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ORIGINAL ARTICLE

Differential Expression of *myb2*, *myb44* and *myb60* gene under Drought stress in *Brassica juncea* L. (Czern and Coss.)

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ABSTRACT

Drought stress decreases the water uptake capacity of plants from the soil, limiting the yield and growth of the plant. The present study was aimed to understand the expression of drought stress induced myb transcription factor genes (myb2, myb44, myb60) in B. juncea cvs. RH8812 (drought sensitive) and RH0116 (drought tolerant) by semi-quantitative reverse transcriptase PCR using actin as internal control. Indian mustard plants were raised in net house and exposed to drought stress condition by withholding water at the flowering stage. We found that myb2 and myb44 genes were upregulated and were expressed in both shoots and roots during drought stress. RH0116 showed higher accumulation of myb2 and myb44 transcripts as compared to RH8812. Whereas, the expression of myb60 gene was down-regulated and limited only to shoots during drought stress condition. The drought stress induced Atmyb44 homologues from RH8812 and RH0116 were cloned, sequenced and the sequences obtained were designated as BjMyb44-1 (872 bp) and BjMyb44-2 (862 bp) respectively. They were found to have much similarity to other myb genes of Arabidopsis thaliana (84%) and B. rapa (94%) and their homology with other known sequences was established when subjected to multiple sequence alignment using CLUSTALW2 program.

Keywords*: Brassica juncea, Drought stress, myb, RT-PCR, sequence alignment*

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INTRODUCTION

Being sessile organisms, plants are constantly exposed to different environmental stresses (e.g. salinity, heat, cold, drought, flood, UV rays, heavy metals, etc.) that retard plant growth, development and yield. These are supposed to be responsible for around half of the crop yield losses globally [1]. Being multigenic trait, it is not so simple to learn the molecular mechanism behind abiotic stress tolerance as compared to biotic stress. Abiotic stresses e.g. high temperature, drought and salinity adversely affect crop development and productivity and impose serious threat to the renewability of crop yields. It accounts for higher losses in crop productivity as compared to any other factor in rainfed agriculture which leads to huge economic losses [2, 3]. As forecasting of drought is not possible, it destabilizes the crop productivity globally leading to severe food scarcity in developing countries [4, 5].

Plant response to multiple environmental stresses involves perception of the stress signal and then signal transduction via ABA dependent or independent pathways, which activates different physiological and metabolic responses [6]. ABA plays an important role in regulating stomatal opening, growth and development. ABA also coordinates signal transduction pathways in response to abiotic stresses in plants [7]. Systematic identification of genes can pave the way for the study of plant drought stress tolerance and create foundation for enhancing the productivity of crop in case of arid conditions and help in raising crop yield as well as the income of farmers.

Transcription factors control the gene clusters like the master regulators [8]. These proteins possess a DNA domain that binds to the cis- acting element in the promoter region and transcriptional regulation region of the target gene. Various transcription factors that are related to diverse families play vital role in stress signaling by serving as positive and negative regulator of stress responsive target genes [9, 10]. Numerous transcription factors regulated by abiotic stress were identified by microarray or next-generation sequencing (NGS) technologies [11].

Myeloblastosis (MYB) is very crucial and largest family amongst transcription factors in plants (12, 13). Based on the position and number of MYB repeats, these are categorized as, 1R-MYB, 2R-MYB, 3R-MYB and 4R-MYB) [14]. The MYB family transcription factors having R2 and R3 are associated with the ABA-induced gene expression [15]. Elucidating the role of *R2R3-MYBs* in the adaptation of stress will contribute in selection and development of crops having higher productivity even in the stress conditions [16]. Numerous MYB transcription factors (TFs) that play important role in the regulation of biological processes in plant have been screened [17]. Large size of MYB family in plants reveals their importance in controlling specific processes [18]. It has been observed differential expression of MYB gene in cotton under the drought and salinity stress condition [19]. It has been demonstrated that an R2R3 MYB TF, *GbMYB5*, plays an important role in the adaptation to drought stress condition in cotton and transgenic tobacco [20]. *AtMyb2* is involved in drought tolerance (21), *AtMyb60* in regulation of movements of stomata and drought tolerance in plants [22] and *AtMyb44* is involved in stomatal closure in response to abiotic stress [23]. As regulatory genes control the expression of several genes, production of transgenic plants for abiotic stress tolerance using regulatory genes under inducible promoters provide a unique opportunity to improve stress tolerance [6].

Brassica ranks third position among the different oilseed crops because of its considerable nutritional and economic importance. The predominant oilseed *Brassica* in Indian subcontinent is *B. juncea* L. (Czern. & Coss.) (Indian mustard; 2n = 36 AABB), an amphidiploid which combines in pairs the chromosome sets of species with low chromosome numbers; *B. nigra* (2n = 16 BB) and *B. rapa* (2n = 20 AA). *Brassica* is a high biomass crop and helps in phytoremediation of heavy metals in polluted soils (24). After groundnut, *Brassica* is the second most commonly grown oilseed crop in India, having an area of approximately six million hectares under cultivation and produces 5-6 million tons of seed anually [25]. One of the major efforts for *Brassica* improvement in India is improving *Brassica* cultivars for drought tolerance. We studied *B. juncea* cvs. RH8812 (drought sensitive) and RH0116 (drought tolerant), developed by CCSHAU, Hisar, Haryana. Comparative *myb* gene expression can help in understanding its function in drought tolerance. Also, it is important to know if this gene modulates certain physiological attributes of the plant to help in drought tolerance.

The development of PCR for transcript identification and characterization revolutionized the identification and analysis of expression of gene. Gene expression studies established upon PCR such as semi-quantitative RT-PCR lead to a quick and highly sensitive approach for analysis of gene expression under drought stress condition and also to semi-quantitate their levels. Thus, one can compare levels of transcripts in different samples. Hence, in the present study we focused to analyse myb gene expression involved in drought stress related tissue specific gene expression which can be useful for improvement of *B. juncea* as enhanced productivity under drought stress conditions can be achieved by characterizing and over-expressing such genes.

MATERIAL AND METHODS

Plant Material

The seeds of *B. juncea* (L. Czern & Coss.) cvs. RH8812 (drought sensitive) and RH0116 (drought tolerant) used for the present investigation were obtained from Oilseed Section, Department of Genetics and Plant Breeding, CCSHAU, Hisar, Haryana. Plants were raised in nethouse and were provided with Hoagland Solution in order to provide sufficient growth nutrients. At the stage of flowering, one-half of the plants of each genotype were considered as control (by maintaining the watering) and the rest of the plants were exposed to drought stress by suspending the watering. Samples of shoot and root tissue were collected from control and stressed plants after the appearance of wilting and were stored at -80 ºC till further use. After sampling the drought stressed plants were rehydrated. The day, rehydrated plants appeared normal and wilting disappeared, sampling were again done for analysis.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from the root and shoot tissues of control, stressed and rehydrated plants using Trizol method (53). RNA pellet was resuspended in nuclease free water, absorbance ratio at 260/280 was observed to quantify and check the purity of isolated RNA using Biophotometer (Eppendorf) and stored at -80 ºC till further use. cDNA was synthesized from the total RNA by reverse transcriptase PCR (RT-

PCR). The conditions for RT-PCR were as total RNA (5 µg), oligo dT primer (0.5 µg), dNTPs mix (2 mM) dNTPs mix (4 mM), RNase inhibitor (30 U) and reverse transcriptase enzyme (200 U), making final volume of 20 µl of the reaction mixture.

PCR amplification of transcripts using gene specific primers

PCR was performed using primers of *BjActin*, *Myb2*, *Myb44* and *Myb60* genes (Table 1). *Bj Actin* was employed as endogenous control. Specific gene was amplified from cDNA by PCR in total reaction volume of 20 µl comprising of 10X PCR buffer (with 2.5 mM $MgCl_2$), 0.6 µM of forward and reverse primers, 1U Taq Polymerase, 200 µM of dNTPs and 20-25 ng of template cDNA). Gene expression experiments were carried out using optimized conditions. The conditions for the PCR were as: initial denaturation at 94 ºC for 5 min; 35 cycles of denaturation at 92 ºC for 45 s, annealing for 1 min and extension at 72 ºC for 1 min; and final extension at 72 ºC for 10 min.

Table 1: Details of gene specific primers used in the present study.

S. No.	Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Annealing Temperature (ºC)
1.	*BjActin*	TGGCATCACACTTTCTACAA	CAACGGAATCTCTCAGCTCC	54.3
2.	*Myb2*	CTGGTAAGAGTTGTAGATTAGG	CTCGGCATCCAAACATTTCTA	64.0
3.	*Myb44*	ATGGCTGATAGGATCAAAGGTCC	CTCGATTCTCCCAACTCCAATTTG	53.5
4.	*Myb60*	AGATGGGTAGGCCTCCATGCTGTGACAAG	AATTAAAGCATATTAGAGAGCTCCATCAA	61.0

The PCR amplified products were resolved on 2.5% agarose gel containing 0.5 µg/ml ethidium bromide (EtBr) and visualized under BioRad Gel Documentation system.

Cloning of *myb44* homologues induced under drought stress

The pJET1.2/blunt cloning vector (2974 bp) (GenBank/EMBL Accession no. EF694056) supplied with CloneJET™ PCR cloning Kit (Thermo Scientific) was used for cloning of the PCR products of *myb44* homologue in RH8812 and RH0116. For the transformation of the recombinant vector, DH5α strain of *E. coli* was used as host system. The cloning strategy was followed as per the manufacturer's instructions. Primer dimers and other ingredients were removed from the PCR products before custom sequencing by purification. The gene specific amplified fragments of cDNA were eluted from the agarose gel by using QIAquick gel extraction kit (Qiagen) following the manufacturers instruction. The eluted cDNA was quantified by using Nanodrop (Thermo Scientific) was used. The eluted cDNA sample was also analyzed on 0.8% agarose gel. T4 DNA ligase was used to ligate the blunted PCR product with the cloning vector pJET1.2. Putatively transformed colonies appeared on the culture plates (Fig. 1) and the recombinant colonies were confirmed by Touch Colony PCR (Fig. 2). Plasmid DNA was isolated and used as template to check the amplification of the specific insert in the putatively transformed colonies.

Sequencing and Comparative analysis of cloned cDNAs

Plasmid DNA samples were sequenced from both the ends (forward and reverse) by using vector specific as well as insert specific primers by automated DNA sequencer (Applied Biosystem 3130 XL Genetic Analyzer, available at Department of Animal Biotechnology, LUVAS, Hisar, Haryana). Contig formation and vector sequences removal was done using DNA BASER Software. For comparative sequence homology and alignment analysis, BLASTN and ClustalW2 bioinformatics tools were used. For ClustalW2 analysis, we used different *myb* genes having role in abiotic stress tolerance, reported in the literature as listed in (supplementary file_ Table 1) [54].

RESULTS

Gene expression under drought stress condition

Expression of *myb* genes in *B. juncea* cvs. RH0116 and RH8812

In case of drought stress, *myb2* transcript accumulation was found in shoot as well as in the root tissues of the drought stressed plants. The *myb2* transcript was not observed in control and rehydrated plants, revealing that it is specifically induced when exposed to drought stress (Fig. 3). However, drought tolerant genotype (RH0116) and shoot samples showed higher transcript accumulation than the roots and sensitive genotype.

Upregulation was noticed during drought stress condition in the homologue of gene *Atmyb44* as it was expressed only in the stressed plants unlike the control experiments and rehydrated seedlings as well. This indicated that *Atmyb44* homologue gene is specifically induced in the drought stress condition (Fig. 4). The expression of the *Atmyb44* gene was present in both root and shoot tissues. However, the expression of the gene was relatively higher in the shoots. RH0116 showed higher expression than RH8812 genotype under stress conditions.

Induction of *myb60* gene in *Brassica juncea* (RH0116 and RH8812) was observed in case of control and rehydrated plants and not in the stressed plants, indicating the downregulation of this gene in case of drought stress condition (Fig. 5). However, the transcript was observed only in the shoot tissues and not in the root tissues, which showed that the gene was specifically induced in shoots.

Induction of *actin* transcript in the roots as well as shoot tissues of the control, stressed and rehydrated plants was observed to be at the same level because *actin* is a housekeeping gene and is expressed at all stages (Fig. 6).

Sequence analysis of the *myb44* cDNAs

The sequence data generated using **DNA BASER** Software was used for further analysis. The *mybs* cloned and sequenced in this study from *B. juncea* cvs (RH8812 and RH0116) under drought stress were designated as *BjMyb44-1* (872 bp) and *BjMyb44-2* (862 bp), respectively. The sequences are listed in (Supplementary File_ Fig. 1).

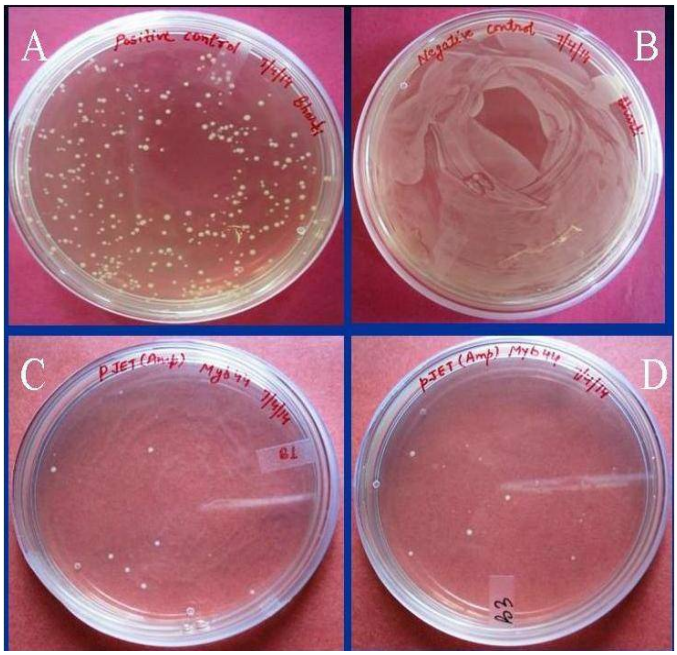

Fig. 1: Bacterial transformation of *myb44* homolgue cDNAs of *B. juncea* L. (A- positive control, B- negative control, i.e. competent cells plated on selection medium, C- RH8812 *myb44*, D- RH0116 *myb44*)

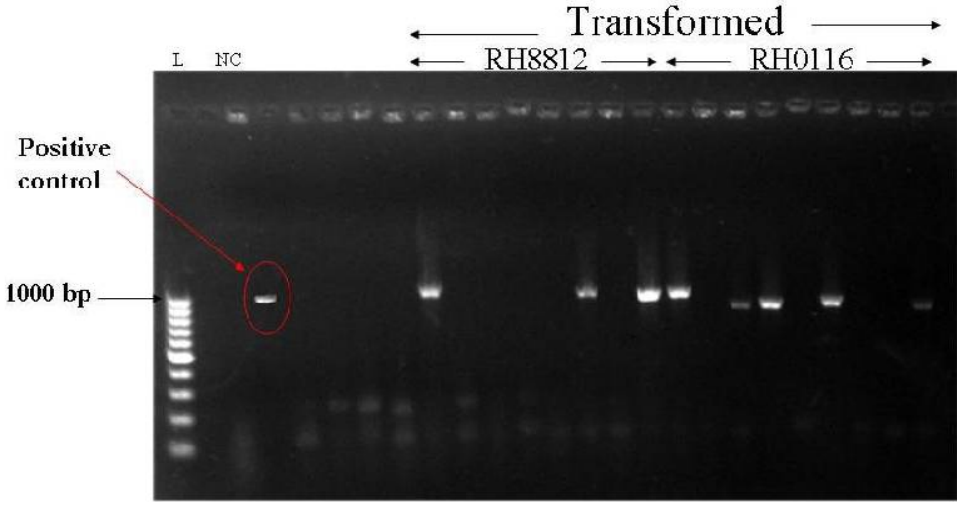

Fig. 2: Confirmation of recombinant colonies by touch colony PCR
(L= 100 bp ladder, NC= negative control)

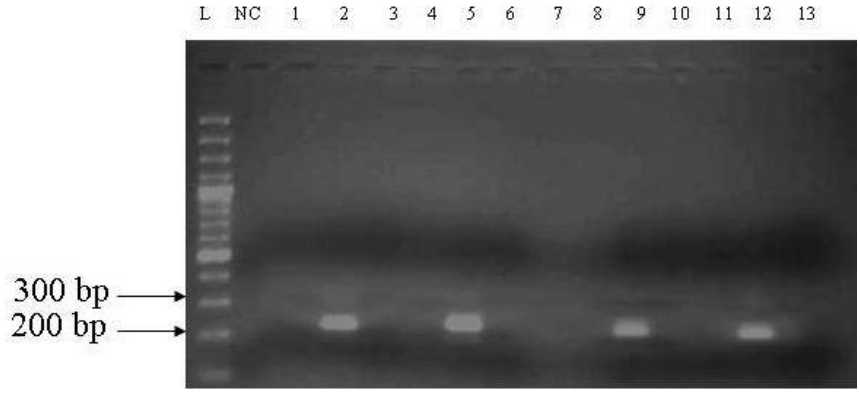

Fig. 3: RT-PCR product of *myb2* transcripts in *B. juncea* genotypes. Lane L-Ladder (100 bp); NC-Negative control; 1-RH8812 (control, shoot); 2-RH8812 (stressed, shoot); 3-RH8812 (rehydrated, shoot); 4-RH0116 (control, shoot); 5-RH0116 (stressed, shoot); 6-RH0116 (rehydrated, shoot); 8-RH8812 (control, root); 9-RH8812 (stressed, root); 10-RH8812 (rehydrated, root); 11-RH0116 (control, root); 12-RH0116 (stressed, root); (5), 13-RH0116 (rehydrated, root).

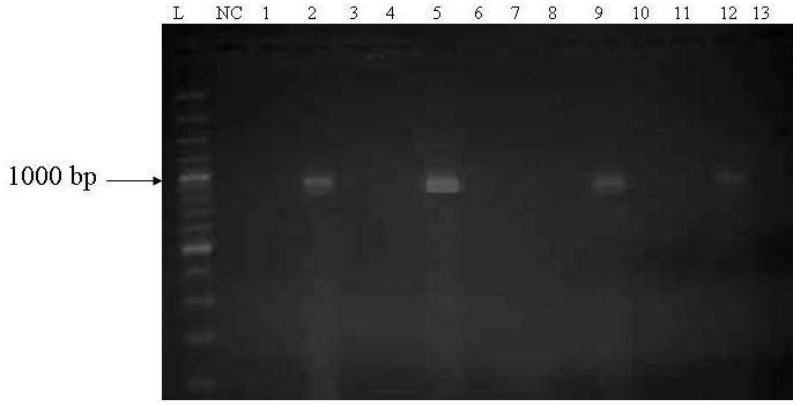

Fig. 4: RT-PCR product of *myb44* transcripts in *B. juncea* genotypes. Lane L-Ladder (100 bp); NC-Negative control; 1-RH8812 (control, shoot); 2-RH8812 (stressed, shoot); 3-RH8812 (rehydrated, shoot); 4-RH0116 (control, shoot); 5-RH0116 (stressed, shoot); 6-RH0116 (rehydrated, shoot); 8-RH8812 (control, root); 9-RH8812 (stressed, root); 10-RH8812 (rehydrated, root); 11-RH0116 (control, root); 12-RH0116 (stressed, root); (5), 13-RH0116 (rehydrated, root).

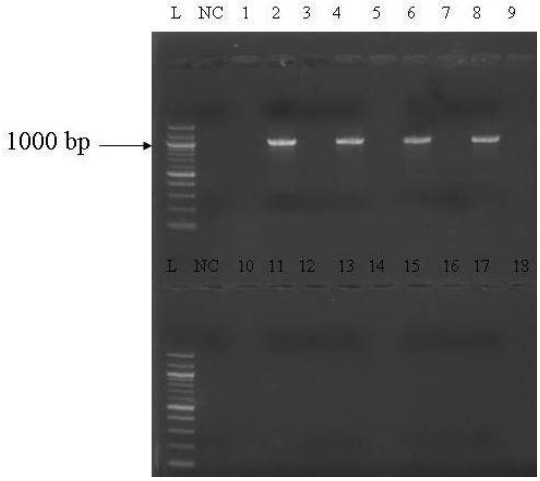

Fig. 5: RT-PCR product of *myb60* transcripts in *B. juncea* genotypes. Lane L-Ladder (100 bp); NC-Negative control; 2-RH8812 (control, shoot); 3-RH8812 (stressed, shoot); 4-RH8812 (rehydrated, shoot); 6-RH0116 (control, shoot); 7-RH0116 (stressed, shoot); 8-RH0116 (rehydrated, shoot); 11-RH8812 (control, root); 12-RH8812 (stressed, root); 13-RH8812 (rehydrated, root); 15-RH0116 (control, root); 16-RH0116 (stressed, root); 17-RH0116 (rehydrated, root).

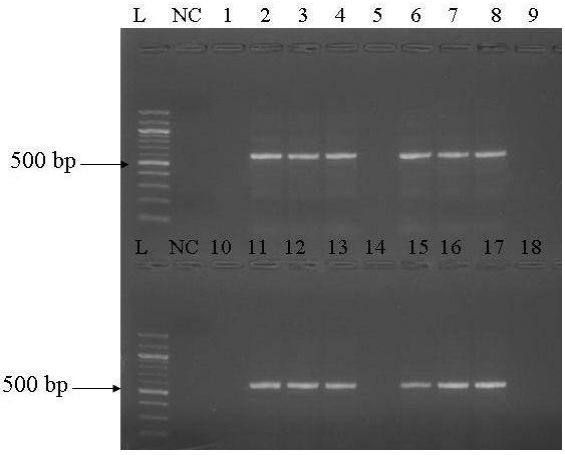

Fig. 6: RT-PCR product of *actin* transcripts in *B. juncea* genotypes. Lane L-Ladder (100 bp); NC-Negative control; 2-RH8812 (control, shoot); 3-RH8812 (stressed, shoot); 4-RH8812 (rehydrated, shoot); 6-RH0116 (control, shoot); 7-RH0116 (stressed, shoot); 8-RH0116 (rehydrated, shoot); 11-RH8812 (control, root); 12-RH8812 (stressed, root); 13-RH8812 (rehydrated, root); 15-RH0116 (control, root); 16-RH0116 (stressed, root); 17-RH0116 (rehydrated, root).

BLASTN analysis of *myb* transcripts

The *myb44* sequences obtained from RH8812 & RH0116 cvs. of *Brassica juncea* were used for BLASTN analysis to know the homology with other genes present in the NCBI database. The sequences obtained were also used for multiple sequence alignment with other related stress induced *mybs* present in different crop plants using clustalW2 (Supplementary File_ Fig.2). BLASTN analysis revealed that **t**he *myb44* cDNAs obtained in this study were novel sequences involved in dehydration response. The *Bjmyb44-1* sequence showed 84% identity and *Bjmyb44-2* sequence showed 81% identity to *Arabidopsis thaliana* transcription factor MYB44 mRNA, complete cds (NM_126130.2) using CLUSTALW2 programme and the bases with * were found to be conserved (Supplementary File_ Fig.3). The *Bjmyb44-1* sequence showed 94% identity to complete cds of the *Brassica rapa* clone 457 R2R3-MYB transcription factor mRNA, (Accession no. EF110971.1). The two sequences *Bjmyb44-1* and *Bjmyb44-2* were subjected to multiple sequence alignment using online CLUSTAL W2 program to know their homology (Supplementary File_ Fig.4). The alignment between the two sequences showed that the transcripts obtained from drought tolerant and drought sensitive genotypes are different but homologous. The bases which were conserved between the two sequences were marked with *. The score of the alignment was found to be 50.58. Multiple sequence alignment was also performed with *Bjmyb44-1, Bjmyb44-2, Arabidopsis thaliana* transcription factor MYB44 mRNA, complete cds (NM_126130.2), complete cds of the *Brassica rapa* clone 457 R2R3-MYB transcription factor mRNA (EF110971.1) and *Brassica juncea* transcription factor Myb (myb1) mRNA, partial sequence (EU233274.1) using CLUSTALW2 programme and the bases marked * were found to be conserved (Supplementary File_ Fig.5).

DISCUSSION

Plants respond to abiotic stress in a complex and dynamic manner [26, 27], which is both reversible and irreversible. Proper supply of water and nutrients affect the growth of the plant and thus crop yield. Water stress is the most severe constraints for the crop growth especially in the arid and semi-arid regions of the world as it plays important role in the growth and development of plants. The plant stress response is dependent on the organ or tissue which is affected by the stress. For instance, transcriptional response to stress condition is cell or tissue specific in roots of the plants and is quite variable according to the type of the stress [28]. Also, the duration and level of the stress (acute or chronic) can pose a significant impact on the complexity of the stress response [29, 30]. Environmental stresses significantly affect the harvestable yield. However, the response multiplicity is a vital aspect of the stress signalling complexity. The genetic and physiological reactions of the plants grown in stressed environments are highly complex and are the main hurdle in the success of the different strategies for the crop improvement [31]. Abiotic stress tolerance, being multigenic in nature, makes adaptation to variable environmental stresses a much complex phenomenon. Recent advances in molecular biology have shown the path to decipher the adaptive mechanisms in plants at the molecular level [32, 33]. Some of the key regulatory pathways in plant response to the abiotic stress have been studied with the help of systems biology and omics approaches [34]. Engineering the plants for abiotic stress tolerance is a viable option.

The goal of genetic engineering strategies is to manipulate gene expression in ways that produce the desired phenotypic effects [24].

B. juncea (Indian Mustard) is an important oilseed crop of India and is amphidiploid between *B. rapa* (2n = 20 AA) and *B. nigra* (2n =16 AA). It contributes approximately 28.6% to the total of oilseed production. India produces approximately 6.7 million tons of *B. juncea* after China (11-12 mt) and EU (10–13 mt), contributing significantly to global rapeseed mustard industry [35]. RH8812 is drought sensitive and RH0116 is drought tolerant variety of *B. juncea* developed by CCSHAU, Hisar. Such cultivars are appropriate to understand the mechanism of stress tolerance and isolation of stress induced genes. A number of genes related to abiotic stress, some transcription factors and regulatory sequences present in the promoters of plants are known to have a great impact on stress tolerance by plants (7). Improved abiotic stress tolerance in relation to the enhanced photosynthetic parameters have been observed in many transgenic plants after the over-expression of such TFs. Transcription factors involved in the abiotic stress response in plants act either by ABA dependent or ABA independent pathway. But both the pathways interact with each other and control to the expression of abiotic stress related gene expression.

MYB transcription factors have regulatory role in the development and defense response in the plants. Variable numbers of R2R3-MYB proteins have been identified and characterized by genetic approaches and have been found to be involved in controlling plant specific processes such as metabolic activities, developmental processes and stress response [36]. MYB-type transcription factors will also be useful to understand the gene regulation of genes in plant and developing new varieties of plants [18].

Semi-quantitative RT-PCR for gene expression studies

We studied *myb* (*myb2*, *myb44*, *myb60*) gene expression under drought stress conditions in *Brassica juncea* using semi-quantitative reverse transcription PCR approach. The two step RT-PCR (using reverse transcriptase for cDNA synthesis using oligo dT primers) is a cost effective and convenient method to analyse gene expression. The protocol employed 200 U of Novascript RT, 40 U of RNase inhibitor (Promega), 0.5 U Taq DNA polymerase for gene amplification and total RNA (5 µg) and 0.2 µg of oligo dT primer were used for cDNA synthesis. We used NovaScript III RNase H minus RT in our experiments and it worked well in carrying out gene expression studies. Inspite of optimization, targets that can't be amplified, can be amplified by the appropriate additive that is included in the PCR master mixture (37). So, we have used DMSO (dimethyl sulfoxide) in our PCR experiments, an enhancing agent which led to higher yield, specific and consistent PCR products by facilitating strand separation. DMSO is usually used as an enhancing agent and is included for standardization of PCR (38). Among several genes involved in response to the abiotic stress, AtMyb2 acts as a positive regulator of abiotic stress induced rd22 gene expression [39]. A knockout mutant of AtMyb2 is insensitive to ABA (40). Trans-activation of a MYB related drought-inducible protein, AtMYB2 in Arabidopsis was reported and it was found that the transcription of reporter genes activates in sequence specific manner (21). It was found that *Atmyb2* homologue is upregulated by mannitol, NaCl, sucrose, air drying and PEG in *Brassica juncea* cvs. RH-0116 and CS-52 as the *myb* transcripts were observed under salt and drought stress conditions [41]. Similar results with *myb* gene were observed in *Brassica carinata* when subjected to drought stress treatment induced by 1 hr and 2 hr air drying [42]. Early induction of myb gene in response to stress has also been reported in Indian mustard [43]. ABA up-regulates the expression of AtMYB2 gene and its overexpression increases the drought tolerance in transgenic plants [44].

The TF AtMYB44 is related to the R2R3 MYB subgroup 22 TF family of Arabidopsis. It is subcellularly located in the nucleus and is expressed in leaves, roots, stems, and inflorescence mostly in stomata and vasculatures during very late stages of embryogenesis. The expression is induced by drought, high salinity, cold, salicylic acid (SA), cadmium ($CdCl_2$), ethylene, jasmonate (JA), gibberellic acid (GA), and ABA (Q9FDW1, UniProtKB/Swiss-Prot 2014). The gene is involved in stress induced stomatal closure as adaptive mechanism providing drought tolerance. Drought stress affects vital metabolic functions and turgor pressure at the cellular level. Hence, Cell wall formation and cell expansion are especially sensitive to water stress. Plants respond to lower water availability by stomatal closure so as to lower the water loss. However, this will also reduce the CO_2 supply within the leaves of the plant and finally hamper photosynthesis process. Crop losses induced by drought pose substantial economic impact and it is expected to increase with global climatic variation [45]. We observed the upregulation of *Myb44* homologue in drought stress condition as it was expressed only in the stressed plants and not in case of the rehydrated plants. AtMYB44 has been identified as a jasmonate-inducible gene in a microarray experiment [46]. The transcript of AtMYB44 was found to be induced within 30 min of ABA, ethylene or methyl jasmonate application to the rosette leaves of *Arabidopsis*. Transgenic *Arabidopsis* plants overexpressing AtMYB44 were observed to be hypersensitive to ABA during seed germination, delayed flowering and dwarfed in early growth stages [23]. BplMYB46 is considered to have role in controlling the

stomatal aperture to reduce water loss [47). It has been studied that ectopic expression of *MYB44* improves the drought and salt stress tolerance in soyabean and such plants were having higher water retention ability as compare to control plants (48).

Number of researchers have shown that *AtMYB60* is another transcription factor gene which is highly expressed in stomatal guard cells [49]. It has been reported that the promoter sequence of *AtMYB60* specifically activates the expression of transgene in *Arabidopsis* stomata (50). Regulation of the guard cell autonomous ABA synthetic pathway plays a vital role in modulating stomatal activity in stress response [51]. We observed the induction of *myb60* homologue in *B. juncea* (RH8812 and RH0116) in control and rehydrated plants while it was absent in drought stressed plants, indicating that this gene is down-regulated in drought stress condition. However the transcript was observed only in the shoot tissues and absent in the root tissues, which shows that the gene is specifically induced in shoots. Absence of its expression in roots is also reported [22]. *AtMYB60* expression is swiftly downregulated by ABA and dehydration stress. As shown by RWC measurements, the expression of AtMYB60 is seen in most plant organs and transpirational water loss. After eight days of drought stress treatment, wild type plants showed chlorosis of rosette leaves and severe wilting, however *AtMYB60-1* remained turgid with green leaves. It clearly demonstrated that the *AtMYB60-1* mutation reduced the stomatal-pore and helped in limiting the water loss during drought stress condition.

Cloning and validation of *myb* cDNAs

In our experiments, we have used pJET1.2/blunt cloning vector. This vector has an additional feature that when recircularized, expresses a lethal restriction enzyme after transformation and is not propagated. Therefore, only recombinant clones carrying the insert appear on culture plates and there is no need of blue/white selection. These vectors use TA cloning procedure for which the first screening strategy for confirmation of recombinants is visual detection of blue-white colonies. Our experiments confirm successful cloning of *AtMyb44* homologues in two genotypes of Indian mustard which was confirmed by touch colony PCR and plasmid DNA profile. The clones were sequenced using both sets of primers (vector specific and insert specific). Sequence analysis further confirmed that these were indeed *AtMyb44* homologues. *AtMyb44* is 1985 bp long with open reading frame of 918 bp. However, using our clones, we have sequence information of 872 bp and 862 bp of the two homologues, *BjMyb44-1* and *BjMyb44-2* respectively. Further experiment is required to complete the cDNA sequence. It has been reported that R2R3-MYB family members from different plants having a common ancestor went through gene duplication events as indicated by the phylogenetic analysis [10].

AtMyb44 has shown potential for engineering drought tolerance as reported by various workers (23, 48). Utilization of cloned Indian mustard with *AtMyb44* homologue will only be possible if we complete the cDNA sequence and overexpress it in a model plant system such as *Arabidopsis* or tobacco to validate their potential. Brassica is the nearest crop relative of the model crucifer plant *A. thaliana* and its sequencing has also created the way to relative study of the complicated *Brassica* genome structures (52). Since Indian mustard also belongs to *Cruciferae* family and much information is already worked out in *Arabidopsis*, engineering this gene in crop plants holds great promise for improving drought tolerance under climate change scenario in targeted crop plants.

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