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Full Length Research Paper

A physiological evaluation of the enhanced osmotic stress tolerance of an asymmetric somatic hybrid introgression line SR3 between bread wheat and couch grass

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The cultivar, Shanrong No.3 (SR3) is the product of an asymmetric somatic hybrid between the bread wheat (*Triticum aestivum* L. cv. Jinan 177) and couch grass (*Thinopyrum ponticum*). After seedlings of SR3 and Jinan 177 were hydroponically subjected to osmotic stress, the accumulation of proline and sugars, the activities of a number of antioxidant enzymes and the relative expression levels of *LEA* and *DHN*-like genes in roots and leaves were examined. Leaf water potential and relative water content were also determined. SR3 leaves were more effective in accumulating proline and soluble sugar than those of Jinan 177, and their osmotic potential was significantly reduced in response to the applied osmotic stress. The mean increase in the activities of SOD, POD and APX in the stressed root and leaf of SR3 were higher than those of Jinan 177, as were the relative expression of two LEA genes and two DHN genes. Overall, SR3 achieved enhanced stress tolerance via an improvement in its osmoregulation, an increase in its enzymatic antioxidant activity and an enhancement in its cellular protection.

Key words: Somatic hybrid, introgression, cultivar-Shanrong No.3, water stress, osmolytes, antioxidant enzymes, semi quantitative RT-PCR.

INTRODUCTION

Abiotic stress, particularly drought, is the primary cause of crop loss worldwide, and is responsible for an average yield loss of more than 50% in the major crops (Boyer, 1982). Drought tolerance mechanisms are typically under multigenic control, and thus, difficult to select for in conventional breeding programmes. Thus, the breeding of crop varieties with improved salinity or drought tolerance has not proved to be very successful to date. The direct transfer of ‘‘candidate’’ tolerance genes has been suggested to offer a more reliable route for the improvement of crop stress tolerance. However, there is, as yet, a very incomplete understanding of the physiological mechanisms determining stress tolerance (Munns and Tester, 2008), which naturally limits the choice of potential target genes. An alternative approach, which avoids the prior need for gene identification and isolation, relies on the existence of naturally evolved stress tolerance in certain non-crop species. Although the majority of these species are typically sexually incompatible with even their related crop species, the technique of somatic hybridization can be exploited to

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Abbreviations: APX, Ascorbate peroxidase; **DHN,** dehydrin; **LEA,** late embryogenesis abundant; **MDA,** malon dialdehyde; **POD,** peroxidase; **Pro,** proline; **ROS,** reactive oxygen species; **RT-PCR,** reverse transcriptase polymerase chain reaction; **RWC,** leaf relative water content; **SOD,** superoxide dismutase; **Ψleaf,** leaf osmotic potential.

transfer desirable traits, as it sidesteps the need for conventional crossing (Gerdemann-Kncörck et al., 1994). The generated somatic hybrids can then be used as parental material from which tolerance can be bred into crop varieties by conventional breeding and phenotypic selection.

Improving the genetic adaptation of wheat (***Triticum aestivum*** L.) to drought stress is one of the main objectives of the regional breeding programmes in China (Zhang et al., 2009). The feasibility of creating somatic hybrids between wheat and some of its wild relatives has been demonstrated for some years (Xia and Chen, 1996; Xia et al., 1996). Specifically, a number of introgression lines have been selected among the progeny of asymmetric somatic hybrids formed between the bread wheat cultivar, Jinan 177, and the couch grass ***Thinopyrum ponticum*** Podp (2n = 10x = 70) which was highly tolerant of salinity and drought (Xia et al., 2003). The inheritance and the nature of the couch grass chromosome segments introgressed by this route into cv. Jinan 177 background have been described (Chen et al., 2004a; Wang et al., 2005), and the enhancement of some of the introgression materials in terms of their salt tolerance has been demonstrated in the field (Chen et al., 2004b; Shan et al., 2006). One derivative in particular, named Shanrong No. 3 (SR3) proved in addition, to be sufficiently improved with respect to drought tolerance that it has been successfully accepted as a commercial cultivar by the Shandong Crop Varieties Examining Committee (Lu-Nong-Shen-Zi No. [2004]030).

The differential response of resistant and susceptible wheat cultivars to drought stress has been well explored (Khanna-Chopra and Selote, 2007). Typically, plants accumulate organic solutes such as proline and sugars in response to desiccation and osmotic stress (Yancey et al., 1982), and these are thought to be helpful to restore the osmotic potential of the cytoplasm and so drive water uptake and maintain cell turgor (Stoop et al., 1996). Much of the damage at the cellular level associated with stress is caused by the action of the reactive oxygen species (ROS) superoxide, peroxide and hydroxyl radical (Mittler, 2002). The ROS formation is controlled by ROS scavenging enzymes including superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (CAT) at a proper level (Apel and Hirt, 2004, Shao et al., 2008). The activities of the scavenging enzymes and the expression of some water stress response genes [late embryogenesis abundant (LEA) proteins and dehydrins] are generally increased during abiotic stress (Mott and Wang, 2007), and these increases are correlated with enhanced cellular protection (Noctor and Foyer, 1998; Goyal et al., 2005).

The objectives of this study were to (a) assess the extent to which SR3 accumulates proline and sugars as a means of lowering leaf osmotic potential during drought stress, (b) determine whether scavenging enzyme activity is higher in SR3 than in the susceptible near isogenic cv. Jinan 177 during osmotic stress, and (c) to elucidate how LEA and DHN genes are involved in this different tolerance.

MATERIALS AND METHODS

Plant materials

Seed of SR3 and its progenitor cv. Jinan 177 were surface sterilized in 2% (w/v) NaClO for 10 min, then washed three times with distilled water and germinated on two layers of damp filter paper in a Petri dish for 2 days at 20°C. Uniformly sized seedlings were transferred to growth chamber (12 h/12 h light/dark, 22/18°C temperature, 300 $\mu molm^{-2}s^{-1}$ light quantum flux, 50% relative humidity), and grown hydroponically in half strength Hoagland's solution for three weeks until they reached the three-leaf stage. The material was then divided into two groups: a control group which was maintained in half strength Hoagland's solution and a test group where 18% (w/v) polyethylene glycol (PEG6000) was added to induce osmotic stress. Solutions were replaced every two days. The actively expanding region of the root and the second fully expanded leaf of 10 seedlings per treatment were sampled from both the control and the test group which were treated with PEG for 3, 6, 12 and 24 h. All these experiments were replicated for 3 times. All samples were immediately frozen in liquid nitrogen after they were cut. For determination of RWC and Ψleaf after stress, the samples were only taken from the plants treated for 24 h.

Measurement of proline and soluble sugars

Proline content was estimated by the method of Bates et al. (1973). Briefly, 0.3 g of plant material was homogenized in 3% (v/v) aqueous sulfosalicylic acid and the homogenate centrifuged at 10,000 ***g*** for 10 min. A reaction mixture consisting of 0.5 ml supernatant, 0.5 ml acid ninhydrin and 0.5 ml glacial acetic acid was boiled for 1 h, cooled in an ice bath, extracted in 2 ml toluene, and the spectral absorbance read at 520 nm. A standard curve was prepared with L-proline. The assay for soluble sugars followed the colorimetric anthrone method of Irigoyen et al. (1992). Briefly, 0.3 g of plant material was homogenized with hot distilled water, the homogenate was centrifuged at 10,000 ***g*** for 10 min. An amount of 100 µl tissue extract was added to 3 ml (final volume) assay media containing 1.08 M H_2SO_4, 1.09 mM thiourea and 2.1 mM anthrone. The mixture was heated at 100°C for 10 min and absorbance read at 620 nm. A calibration curve was plotted with D-glucose as a standard.

Leaf relative water content (RWC)

RWC was calculated as (FW-DW) / (SW-DW) x 100% (Muranaka et al., 2002), where FW is the fresh weight of the leaf, DW is the weight after drying at 85°C for 3 days, and SW is the weight after soaking in distilled water for 4 h in the dark at room temperature (approximately 20°C).

Leaf osmotic potential

Leaf osmotic potential (Ψleaf) was measured using a vapour pressure osmometer (Wescor, Model 5520). The sample was held at -20°C for at least 3 h in a 1.5 ml tube, thawed for 30 min at room temperature and squashed to extract the sap (Clifford et al., 1998). Ψleaf was calculated as -RTC, where R is the gas constant (8.314

Table 1. Sequence information of primers used for RT-PCR analysis.

Accession No.	Possible annotation	Primer
X56882	group 3 LEA	S: 5' GCAAGGACCAGACCGCCAGCAC 3' A: 5' GCCCGCGAACGACCAAACACTA 3'
AB297680	Wrab19 gene for group3 LEA	S: 5' ATGCTCTCTTGGTTTGGCTGCG 3' A: 5' CTGGTGGTGGTGTCCTTGGTGG 3'
AB297677	Wdhn13 gene for group2 LEA	S: 5' TAACTATTGCCCGACTCCCCTT 3' A: 5' TATTGTTTCGCACGCACCTTGA 3'
AY148491	LEA2 protein	S:5' GCCGAGAGCAAGGACCAGACAG 3' A:5' GGCGGAAATCACACAAGAGGGA 3'
AY148490	LEA1 protein	S:5' CAAGGACCAGACCGCCAGCACC 3' A:5' CCCGCGAACGACCAAACACTAC 3'
EU124658	dehydrin WZY1-2	S: 5' AGAAGGCTGAGGAGGACAAGGA 3' A: 5' GTGGGTCTGCACAGACGCGGGG 3'
AB272228	DHN14 mRNA for dehydrin	S: 5' CGCAAGATGGAGCACCAGGGGC 3' A: 5' TGCAAAGGTAGCGGGCAGACCG 3'
AY574032	dehydrin-like gene	S: 5' AAGGGGTCTCAAGGGAAGGAAG 3' A: 5' GTGCTACTGGTGCTGGAGCAGC 3'
AB076807	Wdhn13 mRNA	S: 5' AGCACACCACTGGAATGAGCGG 3' A: 5' ATGCAAATTGTAAACAAACGAA 3'
AF453444	dehydrin WZY1-1 mRNA	S: 5' GGAGCACGGCCAGGCGACCATC 3' A: 5' CCATCGGTGACATGCGTCCCAG 3'

$Jmol^{-1}K^{-1}$), T the absolute temperature (K), and C the value given by the vapor pressure osmometer ($molkg^{-1}$).

Determination of lipid peroxidation

The level of lipid peroxidation was determined by a procedure based on the method of Heath and Packer (1968), in which 0.3 g fresh sample was first ground in 3 ml 5% (w/v) TCA (trichloroacetic acid) and the homogenate centrifuged at 15,000 ***g*** for 10 min. Then, 0.5 ml supernatant was mixed with 0.5 ml of thiobarbituric acid (TBA) solution (0.5% TBA, 20% TCA), held at 100°C for 30 min, chilled on ice, and centrifuged at 1,000 ***g*** for 10 min. The spectral absorbance of the supernatant was measured at 532 nm (taking the extinction coefficient of the MDA-TBA complex as 155 $mM^{-1}cm^{-1}$) and adjusted for non-specific absorbance at 450 and 600 nm.

Antioxidant enzymes

0.3 g sample was frozen in liquid nitrogen and ground in 3 ml of a pre-cooled solution containing 50 mM phosphate buffer (pH 7.8), 1% (w/v) poly-vinylpolypyrrolidone, and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 15,000 ***g*** for 10 min. For the ascorbate peroxidase (APX) assay, a separate extraction was carried out using the same buffer, but containing 0.5 mM ascorbate. The total protein content and the activity of SOD (hydroxylamine assay) were assessed with specific reagent kits (Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's recommendations (Oyanagui, 1984). POD activity was measured using a modification of the method of Chance and Maehly (1955), in which 3 ml of the reaction solution (50 mM phosphate buffer pH 5.0, 20 mM guaiacol, 40 mM H_2O_2) was added to 0.01 ml of the enzyme extract. Spectral absorbance at 470 nm was determined every 30 s. APX activity was measured following Nakano and Asada (1981) with minor modifications. A reaction mixture of 50 mM sodium phosphate buffer pH 7.0, 0.5 mM ascorbate, 0.1 mM sodium EDTA, 1.0 mM H_2O_2 and 0.1 ml of enzyme extract formed an assay volume of 1 ml. To correct for the effect of total protein on the spectral absorbance at 290 nm, a blank sample was prepared lacking H_2O_2. The concentration of oxidized ascorbate was calculated based on an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$.

RT-PCR analysis

Total RNA was isolated from the root and leaf of SR3 and Jinan 177 using Trizol Reagent (Invitrogen, Cat No. 455926-026) and cDNA was synthesized from DNase-treated total RNA with Omniscript RT Kit (Qiagen) using oligo-dT-primer. Primers were prepared according to sequences searched online http://www.ncbi.nlm.nih.gov/ (Table 1). The expression of β-tubulin was used as an internal positive control. The effects of PEG-induced differential expression were screened. The amplification reaction was tested in the exponential phase and the PCR products were applied to 1.5% agarose gel and visualised under Alpha Imager 2000 after ethidium bromide staining.

Statistical analysis of data

The plant materials were arranged in a completely randomized design. Data were subjected to two-way analyses of variance, and differences between means were compared by Fisher's least standard deviation (LSD) test. All determinations were repeated three times, and a type I error probability of < 0.05 was chosen to declare statistical significance. To compare each index between SR3 and Jinan 177, the control value was subtracted from the stressed value. All statistical analyses were performed with the

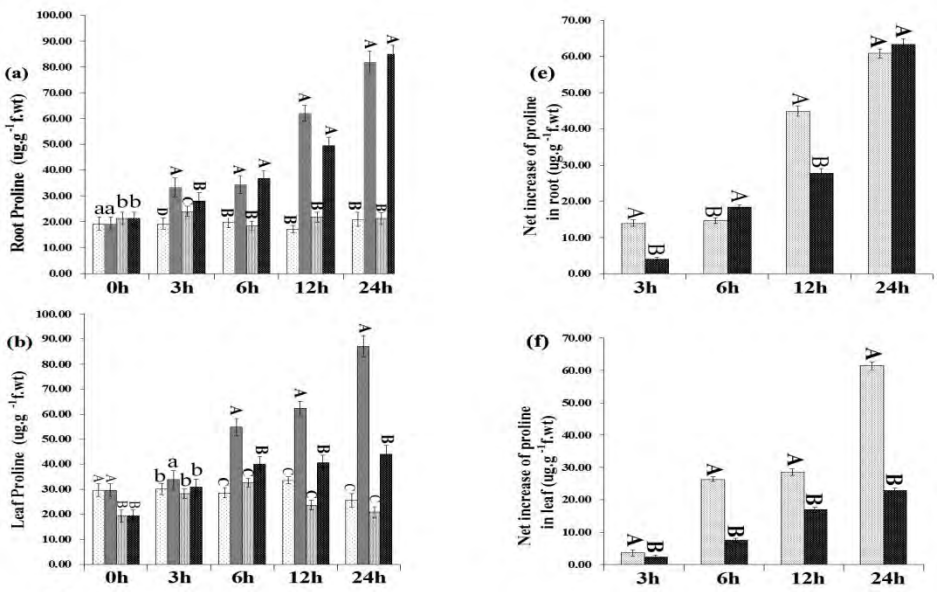


Figure 1. Total (a to d) and mean (e to h) increase in proline and soluble sugar content in the root and leaf of SR3 and Jinan 177 after 0 to 24 h of PEG-induced osmotic water stress. SR3-C, JN177-C, unstressed plants; SR3-S, JN177-S, PEG-stressed plants. Values represent the mean of three replicate measurements in three biological repeats per treatment. Bars represent ±S.D. The significance is expressed as capital letters as $P < 0.01$, while it is expressed as lower case letters as $P < 0.05$. If the letters are different then the averages are significantly different.

software package DPS v9.01 (Tang and Feng, 2002).

RESULTS

Osmolyte accumulation and leaf osmotic potential

The content of proline and soluble sugar in the root and leaf of SR3 and Jinan 177 responded differently to the addition of PEG (Figures 1a to d). The former increased with the time of applied stress in both tissues of both cultivars, while the soluble sugar content reflected the alternation of light and dark. The net accumulations of both solutes in the leaves of SR3 were more than those of Jinan 177 (Figures 1f and h). However, the solute content in the roots of the two genotypes showed no evidence of any differential genotypic response to stress (Figures 1e and g). Leaf RWC had also decreased significantly after this time in both SR3 and Jinan 177, but the reduction was greater in Jinan 177 than in SR3 (Figure 2a). Ψleaf was significantly decreased by PEG stress in both varieties, and reached a significantly lower level in SR3 than in Jinan 177 after 24 h of stress (Figure 2b).

Enzymatic antioxidants

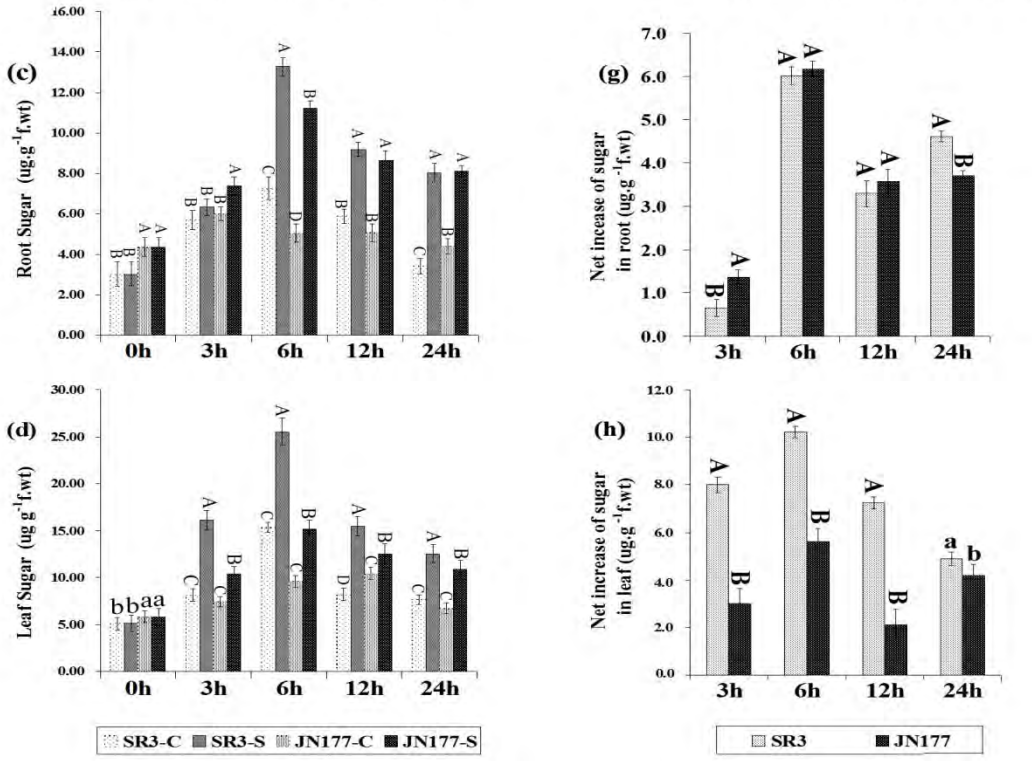
SOD activity in SR3 roots was significantly less than those of Jinan 177 even under control conditions (Figure 3a), but its increase in response to PEG stress was greater in SR3 than in Jinan 177, especially during the initial stages of stress (3 to 6 h, Figure 3c). Leaf SOD activity mirrored that in the root (Figure 3b), but the differential response shown by SR3 was somewhat delayed (12 to 24 h, Figure 3d). In contrast, there was no difference in either POD or APX level between the two genotypes under the control conditions (Figures 4a to d). Water stress significantly increased both root and leaf POD and APX activity more greatly in SR3 than in Jinan 177 (Figures 4e to h).

Level of lipid peroxidation

The level of root and leaf cell membrane lipid peroxidation was greater in SR3 than in Jinan 177 even in the absence of PEG stress (Figures 5a and b). The effect of stress was to significantly increase the MDA concentration in both the root and leaf of both varieties, with the increase experienced by SR3 been significantly less than that of Jinan 177 (Figures 5c and d).

RT-PCR analysis

A total of five LEA protein genes and five dehydrin (DHN)-like genes (Table 1) were selected from GenBank, and differential expressions were screened through RT-PCR



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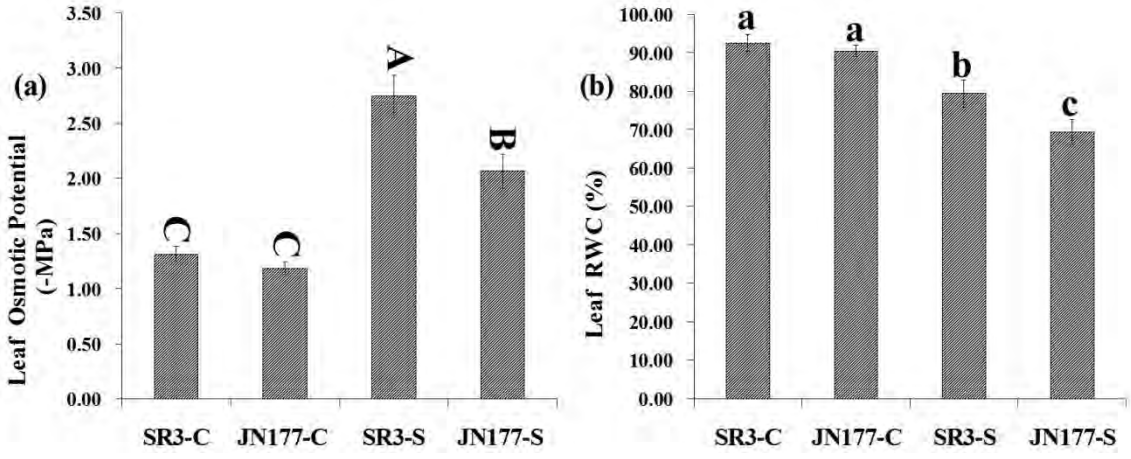


Figure 2. The response of leaf osmotic potential (a) and leaf relative water content (b) of SR3 and Jinan 177 after 24 h of PEG-induced osmotic stress treatment. SR3-C, JN177-C, unstressed plants; SR3-S, JN177-S, PEG-stressed plants. Bars represent ±S.D. The significance is expressed as capital letters as $P < 0.01$, while it is expressed as lower case letters as $P < 0.05$. If the letters are different then the averages are significantly different.

both in leaves and roots of SR3 and JN177. The results show that one LEA protein gene (X56882) and one DHN-like gene (AB272228) were expressed highly in both root and leaf of SR3 than in those of Jinan 177 (Figure 6). Two DHN-like genes (AY574032 and AB076807) showed relative higher expression in SR3 root than in JN177, while two LEA protein genes (AB297680 and AY148491) showed higher expression in SR3 leaf than in JN177 (Figure 6). There were no significant differences between SR3 and Jinan 177 for the other four genes tested (data

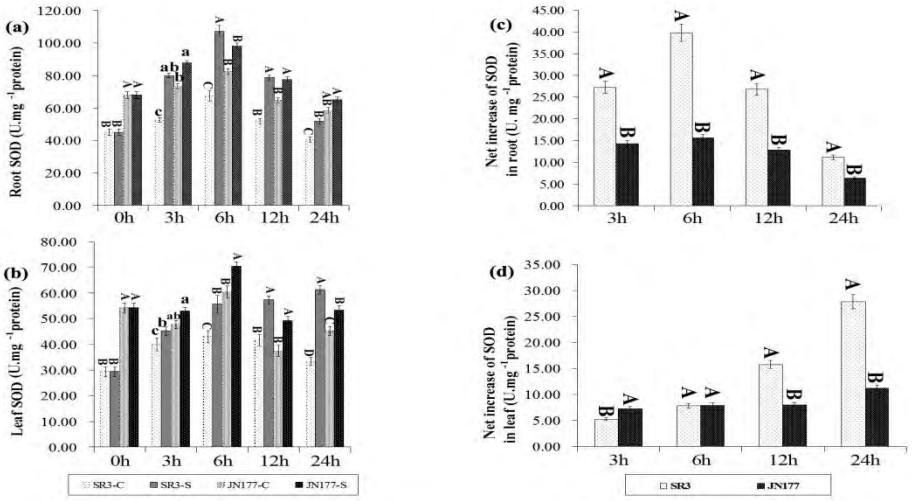


Figure 3. Total (a and b) and mean increase (c and d) in SOD activity in the root and leaf of SR3 and Jinan 177 after 0 to 24 h of PEG-induced osmotic water stress. SR3-C, JN177-C, unstressed plants; SR3-S, JN177-S, PEG-stressed plants. Bars represent ±S.D. The significance is expressed as capital letters as $P < 0.01$, while it is expressed as lower case letters as $P < 0.05$. If the letters are different, then the averages are significantly different.

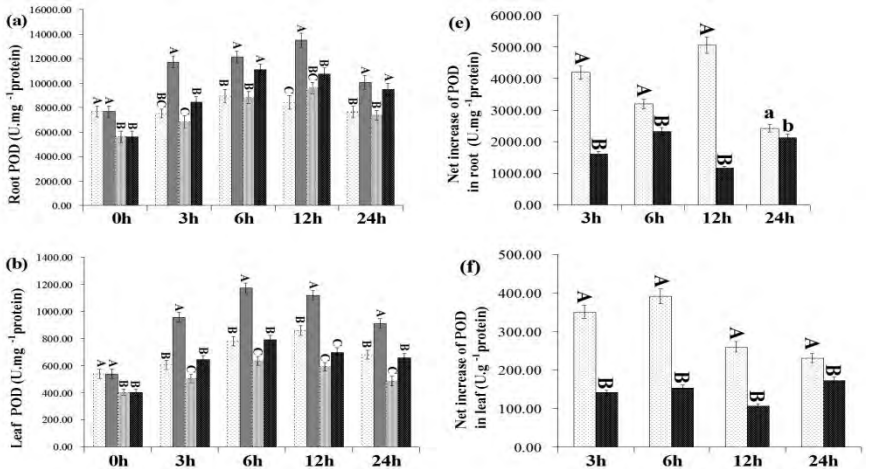


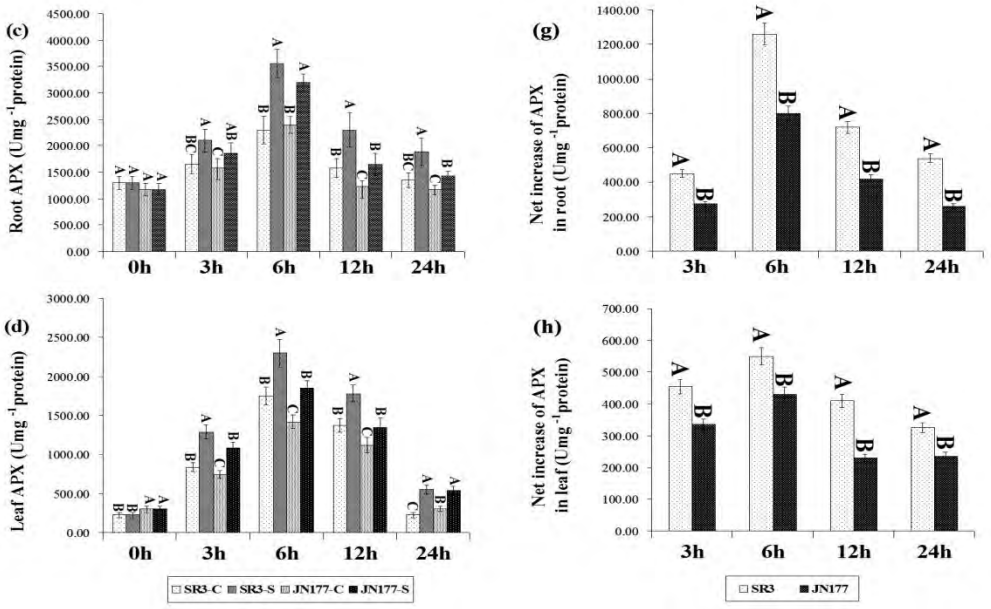
Figure 4. Total (a to d) and mean increase in (e to h) in POD and APX activity in the root and leaf of SR3 and Jinan 177 after 0 to 24 h of PEG-induced osmotic water stress. SR3-C, JN177-C, unstressed plants; SR3-S, JN177-S, PEG-stressed plants. Bars represent ±S.D. The significance is expressed as capital letters as $P < 0.01$, while it is expressed as lower case letters as $P < 0.05$. If the letters are different then the averages are significantly different.

not shown).

DISCUSSION

SR3 displays an enhanced osmoregulation ability compared to Jinan 177

Plants respond to osmotic stress in a number of physiological and biochemical ways. Osmoregulation is considered to be particularly important when in water deficit situations (Xia and Chen, 1996). This can be achieved by the accumulation of solutes as the water potential of the plant environment falls. By lowering the osmotic potential of the cell, turgor pressure can be maintained (Xia and Chen, 1996). Proline and soluble sugars are widely used across the plant kingdom for this purpose. The net accumulation of proline and soluble



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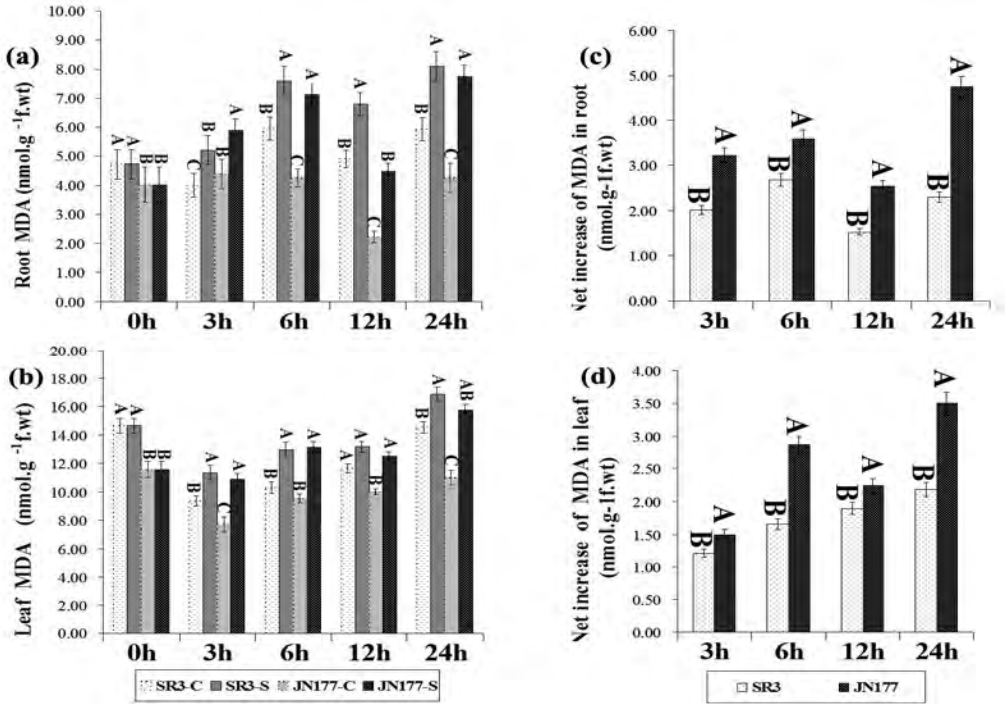

Figure 5. Total (a and b) and mean increase (c and d) in MDA content in the root and leaf of SR3 and Jinan 177 after 0 to 24 h of PEG-induced osmotic water stress. SR3-C, JN177-C, unstressed plants; SR3-S, JN177-S, PEG-stressed plants. Bars represent ±S.D. The significance is expressed as capital letters as $P < 0.01$, while it is expressed as lower case letters as $P < 0.05$. If the letters are different then the averages are significantly different.

sugar in SR3 leaves in response to PEG-induced stress was markedly higher than those in its parental genotype Jinan 177, and allowed them to maintain a lower osmotic potential. This phenotypic difference implies that SR3 has incorporated genetic information from couch grass which enhanced its osmoregulation ability, and therefore

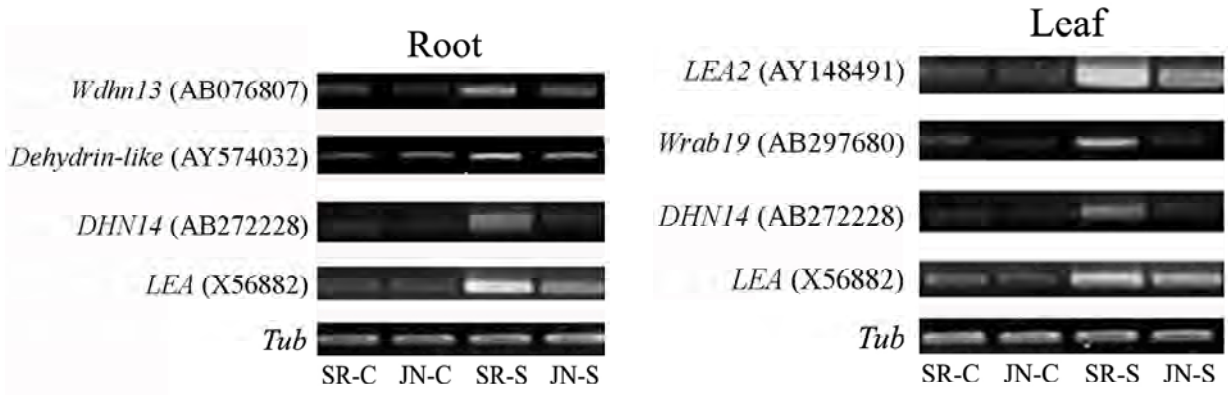

Figure 6. RT-PCR analysis of a few LEA protein genes and DHN-like genes in root and leaf of SR3 and Jinan 177 after 24 h of PEG-induced osmotic stress treatment. SR3-C and JN-C, unstressed SR3 and Jinan 177 plants; SR3-S and JN-S, PEG-stressed SR3 and Jinan 177 plants

improves its ability to extract water from drought stressed soil over that of its parent Jinan 177.

SR3 has a more efficient enzymatic ROS-scavenging system than Jinan 177

Osmotic stress like other abiotic stresses leads to oxidative stress through the increase in reactive oxygen species (ROS) which can damage organelles, oxidize proteins, nick DNA (single-base DNA damage), deplete antioxidant levels, and ultimately trigger cell death (Apel and Hirt, 2004). Therefore, cellular levels of ROS must be tightly controlled and detoxified as efficiently as possible to minimize damage (Moller, 2001). The activity of SOD, POD and APX were all increased greater in the tissue of SR3 than in those of Jinan 177, and the increase in MDA content (a reliable indicator of lipid peroxidation in plants) generated by ROS activity during abiotic stress (Noctor and Foyer, 1998) was lower in SR3 than in Jinan 177. These measurements imply that the enhanced enzymatic ROS-scavenging ability of SR3 is a contributor to its improved osmotic tolerance.

SR3 has a more effective cellular protection system than Jinan 177

LEA proteins and dehydrins in both plants and animals are associated with tolerance to abiotic stress resulting from desiccation or salinity (Goyal et al., 2005; Mott and Wang, 2007). Drought, cold, salt, and the exogenous application of abscisic acid (ABA) all induce an accumulation of dehydrin in plants (Ingram and Bartels, 1996). Although their precise function is still unclear, they are assumed to protect cellular or molecular structures from the damaging effects of water loss. The relative high level expression of a few ***LEAs*** and ***DHNs*** in SR3 leaf and leaf may imply that the molecular structures of SR3 were protected more effectively than JN177 under water stress.

Overall, it is clear that SR3 achieves its enhanced stress tolerance via an improvement in its osmoregulation, an increase in its enzymatic antioxidant activity and an enhancement in its cellular protection. The genetic basis of these enhancements is probably associated with genes present on the chromosome segments introgressed from couch grass. A functional genomic approach would be an appropriate means for exploring the molecular mechanisms underlying the enhanced stress tolerance of SR3.

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