WHY ZAT12 IS CRUCIAL IN STRESS TOLERANCE IN WHEAT TRANSGENICS?

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Abstract - Zat12 encodes a zinc-finger transcription protein and play a significant role in modulating defense and acclimation responses in plants. In order to make the wheat crop tolerant to abiotic stress conditions, Zat12 transformed wheat lines were produced in the background of PBW 621 by agrobacterium mediated gene gun method. To gain indepth knowledge of Zat12 related biochemical activities in stress tolerance, expression of Zat12 gene was analyzed through RT-PCR. Activities of antioxidative enzymes (SOD, POD, GR, GST) alongwith proline and ascorbate content have been studied under non stress and stressed conditions (heat and cold) in transformed lines (Z-8-12 1A, Z-8-12 1B, Z-8-19 and Z-15-10) and wheat cultivars (PBW 621, PBW 550 and HD 3086). It was observed that Zat12 transformed lines led to stimulation of antioxidative defence system, overexpression of antioxidative enzymes was reported under temperature stress in Zat12 transformed wheat lines.

Keywords - transcription factor, proline, ascorbate, superoxide dismutase (SOD), peroxidase (POD), glutathione reductase (GR), glutathione-s-transferase (GST), antioxidative enzymes

I. INTRODUCTION

Wheat (Triticum aestivum L.), is one of the most important staple food crops grown throughout the world with yearly production of about 754.1 million tonnes. Its contribution is about 20% of the total dietary proteins and calories worldwide (Shiferaw et al 2013). Clearly wheat and its products signify a key role in managing food economy of a country. But some wheat growing area is prone to abiotic stresses, being the greatest restraint to production and yield of crop.

Annually about 42% of the global crop productivity is lost owing to these stresses. Several members of the gene families such as zinc finger protein Zat12 in Arabidopsis thaliana (Eulgem et al 2000) were found to regulate diverse process in response to abiotic stress and make it to grow on rocky, sandy and disturbed terrains. A major challenge for current agricultural biotechnology is to satisfy an ever increasing demand in food production facing a constantly increasing world population (Godfray et al 2010, Tester and Langridge 2010). Consequently, development of stress tolerant cultivars is of major concern in wheat breeding programme.

The plant stress tolerance can be improved by various means, the most important of which include the use of transgenics. So, in order to make wheat crop abiotic stress tolerant, Zat12 gene was extracted from Arabidopsis thaliana and wheat transformants were generated using Agrobacterium and particle gun mediated transformation in embryo derived tissues of wheat variety PBW 621. The gene Zat12 encodes a zinc-finger transcription protein (Gupta et al 2013) and Zat 12 has since been found to respond to environmental stress from heat, cold (Fowler et al 2002), heavy metals (Beilen et al 2013), light and UV radiation. As a key transcription factor involved in the activation of ROS-related antioxidant system, Zat12 play a significant role in modulating defense and acclimation responses in plants (Mittler et al 2006). ROS that accumulate in the cytosol sensed by different redox response transcription factors that include members of the zinc finger protein Zat family (Zat12, 10 and 7) (Ciftci-Yilmaz et al 2007, Davletova et al 2005).

Since, Zat12 as a transcription factor, improves stress tolerance, it is quite possible that transformed PBW 621 wheat genotypes might result in switching on some metabolic processes in order to combat oxidative stress. Improvement in plant growth and development under different stresses due to zinc finger protein Zat12 has been observed in some transgenic crops like transgenic Brassica lines for zinc finger protein with LEA promoter showed increase in shoot length over wild type under abiotic. It has also been reported that Zat12 from Brassica carinata when transferred to tomato (Chandra et al 2013) conferred tolerance to abiotic stress conditions. Cheong et al (2002) suggested that Zat12 is concerned in the plants' response to stress as improved expression of Zat12 gene was reported under several abiotic stress conditions. Hence, the improved expression of Zat12 gene in response to various stress conditions makes Zat12 an attractive subject for biochemical analysis.

In the present study, we evaluated the biochemical parameters for stress tolerance in Zat12 transformed lines at early stage of seedling (root, shoot) i.e. 3 week and 6 week and under temperature stress to see how antioxidative defence system activated under unfavorable conditions. Hence this article investigates the stress tolerant behavior of Zat12 gene based on antioxidative defence system.

II. DETAILS EXPERIMENTAL

2.1. Materials and Procedures

Seeds of three wheat (Triticum aestivum L.) cultivars, PBW 621, PBW 550, HD 3086 were obtained from the Department of Plant Breeding and Genetic, Punjab Agricultural University, Ludhiana, Punjab, India. Four Zat12 (Z-8-12 1A, Z-8-12 1B, Z-8-19, Z-15-10) homozygous lines in the background of PBW 621 were used to represent transgenic lines. Seeds were surface sterilized with 0.1 % $HgCl_2$ for 2 mins, rinsed thoroughly with distilled water several times and germinated in dark for 24 h at 25 °C in petri dishes (9 cm) on filter paper moistened with 7 cm^3 of distilled water. After germination, seedlings were transferred to pots containing vermiculite, and Murashige & Skoog media was used as a nutrient medium and placed in growth chamber maintained at 25ºC. The experiment was divided in four sets. One set of seeds was grown at 25 ± 1°C in pots containing vermiculite as a nutrient medium and was taken as a control. After eight days of germination, second set of seeds was maintained to a continuous cold stress (-2°C) for 48 h for expression analysis by RT-PCR. For biochemical analysis, after 2 weeks of germination, third set of seeds was maintained at 30ºC and 32ºC for 7 days and fourth set of seedlings was maintained to a continuous cold stress at (-2°C) and (-4ºC) for 48 h and the measurements were performed on shoots in triplicates.

2.2. RT-PCR analysis

Total RNA was isolated using the phenol/SDS method (Wilkins and Smart 1996). cDNA was synthesized using the Prime Script RT reagent Kit (Takara, Dalian, China) according to manufacturer's instructions. Actin was selected as a housekeeping gene and primers were designed for both Zat12 and housekeeping gene. The sequences of the Zat12-specific primers were as follows: Zat12-F: 5'- GAA GCC TAA CAA CGA CGC TT- 3', Zat12-R: 5'- TTG TCC CAT CGG AAA CTC CA - 3', Actin-F: 5'- CCC AAG GCT AAC AGG GAG AA - 3'and Actin-R: 5'- GAT ACC TGT TGT GCG TCC AC - 3'. A master mix for each PCR run was prepared with SYBR Green PCR Core Reagents (Applied Biosystems, Foster City, USA). 100 µl of stock solution of primer mix was prepared by mixing 20µl forward primer and 20µl reverse primer and RT-PCR amplification reactions were performed in the volume of 20 µl containing the following components in appropriate proportion: 1µl cDNA, 4µl Syber green, 2µl primer mix and 13µl DEPC treated water. Reactions were programmed as follows: initial denature at 94°C for 4 min; followed by 35 cycles of denaturing at 95°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min.

All samples were amplified in triplicate from the same RNA preparation and the mean value was considered. The real-time PCR efficiency was determined for each gene with the slope of a linear regression model (Pfaffl 2001). The corresponding ΔCT values were calculated for Cq values as: ΔCT = Cq sample- corresponding endogenous Cq value. For each gene, PCR efficiency was determined by measuring the CT to a specific threshold for a serial dilution of bulked cDNA. And PCR expression was calculated as:

$$\text{Expression} = \text{PCR efficiency}^{-\Delta CT}$$

2.3. Biochemical analysis of antioxidative defence system

At 3 and 6 week interval stage, fifteen seedlings were used in each experiment and were repeated in triplicates. Seedlings (1 g) were homogenized in 3 cm^3 of ice-cold 50 mM phosphate buffer pH 6.5 (for POD, SOD), pH 7.0 (GR) and pH 7.5 (GST). The homogenate was centrifuged at 10000 g for 20 min at 4°C and clear supernatant was used for assaying activities of POD, SOD, GR and GST. POD (EC 1.11.1.7) activity was measured using method of Shannon et al (1966). SOD (EC 1.15.1.1) activity was determined by measuring its ability to inhibit the autooxidation of pyrogallol by the method of Marklund and Marklund (1974). Glutathione reductase (EC 1.6.4.2) activity was determined by method of Goyal and Asthir (2010). Glutathione S transferase was determined by measuring its ability to catalyze the conversion of hydroperoxide to less toxic dinitrophenyl glutathione using glutathione as substrate by the method of (Habig et al 1974). Content of antioxidants proline and ascorbate was determined by the method of Bates (1973) and Law et al (1983), respectively.

Statistical analysis

The values were statistically analyzed by multifactor ANOVA (Statgraphics plus v. 2.1). Values are presented as a means ± standard deviation (n = 3 or more). Statistically significant differences among values at the significance level $p < 0.05$ are indicated by different letters.

III. RESULTS AND DISCUSSION

3.1. Expression analysis of Zat12 by RT-PCR

Expression of Zat12 gene was analysed in shoot tissue of transformed (Z-8-12 1A, Z-8-12 1B, Z-8-19 and Z-15-10) and non transformed wheat genotype (PBW 621) through RT-PCR. Low levels of expression were detected in shoots of 5 days after germination stage and higher levels were observed in shoots of 10 days after germination stage in Zat12 transformed wheat lines and no such expression was observed in PBW 621. The highest level of expression was observed in shoots under stress conditions (-2ºC) in transformed wheat lines. The Zat12 gene was induced by cold stress of 10-day-old seedlings (Fig 1). Hence Zat12 expression was enhanced under stress condition, thus confirming that

Zat12 gene might help in combating the stress. Moreover, real time PCR (qPCR) analyses revealed the role of Zat12 in stress tolerance and our results of biochemical analysis have provided a basis for further understanding of Zat12 gene in ameliorating the stress tolerance in Zat12 transformed wheat lines.

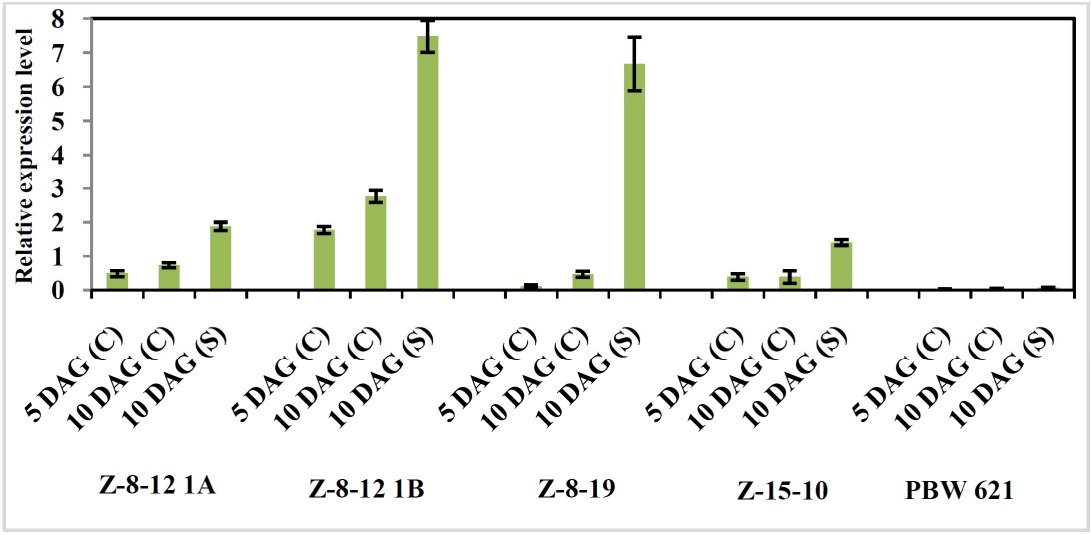
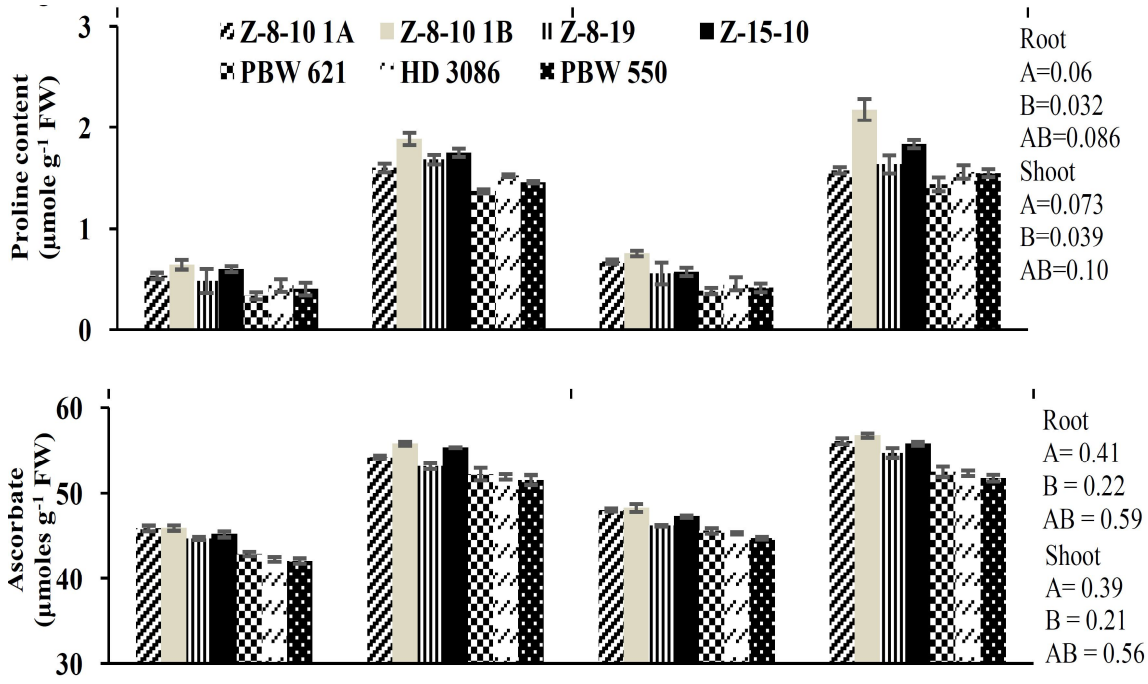


Fig 1 Relative expression of Zat12 gene in various transformed wheat lines and PBW 621 at different seedling stages under non stress (25°) (C) and stress (-2) (S).

Biochemical Analysis

Antioxidatuve enzymes are vital components for assessing stability and cell homeostasis against any kind of stresses. Rizhsky et al (2004) proposed that Zat12 expression is directly linked to reactive oxygen metabolism in Arabidopsis thaliana and in order to know the role of Zat12 in stress tolerance by enhancing antioxidative defence system via antioxidative enzymes and antioxidants, analysis of biochemical parameters were conducted. It was revealed through biochemical analysis that higher content of non enzymatic components (ascorbate and proline) and higher antioxidative enzymes activity was observed in transgenic lines (Z-8-12 1A, Z-8-12 1B, Z-8-19, Z-15-10) as compared to PBW 621. HD 3086 and PBW 550 also showed relatively less activity of antioxidative enzymes. The higher activity in Zat12 transformed lines is due to activation of antioxidative defence system which might help in combating the stress conditions.



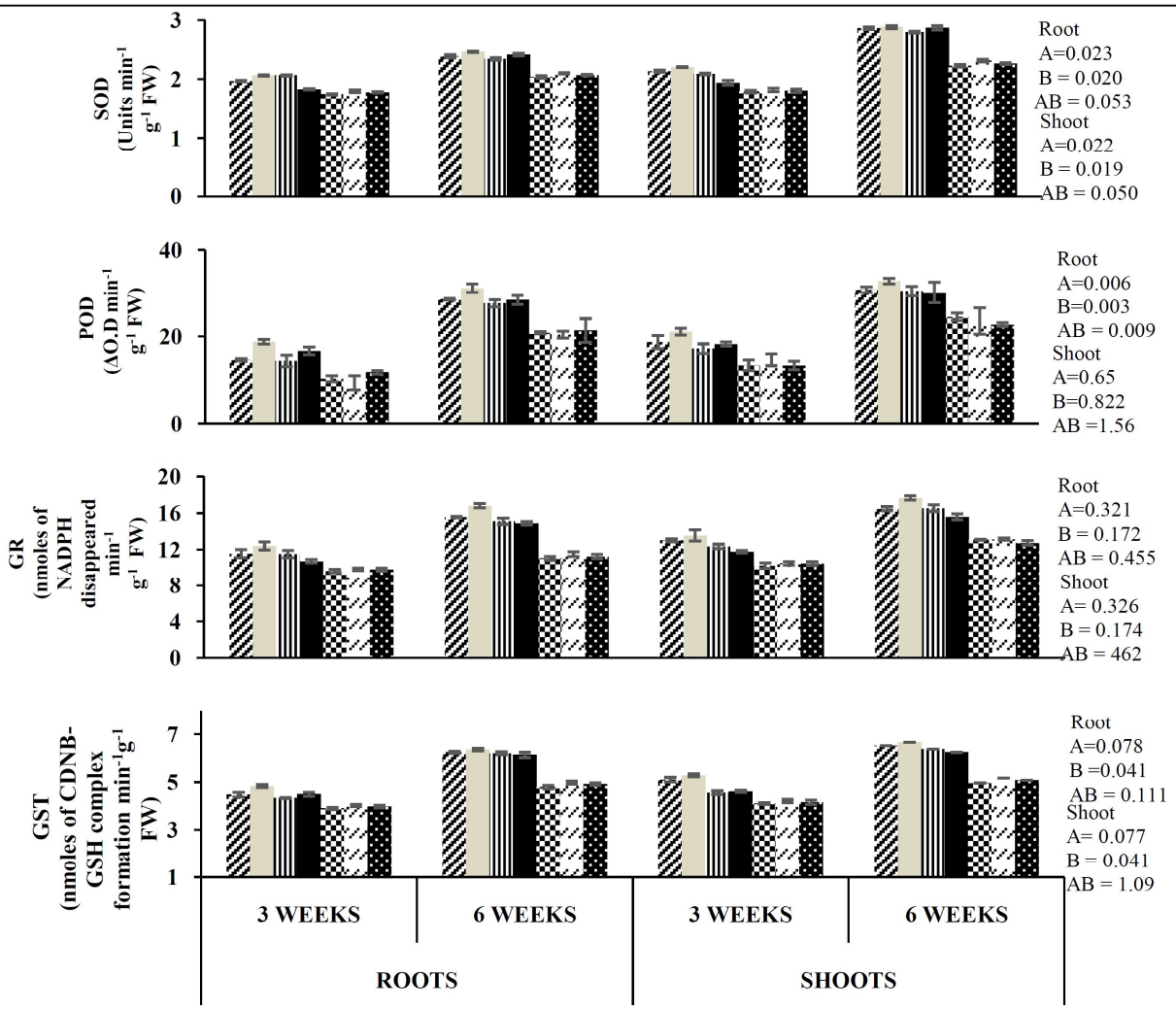


Fig 2 Proline and ascorbate content and antioxidative enzymes (POD, SOD, GR and GST) activity in root and shoot of Zat12 transformed (Z-8-12 1A, Z-8-12 1B, Z-8-19 and Z-15-10) and non transformed (PBW 621, HD 3086 and PBW 550) wheat genotypes at 3 and 6 week interval stage under non stress conditions. A-Genotype, B-treatment, AXB = genotype and treatment interaction.

Under stress conditions

It is well documented that elevated temperature causes biochemical perturbations in plants leading to changes in antioxidant defense system. But the mechanisms of regulation of antioxidant enzymes as well as participation of zinc finger protein in transgenic wheat in amelioration of temperature stress are poorly understood. Our study is focused mainly to identify the salient components among antioxidant defense system conferring unfavourable temperature tolerance in shoot tissues of transformed and non transformed wheat genotypes. The activities of antioxidative enzymes (POD, SOD, GPX and GR) in shoots of 3 week old seedlings of transformed lines (Z-8-12 1A, Z-8-12 1B, Z-8-19, Z-15-10) and non transformed wheat genotypes (PBW 621, PBW 550, HD 3086) under high temperature and cold stress were assayed (Fig. 3). An increase in the SOD enzyme activity in all of the Zat12 transformed wheat lines was observed under stress condition in shoots. Under favorable conditions, SOD enzyme activity in transformed wheat lines lied in the range of 1.82-1.96 units min^{-1} g^{-1} FW, whereas under stress conditions, it lied in the range of 3.28-3.75 units $min^{-1}g^{-1}$ FW. Z-8-12 1B and Z-8-12 1A showed more than 3 fold increase under stress. The activity was more in the seedlings at 32ºC than at the 30ºC. Similarly higher activity was observed in all the transformed wheat lines at -4ºC than at -2ºC. In case of checks, mean enzyme activity was found to be the range of 2.39-2.52 units $min^{-1}g^{-1}$ FW. Our results are in agreement with the result showing significant up regulation of the SOD activity under stress in barley (Turkyilmaz et al 2011). This enhanced activity may be an adaptive reaction to changes in oxidative stress which can be considered as a positive feedback mechanism (Chakrabarti and Patra 2013). Activity of peroxidase in shoots was found to be increased under stress in most of the accessions of Zat12 transformed wheat lines and checks. Z-12-1B showed the highest enzyme activity 57.33 ΔO.D $min^{-1}g^{-1}$ FW at -4ºC. Although activity varied under different stresses but was maximum at -4ºC. At -4ºC cold stress, the peroxidase activity was increased in the range of :

PBW 550 (41.73 ΔO.D $min^{-1}g^{-1}$ FW) < HD 3086 (42.0 ΔO.D $min^{-1}g^{-1}$ FW) < PBW 621 (42.26 ΔO.D $min^{-1}g^{-1}$ FW) < Z-8-19 (53.0 ΔO.D $min^{-1}g^{-1}$ FW) < Z-8-12 1A (54.13 ΔO.D $min^{-1}g^{-1}$ FW) < Z-15-10 (54.8 ΔO.D $min^{-1}g^{-1}$ FW) and Z-8-12 1B (55.66 ΔO.D $min^{-1}g^{-1}$ FW).

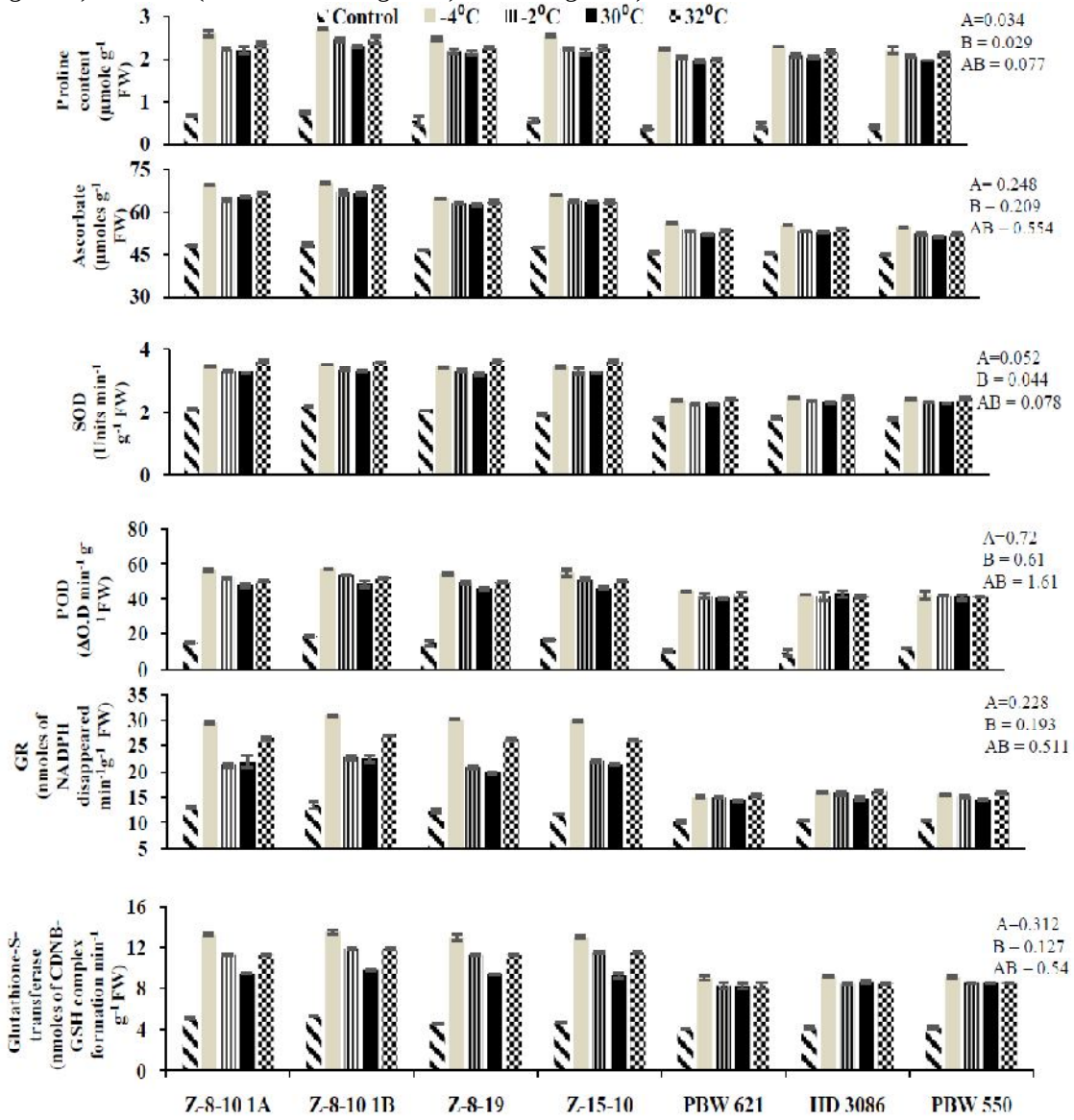


Fig 3 Proline and ascorbate content and antioxidative enzymes (POD, SOD, GR and GST) activity in shoot of Zat12 transformed (Z-8-12 1A, Z-8-12 1B, Z-8-19 and Z-15-10) and non transformed (PBW 621, HD 3086 and PBW 550) wheat genotypes at 3 week interval stage under different temperatures. A-Genotype, B- treatment, AXB = genotype and treatment interaction.

GST is an enzyme known for its ability to catalyze the conjugation of tripeptide glutathione (GSH) to unfamiliar electrophilic and hydrophobic substrates to form less toxic or non-toxic peptide derivatives and play significant role under various stress conditions (Dixon et al 2002). In case of shoots, heat stress at 32ºC and cold stress at -4ºC showed the maximum increase in enzyme activity in the transformed wheat genotypes, where the maximum was recorded in Z-8-12 1B and least in PBW 621. Our results are supported by earlier observations of Gapinska et al (2008), wherein they indicated that GST activity had increased with salt treatment in tolerant tomato cultivars. Glutathione reductase plays a crucial role in regeneration of reduced glutathione which is a reducing equivalent donor for reduction of dehydroascorbate, thus contributing to efficient operation of GSH-ASC cycle. As compared to control conditions, the GR activity at -4ºC increased by 2.2 to 3.2 fold in shoots of all transformed genotypes. Under cold stress condition at -2ºC, the activity of GR was increased by 2.1 to 2.4 fold which is lower as compared to -4ºC cold stress conditions. Similar trend was shown at heat stress of 30ºC and 32ºC. Hence the activity of enzyme is up-regulated under stress conditions, these facts indicate relatively better ability of the enzyme glutathione reductase to not only reveal genotypic variation but also play its part in conferring stress tolerance. A study conducted by Naik and Devraj (2016), reported that GR activity

increased with abiotic stress i.e. salinity treated for 24, 48 and 72 h at 400 mM NaCl concentration. Higher SOD and POD activities in Zat12 transformed lines and under elevated temperature demonstrates its superior tolerance mechanism in terms of H_2O_2 scavenging over PBW 621. It demonstrated the activation of ROS signaling in Zat12 transformed lines under stress conditions and activation of antioxidative enzyme activities in transformed lines might be due to the H_2O_2 which get accumulated in the stress and it further stimulates ROS signaling as mentioned in Fig.1. Significance of POD activity in temperature stress tolerance has also been reported by other workers (Almeselmani et al 2006). Therefore, it was observed that response of antioxidative enzymes was more significant in transformed wheat lines. It may thus be inferred that Zat12 plays a significant role in stress tolerance by enhancing the activities of antioxidative enzymes.

CONCLUSIONS

Biochemical analysis of Zat12 transformed lines was studied and major conclusions are as follows:

1. RT-PCR analysis of Zat12 transformed wheat lines revealed that expression level of Zat12 enhanced under the stress conditions which might play an important role in stress tolerance.
2. Higher antioxidative enzymes activity in Zat12 transformed lines under non stress and stress conditions indicates that Zat12 help in stress tolerance by activating the antioxidative defence system.

ACKNOWLEDGMENTS

Abiotic stress conditions such as heat, cold, drought and salinity effect the crop yield and productivity. As a zinc finger transcription protein, Zat12 respond to several stress conditions and make the crop tolerant to abiotic stress. As Zat12 involved in activation of ROS related antioxidative system, when biochemical parameters analysed in transformed wheat lines, Zat12 play an important role in ameliorating temperature stress by enhancing the activities of antioxidative enzymes.

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