

A Novel Plant Leucine-Rich Repeat Receptor Kinase Regulates the Response of *Medicago truncatula* Roots to Salt Stress ^W

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In plants, a diverse group of cell surface receptor-like protein kinases (RLKs) plays a fundamental role in sensing external signals to regulate gene expression. Roots explore the soil environment to optimize their growth via complex signaling cascades, mainly analyzed in *Arabidopsis thaliana*. However, legume roots have significant physiological differences, notably their capacity to establish symbiotic interactions. These major agricultural crops are affected by environmental stresses such as salinity. Here, we report the identification of a leucine-rich repeat RLK gene, *Srlk*, from the legume *Medicago truncatula*. *Srlk* is rapidly induced by salt stress in roots, and RNA interference (RNAi) assays specifically targeting *Srlk* yielded transgenic roots whose growth was less inhibited by the presence of salt in the medium. Promoter- β -glucuronidase fusions indicate that this gene is expressed in epidermal root tissues in response to salt stress. Two *Srlk*-TILLING mutants also failed to limit root growth in response to salt stress and accumulated fewer sodium ions than controls. Furthermore, early salt-regulated genes are downregulated in *Srlk*-RNAi roots and in the TILLING mutant lines when submitted to salt stress. We propose a role for *Srlk* in the regulation of the adaptation of *M. truncatula* roots to salt stress.

INTRODUCTION

Plants are affected by different environmental conditions, such as cold, drought, and soils with changing salt and nutrient concentrations. Salinity is one of the most important abiotic stresses for crop productivity, and the amount of land affected by salinity is increasing (Wang et al., 2003). Salt stress induces various complex biochemical, molecular, cellular, and physiological changes in plants (Wang et al., 2003; Tuteja, 2007; Munns and Tester, 2008). The fact that related plant genotypes showed large variations in their response to abiotic stresses suggests that activation of specific genes may lead to major changes in their adaptive responses to cope with unfavorable environmental conditions (de Lorenzo et al., 2007).

Because approximately one-third of the food required to feed the world's population depends on industrially produced nitrogen fertilizer, legumes are becoming a strategically important crop for grain and forage purposes (Smil, 1997; Graham and Vance, 2003). Indeed, these plants have the capacity to establish root symbioses with nitrogen-fixing bacteria commonly known as

rhizobia, resulting in the formation of highly specialized root nodules where nitrogen fixation takes place (Limpens and Bisseling, 2003; Jones et al., 2007). Legume production is greatly constrained by numerous abiotic stresses, which can directly affect root growth and symbiotic interactions (Sharma and Lavanya, 2002; Morón et al., 2005). Understanding the different mechanisms by which these plants perceive and react to environmental stresses may lead to novel strategies for crop improvement.

In plants, a diverse group of cell surface receptor-like protein kinases (RLKs) play a fundamental role in sensing external signals and regulating gene expression responses at the cellular level (Stone and Walker, 1995; Lease et al., 1998). The multiplicity of external stimuli perceived by plants may be linked to the large number of RLK genes (at least 610 members in *Arabidopsis thaliana*; Torii, K.U., 2004). Members of this plant family are known to play roles in plant growth and development, plant defense responses against pathogens (Becraft, 2002; Shiu et al., 2004), and legume symbiotic interactions (Stacey et al., 2006). Nevertheless, few RLKs to date have been implicated in abiotic stress. The *Arabidopsis LecRK2* receptor containing an extra-cellular lectin-like domain is salt responsive and regulated by ethylene signaling (He et al., 2004). The *Arabidopsis* cell wall-associated RLK gene *WAK4* is regulated differentially by various biotic and abiotic factors and plays a vital role in cell elongation (Lally et al., 2001). More conclusively, using T-DNA insertion mutants and overexpressing and antisense plants, the RPK1

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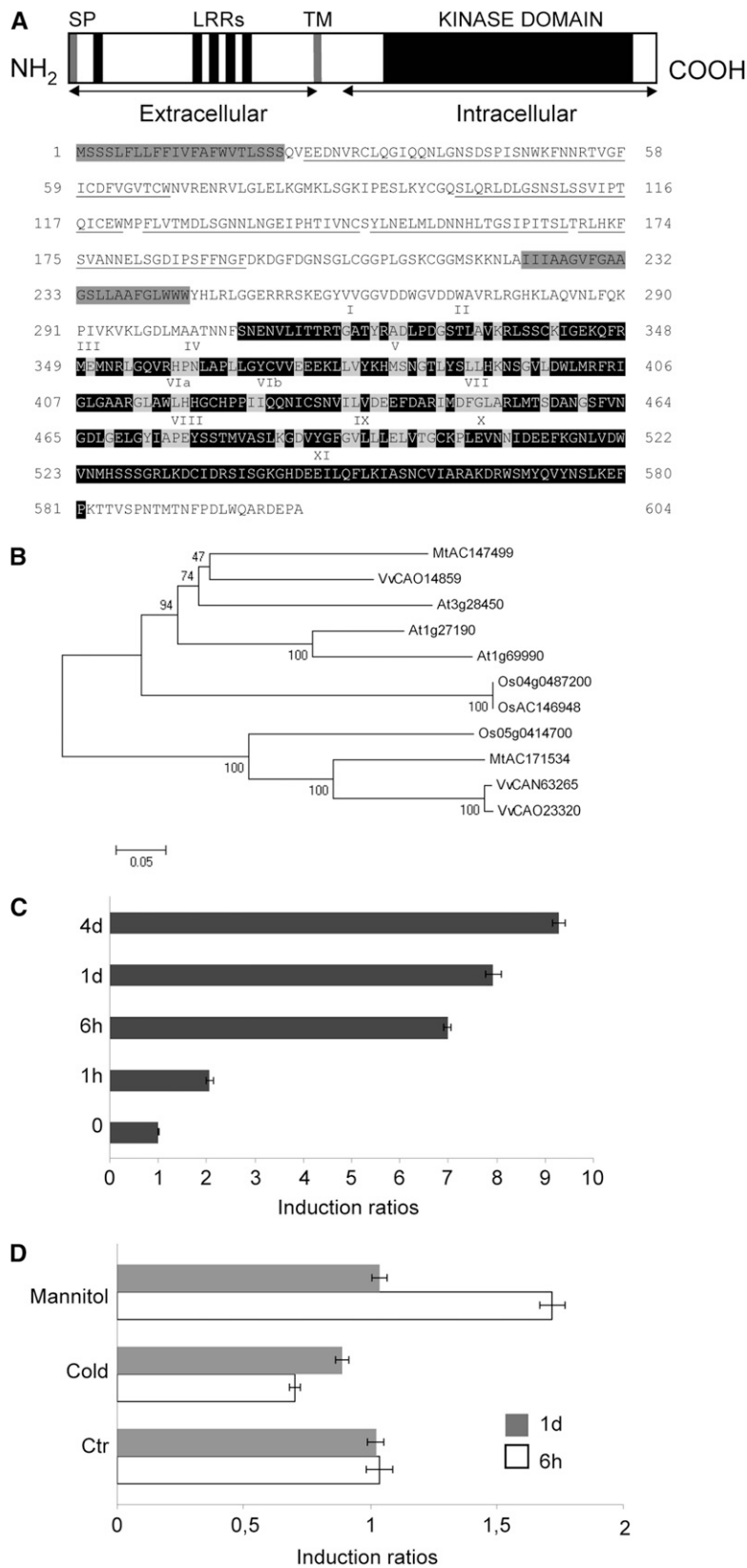


Figure 1. An LRR-RLK Is Induced by Salt Stress in *M. truncatula* Jemalong A17.

gene has been implicated in early abscisic acid (ABA) perception in *Arabidopsis* (Osakabe et al., 2005).

Receptor kinases are typically composed of an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic kinase domain. The extracellular domains of these receptors are quite divergent and enable them to respond selectively to different signals (Walker, 1994; Diévar and Clark, 2003; Torii, 2004; Johnson and Ingram, 2005). Based on the more than 20 structures of the extracellular domains, the plant RLK superfamily has been classified into various groups, such as leucine-rich repeats (LRRs), S domains (homologous to the S [self-incompatibility] locus glycoprotein), domains with epidermal growth factor repeats, and lectin domains (Torii and Clark, 2000; Shiu and Bleecker, 2001a). Among these, LRR-RLKs have been extensively studied in plants, although only a handful of receptors corresponding to mutants with clear phenotypes have been isolated. Thus, out of the 216 LRR-RLKs in *Arabidopsis*, only 10 have known functions, and only four have been studied in detail (Diévar and Clark, 2004; Osakabe et al., 2005). The abundance of plant LRR-RLKs may represent a plant-specific adaptation for the recognition of a wide variety of extracellular signals in these sessile organisms.

Using a suppressive subtractive hybridization (SSH) approach, we have previously identified several genes induced by salt stress in the model legume *Medicago truncatula*, including an LRR-RLK gene (named *Srlk*; de Lorenzo et al., 2007; Merchan et al., 2007). Here, we have evaluated the involvement of this gene in salt stress responses of legume roots and showed that this LRR-RLK plays a role in determining the sensitivity of legume roots to salt stress. Expression of the gene is rapidly and strongly increased in response to salt stress in roots and promoter- β -glucuronidase (*GUS*) fusions, suggesting that *Srlk* is activated in the root epidermis. Functional analysis of *Srlk* using RNA interference (RNAi) yielded roots whose growth was insensitive to the presence of salt in the medium, a phenotype similarly observed in two independent *Srlk*-mutant TILLING alleles. These plants accumulate fewer sodium ions than controls, and several early salt-regulated genes are downregulated after a salt stress. Taken together, these results suggest a role for *Srlk* in mediating early events in the response of *M. truncatula* Jemalong A17 roots to salt stress.

RESULTS

Srlk, a Novel LRR-RLK Induced by Salt Stress in *M. truncatula*

A partial cDNA of *Srlk* (for Salt-induced Receptor-Like Kinase) was previously isolated via an SSH approach for identifying genes involved in the reacquisition of root growth after salt stress in *M. truncatula* (Merchan et al., 2007). A sequence corresponding to *Srlk* was identified in The Institute for Genomic Research (TIGR) database, GenBank accession number TC101212, for tentative consensus sequence derived from several ESTs, corresponding to a transcript of ~ 1.8 kb, and coding for a predicted protein of 604 amino acids. Analyses of structural properties of the *Srlk*-predicted protein using Pfam (Bateman et al., 2004) and SMART programs (Letunic et al., 2004) suggest that this protein encodes an LRR-RLK with four domains: an N-terminal hydrophobic signal peptide (1 to 22), extracellular LRRs (23 to 221), a transmembrane domain (from amino acid 222 to 244), and a cytoplasmic kinase domain (from amino acid 307 to 581) (Figure 1A). The *Srlk* extracellular region contains five predicted LRR domains, whereas the intracellular kinase domain contains the 11 conserved kinase subdomains (identified by roman numerals in Figure 1A). The previously described LRR-RLK extracellular domains (Shiu and Bleecker, 2001a) differ from those of *Srlk*, suggesting that this receptor recognizes a different signal.

To identify *Srlk* homologous proteins, a phylogenetic tree was constructed using the deduced protein sequences of these genes, the MEGA4 program, and the *Arabidopsis* proteins AtMSrkl1 (for *Medicago* Smrkl1-like protein, accession number At3g28450), AtMSrkl2 (accession number At1g27190), and AtMSrkl3 (accession number At1g69990), three rice homologs (accessions numbers Os05g0414700, AC146948.2, and Os04g0487200), two *Vitis vinifera* homologs (Vv/CAN63265.1 and Vv/CAO23320.1), and one *Medicago* homolog (Mt/AC171534.4) as shown in Figure 1B. The closest homolog to Mt *Srlk* was an *Arabidopsis* protein (AtMSrkl1 for *Medicago* Salt Receptor kinase-like 1) that exhibits 62% identity. No function has been assigned to this gene in any species.

Next, we examined expression of the *Srlk* gene in response to salt and other abiotic stresses. The ATLAS of gene expression for

Figure 1. (continued).

(A) Predicted amino acid sequence of *Srlk*. Hydrophobic regions corresponding to the signal peptide sequence and the transmembrane region (gray boxes), the LRRs (amino acids underlined), and the kinase domain (black) are indicated. Roman numerals indicate the 11 characteristic subdomains of protein kinases. Letters shaded in gray indicate amino acids highly conserved among protein kinases.

(B) Phylogenetic relationship among *Srlk* homologs. Phylogram of deduced full-length protein sequences of *Srlk* homologs constructed with the MEGA4 software (Tamura et al., 2007). The bootstrapping value (out of 10,000 samples) for each node, obtained with the same software, is shown. Species are as follows: *Mt*, *Medicago truncatula*; *At*, *Arabidopsis thaliana*; *Vv*, *Vitis vinifera*, and *Os*, *Oryza sativa*.

(C) *Srlk* expression levels in response to salinity treatments. *M. truncatula* Jemalong A17 roots (4-d grown) were transferred to 150 mM NaCl for different times. Real time RT-PCR analysis of *Srlk* expression in roots at 0, 1 h, 6 h, 1 d, and 4 d of treatments is shown.

(D) *Srlk* expression levels in response to mannitol (300 mM) and a cold (4°C) stress during 6 and 24 h. For **(C)** and **(D)**, histogram represents quantification of specific PCR amplification products for *Srlk* gene normalized against the constitutive control *actin11*. Numbers on the x axis indicate fold induction of gene expression in treated compared with untreated samples. A representative example out of two biological experiments is shown, and error bars represent SD between three technical replicates.

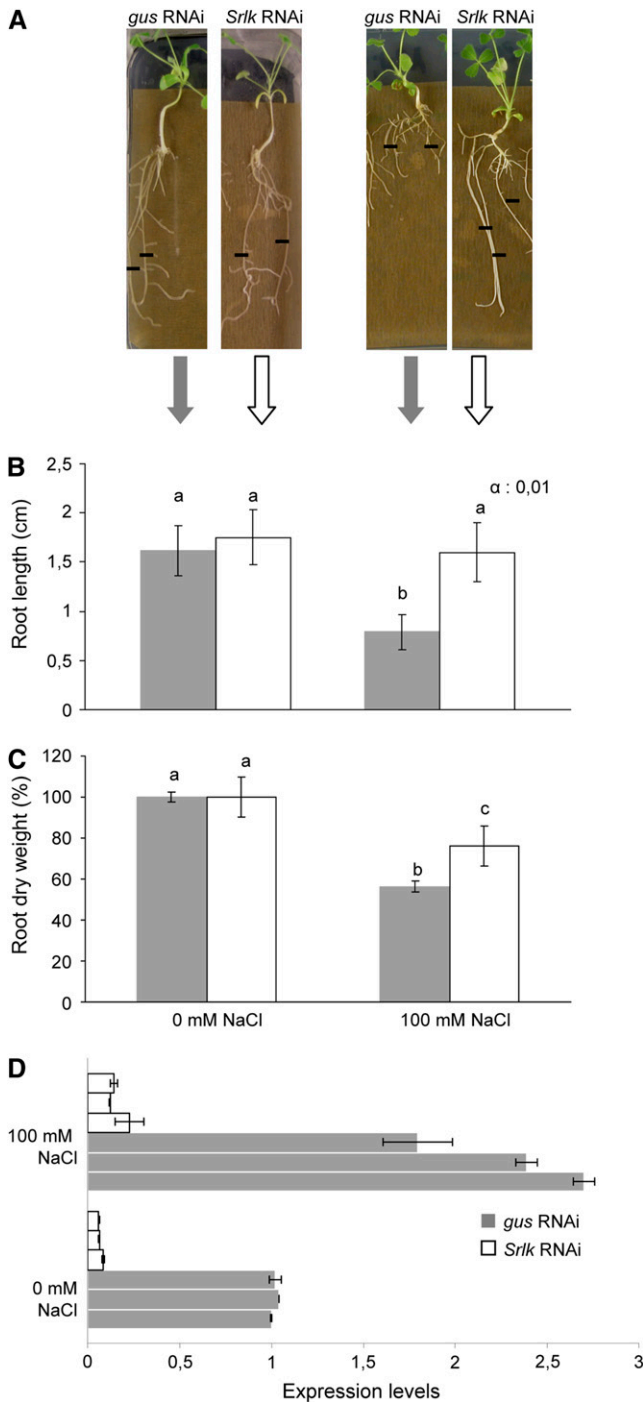


Figure 2. Functional Analysis of the *Srlk* Gene Using RNAi.

(A) Representative images of *gus*-RNAi and *Srlk*-RNAi *A. rhizogenes*-transformed *M. truncatula* roots 6 d after transfer to control medium (left) or 100 mM NaCl (right). Black lines indicate the position of root tips at the moment of transfer to the salt or control medium. Similar root lengths were observed for both plants in control conditions.

(B) Quantification of mean root growth of independent transgenic roots transformed with the constructs mentioned in **(A)** and grown 6 d on media without salt (left graph) or supplemented with 100 mM NaCl (right

M. truncatula recently developed based on Affymetrix chips (<http://bioinfo.noble.org/gene-atlas/>; Benedito et al., 2008) revealed that the *Srlk* gene is mainly expressed in root tissues (see Supplemental Figure 1A online). We determined *Srlk* expression levels in *M. truncatula* Jemalong A17 roots submitted to salt (150 mM NaCl), mannitol (300 mM), and cold stress (4°C) at different time points (0, 1 h, 6 h, 1 d, and 4 d for salt stress, and 6 h and 1 d for mannitol and cold stress treatments). *Srlk* expression levels were already induced at 1 h after salinity treatment and reached their highest level from within 6 h and maintained up to 4 d of salt stress (Figure 1C). In mannitol and cold stress conditions, the *Srlk* expression was not significantly induced (Figure 1D). The *Zpt2-1* and *CorA1* genes (Merchan et al., 2007) were used as positive controls of stress treatments (see Supplemental Figure 1B online). Thus, we have identified a LRR-RLK in *M. truncatula* Jemalong A17 that is induced early in response to salt stress.

Functional Characterization of Salt Signaling Pathways in *M. truncatula* Jemalong A17 Roots

To investigate the putative role of *Srlk* in response to salt stress, an RNAi approach was used in *M. truncatula* Jemalong A17 roots using composite plants (Boisson-Dernier et al., 2001). RNAi knockdown of candidate genes is an efficient way to suppress gene expression (Smith et al., 2000; González-Rizzo et al., 2006). A 203-bp fragment of *Srlk* with maximal RNAi specificity was used for RNAi constructs by selecting a region with <21-bp-long stretches of full complementarity with any other sequence of *M. truncatula*. We compared transgenic *Agrobacterium rhizogenes*-transformed roots carrying an *Srlk*-RNAi construct with a *gus*-RNAi control to rule out any indirect effect induced by the activation of the silencing machinery. Two weeks after *A. rhizogenes* infection in the appropriate medium, similarly grown composite plants were transferred to a medium containing 100 mM NaCl or control medium without salt and incubated for six more days (Figure 2). Root length for each plant was determined from the moment of transfer into these media up to the 6-d period. A significant difference in root growth was detected in the population of *Srlk*-RNAi lines compared with control roots under salt stress conditions (Figures 2A and 2B). By contrast, a similar behavior was observed in the absence of salt stress. Measurements of $n > 100$ independent transgenic roots per construct

graph). A representative example out of three biological experiments is shown ($n > 30$ per construct and condition per experiment). The different letters indicate mean values significantly different between *gus*-RNAi and *Srlk*-RNAi roots (Student's *t* test, $P < 0.001$).

(C) Quantification of root dry weights of *Srlk*-RNAi or *gus*-RNAi plants under salt stress or control conditions after 15 d. The different letters indicate mean values significantly different using the Kruskal and Wallis statistical method ($n = 20$). For **(B)** and **(C)**, error bars indicate the interval of confidence ($\alpha = 0.01$).

(D) Real-time RT-PCR analysis of expression levels of *Srlk* in pools from three independent *Srlk*-RNAi (white bars) and three *gus*-RNAi transgenic lines (gray bars) treated or not with salt. Values were normalized against the actin gene, and error bars are SD.

(from three biological replicates) were examined to evaluate the salinity-dependent effect on root growth (Student's *t* test, $P < 0.001$). The phenotype observed in *Srlk*-RNAi transgenic roots was further confirmed using root dry weight measurements (Figure 2C).

As several LRR-RLKs are involved in symbiosis (Endre et al., 2002; Searle et al., 2003; Torii, 2004; Schnabel et al., 2005; Stacey et al., 2006), we also analyzed a potential role of *Srlk* in nodulation, particularly under salt stress conditions. Nodulation capacity under control and salt stress conditions of several independent transgenic root lines expressing *gus*-RNAi and *Srlk*-RNAi constructs was analyzed. After 3 weeks of growth in control medium, these composite plants were transferred to a nitrogen-depleted medium without or with salt (100 mM NaCl) and subsequently inoculated with the *Sinorhizobium meliloti* 2011 wild-type strain. We determined the total number of nodules per plant 21 d after inoculation, and no obvious differences were observed. A similar inhibitory effect of salt stress on the symbiotic interaction was detected both in *gus*-RNAi and *Srlk*-RNAi composite plants (see Supplemental Figure 2 online).

The effect of the RNAi construct on *Srlk* expression in transgenic roots was analyzed. A strong and significant decrease of *Srlk* expression was observed both under normal or salt stress conditions (Figure 2D) in *Srlk*-RNAi roots compared with *gus*-RNAi controls. These results confirmed the downregulation of *Srlk* gene expression by RNAi in these transgenic roots.

Therefore, repression of *Srlk* expression in *M. truncatula* Jemalong A17 plants prevents the inhibition of root growth by salt, suggesting that *Srlk*-RNAi roots may be less sensitive to this environmental constraint.

Several Salt-Responsive Genes Are Downregulated in *Srlk*-RNAi Roots under Salt Stress

Our previous work (de Lorenzo et al., 2007; Merchan et al., 2007) identified several early markers of salt stress responses for *M. truncatula* Jemalong A17 roots. Five genes induced by short-term salinity treatments were selected to investigate their behavior in *Srlk*-RNAi roots. A calcium-dependent protein kinase (*CDPK3*) gene was induced at 1 and 6 h after salt stress (Gargantini et al., 2006), whereas a cytokinin-related response regulator (*RR4*; Merchan et al., 2007) was induced within 1 h for up to 4 d. In addition, two transcription factors (*Zpt2-1* and *Zpt2-2*; de Lorenzo et al., 2007; Merchan et al., 2007) and an RNA binding protein (*Rbp2*) gene showed increased levels from 1 h of salt treatment (see Supplemental Figure 3 online). Expression of these five genes in *Srlk*-RNAi and *gus*-RNAi transgenic roots treated with or without salt for 6 h (see Methods) was analyzed using real-time RT-PCR. Four (*CDPK3*, *RR4*, *Zpt2-1*, and *Zpt2-2*) were significantly downregulated in *Srlk*-RNAi plants under salt stress (Figure 3), whereas the *Rbp2* gene was less affected. Expression levels are presented as induction ratios between salt and control conditions.

These results strongly suggest that *Srlk*-RNAi roots are less sensitive to the external addition of salt and that the genes tested (except for *Rbp2*) may be involved in a salt-responsive signaling pathway activated by this RLK.

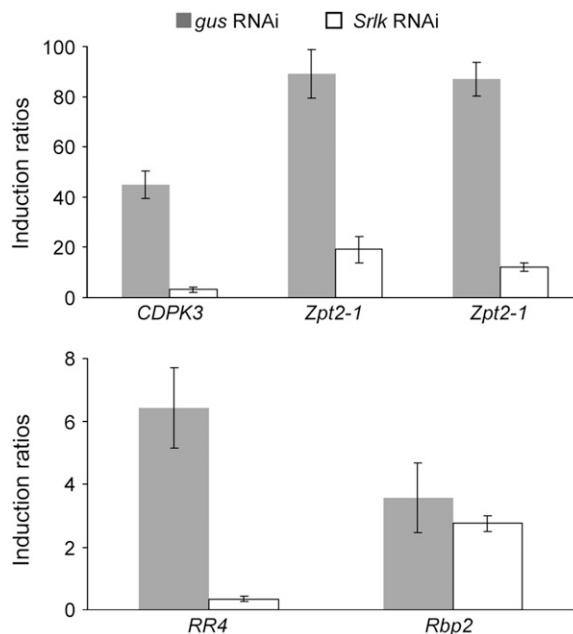


Figure 3. Expression of Salt-Regulated Genes in *Srlk*-RNAi Plants under Salt Stress.

The effect of *Srlk* suppression on the expression of early induced salt-responsive genes was determined in transgenic root apices treated with 150 mM NaCl during 6 h. RNA samples from three independent *Srlk*-RNAi transgenic lines and their corresponding controls were analyzed by real-time RT-PCR. Histograms represent the induction ratios between salt and control conditions of the expression levels of each gene in *Srlk*-RNAi and in *gus*-RNAi roots. The salt-regulated genes studied were *CDPK3*, *RR4*, *Zpt2-1*, *Zpt2-2*, and *Rbp2*. Values were normalized against the actin gene.

Spatial Expression of *Srlk* in *M. truncatula* Roots

The temporal and spatial expression patterns of *Srlk* in *M. truncatula* roots and its regulation by salt stress were investigated using a 2.2-kb *Srlk* promoter:*GUS* fusion. Two weeks after *A. rhizogenes* infection, the histological *GUS* activity of the resulting transgenic root was determined. The *Srlk* promoter was only weakly active in the root epidermis under control conditions.

Based on results obtained for *Srlk* expression levels at different time points (Figure 1C), we evaluated the effect of salt stress conditions on *GUS* gene expression directed by the *Srlk* promoter. After 6 h of NaCl treatment, we observed an increase in *GUS* expression in the root epidermis (Figure 4). Whole-mount longitudinal views and transversal sections of transgenic roots in the absence (Figures 4A and 4B) or presence of salt revealed strong staining in epidermal cells and root hairs (Figures 4C to 4E). In half of the observed roots, an additional staining of vascular tissues could be observed after a NaCl treatment (Figure 4D).

As the root apex determines root growth in the soil and is known to be particularly sensitive to a variety of environmental stimuli (Dinneny et al., 2008; Gruber et al., 2009), we evaluated *Srlk* expression in this zone. Under salt stress, *GUS* expression driven from the *Srlk* promoter was also strongly detected in the basal meristematic region of the lateral root tip and vascular

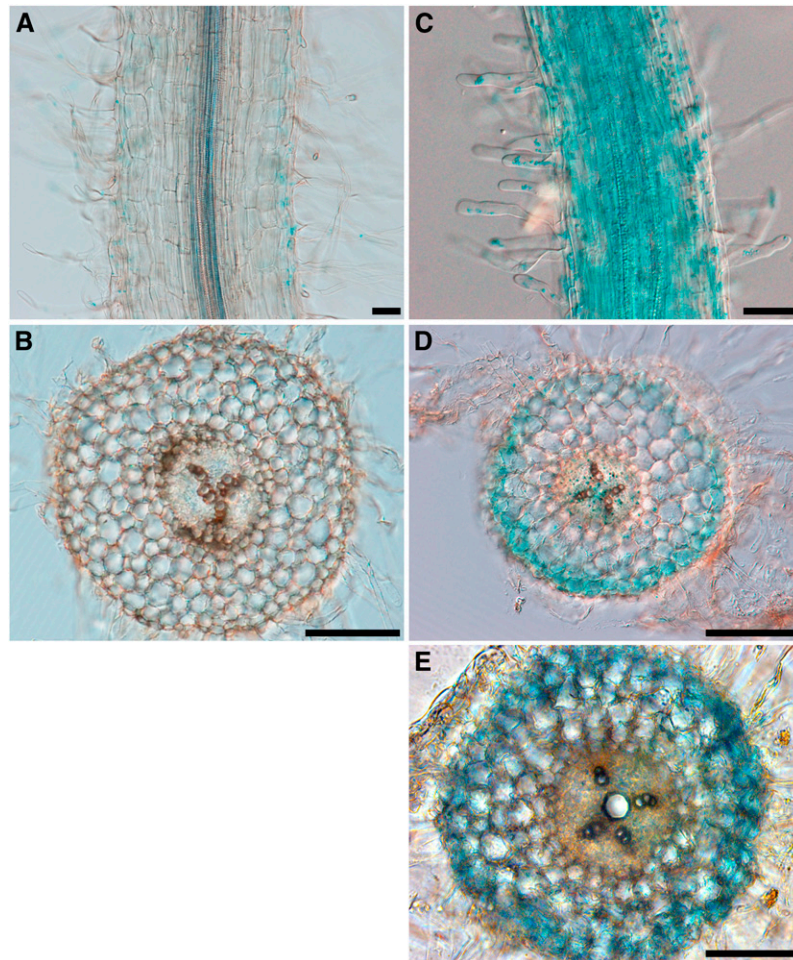


Figure 4. Spatial Expression Pattern of the *Srlk* Promoter.

Histochemical localization of *GUS* activity in *M. truncatula* transgenic roots expressing the *Srlk* promoter:*GUS* fusion. Plants in (C) to (E) were treated for 6 h with salt (150 mM NaCl) before *GUS* staining. Bars = 250 μ m in (A) and (C) and 500 μ m in (B), (D), and (E).

(A) and (B) Whole-mount staining (A) of untreated roots allowed weak detection of blue staining in the epidermis that is barely detected in transverse sections (B).

(C) Whole-mount staining of NaCl-treated roots revealed strong staining of the epidermis and root hairs.

(D) and (E) Transverse sections of the transgenic roots after NaCl treatment showing strong blue staining, representative of *GUS* activity, in the root epidermis. In half of the cases, weak staining was observed in vascular tissues (E). *GUS* staining data are representative of at least 25 independent transgenic roots.

tissues in contrast with untreated root apices (see Supplemental Figure 4 online). Transgenic roots carrying a *GUS* transgene without promoter did not show any *GUS* activity (see Supplemental Figure 4A online).

Expression of the *Srlk* promoter was predominantly localized in the root epidermis and is induced in this tissue as well as in the root apex, two regions potentially linked to the perception of soil environmental conditions.

Two Mutants Carrying TILLING-Derived Alleles of *Srlk* Are Affected in Salt Stress Responses

We next characterized two alleles of the *Srlk* genes through TILLING (for targeting-induced local lesions in genomes) in *M.*

truncatula (Comai and Henikoff, 2006). Mutations predicted by CODDLE (for codons optimized to discover deleterious lesions) as potentially deleterious for gene function in the LRR region of the receptor were screened and two lines carrying different alleles of the *Srlk* gene were characterized. One corresponded to an S-to-L substitution at position S47 in the N-terminal LRR region (*srlk1-1*), while the second line had a single G-to-A base change in the N-terminal LRR sequence, creating a stop codon (*srlk1-2*). Both mutations are indicated in Figure 5A. We have identified heterozygous and homozygous mutants for the null and the S-to-L allele, respectively.

These *Srlk*-mutants were assayed for their root growth under control and salt stress conditions (100 mM NaCl). After germination, root length was measured after 6 d in each medium, and the

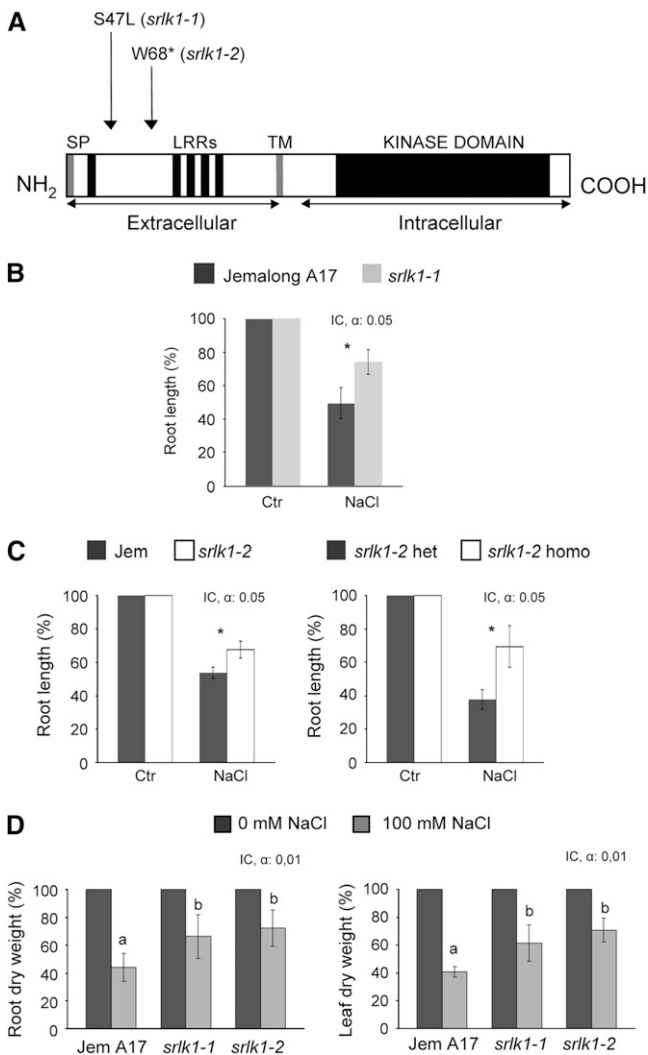


Figure 5. Phenotypic Analysis of TILLING *Srlk* Mutants.

(A) Schematic representation of *Srlk*, as shown in Figure 1, indicating the positions of the changed amino acids in the TILLING lines.

(B) and **(C)** Quantification of root length after salt stress treatments (100 mM NaCl) of different TILLING *Srlk*-mutants and control plants (*M. truncatula* Jemalong A17 or heterozygous siblings). Plants were scored at 6 d after germination in an in vitro system to monitor root growth under salt stress or control medium. The relative root length at 100 mM NaCl was calculated as percentage of corresponding untreated plants (homozygous, segregating heterozygous, or wild-type Jemalong A17). In **(B)**, a representative example of three biological replicates is shown. Asterisks indicate mean values statistically different between pairwise plants at $P < 0.05$. Error bars indicated the interval of confidence ($\alpha = 0.05$), and the Kruskal and Wallis test has been used ($n = 20$ per plant line).

(D) Five-day-old plants of the two *Srlk* mutants and Jemalong A17 were grown in the growth chamber in the presence of 0 and 100 mM of NaCl. Relative dry weights of roots (left) and leaves (right) from *M. truncatula* Jemalong A17 and *srk1-1* and *srk1-2* TILLING homozygous mutants after 12 d of growth in “i” medium submitted to 100 mM NaCl are shown. Results are represented as percentage of control without salt. IC, interval of confidence. Error bars indicate the interval of confidence at the indicated probability. The Kruskal and Wallis test has been used ($P < 0.01$ and $n < 20$).

effect of salt stress was measured as the percentage of root length in control conditions (percentage of control). This allowed comparison of root growth between different alleles independently of the initial growth for each plant. Figure 5B shows a comparison of root growth for Jemalong A17 plants and *srk1-1* homozygous lines under salt stress conditions. A significantly enhanced root length in the *srk1-1* lines under salt stress was observed ($n = 20$, Kruskal and Wallis test, $P < 0.05$). The *srk1-2* homozygous mutants were compared with Jemalong A17 and also segregating siblings heterozygous for the *Srlk* mutation. A statistically significant difference in root length was observed in both cases (Figure 5C).

A role of *Srlk* in the response of *M. truncatula* to salt stress was further examined by measuring root and leaf dry weights in these plants. Significant differences were found for both parameters between Jemalong A17 and the *Srlk* mutants under salt stress conditions (Figure 5D; see Supplemental Figure 5 online). The accumulation of sodium was analyzed in roots and leaves of mutant and control plants after exposure to 100 mM NaCl during 14 d. Roots and leaves of *srk1-1* and *srk1-2* plants had reduced levels of Na⁺ (up to 50 and 65% reduction) than control Jemalong A17 plants (Figures 6A and 6B, roots and leaves, respectively). These results suggest that the *Srlk* mutants are less sensitive to salt stress due to a differential accumulation of sodium inside plant tissues.

Gene expression was analyzed in these mutant plants in comparison to levels found in segregating heterozygous siblings to avoid any origin-driven variation with Jemalong A17 plants. Roots of *srk1-1* and *srk1-2* had reduced levels of *Srlk* transcripts, respectively (Figure 7A, 35 and 9% for the S-to-L and null mutants, respectively). The S-to-L substitution did not drastically affect accumulation of *Srlk*, whereas a strong reduction was observed in homozygous null alleles. The presence of a 5' stop codon mutation may change its mRNA stability. Under salt stress and control conditions, the expression levels of the two genes tested, *Zpt2-1* and *CDPK3* (selected due to their differential expression levels between *gus*-RNAi and *Srlk*-RNAi), were significantly downregulated in homozygous lines, when compared with heterozygous lines (Figures 7A and 7B, respectively). The same results were obtained when the aerial parts of these lines were analyzed (see Supplemental Figure 6 online).

These results demonstrate that two different mutant alleles of *Srlk* are affected in their responses to salt stress at both phenotypic (root growth) and molecular levels in a similar manner to the RNAi composite plants. These results indicate a major role of *Srlk* in the regulation of early salt stress responses of *M. truncatula* roots.

DISCUSSION

To cope with abiotic stresses, plants have developed various strategies that usually involve three steps: recognition of the stress condition, signal transduction, and activation of gene expression leading to adaptive or protective physiological responses, such as metabolite accumulation (Rathinasabapathi, 2000; Denby and Gehring, 2005; Valliyodan and Nguyen, 2006). Membrane-located receptor kinases play important roles in

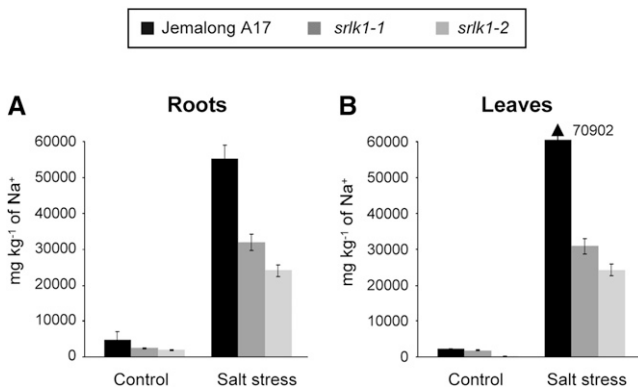


Figure 6. Sodium Accumulation in Different Tissues of *Srlk* Mutants.

Sodium concentrations were determined in roots (A) and leaves (B) of plants treated twice with 100 mM NaCl during 7 d (a total of 14 d) and in untreated plants using inducted coupled plasma optical spectrophotometry. Results are expressed in mg kg⁻¹ ($n = 20$ per plant line), and two biological replicate experiments were performed. Error bars indicate the interval of confidence ($\alpha = 0.01$).

many plant signal transduction pathways (Tichtinsky et al., 2003). In this study, we report the characterization and functional analysis of a novel LRR-RLK gene, *Srlk*, involved in the regulation of early *M. truncatula* root responses to salt stress.

RLKs constitute one of the largest gene families in plant genomes (Shiu and Bleecker, 2001a, 2001b). The basic structure of RLKs can be adapted through diverse extracellular domains to recognize an extremely diverse array of ligands (Johnson and

Ingram, 2005). The SRLK putative extracellular domain contains five LRRs, placing this receptor into the LRR-RLK family. The *Srlk* gene shows high sequence similarity with the kinase domain of three *Arabidopsis* genes (At3g28450, At1g27190, and At1g69990) but contains a different extracellular LRR domain.

Many receptor kinases have been shown to be regulated by abiotic stresses, and, interestingly, the closest *Arabidopsis* homolog of Mt *Srlk* is apparently induced by salt stress (Zimmermann et al., 2004; GENEVESTIGATOR, <https://www.genevestigator.ethz.ch/>). However very few LRR-RLK genes have known functions in stress responses. In *Arabidopsis*, the cell wall-associated RLK gene *WAK4* is regulated differentially by various biotic and abiotic factors and plays a vital role in cell elongation (Lally et al., 2001), and RPK1, an LRR-RLK upregulated by ABA, dehydration, high salt, and low temperature (Hong et al., 1997), regulates ABA early signaling (Osakabe et al., 2005). The involvement of a novel LRR-RLK in regulating a salt signaling pathway in *M. truncatula* further demonstrates potential roles of these genes in abiotic stress responses, notably in the agriculturally important legumes.

Recent genetic and biochemical studies have revealed mechanisms that underlie ligand recognition, dimerization, activation, regulation of activity, and signaling specificity of some LRR-RLK receptors (Diévar and Clark, 2003). The *srlk1-1* allele carrying an S-to-L transition within an LRR domain-coding region suggests that this missense allele compromises its function. Mutations that affect the β -sheet sequence of LRRs of *d61-2* in rice (*Oryza sativa*; Yamamoto et al., 2000), *br1-9* in *Arabidopsis* (Noguchi et al., 1999), and the FLS2 receptor, *fls2-24* (Gómez-Gómez et al., 1999; Gómez-Gómez and Boller, 2000), interfere with receptor activation, suggesting that this region may be involved in

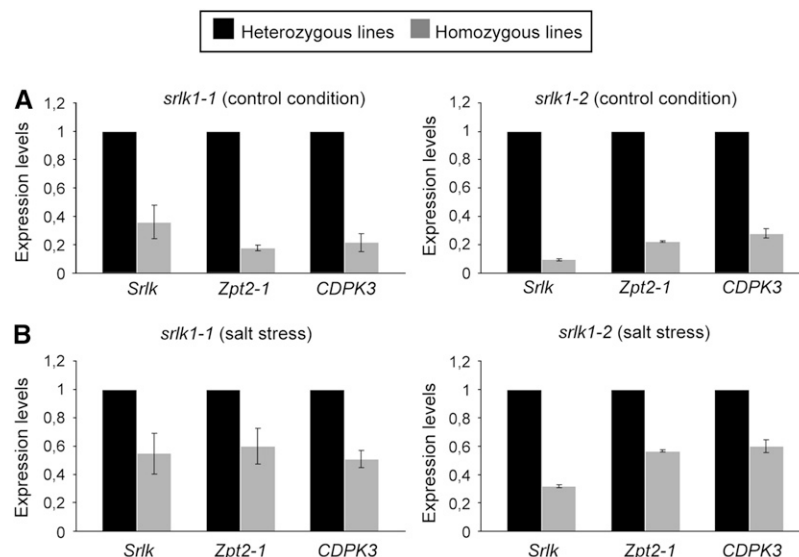


Figure 7. Expression of Salt-Regulated Genes in *Srlk* Mutants.

Effect of *Srlk* mutation on the expression of early induced salt-responsive genes was determined in roots treated with 150 mM NaCl during 6 h. RNA samples from *srlk1-1* and *srlk1-2* homozygous lines and their corresponding segregating heterozygous siblings were analyzed by real-time RT-PCR. Histograms represent the ratio of expression levels of each gene in homozygous mutant lines in relation to their heterozygous sibling (black columns). The genes studied were *Zpt2-1*, *CDPK3*, and *Srlk* in control (A) and salt stress conditions (B). Values were normalized against the actin gene.

brassinosteroid hormone or flagellin recognition, respectively. In addition, the extracellular domains of the LRR-RLKs play a role in receptor dimerization in different plants (Koka et al., 2000; Shah et al., 2001). The *srlk1-2* allele contains a stop codon in the N-terminal LRR sequence and encodes a severely truncated protein. Interestingly, *Srlk* expression was barely detectable in root and leaves of *M. truncatula srlk1-2* mutants even under salt stress. This null-mutation may affect *Srlk* mRNA stability and, eventually, initiate a non-sense-mediated RNA decay that (Conti and Izaurralde, 2005) as the other inactive *Srlk1-1* allele did not show any significant reduction in its own transcript levels.

High salinity causes an imbalance in sodium and can lead to toxic accumulation of this ion in the cytosol and negatively impact the acquisition and homeostasis of essential nutrients, such as K^+ and Ca^{2+} (Manchanda and Garg, 2008). This imbalance can be compensated by the coordinated action of various pumps, ions, Ca^{2+} sensors, and its downstream interacting partners. The roles of various ion pumps/channels in salinity tolerance have been described (Zhu, 2002; Mahajan et al., 2006). Among them, the SOS pathway results in the exclusion of excess Na^+ ions of the cell via a plasma membrane Na^+/H^+ antiporter to maintain cellular ion homeostasis (Mahajan and Tuteja, 2005; Martínez-Atienza et al., 2007; Manchanda and Garg, 2008). We could not reliably identify an SOS1 homolog in *M. truncatula* to establish any link between *Srlk* and SOS pathways. These diverse mechanisms ultimately lead to the efflux of excess Na^+ ions, to the reduction of Na^+ entry into the cell, to Na^+ sequestration in the vacuole, and/or in the closure of stomata to prevent water loss and reduce water (and Na^+) uptake (Zhu, 2003; Munns and Tester, 2008). Differential sodium ion accumulation in plant tissues of *Srlk* mutants suggests that they limit Na^+ entry into the cell or have reduced stomatal conductance (hydroactive closure; Mahajan and Tuteja, 2005). As the phenotype was initially observed in RNAi composite plants, where the transgene is only present in root tissues and its downregulation blocks early salt stress molecular responses in roots, we think that a change in stomatal conductance is unlikely. Nonetheless, the *Srlk* kinase seems to control a salt avoidance response in the model legume *M. truncatula*.

A signal transduction pathway mediated by *Srlk* is linked to the activation of a *Zpt2-1* Transcription Factor and a *CDPK* gene. CDPKs regulate plant adaptation responses to biotic and abiotic stresses by sensing Ca^{2+} and phosphorylating downstream components (Ludwig et al., 2004; Zhu et al., 2007). For example, in *Arabidopsis*, the roots of plant affected in CPK4 and CPK11 function are insensitive to salt (Zhu et al., 2007). CPK11 was found to interact and phosphorylate in vitro a zinc-finger transcription factor At Di19 (Milla et al., 2006). In addition, the SOS3 gene is a Ca^{2+} binding protein that senses the change in Ca^{2+} concentration and activates SOS2 protein kinase activity in a calcium-dependent manner. This complex was found to phosphorylate SOS1, a plasma membrane Na^+/H^+ antiporter, to maintain cellular ion homeostasis (Mahajan and Tuteja, 2005; Martínez-Atienza et al., 2007; Manchanda and Garg, 2008).

Among salt-responsive transcription factors, TFIIIA zinc finger proteins play an important role in plant responses to salt stress in *M. truncatula* roots (de Lorenzo et al., 2007; Merchan et al., 2007). SSH approaches using salt-treated roots have revealed several

salt-regulated genes in *M. truncatula* (Gargantini et al., 2006; de Lorenzo et al., 2007; Merchan et al., 2007). Similarly, the RPK1 mutant and antisense RPK1 transgenic plants showed reduced expression levels of various ABA-inducible genes (Osakabe et al., 2005). *Srlk* may be a new candidate receptor to function in an early step of a salt-dependent signal transduction pathway leading to expression of downstream genes, such as *Zpt2-1*, *Zpt2-2*, *RR4*, and *CDPK*. Promoter-GUS fusion analyses suggest that the *Srlk* is expressed in epidermal tissues, as are the HAK-type K^+ transporters and the *At KC1* and *AKT1* (*Arabidopsis* Shaker K^+ channel genes) (Lagarde et al., 1996; Su et al., 2002; Pilot et al., 2003). These results may link the *Srlk* receptor with perception of high salinity and activation of a signaling pathway leading to plant avoidance of deleterious effects of salt in internal tissues.

Further research should focus on identifying the extracellular ligand/s recognized by *Srlk* to activate this pathway. This may provide tools to reduce the negative impact of high salinity in agriculture, particularly in legume crops.

METHODS

Plant Material and *Agrobacterium rhizogenes* Transformation

Medicago truncatula genotype Jemalong A17 seeds were sterilized as described by de Lorenzo et al. (2007). After washing with sterilized water, seeds were sown on 1% agar plates and stored for 2 d at 4°C before being incubated overnight at 24°C in the dark to ensure uniform germination. Germinated seedlings were transferred to square plates containing appropriate medium for treatment (see below) and grown vertically in chambers at 24°C under long-day conditions (16 h light/8 h dark). Different constructs were introduced into *A. rhizogenes* ARqua1 (*Sm^r*-derivative strain of A4T) and used for transformation of *M. truncatula* Jemalong A17 as described by Boisson-Dernier et al. (2001).

M. truncatula TILLING *Srlk*-Mutants

M. truncatula TILLING mutants of *Srlk* were obtained from the TILLING platform (J. Clarke and D. Baker, John Innes Centre Genome Laboratory, Norwich, UK; <http://jicgenomelab.co.uk/services/mutation-detection/tilling.html>). Genomic DNA was isolated from each line according to Sambrook et al. (1989), and the mutation of each line was confirmed by PCR, sequencing, and digestion of the amplified fragment. In the same way, the segregation of the mutation was checked to identify heterozygous and homozygous siblings of the same progeny.

Phenotypes (root growth and dry weights) were scored on low-nitrogen medium containing or not 100 mM NaCl as described previously (de Lorenzo et al., 2007). Root length under salt stress was expressed as percentage of root growth without salt using homozygous lines for the mutation in *Srlk* and wild-type Jemalong A17 or segregating heterozygous siblings as control. Significant differences were tested at 5% probability by the Kruskal and Wallis statistical method (Georgin and Gouet, 2000).

Salinity Treatments

The salt sensitivity of *M. truncatula* Jemalong A17 was determined as previously described (de Lorenzo et al., 2007). NaCl at 150 mM severely inhibits root growth, whereas at 100 mM NaCl, primary root elongation was inhibited ~50% when compared with control without salt. For this reason, the phenotype of *Srlk*-RNAi transgenic roots and TILLING *Srlk*-mutants was evaluated at 100 mM NaCl.

Root responses to salt stress in composite plants were tested on growth papers (Mega International) placed on low-nitrogen medium supplemented or not with 100 mM NaCl (Merchan et al., 2007). The position of root tips was marked at the time of transfer, and root growth from this point was measured after 6 d. Three biological experiments were performed, and at least 100 independent transgenic roots per construct and per condition were analyzed. In a second set of experiments, root dry weights of composite plants were analyzed at 15 d after the start of salt treatment. Roots of each plant were harvested and dried at 60°C for 48 h, and dry weight under salt stress was represented as percentage of untreated roots dry weight.

Nodulation assays were performed as described by de Lorenzo et al. (2007). Two biological replicate experiments were performed, and a minimum of 60 independent transgenic roots per construct and per condition were analyzed. In both experiments, the statistical method used for evaluation of samples was the Student's *t* test ($P < 0.001$).

Salt stress treatment for determination of sodium concentrations in different plant tissues (root and leaf) was performed on 10-d-old plants grown and then exposed twice to a 100 mM NaCl treatment during 7 d (for a total of 14 d).

Determination of Sodium in Plant Tissues

Samples of different plant tissues were dried for 48 h at 60°C, homogenized with a mortar, and filtered. Sodium element was determined by inducted coupled plasma optical spectrophotometry after treatment in a microwave system (Murillo et al., 1999). A sample of untreated plants was also processed in the same way and used as a control. A BCR Certified Reference Material was used as quality control of the analytical procedures. Two biological replicate experiments were performed, and 20 independent plant tissues were analyzed.

Gene Expression Analysis

Total RNAs were extracted from frozen roots using the RNeasy plant mini kit (Qiagen). First-strand cDNA was synthesized from 1.5 µg of total RNA, using the SUPERScript II first-strand synthesis system (Invitrogen). One-tenth of the cDNAs was used as a template in 10-µL PCR reactions. PCR was performed with a Light Cycler apparatus and the LC FastStart DNA Master SYBR Green IR (Roche Diagnostics) in a standard PCR reaction according to the manufacturer's instructions. A negative control without cDNA template was always included for each primer combination. Technical replicates in three independent syntheses of cDNA (derived from the same RNA sample) and two independent biological replicate experiments were performed. Parallel reactions to amplify *actin11* were used to normalize the amount of template cDNA (see Supplemental Table 1 online for gene-specific primers).

Gene Constructions

An *Srlk* promoter-*GUS* fusion was constructed. A 2.2-kb genomic DNA fragment lying upstream of the *Srlk* translation initiation codon was amplified using *Srlk* promoter primers (see Supplemental Table 1 online) and Pfu DNA polymerase (Promega). The PCR product was sequenced and cloned between the *EcoRI* and *BamHI* polylinker sites of the binary vector pPR97 (Szabados et al., 1995). The resulting chimeric construct was transformed into *M. truncatula* Jemalong A17 roots as described (Boisson-Dernier et al., 2001). A histochemical assay of *GUS* activity (overnight) was performed on roots of *M. truncatula* Jemalong A17 composite plants as described by Pichon et al. (1992). Briefly, *GUS* expression was visualized after soaking the tissues in staining buffer and washing several times in 70% ethanol. To improve the contrast between stained and nonreactive tissues, the samples were briefly cleared with

sodium hypochlorite (Boivin et al., 1990). Using this method, endogenous *GUS* activity was not observed in root tissues from untransformed *M. truncatula* Jemalong A17 plants. Transversal root sections (100 µm) were done on material embedded in 6% agarose using a Leica VT1200S vibratome. Observations were performed using a Nikon AZ100 microscope equipped with a Nikon DS-Ri1 digital camera. *GUS* staining data were representative of at least 25 independent transgenic roots.

To construct *Srlk*-RNAi and *gus*-RNAi plasmids, specific fragments of *Srlk* and *uidA* genes were cloned into a pFRN destination vector (González-Rizzo et al., 2006). Primers used were *Srlk*-LC and *UidA* (*GUS*) (see Supplemental Table 1 online for primer sequences). Efficiency of silencing was verified using real-time RT-PCR on various individual clones.

Gene Expression in Response to Salt Stress

For gene expression studies in *M. truncatula* plants, 15 germinated seedlings were placed in flasks with 30 mL of low-nitrogen liquid medium (Blondon, 1964) and grown in a shaking incubator (125 rpm) at 24°C under long-day conditions (16 h light/8 h dark). After 4 d, seedlings were treated with 150 mM NaCl for various incubation times (0, 1 h, 6 h, 1 d, and 4 d) under the same growth conditions. Plants were similarly treated with mannitol (300 mM) or exposed to cold stress (4°C) during 6 h and 1 day. Roots were collected at the indicated time points and immediately frozen in liquid nitrogen for RNA extraction.

In RNAi-transformed roots, 2-week-old composite plants were placed in sterile plastic pots containing perlite/sand (3:1, v/v) as mixed substrate. Plants were grown in control low-nitrogen solution until the root tips emerged out of each pot ~2 cm. Then, the emerging root tips were immersed in salt or control solution for 6 h and immediately frozen in liquid nitrogen for RNA extraction. A minimum of 10 independent transgenic roots per construction and condition were pooled to perform quantitative RT-PCR analyses, and measurements of five pools of transgenic roots per construct and conditions were examined.

For analysis of salt-regulated gene expression in TILLING mutants, these plants were grown in agar plates and then treated for 6 h with 150 mM NaCl. A minimum of 10 independent transgenic roots per line and condition were pooled to perform quantitative RT-PCR analyses.

Sequencing and Data Analysis

DNA was sequenced with the BigDye terminator system (GenPak), and nucleotide sequences were determined in an automatic laser sequencer 373A (Applied Biosystems).

Srlk sequence was used to query the TIGR *Medicago* Gene Index (<http://www.tigr.org/>) and National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/BLAST/>) using the BlastN and BlastX sequence comparison algorithms. Conserved protein domains were examined using the Conserved Domains at NCBI (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) in conjunction with the Pfam Protein Families database (<http://pfam.wustl.edu/>) and SMART programs (<http://smart.embl-heidelberg.de/>). Related LRR-RLKs from *Arabidopsis* and others plant species were identified by BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) algorithms. Sequence alignment was made using the ClustalW program (<http://www.ebi.ac.uk/clustalw/>). The phylogenetic tree was produced using the neighbor-joining method (Saitou and Nei, 1987) with midpoint rooting. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to each branch (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There were a total of 522 positions in the final data set. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: TC101212 (for *SrIk* gene), At3g28450 (for At MSrIk1), At1g27190 (for At MSrIk2), At1g69990 (for At MSrIk3), Os05g0414700 (for Os MSrIk1), AC146948.2 (for Os MSrIk2), Os04g0487200 (for Os MSrIk3), Vv/CAN63265.1 (for Vv MSrIk1), Vv/CAO23320.1 (for Vv MSrIk2), Mt/AC171534.4 (for MtSrlk), TC77176 (for *Zpt2-1*), TC86494 (for *Zpt2-2*), TC76706 (for *CorA1*), TC69270 (for *CDPK3*), TC64310 (for *RR4*), TC87010 (for *Rbp2*), and TC85697 (for *actin11*).

Supplemental Data

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

Supplemental Figure 1. Expression Levels of *SrIk* in Different Plant Tissues According to the Affymetrix Gene ATLAS of *M. truncatula* (<http://bioinfo.noble.org/gene-atlas/>) and of *Zpt2-1* and *CorA1* in Response to Mannitol and a Cold Stress, Respectively.

Supplemental Figure 2. Evaluation of Nodulation Capacity of Transgenic *gus*-RNAi and *SrIk*-RNAi *M. truncatula* Roots.

Supplemental Figure 3. Expression Levels of Different Salt-Related Genes in Response to Short-Term Salinity Treatments.

Supplemental Figure 4. Expression of the *SrIk* Promoter in Lateral Root Apexes with or without Salt and a Control Carrying the Vector without Promoter.

Supplemental Figure 5. Effect of a Long-Term NaCl Treatment on *M. truncatula* Jemalong A17 and Two Mutants of *SrIk*.

Supplemental Figure 6. Expression of Salt-Regulated Genes in *SrIk* Mutants in Salt Stress Condition.

Supplemental Table 1. Sequences of Oligonucleotides Used for PCR and Quantitative RT-PCR.

Supplemental Data Set 1. Text File of Alignment of *SrIk* Homologs in Several Plant Species.

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