for *LecRK-II.2* as well as for the two lines mutated in *LecRK-V.6* and the two in *LecRK-S.5*. The consistency among the two independent *LecRK-V.6* lines and the two independent *LecRK-S.5* lines suggests that a complete knockout of these genes is lethal, raising the possibility that these *LecRK* have a crucial role in development. In total, 54 lines representing 36 individual *LecRK* were confirmed as homozygous T-DNA insertion lines and used for further analysis (Table 1).

Arabidopsis LecRK T-DNA insertion lines showed no phenotypic changes in growth and development.

To determine whether *LecRK* are involved in plant growth and development, *LecRK* T-DNA insertion lines were grown in soil and analyzed for phenotypic changes in rosette size, inflorescence emergence, and flower and silique morphology for up to 8 weeks. In addition, seed were grown in vitro on vertical 0.5 Murashige Skoog (MS) plates to monitor shoot and root growth. Under these conditions, no consistent growth alterations were observed compared with Col-0 (Table 1). Previously, Wan and associates (2008) described that a homozygous mutant with two insertions in the region encoding the lectin do-

main of *LecRK-IV.2* developed abnormal siliques and they concluded that *LecRK-IV.2* is required for proper pollen development. The T-DNA insertion line *lecrk-IV.2* that we analyzed harbors a T-DNA insertion in the 5' UTR, at approximately 70 bp before the start codon. Because this line showed no defects in silique development, we analyzed the *LecRK-IV.2* expression by quantitative reverse-transcriptase (qRT)-PCR and found that the transcript level is only slightly reduced when compared with Col-0 (approximately 40%) (Supplementary Fig. S1A and B). This reduction is apparently not sufficient to disturb normal silique development. Unfortunately, the mutant analyzed by Wan and associates (2008) is no longer available in public resources, thus hampering further functional analysis of *LecRK-IV.2*.

Response of *LecRK* T-DNA insertion lines to abiotic stress.

To evaluate the role of *LecRK* in resistance to abiotic stresses, the *LecRK* T-DNA insertion lines were grown on 0.5 MS medium supplemented with 100 mM NaCl or 200 mM mannitol. Plants were checked for changes in rosette size and growth of shoots and roots. No consistent differences were observed between any of the *LecRK* T-DNA insertion lines and Col-0. Previously, Deng and associates (2009) observed that a line with a T-DNA insertion in *LecRK-V.2* showed enhanced tolerance to NaCl and mannitol and developed longer primary roots than Col-0, and concluded that *LecRK-V.2* is involved in salt and osmotic stress. In our study, *lecrk-V.2*, a T-DNA insertion line with an insertion in the 3' UTR, approximately 60 bp after the stop codon, showed growth similar to that of Col-0 upon treatment with NaCl or mannitol, also when treated using exactly the same conditions as described by Deng and associates (2009) (Table 1; Supplementary Fig. S2A and B). To find a reason for this discrepancy, we determined the *LecRK-V.2* expression in *lecrk-V.2* and Col-0 by qRT-PCR. Remarkably, the transcript level in *lecrk-V.2* was higher than in Col-0, instead of lower. Possibly the T-DNA insertion in the 3' UTR in one way or another stabilizes the mRNA and this may explain why we did not find defects in salt and osmotic stress responses. T-DNA insertion line *lecrk-V.2* was excluded from further analysis.

Response of *LecRK* T-DNA insertion lines to *Phytophthora* pathogens.

Previously, *LecRK-I.9* was reported as a novel resistance component against *Phytophthora brassicae* in *Arabidopsis* (Bouwmeester et al. 2011). To determine the involvement of other *LecRK* in *Phytophthora* resistance, we performed infection assays on the *LecRK* T-DNA insertion lines with *P. brassicae*, *P. capsici*, and *P. parasitica*. Recently it was reported that the latter two are able to infect *Arabidopsis* (Wang et al. 2011, 2013).

In order to identify *LecRK* contributing to *Phytophthora* resistance, infection assays were performed with isolates that are not able to infect *Arabidopsis* accession Col-0 (i.e., *P. brassicae* HH and *P. capsici* LT123). As shown by Bouwmeester and associates (2011), isolate HH can successfully infect *LecRK-I.9* mutants. Inoculation of *lecrk-I.9-1* and *lecrk-I.9-2* with *P. brassicae* HH resulted in water-soaked lesions at 4 days after inoculation (dpi), with a mean disease severity index (DSI) of 2.2 and 2.4, respectively (Supplementary Fig. S3A). *P. capsici* LT123 caused similar disease symptoms on these mutants and, at 4 dpi, the mean DSI was 2.0 and 1.9, respectively. This shows that *LecRK-I.9* also contributes to resistance against *P. capsici*.

Inoculation of all available *LecRK* T-DNA insertion lines with *P. brassicae* HH and *P. capsici* LT123 resulted in evident disease symptoms on several lines. For example, *lecrk-S.1*,

which contains a T-DNA insertion in the region encoding the extracellular lectin domain and has a 90% reduction in *LecRK-S.1* expression level (Fig. 1A and B), showed a mean DSI of 2.5 and 2.1 with HH and LT123, respectively (Fig. 1C). In total, 19 T-DNA insertion lines representing 13 *LecRK* from different clades showed a significantly higher DSI at 4 dpi than Col-0 (Table 1). Of these 13 *LecRK*, three are from clade V, two from clade IX, and four are singletons. The four remaining *LecRK* are from clades I, III, IV, and VIII, respectively. For four of 13, two T-DNA insertion lines are available which both showed enhanced disease severity when challenged with *Phytophthora* spp., with at least one of the two having an insertion in the coding region (Table 1). For two other *LecRK* for which two or more T-DNA insertion lines are available, T-DNA insertion lines with different insertion sites showed discrepancy in susceptibility. In the case of *LecRK-VIII.2*, with three lines

having insertions in the coding region, only *lecrk-VIII.2-3* developed rapid spreading lesions, resulting in a DSI ≥ 2 on approximately 80% of the inoculated leaves at 4 dpi. The other two lines, *lecrk-VIII.2-1* and *lecrk-VIII.2-2*, also showed disease symptoms but the disease severity was less pronounced (Table 1). In the case of *LecRK-S.7*, the two T-DNA insertion lines showed opposite responses. Line *lecrk-S.7-1* is fully resistant to both *P. brassicae* HH and *P. capsici* LT123, whereas *lecrk-S.7-2* showed clear disease symptoms with a mean DSI of 2.0 and 1.9, respectively (Table 1). Both lines have the T-DNA insertion in the 5' UTR but at different positions. Expression analysis revealed that the difference in phenotype between *lecrk-S.7-1* and *lecrk-S.7-2* correlates with *LecRK-S.7* expression levels. In *lecrk-S.7-2*, expression is reduced compared with Col-0 whereas, in *lecrk-S.7-1*, it is increased (Supplementary Fig. S4B). For the seven remaining *LecRK* of which T-DNA

Table 1. Overview of the clades and members of the *LecRK* gene family in *Arabidopsis*, the T-DNA insertion lines used in this study, and their phenotypes in response to pathogen infection and abiotic stress

Clade	Gene name ^a	Locus	T-DNA insertion line	Insertion site	Zygoty of insertion ^b			Proposed mutant name ^a	Biotic stress ^c					Plant growth ^c		Abiotic stress (seedling growth) ^c			
					According to NASC catalog	Based on our analysis	Obtained/tested		<i>P. brassicae</i>		<i>P. capsici</i>		<i>P. parasitica</i>	<i>A. brassicicola</i>	<i>Pst</i>	Plant size, inflorescence, flowering, silique size	Abiotic stress (seedling growth) ^c		
									CBS686.95 (3 dpi)	HH (4 dpi)	LT263 (3 dpi)	LT123 (4 dpi)					LT62 (4 dpi)	Pp009 (4 dpi)	MUCL20297 (4 dpi)
I	<i>LecRK-I.1</i>	At3g45330	SALK_069415C	5'UTR	>>	>	>>	<i>lecrk-I.1</i>	•	•	•	•	•	•	•	•	•	•	•
	<i>LecRK-I.2</i>	At3g45390	SAIL_847_F07	Exon	>>	>>	>>	<i>lecrk-I.2</i>	•	•	•	•	•	•	•	•	•	•	•
	<i>LecRK-I.3</i>	At3g45410	SALK_089591C	5'UTR	>>	>>	>>	<i>lecrk-I.3-1</i>	•	•	•	•	•	•	•	•	•	•	•
			SALK_025070C	5'UTR	>>	>>	>>	<i>lecrk-I.3-2</i>	•	•	•	•	•	•	•	•	•	•	•
			SALK_087804C	Exon	>>	>>	>>	<i>lecrk-I.3-3</i>	•	•	•	•	•	•	•	•	•	•	•
	<i>LecRK-I.4</i>	At3g45420	SALK_091901	Exon	>	>	>	<i>lecrk-I.4</i>	•	•	•	•	•	•	•	•	•	•	•
	<i>LecRK-I.5</i>	At3g45430	— ^d	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	<i>LecRK-I.6</i>	At3g45440	SALK_135783	Promotor	>	>	>>	<i>lecrk-I.6</i>	•	•	•	•	•	•	•	•	•	•	•
	<i>LecRK-I.7</i>	At5g60270	SAIL_717_B11	Exon	>	>	>>	<i>lecrk-I.7</i>	•	•	•	•	•	•	•	•	•	•	•
	<i>LecRK-I.8</i>	At5g60280	SALK_147846C	Promotor	>>	>>	>>	<i>lecrk-I.8-1</i>	•	•	•	•	•	•	•	•	•	•	•
SALK_066416C			Exon	>>	>>	>>	<i>lecrk-I.8-2</i>	•	•	•	•	•	•	•	•	•	•	•	
<i>LecRK-I.9</i>	At5g60300	SALK_042209	Exon	>>	>>	>>	<i>lecrk-I.9-1</i>	•	•	•	•	•	•	•	•	•	•	•	
		SALK_024581	Exon	>>	>>	>>	<i>lecrk-I.9-2</i>	•	•	•	•	•	•	•	•	•	•	•	
<i>LecRK-I.10</i>	At5g60310	SALK_088306	Exon	>	>	>>	<i>lecrk-I.10</i>	•	•	•	•	•	•	•	•	•	•	•	
<i>LecRK-I.11</i>	At5g60320	— ^d	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>LecRK-II.1</i>	At5g59260	— ^d	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>LecRK-II.2</i>	At5g59270	SALK_096198	Exon	>	>	>	<i>lecrk-II.2</i>	•	•	•	•	•	•	•	•	•	•	•	
III	<i>LecRK-III.1</i>	At2g29220	SALK_027663C	Exon	>>	>>	>>	<i>lecrk-III.1-1</i>	•	•	•	•	•	•	•	•	•	•	•
			SALK_070736C	Exon	>>	>>	>>	<i>lecrk-III.1-2</i>	•	•	•	•	•	•	•	•	•	•	•
			SAIL_K_149292	Exon	>>	>>	>>	<i>lecrk-III.1-3</i>	•	•	•	•	•	•	•	•	•	•	•
<i>LecRK-III.2</i>	At2g29250	SALK_010738	Exon	>	>	>>	<i>lecrk-III.2</i>	•	•	•	•	•	•	•	•	•	•	•	
IV	<i>LecRK-IV.1</i>	At2g37710 ^f	SALK_053703C	Promotor	>>	/	/	<i>lecrk-IV.1-1</i>	—	—	—	—	—	—	—	—	—	—	—
			SALK_019496C	Exon	>>	/	/	<i>lecrk-IV.1-2</i>	—	—	—	—	—	—	—	—	—	—	—
<i>LecRK-IV.2</i>	At3g53810	SALK_119402C	5'UTR	>>	>>	>>	<i>lecrk-IV.2-1</i>	•	•	•	•	•	•	•	•	•	•	•	
<i>LecRK-IV.3</i>	At4g02410 ^g	SAIL_529_D07	Exon	>>	Nd	Nd	<i>lecrk-IV.3</i>	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	
<i>LecRK-IV.4</i>	At4g02420	SALK_07767C	5'UTR	>>	>>	>>	<i>lecrk-IV.4-1</i>	•	•	•	•	•	•	•	•	•	•	•	
		SALK_128001C	Exon	>>	>>	>>	<i>lecrk-IV.4-2</i>	•	•	•	•	•	•	•	•	•	•	•	

Continued on following page

^a Alternative gene names and alternative mutant names are provided in Supplementary Table S1.

^b >>: homozygous; >: segregating; /: no insertion in the gene under study.

^c The response to *Phytophthora brassicae* and *P. capsici* is depicted by bars that show the disease severity index (DSI) obtained from 20 to 40 inoculated leaves in three independent experiments. The DSI scale ranges from 0 (light gray) to 4 (dark gray). An asterisk (*) indicates T-DNA insertion lines showing significantly higher or lower DSI as compared with Col-0, based on a *t* test, $P < 0.05$. Response to infection with the remaining pathogens, plant growth, and response to abiotic stress are depicted by a black dot (•) when there is no difference compared with Col-0 and by S (susceptible), R (resistant), EN (enhanced necrotic symptoms but no increased bacterial proliferation), or ES (enhanced necrotic symptoms and increased bacterial proliferation) to indicate the disease phenotype. *Pst* = *Pseudomonas syringae* pv. *tomato*. Infection assays with *P. capsici* LT62 were performed once while infection assays with all other pathogens were repeated at least three times.

^d No T-DNA insertion lines available.

^e With 10-fold higher inoculation densities, *lecrk-I.9-1* and *lecrk-I.9-2* show enhanced susceptibility to *Pseudomonas syringae* pv. *tomato*, as reported by Bouwmeester and associates (2011).

^f SALK_053703C and SALK_019496C were indicated as lines with T-DNA insertions in *At2g37710*, but in our analysis, this could not be confirmed.

^g The obtained SAIL_529_D07 line did not germinate.

^h Similar results were obtained as described by Desclos-Theveniau and associates (2012) and Singh and associates (2012).

insertion lines showed a change in phenotype in the *Phytophthora* disease assays, only a single T-DNA insertion line was available, with six (i.e., *lecrk-III.2*, *lecrk-V.4*, *lecrk-V.5*, *lecrk-V.7*, *lecrk-IX.2*, *lecrk-S.1*, and *lecrk-S.6*) having a T-DNA insertion in the coding region and one (i.e., *lecrk-V.5-3*) in the 5' UTR (Table 1).

When challenging the *LecRK* T-DNA insertion lines with *Phytophthora* isolates that are less virulent (in this case, *P. capsici* LT62 and *P. parasitica* Pp009), none of the *LecRK* T-DNA insertion lines showed lesions (Table 1). They remained resistant, similar to Col-0. This suggests that these *LecRK* are not essential to stop less virulent isolates but might function as an extra barrier for more virulent isolates.

To investigate the potential role of *LecRK* in suppressing *Phytophthora* resistance, *LecRK* T-DNA insertion lines were challenged with *Phytophthora* isolates that are capable of infecting Col-0 (i.e., *P. brassicae* CBS686.95 and *P. capsici*

LT263). Out of all tested *LecRK* T-DNA insertion lines, only *lecrk-I.7*, with an insertion in the region encoding the kinase, showed slightly smaller lesions when compared with Col-0 (Fig. 1D). All the other T-DNA insertion lines showed disease severity comparable with or slightly higher than Col-0 (Table 1).

Response of *LecRK* T-DNA insertion lines to *Pseudomonas syringae* and *Alternaria brassicicola*.

Previously, *LecRK-VI.2* was reported to play a role in resistance to bacterial pathogens (Singh et al. 2012). To determine the involvement of other *LecRK* in bacterial resistance, we performed infection assays with *Pseudomonas syringae* DC3000. Upon spray inoculation, T-DNA insertion lines of eight *LecRK* showed enhanced yellowing and necrosis at 4 dpi when compared with Col-0 (Table 1; Fig. 2A). For three of these, two T-DNA insertion lines are available which both showed enhanced symptoms when challenged with *Pseudomo-*

Table 1. Continued from previous page

Clade	Gene name ^b	Locus	T-DNA insertion line	Insertion site	Zygosity of insertion ^c			Proposed mutant name	Biotic stress					Plant growth		Abiotic stress (seedling growth)			
					According to NASC catalog	Based on our analysis	Obtained/tested		<i>P. brassicae</i> ^d		<i>P. capsici</i> ^d		<i>P. parasitica</i>	<i>A. brassicicola</i>	<i>Pst</i>	soil	0.5 MS +	200 mM mannitol	100 mM NaCl
									CBS686.95 (3 dpi)	HH (4 dpi)	LT263 (3 dpi)	LT123 (4 dpi)	LT62 (4 dpi)	Pp009 (4 dpi)	MUCL20287 (4 dpi)				
V	<i>LecRK-V.1</i>	At1g70110	SALK_071152	Promotor	>>	>>	>>	<i>lecrk-V.1</i>	■	■	■	■	■	■	■	■	■	■	
	<i>LecRK-V.2</i>	At1g70130	SALK_014678C	3'UTR	>>	>>	>>	<i>lecrk-V.2</i>	—	—	—	—	—	—	—	—	—	—	
	<i>LecRK-V.3</i>	At2g43690	SALK_013197C	5'UTR	>>	>>	>>	<i>lecrk-V.3</i>	■	■	■	■	■	■	■	■	■	■	
	<i>LecRK-V.4</i>	At2g43700	SALK_119422C	Exon	>>	>>	>>	<i>lecrk-V.4</i>	■	■	■	■	■	■	■	■	■	■	
	<i>LecRK-V.5</i>	At3g59700	SALK_133163C	5'UTR	>>	>>	>>	<i>lecrk-V.5-3</i>	■	■	■	■	■	■	■	■	■	■	
	<i>LecRK-V.6</i>	At3g59730	SALK_009623C	Exon	>>	>	>	<i>lecrk-V.6-1</i>	■	■	■	■	■	■	■	■	■	■	■
			SALK_083045C	Exon	>>	>	>	<i>lecrk-V.6-2</i>	■	■	■	■	■	■	■	■	■	■	■
	<i>LecRK-V.7</i>	At3g59740	SALK_151041	Exon	>	>	>>	<i>lecrk-V.7</i>	■	■	■	■	■	■	■	■	■	■	
	<i>LecRK-V.8</i>	At3g59750	WiscDslOx381A8	Promotor	>>	>>	>>	<i>lecrk-V.8</i>	■	■	■	■	■	■	■	■	■	■	
<i>LecRK-V.9</i>	At4g29050	SALK_092640C	5'UTR	>>	>>	>>	<i>lecrk-V.9-1</i>	■	■	■	■	■	■	■	■	■	■	■	
		SALK_009531C	Exon	>>	>>	>>	<i>lecrk-V.9-2</i>	■	■	■	■	■	■	■	■	■	■	■	
		SAIL_917_A03	Exon	>>	>>	>>	<i>lecrk-V.9-3</i>	■	■	■	■	■	■	■	■	■	■	■	
VI	<i>LecRK-VI.1</i>	At3g08870	SALK_058958C	Exon	>>	>	>>	<i>lecrk-VI.1</i>	■	■	■	■	■	■	■	■	■	■	
			SALK_077782	Exon	>>	>>	>>	<i>lecrk-VI.2-1</i>	■	■	■	■	■	■	■	■	■	■	
			SALK_070801	Exon	>>	>>	>>	<i>lecrk-VI.2-2</i>	■	■	■	■	■	■	■	■	■	■	
<i>LecRK-VI.3</i>	At5g01550	SALK_151042C	5'UTR	>>	>>	>>	<i>lecrk-VI.3-1</i>	■	■	■	■	■	■	■	■	■	■	■	
		SALK_108000C	Exon	>>	>>	>>	<i>lecrk-VI.3-2</i>	■	■	■	■	■	■	■	■	■	■	■	
		SALK_028891C	5'UTR	>>	>>	>>	<i>lecrk-VI.4-1</i>	■	■	■	■	■	■	■	■	■	■		
<i>LecRK-VI.4</i>	At5g01560	SALK_122197	Exon	>	>	>	<i>lecrk-VI.4-2</i>	■	■	■	■	■	■	■	■	■	■	■	
		SALK_093876C	Exon	>>	>>	>>	<i>lecrk-VII.1</i>	■	■	■	■	■	■	■	■	■	■		
VII	<i>LecRK-VII.2</i>	At4g28350	SALK_141841C	3'UTR	>>	>>	>>	<i>lecrk-VII.2</i>	■	■	■	■	■	■	■	■	■	■	
			SALK_092365C	Exon	>>	>>	>>	<i>lecrk-VIII.1-1</i>	■	■	■	■	■	■	■	■	■	■	
VIII	<i>LecRK-VIII.1</i>	At3g53380	SALK_056492C	Exon	>>	>>	>>	<i>lecrk-VIII.1-2</i>	■	■	■	■	■	■	■	■	■	■	
			SALK_051706C	Exon	>>	>	>>	<i>lecrk-VIII.2-1</i>	■	■	■	■	■	■	■	■	■	■	
			SALK_053278C	Exon	>>	>	>>	<i>lecrk-VIII.2-2</i>	■	■	■	■	■	■	■	■	■	■	
IX.1	<i>LecRK-IX.1</i>	At5g10530	SALK_127554C	Exon	>>	>>	>>	<i>lecrk-IX.1-1</i>	■	■	■	■	■	■	■	■	■	■	
			SALK_042414C	Exon	>>	>>	>>	<i>lecrk-IX.1-2</i>	■	■	■	■	■	■	■	■	■	■	
			SALK_111817	Exon	>>	>>	>>	<i>lecrk-IX.2</i>	■	■	■	■	■	■	■	■	■	■	
Singletons	<i>LecRK-S.1</i>	At1g15530	SALK_033248C	Exon	>>	>>	>>	<i>lecrk-S.1</i>	■	■	■	■	■	■	■	■	■	■	
	<i>LecRK-S.2</i>	At2g32800	SAIL_510_C08	Exon	>>	>>	>>	<i>lecrk-S.2</i>	■	■	■	■	■	■	■	■	■	■	
	<i>LecRK-S.3</i>	At3g46760	— ^e	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	<i>LecRK-S.4</i>	At3g55550	SALK_059967C	5'UTR	>>	>>	>>	<i>lecrk-S.4-1</i>	■	■	■	■	■	■	■	■	■	■	
			SAIL_795_F11	Exon	>>	>>	>>	<i>lecrk-S.4-2</i>	■	■	■	■	■	■	■	■	■	■	
	<i>LecRK-S.5</i>	At5g06740	SALK_088076C	5'UTR	>>	>	>	<i>lecrk-S.5-1</i>	—	—	—	—	—	—	—	—	—	—	
			SAIL_1278_B07	Exon	>	>	>	<i>lecrk-S.5-2</i>	—	—	—	—	—	—	—	—	—	—	
<i>LecRK-S.6</i>	At5g42120	SAIL_K_151974C	Exon	>>	>	>>	<i>lecrk-S.6</i>	■	■	■	■	■	■	■	■	■	■		
<i>LecRK-S.7</i>	At5g55830	SALK_140480C	5'UTR	>>	>>	>>	<i>lecrk-S.7-1</i>	■	■	■	■	■	■	■	■	■	■		
		SALK_008479C	5'UTR	>>	>>	>>	<i>lecrk-S.7-2</i>	■	■	■	■	■	■	■	■	■	■		
Col-0								■	■	■	■	■	■	■	■	■	■		

nas syringae DC3000, with at least one of the two having an insertion in the coding region (Table 1). To determine to what extent the visible symptoms are correlated with enhanced *Arabidopsis* susceptibility, bacterial colonization was quantified in rosette leaves. In line with Singh and associates (2012), we detected significantly higher bacterial titers in *lecrk-VI.2-2* compared with Col-0 (Fig. 2B). Also, in lesions on the two T-DNA insertion lines from *LecRK-IV.4*, the one from *LecRK-S.1*, and two from *LecRK-S.4*, significantly higher bacterial titers were detected and this was reproducible (Fig. 2B). Thus, these T-DNA insertion lines were scored as enhanced susceptibility (ES). The phenotype of the remaining four T-DNA insertion lines (i.e., *lecrk-IV.2*, *lecrk-V.4*, *lecrk-V.7*, and *lecrk-S.6*) was scored as enhanced yellowing and necrosis (EN) rather than ES (Table 1). This is because the bacterial titers were not consistently higher in all assays, despite the fact that the enhanced susceptibility phenotypes were reproducible.

To determine the potential involvement of *LecRK* in nonhost resistance, we tested the response of the *LecRK* T-DNA insertion lines to *Alternaria brassicicola*, a fungal pathogen that is not pathogenic on Col-0 but is capable of causing disease symptoms on the camalexin-deficient mutant *pad3* (Van Wees et al. 2003). The majority of the *LecRK* T-DNA insertion lines showed no visible symptoms up to 5 dpi. Five T-DNA insertion lines, however, representing four clade I *LecRK*, showed a slight gain of susceptibility (Table 1; Fig. 2C). Two of three *LecRK-I.3* T-DNA insertion lines showed the phenotype, one with an insertion in the exon (*lecrk-I.3-3*) and the other in the

5' UTR (*lecrk-I.3-1*). The third (i.e., *lecrk-I.3-2*), also with a 5' UTR insertion, was not infected. Also, in the case of *LecRK-I.8*, the exon mutant (*lecrk-I.8-2*) showed gain of susceptibility but not the T-DNA insertion line, with a 5' UTR insertion (*lecrk-I.8-1*).

LecRK-V.5 plays a dual role in resistance to *Pseudomonas syringae* and *Phytophthora* pathogens.

Our infection assays revealed that the mutant *lecrk-V.5-3* showed gain of susceptibility to *Phytophthora brassicae* HH and *P. capsici* LT123 (Table 1; Fig. 3A to C). In contrast, Desclos-Theveniau and associates (2012) reported that mutants of *LecRK-V.5* showed enhanced resistance toward DC3000. Because of these opposite phenotypes with respect to different pathogens, we decided to investigate the function of *LecRK-V.5* in more detail.

Gain of *Phytophthora* susceptibility in *lecrk-V.5-3* points to a role of *LecRK-V.5* in *Phytophthora* resistance. To confirm this, we generated transgenic *Arabidopsis* lines overexpressing *LecRK-V.5* in a Col-0 background. Two lines (i.e., *35S-LecRK-V.5-1/2*) with constitutive and strongly enhanced *LecRK-V.5* expression were selected (Fig. 3B). Neither of the *35S-LecRK-V.5* lines showed any visible developmental defect. Inoculation of Col-0 with *P. brassicae* CBS686.95 and *P. capsici* LT263 resulted in almost fully colonized leaves at 3 dpi (Fig. 3D). In contrast, the two *35S-LecRK-V.5* lines showed reduced lesion sizes, with a DSI of approximately 2.0 (Fig. 3D). To determine whether the reduced disease severity on the *LecRK-V.5* overex-

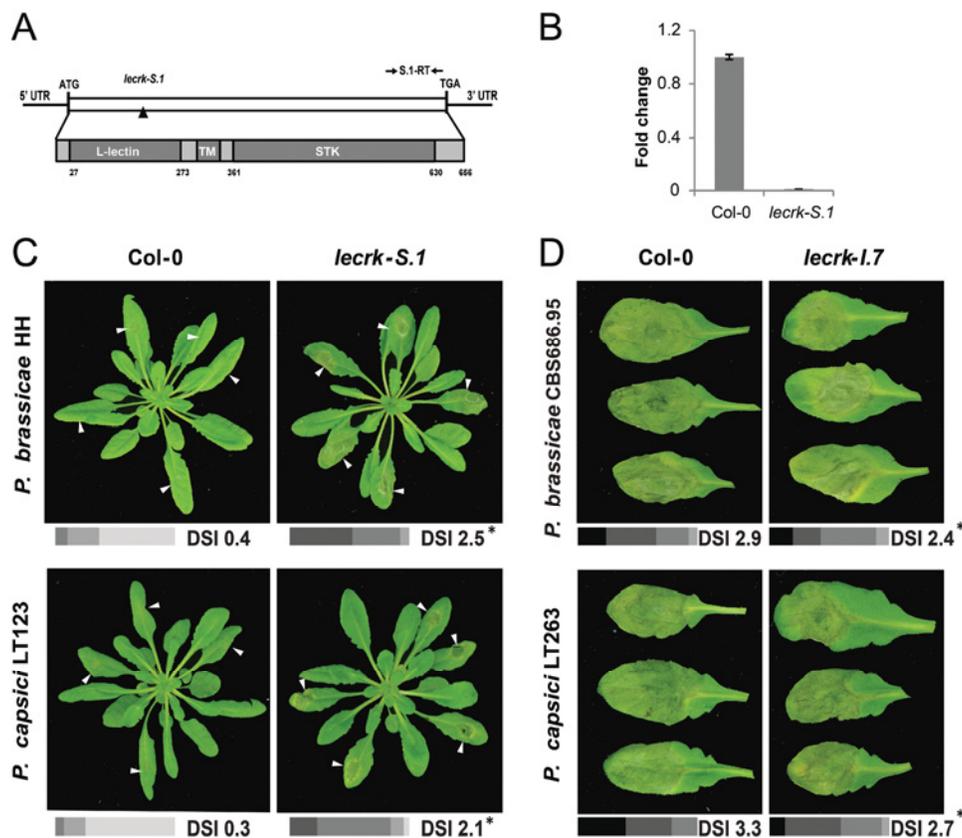


Fig. 1. Response of *Arabidopsis* *LecRK* T-DNA insertion lines to infection by *Phytophthora* spp. **A**, Schematic model representing *LecRK-S.1*. The black arrowhead points to the T-DNA insertion site in mutant *lecrk-S.1* while the head-to-head arrow pairs indicate the position of the primers (S.1-RT) used for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). **B**, Relative quantification of *LecRK-S.1* transcript levels in Col-0 and *lecrk-S.1* by qRT-PCR. **C**, Disease symptoms on Col-0 and *lecrk-S.1* at 4 days after plug-inoculation (dpi) with *P. brassicae* HH and *P. capsici* LT123. White arrowheads point to inoculated leaves. Each bar represents the disease severity index (DSI) obtained from at least 30 leaves in three independent experiments, with the number showing the average DSI and an asterisk (*) indicating a significant difference ($P < 0.05$) according to a *t* test. **D**, Disease symptoms on T-DNA insertion line *lecrk-I.7* and Col-0 inoculated with *P. brassicae* CBS686.95 and *P. capsici* LT263 at 3 dpi; * indicates significant difference in DSI ($P < 0.05$) according to a *t* test.

pression lines is indeed correlated with enhanced resistance, *Phytophthora* biomass was quantified by qRT-PCR in leaves inoculated with *P. capsici* LT263 at 3 dpi. This showed that the *P. capsici* biomass in the 35S-*LecRK-V.5* lines was reduced by at least 50% compared with Col-0 (Fig. 3E).

To confirm the role of *LecRK-V.5* in resistance to DC3000 as postulated by Desclos-Theveniau and associates (2012), we compared the phenotype of *lecrk-V.5-3* with the phenotypes of the previously analyzed mutants *lecrk-V.5-1* and *lecrk-V.5-2*. Similar to *lecrk-V.5-1* and *lecrk-V.5-2*, *lecrk-V.5-3* showed fewer necrotic symptoms and reduced bacterial proliferation compared with Col-0 (Fig. 3F and G). Moreover, both 35S-*LecRK-V.5* lines showed enhanced susceptibility to DC3000, with increased necrosis and higher bacterial titers than Col-0 (Fig. 3F to G). Taken together, these results demonstrate that *LecRK-V.5* has a dual role in regulating defense to different pathogens.

Are there associations between *LecRK* mutant phenotypes and *LecRK* expression profiles?

Expression profiling analysis has been widely used to predict potential biological functions of genes (Fukushima et al. 2012; Li et al. 2006). Here, we explored the transcriptome-to-phenotype relations by comparing *LecRK* expression profiles retrieved from publicly available microarray data with the mutant phenotypes obtained in this study (Fig. 4). Because there is no genome-wide expression data available from *Arabidopsis* inoculated with *P. brassicae* or *P. capsici*, we used the expression data derived from *Arabidopsis* leaves inoculated with *P. infestans*, a *Phytophthora* sp. that is not capable of infecting *Arabidopsis* (Huitema et al. 2003). The 13 *LecRK*, of which T-DNA insertion lines showed compromised resistance to *P. brassicae* HH and *P. capsici* LT123, were found to be differen-

tially expressed during *P. infestans* infection (Fig. 4A). *LecRK-IX.2* was highly induced and the expression level was increased up to 50-fold (\log_2 value of 5.7) at 6 h postinfection (hpi). *LecRK-I.9*, *LecRK-V.5*, and *LecRK-V.7* showed induced expression with a maximum of fourfold-increase, whereas the other nine *LecRK* were either hardly expressed or even downregulated during infection. The same holds for *LecRK-I.7*, the only *LecRK* of which the mutant showed slightly enhanced *Phytophthora* resistance.

The nine *LecRK* of which T-DNA insertion lines showed altered phenotypes in response to DC3000 (EN, ES, or resistant [R]) were all downregulated at one or more time points after inoculation with DC3000 (Fig. 4A). The five *LecRK* with T-DNA insertion lines showing significantly altered bacterial colonization (ES or R) were downregulated at 12 hpi, with \log_2 values ranging from -0.7 to -1.4.

Despite the fact that several *LecRK* were differently expressed in various tissues and in response to NaCl or mannitol treatment, none of the tested T-DNA insertion lines showed consistent phenotypic changes during growth and development or when exposed to abiotic stress. Hence, it is difficult to reconcile the biological function of these *LecRK* with expression profiles. However, lack of phenotypic changes in mutants does not exclude the possibility that certain *LecRK* have a function in these processes. It could well be that functional redundancy masks the phenotypes caused by a single gene disruption.

Genes that exhibit similar expression patterns are often expected to be involved in the same biological processes (Eisen et al. 1998; Fukushima et al. 2012). We computed coexpression profiles of all *LecRK* in leaves inoculated with *P. infestans* and DC3000 over time. In order to easily visualize *LecRK* expression patterns, expression levels (\log_2 values) were normalized by Z-score transformation (Cheadle et al. 2003). *LecRK*

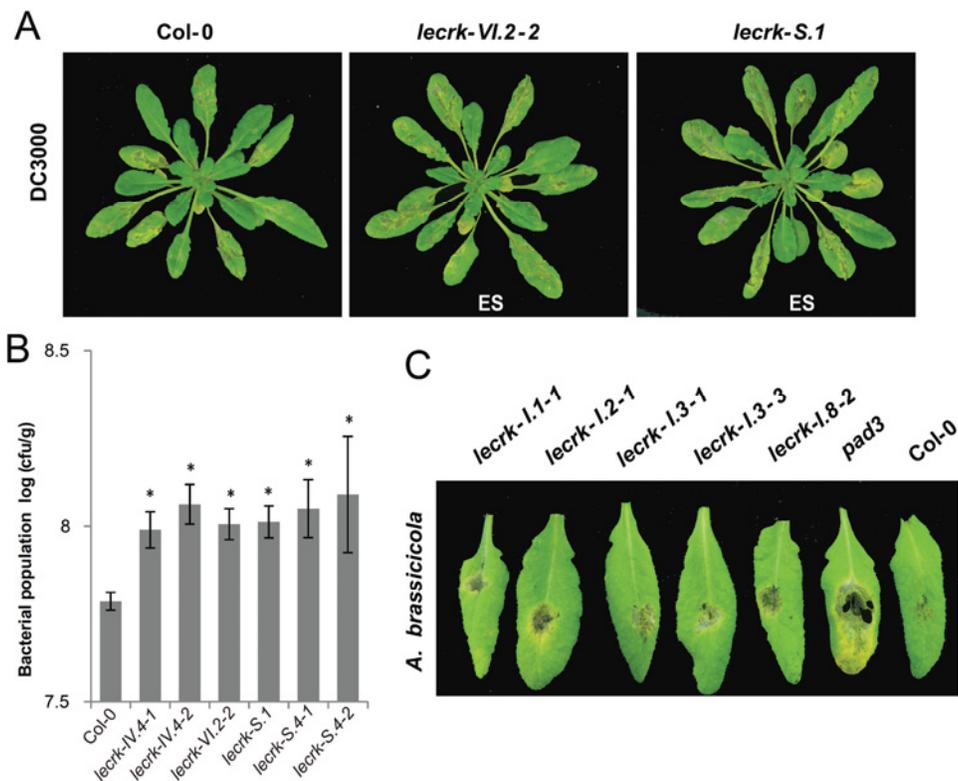


Fig. 2. Response of *Arabidopsis LecRK* T-DNA insertion lines to infection by *Pseudomonas syringae* and *Alternaria brassicicola*. **A**, Disease symptoms on Col-0, *lecrk-S.1*, and *lecrk-VI.2-2* 4 days after inoculation (dpi) with *P. syringae* DC3000 (10^8 CFU ml^{-1}). **B**, Bacterial growth in Col-0 and various *LecRK* T-DNA insertion lines at 3 dpi. Bars depict mean bacterial titers of four plants (\pm standard deviation); * indicates $P < 0.05$ according to a *t* test for comparisons between Col-0 and *LecRK* T-DNA insertion lines. Experiments were repeated three times with similar results. **C**, Disease symptoms on Col-0, *pad3* and various *LecRK* T-DNA insertion lines 5 dpi with *A. brassicicola* MUCL20297.

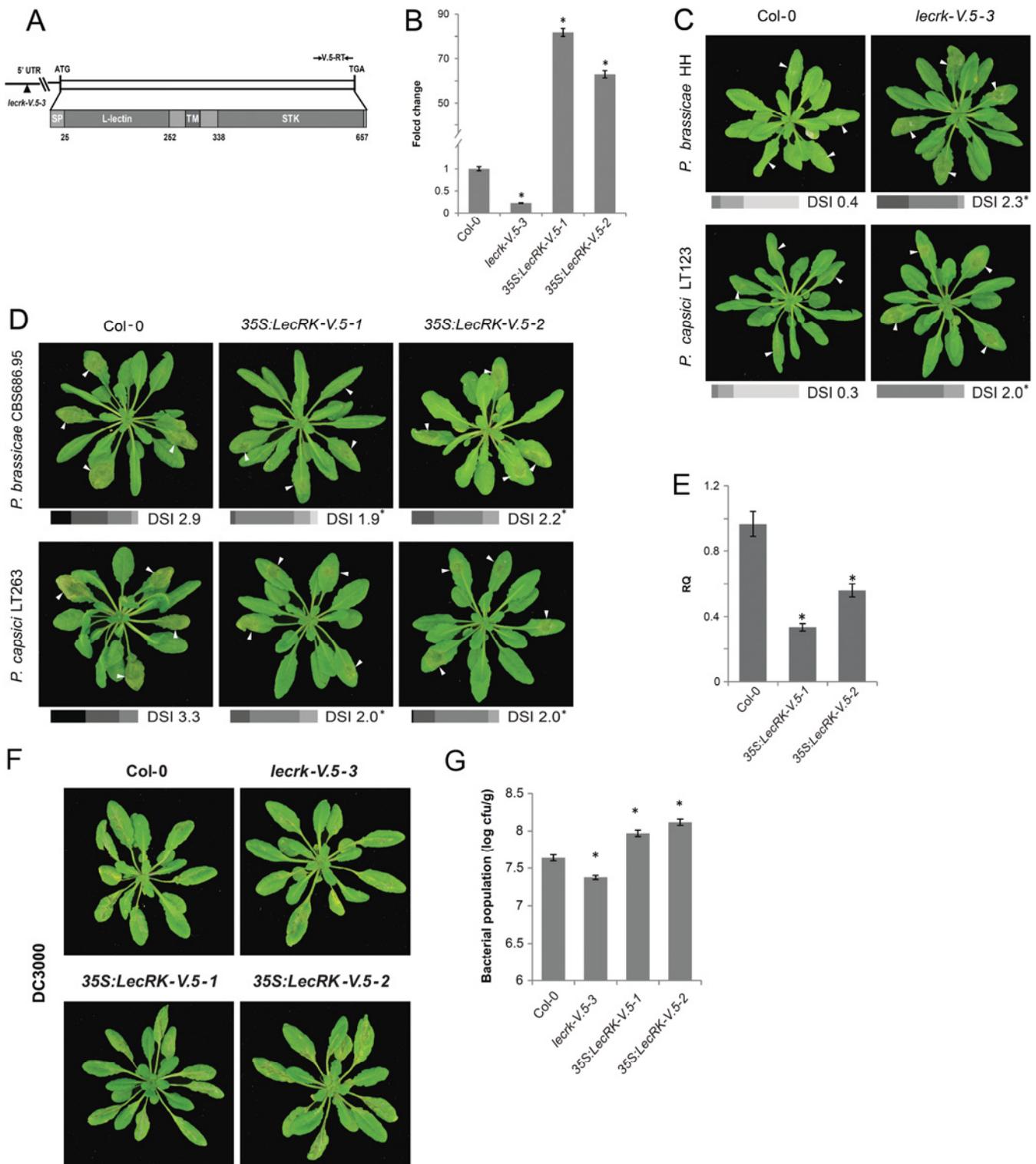


Fig. 3. LecRK-V.5 plays opposite roles in resistance to *Phytophthora* spp. and *Pseudomonas syringae*. **A**, Schematic model representing LecRK-V.5. The black arrowhead points to the T-DNA insertion site in *lecrk-V.5-3* while the head-to-head arrow pairs indicate the position of the primers (V.5-RT) used for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). **B**, qRT-PCR analysis of *LecRK-V.5* transcript levels in Col-0, *lecrk-V.5-3*, and two overexpression lines; * indicates a significant difference ($P < 0.05$) according to a *t* test. **C**, Disease symptoms on Col-0 and *lecrk-V.5-3* 4 days after inoculation (dpi) with *Phytophthora brassicae* HH and *P. capsici* LT123. White arrowheads point to inoculated leaves. Each bar represents the disease severity index (DSI) obtained from at least 30 leaves in three independent experiments, with the number showing the average DSI and an asterisk (*) indicating a significant difference ($P < 0.05$) according to a *t* test. **D**, Disease symptoms on Col-0 and two *LecRK-V.5* overexpression lines 3 dpi with *P. brassicae* CBS686.95 and *P. capsici* LT263. **E**, Relative quantification of *P. capsici* biomass (RQ) in Col-0 and overexpression lines at 3 dpi by qRT-PCR. Bars represent mean values (\pm standard deviation [SD]) relative to that of infected Col-0 leaves; * indicates significantly less biomass ($P < 0.05$), according to a *t* test. Experiments were repeated twice with similar results. **F**, Disease symptoms on Col-0, *lecrk-V.5-3*, and two overexpression lines 4 dpi with *Pseudomonas syringae* DC3000. **G**, Bacterial growth in Col-0, *lecrk-V.5-3*, and two overexpression lines at 3 dpi. Bars shown are mean values (\pm SD); * indicates significantly lower or higher bacterial titers ($P < 0.05$) according to a *t* test. Experiments were repeated twice with similar results.

with similar expression patterns were grouped together using hierarchical clustering with Pearson's correlation coefficient (r) and average linkage. Pearson's r is a metric to evaluate the strength of the correlation between variables, ranging from -1 to 1 (Usadel et al. 2009); therefore, we used the cut-off value of $r \geq 0.6$ for clustering which, in general, indicates strong positive correlation (Aoki et al. 2007; Fukushima et al. 2012). With this criterion, *LecRK* could be grouped into five clusters based on their expression patterns upon infection with *P. infestans* (Fig. 4C) and another five clusters upon infection with DC3000 (Fig. 4D). Of the 14 *LecRK* of which T-DNA insertion lines showed altered susceptibility to *Phytophthora* spp., five were grouped in cluster II and showed a slight reduction in expression between 6 and 24 hpi. The four *LecRK* that grouped in cluster V showed an opposite expression pattern compared with *LecRK* in cluster II. Of the remaining *LecRK*, two are in cluster I (i.e., *LecRK-I.9* and *LecRK-S.1*), two in cluster III (i.e., *LecRK-S.4* and *LecRK-S.7*), and one in cluster IV (i.e., *LecRK-III.2*). Similar transcriptome-to-phenotype patterns were observed upon challenging with DC3000 (Fig. 4D). Of the five *LecRK* of which T-DNA insertion lines showed altered susceptibility to DC3000, *LecRK-V.5* and *LecRK-VI.2* are combined in cluster I, *LecRK-S.1* and *LecRK-S.4* are in cluster II, while *LecRK-IV.4* was allocated to cluster III. When combining the expression patterns of the two time courses obtained from leaves inoculated with the two different pathogens, 27 of the 45 *LecRK* grouped in clusters, 11 in total, while the remaining 18 did not share their expression pattern with any other *LecRK* (Fig. 4B). Although the four *LecRK* that are implicated in resistance to both *Phytophthora* pathogens and DC3000 do not group in one cluster and are far apart in the dendrogram, the clustering of two and three relevant *LecRK* is maintained (i.e., *LecRK-V.4* and *LecRK-VIII.2* and the combination of *LecRK-V.5*, *LecRK-VI.2*, and *LecRK-IX.2*).

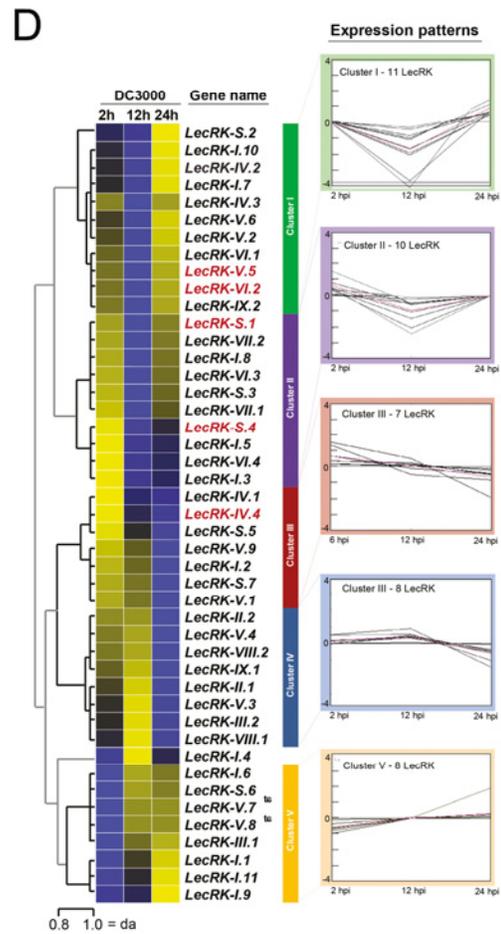
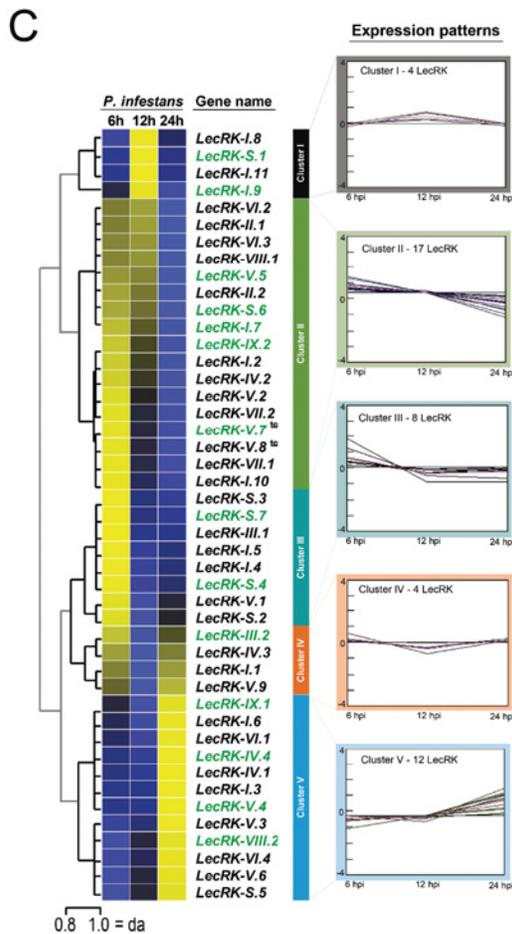
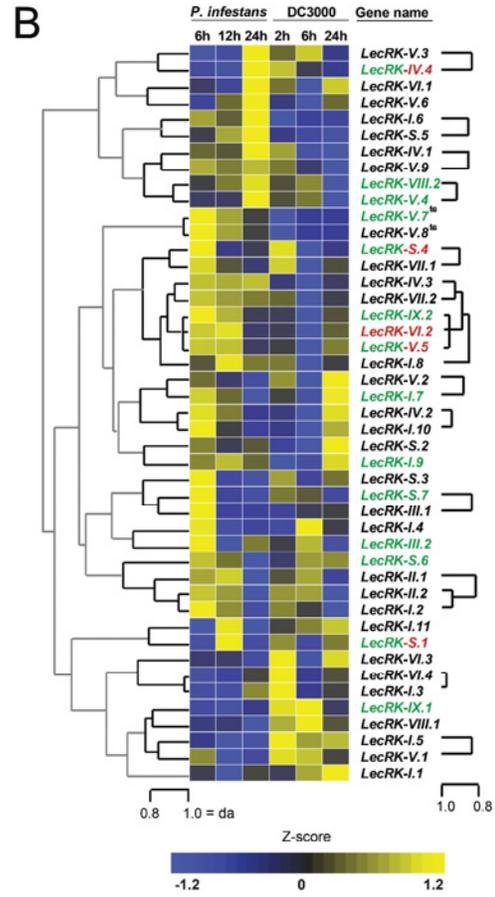
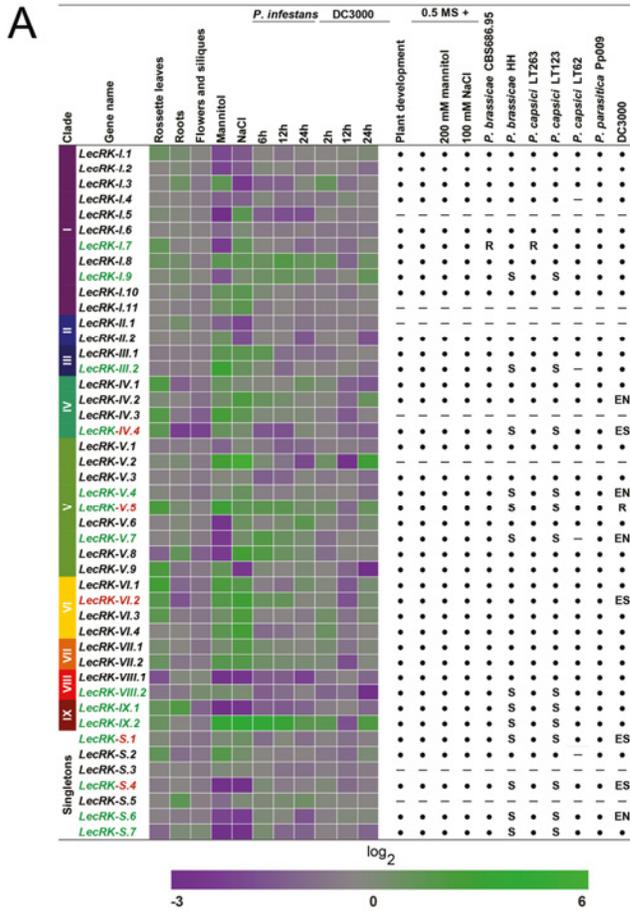
DISCUSSION

Most of the plant immune receptors that have been identified to date are either plasma membrane-localized receptors, including RLK and receptor-like proteins (RLP, or intracellular nucleotide-binding leucine-rich repeat proteins (NLR) (Spoel and Dong 2012). Most were discovered based on their ability to activate defense responses upon recognition of MAMPs or pathogen effectors. For example, the *RLK* gene encoding the MAMP receptor FLS2 was isolated by a forward genetics approach based on screening random mutants for flagellin-insensitive mutants, followed by map-based cloning of the locus harboring the mutation (Gómez-Gómez and Boller 2000). Also, the isolation of several NLR genes that function as major resistance genes in gene-for-gene interactions was the result of combining phenotype (i.e., pathogen recognition) and map-based cloning; in this case, facilitated by linkage mapping using segregating progenies of resistant and susceptible parents (Marone et al. 2013; Rodewald and Trognitz 2013). Genome sequencing has revealed that defense-related RLK and NLR often have many homologs of which the function is unknown. They are grouped into families and subfamilies based on sequence similarity and shared domain architecture.

In this study, we focused on one such RLK subfamily in *Arabidopsis*, namely, the *LecRK* family, of which three of 45 members were previously identified as critical players in *Arabidopsis* innate immunity. Two of the three were selected for functional studies based on their increased expression in plants treated with priming agent BABA (*LecRK-VI.2*) (Singh et al. 2012) or oligogalacturonic acid (*LecRK-V.5*) (Desclos-Theveniau et al. 2012). The third (*LecRK-I.9*) was selected via a phage display aimed at identifying RGD-binding pro-

teins in plants (Gouget et al. 2006) and appeared to be a potential host target of a *P. infestans* RXLR effector (*LecRK-I.9*) (Bouwmeester et al. 2011). We anticipated that functional screening of other members of the *LecRK* family would reveal several novel candidates with a role in disease resistance. In an unbiased strategy, we monitored all available *LecRK* T-DNA insertion lines and, indeed, in addition to *LecRK-I.9*, *LecRK-V.5*, and *LecRK-VI.2* mentioned above, we found 16 additional *LecRK* of which mutants showed phenotypic changes in response to pathogens. T-DNA insertion lines are effective tools for determining gene function and linking genotype to phenotype (Lloyd and Meinke 2012; O'Malley and Ecker 2010). In other studies, similar unbiased screenings of T-DNA insertion line collections resulted in novel insight into gene function. An example is the study of ten Hove and associates (2011), who performed large-scale screening of T-DNA insertion lines of root-expressed leucine-rich repeat RLK and identified RLK implicated in hormone and abiotic stress responses. In a similar way, Wang and associates (2008) performed a screening of a broad range of T-DNA insertion lines in 56 genes encoding RLP and assigned functions to these RLP in plant development and resistance. It should be noted that the success rate in these two studies was relatively low; only a few T-DNA insertion lines were found that could be linked to the phenotype of interest. This could well be due to redundancy in function among members of these subfamilies. When one gene is inactivated because of the T-DNA insertion, another family member might take over its function, resulting in the wild-type phenotype. For example, SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (*SERK1*) and the closest homolog *SERK2* were concluded to function redundantly in controlling anther development and male gametophyte maturation based on the observation that the single mutants *serk1* and *serk2* did not show any developmental defects, whereas *serk1serk2* double mutants were defective in pollen development (Colcombet et al. 2005). This implies that such screenings, including ours, are by definition not conclusive for all members of the family; the ones of which mutants don't show a phenotype could still have an important role. Another issue to keep in mind is the variation in knock-out efficiency among mutants with a T-DNA insertion in the same gene. According to a survey on 1,084 published *Arabidopsis* insertion lines, an insertion in the coding region leads to inactivation of the target gene in over 98% of the cases; in mutants with an insertion before the start codon or after the stop codon, this percentage decreases but is still high (86 and 75%, respectively) (Wang 2008). Variation in knock-out efficiency might explain why, in some cases, we found differences in phenotype among T-DNA insertion lines of the same gene such as, for example, the three *LecRK-I.3* lines. In one case, we analyzed expression of the target gene (i.e., *LecRK-IV.2*), and we, indeed, detected residual expression in a mutant that contains a T-DNA insertion in the 5' UTR, and no change in phenotype, whereas a line with insertions in the exon of that same gene showed a phenotype (Wan et al. 2008). In the case of *LecRK-V.2*, we even found that a T-DNA insertion in the 3' UTR led to increased transcript levels of the target gene, a phenomenon that was also noted in the survey of Wang (2008). Thus, it is not surprising that we could not confirm the phenotype of a *LecRK-V.2* mutant observed by Deng and associates (2009). Because we have not systematically checked all T-DNA insertion lines for expression, there is a chance that some of them are not true mutants, in the sense that they lost gene function. This implies that *LecRK* with T-DNA insertion lines showing no phenotype might still be relevant for pathogen resistance.

In the unbiased strategy that we used here, successful identification of mutants is highly dependent on the design and



methodology of the phenotypic screening. In our screening, none of the *LecRK* T-DNA insertion lines showed phenotypic changes upon exposure to mannitol or NaCl, despite the fact that multiple *LecRK* are induced during abiotic stress. It could well be that the conditions that we used for screening were not optimal. We only tested one concentration of mannitol and one of NaCl instead of a whole range of concentrations. When screening for disease resistance, the choice of pathogen isolates is extremely important. In the large-scale screening performed by Wang and associates (2008), none of the four *RLP30* T-DNA insertion lines showed altered susceptibility toward the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *B. cinerea*. Recently, however, it was found that two of these *RLP30* T-DNA insertion lines showed increased susceptibility to *S. sclerotiorum* and *B. cinerea*, which is likely due to the fact that Zhang and associates (2013) used other isolates that are more virulent. In our screening, we choose to use different *Phytophthora* spp. and different isolates next to each other. The *P. capsici* and *P. brassicae* isolates were selected based on their differential behavior on *Arabidopsis* (Roetschi et al. 2001; Wang et al. 2013). We confirmed the role of LecRK-I.9 in resistance to *P. brassicae* and showed that this LecRK also inhibits *P. capsici*. In addition, we found several T-DNA insertion lines representing 12 *LecRK* with the same phenotype as *lecrk-I.9-1* and *lecrk-I.9-2* (i.e., compromised resistance to both *Phytophthora* spp.). For four of these LecRK (i.e., LecRK-IV.4, LecRK-VIII.2, LecRK-IX.1, and LecRK-S.4), two or more independent T-DNA insertion lines showed consistent gain of susceptibility, demonstrating that they function as *Phytophthora* resistance components. For another one (i.e., LecRK-V.5), we confirmed the mutant phenotype by showing that overexpression of *LecRK-V.5* enhances *Phytophthora* resistance. For the remaining ones, we have to be more cautious; in the phenotypic analysis, only a single T-DNA insertion line was included (the only one available) or, in the case of LecRK-S.7, only one of the two lines was a reliable mutant. Therefore, for these LecRK, only a potential role in resistance against *Phytophthora* pathogens can be suggested. With respect to *P. brassicae* and *P. capsici*, *LecRK* T-DNA insertion lines showed no specificity, suggesting that LecRK do not discriminate between *Phytophthora* spp. Overall, the T-DNA insertion lines are more susceptible to *P. brassicae* HH than to *P. capsici* LT123 and less susceptible to *P. brassicae* CBS686.95 than to *P. capsici* LT263. None of the *LecRK* T-DNA insertion lines was infected by the less virulent isolates *P. capsici* LT62 and *P. parasitica* Pp009. These two isolates are probably too weak to reach the stage at which LecRK come into play as a barrier for infection.

Four of the LecRK that have a function in *Phytophthora* resistance were also found to have a role in the interaction of *Arabidopsis* with the bacterial pathogen DC3000. Three of these promote resistance, suggesting that they can monitor dif-

ferent types of pathogens and potentially confer a broad-spectrum resistance. The fourth one, however, *LecRK-V.5*, was found to have opposite roles in these two pathosystems (i.e., a resistance component against *Phytophthora* pathogens but a susceptibility factor for DC3000). One explanation for this dual role is that *Phytophthora* spp. and bacteria have different modes to enter host tissues. Bacteria usually penetrate via natural opening such as stomata, and stomatal closure can essentially restrict bacterial invasion (Melotto et al. 2006). *Phytophthora* pathogens can also penetrate via stomata but, instead, they prefer to penetrate at the junctions between epidermal cells (Hardham 2007; Wang et al. 2011). Therefore, the function of *LecRK-V.5* in suppressing stomatal immunity is dispensable for preventing *Phytophthora* penetration. In addition, *LecRK-V.5* was found negatively regulating the ABA response in *Arabidopsis* (Desclos-Theveniau et al. 2012). ABA plays a multifaceted role in regulating plant resistance (Ton et al. 2009). On the one hand, ABA enhances resistance against bacterial pathogens by regulating stomatal closure; on the other hand, ABA compromises defense to various pathogens via suppression of salicylic acid-signaling (Cao et al. 2011; Mohr and Cahill 2007; Sanchez-Vallet et al. 2012), a process that in *Arabidopsis* is essential for resistance to *P. capsici* (Wang et al. 2013). Hence, the gain of susceptibility found in *lecrk-V.5-3* upon *Phytophthora* infection might be linked to ABA signaling.

Previously, LecRK-VI.2 was found to be required for resistance to various bacterial pathogens but not to the fungal pathogen *B. cinerea* (Huang et al. 2014; Singh et al. 2013). In our study, we confirmed the role of LecRK-VI.2 in bacterial resistance and found that it is not required for resistance to either *Phytophthora* pathogens or the fungal pathogen *A. brassicicola*. This specificity could be explained by the recent finding that LecRK-VI.2 associates with FLS2 and participates in flg22-mediated defense (Huang et al. 2014), which is essential for bacterial pathogen resistance but might be dispensable for resistance to *Phytophthora* or fungal pathogens.

Considering the fact that the primary sequence of a protein is a prime determinant of biological function, LecRK with high sequence similarity might share similar biological function. This might be the case for the four LecRK in clade I of the T-DNA insertion lines that showed compromised resistance to *A. brassicicola*. This clustering was not observed for LecRK involved in *Phytophthora* or DC3000 resistance. They are dispersed over different clades and, thus, sequence similarity as such does not account for a similar function. Moreover, the computed expression profiling revealed that LecRK with similar function displayed rather different expression patterns upon pathogen infection and, therefore, expression is also not a determinant of the functional similarity among *LecRK*.

In summary, phenotypic analysis of a large set of T-DNA insertion lines uncovered multiple *Arabidopsis* LecRK with



Fig. 4. Compilation of expression profiles of *LecRK* and phenotypes of *LecRK* T-DNA insertion lines. **A**, The heat map in the left panel shows *LecRK* expression levels in different plant tissues and in response to various biotic and abiotic stresses. Signal intensity \log_2 ratios are color-coded in the range of -3 (purple) to 6 (green), as indicated in the scale bar, and represent the relative expression levels compared with corresponding controls. The table on the right shows the phenotypes of the *LecRK* T-DNA insertion lines, with 'R' standing for resistant, 'S' for susceptible, 'EN' for enhanced necrotic symptoms but no increased bacterial proliferation, and 'ES' for enhanced necrotic symptoms and increased bacterial proliferation; a black dot (●) indicates no consistent phenotypic changes and – indicates not tested. **B** to **D**, Hierarchical clustering of *LecRK* based on expression patterns at **C**, 6, 12, and 24 h after inoculation with *Phytophthora infestans*; **D**, 2, 6, and 24 h after inoculation with *Pseudomonas syringae* DC3000; or **B**, combined using Pearson's correlation coefficient (r) of per gene Z-score transformed \log_2 values (sample relative to control). Clusters were created using a cut-off value of $r \geq 0.6$. **C**, and **D**, Clusters are indicated by vertical colored bars connected to panels showing *LecRK* expression patterns in each cluster. The lengths of the branches (da) are the scaled (black lines) and unscaled (gray lines) average Pearson correlation distances ($dp = 2da = 1 - r$). Normalized expression data are color coded to indicate higher (yellow, saturated at 1.2) and lower (blue, saturated at -1.2) expression relative to the respective controls. On the microarray, the same probes represent *LecRK-V.7* and *LecRK-V.8* and, thus, the expression values are identical. **A** to **D**, *LecRK* involved in *Phytophthora* resistance (S or R) are depicted in green and those involved in DC3000 resistance (ES or R) in dark red. *LecRK* involved in both are depicted in combined green-red.

thus-far-unknown function that contribute to defense against pathogens, thus pointing to LecRK as potential immune receptors. The incentive for this screening was the finding that LecRK-I.9 is a *Phytophthora* resistance component and, as importantly, that transgenic potato lines expressing *Arabidopsis* *LecRK-I.9* are more tolerant to *P. infestans*, the causal agent of potato late blight (Bouwmeester et al. 2014). This suggests that LecRK can be exploited to confer resistance in crops. Because LecRK are widespread in the plant kingdom, understanding the function of LecRK in *Arabidopsis* will facilitate the functional analysis of LecRK in crop species, and this will be helpful in exploring novel resistance sources against damaging plant pathogens. Further studies should reveal how the different LecRK are activated, what their ligands are, and how they feed into downstream signaling and activate defense. The overall importance of LecRK in plants is demonstrated by the recent finding that LecRK-I.9 (alias DORN1) is capable of binding extracellular ATP (Choi et al. 2014). It is the first eATP receptor identified in plants and has a completely different structure than eATP receptors in animals. The collection of *LecRK* T-DNA insertion lines described in this study will be instrumental to further explore the role of LecRK as eATP receptors.

MATERIALS AND METHODS

Analysis of T-DNA insertion lines.

LecRK T-DNA insertion lines were obtained from the European *Arabidopsis* Stock Center (Alonso et al. 2003). T-DNA insertion sites and homozygosity were checked by PCR using T-DNA primers LBB1.3 (SALK lines), LB1 (SAIL lines), or p745 (WiscDsLox lines) in combination with gene-specific primers.

Plant growth conditions.

Arabidopsis seed were sown on soil or in vitro on 0.5 MS medium, stratified in the dark at 4°C for 3 days, and subsequently placed in a growth chamber at 19 to 21°C with 75 to 78% relative humidity and a 12-h photoperiod. Alternatively, seed were grown on vertically orientated 0.5 MS plates or 0.5 MS plates supplemented with 100 mM NaCl or 200 mM mannitol or under the same conditions as described previously (Deng et al. 2009). Seedling growth was evaluated from the 7th to 16th day after seed sowing. Each treatment included at least three replicates and the experiment was repeated four times.

Pathogen growth and infection assays.

P. brassicae, *P. capsici*, and *P. parasitica* were grown in the dark on 20% (vol/vol) V8 agar plates at appropriate temperatures. Infection assays on *Arabidopsis* with fresh mycelial plugs were carried out as described previously (Bouwmeester et al. 2011). Disease symptom scoring using a DSI and the *Phytophthora* biomass quantification were conducted as described by Wang and associates (2013).

Pseudomonas syringae DC3000 was grown on King's B medium supplemented with rifampicin at 50 µg ml⁻¹. *Arabidopsis* plants were spray inoculated with bacterial suspensions with a concentration of 10⁸ CFU ml⁻¹ in 10 mM MgSO₄ and 0.02% (vol/vol) Silwet-L77, or mock treated with 10 mM MgSO₄ and 0.02% (vol/vol) Silwet-L77. Bacterial growth was determined at 3 dpi. Whole rosettes were collected and measured for fresh weight and subsequently homogenized in 10 mM MgSO₄. Homogenates were serially diluted in 10 mM MgSO₄ and were plated on *Pseudomonas* agar (BD Biosciences) containing rifampicin at 50 µg ml⁻¹. Bacterial growth was evaluated by counting bacterial CFU 2 days after incubation

at 28°C and normalized as CFU per gram using the total weight of the inoculated rosette leaves. This experiment was repeated three times, each with at least three replicates.

A. brassicicola MUCL20297 was cultured in darkness at 22°C on potato dextrose agar. Four- to five-week-old *Arabidopsis* rosette leaves were drop inoculated with 5 µl of spore suspensions (10⁶ spores ml⁻¹) or mock inoculated with 5 µl of water. Plants were placed in trays covered with lids and kept in the dark during the first 24 h. Thereafter, they were moved to a climate chamber with the aforementioned settings. Disease symptoms were checked from 3 to 5 dpi.

Plasmid construction and plant transformation.

The coding sequence of LecRK-V.5 was amplified by PCR using Pfu DNA polymerase (Promega) with primers listed in Supplementary Table S2. Purified PCR products were cloned into the vector pENTR/D-TOPO (Invitrogen). Plasmids with the correct sequence were recombined by LR reaction into the binary vector pGWB2. The resulting vector was transformed into *Agrobacterium tumefaciens* GV3101. *Arabidopsis* Col-0 plants were transformed using the floral dip method, as previously described (Zhang et al. 2006).

RNA isolation and qRT-PCR.

Total RNA was isolated from 4-week-old *Arabidopsis* leaves with a NucleoSpin RNA plant kit (Macherey-Nagel) and thereafter used as the template for cDNA synthesis with an oligo-dT primer using an M-MLV reverse transcriptase kit (Promega). qRT-PCR was performed using an ABI7300 real-time PCR system (Applied Biosystems). Data were analyzed using a 2^{-ΔΔCT} method (Livak and Schmittgen 2001). Transcript levels of *LecRK* were normalized with *Arabidopsis Actin2* and expressed as mean fold changes (±standard deviation) relative to that in Col-0 leaves, which was arbitrarily set as 1.

Expression data analysis.

LecRK expression data during plant growth or upon treatment with different stimuli were obtained from the public microarray datasets using eFP-Browser at the Bio-Array Resource (Winter et al. 2007). Signal intensity log₂ ratios of the samples relative to respective controls were used as a measure for *LecRK* expression. Of the datasets sampled from *Arabidopsis* in different growth stages and exposed to abiotic stresses, the maximal (highest or lowest) signal intensity relative to the mean throughout all arrays in the database was retrieved. Expression data of *Arabidopsis* treated with biotic stresses were derived from 5-week-old *Arabidopsis* Col-0 rosette leaves inoculated with *Phytophthora infestans* or infiltrated with DC3000. *LecRK-V.7* (*At3g59740*) and *LecRK-V.8* (*At3g59750*) share the same probe-set and obtained the same values. For expression pattern analysis, per-gene log₂ values were normalized by the Z-score method according to Cheadle and associates (2003). Coexpression analysis of *LecRK* was conducted using hierarchical clustering with Pearson's correlation coefficient (*r*) and average linkage (da), as implemented in MultiExperiment Viewer (MeV4.0).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

SALK Institute Genome Analysis Laboratory: signal.salk.edu
 European *Arabidopsis* Stock Center: Arabidopsis.info
 Bio-Array Resource: www.bar.utoronto.ca