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Transgenic Note

Accumulation of raffinose in rice seedlings overexpressing *OsWRKY11* in relation to desiccation tolerance

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Abstract We previously reported that transgenic rice (*Oryza sativa* L.) lines overexpressing *OsWRKY11* showed significant desiccation tolerance, as indicated by their slower water loss in detached leaves. Here we examined the contents of sucrose, glucose, fructose and raffinose. Raffinose was shown to accumulate at a significantly higher level in the transgenic plants overexpressing *OsWRKY11*. Microarray analysis of gene expression profile indicated that the gene expression of Os07g0209100 encoding raffinose synthase and that of Os07g0687900 encoding galactinol synthase were up-regulated. These results suggest that *OsWRKY11* induced activation of these genes involved in raffinose synthesis and the accumulated raffinose played an important role in the desiccation tolerance of the *OsWRKY11*-overexpressed plants.

Key words: Desiccation tolerance, raffinose, transgenic rice, WRKY.

An *OsWRKY11* gene, which encodes a transcription factor with the WRKY domain, was identified as one of the genes induced by both heat shock and drought stresses in seedlings of rice (Shiroto et al. 2004). To determine if overexpression of *OsWRKY11* confers combined heat/drought tolerance, *OsWRKY11* cDNA was fused to the promoter of *HSP101* of rice and introduced into a rice cultivar Sasanishiki (Wu et al. 2009). Overexpression of *OsWRKY11* was induced by heat pretreatment. After heat pretreatment, the transgenic lines showed significant combined heat/drought tolerance, as indicated by the slower leaf-wilting and less-impaired survival rate of green parts of the plants. They also showed significant desiccation tolerance, as indicated by the slower water loss in their detached leaves (Wu et al. 2009).

To adapt to a reduction in external water activity, cells accumulate low-molecular-weight solutes, called osmoprotectants, to maintain proper intracellular osmotic balance. Osmoprotectants can mitigate the adverse effects of high osmolarity by increasing the free water content of the cytoplasm, and they may also increase the stability of macromolecules in solutions of low water activity (Arakawa and Timasheff 1985; Ignatova and Gierasch 2006). Major osmoprotectants in plants are sugars. Here we examined the contents of sucrose, glucose, fructose and raffinose in transgenic plants overexpressing *OsWRKY11* and their gene expression profile.

Transgenic lines (i.e., ox2 and ox3) with a single copy of the T-DNA containing HSP101 promoter::*OsWRKY11* were previously produced (Wu et al. 2009). Two-week-old seedlings grown in soil at 22/18°C (12/12 h) were exposed to heat pretreatment at 37/22°C (12/12 h) for 2 weeks for the induction of *OsWRKY11* overexpression. For the control condition of no-heat pretreatment, plants were grown at 22/18°C (12/12 h).

For analysis of sugar content, leaf blades of fully expanded top leaves (60 mg) were sampled in the morning and immediately frozen in liquid nitrogen after being treated for 2 weeks with or without heat pretreatment. Then they were crushed and added to 3.5 ml 80% ethanol preheated to 80°C, then centrifuged at 3,000 rpm for 5 min. This series of steps was repeated four times, followed by vacuum drying and dissolving in 2.5 ml water. Then, sugars of the extracts were analyzed using Saccharose/D-Glucose/D-Fructose F-kit (J.K. International, Tokyo, Japan) for sucrose, glucose and fructose content; and Raffinose F-kit (J. K. International, Tokyo, Japan) for raffinose content. Statistic analysis was carried out using a multiple comparison test analogous to the Tukey test at the 5% level.

For microarray analysis, total RNA was isolated from leaf blades using RNeasy (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Poly $(A)^+$ RNA was isolated using the Dynabeads mRNA

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Purification Kit (Invitrogen, Carlsbad, USA). cDNA synthesis was accomplished using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbud, USA). The RNAs (400-ng aliquots) were labeled with a Low RNA Input Linear Amplification/Labeling Kit (Agilent Technologies, Tokyo, Japan) according to the manufacturer's instructions. Aliquots of Cy3-labeled cRNAs (1 μg each) of the *OsWRKY11* overexpressing plant (ox3) were used for hybridization in an Agilent Rice 44 K Oligo Microarray Agilent Technologies). Three biological replicate samples sets (of both heat pretreatment and no-heat pretreatment) were analyzed. After hybridization, microarray slides were scanned (scanner model G2505B and software G2565BA; Agilent Technologies). All microarray procedures and data analyses were performed according to the manufacturer's manual at the Rice Genome Resource Center, Tsukuba, Japan. The data analysis using GeneSpring (Agilent Technologies) was conducted by Hokkaido System Science (Sapporo, Japan).

Contents of sucrose, glucose, fructose and raffinose in plants with no-heat pretreatment (C) and those with heat pretreatment (H) were shown in Figure 1. With heat-pretreatment, contents of sucrose, fructose and raffinose were significantly higher in ox2-H and ox3-H plants than in wild-type (WT-H) plants. In particular, raffinose accumulated at a significantly higher level in the heat-pretreated ox2 and ox3 plants overexpressing *OsWRKY11*, while the unstressed WT had no detectable amount of raffinose. Raffinose content was significantly higher in the plants with heat-pretreatment (ox2-H and ox3-H) than in those with no-heat pretreatment (ox2-C and ox3-C), while such significant difference was not detected in sucrose, glucose or fructose, indicating that overexpression of *OsWRKY11* increased raffinose accumulation in these transgenic plants. With no-heat pretreatment, fructose significantly highly accumulated in ox2-C and ox3-C plants than in WT-C, suggesting that accumulation of fructose was induced by some leaky expression of *OsWRKY11* under the unstressed condition as described before (Wu et al. 2009). These results suggest an important role of raffinose accumulation in the desiccation tolerance of the *OsWRKY11*-overexpressed plants. It is also possible that accumulation of sucrose and/or fructose is partially involved in the desiccation tolerance.

Accumulation of sugars such as raffinose, sucrose, glucose and fructose in transgenic rice showing increased tolerance to drought, high-salt and low-temperature stresses has been reported in the transgenic plants overexpressing the *OsDREB1* or *AtDREB1* genes (Ito et al. 2006). The content of raffinose of WT plants under NaCl stress was reported to be 1.1 μg mg^{-1} fresh weight (Ito et al. 2006). This value was almost equal to that of the raffinose content in WT plants with heat-pretreatment in our current study (Figure 1, WT-H). The raffinose content in *OsWRKY11*-overexpressing line (ox2-H and ox3-H) was 16.5 and 19.9 μg mg^{-1} fresh weight, respectively (Figure 1), and was more than10-fold higher than that of transgenic line DREB1A26-C, which was reported to be 1.3 μg mg^{-1} fresh weight (Ito et al. 2006). Accumulation of a higher content of raffinose was prominent in the *OsWRKY11*-overexpressing transgenic plants.

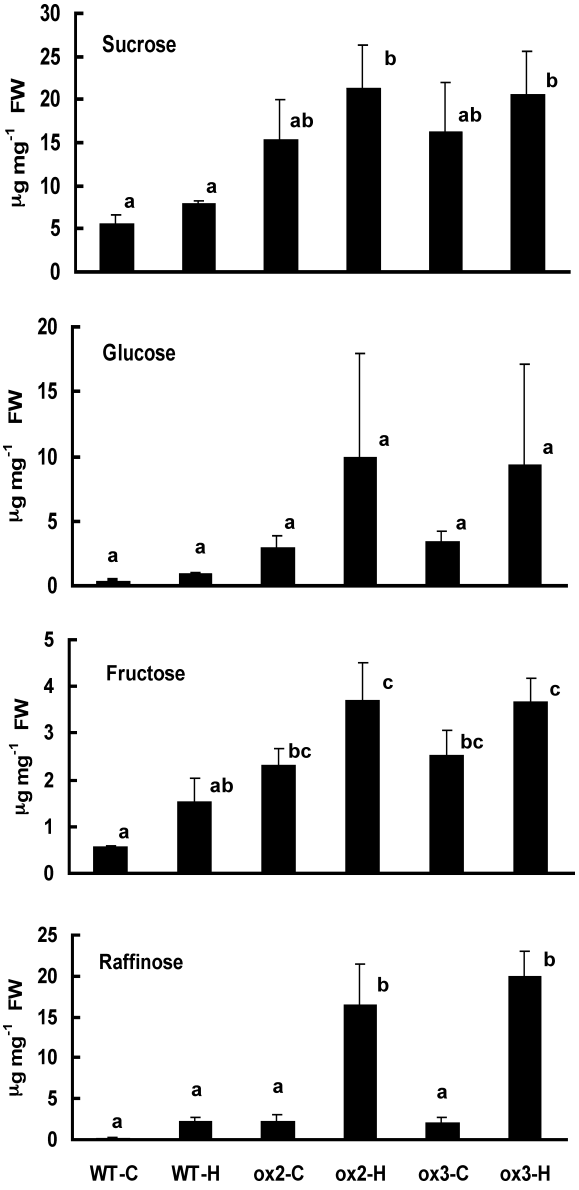

Figure 1. Sugar contents per fresh weight (FW) of rice plants with no-heat pretreatment (C) and with heat pretreatment (H) for the induction of *OsWRKY11* overexpression. ox2 and ox3, transgenic plants with *HSP101* promoter::*OsWRKY11*. Values followed by a different letter are significantly different based on a multiple comparison test analogous to the Tukey test ($P<0.05$).

Raffinose family oligosaccharides (RFOs, i.e., raffinose, stachyose, and verbascose) have long been suggested to act as anti-stress agents in both generative

and vegetative tissues (Koster and Leopold 1988; Bachmann et al. 1994; Brenac et al. 1997; Taji et al. 2002; Pennycooke et al. 2003). Seed research has revealed strong correlations between accumulation of RFOs and desiccation tolerance (Horbowicz and Obendorf, 1994; Hoekstra et al. 1997). RFOs are synthesized from sucrose by the subsequent addition of activated galactinol moieties donated by galactinol (Peterbauer and Richter 2001). Galactinol synthase (GolS) catalyzes the first committed step in the biosynthesis of RFOs and plays a key regulatory role in the carbon partitioning between sucrose and RFOs (Saravitz et al. 1987). Raffinose synthase (RS) catalyzes the transfer of galactosyl residue from galactinol to sucrose to form raffinose. Overexpression of *Arabidopsis GolS2* in transgenic *Arabidopsis* plants has been found to cause an increase in the levels of endogenous galactinol and raffinose under normal conditions (Taji et al. 2002). Other than the function of osmoprotectants, galactinol and raffinose have been reported to constitute a novel function to scavenge hydroxyl radicals to protect plant cells from oxidative damage caused by methylviologen treatment, salinity, or chilling (Nishizawa et al. 2008). Such scavenging function of raffinose might also take part in enhancing combined heat/drought tolerance, as indicated by the slower leaf-wilting and less-impaired survival rate of green parts of the *OsWRKY11*-overexpressing plants (Wu et al. 2009).

As shown in Table 1, we identified four genes as rice orthologous genes for *Arabidopsis RS*, and two genes for *GolS*, based on SALAD database (http://salad.dna.affrc.go.jp/salad/). Gene expression profiles were compared between ox3 and WT plants with heat or pretreatment at 37/22°C or no-heat pretreatment using the data of 44K Agilent microarray. A gene, Os07g0209100 (AK120944, ortholog of *Arabidopsis RS2*) was found to be up-regulated 6.2-fold in ox3 plants with heat pretreatment (Table 1). Another gene, Os07g0687900 (AK107065, ortholog of *Arabidopsis GolS1*) was 3-fold up-regulated (Table 1). Up-regulation of these genes are considered to be caused by *OsWRKY11* overexpression, which was driven by the *HSP101* promoter. The heat pretreatment, by itself, might possibly induce the expression of the *GolS* gene and the *RS* gene, since *GolS1*, *GolS2*, *GolS4* and *RS2* of *Arabidopsis* have been reported to be up-regulated by

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GAATTCTAGAACCACCTACAAACTCCATCGAACATACTTGATGAGTTGAGGGTTTCCCTGTAATAATAACTTTGTATTATTATATATTTT  90

CATAAAATTATGTATTGTTAATTTCTTCTCCCACCAGTACGTGCGTAAAAAATTTATTGCTAGCTAATCATTAGCCCCATTTGCAATGAT  180

AGAATTCTAAAGGGGGAAAAATCATTCGATTTAAATAATAGAGAATTACCGATGTCATAGTTGTCCAAAACACGATTTTTCTTCCTACGT  270

ACGTGAAAGTAACTTAGTCACGAAGAATTTGAGAAAAATAATTTACACCGACGCAATCGCTTGCTCATCGACGACAACCTACAAGTAACA  360

ATATAACAAATAGTGAGGAAAGGAAAAATATAAAGCAGCATTCAATCAGTCAACACAGGTGTTCTAGCCATATATATGCAACCCATCAAC  450
                                                ←
ATGGGGAATGCAACAATATATATAGCAGCCTAGCTAGTCTTCAAAATGATGAAGCCCACAGAAAATGCACACAAGCAAACAAACGATATC  540

TCAAAATATAAAATCACCAGATATAAGACAGACGCTCCCATACCTGAATTTTTGTTTCTTGCAAGAAATCAATGCATGATATAACTGTAA  630

CACATCAAGTTGTCTGACCATATCATATATCATCATGCATGCACAGCCTAATTAAATCTTACACACAAAGAAACCAAAATTGGAGAAATC  720

AAATTCCTCCTCTCCCAGGGGCAAAATAGGAAGAGAGATATTTCTTTTTGCAAGAAGAAGAAACAGTTACAAAATCTCATGGAAACTTAC  810

ACCTGTCCCTTGGCCATTTTGATCACCATGTGATGTGAGTCCCCCCACTTACATTAATCTCATGCTAAAAAACAAACAGATAAATAAATA  900

ATTGCCATTGCCATGCTGCCACGTGTCATGGGACACGTCACCTCCTCTTCCTCTCCCGCGAGTATATAAACCCCTCGCTCCCCTCTCCTT  990

CCTCCCGCCATCTATCTCCCTCTCTTTTGAGGAGGGCACCGCCATTTAGTTAATTAGTAGTTGTTAATTATCTTCACTAACCTTAATTAC  1080

CCTCACGCAAAGCACTCTCTAATCCTCCTCGGCGCTCGCATAATCTTGTATCCCTCATCTCAGATTATCATCACCTCGCCGTGTTAGAAA  1170

AAAGGTACTATGTTTGGTTGATTTGTAATAATTTTCTTGGTTGGTGGTTTGGTTGATTTCTTGATTAGTGTGATGCTAATTGTTTTCGTT  1260

TGAAGGGAGTAAGTTCGTTTTATCGTCGGATTTGGTGAGAGTTCGCCGGAGTAGAAGAAGATGACGGTGACGCCGCAGATCACGGTGAGC  1350
                                                M  T  V  T  P  Q  I  T  V  S
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Figure 2. W box within 1 kb of the promoter region of Os07g0209100 encoding raffinose synthase. W box is boxed. The transcribed region is indicated by shadow with translated amino acids.

Table 1. Fold change of transcripts (ox3/WT) for raffinose synthase and galactinol synthase.

	RAP locus ID	Orthologous gene in *Arabidopsis* thaliana (amino acid identity %)	Fold Change No heat pretreatment	Fold Change Heat pretreatment
Raffinose synthase	Os08g0495800	*RS1*, At1g55740 (69%)	1.2	0.7
	Os07g0209100	*RS2*, At3g57520 (67%)	2.1	6.2
	Os01g0170000	*RS5*, At5g40390 (62%)	0.8	0.4
	Os06g0172800	*RS6*, At5g20250 (66%)	1.1	1.2
Galactinol synthase	Os03g0316200	*GOLS1*, At2g47180 (72%)	0.7	0.5
	Os07g0687900	*GOLS2*, At1g56600 (72%)	1.1	3.0

Heat pretreatment induced *OsWRKY11* overexpression. Rice genes are presented as RAP locus ID (http://rapdb.dna.affrc.go.jp/). Corresponding orthologous genes of *Arabidopsis thaliana* are also shown.

heat shock factors (Panikulangara et al. 2004; Nishizawa et al. 2008). Up-regulation by the heat pretreatment, however, did not induce significant increase of raffinose content in a WT control, as observed in WT-C and WT-H (Figure 1). We, therefore, concluded that expression of *OsWRKY11* induced the gene expression of Os07g0209100 encoding RS and Os07g0687900 encoding GolS, which likely functioned to accumulate raffinose and conferred desiccation tolerance in the transgenic plants overexpressing *OsWRKY11*.

A WRKY transcription factor is expected to bind specifically to the W box, TTGAC(C/T), in the promoter regions and to activate certain gene expressions (Eulgem et al. 2000; Maleak et al. 2000). In the analyses of the promoter sequence (1 kb upstream region) of the up-regulated genes, the W box, TTGAC(C/T) was found in the *RS* promoter of Os07g0209100, as shown in Figure 2, but not in the *GolS* promoter of Os07g0687900. It is expected that *OsWRKY11* binds to the W box sequence and activates the *RS* gene expression. It is also possible that *OsWRKY11* binds to some other genes that activate the *RS* genes indirectly. Further experiments such as gel-shift assay are necessary to determine if the *OsWRKY11* directly binds to the W box sequences in the promoter regions.

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