A transcriptomic analysis reveals the nature of salinity tolerance of a wheat introgression line

Chun Liu · Shuo Li · Mengcheng Wang · Guangmin Xia

Abstract The bread wheat cultivar Shanrong No.3 (SR3) is a salinity tolerant derivative of an asymmetric somatic hybrid between cultivar Jinan 177 (JN177) and tall wheatgrass (*Thinopyrum ponticum*). To reveal some of the mechanisms underlying its elevated abiotic stress tolerance, both SR3 and JN177 were exposed to iso-osmotic NaCl and PEG stress, and the resulting gene expression was analysed using a customized microarray. Some genes associated with stress response proved to be more highly expressed in SR3 than in JN177 in non-stressed conditions. Its unsaturated fatty acid and flavonoid synthesis ability was also enhanced, and its pentose phosphate metabolism was more active than in JN177. These alterations in part accounted for the observed shift in the homeostasis related to reactive oxygen species (ROS). The specific down-regulation of certain ion transporters after a 0.5 h exposure to 340 mM NaCl demonstrated that Na^+ uptake occurred rapidly, so that the early phase of salinity stress imposes more than simply an osmotic stress. We discussed the possible effect of the introgression of new genetic materials in wheat genome on stress tolerance.

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Introduction

Soil salinity is one of the most common of the abiotic stresses encountered by plants, and can severely compromise plant growth and crop productivity (Askari et al. 2006). Wheat, a crop which has been subjected to intensive breeding and selection over at least a century, remains a glycophyte with little prospect for the discovery of salinity tolerance genes within the cultivated gene pool. However, some of its close relatives are well adapted to saline environments, and are thus considered to represent a potential source of genetic variation for the improvement of wheat's salinity tolerance. We have bred the cultivar Shanrong No.3 (SR3), a derivative of an asymmetric somatic hybrid between the bread wheat cultivar Jinan 177 (JN177) and the highly tolerant species tall wheatgrass (*Thinopyrum ponticum*) (Xia et al. 2003; Shan et al. 2006). SR3 has demonstrated an exceptional level of salinity tolerance (Peng et al. 2009), expressed both at the level of germination (www.seedsd.com/news/news_view.asp?id=511) and the rate of seedling survival under 340 mM NaCl treatment (unpublished data). Cytogenetic analysis has shown that it carries a small number of short tall wheatgrass chromosome segments dispersed throughout its genome (Wang et al. 2005; Chen and Xia 2003), and it is likely that some at least of its salinity tolerance is derived from the tall wheatgrass genes mapping to these segments (Peng et al. 2009; Shan et al. 2006). Comparisons between SR3 and JN177 have also revealed whole-scale changes at both the genetic and epigenetic level (Liu et al. 2007, 2010), some of which, it is assumed, must be responsible for the enhanced salinity and

drought tolerance of SR3. A proteomic analysis of seedlings subjected to salinity stress up to a level of 200 mM NaCl has suggested that the performance of SR3 reflects its more effective capacity to ensure osmotic and ionic homeostasis, its superior ability to remove toxic by-products, and its better potential to recover when the stress is alleviated (Peng et al. 2009; Wang et al. 2008).

Microarray analysis has been proven to be an informative means of identifying the molecular basis of the plant stress response [see reviews by Deyholos (2010) and van Baarlen et al. (2008)]. In barley, the response to salinity stress includes the synthesis of jasmonate and the induction of the signalling transduction pathways associated with this phytohormone (Walia et al. 2006, 2007). In wheat, transcriptomic analysis has largely focused on the differential expression of stress-responsive genes and difference between common cultivars and their derivatives such as near isogenic lines and deletion lines. For example, Mohammadi et al. (2007) showed that drought stress induced a number of transcriptomic changes, among which were included several dehydration-responsive genes. A similar approach reported by Aprile et al. (2009) suggested that genome organization can influence the adaptive response to drought in wheat terminal deletion lines. Few transcriptomics-based studies of the introgression lines of wheat (and other plant species) response to salinity tolerance have, however, been reported to date.

The majority of experiments testing the salinity response of barley and wheat have been operated at relatively low concentrations and/or gradient-increment of salt (generally below 200 mM NaCl), as this level represents about the limit which is tolerated by these glycophytic species. However, the threshold for SR3 lies somewhat above this level, providing therefore an opportunity to study mechanisms of tolerance to higher concentrations of salinity. While salinity stress includes both an ionic and an osmotic pressure component, drought stress involves only the latter (Munns and Tester 2008). However, typically, the effect of osmotic pressure has not been taken into account in transcriptomic studies of the difference in response to salinity from that to drought.

Since the SR3 genome includes a number of tall wheatgrass genes (Wang et al. 2005), a standard wheat microarray would be unlikely to provide an adequate means of capturing its global transcriptome. Thus here, we have developed a customized microarray based on public domain EST sequences and a suppression subtraction hybridization (SSH)/full-length cDNA library built from SR3. Our aim was to identify the genes which were induced in SR3 plants exposed to either high salinity or drought stress, and to use this information to aid in the understanding of the molecular basis of the enhanced stress tolerance of this cultivar.

Materials and methods

Design of the customized microarray

The 15,172 60-mer microarray assembled by Agilent according to sequences derived from two sources (Table 1). The first were 2,340 sequences from our constructed SSH cDNAs extracted from SR3 plants exposed to 340 mM NaCl and full length cDNAs derived from SR3 plants treated variously with either NaCl or PEG. The second were sequences from several public databases, including those with annotations in the DFCI wheat gene index (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=Wheat, release 10.0), those associated with stress-tolerance in the Genbank wheat EST collection, and those in the TIGR wheat transcript assemblies release 2 (http://www.tigr.org/tdb/e2k1/tae1/wheat_downloads.shtml). ESTs of length <100 bp were omitted. These probes harbored 15,000 wheat unigenes.

Salinity and osmotic stress treatment

SR3 and JN177 seedlings were initially grown at 22°C in 1/2 strength Hoagland's culture solution under a 16 h light/8 h dark cycle, and were exposed to 340 mM NaCl (equivalent to an osmotic pressure of −1.25 MPa) at the three leaf stage. A similar treatment based on 30% PEG6000 was applied to impose an equivalent severity of purely osmotic stress. The response to the stress treatment was gauged after 0, 0.5 and 24 h, and the plants were sampled at each of these time points to perform an RNA extraction (root) and a malondialdehyde (MDA) assay (leaf). Each sample represented three to five plants, and the samples were replicated 3 or 4 times.

MDA assay

The assessment of MDA content followed the method described by Li et al. (2010). Each 0.1–0.2 g leaf sample was homogenized in 2 mL 5% w/v trichloroacetic acid, and the material was centrifuged at $13,000 \times g$ for 10 min at

Table 1 Source of the sequences included on the microarray

Source	Number	Proportion (%)
SSH cDNAlibrary	171	1.1
SR3 full-length cDNA library	2,169	14.3
DFCI Wheat Gene Index database	12,589	83.0
Genbank wheat EST database	211	1.4
TIGR wheat transcript assemblies database	32	0.2
Total	15,172	100.0

room temperature. A 1.6 mL volume of 20% w/v trichloroacetic acid containing 0.5% w/v thiobarbituric acid was added to the resulting supernatant, and the mixture was boiled for 15 min. Following cooling and filtration, the absorbance of the solution was measured at 450, 532 and 600 nm, and the MDA content (μmol/g fresh weight) was calculated as $[6.45 \times (A_{532}–A_{600})–0.56 \times A_{450}] \times 4$.

Microarray processing, scanning and analysis

Total RNA was extracted from root tissue using the TriZOL reagent as recommended by the manufacturer (Invitrogen), and a 2 μg aliquot was used to synthesize the cRNA. The hybridization probe consisted of 875 ng Cy3- and Cy5-labelled fragmented cRNA, which was used to challenge the microarray according to the Agilent protocol (17 h, 65°C in a rotating hybridization oven set at 10 rpm). The microarrays were scanned using an Agilent Array scanner at 100 and 10% photomultiplier tube (PMT), with a scan resolution of 5 μm. The microarray data were manipulated in an R (v 2.7.0) environment, using the LIMMA package (Smyth et al. 2003). Quantile normalization was performed based on the JN177 control present on each array, and LOWESS normalization was used within each slide. The Student's t test was used to assign statistical significance to between treatment expression level differences, with fold change >2 and a P value <0.03 considered as indicating differential expression. Hierarchical clustering was performed using Cluster v3.0 software (University of Tokyo, Human Genome Center), and visualized by Java TreeView (v1.1.1) (Saldanha 2004). The normalized hybridization signal dataset was subject to principal component analysis and tree clustering using, respectively, SAS v8.0 and Statisitica v6.0. Blast2Go (Conesa et al. 2005) and KOBAS (Wu et al. 2006) analyses were used to determine pathway enrichment based on Fisher's enrichment test method, where the full probe set mounted on the microarray was taken as background.

Semi-quantitative RT-PCR (sqRT-PCR) and Real-time PCR

Some expression profiles were validated using either sqRT-PCR or real-time PCR, based on a template of the first cDNA strand, synthesized using Reverse Transcriptase M-MLV (TaKaRa) according to the manufacturer's protocol. For sqRT-PCR, each 20μL reaction was denatured at 95°C for 5 min, followed by the imposition of 20–35 cycles of 95°C/30 s, 60°C/30 s, 72°C/90 s, with a final extension of 72°C/5 min. Real-time PCRs were performed in a 20 μL volume containing 10 μL SYBR Premix Ex Taq mix (Takara), 0.2 mM forward and 0.2 mM reverse primers and 1 μL diluted (1:10) template, with a cycling regime comprising an initial denaturation step (95°C/2 min), followed by 40 cycles of 95°C/10 s, 60°C/20 s, 72°C/20 s. A melting curve analysis was performed over the range 80–95°C at 0.5°C intervals. The constitutively expressed wheat actin gene was used as a reference. Each sqRT-PCR and real-time PCR was run in triplicate. The relevant primer sequences are listed in Table S1.

Comparison between transcriptome and proteome

To compare the correlation between transcriptome and proteome, we built a local BLAST server according to the guide at NCBI (http://blast.ncbi.nlm.nih.gov/). Sequences of differentially expressed proteins (DEPs) were subject to TBLASTN against EST sequences (Table 1) for microarray probe design (expect value <e-20 and identities >60%). A certain DEP sequence and the microarray probe referring to the aligned EST sequence of the DEP were believed to match each other. These matched DEP sequences and microarray probes were selected for transcriptomic and proteomic correlation comparison.

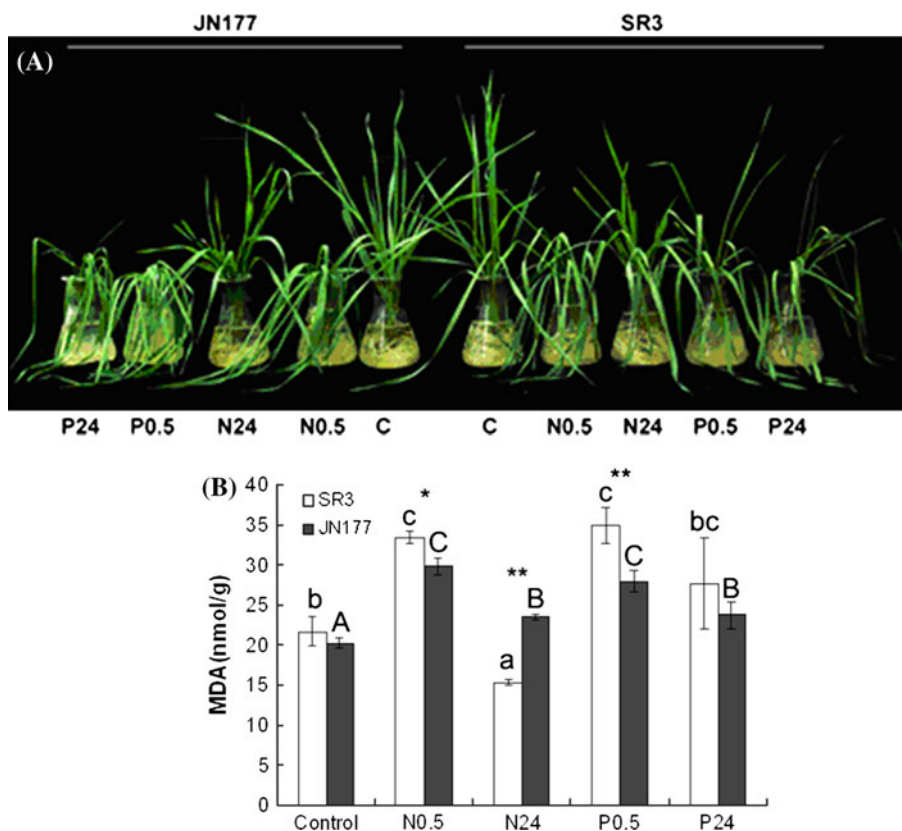## BLAST of probe corresponding sequences

To know whether the microarray covered the introgressed new genetic materials, the sequences of SR3 cDNA library as well as those of SSH library that were selected for probe design (Table 1) were subject to BLASTN against wheat EST sequences downloaded from NCBI (expect value <e-10 to as far as assume the unaligned sequences were possibly come from tall wheatgrass). The transcriptomic patterns of probes referring to these unaligned sequences (expect value >e-10) were compared.

Results

The response to NaCl and PEG treatment

Under the non-stressed conditions, SR3 seedlings formed longer shoots and roots than those of JN177 (Fig. 1a). Within 0.5 h of the imposition of NaCl stress, both cultivars wilted, but the effect was more severe for JN177 (Fig. 1a) and both the leaf and root growth rate was inhibited (data not shown). The plants recovered from wilting by the 24 h time point (Fig. 1a). Under PEG stress, the leaves of JN177 withered within 0.5 h and remained so throughout, but SR3 continued to grow (Fig. 1a). In order to know the effect of ROS produced under these stresses on the plasma membrane, MDA content was examined. After 0.5 h of either stress, the MDA content of the SR3 leaves was higher than that of the JN177 leaves, but the 24 h NaCl treatment had the opposite effect (Fig. 1b). Thus, PEG

Fig. 1 Phenotypic and physiological characteristics of JN177 and SR3 subjected to iso-osmotic NaCl and PEG stress. Columns marked with an identical *upper* or *lower* case *letter* indicate the absence of a significant difference. *, ** differences between SR3 and JN177 significant at, respectively, $P < 0.05$ and <0.01. *Bar* standard deviation

stress appeared to inhibit growth more severely than NaCl stress, and SR3 showed more tolerance than JN177 to these high levels of stress.

Microarray annotation

A BLASTN scan of the 60mer probe corresponding sequences against the *Arabidopsis* CDS database was able to annotate 87.4% of the sequences on the microarray at an E value <-20. Based on these annotations, 81.4% of the sequences were assigned a GO function (Table S2). 42.5% of probe corresponding sequences produced BLASTX hits against the non-redundant rice protein databases using the KOBAS system (Table S2). Furthermore, 2,340 SSH/full-length cDNA sequences of SR3 were subject to BLASTN against the TIGR wheat EST database, 1,089 produced no hit (of these, 407 could not be annotated) (Table S3).

Analysis and validation of microarray-based expression data

Signal was detected for 81.3–93.3% of the microarray features. The principal component and tree clustering analyses showed that the 24 h exposure to NaCl induced the greatest changes in gene expression (Fig. S1A), followed by the 0.5 h PEG treatment. The control and 0.5 h NaCl treatment transcriptomes were largely similar to

another (Fig. S1B). A sample of 14 differentially induced genes was carried forward for more detailed expression analysis revealed a high level of consistency between the real-time/sqRT-PCR and microarray outputs (Fig. 2).

The short-term response to salinity and drought stress

The comparison between the iso-osmotic NaCl and PEG 0.5 h treatments revealed 447 PEG responsive (323 up- and 124 down-regulated) and 310 NaCl responsive (154 up- and 156 down-regulated) genes (Table 2, S4). Some of these genes were stress-specific, and others were induced by both stresses (co-responsive) (Fig. 3a). Of the latter, 107 were up-regulated, and 57 down-regulated by two stresses, and generally the dynamic response to PEG was greater than to NaCl (Table S4). The transcription factors WRKY, MYB, DREB and ERF, along with a number of hormone responsive proteins fell into this category. Among the stress-specific genes, a greater number was up-regulated by PEG than by NaCl, and vice versa for those which were down-regulated (Table 2). Among the former class were 18 serine-type endopeptidase inhibitors, and genes encoding various signal transduction associated proteins, such as casein kinases (ta_01844, ta_12877) and C2 domain-containing proteins (ta_10645, ta_10646), as well as eight genes encoding phenylalanine ammonia lyase (Table S5). Among the up-regulated NaCl-specific genes were the

Fig. 2 Validation of microarray results using quantitative real-time PCR and semi-quantitative RT-PCR. Fourteen probes with different expression patterns were randomly selected. The *line charts* were quantitative real-time PCR (*upper*) and microarray data (*lower*). The *y* axis indicates the relative expression levels and signal values to JN177 control. *Arrows* indicate significant up- and down-regulation. N0.5, N24 and P0.5 represent treatments of NaCl for 0.5 h, NaCl for 24 h and PEG for 0.5 h, respectively
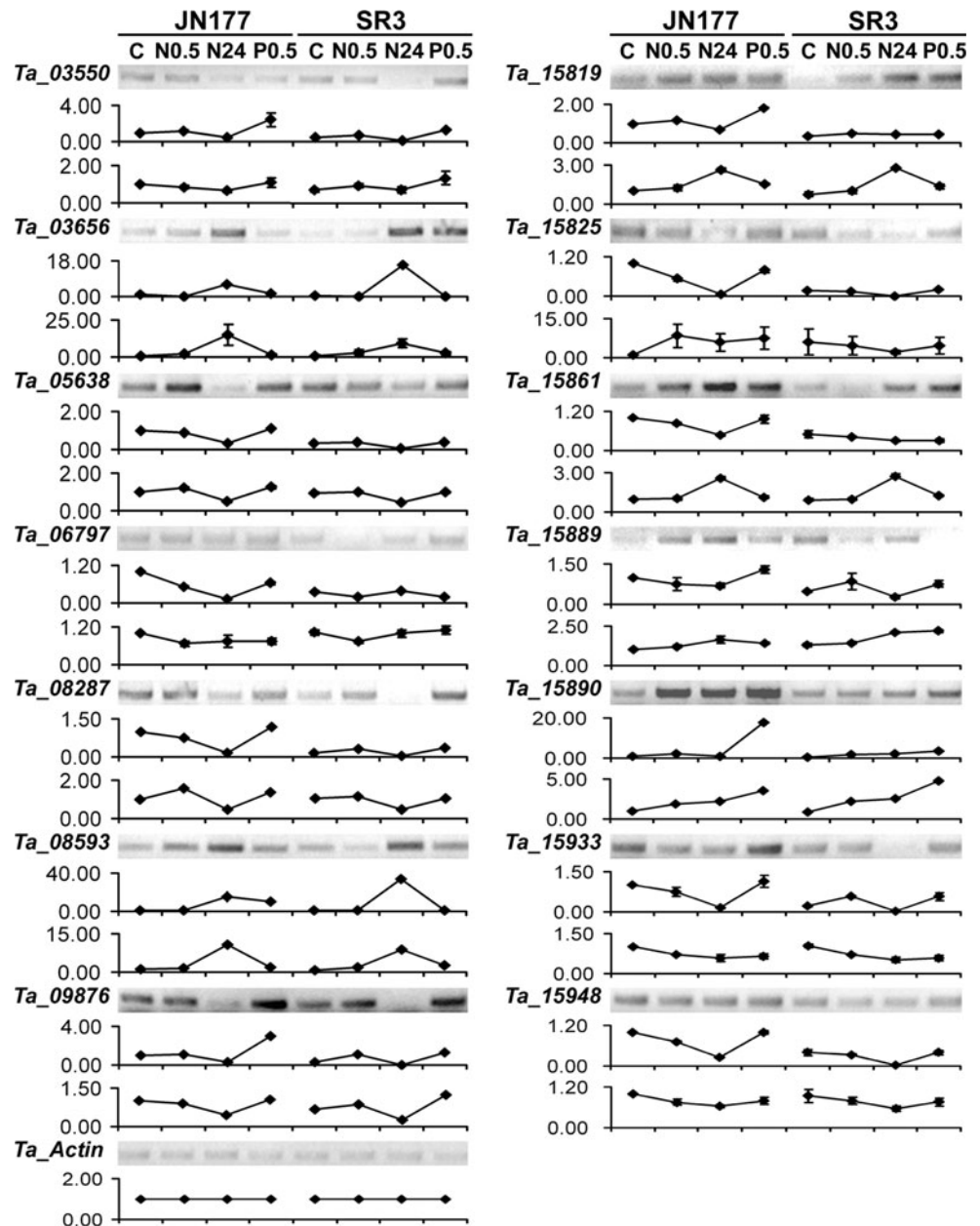
stress-associated gene *GST* (ta_07226) and the signal transduction module gene encoding diacylglycerol kinase (ta_07191). Transporter showed predominant difference among stress-specific genes. An ABC transporter (ta_13205) and a vesicle-mediated transporter SYP121 (ta_01966) were both up-regulated by PEG stress, while two sugar transporters ta_00085 and ta_14781 were both down-regulated; The salinity stress induced genes encoding proline transporter 2 (ta_02225), amino acid permease 3 (ta_02638) and a Ca^{2+}-transporting ATPase (ta_04206), while the genes encoding a high affinity K^+ transporter 5 (ta_02223, ta_06874), a zinc transporter 1 precursor (ta_07709), AKT1 (ta_08208, ta_12100), and a pair of

ammonium (ta_11267, ta_11268) and nitrate (ta_03162) transporters were down-regulated (Table S5).

The short-term and the more sustained response to salinity stress

In contrast to the 310 genes differentially expressed as a result of a 0.5 h exposure to NaCl, the longer exposure affected nearly 4,000 genes (2,116 up-, 1,762 down-regulated) (Tables 2, S6). Of these, respectively 108 and 56 were implicated at both the 0.5 h and 24 h time points; 44 were up-regulated early, but down-regulated later, and three behaved in the opposite fashion. A total of 99 genes (43 up- and 56

down-regulated) responded solely to the 0.5 h NaCl treatment (Table 2; Fig. S2). Many of the genes up-regulated after 24 h of NaCl stress were involved in global processes, while the down-regulated ones were mostly implicated in chromatin assembly/disassembly, macromolecule metabolism, lipid transport and transcription (Table S7). Most of the genes in common to both time points were more strongly expressed at the later time. Example of such genes were those encoding glucan endo-1,3-β-D-glucosidase (ta_05704, ta_01991) and α-amylase (ta_07327, ta_02385) (Table S5), which are both involved in the degradation of complex carbohydrates into simpler molecules such as sucrose which have an osmoprotectant and signalling function (Bartels and Sunkar 2005). Notably, RBOHD (ta_06216) functioning in ROS production was induced 2.5 fold at 0.5 h, and 15.2 fold at 24 h after the imposition of salinity stress (Table S5), showing the accumulative effect of stress. However, 18 probes GO-termed as redox homeostasis were up-regulated only at 24 h (Table S8), indicating that ROS scavenging machinery would be triggered until increase of ROS over the damage threshold in cells. Many of the genes specifically induced under the short exposure period were involved in signal transduction or in water and ion transport. The gene encoding an auxin-responsive protein (ta_08021) and AtERF-7 (ta_14361) were also up-regulated at this time point (Table S5). At the later time point, the genes specifically up-regulated were dominated by those encoding the phospholipid signal transduction enzymes phospholipase 2A (ta_00226, ta_00246, ta_00847, ta_13480, ta_13988), C2 (ta_06823), Dα1 (ta_03573) and several phosphatidylinositol kinases (ta_01665, ta_02142), although not the gene encoding diacylglycerol kinase (ta_07191, ta_07728) which was specifically up-regulated after 0.5 h of stress (Table S5). Those genes showing a contrasting response at the early and late time points tended to be involved with transcriptional regulation, energy metabolism and transportation, osmotic protection and the scavenging of oxidative products.

Examples were the genes encoding sugar transport protein 13 (ta_12110) and ADP/ATP carrier 3 (ta_06835, ta_06836), LCR68/PDF2.3 (ta_01304), OSMOTIN 34 (ta_05388) and CYP71a12 (ta_05996, ta_13177) (Table S5).

The differential response of SR3 and JN177

Under non-stressed conditions, 508 genes were differentially expressed between SR3 and JN177, comprising 241 up- and 267 down-regulated in the former cultivar (Table S9). Among the former, 18 were categorized as 'response to stress' genes (Table S10), including examples relevant for the cold, heat and drought response (Table S11). The KOBAS analysis indicated that genes in the pentose phosphate pathway and ascorbate and aldarate metabolism were more likely to be up-regulated in SR3 (Table S10). In addition, genes involved in hormone synthesis and signalling transduction were up-regulated, including those encoding allene oxide synthase (ta_06622) (jasmonate synthesis), GA20 oxidase (ta_08346) and GA3 β hydroxylase (ta_12167) (gibberellic acid metabolism), a nodulin-like protein (ta_07415) (auxin signalling transduction) and the GA receptor GID1L2 (ta_06964) (Table S5). In contrast, the carbon and energy metabolism associated processes, photosynthesis and glyoxylate/dicarboxylate metabolism were largely down-regulated in SR3 (Table S10).

Following the 0.5 h exposure to NaCl, 122 genes were up-regulated in SR3 and 87 in JN177, of which 48 were in common; there were, respectively, 101 and 218 down-regulated genes, of which 75 were in common (Table 3). After 0.5 h exposure to PEG, the numbers of up-regulated genes were, respectively 215 and 210 (71 in common), and 81 and 143 were down-regulated (29 in common) (Table 3). After the 24 h exposure to NaCl, these numbers were, respectively, 1,451 versus 1,556 (950) and 1,579 versus 1,507 (978) (Table 3). The fold change in expression of some of the co-responsive genes varied between the two cultivars. Most of

Table 2 Numbers of genes up- and down-regulated by exposure to iso-osmotic PEG and NaCl stress

	PEG 0.5 h	NaCl 0.5 h	Overlapped	PEG 0.5 h specific	NaCl 0.5 h specific
Up	323	154	107	216	47
Down	124	156	57	67	99
Total	447	310	164	283	146

	NaCl 24 h			NaCl 0.5 h total	NaCl 0.5 h specific
	UP	Down			
NaCl 0.5 h					
UP	108	3		154	43
Down	44	56		156	56
N24total	2,116	1,762			

Up up-regulated genes, *Down* down-regulated genes

Fig. 3 Hierarchical clustering of genes. **a** Genes induced after exposure for 0.5 h to NaCl and/or PEG; **b** Genes induced after exposure for 0.5 or 24 h to NaCl
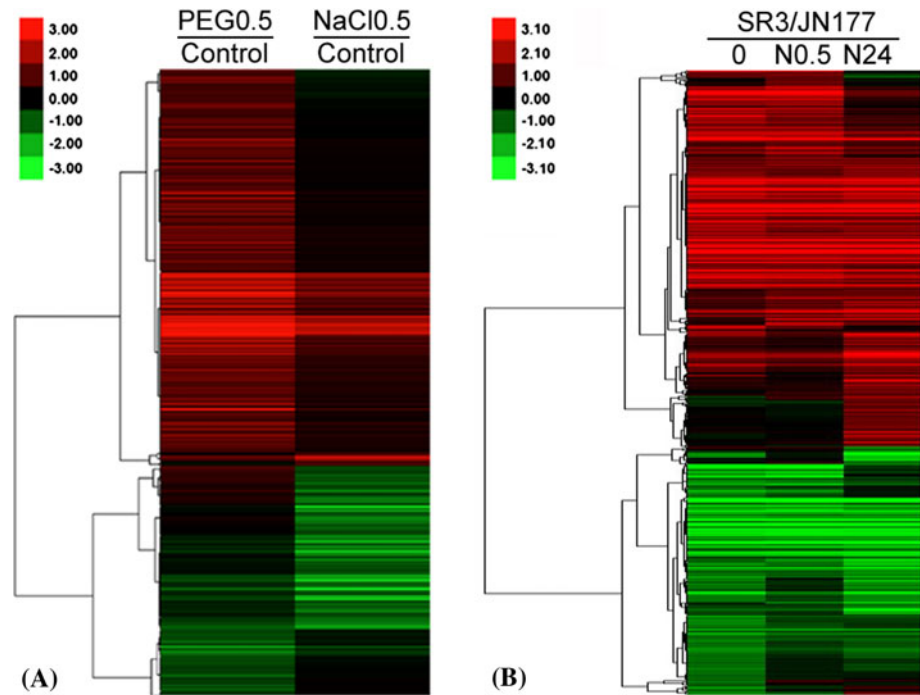
the genes which were stress-specific responded in one cultivar but not in the other; the exceptions, where the gene was up-regulated in SR3 and down-regulated in JN177 (or vice versa) were ta_02995 and ta_09161 (0.5 h PEG treatment), and ta_03492 and ta_03589 (24 h NaCl treatment) (Tables 3, S5, S12). In all, 484 genes were up- and 469 down-regulated in SR3 compared to JN177 (Fig. 4; Table S13). Of these, 60 were more, and 39 less highly expressed independent of the treatment. The NaCl responsive genes clustered into the following six categories (Fig. 3b; Table S12): (1) a set of 83 up-regulated in SR3 under non-stressed and NaCl-stressed conditions; (2) 69 up-regulated after a 0.5 h exposure to NaCl; (3) 263 were more strongly expressed after a 24 h exposure to NaCl; (4) 49 were down-regulated in SR3 under both non- and NaCl-stressed conditions; (5) genes specifically repressed by exposure to either 0.5 h (23 genes) or 24 h (65 genes) of NaCl stress; and (6) genes either up- (143) or down- (191) regulated by a 0.5 h exposure to PEG (of these, 30 and 97, respectively, were responsive only to PGE stress).

Some of the genes involved in the stress response of SR3 were quite distinct from those of JN177 (Table S14). For example, after the 0.5 h exposure to both NaCl and PEG, both unsaturated fatty acid and lipid synthesis as well as α-linolenic acid metabolism were enhanced in SR3, whereas the degradation of other glycans, the citrate cycle and phenylalanine metabolism were more markedly up-regulated in JN177. After the 24 h exposure to NaCl, ascorbate/aldarate and histidine metabolism, glycolysis/

gluconeogenesis and unsaturated fatty acid synthesis were more active in SR3 than in JN177. Among the 140 "hormone synthesis/response" and 718 "transporter" genes, there had more amounts of probes showing differential expression in SR3, which were categorized into more types of clusters (Fig. S5, S15). Some examples were genes within the jasmonate synthesis pathway (*AOC1*, ta_15960; *AOC4*, ta_10106; *AOS*, ta_06622; *OPR1*, ta_02274; *OPR2*, ta_13787), the transcripts of which were all more abundant in SR3 than in JN177 under both non-stressed and stressed conditions (Table S5). *ABF4* (ta_14314), a key component of the ABA signalling pathway with a positive role in stress tolerance (Kang et al. 2002) and the two ABA-inducible genes *LIP30* (ta_13550, ta_00020) and *RAB18* (ta_01732, ta_01730) (Zalejski et al. 2006) were all up-regulated in SR3.

Sequence alignment and low correlation
between transcriptomes and proteomes

Among 2,169 sequences of SR3 cDNA library and 171 of SSH library (Table 1), 104 and six sequences had no identity with wheat ESTs (expect value <e-10). Most of these sequences showed similar or rather differential expression patterns between two cultivars under the control and/or stressful conditions. Interestingly, probe ta_15764 referring to the full-length cDNA HR52 had low transcription level (signal values 377–419) in JN177, while dramatically high level (signal values 9,251–11,625

Table 3 Stress-responsive genes expressed differentially in JN177 and SR3

	SR3			Total
	Up	Constant	Down	
NaCl 0.5 h				
JN177				
Up	48	39	0	87
Constant	74		26	
Down	0	143	75	218
Total	122		101	
PEG 0.5 h				
JN177				
Up	71	139	0	210
Constant	142		52	
Down	2	112	29	143
Total	215		81	
NaCl 24 h				
JN177				
Up	950	606	0	1,556
Constant	499		601	
Down	2	527	978	1,507
Total	1,451		1,579	

Up up-regulated genes, *Down* down-regulated genes, *Constant* genes unaffected by stress

under the control and 0.5 h PEG/24 h NaCl treatments, and up to 23,797 under 0.5 h NaCl treatment) in SR3 (Table S16).

Our previous proteomic study identified 88 differentially expressed proteins (DEPs) in roots (Peng et al. 2009). Of them, 48 DEPs aligned with EST sequences for microarray probe design (Table S17). Under the control, only one DEP between SR3 and JN177 had an opposite transcriptional profile, and the other 33 ones showed no different transcripts. When exposed to NaCl for 24 h, five up- and two down-regulated DEPs in SR3 both had down-regulated transcriptional patterns, and the other 25 ones had constant mRNA abundances (Table 4; S17). Correlation analysis showed that quite small amount of DEP-probe pairs showed positively correlative ($r > 0.6$) under the control and stressful conditions, including seven in proteome control-24 h NaCl treatment/transcriptome control-24 h NaCl treatment (C-N24/C-N24) comparison, six in C-P24/C-P0.5 (P: PEG), two in C-P24-N24/C-P0.5-N24, seven in C-N24/C-N0.5, two in C-P24-N24/C-P0.5-N0.5 (Table S18). This indicated that the correlation between proteome and transcriptome is low, further indicating the complicated regulation mechanism form gene to protein, although conditions were slightly different between this and previous works (Peng et al. 2009).
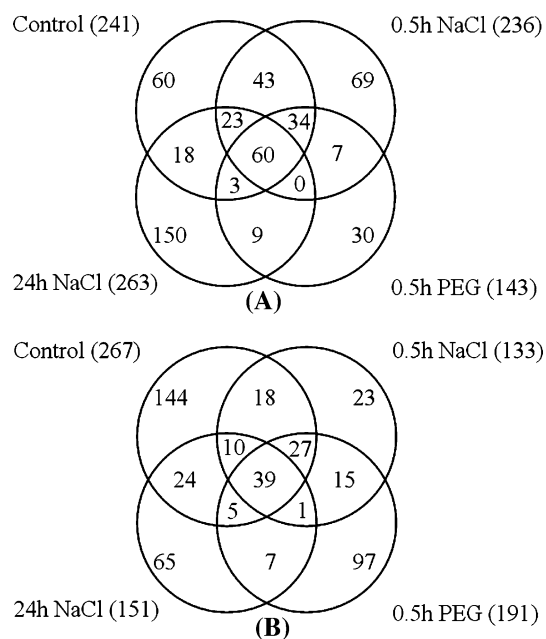
Fig. 4 Numbers of genes showing differential expression between SR3 and JN177 under non-stressed and stressed conditions. **a** Genes up-regulated in SR3; **b** Genes down-regulated in SR3

Discussion

Given the specificity of SR3's genome and the accuracy of probe annotation, a 60-mer customized wheat cDNA microarray with some probes possibly covering exogenous chromatins was used to gain an actual insight into the difference between JN177 and its introgression derivate SR3, as well as the difference between iso-osmotic PEG and salt stress. More importantly, inconsistent with most of published studies, the sudden treatment of higher concentration NaCl was conducted based on the phenotypic and biochemical difference in SR3 and parent JN177, and the response characteristics of SR3 in such condition will largely answer the basis of its tolerance to extreme saline stress.

The immediate effects of salinity stress

Salinity stress exerts both an osmotic and an ionic effect on the plant, and the former is the stress type more rapidly sensed (Munns and Tester 2008). For this reason, the present experiments included two iso-osmotic treatments, one involving salt and the other not, and the earliest measurements were made within 0.5 h of the imposition of stress. The salinity stress rapidly induced the expression of Ca^{2+} transporting ATPase (ta_04206) (Table S5), which may contribute in part to the well-known phenomenon of intracellular Ca^{2+} spiking as a first order response to the presence of excess Na^+ in the growth medium (Hirayama and Shinozaki 2010; Bartels and Sunkar 2005; Zhu 2002). An increase in the intracellular Ca^{2+} concentration is perceived by, among others,

Table 4 Transcriptomic and Proteomic comparison between SR3 and JN177 under the control and 24 h NaCl treatment

	Probe			Total
	Up	Constant	Down	
Control				
DEP				
Up	0	20	1	21
Constant	0	14	0	14
Down	0	13	0	13
Total	0	47	1	48
NaCl 24 h				
DEP				
Up	0	13	5	18
Constant	0	15	1	16
Down	0	12	2	14
Total	0	40	8	48

Up up-regulated genes, *Down* down-regulated genes, *Constant* genes unaffected by stress. *DEP* differentially expressed proteins identified in our previous study (Peng et al. 2009)

the phospholipid and SOS signalling pathways (Hirayama and Shinozaki 2010; Bartels and Sunkar 2005; Zhu 2002). Osmotic and salinity stress often alter membrane fluidity and change phospholipid content (Munnik and Meijer 2001). Phospholipids themselves can be cleaved (by phospholipases) to produce secondary messengers, such as inositol 1,4,5-triphosphate, diacylglycerol (DAG) and phosphatidic acid (PA). Here, diacylglycerol kinase (ta_07191, ta_07728), a key catalyzer of DAG to PA, was specifically up-regulated by NaCl; whereas SOS3 (ta_07772), a component of the SOS pathway (Ishitani et al. 2000) and its enhancer ENH1 (ta_13913) were down-regulated. The expression of its interacting factor CIPK11 (ta_11091) appeared to have been rather constant (Table S4). This behaviour suggests that signals coupled to intracellular Ca^{2+} concentration may have been perceived during the early stages of the stress by the phospholipid pathway rather than by the SOS pathway. The salinity-modulated down-regulation of a set of ionic (K^+, etc.) transporters (Table S4) demonstrated that Na^+ absorption occurred at very early stage of the stress, since it is well understood that K^+ and Na^+ are transported through the plant membrane in a synergistic manner (Munns and Tester 2008). The result of this inflow would have been a rapid development of ionic stress, which may thereby account for the large transcriptomic difference observed between the salinity and the merely osmotic stress treatments. Notably, most of the PEG/NaCl co-induced genes responded more strongly to PEG than to salinity, and the PEG treatment also had a more severe adverse effect on plant growth (Fig. 1a). These observations show that once the roots have sensed the stress, they are able to respond rapidly and discriminatingly to it.

Reactive oxygen species (ROS) homeostasis is important for SR3's tolerance to extreme stress

Up-regulation of genes encoding key enzymes in the production of ROS such as RBOHD (ta_06216) is activated by intracellular Ca^{2+} (Ogasawara et al. 2008). ROS production under stress induced the expression of several oxidative stress responsive genes, one of which was the ozone-responsive stress-related protein (ta_02524, ta_02525), a protein similar to AtOZI1 which accumulates in response to the production of ROS (Sharma and Davis 1995); another induced oxidative stress responsive gene was RCD1 (Radical-Induced Cell Death1) (ta_10067), which participates in the defence pathway in conjunction with jasmonate and ethylene. This gene's activity is correlated with tolerance to oxidative stress, and interacts with many proteins involved in salinity stress tolerance (Overmyer et al. 2000; Fujibe et al. 2006; Katiyar-Agarwal et al. 2006). SR3 has been shown to produce higher levels than JN177 of the ROS detoxifiers peroxidase (ta_01591, ta_01589), superoxide dismutase (ta_08657) and glutathione transferase (ta_01771, ta_13783) (Peng et al. 2009). The expectation is that a more plentiful supply of scavengers would increase the efficiency of ROS removal, as suggested by the lower MDA content of the SR3 leaf after a 24 h exposure to salinity than under non-stressed conditions (Fig. 1b) [Note that MDA is an indicator of ROS content and ROS-mediated lipid peroxidation, see Nankivell et al. (1994).].

Enhancing the efficiency of unsaturated fatty acid (ta_07855, ta_03653, ta_10107) and flavonoid synthesis (ta_13807, ta_09832, ta_09835) may also be an important contributor to SR3's improved level of oxidative stress tolerance. SR3 tissue contains more flavanoids than JN177 tissue (data not shown). Unsaturated fatty acids are important for their antioxidation activity and as precursors of various stress tolerance associated chemicals, and are for the most part synthesized when the plant is subjected to abiotic stress (Sakamo and Murata 2002). Flavonoids are important secondary metabolites associated with stress tolerance, and may also act as signals as part of the comprehensive response (Treutter 2006). The higher transcript abundance of aldehyde dehydranase (ta_14051) in SR3 (Table S5) is suggestive of a more efficient detoxification of aldehyde, which is responsible for oxidative damage to membrane lipids (Kotchoni et al. 2006; Huang et al. 2008). The pentose phosphate pathway was induced in SR3 after just 0.5 h of exposure to NaCl (Table S9). This pathway is coupled to proline synthesis and stimulates the synthesis of NADPH and sugar phosphates (Shetty and Wahlqvist 2004), which are important for the response to abiotic stress (Mittler 2002). Thus, the enhanced ROS detoxification shown by SR3 must be more than simply a matter of it producing a greater quantity of ROS scavenging enzymes.

The putative impact of introgressed genetic materials on transcriptome

Our previous study showed that a few genetic materials of tall wheatgrass were introgressed into SR3 genome (Wang et al. 2005; Chen and Xia 2003). In this work, we found that 110 EST sequences of SR3 for microarray design have no identity to any wheat EST in public database, especially probe ta_15764 has dramatically high transcription level in SR3 (Table S16), and its referring EST sequence has no annotation (Table S2) and no identity to any available wheat EST (Table S16), speculating that some of these sequences (especially ta_15764 referring sequence) are possibly introgressed from tall wheatgrass. Given that there has no EST information except for glutenin genes in tall wheatgrass and the wheat EST sequences are not adequate, this speculation can not be confirmed so far. Beside, we cloned more than 60 genes with differential expression levels between SR3 and JN177 under the salt-stressed conditions based on the cDNA and SSH library data and proteomic results. Two genes have high identity to the homologues of tall wheatgrass but low identity to those of JN177, and some others (over 20%) have allelic variation such as SNPs or other mutations (unpublished data). Out of them, one allele with two amino acid substitutions contributes to vigor growth and salt tolerance, the mainly phenotype of SR3 seedlings (unpublished data). Furthermore, the large-scale sequencing of SR3 and JN177 cDNA libraries showed that a high frequency of variation (SNPs and indels) exists between their genomes (unpublished data). With these findings, we suggest that the introgression of new genetic materials in wheat genome induce genomic variation or regulate expression of wheat genes so as to contribute to the stress tolerance.

In conclusion, the present transcriptomic analysis has indicated that SR3 responded in a distinct manner to iso-osmotic PEG and NaCl stress. This response included both signal perception and adaptation to the stress. The ability to achieve this must have derived from genomic variation or expression alternation of wheat genes brought about by the somatic hybridization process used to introgress tall wheatgrass chromosome segments (Gao et al. 2010). The origin of these differentially expressed genes and the effect of introgressed genetic materials on the regulation of wheat genes is worthy of being investigated. The identity of the genes induced and repressed in SR3 has provided a wealth of leads into the mechanisms of salinity tolerance, both in wheat itself and by extension, to other cereal and non-cereal crop species.

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