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Hydrogen peroxide-scavenging enzymes impart tolerance to high temperature induced oxidative stress in sugarcane

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Abstract

Seventy-one genotypes of sugarcane from diverse agro-climatic zones of India *viz*. peninsular, northwest, north-central and eastern zones, were screened for their tolerance to high temperature stress based on the damage to leaf biomass *i.e.* necrosis of leaf-tips and margins, and rolling of leaves. Nine selected genotypes showing variable response to heat injury were tested for activity pattern of isoforms of two H_2O_2-scavenging enzymes; ascorbate peroxidase (APX) and catalase (CAT), under high temperature induced oxidative stress. Changes in the activity of APX and CAT isozymes in leaves corresponded to the level of tolerance of genotypes towards heat injury which was substantiated by the highly negative correlation coefficients of heat injury levels of leaves *vs.* integrated density of APX and CAT isozyme bands. This indicated that the criteria of higher expression of CATs' and APXs', the two major reactive oxygen species scavenging proteins in leaves may be used to screen large seedling populations and germplasm for high temperature tolerance.

Key words

Saccharum spp. (hybrids), H_2O_2-scavengers, Heat tolerance, Oxidative stress and Reactive oxygen species

Introduction

High temperature stress is a widely occurring problem in many sugarcane-growing countries and affects growth and development of sugarcane. The subtropical North Indian conditions are prone to high temperature stress in the months of April, May and June which generally has an adverse effect on growth and development phase of sugarcane crop. Temperature is an important factor affecting growth, development and distribution of many plants (Grover *et al*., 2001). By influencing photosynthesis, respiration, other metabolic activities, flowering and circadian rhythms etc., higher as well as suboptimal temperatures limit crop productivity. The effect of high temperature on higher plants is primarily on photosynthetic functions (Crafts-Brandner *et al*., 2000). More chloroplast-associated and photosynthesis-related genes would be expressed in the heat-tolerant genotype subjected to high temperature. Although the functions of these genes are not clear, they could play important roles in tolerant genotypes to sustain heat stress. There is increasing evidence for considerable interlinking between the responses to heat stress and oxidative stress. Heat induced oxidative stress in plants (Foyer *et al*., 1994) produces reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide and hydroxyl radicals. Accumulation of hydrogen peroxide has not only negative consequences on living cells, but it is also involved in stress signaling and mediating the cellular redox status (Mittler and Zilinskas, 1993; Neill *et al*., 2001). In contrast to atmospheric oxygen, the ROS are capable of unrestricted oxidation of various cellular components and can lead to the oxidative damage of cellular components. Whereas under normal growth conditions, the production of ROS in cells is low, these stresses that disrupt the cellular homeostasis of cells enhance the production of ROS (Noctor and Foyer, 1998).

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The balance between superoxide dismutase (SOD) and ascorbate peroxidase (APX) or catalase (CAT) activities in cells is crucial for determining the steady-state level of superoxide radicals and hydrogen peroxide. The different affinities of APX and CAT for H_2O_2 suggest that they belong to two different classes of H_2O_2-scavenging enzymes: APX might be responsible for the fine modulation of ROS for signaling, whereas CAT might be responsible for the removal of excess ROS during stress (Noctor and Foyer, 1998).

Sugarcane is one of the leading agricultural crops, which is not only a source of sugar but also gives a large number of by products. In India, sugarcane is cultivated in approximately 4.28 million hectares, producing ~300 million tonnes of cane every year (Nair, 2011). The production and the productivity of sugarcane in the country are limited by various biotic and abiotic factors. Among the abiotic factors, water logging and temperature stress are the major hindrance to the growth of sugarcane crop (Gupta *et al*., 2010; Wahid, 2007). In the crop year 2007-08, the sugarcane cultivation in subtropical North India experienced extremely high temperatures. In order to identify the varieties tolerant to temperature stress conditions, in the present study sugarcane varieties belonging to various agro-climatic zones of India were screened for their tolerance towards high temperature stress and the expression pattern of catalases and ascorbate peroxidase.

Materials and Methods

Seventy-one genotypes of sugarcane belonging to peninsular, northwest, northcentral and eastern agro-climatic zones of India (Table 1) were used for the study. The daily temperatures were recorded for a span of 60 days, starting from 15th April to 15th June, 2007. The impact of high temperature was assessed based on the damage to their leaf biomass at early growth stages of plant (three month crop age) in terms of: (i) necrosis of leaf-tips and margins and (ii) rolling and drying of leaves. The genotypes were rated for injury faced by the leaves on a 0-4 point arbitrary scale with respect to damage to leaf-tip and margins: 0 = no damage to leaf , 1 = up to 2 cm leaf tip damage, 2 = 2-4 cm leaf tip damage and drying of leaf margins, 3 = 4-6 cm leaf tip damage and leaf margin damage/drying, 4 = > 6 cm drying of leaves; and rolling/non rolling of leaves in response to/ escape high temperature stress (0 = no leaf rolling, 1 = Up to 25% leaf rolling, 2 = 26%-50%, 3 = 51%-75%, 4 = > 76%). To calculate percentage of rolled leaves, number of rolled leaves/ genotype as well as total number of leaves/ genotype in a plot of 5.4 m^2 were taken into consideration. Based on this, nine sugarcane genotypes were selected which had heat injury rating as well as leaf rolling/ not rolling rating ranging from 0-4. The expression pattern of two H_2O_2-scavenging enzymes catalase and ascorbate peroxides were studied in these nine genotypes.

Gel-electrophoresis of CAT and APX : The first completely unfurled leaf from the top was harvested as experimental material. For expression of CAT, leaf protein was extracted in chilled extraction buffer (50 mM Tris HCl, pH 7.4 supplemented with leupeptin, DTT (dithiothreitol) and PVP (polyvinyl pyrrolidone) followed by centrifugation at 12000 *g* at 4°C for 15 min. The supernatants were dialyzed in cellophane membrane tubings for 4 -6 hrs in cold against 3-4 changes of the extraction buffer. For ascorbate peroxidase assay, 500 mg fresh leaf samples were homogenized in chilled mortar and pestle in 2 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 1mM ascorbic acid, 1 mM EDTA and 2% polyvinyl pyrrolidone (PVP) which was added just prior to use. The homogenate were centrifuged at 12,000 *g* for 10 min at 4ºC and the dialyzed enzyme extracts were used for the assay. Soluble protein content was determined by the method of Bradford (1976) with bovine serum albumin as a standard. For isozymes, supernatant equivalent to 25 μg protein was electrophoresed on 7% PAGE without SDS. Electrophoresis was carried out in a vertical gel electrophoresis apparatus (Mini Protean III, BioRad, USA) using a discontinuous buffer system. The composition of gels and buffers used for the electrophoresis was according to the method of Laemmli (1970). The gels were photo documented with a CCD camera attached to AlphaImager™ gel documentation system (Alpha Innotech Corporation, San Leandro, USA) and stored in 3% acetic acid.

Catalase isoforms were visualized on gels by the method of Aebi (1984), using stain mixture containing $FeCl_3$ and $K_3Fe(CN)_6$ at room temperature until white bands of CAT appeared on dark green background. To visualize APX isoforms, after electrophoresis gels were incubated at room temperature for 15 min in 0.1 M sodium phosphate buffer (pH 6.4) containing 4 mM ascorbate and 4mM hydrogen peroxide. The gels were washed with water and then stained for 10 min with 0.1% ferric chloride (w/v) in 0.125 N HCl (Mittler and Zilinskas, 1993). Ascorbate peroxidase was located as an achromatic band on a prussian blue background.

Relative mobility, optical density and percent integrated density value of isozyme bands were scored by the AlphaEase software ver 2.5 (Alpha Innotech Corporation, San Leandro, USA). Each band on the gel corresponding to an isozyme was designated using the enzyme code (APX for ascorbate peroxidase and CAT for catalase) followed by a number based on the relative mobility of a particular band. Relative mobility (Rm) of the bands was determined as the ratio of the distance of the band from the origin to the distance of the dye front expressed in percentage. Integrated density value (IDV) denoted the percentage density of isozyme bands that each genotype contributed to the total density measured thus far, for all the isozyme bands of all the genotypes based on the pixel values, taking background correction into consideration. The sum of the ID values is 100. All the data were recorded in three replications.

Results and Discussion

The day-to-day temperature record was maintained for a period of 60 days. During this period the minimum temperature exceeded 25°C for 22 days, whereas, the maximum temperature was more than 40°C for 30 days. Seventy-one genotypes of sugarcane belonging to various agro-climatic zones of India *viz.* peninsular zone (6 genotypes), northwest (58 genotypes),

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Table 1: Level of heat injury and leaf rolling in genotypes of different agro-climatic zones of India

S. No.	Genotype	Heat* injury	Leaf** rolling
North-western zone:			
1.	CoLk 8102	1	0
2.	CoLk 04238	0	0
3.	CoLk 94184	3	0
4.	CoLk 9616	1	0
5.	CoLk 9617	3	0
6.	CoLk 9705	1	0
7.	CoLk 9707	0	0
8.	CoLk 9709	2	1
9.	CoLk 9412	1	1
10.	CoLk 9606	1	1
11.	CoS 88230	1	0
12.	CoS 96268	3	0
13.	CoS 99259	0	0
14.	CoS 95255	1	0
15.	CoS 8436	1	0
16.	CoS 96275	0	0
17.	CoS 767	1	4
18.	CoS 01268	2	1
19.	CoS 02264	1	0
20.	CoS 00221	1	1
21.	CoJ 02191	3	4
22.	CoS 98259	1	0
23.	CoS 96258	1	1
24.	CoS 2252	1	3
25.	CoS 97248	1	3
26.	CoS 95258	0	0
27.	CoS 95222	1	0
28.	CoS 94257	1	2
29.	CoSe 92423	1	0
30.	CoSe 0241	0	3
31.	CoH 110	2	0
32.	CoH 119	2	0
33.	CoH 92201	1	0
34.	CoH 92202	0	0
35.	CoJ 64	1	3
36.	CoJ 83	1	3
37.	CoJ 20193	1	0
38.	CoJ 99192	0	1
39.	Co 99016	1	1
40.	Co 99015	2	0
41.	Co 00240	1	0
42.	Co 00241	1	0
43.	Co 89003	1	0
44.	Co 0238	4	3
45.	Co 0237	0	1
46.	Co 0120	0	0
47.	Co 0230	1	0
48.	CoPant 84212	1	0
49.	CoPant 90223	1	0
50.	CoPant 02217	0	4
51.	CoPant 84211	1	2
52.	CoPant 02218	2	2
53.	CoPant 99213	1	4
54.	CoPant 99214	1	0
55.	CoPant 98224	1	0
56.	CoPk 59	1	2
57.	CoPk 112	1	1
58.	CoPk 78	1	4
Peninsular zone			
1.	CoC 90063	1	3
2.	CoA 93081	1	1
3.	Co 86249	1	1
4.	Co 93009	1	4
5.	CpV 94101	2	1
6.	CoLk 8001	1	2
North-central and Eastern zones			
1	BO 130	1	3
2	BO 128	1	4
3	Co 89029	0	2
4.	CoSe 95422	1	0
5.	CoP 9301	1	0
6.	CoP 9206	0	0
7.	BO 91	1	1

*No damage to leaf -0, Up to 2 cm leaf tip damage -1, 2-4 cm leaf tip damage and drying of leaf margins -2, 4-6 cm leaf tip damage and leaf margin damage/drying -3, > 6 cm drying of leaves -4

** % leaves rolled = Nil -0, Up to 25% -1, 26-50% -2, 51-75% -3,>76% -4

northcentral and eastern zones (7 genotypes) were screened for their tolerance to high temperature and its impact on their leaf biomass (Table 1). These genotypes were rated for injury faced by the leaves at early growth stages of plant on a 0-4 point arbitrary scale with respect to damage to leaf-tip and margins and rolling of leaves in response to or escape high temperature stress. No damage to leaf biomass was observed in 18.31% of the total genotypes. Maximum number of genotypes (64.79%) suffered up to 2 cm leaf tip damage only, followed by 11.27% genotypes showing 2-4 cm leaf tip damage along with drying of leaf margins. Similarly, maximum number of genotypes (50.70%) did not show any rolling of leaves followed by 19.72% genotypes showing up to 25% leaf rolling in response to or to escape high temperature stress. Four genotypes showed up to 4-6 cm damage of leaf tips and margins along with drying of leaves and only one genotype suffered from more than 6 cm drying of leaves. Six genotypes had 26-50% leaf rolling followed by eight genotypes showing 51-75% of leaf rolling and seven genotypes had more than 75% rolled leaves. The pattern showed variable response of sugarcane genotypes towards high temperature stress.

To relate the role of antioxidant isozyme pattern and high temperature tolerance, CAT and APX isozyme pattern of leaves was analyzed on native PAGE with 7% resolving gel, from some sugarcane genotypes showing variable response (from susceptible to tolerant) towards high temperature induced injury to the leaves. The total soluble ascorbate peroxidase and catalase activity was increased in high temperature tolerant sugarcane genotypes as indicated by higher average integrated density of the isoforms of these enzymes on native gel (Table 2).

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Table 2 : Varieties selected, their extent of leaf rolling and heat injury and total integrated density of catalase (CAT) and ascorbate peroxidase (APX) isozymes

Variety	Leaf* rolling	Heat injury**	Total integrated density	
			CAT	APX
CoPant 02217	4	0	23.2	15.4
CoSe 0241	3	0	19.2	11
Co 0237	1	0	10.7	12.3
CoJ 99192	1	0	14.7	11.1
CoS 8436	0	1	12.7	14.5
CoLk 9709	0	2	8	8.9
Co 99015	0	2	4.4	9.5
CoJ 02191	4	3	4.4	9.2
Co 0238	3	4	2.7	8.1

* = % leaves rolled= nil -0, Up to 25% -1, 26-50% -2, 51-75% -3, > 76% -4
**No damage to leaf -0, Up to 2 cm leaf tip damage -1, 2-4 cm leaf tip damage and drying of leaf margins -2, 4-6 cm leaf tip damage and leaf margin damage/drying -3, > 6 cm drying of leaves -4

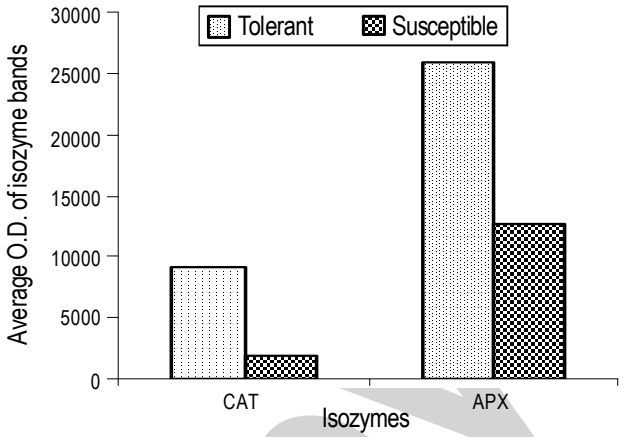

Fig. 1: Average optical density of catalase (CAT) and ascorbate peroxidase (APX) isozyme bands in high temperature tolerant and susceptible varieties of sugarcane

Table 3 : Activity pattern of isozyme bands* of ascorbate peroxidase and catalase on native gel in different sugarcane genotypes

	Rm	Co Pant 02217	CoSe 0241	Co 0237	CoJ 99192	CoS 8436	CoJ 02191	CoLk 9709	Co 99015	Co 0238
Ascorbate peroxidase										
APX-1	0.13	-	-	-	+++	-	++	++	-+	-
APX-2	0.15	+++	+++	+++	-	+++	-	-	-	++
APX-3	0.26	+++	++	-	+++	-	-	-	+	-
APX-4	0.52	++++	-	-	++	-	+	-	-	-
APX-5	0.62	++++	++	++++	-	++++	-	-	+	-
APX-6	0.69	+++	-	++++	+	-	-	+	+	-
APX-7	0.74	+++	+++	-	+	++++	-	-	-	-
Total No. of APX bands		6	4	3	5	3	2	2	4	1
Catalase										
CAT-1	0.15	+++	++	+	++	+	-	-	-	-
CAT-2	0.24	++++	++	+++	+++	+++	++	++	++	-
CAT-3	0.29	+++	+	-	-	-	-	-	-	+
Total No. of CAT bands		3	3	2	2	2	1	1	1	1

* Intensity of bands; - = Band absent; + = Band of low intensity; ++ = Band of medium intensity; +++ = Band of high intensity; ++++ = Band of very high intensity

Seven isoforms of ascorbate peroxidase, APX-1 (Rf = 0.13), APX-2 (Rf = 0.15), APX-3 (Rf = 0.26), APX-4 (Rf = 0.52), APX-5 (Rf = 0.62), APX-6 (Rf = 0. 69), APX-7 (Rf = 0.74) were present (Table 3). Marked variation in APX isozyme pattern was observed among the genotypes. Maximum number of APX isozymes (6 isoforms) was present in CoPant 02217 followed by CoJ 99192 (5 isoforms). The genotypes showing very little or no heat injury expressed 3 to 6 isoforms of APX as compared to the presence of 1 to 4 isoforms in the genotypes severely damaged by high temperature. The activity of most of the isoforms as indicated by the APX band intensity increased in the leaves of the genotypes, which showed very low or no heat injury (Fig. 1). The optical density (O.D.) of APX isozyme bands was also higher in high temperature tolerant genotypes compared to the susceptible ones (Table 2). Changes in the activity of APX, as measured with activity gels, corresponded to the trend of tolerance towards heat injury. The correlation coefficient of heat injury levels of leaves *vs.* Integrated density value (IDV%) of APX isozyme bands was also highly negative (r = -0.83). However, there was no correlation of the rolling/ not rolling of leaves with APX isozyme expression.

Three isoforms of catalase, CAT-1 (Rf = 0.15), CAT-2 (Rf = 0.244), and CAT-3 (Rf = 0.291) were present (Table 3). The heat susceptible genotypes expressed only one isoform of CAT, whereas 2 to 3 isoforms of CAT were resolved in heat tolerant genotypes. CAT-2 isoform was present in all genotypes whether susceptible or tolerant except Co 0238. CAT-3 was expressed in three genotypes CoPant 02217, Co 0238 and CoSe 0241, whereas CAT-1 was expressed in CoJ 99192, CoPant 02217, CoS 8436, Co 0237 and CoSe 0241. Except Co 0238, CAT-1 and CAT-3 were expressed

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in heat tolerant genotypes only. Expression of all the isozymes *i.e.* CAT-1, CAT-2 and CAT-3 was more in high temperature tolerant sugarcane genotypes than in susceptible genotypes (Table 3) and average O.D. values of tolerant and susceptible genotypes (figure 1). The correlation coefficient of heat injury levels of leaves *vs.* IDV% was also negative (r = -0.71). However, the rolling/ not rolling of leaves did not show any correlation with CAT isozyme expression.

Higher expression of CATs' and APXs' under high temperature in the leaves of heat tolerant sugarcane genotypes may protect them from reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radicals (OH^-) produced after exposure to high temperature. Ascorbate peroxidase one of the most important antioxidant enzymes of plants that detoxify hydrogen peroxide using ascorbate for reduction is present in chloroplasts, cytosol, mitochondria, apoplast and peroxisomes. By contrast, CAT is only present in peroxisomes, but it is indispensable for ROI detoxification during stress, when high levels of ROS are produced (Scandalios *et al*., 2000). In addition, oxidative stress causes the proliferation of peroxisomes (Lee *et al*., 1999). Catalase can be used to reduce hydrogen peroxide levels in the peroxisomes but it is absent in chloroplasts. The role of CAT is filled by specific APXs'. This peroxidase uses ascorbic acid as a hydrogen donor to break down hydrogen peroxide (Asada, 1994). There are evidences, which suggest the role of higher expression of some isozymes of catalase and ascorbate peroxidase under high temperature stress. In leaves of *Arabidopsis*, a new APX isoform, APX^S, was identified after activity staining in native protein gels and the total soluble APX activity increased after heat treatment at 34 °C and 37 °C (Lopez-Huertas *et al*., 2000). In plants cultivated at 20 °C, there was only one major isoform, the cytosolic APX-1, detectable in gels. These quantitative effects correlated with the appearance of new isoform of APX (Lopez-Huertas *et al*., 2000). In thermally stressed maize seedlings, the accumulation and/or stability of CAT-1 mRNA compensated for the lack of CAT-2 transcript in a tissue where CAT-2 mRNA normally accumulated during the developmental period examined (Noctor and Foyer, 1998).

The higher expression of CATs' and APXs' in leaves of sugarcane genotypes as observed in the present study may thus be taken as a parameter to screen large seedling populations and germplasm resources of sugarcane in order to breed high temperature tolerant genotypes as well as to identify genetic stocks to be used as parents in future breeding programmes of sugarcane.

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