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DNA methylation involved in proline accumulation in response to osmotic stress in rice (*Oryza sativa*)

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ABSTRACT. Proline accumulation is involved in plant osmotic stress tolerance. Given that DNA methylation is related to almost all metabolic processes through regulation of gene expression, we suspected that this epigenetic modification and proline biosynthesis are probably related. To test this, we investigated whether osmotic stress-induced proline accumulation is associated with DNA methylation modifications in rice. We assessed DNA methylation and expression of 3 key genes (*P5CR*, *P5CS*, and *δ-OAT*) involved in proline biosynthesis, and measured proline content under both osmotic stress (15% polyethylene glycol) and control conditions. After osmotic stress, selfed progenies of osmotic-stressed plants accumulated higher concentrations of proline in leaves under both normal conditions and under osmotic stress than the unstressed control plants. Concomitantly, under osmotic stress, the selfed progeny plants showed higher expression levels of *P5CS* and *δ-OAT* than the control. This up-regulated expression was stably inherited by the subsequent generation. Methylation-sensitive

Southern blotting indicated that 2 of the 3 genes, *P5CS* and *δ-OAT*, had greater DNA demethylation in the selfed progenies than in the control. Apparently DNA demethylation facilitated proline accumulation by up-regulating expression of the *P5CS* and *δ-OAT* genes in response to osmotic stress.

Key words: Rice; Proline; Osmotic stress; DNA methylation

INTRODUCTION

Drought is one of the most prevalent abiotic stresses that limit crop productivity in many regions of the world. Plant response to drought is a very complex network affecting almost all processes in plant metabolism and development, including water balance, nutrient uptake and metabolism, and photosynthetic assimilation. Plant survival and growth under drought conditions result via adaptive processes involving altered ion uptake, stomatal regulation, and the accumulation of osmotic solutes such as proline. Proline accumulation is one of the most frequently reported physiological responses induced by drought and salt stress in plants and is believed to be involved in stress tolerance, although its precise role remains unclear (Lutts et al., 1999).

Proline accumulation during plant responses to osmotic stress is mainly due to increased synthesis and reduced degradation. In higher plants, proline is synthesized via 2 main pathways, the glutamate and ornithine pathways (Delauney and Verma, 1993; Roosens et al., 1998; Nanjo et al., 2003; Verbruggen and Hermans, 2008). Proline is synthesized via the glutamate pathway via 2 key enzymes, Δ^1-pyrroline-5-carboxylate synthetase (P5CS, EC 1.5.1.12) and Δ^1-pyrroline-5-carboxylate reductase (P5CR, EC 1.5.1.2) (Hu et al., 1992; Verbruggen and Hermans, 2008), whereas ornithine-*δ*-aminotransferase (*δ*-OAT, EC 2.6.1.13) is a key enzyme of the ornithine biosynthesis pathway. In the glutamate pathway, reciprocal regulation of P5CS and P5C dehydrogenase (EC 1.5.1.12), a degradation enzyme, plays a key role in the control of proline levels (Kiyosue et al., 1996). Enzymolysis of synthesized proline from both pathways is mediated by proline dehydrogenase (EC 1.5.99.8) (Peng et al., 1996; Verbruggen et al., 1996). Although plant biologists have studied the proline biosynthesis pathway extensively, many questions remain. For example, how do plants regulate proline biosynthesis during growth and development? What are the tuning trajectories for its rapid synthesis in response to abiotic stresses?

Cytosine DNA methylation plays important roles in multiple fundamental cellular activities, including control of gene expression, maintenance of genomic integrity, formation and perpetuation of chromatin structure, and control of genomic imprinting (Rangwala and Richards, 2004; Tariq and Paszkowski, 2004; Chan et al., 2005). Studies in recent years have revealed that DNA methylation is labile and sometimes responsive to both internal and external perturbations and that biotic and abiotic stresses may cause heritable alterations in cytosine methylation patterns, which presumably represent adaptive responses, in both plants and animals (Richards, 2006). Several studies have shown that heritable phenotypic novelties may be related to heritable alterations in DNA methylation (Molinier et al., 2006; Boyko et al., 2007; Chinnusamy and Zhu, 2009). Given that DNA methylation is involved in almost all metabolic processes because it regulates gene expression, we suspected that epigenetic modification and proline biosynthesis are probably intrinsically interlaced. To test whether

osmotic stress-induced proline accumulation is associated with DNA methylation modification, we investigated the transgenerational effects of osmotic stress on DNA methylation, the expression of key genes involved in proline metabolism, and proline accumulation in rice.

MATERIAL AND METHODS

Plant materials

'Matsumae', which used to be a major rice (*Oryza sativa*) cultivar in northeast China, was chosen as the test organism. Seeds were germinated and grown on Petri dishes for 6 days in a growth cabinet (30°C during the day and 25°C during the night, 16-/8-h photoperiod at 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Seedlings were transferred to buckets containing 2000 mL sterile nutrient solution for culture. The solution was replaced daily. The solution used in this study contained the components described by the International Rice Research Institute, including 1.44 mM NH_4NO_3, 0.32 mM NaH_2PO_4, 0.6 mM K_2SO_4, 1.0 mM $CaCl_2$, 1.6 mM $MgSO_4$, 0.072 mM Fe-ethylenediaminetetraacetic acid, 0.2 mM Na_2SiO_3, 9.1 µM $MnCl_2$, 0.154 µM $ZnSO_4$, 0.156 µM $CuSO_4$, 18.5 µM H_3BO_3, and 0.526 µM H_2MoO_4, pH 5.2.

Stress treatment

After 14 days of growth in hydroponic medium, rice plants were subjected to osmotic stress by transferring them to buckets containing 2000-mL treatment solution amended with the above nutrients and 15% polyethylene glycol for 4 days. We selected an osmotically stressed plant that showed the most obvious alteration in DNA methylation and its 16 selfed progenies (S1) to test the methylation state of *P5CR*, *P5CS*, and *δ-OAT* using methylation-sensitive Southern blotting. We also tested the methylation state of an *O. sativa* retrotransposon 42 and the housekeeping gene *Elongator*, which are unrelated to osmotic stress. We compared proline content and the expression levels of *P5CR*, *P5CS*, and *δ-OAT* in S1 plants and 3 S2 plants of this individual plant. Proline was measured using the ninhydrin method.

DNA isolation and digestion

Genomic DNA was isolated from expanded leaves using a cetyltrimethylammonium bromide method and purified with phenol extractions. The DNA was digested with the methylation-sensitive isoschizomer *Msp*I. To ensure complete digestion, we used an excess of enzymes (10 U enzyme per µg DNA) and extended the incubation time to approximately 48 h. Restriction enzymes were purchased from New England Biolabs, Inc. (Beverly, MA, USA). Digested DNA was used for Southern blotting and inter-simple sequence repeat analysis.

Methylation-sensitive amplification polymorphism (MSAP) analysis

The MSAP method was carried out as reported by Dong et al. (2006). Rice genomic DNA was digested with an *Msp*I. In total, 1 pair of pre-selective primers and 19 pairs of selective primers were used for the amplifications (Table S1). The MSAP products were resolved using 5% denaturing polyacrylamide gel electrophoresis and visualized with silver staining.

Only clear and completely reproducible bands in 2 experiments using independent DNA extractions were scored.

Methylation-sensitive Southern blotting

Rice genomic DNA was digested with the *Msp*I. The digested DNA was analyzed on 1% agarose gels and transferred to Hybond N^+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using the alkaline transfer recommended by the supplier. These probes were prepared via sequence-specific polymerase chain reaction (PCR) amplifications using specific primers (Table S2). The authenticity of all PCR-amplified probe fragments was verified through sequencing. The fragments were gel purified and labeled with fluorescein-11-2'-deoxyuridine 5'-triphosphate with the Gene Images Random Prime Labeling Module (Amersham Pharmacia Biotech). A hybridization signal was detected with the Gene Images CDP-Star detection module (Amersham Pharmacia Biotech) after washing at a stringency of 0.2X saline sodium citrate and 0.1% sodium dodecyl sulfate, twice for 50 min. The filters were exposed to X-ray films.

Quantitative real-time PCR analysis

We extracted total RNA from the leaves of seedlings grown under stress and non-stress conditions using TRIzol reagent (Invitrogen). The RNA was treated with DNase I (Invitrogen), reverse-transcribed using SuperScript™ RNase H-Reverse Transcriptase (Invitrogen), and then subjected to real-time PCR analysis using gene-specific primers. The gene-specific primers are listed in Table S3. PCR amplification was conducted with an initial step at 95°C for 1 min followed by 45 cycles of 5 s at 95°C, 10 s at 60°C, and 30 s at 72°C. Amplification of the target gene was monitored using SYBR Green in every cycle. Amplifications of the rice *Ubiquitin 5*, *Eukaryotic elongation factor-1α*, and *Atin 2* messenger RNA were used as an internal quantitative control (Jain et al., 2006; Zang et al., 2010). The relative expression of the target genes was calculated using the ΔCt method (Livak and Schmittgen, 2001). We optimized the PCR system, after which the amplification efficiencies of each target and reference gene were approximately equal.

Statistical analysis

Statistical analysis of the data was performed using the SPSS 13.0 statistical program (SPSS, Chicago, IL, USA). Measurements of proline content and gene expression were represented by an average of 3 replications and their standard errors, and each replicate consisted of a pool of 5 plants.

RESULTS AND DISCUSSION

Alteration in DNA methylation induced by osmotic stress

MSAP is a modified version of the standard amplified fragment length polymorphism technique that substitutes the original *Mse*I enzyme with the *Hpa*II or *Msp*I methylation-sen-

sitive enzymes. Using 19 pairs of selective *Eco*RI + *Msp*I primer combinations (Table S1), we amplified 900 clear bands across the set of plant samples (including control plants and 14 individual osmotic stressed plants; Figure 1). By tabulating the number of bands representing the various MSAP patterns, we calculated the hypermethylation, hypomethylation, and total methylation levels (see Figure 1). Fourteen individual osmotically stressed plants showed small differences in total methylation levels but large difference in hypermethylation and hypomethylation pattern changes (see Figure 1). Compared with the non-stressed control plants, the osmotic stressed rice plants showed clearly altered DNA methylation patterns (see Figure 1).

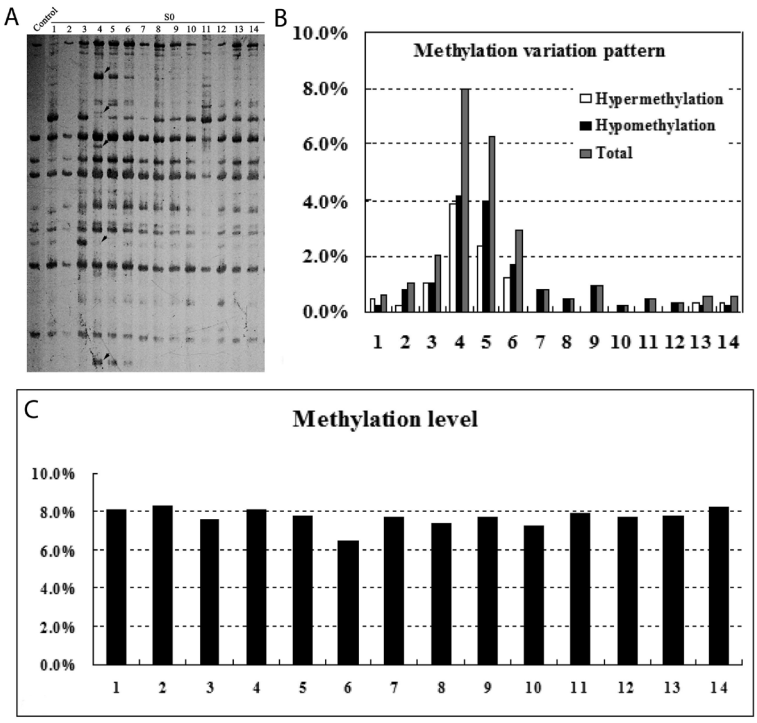

Figure 1. Effect of osmotic stress on DNA methylation state of rice plants revealed by MSAP analysis. **A.** Examples of altered DNA methylation patterns induced by osmotic stress; the methylation change was denoted by arrowheads. *Lanes 1* to *14* = osmotically stressed plants; Control = unstressed plant; S0 = individual osmotic stressed rice plant. The DNA was digested by a methylation-sensitive isoschizomer, *Msp*I. The digested DNA was analyzed by MSAP. **B.** DNA methylation alteration patterns of osmotic stressed plants. **C.** DNA methylation levels of osmotic stressed plants.

Possible role of DNA methylation in proline accumulation

We used methylation-sensitive Southern blotting analysis to investigate the role of DNA methylation in proline accumulation in response to osmotic stress. We selected an osmotically stressed plant that showed the most obvious alteration in DNA methylation patterns and its 16 S1 to test the methylation states of 3 genes, *P5CR*, *P5CS*, and *δ-OAT*, known to be involved in osmotic stress tolerance (Figure 2) (Verbruggen and Hermans, 2008). We

also compared expression levels of the 3 genes and proline content in an S1 plant and its 3 S2 plants. The Southern blotting results showed that under osmotic stress, 2 of the 3 tested genes, *P5CS* and *δ-OAT*, showed DNA demethylation in several stressed S1 plants but not in the immediately stressed plants (see Figure 2). By contrast, osmotic stress did not affect the methylation state of the third gene (*P5CR*), an *O. sativa* retrotransposon 42 and a housekeeping gene (*Elongator*; see Figure 2). This result suggested that the osmotic stress-induced DNA methylation changes are nonrandom.

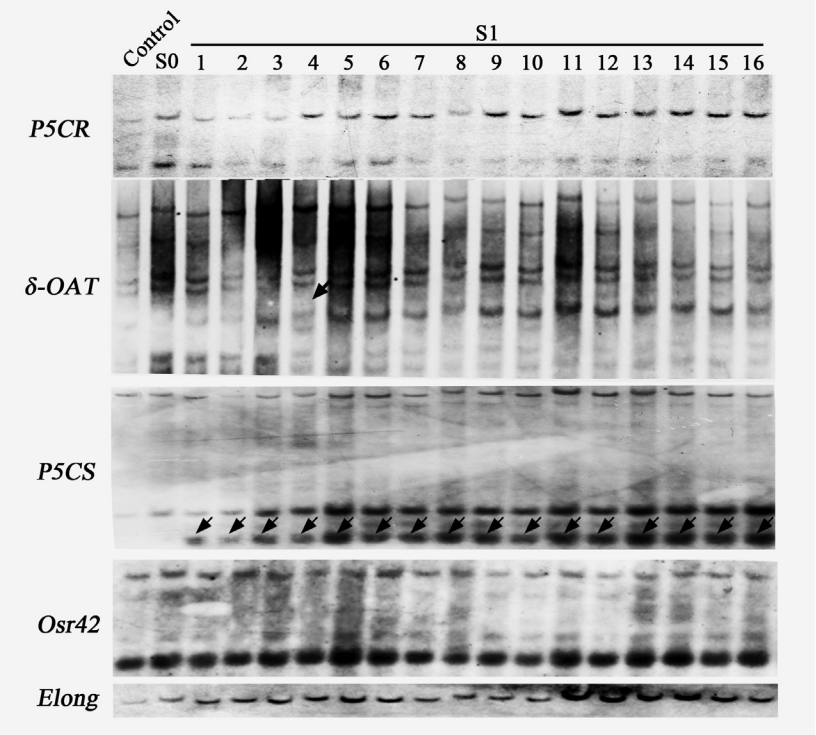

Figure 2. Transgenerational inheritance of altered DNA methylation patterns induced by osmotic stress to selfed progenies (S1) of the immediately stressed plants, revealed by methylation-sensitive Southern blotting. Control = unstressed plant; S0 = individual osmotic stressed rice plant; S1 = selfed progenies (*lanes 1* to *16*) of the stressed plant (S0). The rice plants were treated by 15% polyethylene glycol for 4 days. We selected an osmotic stressed plant that showed the most obvious alteration in DNA methylation and its 16 S1 plants to test methylation state of *P5CR*, *P5CS*, and *δ-OAT* genes by methylation-sensitive Southern-blotting analysis. We also tested methylation state of a retrotransposon *Osr42* and a housekeeping gene *Elongator* (*Elong*), which are known as unrelated to osmotic stress. *P5CR* = Δ^1-pyrroline-5-carboxylase reductase; *P5CS* = Δ^1-pyrroline-5-carboxylate synthetase; *δ-OAT* = ornithine-*δ*-aminotransferase.

P5CR, *P5CS* (glutamic acid pathway), and *δ-OAT* (ornithine acid pathway) are 3 key genes of proline synthesis. Many studies have demonstrated that osmotic stress induces increases in *δ-OAT* and *P5CS* abundance and activity (Roosens et al., 1998; Lutts et al., 1999). Thus, up-regulation of *P5CS* and *δ-OAT* expression may contribute to the accumulation of proline in response to osmotic and salinity stress (Verslues and Sharma, 2010). We observed that in the immediately stressed plant generation, *P5CS* expression in rice leaves was not al-

tered by osmotic stress. However, in selfed progeny of an osmotically stressed plant (S1) that showed DNA methylation changes, *P5CS* expression was also concomitantly up-regulated (see Figure 1). Interestingly, this altered gene expression pattern was also stably inherited in the S2 generation (Figure 3). Under osmotic stress, another proline synthesis key gene, *δ-OAT*, was expressed in S1 and 2 S2 plants at levels much higher than those in the immediately stressed plant generation (control). The osmotic stress response of *P5CR* was similar to that of *δ-OAT*.

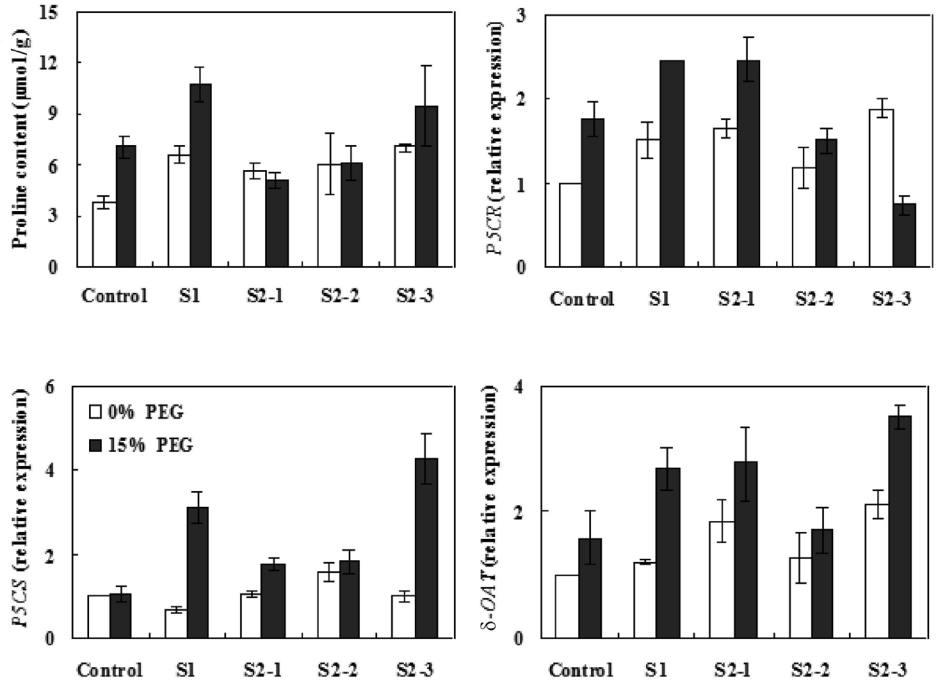

Figure 3. Effects of osmotic stress on proline content and expression of *P5CR*, *P5CS*, and *δ-OAT* genes in rice leaves. Control = normal rice plants grown under the same condition except the osmotic stress (Mock): S1 = selfed progeny of an osmotic stressed plant that showed most obvious alteration in DNA methylation; S2-1, S2-2, and S2-3 = 3 selfed progenies of this S1 plant. All rice plants were treated by 15% polyethylene glycol (PEG) for 4 days. *P5CR* = Δ^1-pyrroline-5-carboxylase reductase; *P5CS* = Δ^1-pyrroline-5-carboxylate synthetase; *δ-OAT* = ornithine-*δ*-aminotransferase.

In accordance with these changes in DNA methylation and gene expression, after the osmotic stress, the leaves of S1 and S2 plants accumulated concentrations of proline that were higher than those of the immediately stressed plant generation (control). In addition, under non-stress conditions, the S1 and S2 plants showed much higher concentrations of proline and higher expression levels of *P5CS* and *δ-OAT* compared with those in the control plants ($P < 0.5$). These results indicated that the heritable up-regulation of *P5CS* and *δ-OAT* may have contributed to the increased accumulation of proline in S1 and S2 plants even under non-stress conditions (see Figures 2 and 3). Namely, osmotic stress quickly generated a heritable phenotype within 1-2 generations that showed higher constitutive expression of relevant genes and

proline accumulation. Epigenetic modification in the form of altered DNA methylation may have played a role in acquiring this heritable phenotype. Indeed, we found that under osmotic stress, both *P5CS* and *δ-OAT* showed DNA demethylation in S1 plants but not in the immediately stressed plant generation, suggesting that DNA demethylation regulated expression of the 2 genes and resulted in their up-regulation.

The modulation in DNA methylation induced by abiotic stress may play a functional role in plant stress tolerance (Richards, 2006; Chinnusamy and Zhu, 2009; Karan et al., 2012). Biotic and abiotic stresses may cause alterations in DNA methylation patterns in both plants and animals (Richards, 2006). Most of these stress-induced epigenetic modifications are reset to the basal level once the stress is relieved, but some of the modifications may be stable - that is, may be carried forward as "stress memory" or organismal progenies (Chinnusamy and Zhu, 2009). Thus, alterations in methylation patterns could be considered part of an evolutionarily meaningful plant protection mechanism in response to environmental stress.

Proline, as a compatible osmolyte, is widely believed to accumulate rapidly in response to water deficiency or osmotic stress (Saradhi et al., 1995; Hare and Cress, 1997; Siripornadulsil et al., 2002; Verbruggen and Hermans, 2008; Mattioli et al., 2009). However, the function of proline in drought or osmotic tolerance is still under debate by plant biologists (Siripornadulsil et al., 2002; Verbruggen and Hermans, 2008). Recent findings have suggested that proline may also play a role in flowering and development both as a metabolite and a signal molecule (Mattioli et al., 2009). In any case, proline clearly plays crucial roles not only in drought tolerance but also in tolerance to other stresses such as salinity, high pH, and cold. Our results suggested that changes in DNA methylation (demethylation) as a result of osmotic stress facilitate proline accumulation via the up-regulation of proline metabolism-related gene expression (e.g., *P5CS* and *δ-OAT*), which is likely an adaptive response to the stress.

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Supplementary material

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