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Expression analysis of candidate genes for abiotic stress tolerance in *Brassica* genotypes with contrasting osmotic stress tolerance

D Phukan[1]*, V Goyal[1], P Palit[1], R Kalia[1], M Koundal[1], SVA Mithra[1], I Ravi[2], DK Yadava[3], V Chinnusamy[4] & T Mohapatra[1,5]*[#]

[1]National Research Centre on Plant Biotechnology (NRCPB), New Delhi-110 012, Delhi, India.

[2]IGNOU Regional Centre, Jaipur, Rajasthan-302 020, India

[3]Division of Genetics; [4]Division of Physiology, [5]ICAR-Indian Agricultural Research Institute, New Delhi-110 012, Delhi, India.

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Drought stresses adversely affect production of mustard as it is cultivated mostly as a rain-fed crop. Drought at critical stages of crop growth is one of the reasons for low productivity of mustard in India. We screened 38 genotypes of Indian mustard [*Brassica juncea* (L.) Czern] and *Sinapis alba* L. (white mustard), and identified genotypes with contrasting drought tolerance. To understand the molecular basis of drought tolerance in mustard, we analyzed expression pattern of orthologs of 24 validated *Arabidopsis* genes in mustard genotypes with contrasting drought tolerance. The candidate genes exhibited distinct expression pattern between stress tolerant and sensitive genotypes. All 24 genes analyzed in this study showed upregulation under drought stress at least in one of the genotypes examined. The present study highlighted the differential expression pattern of several drought responsive candidate genes in five different cultivars of *Brassica juncea* (L.) Czern and its wild relative *S. alba*. Drought stress specifically upregulated the expression of *ERECTA*, *TMM*, *ABA1*, *AREB1*, *CYP707A*, *ROP10*, *SOS4*, *RabG3e*, *STT3a* and *SHN1* genes >2-fold in one or more of the drought tolerant genotypes, but not in any of the susceptible genotypes. Only *AREB1*and *INO1* showed upregulation in all the genotypes under drought stress. Thus, this study revealed considerable association between drought stress induction of candidate genes and abiotic stress tolerance of *B. juncea* and *S. alba* genotypes. These genes will be useful for further understanding of drought tolerance and genetic improvement of oil seed *Brassica* crops.

Keywords: Drought, Gene expression, Indian Mustard, Oilseed, qRT-PCR, *Sinapis alba*

Indian mustard [*Brassica juncea* (L.) Czern] is not only a major edible oilseed crop but also medicinal, and is cultivated on more than 6 million hectares in the Indian subcontinent. It ranks 5th among the economically important crops followed by rice, wheat, maize and cotton[1-3]. Mustard crop is susceptible to many biotic and abiotic stresses which cause moderate to high level of losses depending upon the intensity of the stress and crop growth stage. Hence, it is necessary to improve abiotic and biotic stress tolerance of the crop for enhancing mustard productivity. Developing new cultivars/inbred lines for hybrid breeding in Brassica to improve productivity, Watts *et al*.[4] have recently recommended *AtCENH3* promoter for developing haploid inducer line in *B. juncea*. On the other hand, Lakra *et al*.[5] who studied the impact of salinity and heavy metal stress on Indian mustard reported stress mitigation and >10% improved growth by media supplement putrescine. Increase in temperature by 1°C may reduce rapeseed-mustard yield by 3-7% while the mean temperature in Ganga basin and other mustard growing regions is estimated to rise by 1.0-1.5°C by 2020 to 2030; and 2.0-3.5°C by 2050[6]. In India, due to unfavourable agro climatic conditions, Rape-Mustard (RM) seed witnessed a decline in sowing in major growing areas, and the total production of RM for 2014-15 is estimated to be 55 lakh metric tonnes, down by 19% compared to the previous year[7]. Declining crop production due to challenging stress factors, both biotic and abiotic, has accelerated the research for possible technological solution.

The wild relatives of cultivated crops are often rich in stress-responsive genes, and therefore,

*Correspondence:
Phone: +91 11 25842367
E-mail: tmnrcpb@gmail.com (TM); dharitree19@gmail.com (DP)
[#]Present add.: Indian Council of Agricultural Research (ICAR), New Delhi

investigating these genes may reveal the molecular stress-tolerance mechanisms of cultivated crops. *Sinapis alba* L., a wild relative of mustard, has many desirable agronomic traits including tolerance to drought and high temperatures[8], high seed yield[9] and resistance to shattering.

Drought is one of the major environmental factors influencing plant growth, productivity of crops and worldwide agricultural loss. Development of stress tolerant crops will be greatly advantageous for modern agriculture in many parts of the world. Recently, reasonable advances have been made towards identifying potential genes involved in stress tolerance. In paddy, expression of stress responsive bZIP transcription factors has been shown to be directly correlated to developmental stage and water deficit stress[10]. Mawlong *et al*.[11] who analyzed the biochemical and molecular basis of drought mechanism in rice indicated possible role of stress responsive genes belonging to AP2/ERF family that manipulates cytokinin, a hormone which negatively regulates root growth. Understanding the molecular basis of stress tolerance and identification of genes that confer stress tolerance to *Brassica* species will help enhance stress tolerance of Brassicas including mustard.

To protect cellular, molecular activities and maintain whole plant integrity, various mechanisms were developed by plants to cope up with abiotic stresses. Many stress-induced genes have been identified in model plant *Arabidopsis*, including those encoding key enzymes for abscisic acid (ABA) biosynthesis and signaling, proteins involved in osmotic adaptation and tolerance to cellular dehydration, cellular protective enzymes, numerous signaling proteins such as protein kinases/protein phosphatases, and transcription factors[12]. We selected a set of genes validated for their role in stress tolerance in *Arabidopsis*, and identified their orthologs in *Brassica*. Further, we analyzed the expression pattern of the selected genes in *B. juncea* and its wild relative *S. alba* to assess their role in drought tolerance.

Materials and Methods

Plant materials

Seeds of oilseed *Brassica* genotypes used in this study were obtained from the Division of Genetics, Indian Agricultural Research Institute, New Delhi.

Phenotyping of *S. alba* and *B. juncea* accessions for drought tolerance

In vitro screening

Screening of 38 genotypes of *Brassica* and allied genera was done using *in vitro* methods *viz*. agar solidified high osmotic medium and high osmotic liquid media (filter paper bridges). Seeds were germinated on moist filter paper for 48 h and were transferred to Whatman No. 1 filter paper soaked with one of the following solutions and allowed to grow in culture room with 8/16 h light/dark cycle at 25+1ºC: control, PEG (−0.5 and −0.7 MPa), mannitol (−0.5 and −0.7 MPa), NaCl (−0.5 MPa = 200 mM; −0.7 MPa = 320 mM). Morphological observations were recorded after four days of imposition of stress. Data was recorded on percent seedling survival/mortality periodically. At the end of the stress period, root/shoot ratio, root length, and root volume were measured from the scanned root images using WinRhizo software. Genotypes were ranked based on % change in root growth under various stress treatments [PEG (−0.5 MPa), PEG (−0.7 MPa), Mannitol (−0.5 MPa), Mannitol (−0.7 MPa), NaCl (−0.5 MPa = 200 mM) and NaCl (−0.7 MPa = 320 mM)] over control. Genotype which showed highest increase or stability in stress over control was given the first rank, and as the root length over control decreased, the rank increased. These ranks for six different stresses were averaged to finally rank the genotypes. Rank was assigned to each variety and mean of the ranks of a variety from all experiments was considered as the selection criteria to select lines with contrasting osmotic/drought stress tolerance for gene expression analysis.

Pot experiment

The 38 genotypes of *Brassica* and allied genera screened in the above experiment were also phenotyped for drought tolerance in pot culture under natural environmental conditions during winter season. Sandyloam soil from IARI field where mustard crop is cultivated every year was used to fill the pots. For one set of plants, drought stress was imposed by withholding irrigation at vegetative stage. Control plants were watered as and when required. Data on relative leaf water content (RWC) and soil moisture were recorded after 20 days of imposition of drought stress. RWC was calculated from the following equation: RWC% = [(Fresh weight of leaf tissue-Dry weight of leaf tissue)/(Turgid weigh of leaf tissue − Dry weight of leaf tissue)] × 100. SMC (%)

was calculated by using the following equation = [(Soil fresh weight – Soil dry weight)/Soil dry weight] × 100. Roots were extracted from soil and root length; diameter and surface area were recorded using WinRhizo software. *Brassica* genotypes were ranked based on Root Growth Index (RGI) = (Rc−Rs)/Rc, Shoot Growth Index (SGI)= (Sc−Ss)/Sc, and Root-Shoot length ratio =Rs/Rc (where, Rc and Rs=Root length under control and stress conditions respectively; and Sc and Ss= Shoot length under control and stress conditions, respectively).

Net house experiment

The same sets of 38 genotypes were also grown in one-meter long tubes filled with soil. Stress was imposed by withholding irrigation after 35 days of sowing i.e. vegetative stage. Data for relative water content and soil moisture was recorded at 22 days after imposition of stress.

Expression analysis of candidate genes under drought stress

Plant material and treatment

S. alba and five genotypes of *B. juncea* namely RGN73, Varuna, RLM619, BEC144 and BIOYSR were selected from the phenotyping experiments. Seeds were germinated in soil and grown in the culture room. Drought stress was imposed by withholding irrigation to the plants for 35 days till the soil moisture content reached 4-5%. The leaves of control and drought stress treated plants were collected, frozen in liquid nitrogen and stored in −80ºC for later use in expression analysis.

Quantitative real time PCR (qRT-PCR) analysis

Total RNA was isolated from controlled and stressed plants by using GeneJET^{TM} Plant RNA Purification mini kit (Fermentas, EU) and cDNA was synthesized using SuperScript^{TM}III First-Strand Synthesis System (Invitrogen, USA) following the manufacturer's instructions. The resulted cDNA samples were diluted 5 times (1:5) in RNase-free water and 1 µL of diluted cDNA were used for qRT-PCR analysis. Expression levels of drought responsive candidate genes (Supplementary Table 1)[13-45] were analyzed by qRT-PCR using gene-specific primers (Supplementary Table 2). Using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the closest homologs of candidate genes were identified from *Brassica* spp. The GenBank accession number is given in Supplementary Table 2. The mRNA sequences were downloaded from NCBI. The primers were designed by using Oligo Analyzer 3.1 tool (http://eu.idtdna.com/calc/analyzer). The expression of each gene in various samples was normalized with actin (GenBank: XM_009132876.1) gene as an internal reference to quantify the expression level of the candidate genes. 1 µL of the (1:5) diluted cDNA was used as template in total reaction volume of 25 µL containing Power SYBR Green PCR Master Mix (Applied Biosystem, Life technology). Quantitative RT-PCR analysis was performed using Stratagene MX3005P Q-PCR system. The thermal cycling conditions of 1st segment of 50°C for 5 min, 2nd segment of 95°C for 10 min followed by 3rd segment of 40 cycles of 95°C for 30 s, 55°C for 40 s, 72°C for 30 s and 4th segment of 95°C for 1 min, 55°C for 30 s, 95°C for 30 s were used. The Q-PCR reactions were performed with triplicates. The specificity of the PCR reactions was confirmed by melting curve analysis of the amplicons. The comparative $2^{-\Delta\Delta CT}$ method was used to calculate the normalized fold changes of each transcript in the samples[46].

Results and Discussion

Characterization of *S. alba* and *B. juncea* accessions for drought tolerance

Screening of 38 genotypes of *Brassica* and allied genera for osmotic and drought tolerance was carried out using *in vitro* methods as described earlier. Differential behavior of the genotypes used in the study was clearly evident from the overall phenotypic expression (both shoot and root growth) under stress as compared to the control (Supplementary Fig. 1). The RGI (Root Growth Index) and SGI (Shoot Growth Index) values indicated that RGN 73, *S. alba* and Varuna were tolerant, while RLM619, BEC144 and BioYSR were highly susceptible (Fig. 1). The Root-Shoot ratio also indicated that RGN 73, *S. alba* and Varuna as the most tolerant and RLM619, BEC144 and BioYSR as most susceptible genotypes (Fig. 2A). The mean rank assigned to the genotypes supported the above results (Fig. 2B). Zhang *et al.*[47] suggested root/shoot ratio as reliable parameter for evaluating drought tolerance. We explored the correlation between relationship between rank assigned by our method (RGI & SGI) and root/shoot ratio under osmotic stress and found that the correlation coefficient is significant ($y = -2.408x + 37.23$; $R^2 = 0.37$, $P \leq 0.01$). However, Zhang *et al.*[47] did not find significant correlation between root/shoot ratio and drought stress index (DSI). This suggests

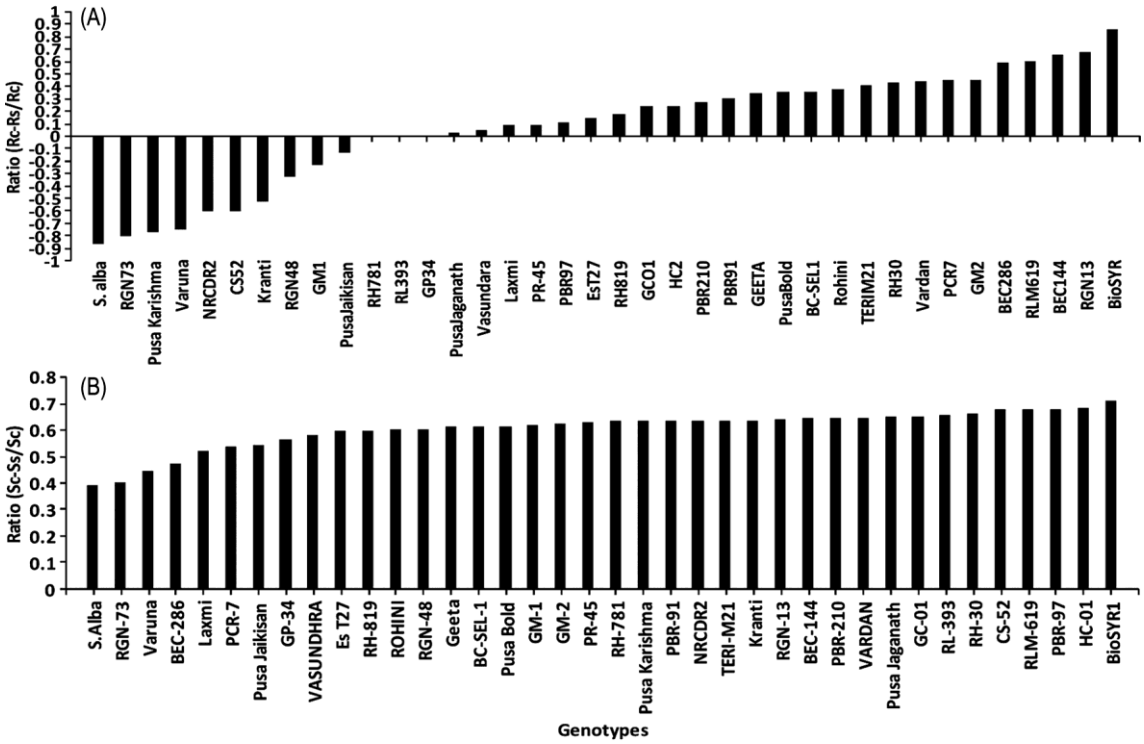

Fig. 1—Ranking of genotypes for osmotic stress tolerance based on (A) Root Growth Index (RGI) = (Rc-Rs)/Rc; and (B) Shoot Growth Index (SGI)= (Sc-Ss)/Sc.

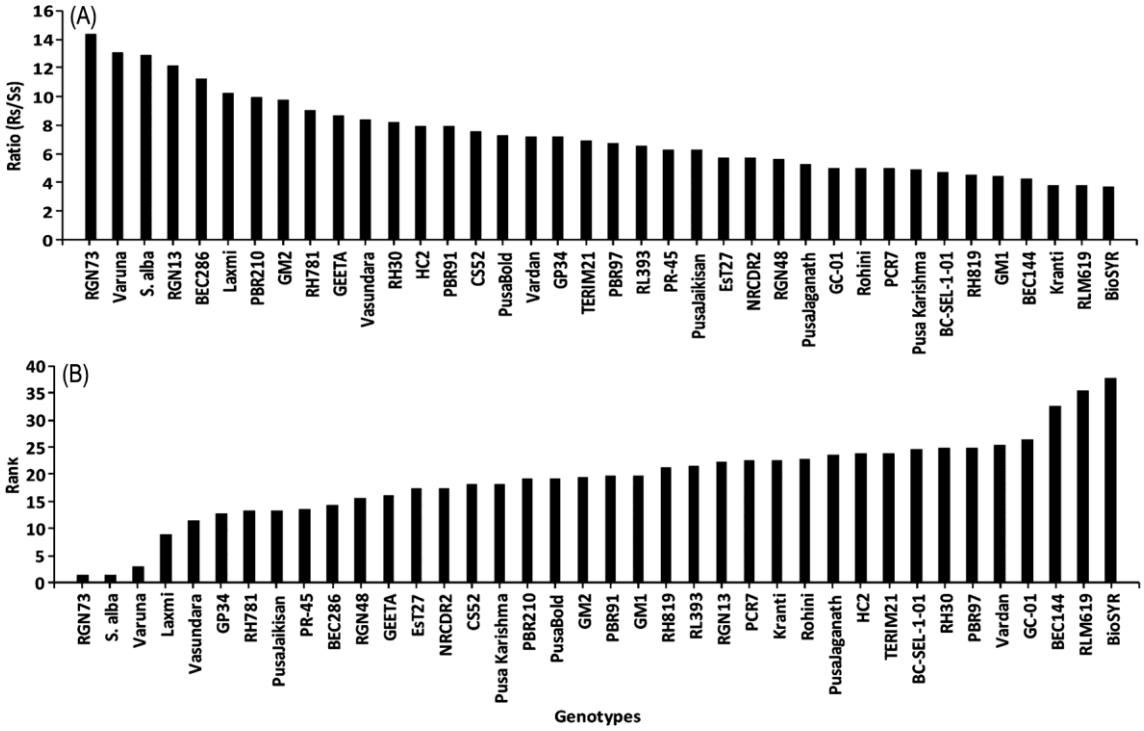

Fig. 2—(A) Root-Shoot length ratio; and (B) Ranking of genotypes for osmotic stress tolerance. [Ranks were assigned to the genotypes based on difference in root length under different stress/root length in control, difference in shoot length under stress/shoot length in control, and mean of root-shoot length ratio under stress]

that the RGI and SGI proposed in this study is better that simple drought stress index (DSI) proposed by Zhang *et al*. [47]. We found root length under osmotic stress has high significance and positive correlation with root/shoot ratio under stress ($y = 0.991x + 0.779$; $R^2 = 0.72$, $P \leq 0.01$). Similarly, root length per se under stress also showed significant correlation with rank ($y = -2.751x + 37.76$; $R^2 = 0.35$, $P \leq 0.01$). The genotypes showing higher root to shoot length ratio among all the genotypes under severe osmotic stress condition is assigned as tolerant genotype exhibiting longer root length under water limitation, an adaptive reaction for water mining and thus, root shoot length ratio has been identified as a reliable parameter of dehydration stress tolerance[48].

The same set of 38 genotypes of *Brassica* and allied genera was also screened for drought tolerance in pot culture during winter season. Drought stress was imposed by withholding irrigation at vegetative stage. With decrease in soil moisture, there was decrease in relative water content (RWC) in all the genotypes. The reduction in RWC was more drastic in susceptible cv. RLM-619 as compared to the tolerant genotypes RGN 73 and *S. alba*. It was observed that when stress was imposed at vegetative stage, highest RWC was seen in RGN 73 under drought stress. Even at very low soil moisture content (<6%), tolerant genotype RGN 73 and *S. alba* maintained high RWC, while the RWC was reduced to similar levels in susceptible genotypes when the soil moisture content was as high as >10%. Similarly, about 55% RWC was maintained by Varuna (tolerant) at very low soil moisture content, while susceptible RLM 619 reached the similar level of RWC at >12% soil moisture content (Fig. 3).

Phenotyping of 38 genotypes of *Brassica* and allied genera was further verified for drought tolerance during the winter season in one meter long PVC tubes filled with soil. *S. alba* maintained highest RWC among the genotypes. High degree of correspondence of the results with that from *in vitro* and pot culture experiments was observed (Fig. 4). High degree of tolerance of *S. alba* has already been reported[49]. The tolerance of popular *B. juncea* variety Varuna observed in this study is supported by earlier studies on Indian mustard[50].

Validation of expression of stress responsive genes under drought stress

We selected a set of genes known to confer abiotic stress tolerance in *Arabidopsis* (Supplementary Table 1), and analyzed their expression in *Brassica* and *S. alba* genotypes differing in their drought tolerance.

The *Arabidopsis ERECTA* gene, which encodes a leucine-rich repeat receptor-like kinase (LRR-RLK), regulates plant architecture, stomatal density, water use efficiency and heat tolerance in *Arabidopsis*[13]. Overexpression of *AtERECTA* in transgenic rice and tomato conferred yield stability under field heat stress conditions[14]. In our study, *ERECTA* showed 2.4 and 16-fold upregulation under drought stress in tolerant genotypes RGN73 and Varuna, respectively, while it was not upregulated in *S. alba* and in non-of the susceptible genotypes (Fig. 5). The peptide hormone STOMAGEN/EPFL9 works in coordination with *ERECTA* and other LRR receptor-like protein TOO MANY MOUTHS (TMM) and positively regulates stomatal development[15]. Hence, we also examined the expression of *TMM* gene. The expression levels of *TMM* was upregulated significantly under drought stress only in RGN73, while it was downregulated in other two tolerant genotypes *viz*., *S. alba* and Varuna (Fig. 5). An optimum combination of drought induced expression levels in *ERECTA* and *TMM* might have contributed to the tolerance. Both the genes did not show any significant change in expression under drought stress in the three susceptible genotypes.

The plant stress hormone Abscisic Acid (ABA) plays key role in plant development and response to

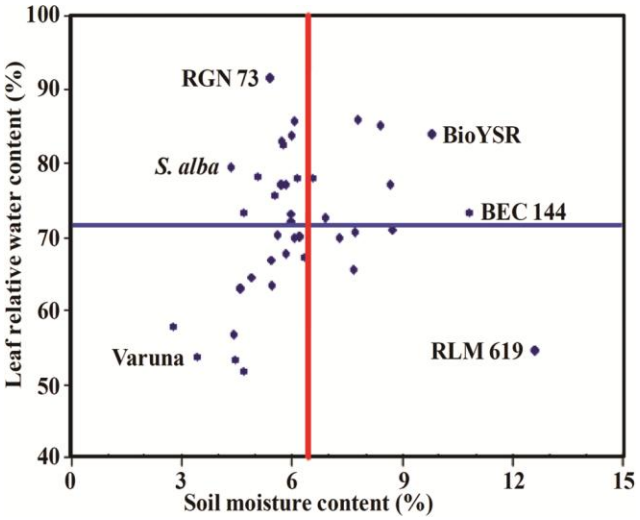

Fig. 3—Relationship between soil moisture content and RWC under drought stress in different *Brassica* genotypes at vegetative stage. The perpendicular line on X-axis shows mean soil moisture content of all genotypes, while the line perpendicular to Y-axis indicates mean RWC of all genotypes, and thus separates genotypes with high and low RWC at similar soil moisture content

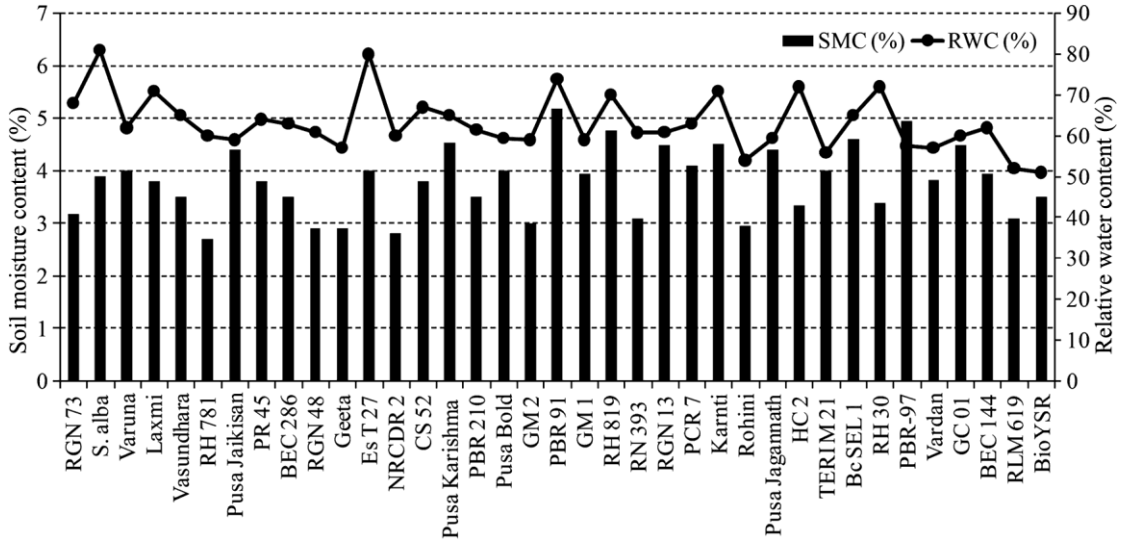

Fig. 4—Effect of drought stress at vegetative stage on relative water content of *Brassica* germplasm. The plants were grown in one meter length soil filled PVC pipes for 35 days with regular irrigation, and then drought stress was imposed by withholding irrigation till the soil moisture content decreased to about 4-5%

biotic and abiotic stresses. We selected *ABA DEFICIENT 1* (*ABA1*) gene coding for zeaxanthin epoxidase involved in ABA synthesis[16], *CYP707A3* (CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE 3) gene coding for ABA 8'-hydroxylase involved in ABA catabolism[17], Protein Phosphatase 2CA (PP2CA), a negative regulator of ABA signaling[18] and ABA activated transcription factor ABA response element (*ABRE*) binding factor1 (*AREB1*)[19] for expression analysis in *Brassica* genotypes. *ABA1* expression was significantly upregulated only in one of the tolerant genotype i.e, Varuna (Fig. 5). Interestingly, *CYP707A3* was upregulated under drought stress only in tolerant genotypes RGN73 and Varuna (Fig. 5). Drought stress did not alter the expression of genes for ABA metabolism in susceptible genotypes (Fig. 5). Expression of *AREB1* TF and *PP2CA* were highly upregulated under drought stress. The fold induction of *AREB1* was highest in tolerant genotypes RGN73 and Varuna, while the negative regulator *PP2CA* expression was highest in susceptible genotype RLM619 (Fig. 5). Further, we examined the expression of negative regulator of ABA signaling such as *PLD* (phospholipase D) and *ROP10* (RHO-RELATED PROTEIN FROM PLANTS 10) genes. The phospholipase D (*PLD*), *PLDα* catalyzes hydrolysis of phosphatidylcholine in to signal molecule phosphatidic acid (PA) in plants. Previous studies have shown that *PLDα* is induced by ABA and osmotic stresses, and the PA released by *PLDα* positively regulates abiotic stress tolerance through inhibition of *PP2C*[20-22], while *PLDδ* is mainly involved in biotic stress tolerance[23,24]. Among *PLDα* and *PLDδ*, *PLDα* expression was upregulated >2 fold in two tolerant cv. RGN73 and Varuna, and in the sensitive cv. BioYSR both *PLDα* and *PLDδ* genes were upregulated >2 fold under drought stress (Fig. 5). Expression of *ROP10*[25] under drought stress was upregulated in RGN73, Varuna and BEC144 (Fig. 5). These results suggest that dynamic regulation of cellular ABA levels by inductive expression of *ABA1* and *CYP707A3*, dynamic regulation of negative regulator (*PP2C* and *ROP10*) levels and activity, and higher expression levels of *AREB1* TF might have contributed to the better stress tolerance of RGN73 and Varuna.

In addition, we analyzed the expression of *INO1* (D myo-inositol-3-phosphate synthase), Salt Overly Sensitive 4 (*SOS4*, pyridoxal kinase) and *CAMBP25* (CALMODULIN-BINDING PROTEIN OF 25 KDA) genes involved in second messenger generation and signaling. *INO1/MIPS* (D-myo-inositol-1-phosphate synthesis) is involved in synthesis of myo-inositol and positively regulates abiotic stress tolerance[26,27]. *INO1* expression was upregulated under drought stress in all the genotypes, with highest expression levels in drought tolerant *S. alba* genotype (Fig. 5). The *SOS4* encoding pyridoxal kinase regulates auxin signaling, root growth and salt tolerance[28]. The *SOS4* was significantly upregulated only in tolerant genotypes RGN73 and Varuna (Fig. 5). The calcium signature

generated by abiotic stresses is sensed by calcium sensor proteins including calmodulin binding proteins. The *AtCAMBP25*, a drought induced calmodulin binding protein, negatively regulates osmotic stress tolerance in *Arabidopsis*[29]. Among the tolerant genotypes, only Varuna showed >2 fold upregulation, while sensitive cultivars BEC144 and BioYSR showed significant upregulation of *CAMBP25* under drought (Fig. 5). This negative regulator expression was highest in drought sensitive BEC144 (Fig. 5). In addition to the *INO1* involved in inositol hexakisphosphate synthesis, we also examined the expression of *MRP5* (MULTI DRUG RESISTANCE PROTEIN 5), a high-affinity inositol hexakisphosphate transporter, which plays a key role in guard cell signaling and phytate storage[30]. *MRP5*

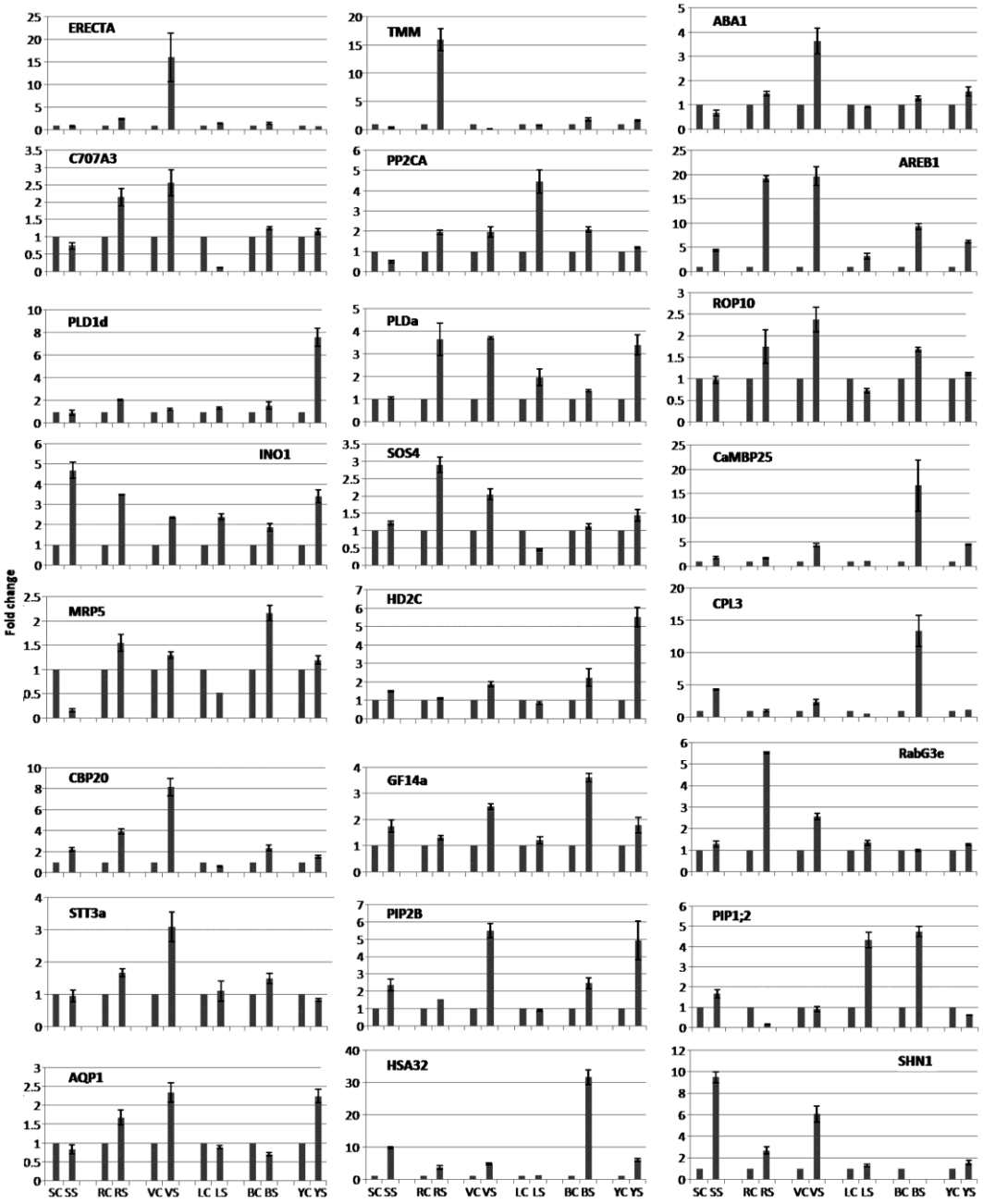

Fig. 5—Quantitative real-time RT-PCR expression analysis of candidate genes (24) in six genotypes of *Brassica* under drought stress conditions [SC, *S. alba* Control; SS, *S. alba* Stress; RC, RGN73 Control; RS, RGN73 Stress; VC, Varuna Control; VS, Varuna Stress; LC, RLM619 Control; LS, RLM619 Stress; BC, BEC144 Control; BS, BEC144 Stress; YC, BioYSR Control; YS, BioYSR Stress; ± Error bars indicate standard error of three replicates]

was upregulated under drought >2 fold only in drought sensitive cv BEC144 (Fig. 5).

We examined the expression of genes involved in transcription, RNA procession and transcription factor in the contrasting *Brassica* genotypes. *HD2C*s (*Histone Deacetylase 2C*) are involved in histone deacetylation mediated epigenetic regulation gene expression in response to abiotic stresses[31]. *Arabidopsis* mutants of DEAD-box RNA helicases, STRESS RESPONSE SUPPRESSOR (STRS1) and STRS2 exhibit abiotic stress tolerance. Proper localization of STRS in response to stress and ABA is impaired in *hd2c* mutant[32]. *HD2C* transcript levels were strongly induced in plants subjected to heat treatment, and the expression of selected heat-responsive genes was upregulated in heat-stressed hd2c mutant, suggesting that *HD2C* acts to downregulate heat-activated genes[33]. *HD2C* expression was significantly upregulated only in drought susceptible BioYSR (Fig. 5). *CPL3* (C-TERMINAL DOMAIN PHOSPHATASE-LIKE 3) dephosphorylates the C-terminal domain of RNA polymerase II (RNAP II). *AtCPL1* and *AtCPL3* negatively regulate stress responsive gene transcription[34]. The *CPL3* gene expression showed upregulation in two drought tolerant cv. *S. alba* and Varuna, and drought susceptible BEC144 (Fig. 5).

The *CBP20* (CAP-BINDING PROTEIN 20), a nuclear cap-binding protein, regulates mRNA splicing. Loss of function mutant of *cbp20* exhibited ABA hypersensitivity and enhanced drought tolerance[35]. Further, *CBP20* also participates in processing of pri-miRNA and thus biogenesis of miRNAs important for development and stress responses[36]. Since abiotic stress induces significant changes in transcription, accurate processing of transcripts is crucial for stress tolerance; we studied the expression of *CBP20* gene. All the three drought tolerant genotypes showed upregulation of *CBP20* gene under drought stress with highest increase of >8-fold in Varuna; while among drought sensitive genotypes, it was upregulated to 2-fold only in BioYSR (Fig. 5).

GF14 (G-box Factor 14-3-3 homologs) or GRF (G-box Regulatory Factor, or General Regulatory Factor) are phosphoserine/threonine binding proteins involved in metabolisms, cell cycle regulation, gene transcription and stress responses[37]. *GF14a* was induced by drought stress in all the genotypes examined (Fig. 5).

We examined two genes involved in protein modification and trafficking as these processes play significant role in abiotic stress tolerance. *AtRabG3e* (ARABIDOPSIS RAB GTPASE HOMOLOG G3E) is a small GTPase involved in membrane trafficking. Transgenic plants overexpressing *RabG3e* gene showed enhanced tolerance to high salt and hyperosmotic stresses[38]. In this study, *RabG3e* expression was upregulated by drought stress only in drought tolerant cultivars RGN73 and Varuna, while it was not induced in drought susceptible genotypes (Fig. 5). Protein N-glycosylation occurs in the endoplasmic reticulum (ER) before it is secreted. It is catalyzed by the oligosaccharyl transferase (OST). Loss of function OST mutants of *Arabidopsis*, encoding *STT3a* and *STT3b* OST subunits, exhibited differential salt stress sensitivity. The *stt3a* mutant but not *stt3b* were sensitive to salt and hyperosmotic stress[39]. In this study, drought stress upregulated *STT3a* expression in two drought tolerant cultivars RGN73 and Varuna and one drought susceptible genotype BioYSR with the highest expression in Varuna (Fig. 5). Thus, drought tolerant genotypes showed higher expression of the genes for protein modification and protein trafficking.

The water channel protein aquaporins regulate transmembrane water transport, and thus regulate hydraulic conductivity and water status of plants[40]. We examined expression of *Brassica* orthologs of two *Arabidopsis* plasmamembrane aquaporin genes namely *PIP1;2* (PLASMA MEMBRANE INTRINSIC PROTEIN 1;2, *PIP1B*) and *PIP2;2* (*PIP2B*), and a tonoplastic aquaporin *AQP1* (*ATTIP2;1*, DELTA TONOPLAST INTEGRAL PROTEIN, *DELTA-TIP*, *DELTA-TIP1*, *TIP2;1*). Previous studies have shown that these genes are generally downregulated upon exposure of *Arabidopsis* plants grown in soil to gradual drought stress[41,42]. However, we found >2 fold upregulation of *PIP1;2* in sensitive cultivars RLM619 and BEC144, while *PIP2;1* was upregulated in all genotypes under drought stress. The *APQ1* was upregulated by drought stress in two tolerant and one susceptible cultivar (Fig. 5). Thus, aquaporin expression did not show clear association with the drought stress response of genotypes.

Several proteins involved in stress damage control and repair are synthesized under abiotic stresses. *HSA32* is one such novel heat shock protein gene highly conserved in land plants and is critical for maintenance of acquired thermo tolerance in *Arabidopsis*[43]. Interestingly, we found that *HSA32*

expression is also induced by drought stress in all *Brassica* genotypes except for one of the drought susceptible cv. RLM619 (Fig. 5). This suggests potential role of *HSA32* in drought tolerance of *Brassica* genotypes. Wax deposition in the epidermal cells is an important drought stress response. In *Arabidopsis SHN1* (*SHINE 1/WAX INDUCER 1*) encodes an ERF/AP2 transcription factor which regulates genes for wax biosynthesis and confers enhanced drought tolerance[44,45]. Drought stress upregulated the expression of *SHN1* gene in all drought tolerant genotypes, while its expression was unaltered or downregulated in susceptible *Brassica* genotypes (Fig. 5).

Conclusion

The model plant *Arabidopsis* is closely related to oil seed Brassicas. Forward and reverse genetic approaches helped unravel molecular mechanisms of physiological processes involved in drought stress and identification of key genes for these mechanisms. Although drought stress adversely affects mustard production, the molecular mechanisms contributing to drought stress responses were poorly understood in mustard. One of the fastest approaches to unravel the molecular basis of mustard is to examine the role of validated *Arabidopsis* gene orthologs in mustard. Towards this goal, we screened 38 genotypes of mustard and *Sinapis alba*, and identified genotypes with contrasting drought tolerance. In these genotypes, we analyzed expression pattern of orthologs of 24 validated *Arabidopsis* genes. Although previous studies have reported stress regulation of these genes in *Arabidopsis*, genotypic difference in drought induced expression was observed in *S. alba* and mustard genotypes. Drought stress specifically upregulated the expression of *ERECTA*, *TMM*, *ABA1*, *AREB1*, *CYP707A*, *ROP10*, *SOS4*, *RabG3e*, *STT3a* and *SHN1* genes >two-fold in one or more of the drought tolerant genotypes, but not in any of the susceptible genotypes. Thus, this study revealed considerable association between drought stress induction of candidate genes and abiotic stress tolerance of *B. juncea* and *S. alba* genotypes. These genes will be useful for further understanding of drought tolerance and genetic improvement of oil seed Brassicas.

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