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Co-expression of ApGSMT and ApDMT promotes biosynthesis of glycine betaine in rice (*Oryza sativa* L.) and enhances salt and cold tolerance

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ABSTRACT

Glycine betaine (GB) is an osmoprotectant that protects plants from abiotic stress. GB is biosynthesized by choline dehydrogenation/oxidation and glycine methylation. The latter pathway involves glycine as a substrate and produces GB via successive methylation of glycine by two different N-methyltransferase enzymes; glycine sarcosine methyltransferase (GSMT), and sarcosine dimethylglycine methyltransferase (SDMT). In this study, *Agrobacterium*-mediated gene transformation was used to produce transgenic rice (*Oryza sativa* L., cv. Nipponbare) plants containing *ApGSMT* and *ApDMT* genes isolated from *Aphanothece halophytica*. The co-expression of both *ApGSMT* and *ApDMT* transgenes resulted in a significant increase of GB biosynthesis and enhanced tolerance to salt and cold stresses in the transgenic rice plants. These results demonstrate the potential of bioengineering for *glycine N-methyltransferase* genes in crop plants tolerance to abiotic stress.

1. Introduction

Plants are exposed to a variety of unfavorable environmental conditions including salinity, cold/heat, and drought. These stresses can have adverse effects on plant growth and development and, if severe enough, cause plant death (Krasensky and Jonak, 2012). Environmental stress causes a considerable yield reduction in agricultural ecosystems and even a complete loss of harvestable crop (Mahajan and Tuteja, 2005). Plants species have evolved a number of strategies to ensure survival and reproduction in response to different abiotic stresses (Perez-Clemente et al., 2013). One of these strategies is to synthesize and accumulate low-molecular-weight compatible compounds that are neutrally charged, highly soluble, and non-toxic to cells even at high concentrations. These compounds include glycine betaine (GB), sugar alcohol, and proline, all of which play essential roles in increasing plant tolerance to abiotic stress (Rhodes and Hanson, 1993; Bohnert et al., 1995; Chen and Murata, 2008). These compatible solutes have been thought to stabilize protein folding or membrane stability under thermodynamically stressed conditions (Arakawa and Timasheff, 1985; Murata et al., 1992) and protect the catalytic activities of enzymes (Manetas, 1990; Nikolopoulos and Manetas, 1991). These properties have attracted great interest because crop plants engineered with selected enzymes involved in osmolytes biosynthesis could be expected to display enhanced stress tolerance (Bohnert and Jensen, 1996).

GB (N,N,N-trimethglycine), a widely studied osmoprotectant, is a quaternary ammonium compound found in many animals, plants, and microorganisms (Pan et al., 1981; Hanson and Wyse, 1982; Rhodes and Hanson, 1993; Chen and Murata, 2002). By stabilizing quaternary structure of proteins and macromolecules (e.g. photosynthetic machinery), and maintaining ROS scavenging capacity, GB protects plants against the synergistic effects of various types of abiotic stresses (Rhodes and Hanson, 1993; Chen and Murata, 2011; Ahmad et al., 2013). GB has been shown to be synthesized

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by choline oxidation and glycine methylation, and both pathways are glycine-dependent. The former pathway begins with choline (converted from glycine) and proceeds through a one- or two-step process of dehydrogenation/oxygenation of choline to produce GB (Apse and Blumwald, 2002; Chen and Murata, 2002). The choline pathway of GB biosynthesis exists in the majority of plants, animals, and microorganisms (Rathinasabapathi et al., 1997; Craig, 2004). The latter pathway is a methylation pathway in which glycine acts as an initiator molecule for the synthesis of GB by a three-step methylation process. This pathway was initially discovered in two extremely halophytic microorganisms, *Actinopolypora halophil*a and *Ectothihodospira halochloris* (Nyyssölä et al., 2000). Two N-methyltransferase enzymes are actively involved in this biosynthetic pathway: (i) glycine sarcosine methyltransferase (GSMT) and (ii) sarcosine dimethyglycine methyltransferase (SDMT). Additionally, S-adenosylmethionine (SAM) is used as a methyl donor throughout the entire pathway. This pathway was later found in *Aphanothece halophytica*, a halotolerant cyanobacterium that synthesizes GB from glycine by using glycine sarcosine methyltransferase (ApGSMT) and dimethylglycine methyltransferase (ApDMT). ApGSMT catalyzes the methylation steps involved in the synthesis of sarcosine (N-monomethyglycine) from glycine and from sarcosine to dimethylglycine, respectively. ApDMT, catalyzes both synthesis of dimethylglycine, from sarcosine as well as the final synthesis of GB from dimethyglycine (Waditee et al., 2003).

Higher plants are either natural GB accumulators or non-accumulators (Stewart et al., 1979; Ishitani et al., 1993; Fitzgerald et al., 2009). In comparison with Chenopodiaceae species such as spinach (*Spinacia oleracea*) and sugar beet (*Beta vulgaris*) that usually accumulate abundant GB in response to water deficit or salt stress (Pan et al., 1981; Hanson and Wyse, 1982), cultivated rice (*Oryza sativa* L.) is considered as a GB non-accumulator (Ishitani et al., 1993; Rathinasabapathi et al., 1993; Nakamura et al., 1997; Shirasawa et al., 2006), although the sequenced rice genome has revealed the presence of orthologous genes for both choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH), which are required for the first and second steps of the catalytic reactions that produce GB from choline (Rathinasabapathi et al., 1997; Matsumoto et al., 2005). Previous studies reported *OsCMO* to be a pseudogene or indicated that its function has been abrogated (Rathinasabapathi et al., 1993; Shirasawa et al., 2006). Recent studies have proposed that the lack of a functional OsCMO protein in rice is caused by short direct repeated (SDR) sequences that lead to defective *OsCMO* mRNAs and hence to a lack of functional protein (Niu et al., 2010; Luo et al., 2012). However, it has been reported that genetic engineering of the production of GB in plants could increase osmotolerance and hence abiotic stress tolerance in non-accumulator plants (Chen and Murata, 2008, 2011; Khan et al., 2009). The methylation pathway transformation, which uses readily obtained glycine as precursor to overcome limited substrate supply in choline oxidation pathway, was confirmed to be a promising strategy against stresses in *Synechococcus*, *Anabaena*, *Nicotiana* and *Arabidopsis* (Waditee et al., 2005, 2012; He et al., 2011).

In the current study, *ApGSMT* and *ApDMT* genes, isolated from *A. halophytica*, were co-expressed in a *japonica* rice variety (*Oryza sativa* L., cv. Nipponbare, a GB non-accumulator, sensitive to abiotic stress) through genetic engineering to determine (i) whether or not transgenic rice plants containing *ApGSMT* and *ApDMT* could enhance GB amounts, and (ii) whether transgenic rice plants exhibit increased stresses tolerance and potential usage in crop plants engineering.

2. Materials and methods

2.1. Construction of pHB-ApGSMT/ApDMT and transformation of rice

The coding region of *ApGSMT* and *ApDMT* genes from *A. halophytica* were synthesized by the Majorbio Company (Shanghai, China) based on GenBank accession nos. AB094497 and AB094498, respectively. The coding sequences were inserted into a pGH vector resulting in pGH-ApGSMT and pGH-ApDMT. To construct the co-expression plasmid, *ApGSMT* cut from pGH-ApGSMT, was inserted into pHB vector, driven by 2× CaMV 35S promoter (Mao et al., 2005). *ApDMT* from pGH-ApDMT was cloned into the pHB-ApGSMT under the control of a single CaMV 35S promoter. The resultant coexpression vector pHB-ApGSMT/ApDMT was introduced into *Agrobacterium tumefaciens* EHA101 (Hood et al., 1993) by the freeze–thaw method.

A *japonica* rice variety (*Oryza sativa* L., cv. Nipponbare) was used for *Agrobacterium*-mediated transformation to generate transgenic rice plants as described by Roy & Wu (2001). After surface sterilization, mature seeds were placed on solid N6 medium amended with 2,4-dichlorophenoxyacetic acid (2 mg L^{-1}) for 3 weeks to induce callus from the scutellum, and subsequently used for transformation. After transformation, rice calli were placed on N6 medium with hygromycin (50 mg L^{-1}) as selective agent for 4 weeks (subcultured every 2 weeks). Resistant calli were transferred to N6 regeneration medium, and then to 1/2 MS medium to induce whole plant production. Regenerated plantlets were transplanted into pots (12 cm × 15 cm) and grown in the greenhouse (30/22 °C, day/night) with supplemental light (16 h photoperiod) for 130 d until maturity. T1 seeds, obtained by self-pollination of primary transgenic (T0) plants, were used in subsequent stress tolerance assays.

2.2. Identification of transgenic plants and analysis of transgene expression

To confirm transgene integration, genomic DNA from leaves of T0 rice plants was extracted using EasyPure Plant Genomic DNA Kit (TransGen Company, Beijing, China), and then were screened by polymerase chain reaction (PCR) for selective gene (*hygromycin phosphotransferase*, *Hyg*) and target genes (*ApGSMT* and *ApDMT*) using the primers Hyg_FR for *Hyg*, ApGSMT_F1R1 for *ApGSMT*, and ApDMT _F1R1 for *ApDMT*, respectively (Table 1).

For *ApGSMT* and *ApDMT* expression analysis, T0 transgenic rice leaves or other organs were harvested and RNA was isolated

Table 1
Primers used for identification of transgenic rice plants and expression analysis of transgenes.

Gene	Primers	Forward primer (5′–3′)	Reverse primer (5′–3′)	Corresponding region
ApGSMT	F1R1	AGAAAAACAAGTTCAAGACTACGGC	CGCAACGTGAATATAAAAGTCGG	+12 to +786
	F2R2	GATTCCACTCCATTCATCTCATTG	GGTTCTTAGGATTTGGTCTTTACG	+218 to +336
ApDMT	F1R1	CAGACGCAGTCGCCAAACAAG	TCATGCGGTCTAAATATTCTTGCGA	+11 to +754
	F1R2	CAGACGCAGTCGCCAAACAAG	ACGAGCGGAGCCACCATAACC	+11 to +243
Actin	FR	AGTGATTGCACCACCAGAAAGA	CAGGACCAGATTCATCATACTCG	+1126 to +1284
Hyg	FR	TCGTTATGTTTATCGGCACTTTG	GCGTCTGCTGCTCCATACAAG	+180 to +739

Corresponding region (Nucleotides are counted from the ATG of the full-length coding sequence, "A" = +1)