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**ORIGINAL ARTICLE**

# Expression analysis of boiling-stable protein (BsCyp) in response to drought, salt and osmotic treatments in drought tolerant and susceptible cultivars of *Triticum aestivum*

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The effect of osmotic, drought and salt stresses on the induction of boiling-stable proteins were studied in C-306 (drought tolerant) and HD-2004 (drought susceptible) cultivars of *Triticum aestivum*. Western blotting analysis, using polyclonal antibodies raised against a 20 kDa *A. thaliana* cyclophilin, revealed the induction of a cross-reacting band (40 kDa, BsCyp) in cv. 306. However no substantial expression was observed in drought sensitive cv. HD-2004, indicating its role in water stress adaptation. Thermostability studies of BsCyp in cv. 306 revealed that BsCyp was stable and can be detected even after 20 min of boiling treatment under all the stresses. Based upon these observations the possible role of BsCyp in water stress tolerance is discussed.

*Key words: Boiling-stable proteins, cyclophilin, water stress, wheat*

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Throughout their life cycle, plants are subjected to many adverse environmental conditions such as drought, heat, cold and flooding etc. that dramatically affect plant survival and limit productivity (Serrano et al. 1999). Most cultivated crop plants are highly sensitive and either die or display reduced productivity after they are exposed to long periods of stress. It was being estimated that two-thirds of the yield potential of major crops routinely lost due to unfavorable conditions (Chaves et al., 2003). Therefore increased drought-tolerance of crop plants has therefore become one of the major objective of the plant breeders on a worldwide scale. It was clear that plants under stress situations undergo a series of adaptive alternations in cell metabolism. These adaptive changes originate at molecular level. Different developmental stages of the plant growth including seed germination, seed maturation, and senescence, are differently affected in response to stress conditions (reviewed in Chaves

et al., 2003). Some environmental factors (such as temperature) can become stressful in a few minutes, others may take days to weeks (soil water) or even months (mineral nutrients) to become stressful. During stress, a plant adapts its metabolism and responds by leading to a verity of biochemical and physiological changes. To cope up with the physical and biochemical challenges accompanying water stress, plants are endowed with an array of protective mechanisms that act synergistically. They include synthesis of protective molecules, the ability to avoid free radical-induced injury during stress and the capacity to repress metabolism in a coordinated fashion (Leprince et al., 2000). Some of these stress-responsive genes encode regulatory proteins, soluble proteins, appearance of new isozymes; whereas others protect cells by causing the accumulation of metabolic proteins and cellular protectants including sugars (Ingram and Bartels 1996). These stress-induced responses enable the plant to adapt its physiology and survive. Stress induced proteins play a definite role in protecting plants from possible damage by these conditions. Concomitant to induced stress tolerance, protein metabolism of the cells undergoes changes in terms of acquiring specific stress proteins, which are either not detected or present in low amounts in the un-induced cells (Ingram and Bartels 1996). A growing body of evidence suggest that stress response involves synthesis of one set of proteins and degradation of the other (Serrano et al. 1999). Therefore, stress responsive changes in gene expression in general and protein profiles in particular have been targeted for intensive investigation. One of these mechanisms that may confer stress tolerance is the activation of a large set of genes, which leads to the accumulation of specific cellular proteins. Heat shock proteins (Hsps), cyclophilins (CyPs) and late embryogenesis abundant proteins (LEA) are the major groups of stress-induced proteins believed to exert cellular protection during stress (Chaves et al. 2003; Chou and Gasser 1997). These proteins also seem to respond similarly to the application of ABA (Chaves et al. 2003; Kullertz et al. 1999). Some drought stress-induced proteins (e.g. LEA, dehydrins) are also produced in response to various environmental stresses such as salt and cold and are highly hydrophilic and remain soluble even after boiling (Close et al. 1989), a characteristic that has been termed “boiling stability” (Close et al., 1989;Jacobsen and Shaw 1989). Some proteins also show heat stability but exits in cells under non stress conditions. It was suggested that some proteins like dehydrins accumulate along with LEA/ sugars and have been proposed to play an important role in membrane stability and osmotic adjustment (Close et al., 1989). Nucleotide sequence analyses of genes from several kingdoms including plant, bacteria and fungi have revealed the conservation of lysine-rich regions in these hydrophilins, thus, suggesting an evolutionary role for these cellular proteins during water-deficits (Arroyo et al. 2000). At present hundreds of genes induced under drought stress have been identified these may allow plants to adapt to water limiting conditions. Because plant responses to environmental stresses are complex and multigenic, the functions of many of the induced genes are still a matter of conjuncture (Bray 2002). These traits are mostly constitutive rather than stress-induced (Passioura 2002). Even some of the proteins detected in total protein extracts, under drought stress, are lost in the boiling-stable fractions (Pelah et al. 1995). Therefore, to better understand the role of these proteins in drought stress tolerance, it is a prerequisite to examine their expression not only under drought stress, but also after boiling. Thereafter, the sequencing of the relevant proteins

and cloning of the corresponding genes will generate probes for early selection of drought resistant genotypes. Most of the these proteins represent a diverse group of distinct proteins. Therefore, to assess the role of these proteins in drought stress adaptation it is imperative that variability in boiling stable proteins (BSPS) should be studied in stress tolerant and susceptible cultivars of a crops. In the light of these observations, the proposed study was undertaken to investigate the abiotic stress-induced changes in the expression of boiling stable cyclophilin (BsCyP) in the drought tolerant and susceptible cultivars of wheat so as to gain an insight into the physiological role of these proteins in water stress adaptation and the possible implication as a marker for drought stress tolerance. To facilitate the detection of BSPs, we focused on heat stable (HS) fractions that resist s coagulation upon heating at 100°C. By this method, the soluble protein extract containing hydrophilic proteins could enriched with BSPS and devoid of storage proteins.

## MATERIALS AND METHODS

### Seed germination and growth conditions

The seeds of *Triticum aestivum* L. cv C-306 (drought tolerant) and HD-2004 (drought sensitive) were procured from PAU Ludiahana, Punjab, India. Seeds were surface sterilized with 1% (w/v) mercuric chloride followed by 70 % (v/v) ethanol (Sharma et al., 2007). Seeds were thoroughly rinsed with deionized water and imbibed for 6 h. After imbibition, seeds were placed in Petri plates containing sterile filter sheets, moistened with water. The plates having seeds were incubated at 25 ±1°C in a seed germinator in darkness and allowed to grow for 4 days. The shoots were harvested and pooled for further analysis. Stress treatments were performed on 3 M Whatman filter paper. Drought stress was imposed by incubating the 3 days germinated seedlings on dry filter paper for 6-h and shoots were harvested for further analysis. Osmotic and salt stresses were imposed to 3 days germinated seedlings by irrigating petriplates with solutions of mannitol (0.75 M) and NaCl (0.42M). After treatments, shoots were harvested and stored at –80°C till further analysis. Tissue Water content (TWC) was measured after imposing stress treatments. Immediately tissues were sealed in a plastic bag and quickly transferred to the laboratory. Fresh weights were determined within 2 h after collection. Dry weights were obtained after oven drying the samples for 72 h at 70° C. TWC was calculated from given equation: TWC( %) = fresh weight-dry weight/fresh weight x 100.

### Analysis of boiling stable proteins

Tissue was homogenized with chilled mortar and pestle in extraction buffer (50 mM Tris-HCl, pH 7.0). Crude extracts were centrifuged at 10,000 g for 10 min. Heat stable (HS) fractions were obtained by boiling the crude extracts at 100°C for 10 min, kept on ice for 5 min, and centrifuged at 10,000 g for 10 min. After centrifugation the supernatant was resolved on SDS-PAGE on 12% (w/v) polyacryalamide gel and visualized by Coomassie brilliant blue as described in Sambrook et al. (1989). Total protein content of boiling stable fractions was determined by the Bradford method (Bradford 1976) using BSA as a standard.

### Western blot analysis

Western blotting analysis was carried out with antibodies (a gift from Dr C.S. Gasser) raised against a 20 kDa *Arabidopsis thaliana* cyclophilin. After electrophoresis, boiling stable proteins were electroblotted to a nitrocellulose membrane. Protein blots were reacted with anti-Cyp20 (1:200 dilution) and developed using an alkaline phosphatase-conjugated secondary antibody (1: 3000 dilution)

and 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt/*p*-nitroblue tetrazolium chloride reagent systems (Sambrook et al. 1989).

**Thermostability studies**

To check the thermostability of extracted BSPS, as described above, tissues were homogenized with mortar and pestle in extraction buffer [50 mM Tris-HCl, pH 7.0]. Heat stable (HS) extracts having equal protein were boiled at 100°C for 10 min, 20 and 30 min, and stored on ice for 10 min followed by centrifugation at 10,000 g for 10 min. Extracts were stored at -20°C for further analysis.

**Statistical analysis**

A statview ANOVA program was used for statistical analysis of the data. Values for different treatments were compared using one-way analysis of variance with repeated measures and student's *t*-test for differences between pairs of data if the ANOVA ($LSD_{0.05}$) revealed significance. Means were tested by LSD at P 0.05 level ($LSD_{0.05}$).

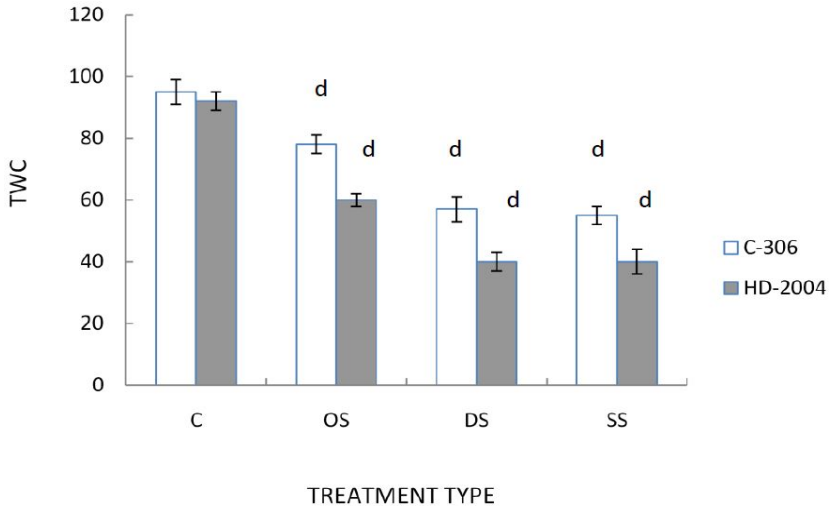


**Figure 1.** Tissue Water Content (TWC, %) of shoots of cv. C-306 (drought tolerant) and cv. HD-2004 (drought sensitive) under different stress treatments. Symbols: C-Control; OS: osmotic stress; DS: drought stress; SS: salt stress. Data shown are average ± SE of three replicates. [d]indicates significant difference vs control at P≤0.05

**RESULT AND DISCUSSION**

Alternation in protein expression is an important part of the ability of the plant to respond to the environmental stresses. Many gene products are induced or repressed by water stress (Ingram and Barteles, 1996). Several gene products that are induced by salt but differ in their response to dehydration, cold and, or heat (Ingram and Barteles, 1996). Therefore, to examine the effect of various abiotic stimuli like drought, salt and osmotic on BsCyp (45 kDa) expression in shoots of drought tolerant and sensitive cultivars of wheat, boiling stable protein (BSPS) fractions were run on a 12% SDS-PAGE and analyzed by immunoblotting. One of the differences in drought resistance between some resistant and sensitive plants is the difference in dehydration avoidance. Previously, Ristic et al (1991) have shown that drought resistant *Zea mays* cultivar had a much greater ability to avoid dehydration under drought and heat stress than sensitive cultivars, because decrease in tissue water content was less pronounced in tolerant cultivar than sensitive under stress conditions. We have also noticed similar observations, as upon imposition of drought, salt and osmotic stresses, a significant decrease in tissue water content was observed in

both the cultivars C-306 and HD-2004, indicating that seedling were under stress. However, cv. C-306 (tolerant) showed much greater capability of avoiding dehydration than cv. H-2004 (susceptible), as indicated by TWC (Fig 1). In cv. C-306, several boiling-stable polypeptides (20 kDa-100 kDa) were induced under osmotic, drought and salt treatments, however, no substantial expression of boiling-stable proteins was observed in the control seedlings (Fig. 2). In cv. C-306, as compared to control, western blot analysis using anti-Cyp20 detected a strong cross-reacting protein band at about 40 kDa (BsCyp) under all the abiotic stimuli. However the expression of BsCyp was significantly higher under drought stress than osmotic and salt stresses (Fig 2A). Further, common responses to different stresses may indicate similar functions of stress-responsive gene products for plants under stress conditions involving water deficit.

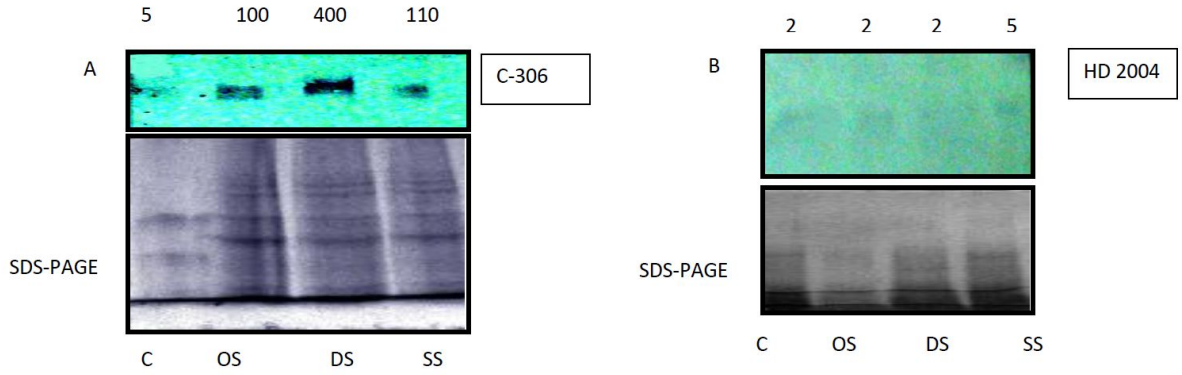


**Figure 2 A**: An SDS-PAGE profile of boiling stable proteins and immunoblot analysis of BsCyp in the shoots of C-306 (drought tolerant) cultivars of wheat after osmotic (OS), drought stress (DS) and salt stress (SS) treatments. Numerical values as shown in the top of Panels, indicates relative band intensities, which were determined using Gel Visualization, Documentation and Analysis system (Bio-Rad, USA). Numerical comparisons are only valid within panels and cannot be made between panels. Each lane loaded with 60µg of boiling stable proteins was resolved on 12% SDS-PAGE and transferred to nitrocellulose membrane and probed with anti-CyP antiserum.

**B**: An SDS-PAGE profile of boiling stable proteins and immunoblot analysis of BsCyp in the shoots HD-2004 (drought sensitive) cultivars of wheat after osmotic (OS), drought stress (DS) and salt stress (SS) treatments. Each lane was loaded with 60 µg of boiling stable proteins. Upper panel on the top of the each figure indicates cyclophilin activity.

Moreover, BsCyp was present in all the heat stable (HS) protein fractions, indicating that BsCyp remains soluble after boiling (Fig. 2B). However, in cv. H-2004, very low or barely detectable cross-reacting protein bands were observed under all the abiotic stimuli, suggesting cultivar specific induction of BSPS. Differences in the expression of specific gene products between stress-sensitive and stress tolerant cultivars indicate that tolerance is conferred by genetically encoded mechanisms (Bray, 1993) so, it is reasonable to expect the inter- and intra-specific differences in the pattern of protein synthesis between plants which differ in their stress resistance.

Notably, no other lower or high molecular mass protein band was recognized by the anti-Cyp antibodies in both the cvs. C-306 and H-2004, suggesting the induction of several other BSPS other than cyclophilins. It has been reported earlier in barley, corn and wheat that several LEA and

dehydrins are induced or accumulate during heat shock and drought stress treatments and remain soluble in aqueous solution during boiling (Close et al. 1989; Danyluk et al. 1991; Jacobsen and Shaw 1989). In cereals, it was reported that the expression of high molecular weight heat stable polypeptides was relatively high but did not cross react with antibodies against dehydrins or RAB (responsive to ABA) (Knight et al. 1995). Rurek (2010) also reported diverse accumulation of high and low molecular weight boiling stable proteins during time course of seedling development as well under abiotic stress conditions.

Further, to gain insight into the thermostability of BSPS expression under abiotic stresses in drought tolerant cv. C-306, western blot analysis was carried out using anti-Cyp20 antibody. The samples analyzed in Fig. 3 were subjected to boiling treatment (100°C) for 10, 20 and 30 min prior to electrophoresis and western blot analysis. Interestingly, BsCyp can be detected even after 30 min of boiling, in drought stress- and osmotic stress-induced proteins, indicating thermostable nature of BsCyp. However, BsCyp band disappeared after 30 min of boiling in the salt stress-induced proteins. So, by virtue of its hydrophilicity, BsCyp belongs to the broad family of boiling-soluble proteins, including those associated with cellular dehydration, either as a result of environmental stress (dehydrins), or during normal seed desiccation (LEA proteins)(Dure et al. 1989). From these observations it is suggested that like other stress regulated proteins (LEA/dehydrins proteins), BsCyp may be playing a significant role in water stress tolerance in drought tolerant cv. C-306, but not in drought sensitive cv. HD-2004. This suggestion has been strengthen by demonstrations that introduction of LEA gene into rice conferred tolerance to drought and salt stresses (Xu et al., 1996). Earlier studies indicated that overexpression of Arabidopsis homolog of MtPM25 in germinating seeds led to improved growth under high NaCl, KCL and sorbitol conditions (Borrell et al., 2002). Liu and Zheng (2005) also reported similar findings by overexpressing PM2 in *E coli*. Recently, Zhu et al. (2007) has reported heat protection in *Arabidopsis thaliana* by overexpressing Aspen sp1. Transgenic rice (TNG67) plants expressing a wheat LEA group 2 protein (PMA80) gene or the wheat LEA group 1 protein (PMA1959) gene resulted in increased tolerance to dehydration and salt stresses (Cheng et al. 2002). In our study, it is plausible that by virtue of chaperonic activity of cyclophilins (Boston et al., 1996), BsCyp may be helping other stress induced proteins to maturation besides regulating the expression of other genes imparting stress tolerance. Due to their hydrophilic nature, cyclophilins may also function specifically in the protection of membranes and proteins against desiccation damage, possibly by binding water tightly or providing hydrophilic interactions in the absence of free water and by preventing the crystallization of cellular components through their ability to act as stabilizing “solvents’ (Close et al., 1989). Another possible role of BsCyp may be is to bind with the accumulated ions (ion sequestering) under water stress and to control solute concentration in the cytoplasm. Earlier studies indicated that at high moisture contents, some BSPS like LEA proteins act as compatible solutes that preferentially exclude chaotropic agents (such as salts) from the surface of macromolecule (Liu and Zheng 2005). Likewise when hydration shell is removed they might exert their protective effects in the dry state by replacing water molecule by hydrogen bonding and/or forming a glass which stabilizes the system in the dried state (Wolkers et al., 2001).

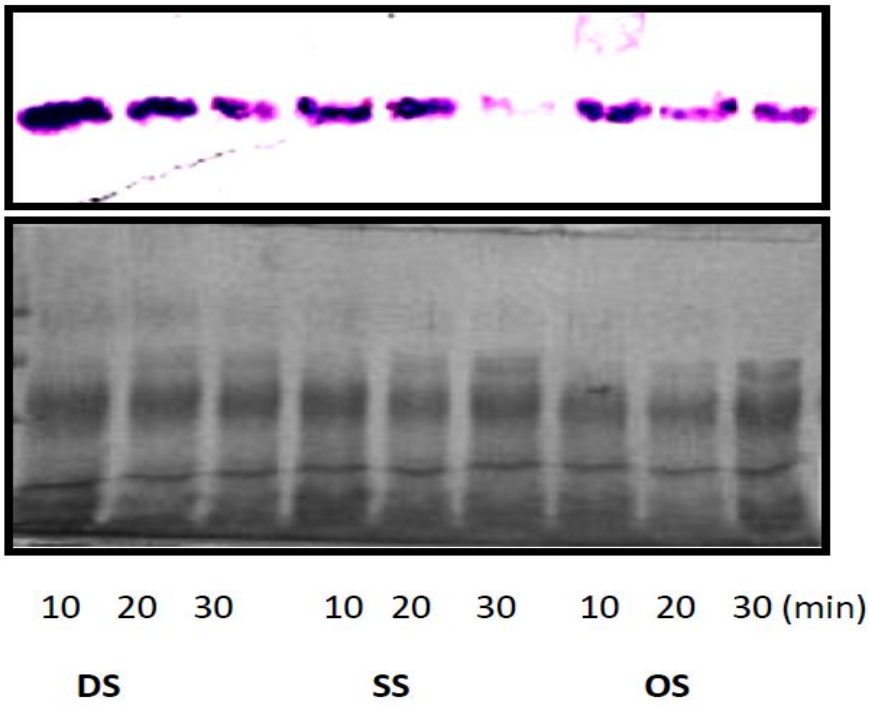


**Figure 3.** Thermostability analysis of BsCyp of drought tolerant cv. C-306. Heat stable (HS) extracts having equal protein were boiled at 100°C for 10 min, 20 and 30 min and subjected to western blot analysis.

In conclusion, BsCyp shares characteristics with two major groups of stress-responsive proteins. They are hydrophilic and remain soluble upon boiling like LEA-type proteins, representing a new class of plant proteins involved in the plant's responses to abiotic stress. Expression analysis of BsCyp from drought tolerant and susceptible cultivars revealed the potential of this protein as a marker for drought tolerance. Likewise, Houde et al 1992 also has made a mention of WCS120 hydrophilin, boiling stable protein as a potential tool for breeders to select for freezing tolerance traits.

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