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Genome-wide analysis of thiourea-modulated salinity stress-responsive transcripts in seeds of *Brassica juncea*: identification of signalling and effector components of stress tolerance

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• *Background and Aims* Abiotic stresses including salinity are the major constraints to crop production. In this regard, the use of thiourea (TU) in imparting salinity-stress tolerance to Indian mustard (*Brassica juncea*) has been demonstrated earlier. To gain an insight into the mechanism of TU action, various molecular and biochemical studies were conducted.
• *Methods* Microarray analysis was performed in seeds subjected to distilled water (control), 1 M NaCl, 1 M NaCl + 6·5 mM TU and 6·5 mM TU alone for 1 h. Real-time PCR validation of selected genes and biochemical studies were conducted under similar treatments at 1 h and 6 h.
• *Key Results* The microarray analysis revealed a differential expression profile of 33 genes in NaCl- and NaCl + TU-treated seeds, most of which are established markers of stress tolerance. The temporal regulation of eight selected genes by real-time PCR indicated their early and co-ordinated induction at 1 h in NaCl + TU only. Besides, NaCl + TU-treated seeds also maintained a higher level of abscisic acid, reduced to oxidized glutathione (GSH : GSSG) ratio and activities of catalase, phenylalanine ammonia lyase and glutathione-*S*-transferases, as compared with that of NaCl treatment. The addition of $LaCl_3$ (a specific calcium-channel blocker) restricted the responses of TU both at molecular and biochemical level suggesting the possible involvement of a cytosolic calcium burst in the TU-mediated response. The TU-alone treatment was comparable to that of the control; however, it reduced the expression of some transcription factors and heat-shock proteins presumably due to the stabilization of the corresponding proteins.
• *Conclusions* The TU treatment co-ordinately regulates different signalling and effector mechanisms at an early stage to alleviate stress even under a high degree of salinity. This also indicates the potential of TU to be used as an effective bioregulator to impart salinity tolerance under field conditions.

Key words: Abscisic acid, calcium signalling, redox state, salinity stress, thiourea, transcriptional profiling.

INTRODUCTION

In the current scenario of increasing interest in sustainable agriculture, soil salinity is a major environmental constraint that limits the use of land. More than 800 million ha of land throughout the world are salt-affected (FAO, 2008) which accounts for >6 % of the world's total land area. Besides, out of 1500 million ha of land farmed by dryland agriculture, 32 million ha (2 %) are affected by salinity to varying degrees and out of the current 230 million ha of irrigated land, 45 million ha (20 %) are salt affected (Munns and Tester, 2008). The problem of soil salinity is becoming severe day by day due to salty rain around the coasts, contamination from the parental rocks and oceanic salts and bad cultivation practices (Mahajan and Tuteja, 2005). Therefore, efforts are underway to increase and/or manage the salinity tolerance in plants through the transfer of individual functional genes either by genetic engineering or plant breeding (Jauhar, 2006; Witcombe *et al.*, 2008; Purty *et al.*, 2008; Visarada *et al.*, 2009). However, these approaches have not been successful as far as consistency, reliability and visible effects at field level are concerned. This is mainly because of the multigenic and complex nature of salinity-stress tolerance (Sairam and Tyagi, 2004; Yamaguchi and Blumwald, 2005). Another viable approach, in this direction, is based on the transfer of transcription factors such as DREB1A (Kasuga *et al.*, 1999), CBF4 (Haake *et al.*, 2002), OSISAP1 (Mukhopadhyay *et al.*, 2004), NAC (Hu *et al.*, 2006) and AP37 (Oh *et al.*, 2009) to activate a set of genes involved in salinity/drought-stress tolerance. However, the limitation of this concept lies in the fact that one transcription factor may regulate several metabolic pathways and one metabolic pathway may need an orchestrated regulation from more than one transcriptional element. Thus, the development of stress-tolerant transgenics using gene transfer approaches needs much more understanding of plant stress-tolerance and gene-regulatory network systems.

Another important factor regulating the key processes in growth and development as well as stress tolerance is the cellular redox state. The maintenance of the redox homeostasis is collectively achieved by redox pairs (e.g. GSH/GSSG), antioxidant systems (catalase and superoxide dismutase, etc.) and other secondary metabolites (flavonoids, alkaloids and carotenoids) (Dietz, 2008). In response to any external stimuli, plants modify their redox state and the extent of change is dependent on the nature of the stimulus itself, the dose, and exposure time to the tissue in question (Miller *et al.*, 2009).

On the basis of these facts, we have hypothesized that if the external application of any molecule can help the plants to maintain their redox homeostasis under stress conditions, it may enhance their stress-tolerance potential. This is entirely a novel concept which is based upon the priming of the existing defence mechanisms and hence avoids any manipulation of the genome. In an earlier study, three thiol compounds, namely, thiourea (TU), dithiothreitol and thioglycollic acid were selected, because of their ability to maintain the redox state, to monitor their effect on the performance of wheat and mustard crops under salinity and drought-affected fields of Rajasthan (India). The field data indicated that the pre-treatment of seeds and foliar spray of the seedlings at later stages with all these thiol compounds could increase the stress tolerance and, more importantly, crop productivity; the performance of TU was found to be the best (Sahu and Singh, 1995; Sahu *et al.*, 2005). Thus, considering the efficiency of TU in alleviating salinity stress and increasing crop yield, studies were undertaken to investigate the molecular mechanism(s) of TU-mediated stress tolerance. Recent research has also established the positive role of TU treatment in maintaining the functioning of seed mitochondria (Srivastava *et al.*, 2009) and root water homeostasis (Srivastava *et al.*, 2010) in *Brassica juncea* under a high degree of salinity stress. In the present study, various molecular and biochemical analyses were conducted at an early time point in *Brassica juncea* seeds to investigate the mechanisms of TU-mediated control of seed germination processes under salinity stress. As far as is known, no such study, offering practical applications at the field level, has been performed where an external bioregulatory molecule is used to decipher different signalling and effector components of salinity-stress tolerance in plants.

MATERIALS AND METHODS

Plant material: stress induction and thiol treatment

The study was performed on Indian mustard, *Brassica juncea* (L.) Czern. For microarray analysis, seeds were surface sterilized with 30 % ethanol for 3 min and washed thoroughly with distilled water to remove the traces of ethanol. The seeds were independently subjected to one of four different treatments [distilled water (DW); NaCl (1 M); NaCl (1 M) + TU (6·5 mM); or TU (6·5 mM)]; after 1 h samples were taken for the microarray analysis. The selection of such a high NaCl concentration (1 M) for microarray analysis was based on preliminary screening of NaCl concentrations ranging from 0·3 M to 1 M in the presence/absence of TU, in terms of seed germination ability (data not shown). In an assay, seeds were soaked in either 1 M NaCl or 1 M NaCl + 6·5 mM TU for 6 h and then allowed to germinate under normal conditions for 1 d and 2 d and it was found that seeds soaked in NaCl exhibited a drastic reduction in their germination ability, whereas in the NaCl + TU treatment, a comparatively better germination pattern was noticed (Fig. S1 in Supplementary Data, available online). Hence, these concentrations were selected for the study. However, an early time point for microarray analysis (1 h) was chosen to get an insight into the regulatory gene network that led to differential seed germination profile in NaCl and NaCl + TU. The selection of time point (1 h) was also supported from a previous study which indicated that the TU-modulated gene-expression pattern becomes detectable as early as 1 h after the stress treatment (Srivastava *et al.*, 2009). Nevertheless, for real-time PCR and biochemical analyses, both time points (1 h and 6 h) were used. For these analyses, surface-sterilized seeds were given one of five different treatments [DW; NaCl (1 M); NaCl (1 M) + TU (6·5 mM); TU (6·5 mM); or NaCl (1 M) + TU (6·5 mM) + $LaCl_3$ (5 mM)].

RNA isolation and quality control

Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen-74903). The integrity and concentration of RNA was measured using an Agilent 2100 bioanalyser with an RNA 6000 nano assay. The RNA integrity number (Schroeder *et al.*, 2006) was developed using an RIN software algorithm and a value greater than eight was treated as a quality control for the RNA to be subjected to the microarray analysis (Fig. S2 in Supplementary Data).

Microarray hybridization and data analysis

Total RNA (100 ng) was used to prepare the cy-3/cy-5-labelled complementary RNA (cRNA), using the Agilent low RNA input linear amplification kit, following the manufacturer's guidelines. The amplification reaction has been optimized to be linear and not introduce bias of the abundant mRNA species over the rare mRNA populations (Fig. S3 in Supplementary Data). The labelled cy3-/cy-5 cRNA was purified by using the Agilent cRNA clean-up module. The quantification of the labelled cRNA was performed using a nanodrop spectrophotometer and the profile of amplified RNA was checked using an Agilent 2100 bioanalyser. An equal amount of labelled cRNA (825 ng) was used for each RNA sample. All hybridizations were performed with a 4X44K arabidopsis array slide. Hybridization and washing steps were carried out using an Agilent gene expression hybridization kit. For each microarray experiment, samples were processed in triplicate with independent dye swap labelling. Separate images (for each hybridization) were acquired using a DNA microarray scanner (Agilent Technologies) by simultaneous two-colour scanning at 5- and 10-μm resolution. Image analysis was performed using Agilent feature extraction software.

Expression data were evaluated using Agilent data analysis software in which the parameters were set as follows: false discovery rate, 5 %; data response type, paired data; data in log scale, $\log_2$; number of permutations, 1000; imputation engine, K-nearest neighbor imputer. A cut-off value of 0·5-fold and a *P*-value of 0·01 were used to select a subset of significant genes for further analysis. Gene clustering analysis was performed with GeneSpring version 6·1 (Silicon Genetics, San Jose, CA, USA).

Identifying biological functions of differentially expressed genes

Using tools on the TAIR website (www.arabidopsis.org/index.jsp), the differentially expressed genes were categorized in terms of their biological function. Expected frequencies for

each category were calculated based on the entire database of annotated arabidopsis genes using Nick's classification superviewer tool (Provert and Zhu, 2003).

Primer designing and quantitative real-time PCR for the validation of microarray data

For quantitative real-time PCR, 15 genes that demonstrated differential expression with respect to NaCl and NaCl + TU treatments were selected. The gene-specific primer pairs were selected from the arabidopsis genome-wide real-time PCR primer pair database (Han and Kim, 2006). All the primer sets were tested by real-time PCR on a dilution series of cDNA (1 : 2; 1 : 4; 1 : 8; 1 : 16; 1 : 32 and 1 : 64) derived from a first-strand cDNA mix, independently from the different treatments in triplicate. Primer efficiencies were calculated using REST-384 version 2 software (http://rest.gene-quantification.info/). Out of 15, only eight primer sets which displayed comparable amplification efficiencies (1·9–2·1) and generated single, specific PCR products and were used further for all the gene expression analyses. The details of these primers are given in Table S1 in Supplementary data. The specificities of all the primer sets were also confirmed by direct sequencing of their cDNA amplified products (data not shown).

For cDNA synthesis, 10 μg of DNA free total RNA from different samples were taken and subjected to a reverse transcription reaction (RT +) with oligo dT primer using a SuperScript first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA), following the manufacturers protocol. Parallel control reactions (RT−; in which the reverse transcriptase enzyme was replaced with water) were also conducted for all RNA samples. Both RT+ and RT− samples were compared for all the primer sets by real-time quantitative PCR to confirm that no DNA contamination is present in any of the samples (data not shown). Real-time PCR was carried out using the Rotor-Gene 6600 (Corbett Life Science; www.corbettlifescience.com). Reactions were set up by combining 10 μL of SyBr green PCR reaction mix (Sigma; S 4320) with 2·5 μL of 1 to 20 diluted cDNA templates, 1·5 μL each of forward and reverse primer (10 mM each), and 4·5 mL of PCR grade water (Sigma W1754). For gene expression analyses, the control gene actin and one target gene were analysed per run, and reactions were carried out on 3 biological and 3 technical replicates for each sample. The following PCR protocols were followed: 95 °C for 15 min; 40 cycles of 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s, sample read; 72 °C for 10 min; and melting curve analysis. The global minimum was subtracted for baseline correction. The threshold line was adjusted to be above early cycle background fluorescence. Data on the threshold cycle at which the fluorescent intensity of each sample first increased above background levels were collected, and normalized to actin levels (which showed very little expression variation among different treatments). The efficiency-adjusted relative expression ratios were calculated using REST-384 version 2 software.

Measurement of glutathione content

To estimate reduced and oxidized glutathione (GSH and GSSG), samples (500 mg) were frozen in liquid nitrogen and homogenized in 0·1 M sodium phosphate-EDTA buffer (pH 8·0) containing 25 % meta-phosphoric acid. The level of GSH and GSSG were determined following the method described by Hissin and Hilf (1976).

Enzyme assays

Seeds given different treatments were ground to a fine powder in liquid nitrogen and then homogenized in buffers specific for each enzyme under chilled conditions. Homogenate was squeezed through four layers of cold cheese cloth and centrifuged at 12 000 *g* for 15 min at 4 °C. The protein content in the supernatant was measured according to Lowry *et al.* (1951). The activities of glutathione-*S*-transferase (GST), catalase (CAT) and phenylalanine ammonia-lyase (PAL) were assayed following the methods described by Habig and Jacoby (1981), Aebi (1984) and Hahlbrock and Ragg (1975), respectively.

Measurement of abscisic acid (ABA) content

Seed samples (200–300 mg f. wt) from different treatments were collected, weighed and immediately frozen in liquid nitrogen, thawed and extracted with distilled water (tissue : water ratio 1 : 20, w/v) for 16 h at 4 °C in the dark. A competitive immunoassay-based quantitative determination of the ABA content was performed in crude aqueous extracts using the Phytodetek ABA test kit, following the procedure described by the manufacturer (Idetek, Sunnyvale, CA, USA).

Statistical analysis

All data were subjected to analysis of variance according to the model for completely randomized design using an SPSS 10·0 program. Differences among treatment means were evaluated by Duncan's multiple range test (DMRT) at a 0·05 probability level.

RESULTS

Global analysis of early TU-responsive gene expression in seeds

All the microarray data, which represent the average ratios of normalized signals in $\log_2$ scale calculated from three independent replicates (Fig. 1), are available under the accession number of E-MEXP-1903 in EBI microarray database (Mukherjee *et al.*, 2005). In TU-alone-treated seeds, 53 genes were down-regulated as compared with that of the control (Table 1). The functional analysis of these genes indicated that most of them are either transcription factors/heat-shock proteins (HSP) or associated with responses such as calcium signalling, protein synthesis/degradation and antioxidant machinery. Eight genes, which are associated with the cellular energetics system, were found to be up-regulated in the TU-alone treatment (Table 2).

In NaCl-stressed seeds, overall 83 down-regulated and 28 up-regulated genes were identified (data not shown). Clustering analysis of these 111 differentially expressed genes resulted in 33 genes being identified whose expression was modulated upon TU supplementation to NaCl. These

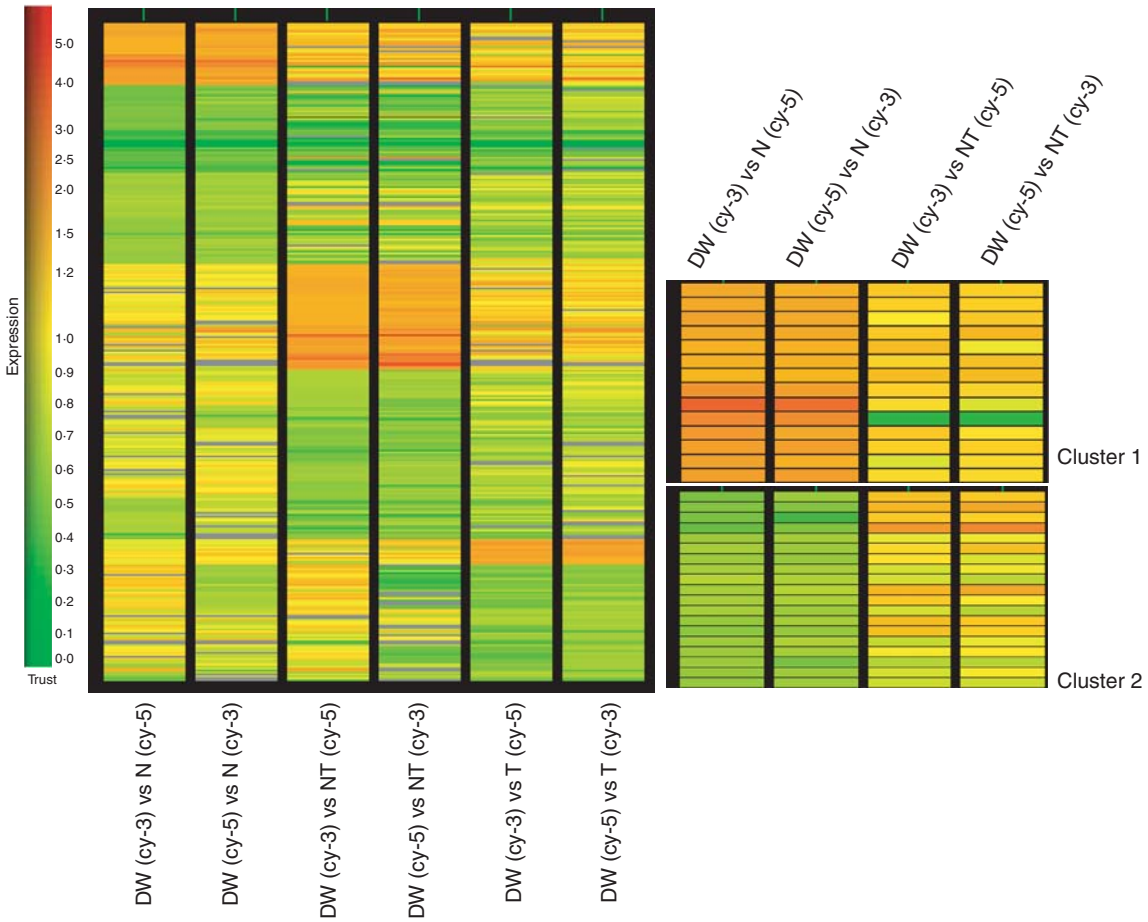

FIG. 1. Microarray analysis in *Brassica juncea* seeds. An overview of microarray analyses (DW vs. N; DW vs. NT and DW vs. T) is represented. Each experiment was performed in triplicate and the average ratios of normalized signals in $\log_2$ scale were calculated from three independent replicate experiments using GENESPRING software. The DW, N, NT and T represent distilled water control, NaCl (1 M), NaCl (1 M) + TU (6·5 mM) and TU (6·5 mM) treatments, respectively. Clusters 1 and 2 represent 19 and 14 genes which were down- and up-regulated, respectively, in NaCl but gets modulated under the NaCl + TU treatment. The attached trust represent the scale of expression fold difference (green and red colours represent the down- and up-regulation, respectively).

genes were divided into Clusters 1 and 2 (Fig. 2) of 14 (Table 3) and 19 (Table 4) genes, respectively, depending on their down-regulation or up-regulation in NaCl + TU as compared with that of NaCl stress. These 33 TU-modulated and salinity stress-responsive genes were then functionally classified (Fig. 2). A total of 27 % genes encoded proteins with a putative regulatory function (transcription factors, hormone and calcium signalling), suggesting a large adjustment in regulatory networks in response to TU treatment under salinity stress, 6 % genes encoded proteins involved in ABA metabolism, 9 % were related to the energy production and 12 % of genes were found to be involved in storage and transport.

Quantitative real-time PCR validation of selected genes under different treatments

Quantitative real-time PCR of the eight selected TU-modulated and salinity-responsive genes was performed to validate the results of microarray (Fig. 3A). In general, the gene-expression pattern obtained under different treatments by real-time PCR correlated well with the data of microarray experiments (as determined by Pearson correlation coefficients; data not shown). However, the values of $\log_2$ ratio obtained by real-time PCR were generally higher than those obtained by microarray analysis. This observation, to some extent, may be attributed to the saturation of fluorescent signals in the microarray. Out of the eight genes analysed by real-time PCR, the $\log_2$ ratio of CAT1 was not correlated with that obtained from the microarray. This could be due to the differential mRNA splicing that can affect the transcript levels detected at different regions of mRNA by two independent techniques (real-time and microarray).

Apart from microarray data validation, real-time PCR analysis was also performed to study the temporal regulation of the same set of genes. The data obtained indicated that the transcripts were under dynamic regulation. At an early phase of imbibition (1 h), the expression level of most of the genes was higher in NaCl + TU it was lower in NaCl, as compared with the control (Fig. 3A). An increase in the expression level of most of the genes in the NaCl treatment as compared with the control was achieved only at 6 h (Fig. 3B). At 6 h, the CYP707A2 and GST-9 were found to be down-regulated in all the treatments. Such a temporal regulation in the expression profile was not observed in TU-alone-treated

TABLE 1. *List of the genes down-regulated in response to TU-treated seeds*

Serial number	ID	Description	D vs. T
Transcription factor, kinases and transporters			
1	*At1g74100*	SOT16 (sulfotransferase 16)	−3·93
2	*At3g29350*	AHP2 (histidine-containing phosphotransmitter-2)	−1·44
3	*At5g59820*	ZAT12 (responsive to high light)	−1·13
4	*At5g53590*	Auxin-responsive family protein	−1·12
5	*At1g06225*	CLE3 (CLAVATA3/ESR-RELATED 3); receptor binding	−1·09
6	*At4g17920*	Zinc finger (C3HC4-type RING finger) family protein	−1·09
7	*At5g61600*	Ethylene-responsive element-binding family protein	−1·09
8	*At1g23740*	Oxidoreductase, zinc-binding dehydrogenase family protein	−1·09
9	*At1g72430*	Auxin-responsive protein-related	−1·04
10	*At3g49530*	Arabidopsis NAC domain containing protein 62; transcription factor	−0·92
11	*At2g19170*	SLP3; serine-type peptidase	−0·86
12	*At4g31800*	ATWRKY18_WRKY18; transcription factor	−0·86
13	*At5g03720*	HSFA3_AT-HSFA3; DNA binding/transcription factor	−0·84
14	*At3g55980*	ATSZF1 (salt-inducible zinc finger 1)	−0·83
15	*At3g16210*	F-box family protein	−0·77
16	*At4g25950*	VATG3 (vacuolar ATP synthase G3)	−0·76
17	*At5g28080*	WNK9; kinase/protein kinase	−0·74
18	*At2g27050*	EIL1 (ethylene-insensitive3-like 1); transcription factor	−0·73
19	*At2g24840*	AGL61 (agamous-like 61); transcription factor	−0·69
20	*At2g23030*	SNRK2·9 (SNF1-related protein kinase 2·9)	−0·68
21	*At5g59340*	WOX2 (Wuschel-related homeobox 2); transcription factor	−0·63
Protective genes			
22	*At3g09640*	APX2 (ascorbate peroxidase 2)	−1·64
23	*At4g10270*	Wound-responsive family protein	−1·16
24	*At1g20620*	CAT3 (catalase 3)	−1·1
25	*At1g59860*	17·6 kDa class I heat-shock protein (HSP17·6A-CI)	−1·04
26	*At1g63940*	Monodehydroascorbate reductase	−0·92
27	*At1g69920*	ATGSTU12 (glutathione *S*-transferase TAU 12)	−0·76
28	*At3g13310*	DNAJ heat-shock N-terminal domain-containing protein	−0·73
29	*At3g53990*	Universal stress protein (USP) family protein	−0·72
30	*At5g59720*	HSP18·2 (heat-shock protein 18·2)	−0·66
31	*At2g29500*	17·6 kDa class I small heat-shock protein (HSP17·6B-CI)	−0·64
Protein synthesis/degradation			
32	*At1g73480*	Hydrolase, alpha/beta fold family protein	−2·26
33	*At5g14980*	Esterase/lipase/thioesterase family protein	−1·27
34	*At5g64660*	U-box domain-containing protein	−1·2
35	*At2g07715*	Ribosomal protein L2, putative	−1·07
36	*At3g18740*	60S ribosomal protein L30 (RPL30C)	−0·88
37	*At1g66160*	U-box domain-containing protein	−0·86
38	*At4g27140*	2S seed storage protein 1/NWMU1-2S albumin 1	−0·84
39	*At3g18080*	BGLU44 (B-S GLUCOSIDASE 44)	−0·79
Calcium signalling-related			
40	*At1g21550*	Calcium-binding protein, putative	−1·16
41	*At1g01140*	CIPK9 (CBL-INTERACTING PROTEIN KINASE 9)	−1·09
42	*At5g25110*	CIPK25 (CBL-INTERACTING PROTEIN KINASE 25)	−1·08
43	*At3g63380*	Calcium-transporting ATPase/putative (ACA12)	−1
44	*At2g41410*	Calmodulin, putative	−0·91
Molecular function unknown			
45	*At4g38060*	Unknown protein	−1·37
46	*At3g07900*	Unknown protein	−1·14
47	*At2g07708*	Unknown protein	−1·1
48	*At2g07772*	Unknown protein	−1·1
49	*At1g62840*	Unknown protein	−1·01
50	*At1g27330*	Unknown protein	−1·01
51	*At3g60990*	Unknown protein	−0·99
52	*At1g33055*	Unknown protein	−0·87
53	*At5g65300*	Unknown protein	−0·71

The microarray analysis was conducted to compare the gene expression profile of TU-treated seeds (T) with that of the control seeds (D). The values represent the average ratios of normalized signals in $\log_2$ scale calculated from three independent replicate experiments using GENESPRING software. Differences in the mean values were found to be statistically significant at $P < 0{\cdot}05$ (one-way ANOVA).

seeds. In TU-treated seeds, the expression of all genes was either at the level of the control or up-regulated at both time points except CAT-1, whose expression decreased at 6 h.

Redox state regulation in seeds under different treatments

At 1 h, the level of GSH decreased by 47 % in the NaCl treatment in comparison with the control, while the decline

TABLE 2. *List of the genes up-regulated in response to TU-treated seeds*

Serial number	ID	Description	D vs. T
1	*At1g13340*	Oxygen-evolving enhancer protein	1·09
2	*At5g44360*	Aldo/keto reductase family protein	0·91
3	*At5g02380*	AHA3; ATPaseohydrogen-exporting ATPase	0·84
4	*At5g13440*	ATOPT6 (oligopeptide transporter 1)	0·83
5	*At1g17710*	FAD-binding domain-containing protein	0·82
6	*At5g56000*	Ubiquinol-cytochrome *c* reductase iron-sulfur subunit	0·74
7	*At1g32470*	MT2B (METALLOTHIONEIN 2B); copper ion binding	0·70
8	*At1g10650*	Unknown protein	0·65

The microarray analysis was conducted to compare the gene expression profile of TU-treated seeds (T) with that of the control seeds (D). The values represent the average ratios of normalized signals in $\log_2$ scale calculated from three independent replicate experiments using GENESPRING software. Differences in the mean values were found to be statistically significant at $P < 0{\cdot}05$ (one-way ANOVA).

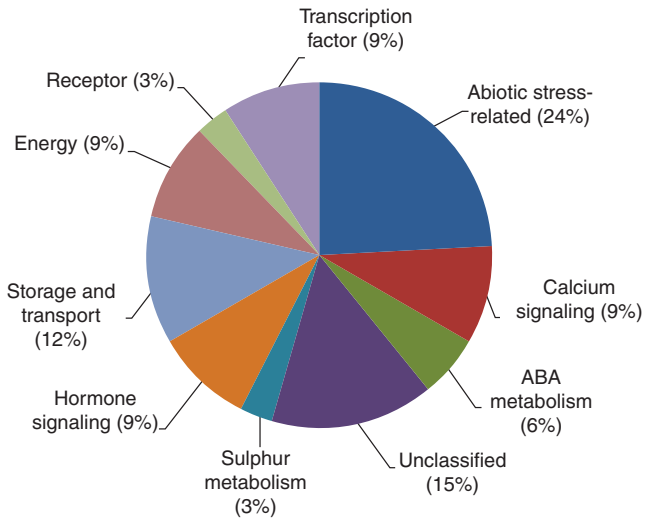

FIG. 2. Functional classification of TU-modulated and salinity stress-responsive genes. Genes were classified according to the function of the protein that they encode (Provart and Zhu, 2003; www.arabidopsis.org).

was only 9 % and 23 % in NaCl + TU and TU treatments, respectively. At 6 h, the level of GSH was at par to the control in TU treatment; while it increased in NaCl and NaCl + TU treatments as compared with the control (Fig. 4A). In the NaCl + TU treatment, the GSH : GSSG ratio was close to the control at 1 h, but was 11 % lower than the control at 6 h. In contrast, in the NaCl treatment, an initial 12 % decrease was seen in the GSH : GSSG ratio at 1 h as compared with the control in which it was maintained for 6 h (Fig. 4B).

Endogenous ABA level in seeds under different treatments

The level of ABA increased only slightly in 1 h of NaCl treatment, while in NaCl + TU- and TU-treated seeds, it increased significantly by 3- and 2·6-fold, respectively, as compared with the control. At 6 h, the level of ABA was close to the control in the NaCl treatment, while it was 20 % lower than the control in the NaCl + TU treatment. However, in the TU treatment, the ABA level remained 43 % higher than the control at 6 h (Fig. 5).

Activities of CAT, PAL and GST in seeds under different treatments

In NaCl-treated seeds, the activity of CAT and PAL decreased significantly both at 1 h and 6 h as compared with the control. In contrast, in the NaCl + TU and TU treatments, both CAT (Fig. 6A) and PAL (Fig. 6B) activities either increased to a higher level or remained close to the control, except in the 1-h TU treatment. The activity of GST decreased significantly as compared with the control in the NaCl treatment for 1 h, whereas at this time point, NaCl + TU- and TU-treated seeds showed an increased GST activity. At 6 h, the GST activity decreased in all treatments, except TU-treated seeds, which still showed about 11 % higher activity than the control (Fig. 6C).

Effect of calcium-channel blocker on the functioning of TU

To reveal whether the functioning of TU is dependent upon a $[Ca^{2+}]_{cyt}$ transient burst, $LaCl_3$ (a specific Ca^{2+} channel blocker) was provided, along with the NaCl + TU treatment. Out of the eight selected TU-responsive genes (whose expression level was measured by real-time PCR), the expression profile of three of them, such as CAT1, EREBP and CAX-2, was found to be almost completely reversed when $LaCl_3$ was added to the NaCl + TU treatment (Fig. 3A and B). In addition, the synthesis of GSH was also slightly affected by $LaCl_3$ in the initial 1 h (Fig. 4A); however, the ratio GSH : GSSG remained unaffected (Fig. 4B). Another critical change was no alteration in the ABA level under the NaCl + TU treatment upon $LaCl_3$ addition (Fig. 5). Further, the $LaCl_3$ treatment was also found to inhibit substantially the increase in the activity of CAT (Fig. 6A), PAL (Fig. 6B) and GST (Fig. 6C), which otherwise increased under the NaCl + TU treatment.

DISCUSSION

In the present study, the mechanism of TU-mediated stress tolerance in the Indian mustard (*Brassica juncea*) was analysed through identification of differentially modulated transcripts using microarray followed by real-time PCR and biochemical assays of selected representative pathways. The present approach to use a high degree of salinity stress was based on previous studies, which demonstrated that TU supplementation

TABLE 3. *List of the genes up-regulated in NaCl but differentially regulated in NaCl + TU-treated seeds*

Serial number	ID	Annotation	Fold D vs. N	Fold D vs. NT
1	*At3g54050*	Fructose-1,6-bisphosphatase, putative	1·97	−0·09
2	*At1g04560*	AWPM-19-like membrane family protein	1·89	0·14
3	*At5g63290*	Coproporphyrinogen oxidase-related	1·8	−0·08
4	*At2g36420*	Unknown protein	1·78	0·12
5	*At2g15580*	Zinc finger (C3HC4-type RING finger) family protein	1·74	−0·09
6	*At2g29090*	CYP707A2 (cytochrome P450, family 707, subfamily A, polypeptide 2)	1·70	0·23
7	*At5g66940*	Dof-type zinc finger domain-containing protein	1·65	−0·27
8	*At4g19170*	NCED4 (9-*cis*-epoxycarotenoid dioxygenase)	1·64	−0·15
9	*At4g36040*	DNAJ heat-shock N-terminal domain-containing protein (J11)	1·58	0·26
10	*At1g20630*	CAT1 (catalase 1); catalase	1·35	0·37
11	*At1g60690*	Aldo/keto reductase family protein	1·3	−2·01
12	*At2g37770*	Aldo/keto reductase family protein	1·3	−2·01
13	*At5g64290*	DCT/DIT2·1 (dicarboxylate transport)	0·74	0·19
14	*At4g33550*	Lipid transfer protein (LTP)	0·61	−0·09

The microarray analysis was conducted to compare the gene expression profile of NaCl + TU- and NaCl-treated seeds (N + T and N, respectively) with that of the control seeds (D). The values represent the average ratios of normalized signals in $\log_2$ scale calculated from three independent replicate experiments using GENESPRING software. Differences in the mean values were found to be statistically significant at $P < 0{\cdot}05$ (one-way ANOVA).

TABLE 4. *List of the genes downregulated in NaCl but differentially regulated in NaCl + TU-treated seeds*

Serial number	ID	Annotation	Fold D vs. N	Fold D vs. NT
1	*At5g61600*	Ethylene-responsive element-binding family protein	−2·46	0·24
2	*At4g36220*	FAH1; ferulate 5-hydroxylase	−2·0	0·98
3	*At2g41410*	Calmodulin, putative	−1·85	1·2
4	*At2g37040*	PAL1; phenylalanine ammonia-lyase	−1·78	1·13
5	*At5g53590*	Auxin-responsive family protein	−1·6	1·49
6	*At2g16060*	AHB1 (arabidopsis haemoglobin 1)	−1·2	1·1
7	*At5g44120*	CRA1 (CRUCIFERINA); nutrient reservoir	−1·04	0·6
8	*At4g02380*	Senescence-associated gene 21 (SAG21)	−1·01	1·2
9	*At5g62480*	ATGSTU9; glutathione transferase	−0·9	2·30
10	*At1g20620*	CAT3 (catalase 3); catalase	−0·8	1·35
11	*At2g06050*	OPR3 (OPDA-reductase 3)	−0·8	1·3
12	*At4g05010*	F-box family protein	−0·8	1·02
13	*AT2G07698*	ATP synthase alpha subunit, mitochondrial	−1·159	0·52
14	*At3g13320*	CAX2 (cation exchanger 2)	−0·7	1·02
15	*At3g22910*	Calcium-transporting ATPase, plasma membrane-type, putative/Ca(2 +)-ATPase, putative (ACA13)	−0·7	1·4
16	*At3g29350*	AHP2 (histidine-containing phosphotransmitter 2)	−0·7	1·85
17	*At3g59970*	MTHFR1; methylenetetrahydrofolate reductase	−0·70	1·81
18	*At3g61440*	ARATH; (BETA-substituted Ala synthase 3;1)	−0·7	0·8
19	*At5g64310*	AGP1 (arabinogalactan-protein 1)	−0·67	1·63

The microarray analysis was conducted to compare the gene expression profile of NaCl + TU- and NaCl-treated seeds (N + T and N, respectively) with that of the control seeds (D). The values represent the average ratios of normalized signals in $\log_2$ scale calculated from three independent replicate experiments using GENESPRING software. Differences in the mean values were found to be statistically significant at $P < 0{\cdot}05$ (one-way ANOVA).

was able to alleviate the damage even at supra-optimal levels (1 M NaCl) so that the viability of seeds was maintained (Srivastava *et al.*, 2009). Hence, it was intriguing to investigate various signalling and effector processes regulated by TU within 1 h of treatment that allowed the seeds to maintain their viability and tolerate the stress. Preliminary experiments conducted to evaluate the differential phenotype of the seedlings under different treatments demonstrated that, firstly, the TU-alone treatment itself did not impose any stress and, secondly, its supplementation to NaCl improved seedlings vigour as compared with that of the NaCl treatment (Fig. S4 in Supplementary Data, available online).

The genome wide transcriptome analysis was performed for the short duration of 1 h in brassica seeds. As, the arabidopsis array is an established tool for gene expression analysis of brassica species due to their close phylogenetic relationship (Hudson *et al.*, 2007), the Agilent-based *Arabidopsis thaliana* microarray platform was used for the transcriptome analysis of brassica seeds. The result of the control versus TU showed that 53 genes were down-regulated (Table 1), while only eight genes were up-regulated (Table 2). However, the fact that the redox state (measured in terms of the GSH : GSSG ratio) was lower than the control, under similar conditions, could be attributed to the decreased synthesis of GSH due to the exogenous supply of a −SH-containing compound, i.e. TU. Therefore, TU-regulated transcripts could be categorized as potential redox-sensitive genes. The TU-mediated decline observed in the expression level of genes under the category

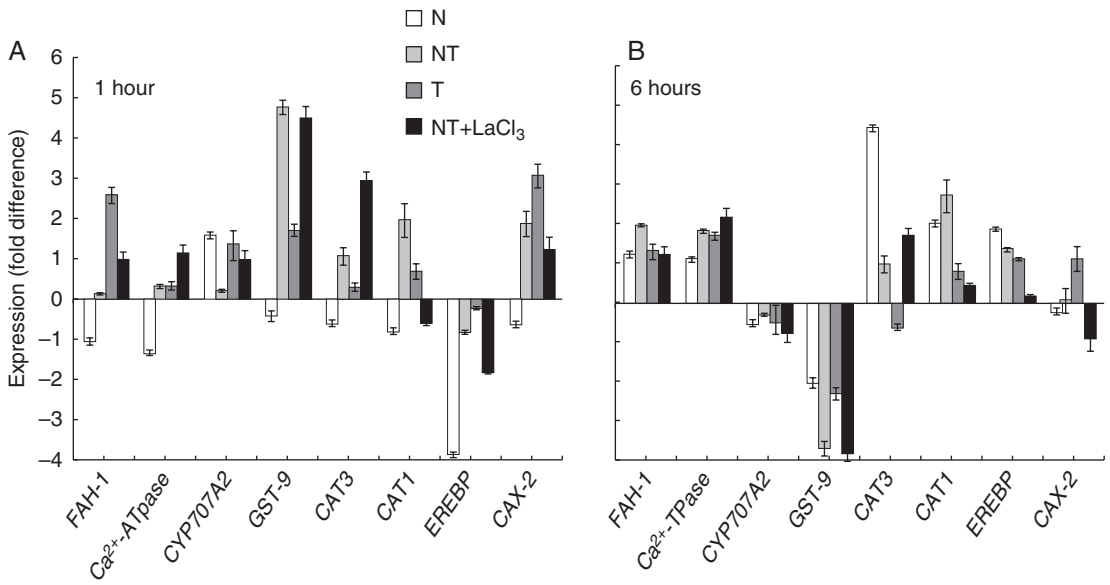

FIG. 3. Real-time PCR validation of the selected TU-modulated salinity-responsive genes. The expression level of the selected TU-responsive genes was measured by quantitative real-time PCR in seeds subjected to different treatments for 1 h (A) and 6 h (B), respectively. N, NT, T and NT + $LaCl_3$ represent the different treatments: NaCl (1 M), NaCl (1 M) + TU (6·5 mM), TU (6·5 mM), and NaCl (1 M) + TU (6·5 mM) + $LaCl_3$ (5 mM), respectively. All real-time PCR results were normalized to that of the actin in $\log_2$ scale ratio as compared with that of the distilled-water control. The data represent the average of three biological and three technical replicates (± s.d.). The differences in the mean were found to be statistically significant at $P < 0{\cdot}05$, in the one-way ANOVA test.

of protein synthesis/degradation might cause a reduction in the overall protein turnover. This may suggest that the TU-controlled redox state presumably helped to stabilize different cytosolic proteins, hence reducing the need for overall protein turnover. The TU-mediated down-regulation of the genes for different HSPs and universal stress proteins (USPs) further indicated that TU-treated seeds probably did not require their *de novo* synthesis. Shelton *et al.* (2005) reported that the mammalian Hsp70 can be activated by the GST-mediated glutathionylation. The higher expression of GST (Fig. 3A and Table 4) and increased GST activity (Fig. 6C) together support the proposition that TU treatment presumably prevented the degradation of HSPs and other proteins through their increased glutathionylation. A reduction in the expression level of various protective genes like ascorbate peroxidase, monodehydroascorbate reductase and CAT was also observed. This might be ascribed to a reduced rate of reactive oxygen species production due to their TU-mediated scavenging via its –SH moiety. Strikingly, a set of transcription factors, such as AP-2/EREBB, NAC, WRKY and WOX-2, the potential upstream signal transducers, i.e. kinases as well as a few genes related to calcium signalling, were also down-regulated in response to the TU-alone treatment, pointing towards the importance of signalling mechanisms under a changed redox state. However, a more detailed analysis is required to clarify how the different components interact with each other to regulate the downstream responses. Among the up-regulated genes, *OPT6* is known to transport glutathione derivatives and to function in stress-resistance responses (Cagnac *et al.*, 2004). Oxygen-evolving enhancer protein has been demonstrated to possess thioredoxin activity (Heide *et al.*, 2004). These results suggest that redox-mediated stress tolerance was presumably improved under the TU-alone treatment. An increase in the expression level of ATPase and ubiquinol-cytochrome *c* reductase suggests an improvement in energy metabolism, probably to induce changes in the redox state and to achieve regulation of the signalling components. Thus, the TU-alone treatment led to a decline in various HSPs, USPs and other stress-responsive genes through complex signalling due to an improved redox state; however, phenotypically, plants were comparable with that of the control (data not shown). In this regard, the emphasis was not on the TU-alone responses, but to an investigation into how NaCl stress was ameliorated upon TU supplementation and to find out the components involved.

For this purpose, the transcript profile of NaCl versus NaCl + TU-treated seeds was analysed. The results revealed that the expression of all 33 genes were differentially regulated between the NaCl and NaCl + TU treatments (Fig. 1, Clusters 1 and 2). These genes were together denoted as 'TU-modulated salinity stress-responsive genes'. Further, real-time PCR analysis of the selected genes at two time points demonstrated their dynamic regulation. An induction in the expression level of most of the genes was achieved as early as 1 h in the NaCl + TU treatment. In contrast, upon the NaCl treatment, an almost similar level of induction in the expression of various genes was observed only at 6 h. Similarly, the data for the GSH : GSSG ratio also depicted the differential change in redox state under NaCl and NaCl + TU treatment at 1 h and 6 h. Thus, these results suggest that in NaCl + TU-treated seeds, there was an early sensing of the stress and the induction of different mechanisms in a co-ordinated manner, as compared with that in NaCl-treated seeds. This early responsiveness in the presence

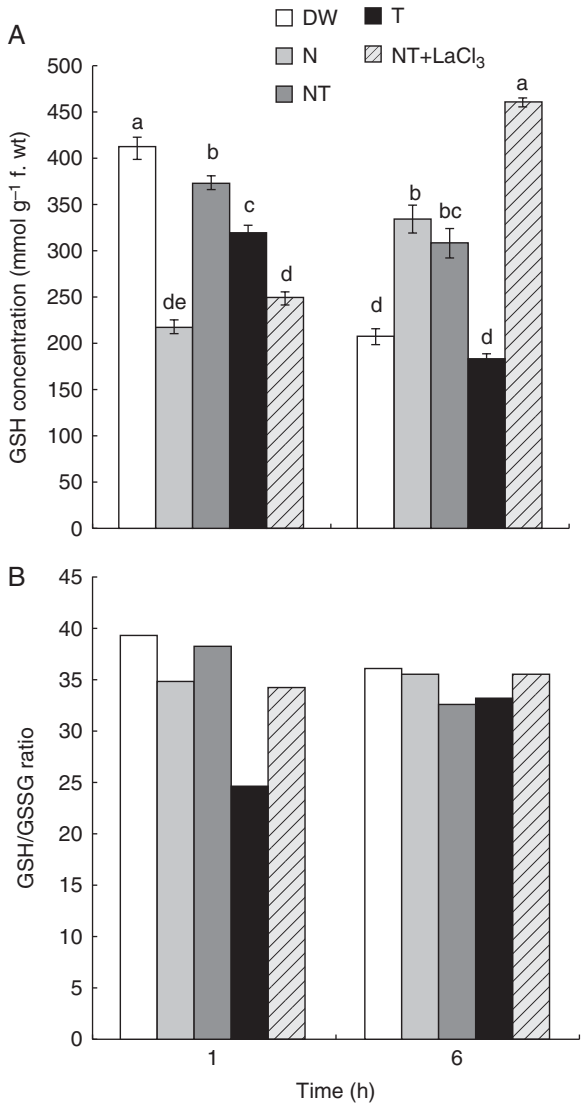

FIG. 4. Measurement of GSH and the GSH : GSSG ratio. The level of GSH (A) and the GSH : GSSG ratio (B) were measured in seeds subjected to different treatments for 1 h and 6 h. DW, N, NT, T and NT + $LaCl_3$ represent the different treatments: distilled water control, NaCl (1 M), NaCl (1 M) + TU (6·5 mM), TU (6·5 mM) and NaCl (1 M) + TU (6·5 mM) + $LaCl_3$ (5 mM), respectively. All the values represent the mean ± s.d. of six technical and three biological replicates. The differences in the mean were found to be statistically significant at $P < 0·05$, in the one-way ANOVA test. Different letters indicate significantly different values at a particular duration (DMRT, $P < 0·05$).

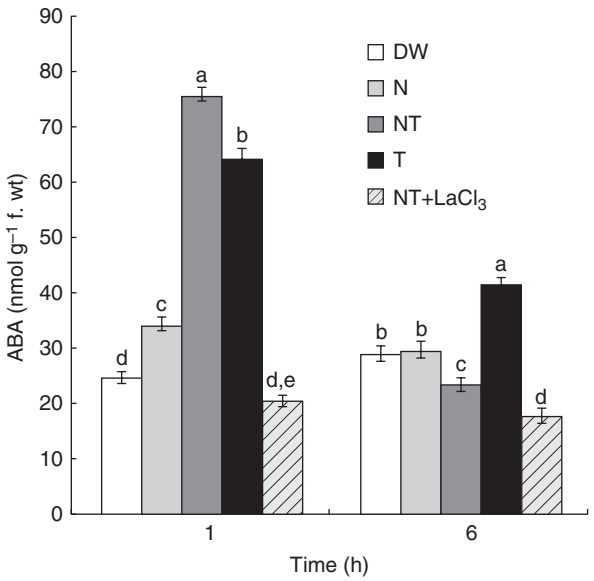

FIG. 5. Measurement of the ABA content. The endogenous level of ABA was measured in seeds subjected to different treatments for 1 h and 6 h. DW, N, NT, T and NT + $LaCl_3$ represent the different treatments: distilled water control, NaCl (1 M), NaCl (1 M) + TU (6·5 mM), TU (6·5 mM) and NaCl (1 M) + TU (6·5 mM) + $LaCl_3$ (5 mM), respectively. All the values represent the mean ± s.d. of six technical and three biological replicates. The differences in the mean were found to be statistically significant at $P < 0·05$, in the one-way ANOVA test. Different letters indicate significantly different values at a particular duration (DMRT, $P < 0·05$).

of TU might be responsible for maintaining the ability of seeds to germinate even under a high degree of salinity stress (Srivastava *et al.*, 2009).

In plants, as the salinity stress signal is perceived, the level of ABA gets enhanced triggering an array of responses including an increase in cytosolic calcium (Jakab *et al.*, 2005). In the present study, the expression of two ABA metabolism genes, such as 9-*cis*-epoxycarotenoid dioxygenase-4 (NCED4), a regulatory enzyme of ABA biosynthesis (Qin and Zeevaart, 1999), and cytochrome P450 sub-family gene (CYP707 A2), a key enzyme of ABA catabolism (Saito *et al.*, 2004), was modulated in response to TU treatment. This suggests the active involvement of ABA in TU-mediated responses. To confirm this, time-dependent measurement of the endogenous ABA level was performed in seeds under different treatments. A significant increase in the level of ABA, as early as 1 h after imbibition, in the NaCl + TU as well as the TU treatment, was observed, which strengthens the proposition that TU-mediated effects are ABA-dependent. With time, the level of ABA was found to decrease in all the treatments which presumably occurred to facilitate the process of seed germination (Penfield and King, 2009). In response to an increase in the level of ABA, other signalling mechanisms are initiated, such as the changes in the calcium signature that help plants adapt under salinity stress. Calcium signature is a condition-specific phenomenon that is governed by the co-ordinated function of different genes, namely, calmodulin, calcium-transporting ATPase and calcium exchanger (Knight *et al.*, 1997). Interestingly, the level of such genes was found to be modulated in seeds treated with NaCl + TU (Table 4) or TU (Table 1), which suggests that TU-mediated signalling changes in the process of stress amelioration probably also involve modulations in the calcium signature pattern.

There is evidence for the role of ABA in triggering the production of H_2O_2 (Xing *et al.*, 2008). In addition, salinity stress itself induces oxidative stress (Borsani *et al.*, 2001). In this context, the role of CATs becomes indispensible for efficiently scavenging the H_2O_2 (Willekens *et al.*, 1997). They are encoded by a small multigene family consisting of CAT1, CAT2 and CAT3 (Frugoli *et al.*, 1996). CAT1 is known to be regulated in an ABA-dependent manner and is mainly involved in regulating the H_2O_2-dependent signalling; while CAT3 is regulated by an unknown mechanism and is mainly

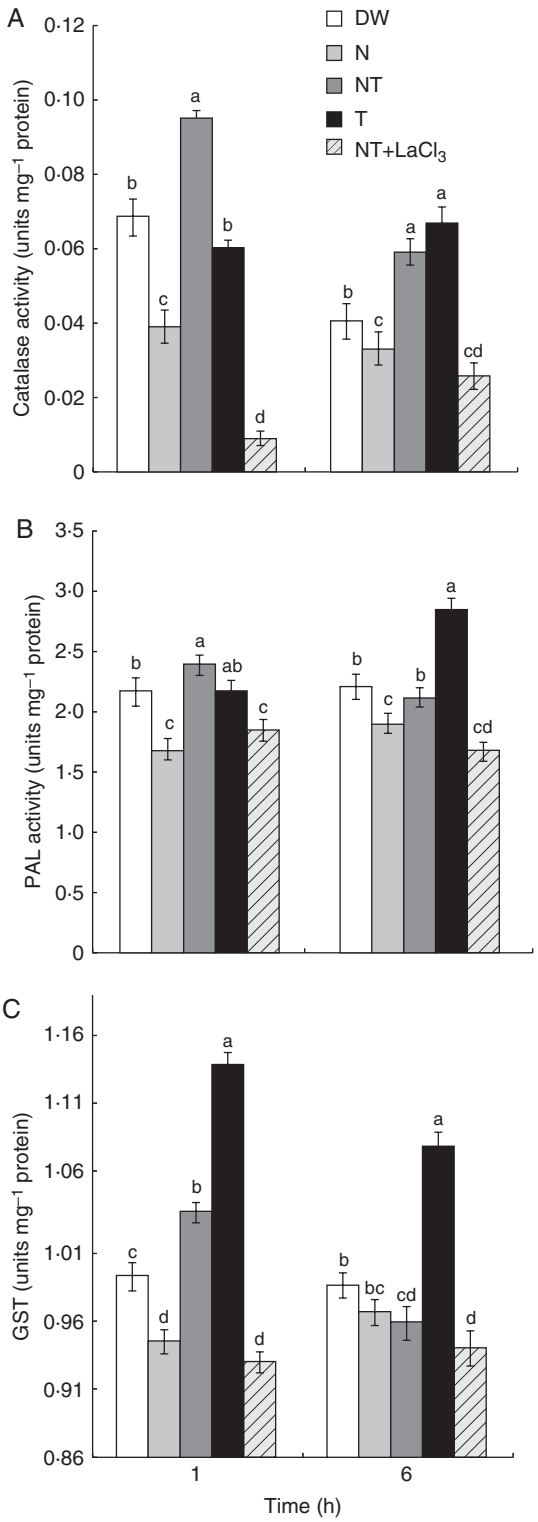

FIG. 6. Measurement of enzyme activities. The activities of CAT (A), PAL (B) and GST (C) were measured in seeds subjected to different treatments for 1 h and 6 h. DW, N, NT, T and NT + $LaCl_3$ represent the different treatments: distilled water control, NaCl (1 M), NaCl (1 M) + TU (6·5 mM), TU (6·5 mM) and NaCl (1 M) + TU (6·5 mM) + $LaCl_3$ (5 mM), respectively. All the values represent the mean ± s.d. of six technical and three biological replicates. The differences in the mean were found to be statistically significant at $P < 0.05$, in the one-way ANOVA test. Different letters indicate significantly different values at a particular duration (DMRT, $P < 0.05$).

associated with scavenging excess H_2O_2. As compared with NaCl, early induction observed in the transcript level of CAT1 and CAT3 (Table 4) as well as in the activity of CAT (Fig. 6A) suggest a change in the early involvement of CAT-mediated signalling and antioxidant defence in NaCl + TU-treated seeds. Besides, TU treatment also modulated the expression of genes related to hormonal signalling. In NaCl + TU-treated seeds, the expression level of an auxin responsive gene (*Aux/IAA*), an AHP family gene (*AHP2*; a mediater of cytokinin signalling), *OPR-3* (12-oxo-phytodienoic acid reductase; a mediater of jasmonate biosynthesis and signalling) as well as *EREB* (ethylene-responsive element-binding gene) were up-regulated, whereas in NaCl-treated seeds, all these genes were down-regulated. These results imply that TU may probably co-ordinately regulate auxin-, cytokinin-, ethylene- and jasmonate-mediated signalling to impart stress tolerance as well as to allow the plants to maintain their normal growth.

In the course of stress management, the phenylpropanoid signalling pathway also plays an important role in the complex but integrated signalling network. PAL catalyses the first step of the phenylpropanoid pathway. The NaCl + TU-treated seeds showed an increased expression of one of the PAL genes, *PAL1* (Anterola and Lewis, 2002) and a homologue of PAL, *FAH1* (ferulate 5-hydroxylase; Costa *et al.*, 2003) as well as a higher PAL activity (both at 1 h and 6 h) than that observed under the NaCl treatment. Both molecular and biochemical data, thus, indicate that TU treatment might lead to an accumulation of major flavonoids, which would assist in reducing the extent of damage in NaCl + TU-treated seeds. The salinity stress also leads to the generation of various metabolic by-products that need to be detoxified in order to maintain the cellular homeostasis. This is achieved via their conjugation with glutathione by the GSTs (Wagnor *et al.*, 2002). In this direction, increased expression and activity of GSTs, during the early time of 1 h, indicated the stimulation of effective detoxification of any stress-induced by-products, such as lipid peroxides from membrane damage, in the NaCl + TU treatment as compared with the NaCl treatment.

An interesting feature associated with most of the TU-modulated transcripts was that they were either directly related to calcium signalling or were the potential targets of the calmodulin/calceneurin B-like proteins (Luan *et al.*, 2002). This suggests that the action of TU might be dependent upon the process of calcium signalling. To confirm this, various molecular and biochemical responses were studied in response to addition of $LaCl_3$ (a calcium-channel blocker) to NaCl + TU. The Ca^{2+}-permeable channels are required to generate the $[Ca^{2+}]_{cyt}$ burst which is necessary for the activation of different cellular responses (Kaplan *et al.*, 2006). When the NaCl + TU treatment was supplemented with $LaCl_3$, the expression profile of a few genes such as *CAT-1*, *EREBP* and *CAX-2* was reversed as compared with their expression in the absence of $LaCl_3$. Besides, the TU-mediated increase in the level of ABA and in the activities of various enzymes, such as CAT, PAL and GST, were also not observed in presence of $LaCl_3$. However, the redox state remained more or less unaltered. Thus, these findings together indicate that the effect of TU might be dependent upon the cytosolic Ca^{+2} burst *per se*.

Taken together, the present results imply that upon stress imposition, TU modulated various signalling pathways such as redox, calcium and ABA, leading to activation of a yet unidentified 'common alarm signal'. This in turn co-ordinately regulated different processes to increase the capacity to combat oxidative stress and to detoxify stress-induced accumulation of any by-products which together help the seeds to maintain their viability even under the high degree of salinity stress. Thus, the application of TU, as a bioregulatory molecule, can augment new approaches/strategies for basic as well as applied research dealing with crop stress management and other productivity constraints at the field level.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Fig. S1: Differential germination response of *Brassica juncea* seeds under different treatments. Fig. S2: Quality control for RNA. Fig. S3: Scatter plots of dye swap labelling. Fig. S4: Differential growth responses in *Brassica juncea* seedlings under different treatments. Table S1: Details of the primers used for the quantitative real-time PCR.

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