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Research article

The *Salix psammophila SpRLCK1* involved in drought and salt tolerance

Huixia Jia[a,1], Jianbo Li[b,1], Jin Zhang[c], Pei Sun[a], Mengzhu Lu[a], Jianjun Hu[a,*]

[a] *State Key Laboratory of Tree Genetics and Breeding, Key Laboratory of Tree Breeding and Cultivation of the National Forestry and Grassland Administration, Research Institute of Forestry, Chinese Academy of Forestry, Beijing, 100091, China*
[b] *Experimental Center of Forestry in North China, Chinese Academy of Forestry, Beijing, 102300, China*
[c] *Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN, 37831, USA*

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ABSTRACT

Receptor-like cytoplasmic kinases (*RLCKs*) play critical roles in biotic and abiotic stress responses in plants. However, the functions of *RLCKs* from the desert shrub willow *Salix psammophila* have not been characterized. Here, we focused on the biological function of *SpRLCK1*, which was previously identified as a potential drought-related gene. Phylogenetic analysis and subcellular localization revealed that *SpRLCK1* was a cytoplasmic-localized protein with a protein kinase domain and belonged to the RLCK VIIa subclass. Gene expression profile revealed that *SpRLCK1* was predominantly expressed in the root, being consistent with the GUS staining of p*SpRLCK1*:GUS transgenic plants. Additionally, the expression of *SpRLCK1* was significantly induced by drought and salt stresses. To verify the function of *SpRLCK1*, we generated its overexpressing transgenic lines in *Arabidopsis thaliana*. The *SpRLCK1*-overexpressing plants exhibited higher tolerance to drought and salt stresses, as evidenced by the higher survival rate, relative water content and antioxidant enzyme activity than those of wild-type plants. The *SpRLCK1*-overexpressing plants enhanced drought and salt tolerance by improving ROS-scavenging activities. A co-expression network for *SpRLCK1* was constructed, and the expression analysis indicated that *SpRLCK1* regulated the expression of a series of stress-related genes. Taken together, our results demonstrate that *SpRLCK1* confers plant drought and salt tolerance through enhancing the activity of antioxidant enzymes and cooperating with stress-related genes.

1. Introduction

Plant cells constantly response to various signals to adapt the change of external environment in the life cycle. As a class of protein kinases superfamily, receptor-like protein kinases (*RLKs*) play important roles in receiving and recognizing these signals. After the first *RLK* gene (*ZmRLK*) was identified in maize in 1990 (Walker and Zhang, 1990), *RLK* members have been successively identified in *Arabidopsis* (Lehti-Shiu et al., 2009), soybean (Liu et al., 2009), rice (Gao and Xue, 2012), tomato (Sakamoto et al., 2012), potato (Dezhsetan, 2017), and other plant species. Typical RLKs are characterized by an extracellular ligand-binding domain, a transmembrane domain, and a conserved intracellular protein kinase domain (Morris and Walker, 2003; Shiu et al., 2004). Some RLKs lacking extracellular or transmembrane domains and being localized in cytoplasm are referred to as receptor-like cytoplasmic kinases (RLCKs). Most RLCKs contain only a protein kinase domain; while the others contain additional domains, such as the epidermal growth factor, leucine-rich repeat and WD40 (Vij et al., 2008; Dezhsetan, 2017). To date, 149, 379 and 130 RLCK members have been identified in *Arabidopsis*, rice and potato, respectively (Vij et al., 2008; Lehti-Shiu et al., 2009; Gao and Xue, 2012; Dezhsetan, 2017).

RLCKs play crucial roles in mediating plant immune responses. As a member of the RLCK VII subfamily, *BOTRYTIS-INDUCED KINASE1* (*BIK1*), regulates the immune response against fungal and bacterial pathogens and defense response to pests through phosphorylation, ethylene signaling, or complex interactions with flagellin sensing 2 and Pep1 receptor kinases PEPR1 and PEPR2 (Veronese et al., 2006; Lu et al., 2010; Laluk et al., 2011; Liu et al., 2013; Lei et al., 2014). Additionally, tomato *ACIK1*, *Arabidopsis PBL27* and rice *NRBB* also have been proved to be involved in plant immunity (Rowland et al., 2005; Guo et al., 2014; Shinya et al., 2014). Except for their roles in immune responses, RLCKs have been confirmed to participate in plant abiotic

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Abbreviations: CAT, Catalase; DAB, 3, 3′-diaminobenzidine; GUS, β-Glucuronidase; MS, Murashige-Skoog; NBT, Nitroblue tetrazolium; NJ, Neighbor-joining; POD, Peroxidase; RLCKs, Receptor-like cytoplasmic kinases; RLKs, Receptor-like protein kinases; ROS, Reactive oxygen species; SOD, Superoxide dismutase

[*] Corresponding author. Research Institute of Forestry, Chinese Academy of Forestry, Beijing, 100091, China.
E-mail address: hujj@caf.ac.cn (J. Hu).

[1] These authors contributed equally to this work.

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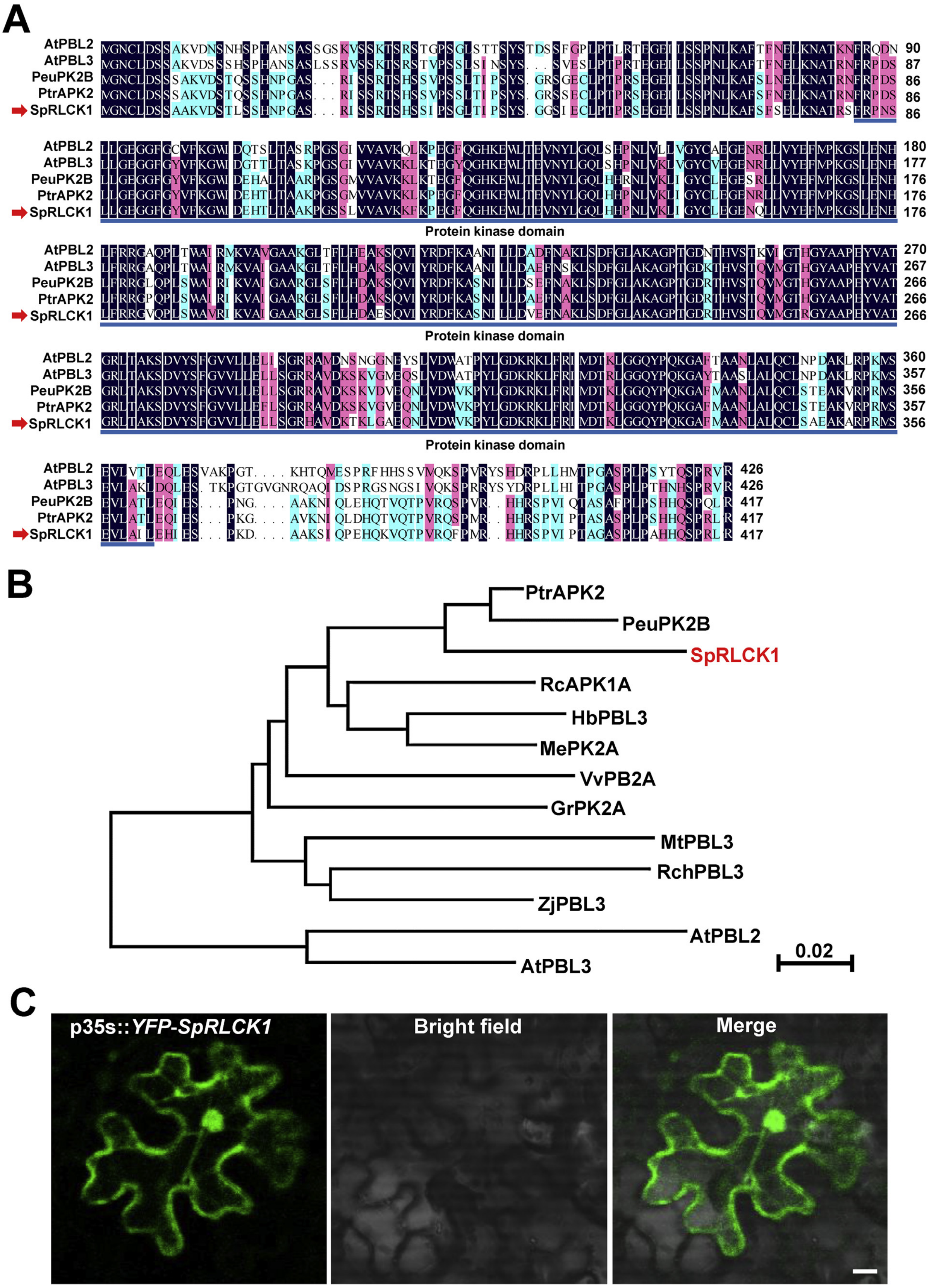

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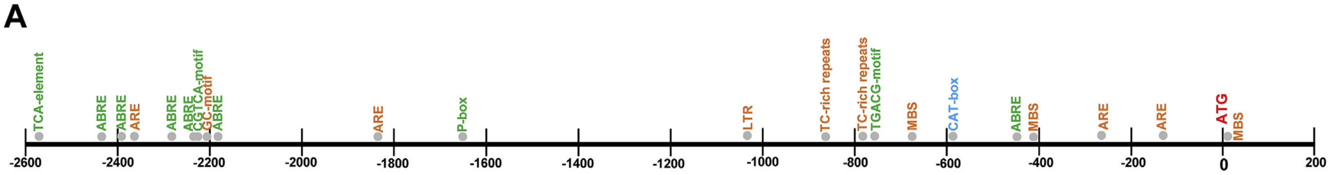
Fig. 1. Phylogenetic analysis and subcellular localization of SpRLCK1. (A) Alignment of the SpRLCK1 protein with its orthologous genes in *Populus* and *Arabidopsis*. The protein kinase domain was underlined. (B) Phylogenetic analysis of the SpRLCK1 protein with its orthologous genes in other plant species. The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates in MEGA 6.0 software. Their GenBank accession numbers were as follows: *S. psammophila* (*SpRLCK1*), *P. trichocarpa* (PtrAPK2, Potri.010G093700), *P. euphratica* (PeuPK2B, CCG032964), *Ricinus communis* (RcAPK1A, 29736.m002016), *Hevea brasiliensis* (HbPBL3, XP_021664922), *Manihot esculenta* (MePK2A, Manes.05G098400), *Vitis vinifera* (VvPB2A, GSVIVT01020041001), *Gossypium raimondii* (GrPK2A, Gorai.008G182000), *Medicago truncatula* (MtPBL3, Medtr5g038870), *Rosa chinensis* (RchPBL3, XP_024193224), *Ziziphus jujuba* (ZjPBL3, XP_015894909), *A. thaliana* (AtPBL2, AT1G17370; AtPBL3, AT2G02800). (C) Subcellular localization of SpRLCK1 protein. A YFP-SpRLCK1 fusion construct was introduced into tobacco epidermal leaf. Scale bar 10 μm.



B

Class	Element	Description	Number
Stress	ARE	*cis*-acting regulatory element essential for the anaerobic induction	4
	LTR	*cis*-acting element involved in low-temperature responsiveness	1
	MBS	MYB binding site involved in drought-inducibility	3
	TC-rich repeats	*cis-acting* element involved in defense and stress responsiveness	2
	GC-motif	Enhancer-like element involved in anoxic specific inducibility	1
Hormone	ABRE	*cis*-acting element involved in the abscisic acid responsiveness	6
	CGTCA-motif	*cis*-acting regulatory element involved in the MeJA-esponsiveness	1
	TCA-element	cis-acting element involved in salicylic acid responsiveness	1
	TGACG-motif	*cis*-acting regulatory element involved in the MeJA-esponsiveness	1
	P-box	Gibberellin-responsive element	1
Development	CAT-box	*cis*-acting regulatory element related to meristem expression	1

Fig. 2. Various *cis*-acting elements in the promoter of *SpRLCK1*. (A) The locations of *cis*-elements in the promoter of *SpRLCK*. (B) The description and statistics of *cis*-elements.

stress adaptation. In rice, OsRLCK253 interacts with stress associated protein 1 (OsSAP1) and OsSAP11, which confer abiotic stress tolerance to plants, and overexpression of *OsRLCK253* in *Arabidopsis* can improve salt and drought tolerance by inducing the expression of stress-related genes (Giri et al., 2011). The rice RLCK *GROWTH UNDER DROUGHT KINASE* (*GUDK*) is involved in drought tolerance through phosphorylation and activation of APETALA2/ETHYLENE RESPONSE FACTOR OsAP37 and then further activating its downstream stress-regulated genes (Ramegowda et al., 2014). RLCK ABA- and osmotic stress-inducible *receptor-like cytosolic kinase1* (*ARCK1*) plays negative roles in abiotic stress signal transduction, and the *arck1* mutant exhibits insensitivity to ABA and osmotic stress (Tanaka et al., 2012). Ectopic expression of *Glycine Soja RLCK* in *Arabidopsis* renders transgenic plants tolerance to drought and salt stresses (Sun et al., 2014). Although some *RLCKs* have been identified and characterized in several plant species, a very limited number of *RLCKs* from woody plant species have been cloned and conducted functional investigation.

Salix psammophila (*Salix*, Salicaceae) is naturally distributed in arid and semi-arid desert regions (Bao and Zhang, 2012). This species is an important desert shrub willow with high tolerance to abiotic stresses (e.g., drought, salt and extreme temperatures) and strong ability in wind-breaking. Thus, *S. psammophila* is widely regarded as perfect a sand-fixation plant species in the surrounding of desert (Li et al., 2016a). A better understanding of the molecular mechanism of *S. psammophila* adaptation to environmental stresses may provide novel insight into genetic improvement breeding of woody plants. In our previous study, the global transcriptome profiles of *S. psammophila* under drought condition have revealed *SpRLCK1* to be a key drought-responsive gene, but its biological function has not been thoroughly dissected, especially in response to unfavorable environmental stimuli.

Here, the major goal of our study was to in-depth understand the biological function of *SpRLCK1* in abiotic stress tolerance. The expression patterns of *SpRLCK1* in *S. psammophila* across different tissues and under various abiotic stresses were examined. Transgenic *Arabidopsis* lines ectopically expressing *SpRLCK1* were generated to compare the phenotypes of wild type (WT) and transgenic *Arabidopsis* plants under normal, drought and salt conditions. We found that overexpression of *SpRLCK1* increased drought and salt tolerance and up-regulated the expression levels of stress-related genes. Overall, these results demonstrate that *SpRLCK1* plays positive roles in regulating drought and salt tolerance and provide a foundation for gene resources that involved in stress tolerance for woody plant species.

2. Materials and methods

2.1. Plant materials and treatments

A. thaliana (ecotype Columbia-0) was grown at 20–22 °C under 16 h/8 h light/dark photoperiod in 1/2 Murashige-Skoog (MS) solid medium or soil conditions. *S. psammophila* clones were cultured at 23–25 °C under a 16 h/8 h light/dark photoperiod using 1/2 Hoagland nutrient solution. For stress treatments, two-month-old seedlings of *S. psammophila* were treated with 22% polyethylene glycol 6000 (PEG 6000, for drought stress), 200 mM NaCl (for salt stress), 4 °C (for cold stress), or 42 °C (for heat stress). The dosages of these treatments were determined based on previous treatments in *S. psammophila* (Li et al.,

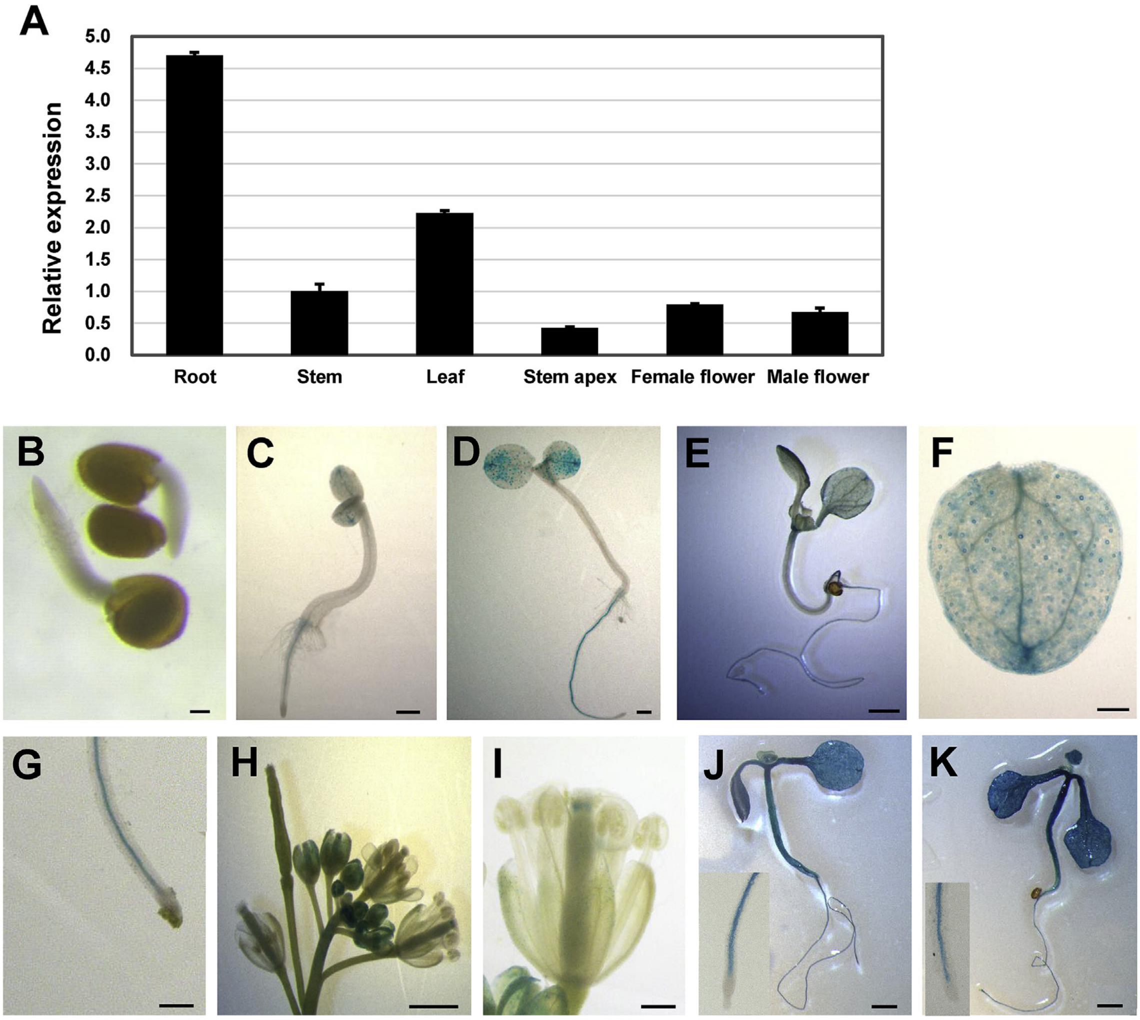


Fig. 3. Expression patterns of *SpRLCK1*. (A) Expression patterns of *SpRLCK* in the root, stem, leaf, stem apex, female flower and male flower of *S. psammophila* measured using qRT-PCR. (B–G) Expression patterns of *SpRLCK1* using GUS staining of p*SpRLCK*:*GUS* transgenic *A. thaliana* at different growth stages and in different organs and tissues: one-day-old seedling (B); three-day-old seedling (C); seven-day-old seedling (D); ten-day-old seedling (E); leaf (F); root (G); flower (H); stigma (I). (J–K) The GUS staining of ten-day-old seedling of p*SpRLCK*:*GUS* were treated with drought (J) and salt (K). Scale bars: (B) 100 μm; (C-E and J-K) 1 mm; (F–I) 500 μm.

2016a). Six time points (0, 3, 6, 24, 48 and 96 h) were selected for sample collection. At the end of each time point, the fully mature leaves from *S. psammophila* plants were quickly harvested, frozen in liquid nitrogen, and stored at −80 °C for further analysis. Three biological replicates were performed for each treatment.

2.2. RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated from plant samples using the RNeasy Plant Mini Kit (Qiagen, Germany). The quality and concentration of the RNA were detected by agarose gel electrophoresis and NanoDrop 8000 (Thermo, USA). First stand cDNA was synthesized using SuperScript III first-strand synthesis system (Life technologies, USA) with 3 μg mRNA. qRT-PCR was performed on the LightCycler 480 (Roche Applied Science, Germany) following manufacturer's instructions. Based on our previous study, *Elongation factor-1 alpha* (*EF1a*), *Histone superfamily protein H3* (*HIS*), and *Actin7* were chosen as reference genes for different tissue, cold treatment and salt treatment, respectively; *Ubiquitin-conjugating enzyme E2* (*UBC*) was used as a reference gene under heat and drought treatments (Li et al., 2016a). Three biological replicates and four technical replicates of each sample were performed for qRT-PCR analysis. All primers used in this study were listed in Supplementary Table S1.

2.3. Cloning and sequence analysis of SpRLCK1

The coding sequence of *SpRLCK1* was amplified from the cDNA of *S. psammophila* using gene-specific primers (Supplementary Table S1). DNAMAN 6.0 software was used to deduce the amino acid sequence of SpRLCK1 and for sequence alignment. MEGA 6.0 software (Tamura et al., 2013) was applied to construct a phylogenetic tree of SpRLCK1 and other plants RLCKs with the neighbor-joining (NJ) method. The compute pI/Mw tool from ExPASy (http://web.expasy.org/compute_pi) was used to predict the isoelectric points (pI) and molecular weight (Mw) of SpRLCK1. The coding sequence and promoter sequence of *SpRLCK1* were provided in Supplementary Table S2.

2.4. Co-expression gene network generation

A co-expression network for *SpRLCK1* was constructed according to the RNA-seq data of *S. psammophila* roots under PEG 6000-simulated drought stress from the NCBI BioProject database under accession number PRJNA485903 (Jia et al., 2019). The co-expression network was analyzed using the weighted gene co-expression network analysis (WGCNA) software (Langfelder and Horvath, 2008) in the R package. The graphic network was created by Cytoscape 3.7.1 software (Shannon et al., 2003).

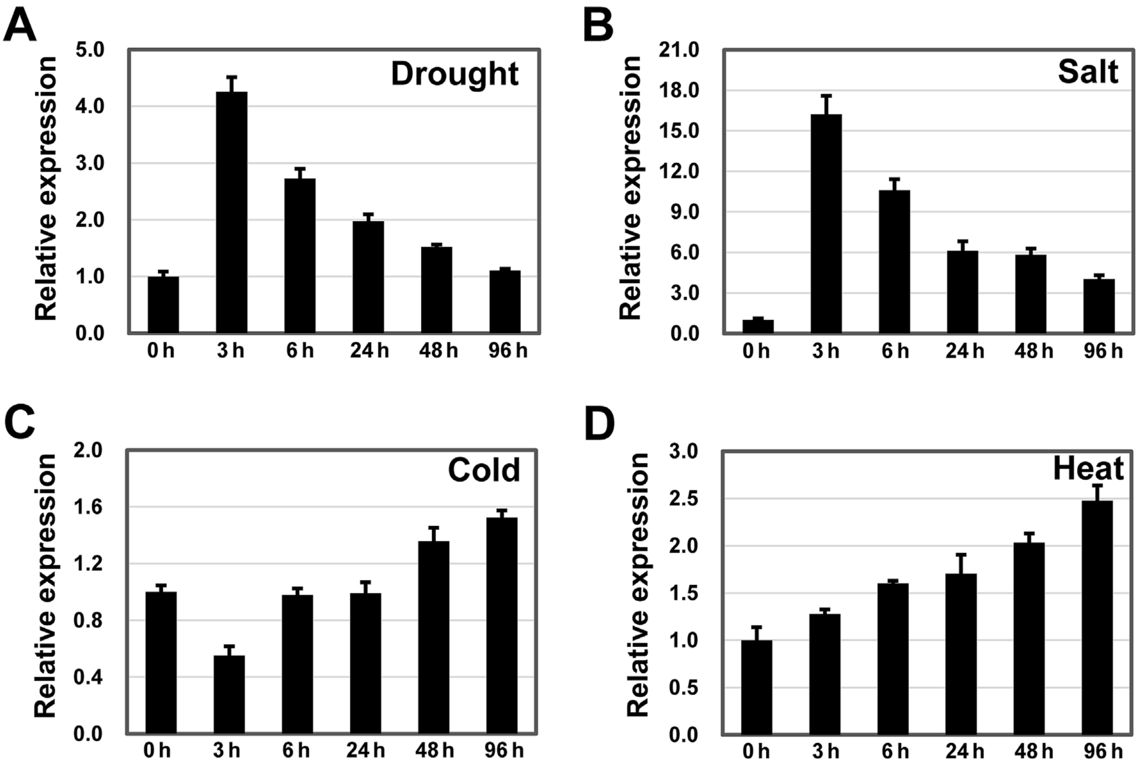


Fig. 4. Expression profiles of *SpRLCK1* in response to various stresses. (A–D) Expression patterns of *SpRLCK* in *S. psammophila* seedlings treated with 22% PEG 6000 (for drought stress) (A), 200 mM NaCl (for salt stress) (B), 4 °C (for cold stress) (C) and 42 °C (for heat stress) (D). Each sample was performed three biological replicates and four technical replicates.

2.5. Plasmid construction and transformation

The coding sequence of *SpRLCK1* was cloned into the vector pMDC32 to produce p*35S*:*SpRLCK1*. The promoter sequence of *SpRLCK1* (~2.6 kb upstream of the translation initiation site) was amplified from the genomic DNA of *S. psammophila* and constructed into the vector pMDC164 to produce p*SpRLCK1*:*GUS*. The resulting constructs were then transformed into *Agrobacterium* GV3101 by electroporation. *Arabidopsis* transformation was subsequently performed using floral dip method (Clough and Bent, 1998). More than 30 independent transgenic lines were obtained. T3 seedlings of three independent transgenic lines with high abundance of *SpRLCK1* were used for stress tolerance analysis, and three independent transgenic lines of p*SpRLCK1*:*GUS* were used for GUS staining according to previous research (Li et al., 2018).

2.6. Stress tolerance analysis

Sterilized seeds from the WT and *SpRLCK1*-overexpressing plants were sown on 1/2 MS solid medium. After being vernalized at 4 °C for three days, the seeds were transferred to normal growth conditions for seven days. The seedlings were then transplanted into 1/2 MS medium (control) or containing 200 mM mannitol (simulated drought stress), or 150 mM NaCl (salt stress). Two weeks later, the fresh weight and root length were compared between the WT and transgenic seedlings.

The two-week-old seedlings were transplanted into soil from 1/2 MS medium, and were well-watered for two weeks before stress treatment experiments. For drought stress, water was withheld for two weeks until the plants showed signs of wilting, after which the plants were flooded to promote recovery for one week. For salt stress, the seedlings were irrigated with 30 mL of 200 mM NaCl solution once two days or pure water as a control. After two weeks, the plants were irrigated with pure water for one week. The physiological parameters including relative water content, relative electrical conductivity and enzyme activity were measured after stress treatment. The relative water content of leaves was measured according to previous method (Smart and Bingham, 1974). The electrical conductivity was measured by DDS-307 conductivity meter (Lei Ci, China). The activities of peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) were calculated according to previous research (Li et al., 2016b). All experiments included five biological replicates and three technical replicates.

2.7. NBT and DAB staining

Four-week-old soil-grown WT and *SpRLCK1*-overexpressing plants were treated with 200 mM mannitol or 150 mM NaCl for two days. Afterward, approximately ten rosette leaves were stained with nitroblue tetrazolium (NBT) (Jabs et al., 1996) or 3, 3′-diaminobenzidine (DAB) solutions (Langebartels et al., 2002) for detecting the superoxide radicals or hydrogen peroxide, respectively.

3. Results

3.1. Isolation and sequence analysis of SpRLCK1 gene

In our previous study, the transcriptome profiles of *S. psammophila* roots (NCBI BioProject database under accession number PRJNA485903) in response to drought stress were analyzed, and we found that *SpRLCK1* was a potential drought-related gene. In this study, the *SpRLCK1* gene was cloned from *S. psammophila*. The coding sequence of *SpRLCK1* was 1254 bp in length, and it encoded an estimated protein of 417 amino acids with a calculated molecular mass of 45.6 kDa and an isoelectric point of 9.49. SMART analysis showed that SpRLCK1 contained a protein kinase domain (residues 82–362), and lacked the extracellular receptor domain and the transmembrane domain (Fig. 1A).

Multiple sequence alignment analysis showed that SpRLCK1 was closely related to PtrAPK2 from *Populus trichocarpa* and PeuPK2B from

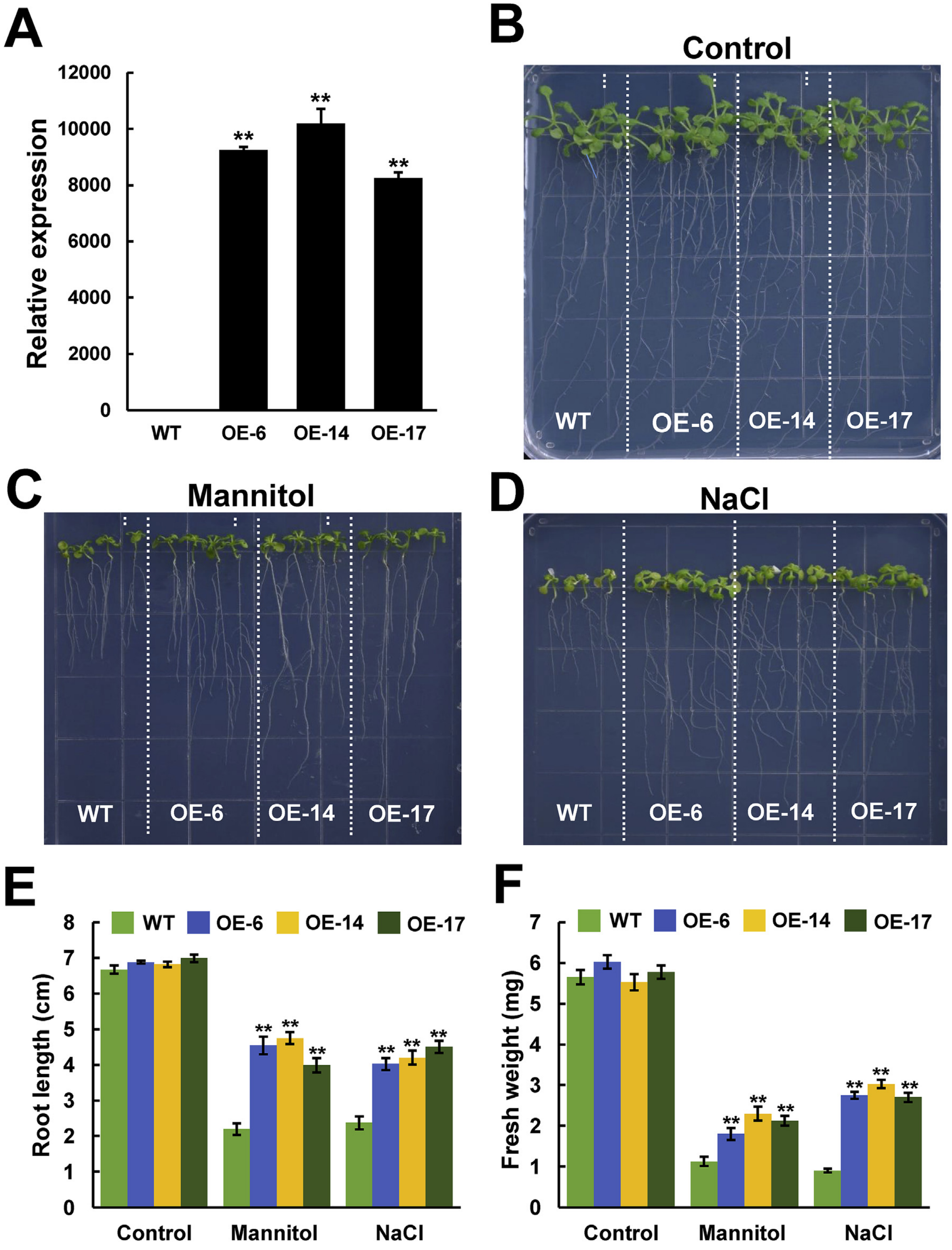


Fig. 5. Drought and salt tolerance analysis in panel growth condition. (A) qRT-PCR analysis of *SpRLCK1* expression in the WT and transgenic plants. (B–D) Photograph of the WT and transgenic seedlings without treatment (B), under mannitol treatment (C) and under salt treatment (D). The WT and three independent transgenic lines (OE-6, OE-14 and OE-17) were plated in per plate, with four seedlings per each genotype. The treatments in the petri plate were inclusive of five biological replicates. (E–F) Measurements of the root lengths (E) and fresh weight (F) of the WT and *SpRLCK1*-overexpressing plants under normal condition and stress treatments. Error bars indicated the standard deviations, and ** indicated significant differences compared with the WT at the $P < 0.01$ level.

P. euphratica (Fig. 1B). SpRLCK1 shared 91.37%, 89.21%, 84.96% and 73.36% identity with PtrAPK2, PeuPK2b, RcAPK1A and AtPBL3, respectively (Supplementary Table S3). It has been reported that AtPBL2 and AtPBL3 belong to the RLCK VII subfamily which is classified into two subclasses in *Arabidopsis* based on sequence similarity (Shiu and Bleecker, 2003). To reveal which subclass SpRLCK1 was in, a phylogenetic analysis was performed using the amino acid sequences of SpRLCK1 and all of *Arabidopsis* RLCK VII members (Supplementary Fig. S1). The results revealed that SpRLCK1 belonged to the RLCK VIIa subclass and was clustered into the same clade with AtPBL2 and AtPBL3 (Supplementary Fig. S1).

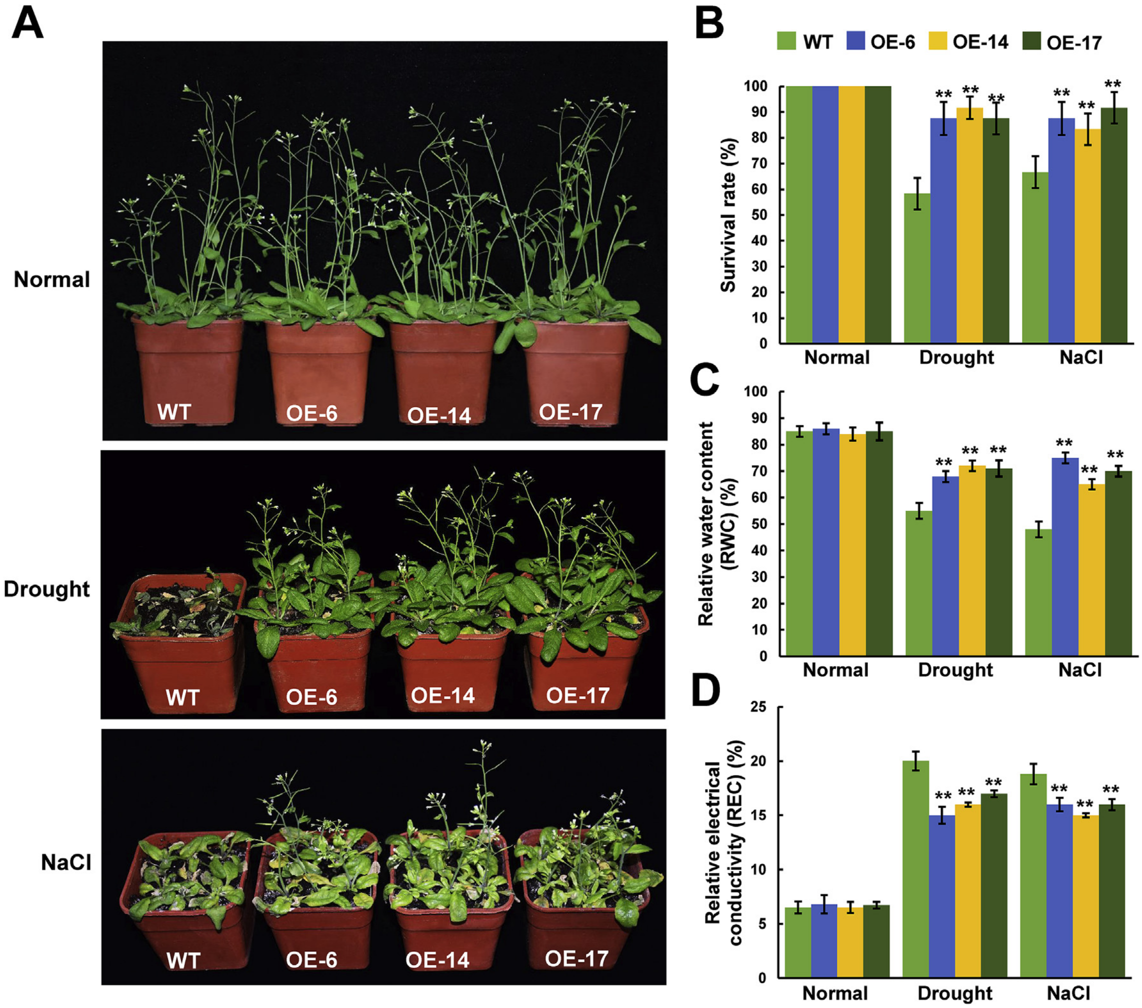


Fig. 6. Drought and salt stress tolerance analysis in soil growth condition. (A) Photograph of the WT and transgenic seedlings under normal condition, drought treatment, and salt treatment. The WT and three independent transgenic lines (OE-6, OE-14 and OE-17) were grown in soil block, with six seedlings per genotype. The treatments in soil were inclusive of five biological replicates. (B) Quantification of the survival rate. (C) Relative water content (RWC). (D) Relative electrical conductivity (REC). Error bars indicated the standard deviations, and ** indicated significant differences compared with the WT at the $P < 0.01$ level.

3.2. *Subcellular localization of SpRLCK1*

The subcellular localization of SpRLCK1 was examined to provide clues to its molecular function. WoLF PSORT (http://www.genscript.com/wolf-psort) predicted that SpRLCK1 resided in the cytoplasm and nucleus. To confirm this prediction, the coding sequence of *SpRLCK1* was fused to the N-terminus of yellow fluorescent protein (YFP) driven by the cauliflower mosaic virus 35S promoter, and the construct was transiently expressed in tobacco leaf epidermal cells. As shown in Fig. 1C, fluorescence from the YFP-SpRLCK1 construct was observed in the cytoplasm and nucleus. This finding indicated that SpRLCK1 was localized in cytoplasm and nucleus.

3.3. *Characterization of cis-elements in SpRLCK1 promoter*

To investigate the stress responses of *SpRLCK1*, the promoter of *SpRLCK1* was firstly analyzed. The upstream sequence (2.6 kb) to the downstream sequence (0.2 kb) of the transcription start site of *SpRLCK1* was used to identify the *cis*-elements using the PlantCARE database. The result showed that a number of stress-related *cis*-acting elements were detected including three MYB binding sites (MBSs), four antioxidant response elements (AREs), two TC-rich repeats, one low-temperature responsiveness (LRT) element and one GC-motif (Fig. 2). Additionally, *cis*-acting elements related to hormone responses in the *SpRLCK1* promoter were identified, including the motifs CGTCA and TGACG involved in MeJA-responsiveness, ABRE involved in abscisic acid (ABA) response, TCA-element involved in salicylic acid response, and P-box involved in gibberellin response (Fig. 2). CAT-box related to meristem expression was also detected in the *SpRLCK1* promoter (Fig. 2). This result indicated that *SpRLCK1* might be involved in stress responses as well as plant development.

3.4. *Expression patterns of SpRLCK1*

Gene expression patterns can provide useful clues for elucidating its gene function. To study the expression profiles of *SpRLCK1*, the transcripts of *SpRLCK1* across different tissues and under various abiotic stresses were examined using qRT-PCR. In different tissues (root, stem, leaf, stem apex, female flower and male flower) of *S. psammophila*, the highest accumulation of *SpRLCK1* was observed in root, following leaf and stem, and low expression level was detected in the stem apex (Fig. 3A). To further investigate the expression patterns of *SpRLCK1*, the promoter of *SpRLCK1* was isolated and fused with GUS, after which the construct was transformed into *A. thaliana*. GUS staining showed that GUS activity was detected throughout the entire plant (Fig. 3B–I), with the highest GUS activity in the root, further confirming the results of the tissue expression profiles of *SpRLCK1*.

The expression of *SpRLCK1* was also detected under different abiotic

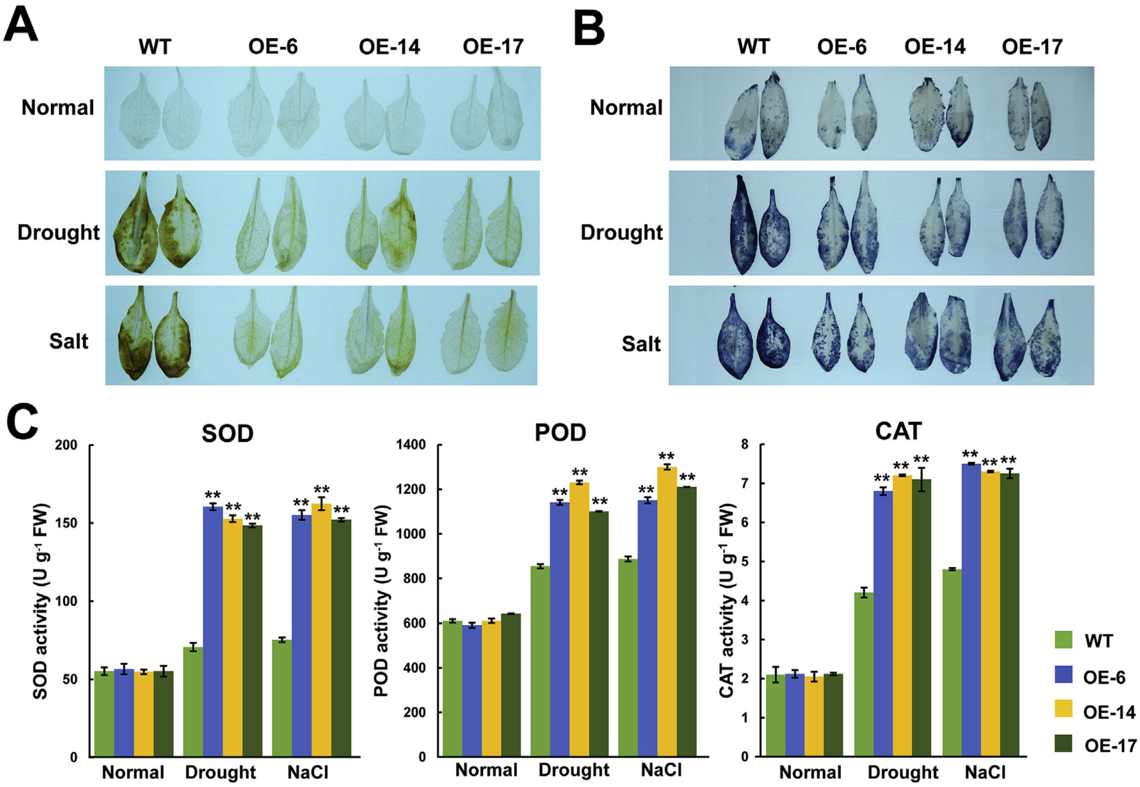


Fig. 7. Activity of antioxidant enzymes in the WT and transgenic plants. Ten rosette leaves from the WT and transgenic seedlings untreated or treated with 200 mM mannitol, 150 mM NaCl were used for (A) NBT and (B) DAB staining. (C) Quantification of the activities of SOD, POD and CAT under normal condition and drought or salt treatment. All experiments were conducted for five biological replicates and three technical replicates. Error bars indicated the standard deviations, and ** indicated significant differences compared with the WT at the $P < 0.01$ level.

stresses (including drought, salt, heat and cold). *SpRLCK1* was significantly up-regulated in response to drought stress, with a peak of nearly 4.2-fold at 3 h; then its expression was gradually reduced (Fig. 4A). In response to high salinity, the expression of *SpRLCK1* was rapidly activated and reached its maximal level (~16-fold) at 3 h, after which it began to decline (Fig. 4B). The expression of *SpRLCK1* declined at 3 h and slightly increased at 48 h and 96 h under cold condition (Fig. 4C). The expression of *SpRLCK1* was gradually induced by high temperature and reached the highest levels (~2.5-fold) at 96 h (Fig. 4D). To further confirm the expression pattern of *SpRLCK1* under stress treatments, ten-day-old *pSpRLCK1::GUS* seedlings were transplanted into 1/2 MS medium containing 200 mM mannitol or 150 mM NaCl. Strong GUS staining was consistently detected in the transgenic seedlings after 3 h of drought and salt treatments (Fig. 3J and K).

3.5. Ectopic expression of SpRLCK1 in Arabidopsis conferred drought and salt tolerance

Considering the inducible expression of *SpRLCK1* under multiple abiotic stresses, we investigated whether *SpRLCK1* contributed to plant stress tolerance. Therefore, transgenic *Arabidopsis* lines with overexpressing *SpRLCK1* were generated to further evaluate the function of *SpRLCK1*. Three independent homozygous *SpRLCK1* transgenic lines (OE-6, OE-14 and OE-17) with high abundance of *SpRLCK1* expression were used for the stress tolerance analysis (Fig. 5A). The phenotypes of the WT and transgenic lines at varying developmental stages were compared under drought and salt treatment.

One-week-old WT and transgenic *Arabidopsis* seedlings were treated with mannitol and NaCl for two weeks on 1/2 MS medium. No phenotypic difference was observed between the WT and transgenic seedlings under normal (control) condition, indicating that overexpression of *SpRLCK1* did not influence plant development (Fig. 5B). Under mannitol and NaCl conditions, the root length and fresh weight of the seedlings were inhibited (Fig. 5C and D). The root length of the transgenic seedlings was 4.0–4.7 cm, and the fresh weight was 1.8–3.1 mg, while the root length and fresh weight of the WT seedlings was only 2.1–2.3 cm and 0.9–1.1 mg, respectively (Fig. 5E and F). This result indicated that the growth inhibition of seedlings under stress was alleviated by overexpression of *SpRLCK1*.

Additionally, four-week-old seedlings planted in soil were also used to test the stress tolerance. The growth of the WT was more inhibited compared to that of the transgenic seedlings (Fig. 6A). The survival rate and relative water content (RWC) of the WT were lower than those of the transgenic plants, whereas the relative electrical conductivity (REC) of the WT was higher than that of the transgenic plants under both drought and salt stresses (Fig. 6B–D). These results demonstrated that the *SpRLCK1*-overexpressing plants exhibited higher drought and salt tolerance than the WT plants.

3.6. Constitutive expression of SpRLCK1 increased the oxidant enzyme activities

Abiotic stress can induce oxidative stress through generation and accumulation of reactive oxygen species (ROS) (Francisco et al., 2010), which causes lipid peroxidation, protein denaturation, DNA mutation and various types of cellular oxidative damage (Smirnoff, 1993; Mittler et al., 2004). In our study, DAB and NBT staining were used to detect their concentrations in the cellular level. There was no significant difference between the WT and transgenic lines under normal condition, whereas the DAB and NBT staining was much stronger in the WT plants than in the transgenic plants under the drought and salt treatments (Fig. 7A and B). These results showed that overexpression of *SpRLCK1* could reduce the oxidative damage under abiotic stress.

Antioxidant enzymes, such as POD, SOD and CAT, play key roles in

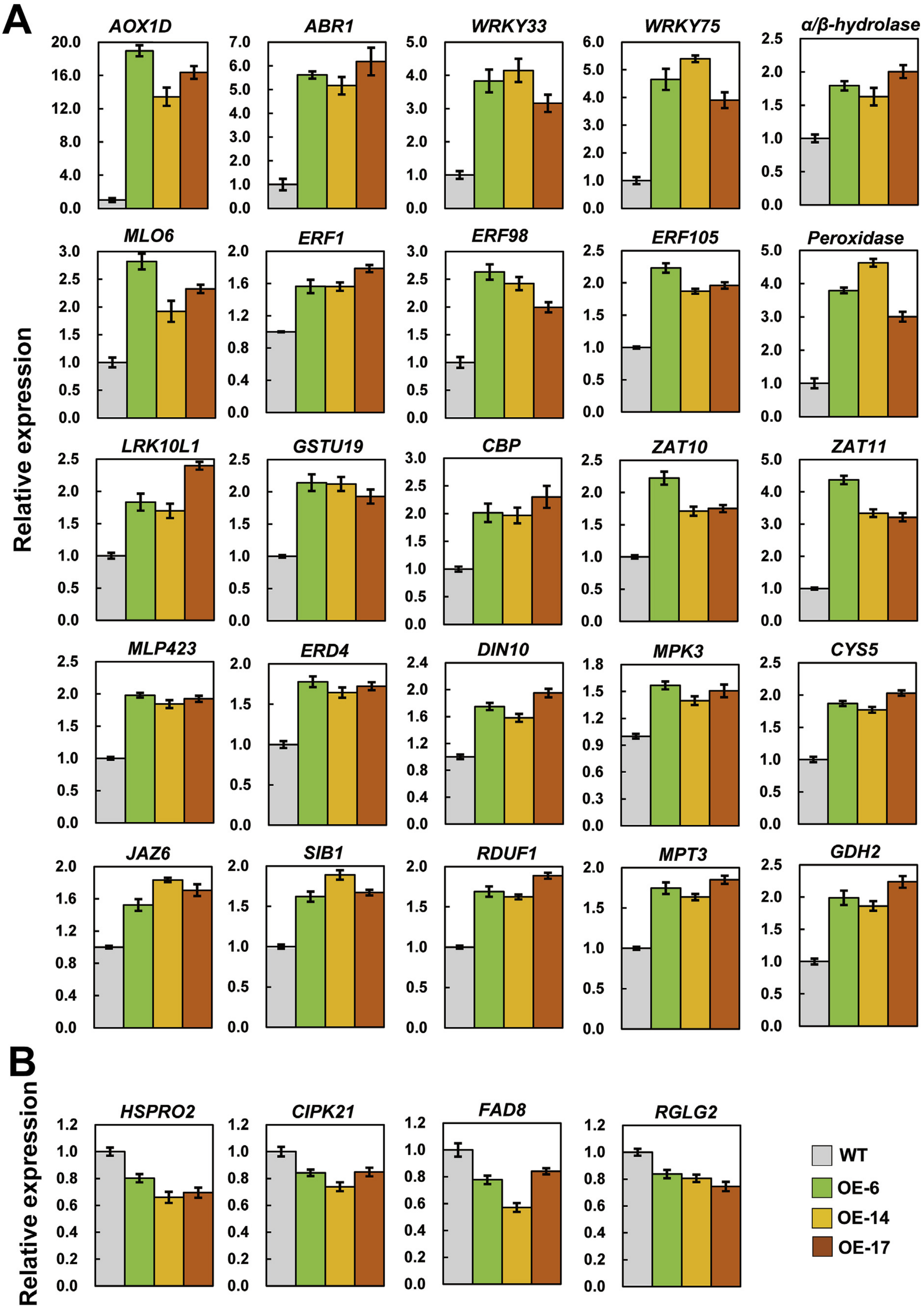


Fig. 8. Expression levels of genes from co-expression network in the WT and *SpRLCK1*-overexpressing plants. Expression patterns of stress-related genes from the co-expression network in the leaves of the WT and *SpRLCK1*-overexpressing plants. (A) Expression patterns of up-regulated stress-related genes in *SpRLCK1*-overexpressing plants. (B) Expression patterns of down-regulated stress-related genes in *SpRLCK1*-overexpressing plants. *AtACTIN* was used as a reference gene. The expression level of each gene in the WT was set to 1. Each sample was inclusive of three biological replicates and four technical replicates.

scavenging ROS and preventing plant from oxidative damage (Mittler et al., 2004). Thus, the activities of these antioxidant enzymes were also measured in the *SpRLCK1*-overexpressing and WT plants. Under normal condition, the activities of POD, SOD, and CAT were not significantly different between the transgenic lines and WT plants (Fig. 7C). However, after drought stress, the POD, SOD and CAT activities in the three transgenic lines were significantly higher than those in the WT plants, as well as in the salt treatment (Fig. 7C). This result suggested that overexpression of *SpRLCK1* improved the drought and salt tolerance of transgenic plants by increasing the antioxidant enzyme activities.

3.7. Expression analysis of stress-related genes in co-expression network for SpRLCK1

Based on the transcriptome profiles of *S. psammophila* under drought stress, a co-expression network for *SpRLCK1* was constructed. A total of 116 genes were identified in the co-expression network, and 43 of which were related to stress tolerance (Supplementary Fig. S2, Supplementary Table S4). The *Arabidopsis* homologous genes of *SpRLCK1*-co-expressed genes were searched through sequence alignment using BLASTp. To verify the co-expression network, the expression patterns of these 43 stress-related *Arabidopsis* homologous genes were compared between the *SpRLCK1*-overexpressing plants and WT plants. The expression levels of 29 genes were detected to change, including 25 up-regulated genes and four down-regulated genes (Fig. 8). Among them, some genes were remarkably induced, such as *AOX1D* (13- to 18-fold), *ABR1* (5- to 6-fold), *WRKY75* (4- to 5-fold), *WRKY33* (3- to 4-fold), *peroxidase* (3- to 4-fold), and *ZAT11* (3- to 4-fold) (Fig. 8).

4. Discussion

Plants often encounter various unfavorable environments including drought, salt, and extreme temperatures which greatly endanger plant growth and productivity. In the long term process of evolution, plants have developed multiple complex regulatory networks to overcome the detrimental effects of these adverse environments. Some key genes in networks were identified based on omics data, and were proved to be involved in plant stress tolerance through transgenic approach. *RLCKs* are a major class of signaling proteins that regulate plant cellular activities in response to biotic/abiotic stress signaling molecules, as well as plant growth. *SpRLCK1* was initially identified as a hub gene that responses to drought stress based on transcriptome data. Our results of phylogenetic analysis and subcellular localization revealed that SpRLCK1 was a cytoplasmic-localized protein from the RLCK VII subfamily. Moreover, SpRLCK1 was also detected in the nucleus, being the same as OsRLCK1 and GSK3/Shaggy-like kinase BR-INSENSITIVE2 (BIN2) which located in cytoplasm and nucleus (Gampala et al., 2007; Kong et al., 2007). In the absence of brassinosteroid (BR), BIN2 promotes the cytoplasmic retention of BRASSINAZOLE RESISTANT1 (BZR1) and BRI1-EMS-SUPPRESSOR1 (BES1) by phosphopeptide-binding 14-3-3 proteins in cytoplasm, and BIN2 can also phosphorylate BZR1 and BES1 and block their DNA binding resulting in the inhibition of BR response gene expression. Upon BR perception, BIN2 is dephosphorylated and results in the derepression of BR response genes in nucleus (Gampala et al., 2007; Belkhadir et al., 2014). Refer to the regulation mechanism of BIN2, we speculated that SpRLCK1 might play different roles in cytoplasm and nucleus to response to and resist the unfavorable environments. Many RLCK VII subfamily members have been reported to function in plant immunity (Liang and Zhou, 2018). For instance, *Arabidopsis* BR SIGNALING KINASE1 (BSK1) interacts with FLAGELLIN SENSING2 and is required for flg22-triggered ROS production. BSK1 is involved in the positive regulation of pathogen-associated molecular pattern-trigged immunity, and the *bsk1* mutant is susceptible to *Golovinomyces cichoracearum*, *Hyaloperonospora arabidopsidis* and *Pseudomonas syringae* (Shi et al., 2013). Tomato *protein kinase 1* (*TPK1b*) is induced by pathogen infection, mechanical wounding and oxidative stress, and plays a signaling role in the ethylene-mediated shared defense mechanism governing resistance to infection by the necrotrophic fungus *Botrytis cinerea* and to infestation by the herbivorous insect *Manduca sexta* (Synan et al., 2008). These results suggest that, as a RLCK-VII member, *SpRLCK1* might be involved not only in abiotic stress tolerance, but also in plant immunity.

Previous studies have shown that transcripts of *RLCKs* are induced by various abiotic stresses. In rice, about 86 *OsRLCKs* were differentially expressed under cold, salt, and drought treatment (Gao and Xue, 2012). The *Arabidopsis RLCK Calmodulin-binding Receptor-like Cytoplasmic kinase 1* is induced by cold, salt, H_2O_2, and ABA (Yang et al., 2004; Tianbao et al., 2010). In *Glycine soja*, *GsRLCK1* also is significantly induced by salt and drought (Sun et al., 2014). Here, qRT-PCR showed that *SpRLCK1* was induced under multiple stress conditions, especially drought and salt stresses. Three drought responsive elements (MBS *cis*-elements) were detected in the *SpRLCK1* promoter, which might be responsible for drought-induced expression of *SpRLCK1*. Phytohormone ABA plays a central role in regulating various stress responses, triggering major changes in gene expression and adaptive physiological responses (Lee and Luan, 2011; Danquah et al., 2014; Kuromori et al., 2018). In the *SpRLCK1* promoter, the ABA-related *cis*-element such as ABREs were detected, indicating that *SpRLCK1* might play a role in the ABA-mediated pathway in stress tolerance.

In this study, multiple parameters including root length, fresh weight, seedling survival rate and relative water content were significantly higher in the *SpRLCK1*-overexpressing plants than in the WT plants under drought and salt conditions, demonstrating that *SpRLCK1* plays positive roles in plant response to osmotic stress. ROS is a class of important signaling molecules that is involved in stress responses and normal plant development, but overproduction of ROS can cause oxidative damage to plants (Apel and Hirt, 2004; Sharma et al., 2012). Increased studies have confirmed that the capacity of ROS scavenging is correlated with plant tolerance to stresses. For example, overexpression of *CHS*, *Eukaryotic translation initiation factor 1A*, and *WRKY14* enhances transgenic plants stress tolerance by improving the activity of ROS scavenging enzymes (Yang et al., 2017; Zhang et al., 2017, 2018). In the present study, compared with the WT plants, the *SpRLCK1*-overexpressing plants exhibited weaker oxidative damage and higher activity of antioxidant enzymes (SOD, POD and CAT) under stress conditions, indicating that *SpRLCK1* conferred plant drought and salt tolerance through enhancing the activity of antioxidant enzymes.

Plant stress tolerance is a complex regulating network. In the co-expression network for *SpRLCK1*, some stress-related genes (e.g., *AOX1D*, *ABR1*, *WRKY33*, *WRKY75*, *ERF98*, *peroxidase*, *GSTU19*, *ZAT11*) were significantly up-regulated in *SpRLCK1*-overexpressing plants. *AOX1D* is putatively involved in the ROS scavenging and signal transduction pathways (Fabio et al., 2005). The up-regulation of *AOX1D* might equip the *SpRLCK1*-overexpressing plants with higher ability to scavenge ROS for protection against cellular damage. *APETALA2-like ABA repressor 1* (*ABR1*) transcription factor serves as a repressor of ABA response during stress condition. Its expression is highly induced by salt, drought and cold stresses; and *abr1* mutant is hypersensitive to mannitol and salt (Pandey et al., 2005). *WRKY33* transcription factor plays important roles in plant biotic and abiotic stress tolerance. *Arabidopsis atwrky33* mutant is susceptible not only to necrotrophic pathogens and *Botrytis cinerea* infection, but also to salt and heat stresses (Zheng et al., 2006; Jiang and Deyholos, 2009; Li et al., 2011; Birkenbihl et al., 2012). As the most abundant water-soluble antioxidant, ascorbic acid (AsA) plays important roles in scavenging ROS. It has been reported that overexpression of *AtERF98* results in increased levels of AsA content, whereas *erf98* mutant shows reduced AsA and decreased the tolerance to salt stress (Zhang et al., 2012). The up-regulated *ERF98* may increase the AsA and reduce the ROS content, enhancing the stress tolerance of *SpRLCK* transgenic plants. As an important antioxidant enzyme, peroxidases reduce peroxide through substrate oxidation, thus it is involved in ROS detoxification to alleviate oxidative stress (Fernanda et al., 2015; Khan and Komatsu, 2016). Increased peroxidase activity is associated with drought tolerance in *Triticum aestivum* (Sheoran et al., 2015) and *Nicotiana tabacum* (Mercadoa et al., 2010). Moreover, overexpression of *Ipomoea batatas swpa4* peroxidase in tobacco can enhance the resistance to pathogens, as well as tolerance to drought and salt (Kim et al., 2008). Overexpressing *Arabidopsis AtRCI3* that encodes an active cationic peroxidase causes increased tolerance to drought and salt, while antisense suppression of *AtRCI3* expression results in opposite phenotypes (Francisco et al., 2010). The increased expression levels of the *peroxidase* gene and enhanced activity of peroxidase enzyme in *SpRLCK1*-overexpressing plants provide increased evidence for a close association between peroxidase and drought tolerance in plants. In both plants and animals, GST function is closely linked to the stress response. *GSTU19* expression is induced by drought stress, oxidative stress, and high doses of auxin and cytokine. Overexpression of *GSTU19* can enhance tolerance to salt, drought and methyl viologen stress (Xu et al., 2016). These results indicate that *SpRLCK1* might collaborate with these stress-related genes to withstand drought and salt stresses.

Overall, *SpRLCK1* was induced by multiple abiotic stresses, especially drought and salt stresses. Overexpression of *SpRLCK1* exhibited higher tolerance to drought and salt stresses than the WT plants, and the transgenic plants exhibited weaker oxidative damage and higher activity of antioxidant enzymes (SOD, POD and CAT). Additionally, we further discovered that *SpRLCK1* could cooperate with a series of stress-related genes that were co-expressed with *SpRLCK1*. Our results demonstrate that *SpRLCK1* confers plant drought and salt tolerance through enhancing the activity of antioxidant enzymes and cooperating with stress-related genes. This investigation provides a foundation for gene resources that involved in stress tolerance for woody plant species.

Authors' contributions

J-HX and L-JB conceived and designed this study. J-HX, L-JB and SP performed the experiments. J-HX, L-JB and ZJ performed the data collection and statistical analysis. J-HX and L-JB wrote the manuscript. L-MZ and H-JJ contributed suggestions. All authors gave the final approval of the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.042.

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