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ORIGINAL ARTICLE

Low Temperature Stress Induced Changes in Biochemical Parameters, Protein Banding Pattern and Expression of Zat12 and Myb Genes in Rice Seedling

Salma Perveen[1], Kamran Iqbal Shinwari[1], Mehmood Jan[1], Ijaz Malook[1], Shafiq Rehman[2], Murad Ali Khan[3] and Muhammad Jamil[1]*

[1] *Department of Biotechnology and Genetic Engineering, Kohat University of Science & Technology (KUST), Kohat 26000, Pakistan*
[2] *Department of Botany, Kohat University of Science & Technology (KUST), Kohat 26000, Pakistan*
[3] *Department of Chemistry, Kohat University of Science & Technology (KUST), Kohat 26000, Pakistan*

*E-Mail: ***dr.jamil@kust.edu.pk**; ***jamilkhattak@yahoo.com***

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Low temperature stress is one of the main abiotic factors that reduce the productivity of many crops in hilly areas around the world. In this study, rice seedling were exposed to low temperature stress (control, 0°C, -2°C, -4°C and -6°C) for 2 hr to observe its effect on two rice varieties (Basmati-385 and Shaheen Basmati) through ion and proline contents, photosynthetic pigments, total protein content, protein banding pattern and expression of Zat12 and Myb genes. Resulted showed different patterns of accumulation of Na^+ K^+ and Ca^{+2} ions with the decrease in temperature in both varieties. Proline accumulation was gradually increased in both varieties with the decrease in temperature. Photosynthetic pigments (Chlorophyll (Chl) a, b and carotene) were negatively affected by low temperature stress in both varieties, however, carotene content was much affected than Chl a and b. Nonsignificant variation in protein contents was observed at all levels of low temperature, but the effects of low temperature stress on protein banding pattern of Basmti-385 and Shaheen Basmati were different at different treatments. RT-PCR results indicated that *ZAT12* was upregulated by short term low temperature stress while *OsMYB* show slight upregulation at -2°C as compared to the other treatments. This study identified that *ZAT12* and *OsMYB* function as a positive regulator to mediate tolerance of rice seedlings at low temperature stress.

Key words: low temperature, biochemical parameters, protein, gene expression, Oryza sativa L.

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Both abiotic and biotic stresses severely affect the rice production in many parts of the world. Biotic factors include insect, pest and diseases while drought, temperature, salinity, cold, pollution and soil pH refer to abiotic stresses (Bashier *et al*., 2007; Oerke *et al*., 2001). Abiotic stresses severely effect production of crops by inducing a series of biochemical and molecular changes (Hasegawa *et*

al., 2000).

Low temperature stress is one of the major abiotic factor that limit the agricultural productivity of crops in hilly areas. Plants respond and survive under stress conditions by bringing changes at the molecular and cellular levels as well as at the biochemical and physiological levels (Xin and Browse, 2000). Low temperature stress inhibits seedling establishment effecting early growth stages of rice and resulting in poor crop maturation. In order to gain stable rice production cold tolerance at the seedling stage is an important character. One of the most effective ways to avoid the low-temperature damage is to develop cold-tolerant genotype (Lou *et al*., 2007; Law and Brandner, 2001). Mineral nutrition acquisition and assimilation are strongly influenced by both high and low temperature stress in plants (Rivero *et al*., 2006). Some essential nutrients such as nitrogen (N), sulfur (S) phosphorus (P), magnesium (Mg), calcium (Ca) are structurally important for the proteins, nucleic acids, chlorophylls, certain secondary metabolites and defense related micro and macromolecules, while others have both structural and functional roles (Epstein and Bloom, 2005; Taiz and Zeiger, 2006).

Genes expressed under stress conditions may increase tolerance to growth retardation, cold, high salt concentration during normal conditions in rice transgenic and Arabidopsis (Dubouzet *et al*., 2003; Ito *et al*., 2006). It has been determined that in comparison with other cereals like wheat and barley, rice is more sensitive to cold stress (Wen *et al*., 2002). Low temperature stress induces many genes, acting either as protectants towards stress-induced damage or regulates expression of other genes and transduce signal (Kang *et al*., 2002; Yu *et al*., 2002; Shinozaki *et al*., 2003). Davletova *et al*. (2011) investigated that in contrast to many signaling and regulatory genes that are stress specific, Zat12 which is a zinc-finger protein gives response to various abiotic and biotic stresses and plays an important role in abiotic stress signaling in Arabidopsis. Similarly, MYB transcription factors play a central role in plant growth and respond to stress conditions (Yang *et al*. 2011). OsMYB2 encodes a MYB transcription factor (stress-responsive element), which is involved in regulation of various pathways leading to tolerance of rice to dehydration stress, salt and cold.

Low temperature exposure often induces a variety of biochemical, physiological and enzymatic change in plant, which can result in an acclimation response (Howard and Ougham, 993; Hughes and Dunn, 1996). Rice is sensitive to chilling stress and its persistence leads to poor germination, stunted seedling, yellowing or withering and decreased tillering (Mukhopadhyay *et al*., 2004). In this study, we had exposed the rice seedling to short duration low temperature to investigate its effect on ion contents, proline, photosynthetic pigments, total protein and nitrogen content, protein banding pattern and expression of Zat12 and Myb genes.

MATERIALS AND METHODS

Two varieties of rice, Shaheen Basmati and Basmati- 385 were obtained from the National Agriculture Research Center (NARC) Islamabad.

Seedling growth

Ten days old seedlings were transferred to plastic pots containing well washed sand and irrigated with Hoagland solution. After three weeks, the seedlings were subjected to low temperature stresses (control, 0°C, -2°C, -4°C and -6°C) for 2 hours in ultra low freezer and the fresh leaves of each replica were cut with sterile scissors and the

pieces of leaves were divided into two parts. The plant material was used for the following tests.

a) Biochemical analysis

b) Molecular analysis

a) Biochemical analysis

Determination of Na^+, K^+ and Ca^{+2} ions

Dried plant material (25 mg) was digested with the help of H_2SO_4 and H_2O_2. The digested material was diluted with 20 ml of distilled water and filtered with the help of Whattman filter paper. Ionic concentrations were determined with the help of flame photometer (Model:Jenway PF5).

Proline analysis

Proline quantification was done following the protocol described by Bates *et al.* (1973) with a slight modification. Fresh plant material (100 mg) was crushed in liquid nitrogen and homogenized in 5 ml of 3% salfosalicylic acid. The homogenate (~1 ml) was mixed with 1 ml of acid ninhydrin for 1 hour at 100 °C in oven. The reaction mixture was vigorously mixed with 2 ml of toluene and placed at room temperature for 30 minutes. The optical density was measured spectrophotometrically at 520 nm using toluene as a blank.

Determination of Chlorophyll and Carotenoids

Lichtenthaler and Wellburn (1985) method was used for the determination of chlorophyll and carotenoids content. Dried plant sample (25 mg) was taken in a test tube. Equal amount of MgO (25 mg) was added to neutralize plant acid and prevent the formation of pheophytin. The turbid pigment extract was centrifuged for 5 minutes at 4000 rpm at room temperature. The absorbance reading was taken against a solvent blank in a (UV-VIS) spectrophotometer at three wavelengths: 666 nm, 653 nm and 470 nm. The chlorophyll "a", "b" and carotenoids in methanol were calculated by the formulas suggested by Lichtenthaler and Wellburn (1985).

Determination of Total Protein Content

Protein content was determined by using the method of Pellett and Young (1980). Dried plant material (100 mg) was taken in a digestion flask containg 100 mg of digestion mixture (potassium sulphate, copper sulphate and iron sulphate) and 2 ml of conc. H_2SO_4. The digest was transferred to the distillation assembly and 10 ml of 50 % NaOH solution was added to the digestion mixture. The distillation was completed in 3 minutes with the change of color of boric acid to colorless. The boric acid having the trapped ammonia was titrated with 0.1N H_2SO_4. The color of boric acid having ammonia changed again into pink. Total protein content was calculated by using the formula's recommended by Pellett and Young (1980).

b) Molecular analysis

Protein extraction

Proteins were analyzed by SDS-PAGE (Sodium dodecyl sulfate-Polyacrylamide Gel Electrophoresis) using the method described by Laemmli, (1970). The plant material (10 mg) was crushed in the presence of liquid nitrogen and homogenate it in 400 ul protein extraction buffer ((Tris-HCL 0.5 M (pH 8), 2.5% SDS, 10% Glycerol, 5% 2-merceaptoethanol). In order to purify the homogenate, samples were centrifuged at 13000 rpm for 10 minutes at room temperature. The supernatant was transferred to new 1.5 ml Eppendorf tubes and stored at 4°C until they were electrophoresised on the PAGE.

Electrophoresis was performed at 80 V for 2 and half hours by using Standard Twin Mini Gel Unit (USA). The gel was stained with Coomassie Brilliant Blue R-250 (Sigma) for 40 minutes and destained

with 20% methanol, and 5% acetic acid. The molecular weight of protein subunits was compared with standard protein molecular weight marker bands (Benchmark Protein Ladder 10 – 220 KDa) in the electrophorogram.

PCR amplification of the desired genes

Plant leaves (140 mg) were crushed in liquid nitrogen. RNA was extracted by using Trizole reagent. cDNA was synthesized with MMLV reverse transcriptase enzyme by using Oligo (dT) primer After cDNA synthesis, the genes were amplified by gene/s-specific primers (Table 1). The following conditions of PCR were used: pre denaturation of 5 min at 95 °C; 45 cycles of 20 Sec at 95°C, 30 Sec at 60°C, 40 Sec at 72°C and a final extension for 15 min at 72°C. The amplified gene/s was separated on 1.5% agarose gel.

Statistical analysis

All the data were analyzed by using statistical software Statistix 9 for ANOVA, followed by least significant difference (LSD).

RESULTS

Biochemical parameters

Ions contents (K^+, Na^+ and Ca^{+2})

Results indicated different pattern of accumulation of Na^+ K^+ and Ca^{+2} ions with the decrease in temperature in both varieties (Fig. 1). In Basmati-385, K^+ ion concentration was increased from control (40 ug/g) to -6°C (43.2 ug/g) (Fig. 1A), while in Shaheen Basmati, the concentration of K^+ ion was maximum at -4°C (45.4 ug/g) under low temperature stress (Fig. 1B). For Na^+ ions, Basmati-385 showed the lowest level (20.2 ug/g) at -6°C (Figure 1A). Shaheen Basmati also showed reduction in the Na^+ ion concentration except at -6°C as compared to control (Fig. 1B). In Basmati-385, Ca^{+2} ion was gradually decreased except at 0°C. In Shaheen Basmati, there was a steady increase in Ca^{+2} ion concentrations from -2°C (8.766 ug/g) to -6°C (15.55ug/g) with the decrease in temperature (Fig. 1B).

Proline content

The results of our study indicated that with the decrease in temperature, the proline accumulation was gradually increased in both varieties (Fig. 2 A, B). In Basmati-385, the maximum amount of proline was found at -6°C (0.65125 µg/g) as compared to control (0.2075 ug/g) (Fig. 2A). In Shaheen Basmati, the amount of proline was increased from control (0.29 ug/g) to -6°C (0.6915 µg/g) under low temperature stress (Fig. 2B).

Chlorophyll Contents

The concentrations of photosynthetic pigments were negatively affected by low temperature stress in both varieties (Fig. 3). In Basmati-385, chlorophyll 'a' content had a maximum value (5.731 ug/g) at -2°C and the lowest value (4.64 ug/g) was seen at -4°C (Fig. 3A). Chlorophyll 'b' content was also decreased under low temperature stress in Basmati-385, showing the lowest value (4.1 ug/g) at 0°C (Fig. 3A). Under low temperature stress, carotene content was gradually decreased from control (5.38 ug/g) to -6°C (1.1 ug/g) in Basmati-385 (Fig. 3A). In Shaheen Basmati, the chlorophyll 'a' content had the minimum value at 0°C (4.33 ug/g), while chlorophyll 'b' content was decreased from control (5.57 ug/g) to -6°C (4.0 ug/g) while carotene content showed minimum concentration at 0°C (3.8 ug/g) (Fig. 3. B).

Protein content

Nonsignificant variation in protein contents was observed at all levels of low temperature treatments in Basmati-385 (Fig. 4.). However in Shaheen Basmati, low temperature stress increased

the protein content at -2°C as compared to control while the other treatments showed the same level of protein content as in control in Shaheen Basmati under low temperature stress (Fig. 4).

Molecular analysis

Protein analysis by SDS-PAGE

The effects of low temperature stress on protein profile on both varieties were different. Protein banding pattern of Basmti-385 and Shaheen Basmati was between 15kDa and 40kDa with reference to the marker. The banding intensity of different polypeptide bands (35, 30, 28, 20 and 15kDa) of Shaheen Basmati was higher than Basmati-385 under low temperature treatment. In Basmati-385, the intensity of these bands was much lower between 15 KDa and 30 KDa at -4 °C as compared to control, however at -6 °C bands between 15 KDa and 30 KDa showed high intensity than other low temperature treatments. In Shaheen Basmati under low temperature stress, there was an induction of the band at 35 KDa which was absent in Basmati-385. It has been observed that in Shaheen Basmati, the intensity of protein bands (35, 30, 28, 20 and 15kDa) was greater in treated plants as compared to untreated plants (Fig. 5).

Expression of Zat and Myb genes

Reverse transcription (RT)-PCR approach was used to find the expression of four stress inducible genes (*ZAT12-1, ZAT12-9, OsMYB2 and OsMYB6*) in Basmati-385 and Shaheen Basmati (Fig. 6) There was a significant difference in gene expression between these two varieties.

In Basmati-385 the expression of ZAT12-9 was upregualted than ZAT12-1 under low temperature treatments. However, ZAT12-1 was slightly expressed at -2°C and -4°C, while there was no expression at -6°C. ZAT12-9 was highly expressed at 0°C, -2°C and -6°C, however no expression was observed at -4°C. In case of *OsMYB2* and *OsMYB6* both showed slight upregulation at -2°C as compared to the other treatments. In Shaheen Basmati, *ZAT12* genes showed high upregulation than *MYB* genes under low temperature treatments. *ZAT12-9* was strongly upregulated than *ZAT12-1*, however *ZAT12-9* was down regulated at -2°C as compared to other treatments. *OsMYB2* showed high upregulation than *OsMYB6* under low temperature stress. *OsMYB2* showed slight expression at 0°C and -2°C and highly expressed at -6°C while *OsMYB6* did not give any expression at control (Fig. 6).

Table 1. Sequences of forward and reverse primers used for amplification of cDNA

Genes	Forward primers	Reverse primers
Actin	5'- TGCTATCCCTCGTCTCGACCT-3'	5'-CGCACTTCATGATGGAGTTGTAT-3'
OsMYB2	5'-CGTGCTTGGATTGGTACGAG-3'	5'-TCAGGCTGTGGCACAGCCCG-3'
OsMYB6	5'-TCCAGCTTTCCAGTTCTTGG-3'	5'-GAAGGTGTAATCCATGGCCG-3'
ZAT12-1	5'-GATCGGCGACGTTAGTGAT-3'	5'-AAGGAAACAATCCAACATGG-3'
ZAT12-9	5'-GCTCGTCATTAAGAGCGAAA-3'	5'-TACAAGAAGGTTAAGTAACT-3'

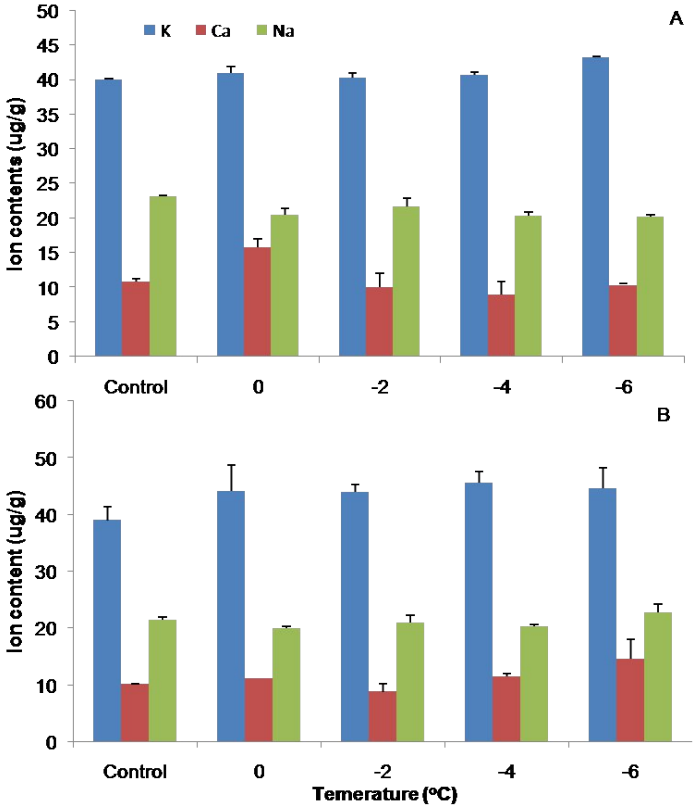

Figure 1. Effect of short term low temperature treatments on K^{+}, Na^{+} and Ca^{+2} ions concentrations of Basmati-385 (A) and Shaheen Basmati (B). Vertical bars are the means of three replication±SD.

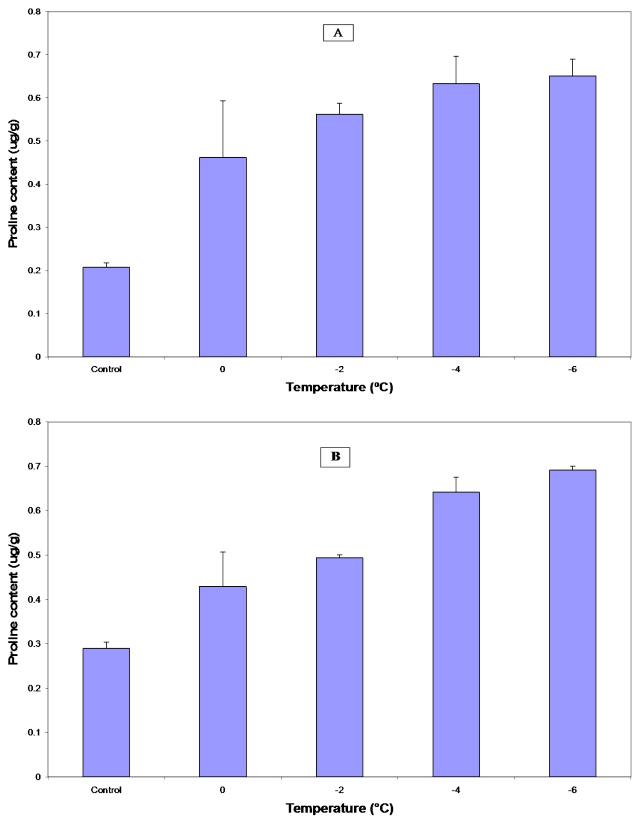

Figure 2. Effect of short term low temperature treatments on proline content of Basmati-385 (**A**) and Shaheen Basmati (**B**). Vertical bars are the means of three replication±SD.

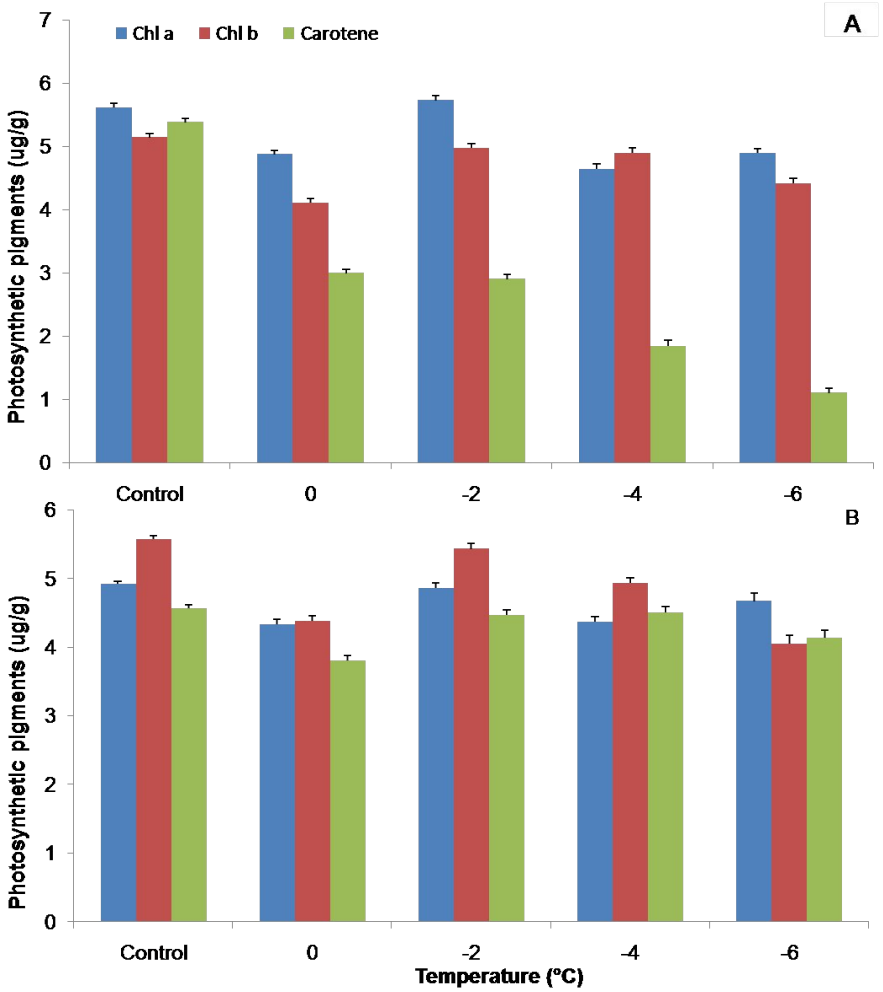

Figure 3. Effect of short term low temperature treatments on Chlorophyll "a, b and carotenoids" contents of Basmati-385 (**A**) and Shaheen Basmati (**B**). Vertical bars are the means of three replication±SD.

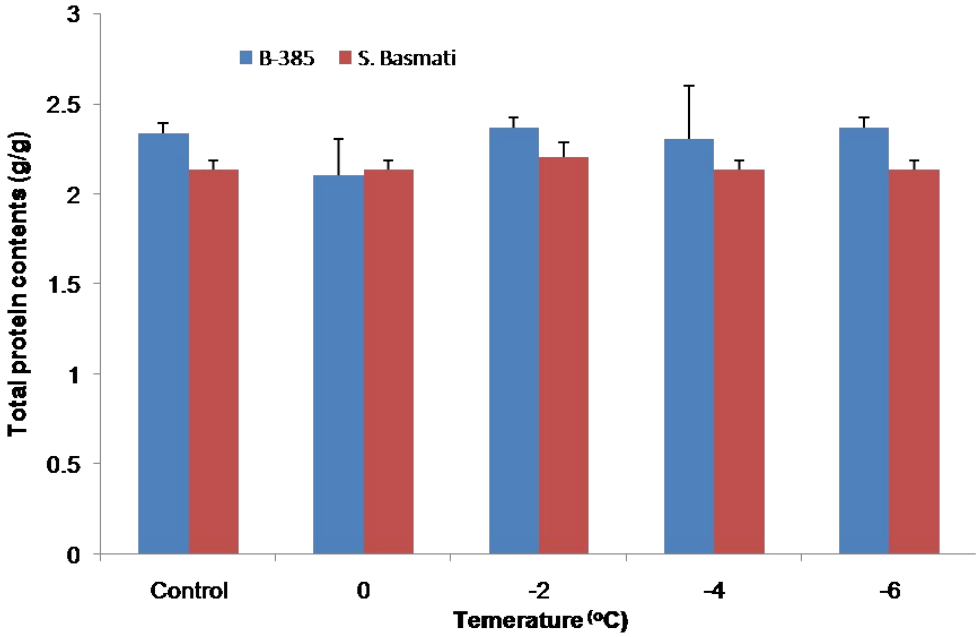

Figure 4. Effect of short term low temperature stress on total protein contents of Basmati-385and shaheen Basmati. Vertical bars are the means of three replication±SD.

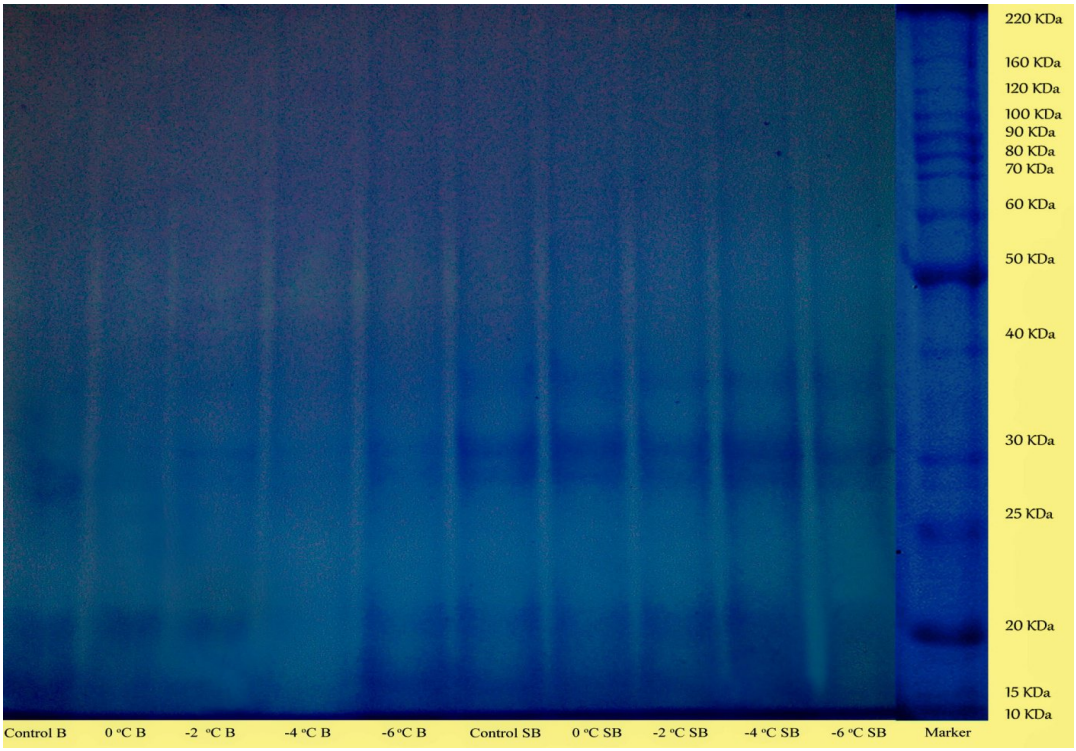

Figure 5. Effect of short term low temperature treatments on protein profile of Basmati-385 and Shaheen Basmati. B represent Basmati-385 while SB for Shaheen Basmati.

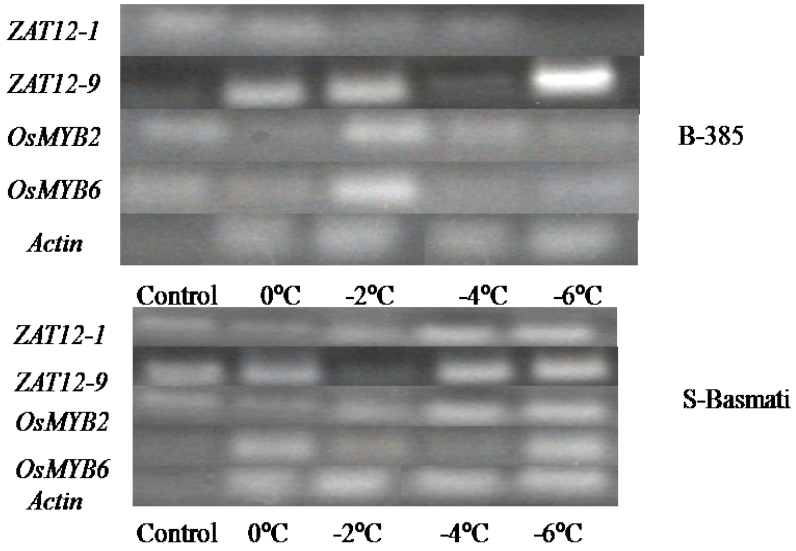

Figure 6. Gene expression of *ZAT12-1, ZAT12-9, OsMYB2, OsMYB6* in Basmati-385 and Shaheen Basmati under short term low temp treatments.

DISCUSSION

It has been reported that mineral nutrition acquisition and assimilation is strongly influenced by both high and low temperature stress in plants (Taiz and Zeiger, 2006). Our study indicated that the accumulation of ions were different at different low temperature treatments. (Fig. 1). Reduction in Na^+ ion helps the plant in the accumulation of other useful compounds. Tuteja and Sopory, (2008) investigated that Na^+ ions maintain membrane potential and K^+ ions keep osmotic balance and act as cofactor for many enzymes but under stress conditions their concentrations change significantly due to increase in electrical conductivity. In plants,

Ca^{+2} ions have role acting as important messengers under the low temperature signaling pathway. Komatsu *et al*. (2007) suggested that Ca^{2+} is increased by cold- stress treatment in the cytosol. Similarly in our study, we had observed that the overall Ca^{2+} content increased in both varieties (Fig. 1). According to Plieth *et al*. (1999), cold-induced Ca^{2+} influx in plants following a rapid temperature drop while Vergnolle *et al*. (2005) determined that calcium signal amplification might be involved in cold-stress signaling.

Proline is one of the main components of defense mechanism of the plant and its accumulation occurs in plant leaves towards abiotic stresses (Shamseddin-saeid and Farahbakhsh, 2008). It has been suggested that proline acts as a signal/regulatory compound which facilitates various physiological or molecular processes (Nowak *et al*., 2010). Our study showed that proline accumulation increased by decreasing low temperature stress, which seems to be associated with adaptation to temperature stress (Fig. 2). Similar patterns of results were also found by Koc *et al*. (2010), they investigated that chilling effectively enhance the proline accumulation under chilling stress conditions. Proline has been shown to be one of the major factors impairing freezing tolerance (Habibi *et al*., 2011).

Reduction of photosynthesis by low temperature is a well known response of chilling sensitive plants and it has been reported that chlorophyll 'a' and 'b' content decrease when plants are subjected to cold stress (Yadegari *et al*., 2007). In our results, slight decrease in photosynthetic pigments was observed under low temperature stress in both varieties as compared to their control values (Fig. 4). The possibility of low chlorophyll and carotenoid content could be oxidative stress caused by low temperature treatment (Yadegari *et al*., 2007). Our results have been confirmed by the research of Aghaee *et al*. (2011) that total chlorophyll concentration of rice leaves was reduced under cold treatment. Habibi *et al*. (2011) observed that the low temperature inducted significant decreases in the chlorophyll a and b content as a result of the total chlorophyll content of leaves.

In this study, we had observed the same level of protein content as compared to control all the treatments (Fig. 4). However, in Shaheen Basmati, low temperature caused an increase in proteins contents at -2°C as compared to control and other treatments (Fig. 4). Kazemitabar *et al*. (2003) subjected rice seedlings of different inbred lines to low temperatures for short periods i.e. for 6 hours at -2°C or -1°C. They found that changes in total leaf protein were detected but no clear difference was observed between susceptible and tolerant individuals. Lee *et al*. (2009) suggested that gene expression at the mRNA level of some selected proteins is not always concomitant to the translational level. Koc *et al*. (2010) investigated that synthesis of specific proteins is an important mechanism involved in increasing cold tolerance. As the present study indicated no significant increase in total protein content, therefore SDS page analysis was done to investigate the protein banding pattern. Protein banding patterns of Basmti-385 (Fig. 5) were between 30 kDa and 15 kDa with reference to the marker under low temperature stress. Different band positions between Shaheen Basmati and Basmati-385 indicated their specific response towards the given low temperature stress (Fig. 5) and also it reflects their different genetic variation. Kazemitabar *et al*. (2003) developed a method to expose rice seedlings of different inbred

lines to low temperatures for short periods i.e. for 6 hours at -2°C or -1°C. The changes in total leaf protein were detected but no clear difference was observed.

It has been observed that genes expressed under stress conditions may increase tolerance to cold, high salt concentration during normal conditions in rice transgenic and Arabidopsis (Dubouzet *et al*., 2003; Ito *et al*., 2006). *ZAT12* was unregulated in response to different low temperature stress (Iida *et al*., 2000; Cheong *et al*., 2002; Fowler and Thomashow, 2002; Kreps *et al*., 2002; Rizhsky *et al*., 2004; Davletova *et al*., 2005). These findings were similar to our study that *ZAT12* was upregulated by short term low temperature stress (Fig. 6). Recently it has been determined using transgenic plants that *ZAT12* is involved in cold acclimation and in the response of plants to oxidative stress (Rizhsky *et al*., 2004).

In case of Basmati-385, *OsMYB2* and *OsMYB6* both show slight upregulation at -2°C as compared to the other treatments while in Shaheen basmati, *OsMYB2* showed more expression than *OsMYB6* (Fig. 6). Yang *et al*. (2012) also found an increase in the *OsMYB2* transcript when rice seedlings were exposed to low temperature (2°C). This study identified that *ZAT12-1, ZAT12-9, OsMYB2, OsMYB6* function as a positive regulator to mediate tolerance of rice seedlings to low temperature stress. The up-regulation of these genes may allow rice plants to effectively osmo-regulate their water potential by accumulating compatible solutes and minimize oxidative damage to plants under low temperature stress.

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