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Research paper

Overexpression of *quinone reductase* from *Salix matsudana* Koidz enhances salt tolerance in transgenic *Arabidopsis thaliana*

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ABSTRACT

Quinone reductase (*QR*) is an oxidative-related gene and few studies have focused on its roles concerning salt stress tolerance in plants. In this study, we cloned and analyzed the *QR* gene from *Salix matsudana*, a willow with tolerance of moderate salinity. The 612-bp cDNA corresponding to *SmQR* encodes 203 amino acids. Expression of *SmQR* in *Escherichia coli* cells enhanced their tolerance under salt stress. In addition, transgenic *Arabidopsis thaliana* lines overexpressing *SmQR* exhibited higher salt tolerance as compared with WT, with higher QR activity and antioxidant enzyme activity as well as higher chlorophyll content, lower methane dicarboxylic aldehyde (MDA) content and electric conductivity under salt stress. Nitro blue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) staining also indicated that the transgenic plants accumulated less reactive oxygen species compared to WT when exposed to salt stress. Overall, our results suggested that *SmQR* plays a significant role in salt tolerance and that this gene may be useful for biotechnological development of plants with improved tolerance of salinity.

1. Introduction

Salinization is a global problem that affects plant growth and crop yields (Boyer, 1982). Salinity has direct effects on plant metabolism, causing ion imbalances and creating hyperosmotic conditions leading to oxidative damage (Munns and Tester, 2008). In response, plants have developed a series of complex mechanisms to defend against salinity-mediated damage and adapt to salinization, including molecular networks involved in stress recognition, information transfer, and activation of various resistance-related genes (Xiong et al., 2002; Vinocur and Altman, 2005). The expression of such resistance-related genes brings about physiological and biochemical changes including activation of detoxification enzymes and systems for removal of reactive oxygen species (ROS) (Foolad, 2007). The salinity stress-induced genes from plants characterized to date include receptors, signaling molecules and transcription factors (Chen et al., 2009). Understanding the functions of these genes serves as the theoretical foundation for efficient engineering strategies to improve salt stress tolerance in agriculturally and environmentally significant species (Kawasaki et al., 2001).

ROS accumulate when plants suffer from salinity stress. This accumulation can cause oxidative damage to the plasma membrane, chlorophyll, proteins and nucleic acids, and ultimately affect metabolism in general (Mittler, 2002). To reduce or avoid these effects, plants have evolved antioxidant systems involving enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD), catalase (CAT), and quinone reductase (QR) (Kärkönen and Kuchitsu, 2015).

QR is found extensively in plants, animals and microbial systems (Martius, 1963). Previous studies have revealed its role in antioxidant processes and detoxification reactions (Prestera et al., 1993; Wang and Maier, 2004; Cuendet et al., 2006; Schopfer et al., 2008; Baxter et al., 2014; Ku et al., 2015). As early as the 1980s, scientists noticed that NAD(P)H:quinone reductase is related to 2(3)-tert-butyl-4-

Abbreviations: BHA, 2(3)-tert-butyl-4-hydroxyanisole; APX, ascorbate peroxidase; CAT, catalase; DAB, 3,3′-diaminobenzidine; H_2O_2, hydrogen peroxide; OH•, hydroxyl radical; IPTG, isopropyl β-D-1-thiogalactopyranoside; MDA, methane dicarboxylic aldehyde; Na^+-NQR, Na^+-translocating NADH: quinone oxidoreductase; NQO1, NAD(P)H:quinone oxidoreductase; NCBI, National Center for Biotechnology Information; NBT, nitro blue tetrazolium; ORF, open reading frame; POD, peroxidase; PCR, polymerase chain reaction; PVPP, polyvinylpyrrolidone; QR, quinone reductase; ROS, reactive oxygen species; *SmQR*, *S. matsudana quinone reductase*; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gels electrophoresis; $^1O_2$, singlet oxygen; O_2^-, superoxide anion; SOD, superoxide dismutase; UTR, untranslated region; WT, wild-type.

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hydroxyanisole (BHA), an antioxidant food additive, in several mouse tissues (Benson et al., 1980). The activity of NAD(P)H:quinone reductase is greatly increased in response to BHA, similar to epoxide hydratase and glutathione S-transferase activities. The findings preliminarily imply that QR is associated with resistance to oxidative damage since this and other studies have found that QR protects the cell from quinone-related oxidative damage. The oxidative effects of quinones are thought to be due to their semiquinone radical intermediates, which generate ROS (Cadenas, 1995; Kishikawa and Kuroda, 2014; Ku et al., 2015). Cytotoxic effects of the one-electron reduction of quinones and free radicals have been shown to be suppressed by QR (Lind et al., 1982; Thor et al., 1982; Chesis et al., 1984). When the cellular quinone content increases, the expression level of NAD(P)H:quinone oxidoreductase (NQO1) increases (Dinkova-Kostova and Talalay, 2010). NQO1, also called DT-diaphorase, uses NAD(P)H as the receptor and catalyzes a two-electron reduction reaction of quinones and quinone derivatives (Ross et al., 2000; Schopfer et al., 2008) such as benzpyrene-3,6-quinone to hydroquinones (Conover and Ernster, 1960).

NQO1 can also remove endogenous quinones and help protect cellular membranes during antioxidant defense (Ross et al., 2000). NQO1 generates ubiquinol from ubiquinone (coenzyme Q) to protect membrane phospholipids in rat livers from oxidative damage, and it also can catalyze reduction of α-tocopherol quinone (vitamin E oxidation products) to α-tocopherol hydroquinone during antioxidant protection in human (Landi et al., 1997; Siegel et al., 1997). In addition to this, interestingly, a Na^+-translocating NADH: quinone oxidoreductase (Na^+-NQR) is thought to serve as a redox-driven sodium pump (involving sodium cycle and respiratory chain) in some marine and halophilic bacteria (Hayashi et al., 1992; Unemoto and Hayashi, 1993; Nakayama et al., 1998; Häse and Barquera, 2001). These findings further suggest that QR is important for salt stress response.

Due to their ability to live under high salinity conditions, halophytes served as a rich source of salt-responsive genes that can be useful for bioengineering (Rajalakshmi and Parida, 2012). Among halophytes, *Salix matsudana* Koidz (*S. matsudana*) is a salt-tolerant tree species that can occupy broad habitats through asexual propagation. Here, the cloning and characterization of *S. matsudana quinone reductase* (*SmQR*) were performed to identify its roles concerning salt tolerance. Our results revealed that *SmQR* may be useful for biotechnological development of plants with improved tolerance of salinity.

2. Materials and methods

2.1. Plant materials and growth conditions

The *S. matsudana* clone I-32 was water-cultivated in an artificial climate chamber at 25 °C with 16 h light/8 h dark at the Research Institute of Subtropical Forestry, Chinese Academy of Forestry, Zhejiang Province, China.

2.2. Isolation of SmQR cDNA

An *SmQR* fragment previously identified by 2-DE and MALDI-TOF/TOF analysis of a salt stress-induced cDNA library from *S. matsudana* was predicted to encode a protein with 95% amino acid identity with *Populus trichocarpa quinone reductase* (Qiao et al., 2014). Here, the full length cDNA was cloned from a cDNA library by polymerase chain reaction (PCR) with primers M13-F, M13-R, *SmQR*-1 and *SmQR*-2 (Table 1). PCR was conducted with 10 min at 95 °C followed by 30 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min. The final extension step was 72 °C for 10 min. The resulting product was gel-purified, cloned into pMD-19T vector (TaKaRa, Dalian, China) and sequenced at Sangon Biotech (Shanghai, China).

Table 1
Specific primers used in this work.

Primers	Sequence (5′–3′)	Description
SmQR-1	CAAAAGTTCCGGCACCATAAG	Amplification
SmQR-2	CCCATGAGCTATGGGCAAC	Amplification
M13-F	TGTAAAACGACGGCCAGT	Amplification
M13-R	CAGGAAACAGCTATGACC	Amplification
SmQR-F	ATGGGGAACACAAAGATCTAC	ORF primer
SmQR-R	CTAGCCTTTGAGCTTCTTCG	ORF primer
SmQRGO-F	TCCCCCGGGATGGGGAACACAAAGATCTAC	Amplification
SmQRGO-R	GCTCTAGA CTAGCCTTTGAGCTTCTTCG	Amplification
QRm-F	CCGGAATTCATGGGGAACACAAAGATCTAC	Amplification
QRm-R	CCCAAGCTTCTAGCCTTTGAGCTTCTTCG	Amplification
SmQR-RT-F	TGCATTCACAGCCATAACGCA	RT-qPCR
SmQR-RT-R	TAATTCGGTGGGTTGACGGCT	RT-qPCR
AtActin-F	GCACCCTGTTCTTCTTACCG	RT-qPCR
AtActin-R	AACCCTCGTAGATTGGCACA	RT-qPCR

2.3. Isolation of SmQR genomic clone

S. matsudana genomic DNA was extracted by the cetyltrimethylammonium bromide (CTAB)-based method (Murray and Thompson, 1980). Using *S. matsudana* genomic DNA as template, primers *SmQR*-F and *SmQR*-R were designed according to the sequence of the ORF used to amplify the complete *SmQR* genomic fragment. The amplified product was gel-purified and sequenced.

2.4. Bioinformatic analysis of SmQR

The *SmQR* sequence was analyzed by the BLAST program National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Translation and protein analysis of *SmQR* was performed using ExPASy tools (http://www.expasy.org/tools/). The subcellular localization was predicted using CELLO v.2.5: subCELlular LOcalization predictor (http://cello.life.nctu.edu.tw/) (Yu et al., 2004). Based on amino acid sequences in plants, comparison and phylogenetic analysis were performed with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/) and MEGA package (version 5.2) (Tamura et al., 2011) using the neighbor-joining method and performing 1000 bootstrap replicates.

2.5. Functional analysis of SmQR protein in E. coli cells under salt stress

Based on the cDNA sequence, primers were designed with flanking restriction sites *EcoR* I in the forward primer *SmQR*-F and *Hind* III in the reverse primer *SmQR*-R (Table 1). The PCR-amplified product encoding SmQR was gel-purified, digested with *Eco*R I and *Hind* III restriction enzymes, and ligated with pET28a expression vector (Life Technologies, Carlsbad, CA, USA) linearized with the same restriction endonucleases. The verified recombinant plasmid pET28a-*SmQR* and pET28a vector were introduced into *E. coli* BL21 Star (DE3).

Liquid culture assays were performed to study the salt stress tolerance of *E. coli* cells transformed with pET28a-*SmQR* recombinant plasmid or vector alone. For protein expression, bacteria were cultured in LB (Luria-Bertani) medium until the OD_{600} reached 0.6 and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the final concentration of 0.1 mM.

After growth for 6 h at 37 °C, 1 mL cells were collected by centrifugation. Proteins were extracted for denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). resuspended in 1× sample loading buffer then subjected to 12% acrylamide gels with 4% stacking gels. Proteins were stained with Coomassie Brilliant Blue R250 (Makino et al., 1986).

After growth for 12 h at 37 °C, cultures were diluted to OD_{600} of 0.6. Then, 4 mL cells were inoculated into 100 mL LB medium supplemented with 800 mM NaCl. The bacterial suspension was harvested for growth

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