HEAT STRESS ASSOCIATED ANTIOXIDANT ISOENZYMES IN WHEAT: EXPRESSION AND PROTEOMICS

Ranjeet R. Kumar*1, Suneha Goswami, Sushil K. Sharma, Kritika A. Gadpayle, Khushboo Singh, Narender Kumar, Gyanendra K. Rai1 and Raj D. Rai

Division of Biochemistry,
Indian Agricultural Research Institute, New Delhi-110 012, India

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ABSTRACT

Abiotic stress leads to change in the expression and activities of number of proteins which are involved in defense mechanism against stresses. Qualitative and quantitative changes in proteins might play a role in signal transduction, antioxidative defence, heat shock, metal binding or osmolyte synthesis. Here, 22 wheat genotypes were screened for its cell membrane stability (CMS), out of which C-306 showed the maximum CMS value of 74%. Protein profiling revealed the expression of many new and existing proteins in C-306 cultivar compared to PBW343 under differential heat shock (HS). An altered protein expression was also observed in tolerant and susceptible cultivars at different stages of growth. Isoenzymic profiling revealed the expression of many new isoenzymes of ascorbate peroxidase and superoxide dismutase in C-306 compared to PBW343 in response to heat shock. A stage specific analysis showed the maximum activity of different antioxidant isoenzymes at pollination and seed hardening stages. SODs plays central role in regulating defense mechanism and need to be further characterized.

Key words: Antioxidant enzymes, Heat shock protein, Heat stress, Isoenzymes, Protein profiling.

INTRODUCTION

All living organisms have evolved endogenous mechanism to cope with different environmental stresses. Abiotic stresses, such as drought, salinity, high/low temperature represent serious threat to agriculture and cause the huge loss of crop yield worldwide by more than 50% annually. Plants growth and yield are strongly affected by heat stress as it damages the functions of cells, tissues, and whole plants. The predicted levels of global warming are likely to increase the constraint to plant productivity imposed by high temperature. Differences in the sensitivity to high temperatures have been observed in crop species, due to differences at the genetic level. Several physiological and biochemical mechanisms have to be modified by plants to overcome heat stress. For instance, high temperature causes modifications of membrane fluidity, permeability and stability (Ismail and Hall, 1999; Sangwan et al., 2002), and electrolyte loss resulting from heat-induced cell membrane leakage is considered a measure of stress cellular damage (Saadalla et al., 1990; Wahid et al., 2007). Plant responses to high temperatures are mediated by both their inherent ability to survive (basal tolerance), and their ability to acquire tolerance to otherwise lethal temperatures (acclimation). These two mechanisms in cereals are due to the activation of different genetic systems (Maestri et al., 2002). High temperature represents a significant constraint to the cultivation of important crops, such as wheat in large areas of the world. Several studies have provided evidence that the genetic variability in stress response among wheat genotypes is mainly due to differential expression of stress-responsive genes and have reported correlations between the acquisition of thermotolerance and the synthesis and accumulation of heat shock proteins (HSPs) and antioxidant enzymes, although the mechanisms underlying the thermal tolerance are not yet

*Corresponding author’s e-mail ranjeetranjaniari@gmail.com
1Sher-e-Kashmir University of Agricultural Sciences and Technology, Jammu, India
Antioxidant enzymes like superoxide dismutase (SOD), catalase, ascorbate peroxidase (APX), glutathione reductase (GR) and peroxidase (POX) also play a very important role in creating tolerance against abiotic stress in plants. They help in scavenging the active oxygen species (AOS) which are produced in response to heat shock and are dangerous for the plant system. Superoxide dismutase (SOD) is a group of metalloenzymes that alter its activity under different environmental conditions (Bowler et al., 1992). It is a highly efficient catalyst mediating a pivotal reaction in the antioxidant pathway (Foyer et al., 1994).

Ascorbate peroxidase uses ascorbic acid as a hydrogen donor to break down hydrogen peroxide (Asada, 2006). APX isoenzymes are distributed in at least four distinct cell compartments i.e. stroma (sAPX), thylakoid membrane (tAPX), mitochondria (mAPX) and cytosol (cAPX) (Asada, 1992; Ishikawa et al., 1998). Different isoforms of APX behave differently under different types of stress (Yoshimura et al., 1999).

The synthesis of stress associated proteins is known to be part of the stress tolerance strategy resulting in the ability of plants to cope with the heat stress (Iba, 2002). The heat stress (HS) response is transient in nature, usually peaking 1 to 2 hr after onset, providing protection from acute episodes of thermal stress. The present investigation has been undertaken with a view to characterize the expression and activities of antioxidant isoenzymes (stress proteins) in response to differential heat shock in wheat. The information generated will be helpful in future to decipher the mechanism of thermotolerance in plants.

**MATERIALS AND METHODS**

**Evaluation of cell membrane stability (CMS)**

Seeds of 22 different genotypes of wheat (collected from Division of Genetics, IARI) were germinated inside BOD at 25°C under a constant light/dark regime with 16 h light and 8 h darkness and watered with tap water. Eight-day-old seedlings with similar leaf size were selected and used to measure cell membrane stability (CMS) using the method of Fokar et al. (1998) with some modifications.

**Germination and heat shock treatment**

The seeds of thermostolerant (C-306) and susceptible (PBW343) wheat cultivars were collected from Division of Genetics, IARI, New Delhi. The seeds were germinated in 40 pots (in group of two) at 23°C ± 2°C under a constant light/dark regime with 16 h light and 8 h darkness inside phytotron chamber. The whole experiment was planned during the year 2010-11 at National phytotron facility, IARI, New Delhi. Samples of both the cultivars were collected from I group at different stages of growth i.e. vegetative, pollination, milky dough and seed hardening. Second group was used for the study of effect of differential heat shock treatment. Samples were collected from both the cultivars exposed to differential HS of 30°C, 35°C and 40°C for 2 hr at vegetative stage. Samples were collected in 3 replications and were immediately frozen in liq. nitrogen before storing at -70°C.

**Crude protein extraction**

1g of samples from C-306 and PBW343 cultivars were taken and it was crushed into powder form using liquid nitrogen in pestle and mortar. 5 ml of extraction buffer (Tris-HCl 100mM, pH 6.8) was added and the mixture was passed through 3 layers of muslin cloths and the extract obtained was centrifuged at 18,000 rpm for 20 min. The supernatant obtained was separated and was further used as a protein crude extract.

**1D SDS polyacrylamide gel electrophoresis**

Crude extract prepared from C-306 and PBW343 was used for the 1D SDS PAGE in order to know the protein profile against different heat stress treatment.1D SDS PAGE was carried out using the standard protocols of Lammeli’s et al. (1970) with some modifications. Mid-range protein marker was used along with the different samples for PAGE. The PAGE run was carried out at 50 V for 3 to 4 hrs. The polyacrylamide gel was stained using CBB R250 for 2 hrs. and the destaining were carried out using glacial acetic acid: methanol: water in the ratio of 3:6:51.

**Electrophoretic mobility profiling of superoxide dismutase isoenzymes**

The crude extract of collected samples was prepared in phosphate buffer (pH 7.5) and the protein content was estimated using Bradford method (Bradford, 1976). 20lg of proteins were...
loaded on to each well of polyacrylamide gel (10%) for native PAGE and further the protocol of Roychaudhari et al., (2003) was followed for the isoenzymic study of SOD. Illumination was discontinued when maximum contrast between achromatic zones and the general blue color had been achieved. The gel was maintained in distilled water till photographed.

**Isoenzymic profiling of ascorbate peroxidase isoenzymes**

Isoenzymes of ascorbate peroxidase were detected by the procedure as described by Mittler and Zilinskas (1991). 20ìg of proteins prepared in phosphate buffer (pH 7.0) was used in this method for native PAGE (10%). Electrode buffer was prepared using 2mM ascorbate and the gel was run for 30 minutes before the samples were loaded. White colored achromatic zones against blue background represent APX activity.

**RESULTS AND DISCUSSION**

**Cell membrane stability (CMS) index**

Twenty two wheat genotypes were characterized for both basal and acquired thermotolerance at seedling stage by evaluation of cell membrane stability (CMS) that is considered as standard method to evaluate thermotolerance (Fokar et al., 1998; Blum et al., 2001; Wahid et al., 2007). The CMS (%) of cultivars (ID 55-65) of wheat is between the ranges of 60 to 70% which is far better than the CMS (%) of cultivars (ID 41-53) of wheat which lies below the 50% (Fig. 1). The wheat cultivars (ID 53-65) showing higher CMS (%) must have higher ability to tolerate the heat stress compared to the varieties having lower (below 50%) value. This can be used as a screening tool to characterize different genotypes of wheat into two groups: a) more than 50% CMS value (thermotolerant) and, b) Less than 50% CMS value (thermosusceptible). Heat stress accelerates the kinetic energy and movement of molecules across membranes, thereby loosening chemical bonds within molecules of biological membranes. This makes the lipid bilayer of biological membranes more fluid by either denaturation of proteins or an increase in unsaturated fatty acids (Savchenko et al., 2002). The heat-stress conditions used in our experiments are in close proximity with that of stress condition which plants experiences during their milky dough and seed hardening stages. The increased solute leakage, as an indication of decreased cell membrane thermostability (CMT), has

long been used as an indirect measure of heat-stress tolerance in diverse plant species, including potato and tomato (Chen et al., 1982), wheat (Blum et al., 2001), cotton (Ashraf et al., 1994), sorghum (Marcum, 1998), cowpea (Ismail et al., 1999), and barley (Wahid and Shabbir, 2005). In Arabidopsis plants grown under high temperature, total lipid content in membranes decreased to about one-half and the ratio of unsaturated to saturated fatty acids decreased to one-third of the levels at normal temperatures (Browse and Somerville, 1991).

**Differential protein profiling**

1D SDS PAGE showed the expression of many new as well as existing proteins in C-306 (thermotolerant) cultivar compared to PBW343 (susceptible) under differential heat shock treatment (Fig. 2). An increase in the expression of existing proteins was observed in C-306 in the range of ~15 KDa to ~100 KDa. New proteins of ~40 to 75 KDa were also observed in C-306. In case of PBW343, increase in the expression of existing proteins was observed in the range of ~25 to ~80 KDa. New proteins were also observed at ~45 and ~75 KDa. It is the change in the expression profile of existing as well as new proteins which play very important role in modulating the defense mechanism of plant under different abiotic stresses. The expression of new proteins of different molecular weight in C-306 might be playing the role in creating tolerance against heat shock.

In order to understand the mechanism of tolerance, a stage specific protein profiling was also carried out in C-306 (tolerant) and PBW343 (susceptible) wheat cultivars. Presence of new proteins was observed whose expression was more at fertilization and seed hardening stages in case of C-306 whereas, a slight increase in the expression of existing...
proteins were observed in case of PBW343 with few new proteins at seed hardening stage (Fig. 3). It is the expression of these proteins which are predicted to be HSPs, antioxidant enzymes, signaling molecules or metabolites which plays very important role in enhancing tolerance in plants against abiotic stresses at different stages of growth.

Electrophoretic mobility profiling of antioxidant isoenzymes

Superoxide dismutase isoenzyme analysis

Electrophoretic mobility profiling of superoxide dismutase (SOD) isoenzymes showed the expression of many new isoenzymes in C-306 cultivar compared to PBW343 under heat stress (Fig. 4). Heat stress leads to increase in the activity of SOD isoenzymes which are predicted to counter the harmful effect of active oxygen species (AOS) produced because of heat shock. An expression of three prominent SODs was observed in C-306 (Lane 7, 8, 9 & 10) compared to PBW343 where two SOD isoenzymes was observed under heat shock of 30°C for 2h.

SODs profiling was also carried out in wheat at different stages of growth and development. An expression of three prominent SOD isoenzymes were observed at vegetative stage and further, a decrease in the activity of isoenzymes were observed especially at pollination, milky dough and seed hardening stages (Fig. 5). The activities of three prominent isoenzymes observed in C-306 at all the stages of growth and development defines the thermotolerant nature of the cultivar compared to PBW343. Similarly, the levels of different SOD isoforms were found up-regulated under drought (Hajheidari et al., 2005), ozone stress (Agarwal et al., 2002), high light (HL) (Nam et al., 2003), As stress (Requejo and Tena, 2005) and plant hormone treatment (Rakwal and Komatsu, 2004). Among all antioxidant isoenzymes, SODs plays central role in regulating the defense mechanism of wheat under differential HS.
Ascorbate peroxidase isoenzyme analysis

Isoenzymic profile of ascorbate peroxidase (APX) showed the expression of 9 APX isoenzymes in C-306 and 6 in case of PBW343 in response to differential heat shock treatment. Increase in the expression of APX isoenzymes was observed both in case of thermostolerant and susceptible cultivars in response to increase in heat shock temperature (Fig. 6). Maximum APX isoenzymes were observed when heat stress treatment of 40°C was given for 2h in case of C-306 (tolerant) wheat cultivar.

APX isoenzyme profiling was also carried out at different stages of growth (Fig. 7). Six APX isoenzymes were observed at seed hardening stage in C-306 and three in case of PBW343. Maximum APX isoenzymes (9 APX) were observed at seed hardening stage in C-306 and milky dough stages in case of PBW343. An continuous increase in the expression of isoenzymes were observed in C-306 at different stages of growth whereas, in case of PBW343 same pattern was observed till milky dough stage and further, a decrease in the activity was observed during seed hardening stage. This makes us to conclude that the APX isoenzymes present in PBW343 is heat sensitive whereas, in case of C-306, it is heat stable. Several proteomic studies showed an up-regulation in different isoforms of APX and other peroxidases, e.g., under drought (Hajheidari et al., 2005), salinity (Yan et al., 2011), high temperature (Sule et al., 2004), Cd stress (Sarry et al., 2006) and ozone stress (Agarwal et al., 2002).
Fig. 6: Ascorbate peroxidase (APX) isoenzymes pattern analysis in thermosusceptible (PBW343) and thermotolerant (C306) cultivars of wheat in response to differential heat shock treatment, arrow shows the expression of APX isoenzymes.

CONCLUSIONS
Abiotic stresses causes altered expression of various stress associated proteins in plants. Here, we report screening of 22 different germplasm of wheat based on CMS where C-306 showed maximum CMS value. An expression of many unknown proteins was observed in C-306 against heat stress. Antioxidant isoenzymes profiling of SOD and APX showed increase in the expression of existing as well as novel isoenzymes in response to heat shock. There is a need to further characterize different stress associated proteins, signaling molecules and metabolites in wheat, so that they can be manipulated for enhancing the thermotolerance capacity against different abiotic stresses.

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Fig. 7: Ascorbate peroxidase (APX) isoenzymes pattern analysis in thermosusceptible (PBW343) and thermotolerant (C-306) cultivars of wheat at different stages of growth, arrow shows the expression of APX isoenzymes.

REFERENCES


