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MOLECULAR CHARACTERIZATION OF GROWTH AND PROTEOLYSIS RELATED GENES IN MAIZE UNDER DROUGHT STRESS

KOMAL ARIF[1], RAFIQ AHMAD[1*], SABAZ ALI KHAN[1], SAEED AHMAD ASAD[2], TOQEER AHMAD[2], GHULAM HASSAN ABBASI[3] AND MUHAMMAD SHAHZAD[1]

[1]Department of Environmental Sciences, COMSATS Institute of Information Technology, 22060, Abbottabad, Pakistan
[2]Centre for Climate Research & Development (CCRD), COMSATS Institute of Information Technology, Islamabad Campus, Park Road, Chak Shahzad, 44000, Islamabad, Pakistan
[3]University College of Agriculture and Environmental Sciences, The Islamia University of Bahawalpur, Pakistan
**For correspondence: drrafiq@ciit.net.pk, jamrafiqe@yahoo.com*
Phone: + 92 992 383 591-6

Abstract

Drought is one of the major environmental stresses that cause severe reduction in growth and yield of maize crop worldwide. Current Research was executed to evaluate for the first time the expression pattern of growth related *expansin* genes, proteolysis related *cysteine protease* and their natural inhibitors "*cystatins*" genes in two maize varieties (Syngenta 8441 and Islamabad gold) under three different water levels of drought stress *i.e.*, control, moderate and sever stress. The results of the molecular analyses demonstrated that *Exp1* gene over expressed in variety Syngenta 8441 as compared to variety Islamabad gold. In contrast, the expression of *ExpB2* was very low in both the varieties. However, the expression of *metacaspase 2* and *metacaspase 3* genes was higher in variety Islamabad gold whereas the expression of their inhibitors *CC8* and *CC9* was found to be very low. It is hypothesized that the expression of *metacaspase 2* and *metacaspase 3* was suppressed by the inhibitors *CC8* and *CC9* under severe drought stress in variety Syngenta 8441. According to these results, Syngenta 8441 is seemed to be a drought resistant variety while Islamabad gold is a drought sensitive variety. Further, bioinformatics analyses of these genes revealed important conserved protein domains that are involved in the drought stress. As *Exp1*, *CC8* and *CC9* genes were highly expressed in maize plant under drought stress, so in future expression of one or more of these genes could be used not only to screen drought sensitive and drought tolerant or resistant maize varieties but also for the production of transgenic maize as well as other crop to enhance their drought stress tolerance.

Key words: Maize; Drought; Genes; Metacaspase; Cystatins.

Introduction

Abiotic stresses including drought severely limit plant growth, development and productivity (Umezawa *et al.,* 2006). Plants respond to drought stress at molecular level by quickly modifying the expression of the important involved genes (Chaves *et al.,* 2009). For example, the modulated expression of *expansin* genes is usually the earliest and most prominent impact of drought stress that causes reduction in the plant growth. Expansins are cell wall proteins that play a crucial role in regulating cell wall extension which then mediates the expansion and enlargement of cells. Expansins are much more specific that particularly induces *In vitro* cell wall extension and *In vivo* cell expansion (Cosgrove, 1997). Currently four families of expansins exist in plants, α-expansin (EXPA), ß-expansin (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB). Expansins in maize form a diverse group of at least 30 genes, ß-expansins are large in number and more abundantly expressed than α-expansins (Wu *et al.,* 2001). Expansin proteins have highly conserved sequences having weight of ~25-30 kilo Dalton with 250-300 amino acids. Expansins are active at low pH thus causing pH dependent cell wall extension called as 'acid growth' (Cosgrove, 2005). Other plant developmental processes which show expansin activity involve fruit softening (Brummell *et al.,*, 1999), appearance of root hairs, invasion of pollen tubes through the stigma and style (Cosgrove, 1997), and abscission and rehydration of 'resurrection' plants (Jones and McQueen-Mason, 2004).

Programmed cell death (PCD) also plays a critical role during drought stress (Mittler & Blumwald, 2010). Proteolytic enzymes are thought to be the principal participants in PCD though little is known about their role in plants. Proteases, the proteolytic enzymes, together with their particular inhibitors that control their actions, are the primary players in executing and monitoring intracellular breakdown of proteins. These are serine protease, cysteine protease, aspartic protease, metalloprotease and threonine protease. Among these, cysteine proteases are best studied proteases with a maximum identification and characterization in plants (Rawlings *et al.,* 2010). Metacaspases, a type of cysteine protease, are essential component and also regulators of the PCD during plant responses to drought stress (Huang *et al.,* 2015).

Depending on the structure of protein, metacaspases can be divided into two types, type I and type II. Type I have an N-terminus prodomain of 80 to 120 amino acids with a Zn finger motif, these structures are not found in type II metacaspases but they do possess a linker region between their two subunits (Tsiatsiani *et al.,*2011). Plants contain both types of metacaspases but their number differs greatly in different species for example, Arabidopsis has nine metacaspases (Tsiatsiani *et al.,* 2011), rice carries eight or nine metacaspases (Wang & Zhang, 2014) while grapevine contains 6 metacaspases (Zhang *et al.,* 2013). Hydrolysis of peptide bond is irreversible so the activity of metacaspases is tightly controlled by their inhibitors 'cystatins', to inhibit the incorrect and irrelevant activated metacaspases (Brady & Duckett, 2009). Cystatins are the proteins that specifically control and suppress activity of

cysteine proteases. On the basis of sequence homology, molecular mass, position of disulfide bonds and their presence or absence, cystatins can be subdivided into four different families *i.e.,* stefin, cystatin, kininogens and phytocystatin (Turk & Bode, 1991). Cystatin inhibitors are expressed only in special organs or at specific stages of plant development for example during germination, early senescence of leaves or drought stress.

Maize (*Zea mays* L.), the 'Queen of Cereals' is the most essential crop that ranges third after wheat and rice in its significance (Mahesh, 2015). Different abiotic stresses reduce the growth and yield of this important crop (Ahmad *et al.,* 2012; Abbasi *et al.,* 2015). Among these, drought that may be the result of overall shortage of water and irregular rainfall, is the most important. Under such conditions development of drought resistant maize varieties is the only option to cope with the shortage of water. New drought tolerant crop varieties having better productivity could be created by understanding the basis of plants morphological, physiological and molecular changes in response to water stress (Martinez *et al.,* 2007).Therefore, the present research was conducted to evaluate the sensitivity of maize to drought stress at molecular level by measuring the expression of growth related *expansin* genes and proteolysis related *cysteine proteases* genes and their natural inhibitors *cystatin* genes in two maize varieties *i.e.,* V1 (Syngenta 8441) and V2 (Islamabad gold). This research of expression pattern of drought stress related genes could be helpful for developing drought tolerant maize varieties in future. The target genes of the current study were *Exp1, ExpB2, metacaspase 2, metacaspase 3, CC8* and *CC9*.

Materials and Methods

Sowing of seeds and application of drought stress: The seeds of two maize varieties *i.e.,* V1 (Syngenta 8441) and V2 (Islamabad gold) were used in this study. Syngenta 8441 was collected from the seed stock of CIIT Abbottabad while Islamabad gold was obtained from seed bank of National Agriculture Research Centre (NARC), Islamabad, Pakistan. Healthy and fresh seeds of these varieties were selected for sowing. Pots of same size were used in the experiment and equally filled with 250g of soil collected from research field of COMSATS, Abbottabad. Seeds of both varieties were sown in plastic pots, 2-3 inches deep in the soil with two replicates. A total of fifteen seeds were sown per pot and pots were properly covered with transparent plastic sheet to maintain temperature and to avoid interruption of rain water. Seeds were then allowed to grow under field conditions until shoot emergence. After three weeks, seedlings were randomly selected and transferred to small plastic cups filled with soil. After shifting in pots, seedlings were exposed to drought stress by providing water in different concentrations. Three different levels of water used in the experiment were 8 ml (control), 5 ml (T1, moderate stress), and 3 ml (T2, severe stress). Seedlings (control and treatments) were watered every third day and placed in growth chamber having temperature of 25°C and 16/8hours light dark period respectively for two weeks.

Molecular analysis

Total RNA extraction and quantification: Leaves after two weeks of treatment were harvested from all treatments and immediately stored at -80°C freezer. CTAB method was used for total RNA was extracted from maize leaves (Kim & Hamada, 2005). Further procedure for RNA isolation was same as reported in our previous research (Ahmad *et al*., 2016). Briefly, the leaf samples were first ground into liquid nitrogen, and then standard procedure of CTAB method was used to isolate the RNA. Pellet of RNA was eluted with 70% ethanol and 50µl RNase free water was added in it. RNA quality and quantity was checked according to protocol mentioned by Ahmad *et al.,* 2015.

Primers designing: Primers of target genes were designed through primer 3 software. The primers and accession numbers of *Exp1*, *ExpB2*, *metacaspase 2*, *metacaspase 3*, *CC8, CC9*genes and *S19* (reference gene) are in Table 1.

Complementary DNA (cDNA) synthesis, polymerase chain reaction (PCR) and analysis of PCR products: For genes expression study cDNA was synthesized from 4 µg total RNA by using the TOP script™ cDNA Synthesis Kit (Enzynomics, Seo µL) according the procedure described by Ahmad *et al.,* 2016. The PCR analysis was performed in the same way as mentioned in Ahmad et al. 2016. Briefly, the total volume used for PCR reaction was 10 µl containing 50ng of cDNA, 0.5 µM of each pair of specific primers, 5 µl of 2X Master Mix and 2 µl of double distilled water. PCR was performed in a thermal cycler "Master cycler gradient" (Applied biosystem). The annealing temperature was different for each primer, for *Exp1* (53°C), *ExpB2* (57°C), *metacaspase 2*(59°C), *metacaspase 3*(59°C), *CC8*(56°C)and *CC9*(56°C), with a final extension (72°C, 10 min).The amplified PCR product of each gene was analyzed using protocol as described by Ahmad *et al.,* 2016.

Table 1. List of primers for the selected genes.

Gene name	Gene bank accession number	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Exp1	AI001321	5'-CTACTACTACTCCATCGACG-3'	5'-AATAAGTTGCACGACACC-3'
ExpB2	AF332175	5'-AGCTAGCTGGTTTGCGCC-3'	5'-AAGCAACAGTGGGCGGG-3'
Metacaspase 2	ACF83610	5'-GAACTTGAGGAACGGTCTCCT-3'	5'-TTGACGTGGGCATCGGCTGTG-3'
Metacaspase 3	ACF88387	5'-GACATTGATGTGGGTTCTATCC-3'	5'-ACTGCCTTGCTCACCTGACTGG-3'
CC8	BN000515	5'-TGGTTTGTTACACGCGGTACTT-3'	5'-AGATCGTCGCCGGGAAAAACT-3'
CC9	BN000513	5'-CAGCAGCACAAGAACCAGAGT-3'	5'-CTACGGATGATCCAGTGACAG-3'
S19	EU970864.1	5'-AAATGGCACCACTGCTCTTGCA-3'	5'-TGCTCAAGCTTCAACAGCAACC-3'

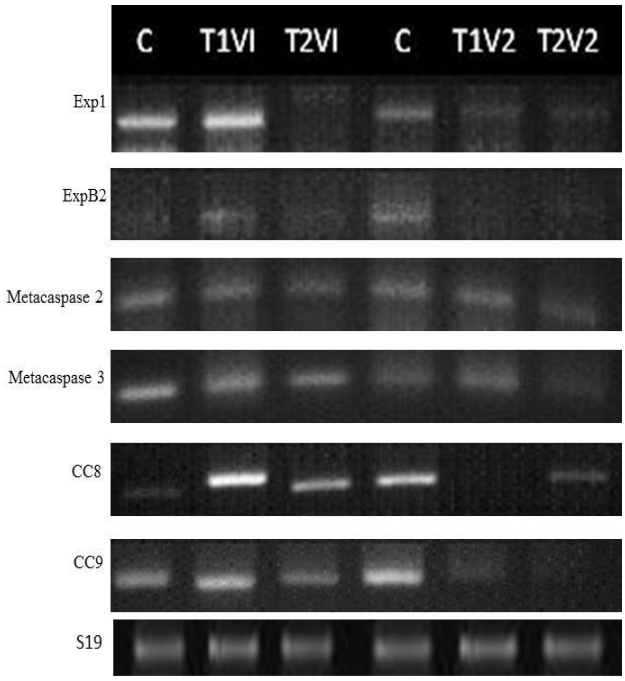

Fig. 1. Expression of *Exp1*, *ExpB2*, *metacaspase 2*, *metacaspase 3*, *CC8*, *CC9*and S19 (reference gene) genes in control (C), T1 (moderate stress) and T2 (Severe stress). V1 indicates variety Syngenta 8441 while V2 indicates variety Islamabad gold.

Bioinformatic analysis: The protein sequences of *Exp1*, *ExpB2*, *metacaspase 2*, *metacaspase 3*, *CC8* and *CC9* genes and proteins sequences of other plants having more than 70% similarity were analyzed through bioinformatics tools. Clustal omega was used for sequences alignment. Phylogenetic tree was constructed using on line phylogeny software. Conserved domains were searched and found with NCBI-CD searches. The low-complexity filters were turned off and expected value was set at 1.0. DOG 2.0 software was used for the visualization of the conserved domains

Results

Expression analysis of *Exp1, ExpB2, metacaspase 2* and *metacaspase 3, CC8* and *CC9* genes: The expression of growth related expansin genes (*Exp1*, *ExpB2*), proteolysis related cysteine proteases (*metacaspase 2* and *metacaspase 3*) and their inhibitors cystatins (*CC8* and *CC9*) were analyzed in two maize varieties (Syngenta 8441 and Islamabad gold) under drought stress. Maize *S19* was used as an internal control. The results of the molecular analysis demonstrated that *Exp1* gene was overexpressed in variety Syngenta 8441 as compared to variety Islamabad gold. In contrast, the expression of *ExpB2* was very low in both of the varieties. Expression of *metacaspase 2* and *metacaspase 3* was suppressed in variety Syngenta 8441 probably due to overexpression of cystatin*CC8* and *CC9* genes under severe drought stress. However, the expression of *metacaspase 2* and *metacaspase 3* was higher in variety Islamabad gold whereas the expression of their inhibitors *CC8* and *CC9* was found to be very low (Fig. 1).

***In silico* characterization of *Exp1, ExpB2, metacaspase 2* and *metacaspase 3, CC8* and *CC9*genes:** Conserved domains of *ExpB2*, alignment of ExpB2 protein sequences in maize and that of other closely plants were identified as shown in Fig. 2a. DPBB-1 superfamily domain (double-psi beta-barrel domain) and Pollen-allerg-1 super family domains are highly conserved in maize ExpB2 and proteins of other selected plants. Similarly CASc super family domain (caspase domain) is highly conserved in metacaspase 2 and metacaspase 3 proteins of maize and proteins of other plants (Fig. 2b & 2c).In the same way, CY super family domain (cystatin like domain) is highly conserved in maize CC8 and CC9 protein and proteins of other plants (Fig. 2d & 2e).

Each sequence of our study was BLAST separately and four closely related sequences of each gene were aligned for the phylogenetic analysis as shown in Fig. 3. Phylogenetic tree showed that ExpB2 of maize is closely related to rice ExpB2 (Fig. 3A) and maize metacaspase 2 and 3 are closely related with sorghum metacapases (Fig. 3B, C). Similarly, cystatin (CC8) of maize has the highest homology with cystatin of *Lantana camara* (Fig. 3D) and cystatin 9 (CC9) has maximum similarity with cystatin of *Vigna unguiculata* (Fig. 3E).

Discussion

Maize is often recognized as one of the oldest cultivated and the most versatile emerging crop (Mahesh, 2015). Maize crop is used to make food for humans and is a source of feed for animals such as poultry and livestock. Besides this, it also serves as the crude material for industrial use. It is environment friendly in terms of its cultivation and can significantly adapt to new environments. Except potato, maize serves as the most common cash and commercial crop in Pakistan's today's history (Tariq & Iqbal, 2010). Due to a serious damage caused by drought stress (losses may range between 78%-87% of maximum yield), the economically important crops as wheat, corn, soybean and tomato raise a major issue for producers, consumers and governments. Due to this, all the characteristic features of plant growth and development (ranging from seed germination to yield) are affected. Drought is recognized as the most dominant abiotic stress that resulted worldwide yield reduction in agricultural production. Water is a principal component of all life and comprises about 90% of fresh weight in physiologically active plants, so its deficiency affects various plants physiological and biochemical processes involved in maintaining plant growth (Hamayun *et al.*, 2010). The adaptation of plant to drought stress is managed by cascades of molecular networks resulting in a combination of metabolic, physiological and morphological changes. Due to this reason, this problem has been reviewed by various researchers in agriculturally important crops such as tomato (Taylor *et al.*, 1982), rice (Singh & Singh, 1983), soybean (Ribas-Carbo *et al.*,2005), wheat (Kerepesi & Galiba, 2000) and grass (Emmerich & Hardegree, 1990).

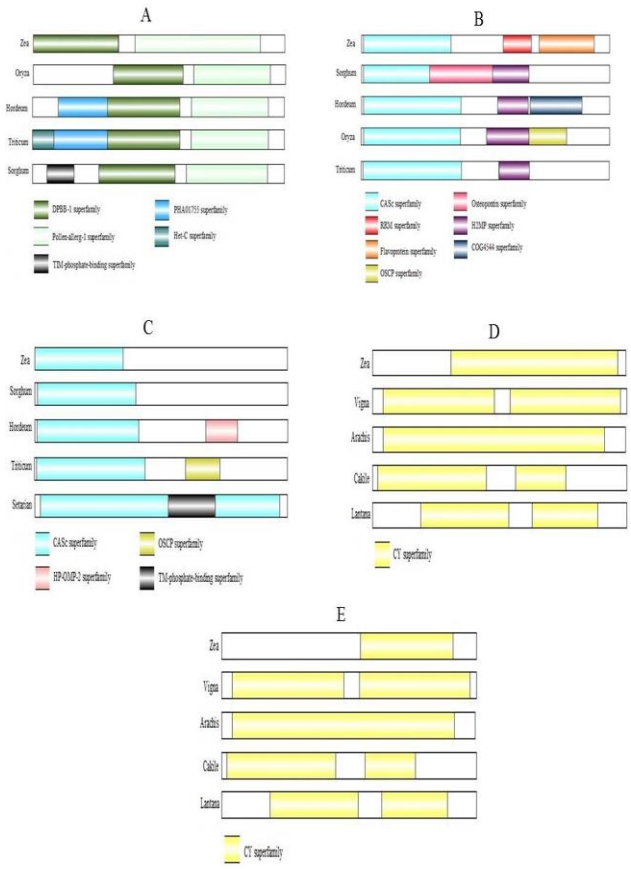

Fig. 2. Schematic diagram showing the arrangement of conserved domains of ExpB2 (a), metacaspase 2 (b), metacaspase 3 (c), CC8 (d) and CC9 (e) protein and proteins of other plants.

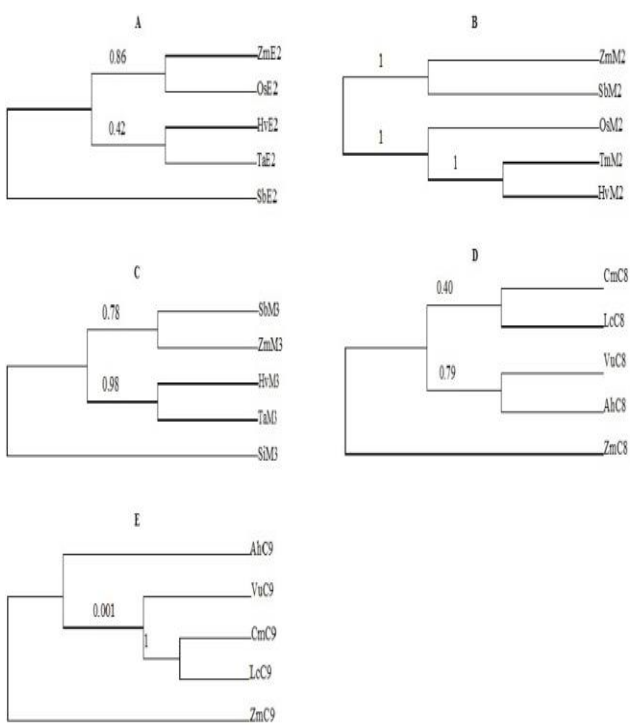

Fig. 3. Phylogenetic trees of *ExpB2* (A), *metacaspase 2* (B), *metacaspase 3* (C), *CC8* (D) and *CC9* (E). E2 (ExpB2), M2 (metacaspase 2), M3 (metacaspase 3), C8 (CC8), C9 (CC9), *Zea mays* (Zm), *Oryza sativa* (Os), *Hordeum vulgare* (Hv), *Triticum aestivum* (Ta), *Triticum monococcum* (Tm), *Sorghum bicolor* (Sb), *Setaria italica* (Si), *Vigna unguiculata*(Vu), *Arachis hypogaea* (Ah), *Cakile maritima* (Cm), and *Lantana camara* (Lc).

Proteolysis occurs naturally in plants to maintain the biological and physiological processes. Proteolysis during biotic stresses and plant senescence is well documented but little is known about the role of cysteine proteases and their natural inhibitors cystatins in proteolysis in response to drought stress conditions. Expansin, important cell wall enzyme, is involved in cell wall loosening and cell growth (Li *et al.,* 2013). Increased expansin activity required for maintaining root growth was observed in maize seedlings against drought stress (Wu *et al.,* 1996).The results of current study indicated that *Exp1*expression under mild stress condition (T1) was greater while under severe drought, a reduction of *Exp1* gene was observed in case of Syngenta 8441. The comparison of *Exp1* gene in both varieties showed that *Exp1* was most strongly expressed with its transcript level being higher in Syngenta 8441 than Islamabad gold. As *Exp1* was more efficiently expressed in Syngenta 8441 as compared to Islamabad gold so Syngenta 8441may be recognized as drought tolerant, while Islamabad gold as drought sensitive. This was consistent with a previous report (Zhao *et al.,* 2011) according to which the expansin activity in drought-tolerant wheat cultivar was greater than that in drought-sensitive wheat cultivar. The drought-tolerant cultivar of wheat can sustain better absorption of water than the drought-susceptible cultivar (Bajji *et al.,* 2001).

The expression of *ExpB2* gene in the present study was found to be very low in case of both varieties although a study reported that *ExpB2* plays a role in the elongation of maize roots and its expression was much higher than other expansin in plant responses towards environmental stimulus (Kam *et al.,* 2005). Another study showed that *TaEXPB23* was overexpressed in transgenic tobacco under drought stress (Li *et al.,* 2011). Wu *et al.* (2001) observed that organ specific changes are induced in the expression of expansin proteins which are of crucial importance for showing response towards drought stress. Considering that reported studies and our results, it is not surprising that expansins are differentially regulated in an organ specific manner. Thus we can conclude that *ExpB2* expression in the leaves of two maize varieties (Syngenta 8841 and Islamabad gold) was not very sensitive to drought stress conditions. Metacaspases are cysteine proteinases which play important role in signaling and executing PCD in plants. Abiotic stress induces PCD that affects growth and development of plants. Abiotic stresses *e.g.,* ultraviolet light and ozone has beenreported to induce the expression of proteases genes in Arabidopsis and maize (He *et al.,* 2008; Ahmad *et al.,* 2012; 2014).

In the present study, we found that *metacaspase 2* was down-regulated at severe drought level in both varieties as compared to control moderate stress. However, *metacaspase 3* showed differential expression at different drought stress levels. It showed expression at moderate stress level in Syngenta 8841 but its expression was suppressed at moderate stress level in variety Islamabad gold. Similar expression of *metacaspase 3* was observed at moderate stress level in both varieties. However, the expression of *metacaspase 3* at control was found to be opposite in the two varieties and these results are almost similar as published by Huang *et al.* (2015).

They studied that under abiotic stresses, the range of changes in the expression of rice *metacaspases* varied greatly. Most *OsMC* genes were down-regulated by drought stress but some of these genes showed overexpression, indicating their possible involvement in various abiotic stresses.

Plant cystatins are highly responsive to various abiotic stresses (Zhang *et al.,* 2008; Ahmad *et al.,* 2014); they regulate and inhibit the activity of endogenous cysteine protease. The expression of cysteine protease inhibitor '*cystatins'* was also analyzed in the present study. According to our results expression of *CC8* was more pronounced in Syngenta 8841 as compared to Islamabad gold. In the same way, *CC8* expression was greater Syngenta 8841 in those plants that were exposed to moderate stress than control plants. Literature study also reveals that drought stress causes the accumulation of cystatins followed by rapid decrease in cystatin induction levels after re-watering. In *Arabidopsis thaliana, AtCYSa* and *AtCYSb* cystatins were expressed by multiple abiotic stresses such as high salt and drought stress (Zhang *et al.,* 2008). In our study, expression of *CC8* was observed to be reduced in plants of both varieties that were exposed to severe stress. These results were according to a published data in which Massonneau *et al.* (2005) observed down-regulation of some cystatin genes in response to severe water deficit in maize and linked the observed data with higher activities of cysteine proteases under severe drought stress. In case of *CC9*, its expression was lowered with increasing drought stress level in both varieties. Our results are in line with those of Massonneau *et al*. who observed down-regulation in the expression of *CC9* in response to water deficiency (Massonneau *et al.,* 2005). It has also been found that different cystatin genes in *Arabidopsis thaliana* show different patterns of expression in response to abiotic stresses, indicating that individual cystatins may have distinct functions under abiotic stress conditions (Hwang *et al.,* 2010).

In the current study, *in silico* characterization of the selected genes (*Exp1*, *ExpB2*, *metacaspase 2*, *metacaspase 3*, *CC8*, *and CC9*) was also performed. Alignment of *ExpB2* sequences of *Zea mays* with *Sorghum bicolor, Oryza sativa*, *Hordeum vulgare* and *Triticum aestivum* protein sequences show that many amino acid residues are conserved in all plants. Conserved and semi-conserved region were also observed (Fig. 3). The sequence similarity of ZmE2 was highest with OsE2 (90.86%) therefore these two are present in the same cluster. However ZmE2 was least similar with SbE2 (67.43%) so these two not only are far away from each other in the dendrogram but also in different clusters. Two domains found to be conserved in *ExpB2* sequences of *Zea mays* with *Sorghum bicolor, Oryza sativa, Hordeum vulgare* and *Triticum aestivum* which are DPBB-1 super family and Pollen-allerg-1 super family.

A large number of conserved amino acid sequences were found when *metacaspase 2* sequences were aligned with *Oryza sativa*, *Hordeum vulgare*, *Triticum monococcum* and *Sorghum bicolor* sequences. Conserved and semi-conserved substitutions were also present in this case. In phylogenetic tree, ZmM2 was present very close to SbM2 and their sequence similarity rate was also maximum *i.e.,* 92.27%. Results showed that, CASc super family domain was present and conserved in all the aligned sequences of *Zea mays*, *Oryza sativa*, *Hordeum vulgare*, *Triticum monococcum* and *Sorghum bicolor*. The conserved CASc domain was present near to the N-terminus. The protein sequence of *metacaspase 3* was aligned with protein sequences of four other plants *i.e., Hordeum vulgare, Triticum aestivum, Setaria italica* and *Sorghum bicolor*. Alignment revealed conserved amino acid sequences with conserved and semi-conserved substitutions. The phylogenetic analysis showed that ZmM3 and SbM3 were present in the same cluster so highest similarity (94.34%) was found between these sequences. The least similarity of ZmM3 was found with SiM3 (50.18%). Single domain *i.e.,* CASs superfamily was observed to be conserved when *metacaspase 3* sequences were aligned with four other selected plants (*Hordeum vulgare, Triticum aestivum, Setaria italica, Sorghum bicolor*).

The alignment analysis of maize CC8 and CC9 was performed with same plants sequences *i.e., Cakile maritima*, *Lantana camara*, *Vigna unguiculata* and *Arachis hypogaea*. Few amino acids were conserved in all the plants. In the phylogenetic tree, maize formed an independent cluster showing that maize was least similar to the selected plants. Percent similarity index also confirmed this and the similarity percentages found to be very low. CY super family domain was identified and conserved in all the selected plants.

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Conflict of interest: The authors declare that they have no conflict of interest.

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