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Research Paper

Pathogenesis-related protein PR10 from *Salix matsudana* Koidz exhibits resistance to salt stress in transgenic *Arabidopsis thaliana*

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ABSTRACT

Pathogenesis-related (PR) proteins are involved in plant defense and have multiple functional adaptations that assist in resisting various pathogens and tolerating environmental stress. *Salix matsudana* Koidz, a deciduous, rapidly growing willow species, can tolerate a range of adverse conditions. Comparative proteomic analysis previously revealed that the *S. matsudana* PR protein SmPR10 was abundant and up-regulated with 100 mM NaCl treatment. In this study, the *SmPR10* gene from *S. matsudana* was cloned and characterized to determine its role in salt tolerance. The amino acid sequence of SmPR10 showed 98% and 93% sequence homology with PR proteins from *S. purpurea* and *Populus trichocarpa*, respectively. SmPR10 was localized in the cytoplasm of *Arabidopsis* protoplasts. *SmPR10* transcript and protein levels were high in roots, and its expression was up-regulated in roots treated with 100 mM NaCl. SmPR10 was detected specifically in phloem fiber cells and root xylem by immunolocalization analysis. Moreover, heterogeneous overexpression of *SmPR10* enhanced the salt tolerance of transgenic *Arabidopsis* plants as shown by analysis of root length, root number, and Na^+ flux, as well as physiological parameters such as chlorophyll content, MDA content, electrical conductivity, and SOD and POD enzyme activity levels. Our results reveal that *SmPR10* plays an important role in salt tolerance and could serve as an important candidate gene to improve salt tolerance in woody species through genetic engineering.

1. Introduction

Soil salinization is a significant issue worldwide and is the main factor limiting soil resource utilization and agricultural development. Salt stress can change plant morphology, physiology, biochemistry, and molecular biology, and these changes can lead to reduce plant growth and yield (Yang et al., 2006). Over long periods of exposure to saline environments, plants have gradually evolved complex physiological mechanisms to resist salt stress. These mechanisms operate mainly involved in osmotic regulation, ion compartmentation, antioxidant production, and signal transduction. As human populations increase and arable land area decreases, breeding new salt-resistant plant varieties via biotechnology is an important approach utilizing saline-alkali land.

Pathogenesis-related proteins (PRs) are a type of protein induced by pathogens and specific chemicals, and play an important role in plant defense. The PRs were first isolated in the 1970s from tobacco leaves that were infected with the tobacco mosaic virus (Van Loon and Van Strien, 1999). PRs have been classified into 17 families according to their electrophoretic mobility, plant of origin, serological relationships and amino acid sequence homology (Sels et al., 2008; Van Loon et al., 2006).

PR10 is one of the most important PR proteins and was first discovered in cultured parsley cells (Somssich et al., 1988); PR10 has been studied in many plants, such as *Lilium brownie* (Huang et al., 1997), *Gossypium* spp. (Zhou et al., 2002), *Oryza sativa* (Hashimoto et al., 2004), *Zea mays* (Xie et al., 2010) and *Glycine max* (Xu et al., 2014). Unlike most other PR protein family members, PR10 protein cannot be secreted outside the cell because it has no signal peptide sequence. This protein is, therefore, generally located in intracellular fluid and cytoplasm (Ziadi et al., 2001). Previously, the biological defense functions of PR10 proteins were mainly reflected by their antibacterial activity and RNase activities (Park et al., 2004). In recent years, however, some reports have found that expression of the genes encoding PR10 proteins was up-regulated in plants under abiotic stresses like drought and

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salinity (Liu and Ekramoddoullah, 2006), indicating that PRl0 proteins may play an important role in plant resistance to abiotic stresses. It was also reported that the expression of *JcPR10* genes from *Jatropha curcas* greatly improved salt tolerance in *E. coli* (Agarwal et al., 2013) while expression of soybean *GmPR10* in transgenic rice plants resulted in increased cell integrity and promoted plants growth under salt stress conditions (Kim et al., 2010). Moreover, expression of the pea *PR10* gene in *Brassica napus* enhanced seeds germination rates and seeding growth under salt-stress conditions (Srivastava et al., 2004). However, the precise role of PR10 in salt stress has remained elusive in woody plants.

Salix matsudana Koidz is a deciduous, rapidly growing willow species that reproduces via asexual reproduction and, can tolerate adverse conditions. In a previous study by our group, comparative proteomes in *S. matsudana* were analyzed to investigate the basis of salt tolerance in this species (Qiao et al., 2013). According to the proteome data, a PR10 protein was found to be abundant and up-regulated under 100 mM NaCl stress. In this study, *SmPR10* from *S. matsudana* was cloned and characterized to identify its role in salt tolerance. The results showed that *SmPR10* can enhance salt tolerance in transgenic *Arabidopsis thaliana* and is, therefore, an important candidate gene to develop salt-tolerant plant species through genetic engineering.

2. Materials and methods

2.1. Plant materials and growth conditions

Stem cuttings approximately 15 cm in length were collected from annual branches of *S. matsudana* and grown in hydroponics. Plants were supplemented with water containing 1/2 strength Hoagland solution every 3 d in the artificial climate box RDN-1000E (25 °C, 16 h light/8 h dark). Seeds of the *Arabidopsis* ecotype Columbia from our laboratory were used.

2.2. Isolation of the full-length SmPR10 cDNA and genomic sequence

According to PR10 peptide fragments from the previously published proteome data, we predicted the nucleotide sequence of *SmPR10*; this was found to be similar to *Populus trichocarpa PR10* (*PtPR10*). Primers PR10-F and PR10-R (Table 1) were designed according to the *PtPR10* sequence and, *PR10* was cloned from a willow cDNA library using the polymerase chain reaction (PCR). The PCR reaction conditions were as follows: 95 °C for 7 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 45 s before a final extension at 72 °C for 7 min. The PCR product of the expected size was gel-purified and cloned into the pMD19-T vector (TaKaRa, Dalian, China) for sequencing at Biosune Biotechnology (Hangzhou, China).

Table 1
Primers used in this study.

Primers	Sequence (5′-3′)	Explanation
PR10-F	ACCAACAAAAACATCTTGC	for cDNA amplification
PR10-R	GGTACAAATAACCACACCA	for cDNA amplification
PR10ORF-F	ATGGGTGTCATCACTTTGG	for full-length of genome
PR10ORF-R	TCAGGCATCTGGATTGGCC	for full-length of genome
PR10ORF-sF	CACC ATGGGTGTCATCACTTTGG	for subcellular localization
PR10ORF-sR	GGCATCTGGATTGGCC	for subcellular localization
SmPR10-RT-F	AAGTTCGAGCCAACTCCTGA	for qRT-PCR
SmPR10-RT-R	TGGATTGGCCAAGAAGTAGG	for qRT-PCR
SmDnaJ-F	GCACCAAATTTGAGCAGGAT	Reference gene in *S. matsudana*
SmDnaJ-R	GGAAGCAGTGGGGTTTTGTA	Reference gene in *S. matsudana*
NPT II-F	ATCTCCTGTCATCTCACCTTGCTCCT	for Kan resistance screening
NPT II-R	TCAGAAGAACTCGTCAAGAAG	for Kan resistance screening
AtActin-F	GCACCCTGTTCTTCTTACCG	Reference gene in *Arabidopsis*
AtActin-R	AACCCTCGTAGATTGGCACA	Reference gene in *Arabidopsis*

To acquire the genomic sequence of *SmPR10*, we designed primers PR10ORF-F and PR10ORF-R according to the open reading frame (ORF) of *SmPR10* (Table 1). *S. matsudana* genomic DNA was used as a template together with this primer pair to amplify the full-length *SmPR10* genomic sequence.

2.3. Bioinformatic analysis of SmPR10

Online ExPASy tools (http://web.expasy.org/tools/) were used to the predict molecular weight and isoelectric point of the SmPR10 protein; the subcellular localization of SmPR10 was predicted using the online tool Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/). SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used to test for signal peptide signatures. A phylogenetic cluster tree was constructed via the neighbor-joining method using MEGA 6.0 software with 1000 bootstrap replicates based on the amino acid sequences of SmPR10 proteins and PR10 proteins in other plant species.

2.4. Subcellular localization of SmPR10

The ORF of *SmPR10* was obtained by PCR amplification using the specific primers PR10ORF-sF and PR10ORF-sR. The purified PCR products were cloned into the Gateway entry vector pENTR/D-Topo (Invitrogen, Carlsbad, USA) and were, then LR-recombined into the N-terminus of an enhanced green fluorescent protein (GFP) in the small binary vector p2GWF7.0. The correct plasmid was extracted by Plasmid Maxprep Kit (Vigorous, Beijing, China). Free vector p35S-GFP containing the 35S CaMV promoter was used as a control. Isolation and PEG-mediated transformation of *Arabidopsis* were carried as previously described (Wu et al., 2009). After incubation for 15 h at 25 °C, the transfected protoplasts were viewed with a Zeiss LSM700 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

2.5. Transcriptional levels of SmPR10 in S. matsudana under salt stress

Six different plant tissues including roots, stems (internodes 2–5), leaves, xylem, phloem, and flowers, were collected from perennial *S. matsudana* plants. After 45 d in water culture, the *S. matsudana* seedlings were subjected to 100 mM NaCl; untreated seedlings were used as the control. The roots, stems and leaves of the treated plants were sampled at 0 h, 12 h, 24 h, 48 h, 72 h and 96 h. All samples were promptly frozen in liquid nitrogen followed by storage at −80 °C until total RNA extraction.

Total RNA of all samples were extracted using the Total RNA Purification Kit (Norgan Biotek Corp., Thorold, Canada). After the concentration of total RNA was detected, PrimeScriptTM RT Master Mix (TaKaRa, Dalian, China) was used for cDNA first-strand synthesis. The resulting cDNA was diluted 10-fold and used as the template for quantitative real-time fluorescent PCR (qRT-PCR) analysis using the primers PR10RT-F and PR10RT-R (Table 1). To normalize the expression level, the *S. matsudana* endogenous gene *DnaJ* was selected as a reference gene (Zhang et al., 2017), amplified using the primers DnaJ-F and DnaJ-R (Table 1).

2.6. Antibody production, western blot analysis, and immunolocalization

A peptide (EHIKSSEIIEGNGGAGTIRK) specific for the SmPR10 protein was synthesized and injected into healthy rabbits to raise polyclonal antibodies. Following the established immunization protocol, crude rabbit serum was collected and purified by the Beijing Protein Innovation Co., Ltd. (Beijing, China). For western blot analysis,