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Full Length Research Paper

Expression of novel ascorbate peroxidase isoenzymes of wheat (*TRITICUM AESTIVUM* L.) in response to heat stress

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Active oxygen species are produced by some of the important organelles like chloroplast and mitochondria through their electron transport chain even under normal environmental condition but the rate of active oxygen species (AOS) production increases many times in response to different abiotic stresses. Plants has antioxidant enzymes system like ascorbate peroxidase (APX), catalase, SOD, GR etc. which helps in reducing the AOS produced inside the plant system in order to protect the key enzymes from AOS. In present investigation, expressions of many new proteins were observed in thermo tolerant (8 in case of C306) and susceptible cultivars (2 in case of PBW343) at different stages in response to heat stress. Isoenzymic profile of APX in response to differential heat shock revealed the expression of 8 APX isoenzymes in C306 (thermo tolerant) compare to 2 in PBW343 (susceptible) cultivars of wheat. A stage specific study revealed the expression of 5 APX isoenzymes at vegetative, pollination, milky dough and seed hardening stages (C306) whereas, 3 APX isoenzymes were observed during milky dough and seed hardening stages in PBW343. It is clear now that high expression of endogenous APX isoenzymes supplements the antioxidant enzyme systems of the plant and protects the plant from oxidative damage in response to biotic and abiotic stresses.

Key words: Heat shock proteins (HSPs), antioxidant isoenzymes, ascorbate peroxidase (APX), active oxygen species, abiotic stress, wheat.

INTRODUCTION

Abiotic stresses can result in oxidative stress, which induces genes involved in the oxidative stress defense. Under stress conditions, excessive active oxygen species (AOS) such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals are formed which cause oxidative damage of cell constituents (Asada, 1997). APX is an integral component of the glutathione-ascorbate cycle. These enzymes are commonly hemoproteins and the co-factor is the site of the oxidation-reduction reaction.

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Abbreviations: HSP, Heat shock protein; **APX,** ascorbate peroxidase.

Ascorbate peroxidases use ascorbate as a reducing agent to catalyze the conversion of H_2O_2 to water; in this reaction ascorbate is oxidized to monodehydroascorbate The later is then reduced back to ascorbate by monodehydroascorbate reductase while converting NAD (P)H to NAD(P). Even under optimal conditions, many metabolic processes, including chloroplastic, mitochondrial, and plasma membrane-linked electron transport systems, produce active oxygen species such as the superoxide radical, H_2O_2, and the hydroxyl radical (Asada, 1997). Furthermore, the imposition of biotic and abiotic stress conditions can give rise to excess concentrations of active oxygen species, resulting in oxidative damage at the cellular level. Therefore, antioxidants and antioxidant enzymes such as ascorbate, glutathione, superoxide dismutase, ascorbate peroxidase (APX; EC 1.11.1.11), and Catalase function to interrupt the

cascades of uncontrolled oxidation in some organelles (Noctor and Foyer, 1998).

Gur et al. (2010) observed an increase in the activity of Ascorbate peroxidase (APX) in response to heat shock treatment of 38 and 45°C in case of cotton. APX isoenzymes play an important role in eliminating H_2O_2 and are distributed in at least four distinct cell compartments, the stroma (sAPX) and thylakoid membrane (tAPX) in chloroplasts, the microbody (mAPX), and the cytosol (cAPX) (Asada, 1992; Ishikawa et al., 1996). A second family of cAPX has also been reported in various plant species such as spinach, Arabidopsis, soybean, and rice (Jespersen et al., 1997). More recently (Jimenez et al., 1997) reported the detection of APX activity in pea mitochondria, but the corresponding protein and cDNA have not yet been identified.

Recent studies have focused on the changes in the cAPX expression level under environmental stresses such as ozone, UV-B radiation, low temperature, high-light stress, salinity, water stress including drought, and pathogen infection (Mishra et al., 1993;). Considering the specific distributions and roles of the APX isoenzymes and the potential for active oxygen species production in each organelle of higher plants, it seems likely that the APX isoenzymes are expressed by distinct regulatory mechanisms. However, no studies have presented simultaneous analysis of the stress responses of all of the APX isoenzymes. In fact, the lack of specific probes to detect the APX isoenzymes at the mRNA and protein levels has limited the understanding of the expression of the respective isoenzymes. In this study, we report the responses of all of the APX isoenzymes in germinated wheat seedling to heat stress conditions.

MATERIALS AND METHODS

Two wheat cultivars C306 (thermotolerant) and PBW343 (susceptible) seeds were collected from Division of Genetics, Indian Agricultural Research Institute, Pusa India. The seeds were grown inside BOD at 22°C under regulated condition. 8 days old germinated seedlings were given four level of heat shock treatment of 22, 30, 35 and 40°C for 2 h. Samples were collected from different stages of growth and development, that is, vegetative, pollination, milky dough and seed hardening stages.

Crude protein extraction for PAGE

1 g of freezed germinated seedlings from thermotolerant (C306) and susceptible (PBW343) wheat cultivars were taken in pestle and mortar and it was crushed into powder form using liquid nitrogen. 5 ml of extraction buffer (100 mM Tris-HCl, pH 7.3) was added in pestle and mortar. The crude extract was passed through 4 layers of muslin cloths and was centrifuged at 18,000 rpm for 20 min. The supernatant obtained was used for 1D SDS PAGE.

Protein profiling in response to heat shock

Protein profiling was carried out by 1D SDS PAGE using the methods of (Laemmli's 1970) with some modifications. The protein of the crude extract was estimated using the Bradford method (Bradford, 1976) and equal amount of protein (20 µg) was loaded onto each well. The sample was run along with mid-range protein marker. The PAGE run was carried out at 50 V for 5 h. The Poly-acrylamide gel was stained using CBB R250 for 2 h and the de-staining were carried out using glacial acetic acid: methanol: water in the ratio of 3:6: 51.

Electrophoretic mobility profiling of APX isoenzymes

Enzyme extract for APX was prepared by first freezing the 1 g of germinated wheat seedling in liquid nitrogen to prevent proteolytic activity followed by grinding with 5 ml of extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA and 1 mM ascorbic acid). The crude extract was passed through 4 layers of cheese cloth and filtrate was centrifuged for 20 min at 15000 g and the supernatant was used as enzyme. 15 µg of extracted APX enzyme was loaded on to the 1D native PAGE. 10% resolving gel was used along with electrode buffer containing 2 mM ascorbate and pre-run of 30 min, was given before the sample was loaded. Isoenzymes of APX were detected by the procedure as described by Mittler and Zilinskas (1993). The gel was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min. Then the gel was incubated in a solution containing 50 mM sodium phosphate buffer (pH 7.0), 4 mM ascorbate and 2 mM H_2O_2 for 20 min. Further, the gel was washed in the buffer for 10 min and finally submerged in a solution of 50 mM sodium phosphate buffer (pH 7.8) containing 2.45 mM NBT for 15 min with gentle agitation. After 10 min, 28 mM TEMED was added into the solution. White colored achromatic zones against bluish background represented APX activity. The gel was washed with distilled water to remove excess stain and was photographed.

RESULTS AND DISCUSSION

Wheat is a staple crop in many region of the world and large areas of the total cultivated land is devoted to this crop. Wheat is very much prone to different abiotic stresses and particularly heat stress which is causing major loss in total wheat grain production all round the world. Even though we have many thermo tolerant cultivars of wheat which can sustain heat stress but still we have not succeeded in deciphering the mechanism of thermo tolerance in these cultivars. Antioxidant enzyme system and HSPs are the key factors which help the plant in creating tolerance against heat stress. Ascorbate peroxidase is one of the important antioxidant enzyme which plays major role in neutralizing the free radicals produced inside the plant cells because of abiotic stresses.

In present investigation, protein profiling of thermo tolerant (C306) and susceptible (PBW43) was carried out using ID SDS PAGE at different stages of growth and development and under differential level of heat shock treatment.

Germinated seedlings of thermotolerant (C306) and susceptible (PBW343) wheat cultivars were used for protein profiling study as well as electrophoretic mobility profiling of ascorbate peroxidase isoenzymes in response to heat stress (heat shock treatment of 22, 30, 35 and

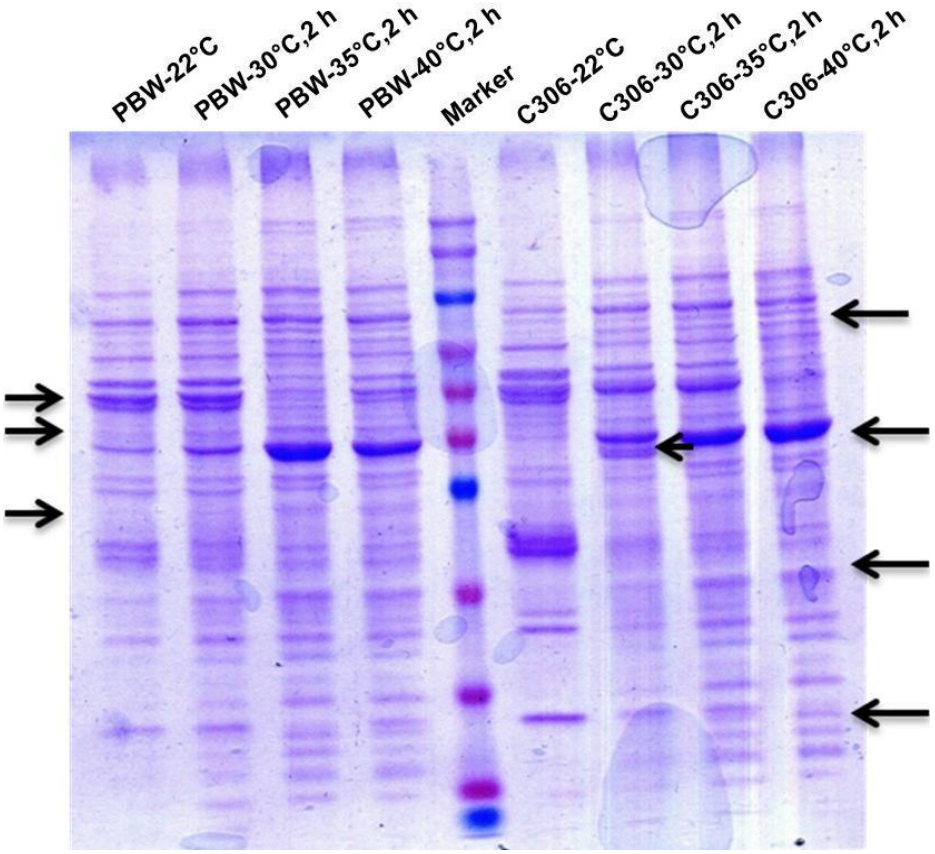

Figure 1. Protein profiling of thermosusceptible (PBW343) and thermotolerant (C306) cultivars of wheat in response to differential heat shock treatment (22, 30°C for 2 h, 35°C for 2 h, 40°C for 2 h) M-mid range SDS molecular weight protein marker, Arrow shows the expression of new protein band.

40°C for 2 h) and at different stages of growth.

Protein profiling

Protein profiling of thermotolerant (C306) and susceptible (PBW343) wheat cultivars was carried out using 1D SDS PAGE (Laemmli, 1970) as shown in (Figure 1). Protein profiling revealed the increase in the expression of existing proteins as well as new proteins in case of thermo tolerant cultivar (C306) in response to differential heat shock compare to susceptible one (PBW343) where very few new bands were observed.

In case of thermotolerant cultivar (C306), there was increase in expression of new protein bands of different molecular weight with the increase in the temperature of heat shock treatment (22, 30, 35 and 40°C for 2 h) An expression of 5 new proteins were observed (thermo tolerant) compare to 2 new protein band in case of (susceptible) as shown in Figure 1.

Similarly, samples collected from C306 and PBW343 at different stages of growth and development were also used for protein profiling study as shown in Figure 2. The profile shows the expression of 6 new protein bands of different molecular weight at milky dough and seed hardening stage of C306 cultivar whereas, only 2 new proteins were expressed in PBW343 cultivar of growth and developmental stages. A gradual increase in the expression of existing proteins was also observed with the increase in number of day of germination. Milky dough stage was observed to have a maximum number of expressions of new proteins in both thermo tolerant as well as susceptible cultivars of wheat. These new protein bands are predicted to be the HSPs or antioxidant enzymes or signaling molecules or metabolites which impart tolerance to the plant against abiotic stresses.

APX Isoenzymes profiling

Electrophoretic mobility profiling of APX isoenzymes was carried out using the enzyme extract prepared from thermotolerant (C306) and susceptible (PBW343) wheat cultivars (*Triticum aestivum* L.) after giving heat shock treatment of 22, 30, 35 and 40°C for 2 h. In case of PBW343, 1 prominent APX isoenzymes was observed having very high activity along with 6 different isoenzymes having very low activity at 22°C whereas, 2

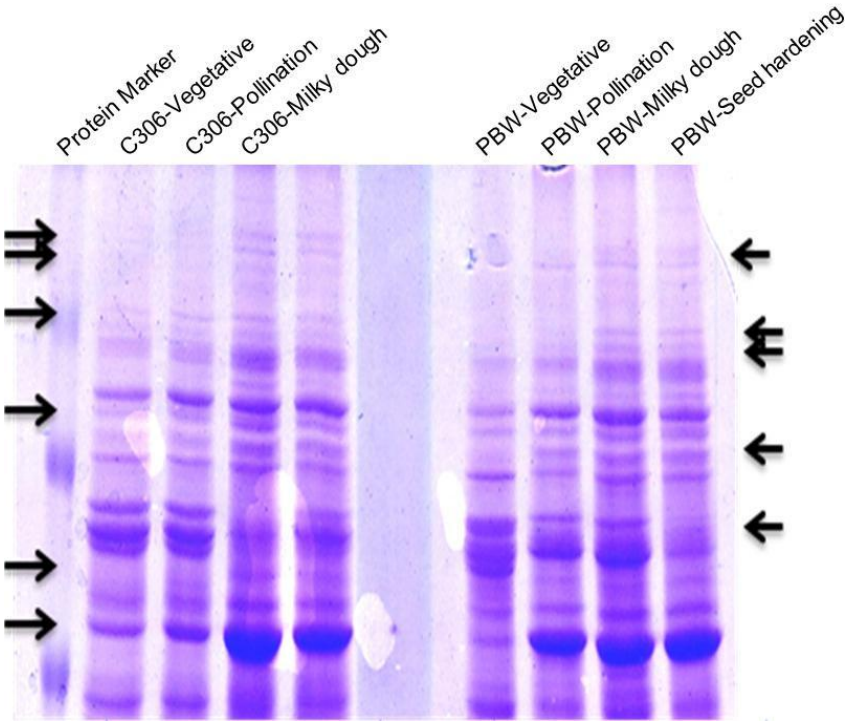

Figure 2. Protein profiling of thermotolerant (C306) and susceptible (PBW343) cultivars of wheat at different stages of growth (Vegetative, Pollination, Milky dough and Seed hardening stages). M-mid range molecular weight SDS protein marker, Arrows shows the expression of new protein bands.

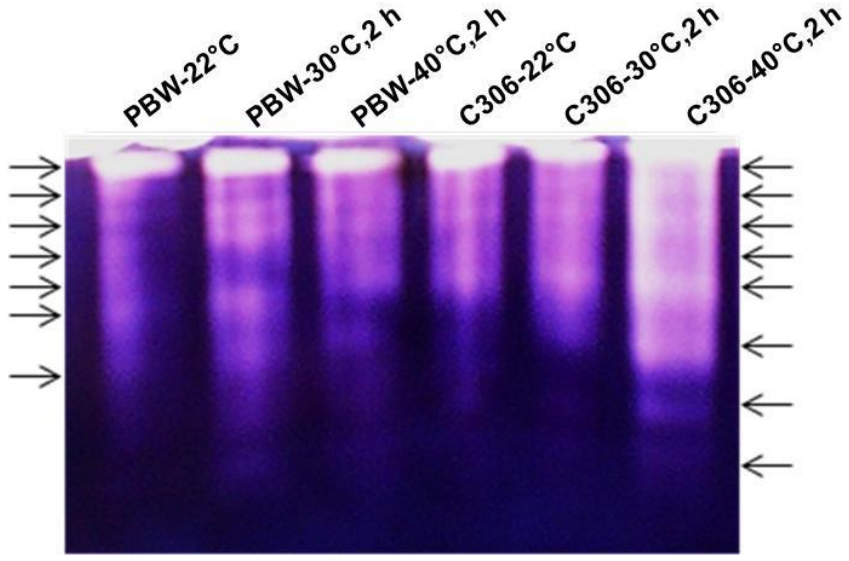

Figure 3. APX isoenzymic pattern analysis in thermosusceptible (PBW343) and thermo tolerant (C306) cultivars of wheat in response to different heat shock treatment (22^{o}C, 30^{o}C for 2h, 40^{o}C for 2h; Arrow shows the expression of APX isoenzymes.

APX isoenzymes having very high activity was observed in C306 at 22°C along with 6 other very low activity isoenzymes as shown in Figure 3. Maximum APX isoenzymes (6 having very high activity and 3 having low activity) PBW343 were observed when heat stress treatment of 30°C was given for 2 h, whereas, at 40°C,

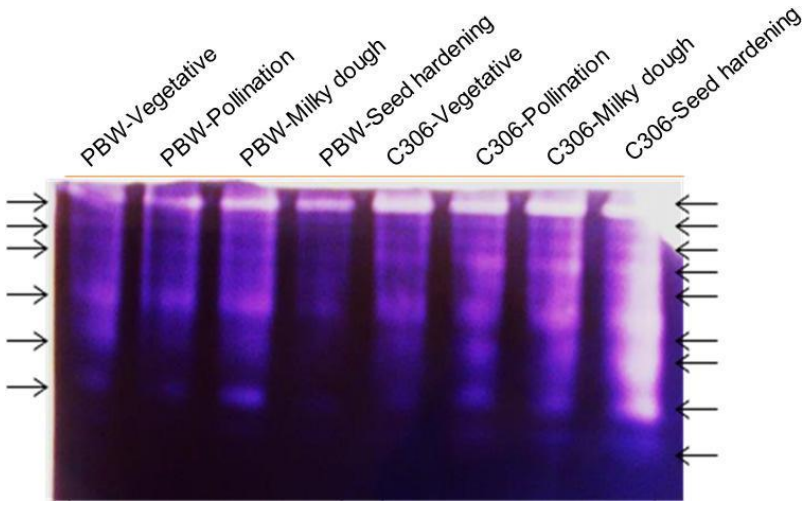

Figure 4. APX isoenzymic pattern analysis in thermo susceptible (PBW343) and tolerant (C306) cultivars of wheat at different stages of growth (Vegetative, Pollination, Milky dough and Seed hardening stages), Arrow shows the expression of APX isoenzymes.

there was denaturation of APX isoenzymes and only 2 isoenzymes with high activity were observed along with 3 isoenzymes having low activity. In case of C306, 3APX isoenzymes having high activity were observed at 22°C followed by 4 APX isoenzymes at 30°C. 5 APX isoenzymes having low isoenzymes activity was also observed at 30°C. Maximum APX isoenzymes in case of C306 were observed when it was given heat shock treatment of 40°C for 2 h as shown in Figure 3. Altogether, 7 prominent APX isoenzymes were observed having very high activity along with 3 isoenzymes having low activity at 40°C as depicted in (Figure 3). It is the expression of more number of APX isoenzymes inside the plant system which plays very important role in controlling the oxidative burst inside the cells when the plant experienced heat stress.

A stage specific electrophoretic mobility profiling of APX isoenzymes were also carried out in thermotolerant and susceptible cultivars of wheat as shown in Figure 4. At germination stage there was not much difference in APX isoenzymes in thermotolerant (C306) and suscepti-ble (PBW343) cultivars (Lane 1, 5). At pollination stage, 5 APX isoenzymes were observed in thermo tolerant compare to 2 APX isoenzymes in case of susceptible (Lane 2,6).At Milky dough stage, 5 APX were observed in case of thermo tolerant compare to 3 APX in susceptible (Lane 3,7). Only 2 APX were observed in susceptible compare to 6 APX in tolerant cultivars at seed hardening stage. A drastic decrease in the expression of APX isoenzymes at seed hardening stage in case of susceptible may be the reason for its sensitivity towards abiotic stresses.

Protein profiling of thermotolerant and susceptible wheat cultivars against heat stress treatment revealed the expression of many new protein bands in case of tolerant compare to susceptible one which was also observed by (Amako et al., 1994). There was gradual increase in the number of new protein bands with the increase in heat shock temperature and the maximum protein band was observed when the heat shock treat-ment of 40°C was given for 2 h. These new protein bands may be the HSPs or the enzyme of the antioxidant systems which plays very important role in providing tolerance against oxidative burst which is in conformity with the observation made by (Yamaguchih et al., 1995). An *Arabidopsis* mutant (*Pst*1) showed enhanced expression of APX and SOD when grown on NaCl containing media (Tsugane et al., 1999). In case of susceptible cultivars only few new protein bands were observed when heat shock treatment of 30 and 35°C was given for 2 h. At 40°C no new protein band was observed in susceptible cultivars which may be due to denaturation of the new protein band expressed earlier. This may be the reason for the susceptible character of these varieties because of less number of different antioxidant isoe-nzymes at particular stages of growth and development as observed by Pastori and Trippi (1992) who reported that drought resistant maize showed greater induction of APX activity than sensitive plants. A variety behave as a tolerant only when it has sufficient compatibility to express different HSPs as well as antioxidant enzymes in response to different abiotic stresses and provide the favorable condition to these proteins in order to have their maximum activity to neutralize the free radicals which are produced because of oxidative burst. The HSPs present at particular stages also helps in protecting the important key enzymes of metabolic pathways from denaturation in response to heat stress. In present investigation, the tolerant varieties has shown very high activity of APX isoenzymes against different heat shock treatment which

was also reported by (Mittler et al., 1994). The transcript level of APX increases 4 fold in response to drought and 15 fold during recovery from stress and many environmental factors like abiotic stresses enhance the transcript level of APX and other antioxidant enzymes as reported by Mittler and Zilinskas (1994). In case of susceptible cultivars, very few APX isoenzymes were observed which may be because of complete denaturation of isoenzymes when 40°C heat shock treatment was given for 2 h. It revealed the fact that the APX isoenzymes present in case of susceptible cultivars are very sensitive to heat stress treatment and has very low activity at high heat shock compare to thermotolerant cultivars where the tolerance level of APX isoenzymes are very high. This result is in conformity with the observation made by Yamaguchi et al. (1995), Schantz et al. (1995) and Ishikawa et al. (1996) who reported that there is change in the activities of APX isoenzymes in response to different abiotic stresses. APX activity has been shown to increase in response to a number of stress conditions, such as drought, salt and deficiency in microelements. So far, several different protein isoforms have been identified: two soluble cytosolic forms (Mittler and Zilinskas, 1991), and two chloroplast forms (Ishikawa et al., 1996), one of which is stromal and the other thylakoid bound. Recently, several isoforms bound to membranes of glyoxysomes and peroxisomes were reported by Yoshimura et al. (1998).

Even the expression of all the APX isoenzymes observed in present investigation on the gel are very high in case of tolerant cultivars compare to susceptible one in response to different heat shock treatment. The APX isoenzymes reported in cytosol has highest activity in response to short heat shock treatment and it acts as a redox buffer for the whole cells as reported by Locato et al. (2009).

This is the first time that so many APX isoenzymes have been observed in response to heat stress at germinating stage. APX expression generally increases along with other enzymes of antioxidant system like Catalase, SOD and Glutathione reductase against environmental stress factors which gives impression that component of AOS scavenging systems are co-regulated (Mittler et al., 1994; Leonardis et al., 2000).

Hence, by maintaining high APX expression level inside the plant system through the genetic engineering tools will definitely supplements the antioxidant defense systems of the plants against AOS and will help in enhancing the tolerance level of the plant. This mechanism can be used in case of thermosusceptible cultivars to enhance their tolerance level by increasing the transcript level of different antioxidant systems.

Conclusion

The mechanism of heat stress tolerance in wheat has not been fully discovered. The HSPs and antioxidant systems are playing the key role in providing the tolerance against heat stress. In present investigation protein profiling of C306 and PBW343 revealed the expression of many new proteins in response to heat shock. Isoenzymic profile of APX showed the expression of 8 isoenzymes in C306 and 2 in case of PBW343 against differential heat shock. A stage specific profiling showed maximum number of APX isoenzymes at vegetative, pollination, milky dough and seed hardening stage in case of C306 and milky dough stage in case of PBW343. The transcript level and number of different isoenzymes are playing very important role in providing tolerance against heat shock treatment. Hence, there is a need to identify, isolate and characterize these different isoenzymes, so that they can be manipulated to increase the endogenous transcript level and ultimately enhancing the tolerance capacity of susceptible crops. This can be used as a means to get a sustainable yield in present situation where we are facing the menace of global warming. This approach will help us to create a model crops which can sustain the effect of global climate change.

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