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NtRLK5, a novel RLK-like protein kinase from *Nitotiana tobacum*, positively regulates drought tolerance in transgenic Arabidopsis

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ABSTRACT

Receptor-like protein kinase (RLKs) plays pivotal roles in plant growth and development as well as stress responses. However, little is known about the function of RLKs in *Nitotiana tobacum*. In the present study, we present data on *NtRLK5*, a novel RLK-like gene isolated from Hongda (*Nitotiana tobacum* L.). Expression profile analysis revealed that *NtRLK5* was strongly induced by drought and salt stresses. Transient expression of NtRLK5-GFP fusion protein in protoplast showed that NtRLK5 was localized to plasma membrane. Overexpression of *NtRLK5* conferred enhanced drought tolerance in transgenic Arabidopsis plants, which was attributed to not only the lower malondialdehyde (MDA) and H_2O_2 contents, but also the higher antioxidant enzymes activities. Moreover, the expression of several anti-oxidation- and stress-related genes was also significantly up-regulated in *NtRLK5* transgenic plants under drought condition. Taken together, the results suggest that *NtRLK5* functions as a positive regulator in drought tolerance.

1. Introduction

As a sessile organism, plant will suffer various environmental stresses (*e. g.* drought, salt, and extreme temperature), all of which result in osmotic stress in cells. It is well established that plant has evolved many sophisticated mechanisms at morphological, physiological, cellular and molecular levels to overcome unfavorable conditions [1–3]. Drought is a major environmental factor that causes considerable agronomic problem by limiting crop yield and distribution world-wide [4]. Plants adapt to drought stress typically through physiological and biochemical responses, including photosynthesis repression, stomatal closure, osmolyte accumulation, and reactive oxygen species (ROS) production [5].

Perception and proper signal transduction are pivotal for plant to adapt external environmental stimulus. Plant perceives and recognizes external stimulus through various classes of receptors, such as receptor-like protein kinases (RLKs). RLK is one of the largest gene families in plants. The Arabidopsis and rice genomes harbor 610 and approximately 1131 RLK members, respectively [6,7]. Extensive researches have demonstrated that RLKs play critical roles in the regulation of diverse signaling pathways, developmental processes, and disease resistance [8,9]. Moreover, RLKs also function as important regulators to activate signaling pathways via perceiving and processing external stimulus to cellular signaling molecules [10]. Overexpression or knockout is two effective strategies in characterising RLKs functions under stresses [11,12]. To date, function of RLKs has been characterized to be involved in the stress response in various plant species, such as rice, Arabidopsis, maize, soybean, and wheat. *PnLRR-RLK27*, a RLK from Antarctic moss *Pohlia nutans*, was shown to play a positive role in salt and oxidation-stress tolerance [13]. *FON1* is a rice RLK gene that induced by drought and ABA. Constitutive expression of *FON1* enhanced the drought tolerance and sensitivity to ABA in transgenic rice plants, whereas *FON1*-RNAi transgenic rice showed Reciprocal Phenotypes [11]. *GsRLCK*, a *Glycine soja* receptor-like cytoplasmic kinase (RLCK), decreased ABA sensitivity and altered

Abbreviations: ABA, abscisic acid; CAT, catalase; GFP, green fluorescent protein; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; MS, murashige and skoog medium; O_2^-, superoxide free radicals; PEG, polyethylene glycol; POD, peroxidase; qRT-PCR, quantitative real-time PCR; REL, relative electrolyte leakage; RLK, receptor-like protein kinase; RNAi, RNA interference; ROS, reactive oxygen species; SOD, superoxide dismutase; WT, wild type.

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the transcript level of ABA-responsive genes in *G. soja* under drought condition [14]. To date, the functions of RLKs in tobacco are poorly understood.

To decipher the relevant genes that related to stresses in tobacco, we analyzed the genome expression profiles of Hongda (*Nitotiana tobacum* L.) under stress conditions by RNA-sequencing. One such gene, which designated as *NtRLK5* in China Tobacco Genome Database, was highly upregulated by drought stress (http://10.6.0.76/). Bioinformatics analysis revealed that *NtRLK5* encodes a protein of 881 amino acid residues with a molecular mass of 96.79 kDa. Transgenic Arabidopsis with overexpressing *NtRLK5* enhanced the drought tolerance, with improved antioxidative enzyme activity and low cell membrane damage.

2. Materials and methods

2.1. Plant materials and growth condition

Arabidopsis *thaliana* ecotype Columbia (Col-0) was used as wild type (WT). Seeds were surface-sterilized with 75% ethanol and 10% bleach (v/v), washed at least three times with sterile water, and then stratified for 2 days at 4 °C in the dark. Plants were grown on 1/2 MS medium containing 0.8% (w/v) sucrose and 0.75% (w/v) agar in a growth chamber maintained at 23 °C and 65% relative humidity under long-day conditions (16-h light/8-h dark).

2.2. Plasmids construction and Arabidopsis transformation

To construct the vector overexpressing *NtRLK5*, cDNA fragment containing the whole open reading frame of *NtRLK5* was amplified from total RNAs of Hongda with the primer *NtRLK5*-F and *NtRLK5*-R. The primers were listed in Supplementary Table S1. The verified PCR products were then ligated into binary expression vector p1300-pJITl63, which was derived from pCAMBIA1300 and pJITl63, to generate p1300-pJITl63-*NtRLK5* plasmid, rendering the expression of *NtRLK5* to be driven by the CaMV 35S promoter. The yield plasmid was introduced into *Agrobacterium tumefaciens* GV3101 (pMP90) cells. Arabidopsis transformation was carried out by floral dip method [15]. The procedure for selecting homozygous was performed as previously described by Xu et al. [16].

2.3. RNA extraction and quantitative real-time PCR

Total RNAs were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed by PrimeScript™ 1st Strand cDNA synthesis kit (Takara) following the manufacturer's protocol. The qRT-PCR analysis was conducted with a Roche LightCycle 96 engine (Roche) using the FastStart Universal SYBR Green Master (Roche). *AtTubulin* was used as the internal control, and the relative amounts of mRNA were calculated. For each sample, at least three biological replicates and three technical replicates were carried out. The data were analyzed using the comparative *Ct* method. The gene-specific primers used to detect transcripts are listed in Supplementary Table S1.

2.4. Subcellular localization

The GFP-fusion constructs that were used in the subcellular localization analysis were created by cloning the full-length coding sequence without termination codon of *NtRLK5* into the pFF19 backbone containing the CaMV35S promoter. The PCR amplification was carried out with primer pairs *NtRLK5-GFP*-F and *NtRLK5-GFP*-R. The primers were listed in Supplementary Table S1. Preparation of tobacco protoplasts was performed as described previously by Chen et al. [17] and the polyethylene glycol (PEG)-mediated transformation was performed as described previously [18]. After incubation in the dark for 12–18 h at 23 °C, fluorescence was observed using a confocal laser microscope (TCS STED CW, LEICA).

2.5. Drought tolerance assays

For the cotyledon greening assay, sterilized seeds of WT and transgenic plants were sown in triplicate on 1/2 MS medium in the same Petri dish in the absence or presence of 300 mM Mannitol (12 days), and the cotyledon greening rates were recorded at the end of the experiment. For drought assays, three-week-old plants grown in soil were withheld water for 14 days. Photographs were taken 7 days after watering was resumed, and survival rates were determined. For qRT-PCR analysis of antioxidation- and stress-related genes, two-week-old plants were treated with 300 mM mannitol. After 48 h treatment, plant materials were prepared for further qRT-PCR analysis.

2.6. Determination of biochemical parameters

Three-week-old plants were used for biochemical analysis. WT and transgenic plants were treated with 300 mM mannitol for 2 days. At the end of the treatment, biochemical parameters were determined. MDA accumulation was measured using the thiobarbituric acid-based method as described by Kramer et al. [19]. Electrolyte leakage was determined by relative conductivity as described by Hu. et al. [20]. After extraction of H_2O_2, CAT, SOD, and POD using PBS solution (0.1 M, pH 7.4), the content of H_2O_2, and the activities of CAT, SOD, and POD were immediately determined by enzyme-linked immunosorbent assay (ELISA), using the corresponding detection kits (Jiancheng, Nanjing, China).

2.7. Statistical analysis

Statistical analyses were performed with SPSS software (SPSS 13.0). Analysis of variance was used to compare the significant difference based on Student's *t*-test.

3. Results and discussion

3.1. Expression pattern of NtRLK5 and its subcellular localization

To elucidate the physiological and functional relevance of *NtRLK5*, qRT-PCR was used to monitor its expression profile, including root, leaf, stem, petal, sepal and axillary bud. Result revealed that *NtRLK5* was constitutively expressed in almost tissues examined, with highest and lowest expression in leaf and in sepal, respectively (Fig. 1A). Kilian et al. analyzed the drought transcript profiling data set and found that expression of upregulated RLKs reached peak level at the 1 h time point after exposure to drought, indicating that RLKs may be essential for a rapid response to drought stress [21]. We further examined the expression profiles of *NtRLK5* under drought and salt conditions. As shown in Fig. 1B, the expression of *NtRLK5* was rapidly induced and reached to maximum level at 1 h after exposure to drought and salt, and gradually declined to basal level at 24 h. *NtRLK5* showed similar expression pattern when exposed to drought and salt stresses (Fig. 1B). Since osmotic stress can be a common consequence of drought and salt stresses [22,23], this may explain the reason why *NtRLK5* displayed similar expression pattern under drought and salt conditions.

To determine the subcellular localization of NtRLK5, p35S:*NtRLK5-GFP* and p35S:*GFP* plasmids were built, and transiently introduced into tobacco protoplast cells. As depicted in

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