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BRIEF COMMUNICATION

Calcium-dependent signaling pathway in the heat-induced oxidative injury in *Amaranthus lividus*

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Abstract

Heat caused reduction in membrane protein thiol content, increased accumulation of thiobarbituric acid reactive substances and reduced germination rate and early growth in germinating *Amaranthus lividus* seeds. Imposition of heat stress during early germination also causes accumulation of reactive oxygen species like superoxide and hydrogen peroxide while activities of antioxidative enzymes catalase, ascorbate peroxidase, and glutathione reductase decreased. Calcium chelator (EGTA), calcium channel blocker ($LaCl_3$) and calmodulin inhibitor (trifluroperazine) aggravated these effects. Added calcium reversed the effect of heat, implying that protection against heat induced oxidative damage and improvement of germination requires calcium and calmodulin during the recovery phase of post-germination events in *Amaranthus lividus*.

Additional key words: asorbate peroxidase, calmodulin, catalase, glutathione reductase, reactive oxygen species.

In nature plants are subjected to changes in temperature both during seasons and individual days. Heat stress limits growth and development and induces many physiological and biochemical changes including oxidative stress (Jiang and Huang 2001, Bhattacharjee and Mukherjee 2006). High temperature influences germination and subsequent seed-ling growth (Alka and Khanna-Chopra 1995, Bhattacharjee and Mukherjee 2003/2004). Imbibition and early germination events continue even at supra-optimum temperature but embryo growth in most of the cases is inhibited.

Several studies showed that Ca^{2+} is involved in the regulation of plant responses to various environmental stresses including heat (Knight and Knight 1993, Gong *et al.* 1998, Jiang and Huang 2001, Nayyar 2003, Cousson 2007). Increased cytosolic Ca^{2+} content under heat stress may alleviate heat injury and enable plant cells to better survive (Knight and Knight 1993, Wang and Li 1999). However, excessive Ca^{2+} released into cytosol and maintaining high Ca^{2+} concentration might be cytotoxic (Wang and Li 1999, Jiang and Huang 2001).

Contradictory results are available concerning the effects of exogenous Ca^{2+} on heat tolerance. It was found that treatment of *Zea mays* and *Amaranthus* seeds with 15 or 20 mM Ca^{2+} solution enhanced intrinsic heat tolerance of seedlings (Gong *et al.* 1997a,b, Bhattacharjee 2001). In contrast, heat induced growth retardation in excised coleoptile of wheat was not alleviated by external Ca^{2+} treatment (Onwueme and Laude 1972).

Therefore, the role of Ca^{2+} in regulation of heat tolerance is still unclear. Some authors suggest that it may be involved in signal transduction involving new gene expression (Trofimova *et al.* 1999) under oxidative and heat stress. Others reported that Ca^{2+} control guard cell pressure potential and stomatal apperture (Mansfield *et al.* 1990, Webb *et al.* 1996), and helps in pressure potential maintenance (Hare *et al.* 1998).

The objective of the present study is to investigate the

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Abbreviations: APOX - ascorbate peroxidase; CAT - catalase, DTNB - 5,5-dithio-bis-2-nitrobenzoic acid; GR - glutathione reductase; HS - heat stress; RGI - relative growth index; MPTL - membrane protein thiol level; RGP - relative germination performance; ROS - reactive oxygen species; SOD - superoxide dismutase; TFP - trifluroperazine; TBARS - thiobarbituric acid reactive substances.

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involvement of Ca^{2+} and calmodulin on heat tolerance and survival during early germination in a tropical leaf crop *Amaranthus lividus* L. and also to examine their effects on reactive oxygen species metabolism and subsequent oxidative injury of the germinating tissues.

Surface sterilized seeds of the tropical leaf crop *Amaranthus lividus* L were imbibed in darkness for 20 h in the following solutions: *1*) 20 mM $CaCl_2$, *2*) 1 mM $LaCl_3$, *3*) 2 mM EGTA, *4*) 200 μM trifluroperazine, *5*) distilled water. Seeds were then air dried at room temperature and finally sown in Petri dishes on moist filter paper (30 seeds per plate) and subsequently kept either at 40 ± 2 °C or at room temperature for 24 h (control). Finally they were allowed to grow at 25 ± 2 °C, 12-h photoperiod (270 μmol m^{-2} s^{-1}) and 78 ± 2 % RH for 72 h.

For studying survival and growth, relative germination performance (RGP) and relative growth index (RGI) were calculated (Bhattacharjee and Mukherjee 2003/2004): RGP = (number of treated seeds germinated after 72 h/number of control seeds germinated after 72 h) × 100; RGI = (average dry mass of ten treated seedlings/ average dry mass of ten control seedlings) × 100.

To estimate membrane lipid peroxidation test for thiobarbituric acid reactive substances (TBARS) was performed using the procedure of Heath and Packer (1968). For the determination of membrane protein thiol content, the membrane was prepared according to Singh (1997) with some necessary modifications. Plant tissue (1 g) was homogenized in 10 cm^3 ice cold buffer (0.05 M Tris-HCl, pH 7.0). The homogenate was centrifuged at 10 000 *g* at 4 °C for 30 min and the pellet was discarded. The membranes were then sedimented at 100 000 *g* at 4 °C for 3 h and the pellet containing the membrane fractions was suspended in ice cold buffer (0.05 M Tris-HCl, pH 7.0). The membrane fractions were stored under ice. The membrane associated protein bound thiol groups were assayed after protein precipitation with 10 % (m/v) trichloroacetic acid (TCA) and quantified with 5,5-dithio-bis-2-nitro-benzoic acid (DTNB) following the procedures of Ellman (1959) and Dekok and Kuiper (1986). H_2O_2 was extracted and estimated following the procedure of MacNevin and Uron (1953) using titanic sulfate. For the estimation of total thiol content the method of Tietze (1969) was followed. Total -SH content was assayed in acid soluble extract (in 3 % m/v TCA solution) followed by a brief centrifugation. The supernatant was then diluted 10-fold in 100 mM phosphate buffer (pH 7.5). Thiol contents was determined measuring absorbance at 412 nm in presence of 0.5 mM DTNB, 0.5 U cm^{-3} glutathione reductase and 0.2 mM NADPH. For the determination of superoxide, the method of Chaitanya and Naithani (1994) was followed with some necessary modifications. Tissue (500 mg) was homogenized in 5 cm^3 of 0.2 M sodium phosphate buffer, pH 7.2, with addition of diethyldithiolcarbamate (10^{-3} M) to inhibit SOD activity. The homogenates was immediately centrifuged at 2 000 *g* at 4 °C for 1 min. In the supernatant, superoxide anion was measured by its capacity to reduce nitrobluetetrazolium (0.25 mM). The absorbance of the end product was measured at 540 nm.

For the extraction and estimation of catalase and superoxide dismutase the methods of Snell and Snell (1971) and Giannopolitis and Ries (1977) were followed. Ascorbate peroxidase (APOX) activity was determined according to Nakano and Asada (1981) using homogenates previously supplemented with 0.5 mM ascorbic acid and 0.1 mM EDTA. Parallel experiments in presence of *p*-chloromercuribenzoate (50 μM) were performed to rule out any interference from guaiacol peroxidases. Glutathione reductase (GR) activity was measured according to Schaedle and Bassham (1977). The reaction mixture contained 50 mM Tris-HCl (pH 7.6), 0.15 mM NADPH, 0.5 mM oxidized glutathione (GSSG), 3 mM $MgCl_2$ and 0.1 cm^3 homogenate (7 mg protein cm^{-3}). NADPH oxidation was determined at 340 nm.

When germinating *Amaranthus* seeds were treated at 40 °C for 24 h, they exhibit less survival (measured in terms of RGP) as well as reduced early growth (measured in terms RGI). RGP and RGI for heat stress seedlings have been reduced to 54 and 56 %, respectively, as compared to control (Table 1). In order to ascertain whether the damage caused by elevated temperature was due to the oxidative stress the accumulation of TBARS was measured. Heat treatment caused significant increase in TBARS content accompanied with decline in membrane protein thiol level (MPTL) (Table 1). To test the hypothesis whether Ca^{2+} plays any role in mitigating oxidative stress and improving survival and growth performances Ca^{2+} (20 mM $CaCl_2$) pretreatment has been done during early imbibitional phase of germination and subsequently exposed to elevated temperature (40 °C for 24 h). Ca^{2+} pretreated seedlings clearly shows reversal in oxidative damage (reduction in the accumulation of TBARS and restoration of MPTL) as well as improved survival and early growth performance (Table 1).

Effect of calcium channel blocker ($LaCl_3$), calcium chelator (EGTA) and inhibitors of calmodulin (trifluroperazine) pretreatment on subsequently heat stressed *Amaranthus* was also assessed in terms of germination and growth performances as well as oxidative membrane injury. All the inhibitors tested increased heat shock induced accumulation of TBARS with a concomitant reduction in MPTL (Table 1). Their effects on survival and early growth performance were also negative. La^{3+} had the greatest effect on increasing the oxidative membrane damage with a corresponding reduction in RGP and RGI.

Heat caused significant increase in the content of ROS (superoxide and H_2O_2) as compared to control. Ca^{2+} pretreatment prior to heat reduced the accumulation of both the ROS, whereas all the inhibitors enhanced their

Table 1. Effect of calcium (20 mM $CaCl_2$), calcium channel blocker (1 mM $LaCl_3$), calcium chelator (2 mM EGTA) and calmodulin inhibitor (250 μM trifluroperazine, TFP) on survival (RGP), early growth (RGI), and parameters of oxidative stress [thiobarbituric acid reactive substances (TBARS), membrane protein thiol level (MPTL), total thiol content, superoxide content, hydrogen peroxide content and activities of ascorbate peroxidase (APOX), catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR)] in response to heat stress (40 °C for 24 h) in *Amaranthus lividus* L. Values are mean of three independent replicates ± SE. CD values are calculated by *ANOVA*.

Parameters		Control	Heat	Ca^{2+} + heat	$LaCl_3$ + heat	EGTA + heat	TFP + heat	CD at 5 %
RGP [%]		100	54.7 ± 0.11	71.5 ± 0.17	44.4 ± 0.08	46.4 ± 0.04	48.0 ± 0.03	0.720
RGI [%]		100	56.1 ± 0.19	72.0 ± 0.10	44.0 ± 0.05	48.2 ± 0.04	46.2 ± 0.04	0.742
TBARS	root	68.0 ± 0.11	88.1 ± 0.14	72.6 ± 0.12	136.5 ± 0.11	101.7 ± 0.19	98.4 ± 0.08	0.864
[mmol g^{-1}(d.m.)]	shoot	44.6 ± 0.09	71.6 ± 0.12	53.5 ± 0.10	122.5 ± 0.13	92.1 ± 0.21	79.9 ± 0.10	1.423
MPTL	root	114.0 ± 0.28	58.5 ± 0.11	83.1 ± 0.20	48.8 ± 0.09	52.7 ± 0.12	48.6 ± 0.08	1.125
[mmol g^{-1}(d.m.)]	shoot	158.0 ± 0.30	85.8 ± 0.21	110.5 ± 0.26	72.6 ± 0.17	83.2 ± 0.17	78.3 ± 0.11	0.834
Total thiols	root	1.07 ± 0.007	0.75 ± 0.002	0.91 ± 0.003	0.63 ± 0.001	0.70 ± 0.01	0.72 ± 0.004	0.058
[μmol g^{-1}(d.m.)]	shoot	0.85 ± 0.002	0.48 ± 0.001	0.60 ± 0.08	0.35 ± 0.004	0.04 ± 0.003	0.38 ± 0.001	0.054
Superoxide	root	14.5 ± 0.07	60.0 ± 0.12	41.8 ± 0.10	68.4 ± 0.17	76.2 ± 0.14	71.5 ± 0.11	0.809
[ΔA_{540} g^{-1}(d.m.)]	shoot	10.1 ± 0.03	58.8 ± 0.19	50.0 ± 0.20	66.8 ± 0.17	68.0 ± 0.18	68.2 ± 0.11	1.287
H_2O_2	root	81.4 ± 0.20	166.4 ± 0.30	138.0 ± 0.28	181.4 ± 0.33	179.0 ± 0.30	180.0 ± 0.31	0.853
[mmol g^{-1}(d.m.)]	shoot	98.2 ± 0.27	198.2 ± 0.61	146.1 ± 0.38	216.4 ± 0.30	224.0 ± 0.33	218.0 ± 0.32	1.006
APOX	root	9.8 ± 0.04	8.06 ± 0.03	8.50 ± 0.07	8.00 ± 0.03	8.00 ± 0.02	7.90 ± 0.02	0.279
[U g^{-1}(d.m.) min^{-1}]	shoot	8.3 ± 0.03	7.00 ± 0.01	7.90 ± 0.04	6.75 ± 0.02	6.80 ± 0.01	6.77 ± 0.01	0.239
CAT	root	4.14 ± 0.01	3.30 ± 0.02	3.79 ± 0.01	3.20 ± 0.01	3.10 ± 0.01	3.15 ± 0.01	0.035
[U g^{-1}(d.m.) min^{-1}]	shoot	3.28 ± 0.01	2.60 ± 0.05	2.98 ± 0.02	2.41 ± 0.007	2.50 ± 0.002	2.54 ± 0.03	0.072
SOD	root	4.80 ± 0.02	3.70 ± 0.02	3.71 ± 0.01	3.50 ± 0.01	3.40 ± 0.03	3.50 ± 0.03	0.173
[U g^{-1}(d.m.) min^{-1}]	shoot	4.00 ± 0.02	3.64 ± 0.02	3.60 ± 0.03	3.10 ± 0.02	3.10 ± 0.02	3.20 ± 0.03	0.066
GR	root	0.17 ± 0.01	0.10 ± 0.01	0.16 ± 0.006	0.10 ± 0.002	0.10 ± 0.002	0.01 ± 0.002	0.019
[U g^{-1}(d.m.) min^{-1}]	shoot	0.13 ± 0.01	0.10 ± 0.01	0.19 ± 0.002	0.11 ± 0.001	0.07 ± 0001	0.09 ± 0.001	0.022

accumulation. Heat stress during early phase of germination induced oxidative injury and significantly reduced the activities of antioxidative enzymes CAT, APOX, SOD, GR as well as total thiol level (Table 1). Ca^{2+} pretreatment helped to maintain higher APOX, CAT and GR activities and total thiol level, however, did not affect SOD activity. Activities of all these antioxidant enzymes and total thiol level decreased significantly when seeds were treated with Ca^{2+} channel blocker, Ca^{2+} chelator or calmodulin inhibitor (Table 1).

Data showed a secondary oxidative stress and related oxidative membrane injury in *Amaranthus lividus* after heating during early germination which continued 3 d post heating. This concurs with the results described by Gong *et al.* (1998), Larkindale and Knight (2002), Bhattacharjee and Mukherjee (2003/2004, 2006), and Bhattacharjee (2005). The levels of oxidative damage generally correlated with survival (germination) and early growth performance. Although this two variables are not completely linked, in all experiments survival declined with higher contents of TBARS and lower MPTL. This suggests that survival after heat shock requires the ability to tolerate or repair oxidative damage and it also requires the ability to tolerate or minimize other kinds of heat induced damage (Woodstock *et al.* 1983, Bhattacharjee 2001, Gong *et al.* 1998, Larkindale and Knight 2002, Bhattacharjee 2005, Bhattacharjee and Mukherjee 2006).

The increase in heat-tolerance in Ca^{2+} pretreated and subsequently heat stress seedlings reinforced the idea that Ca^{2+} might be required in some signaling pathway in *Amaranthus* leading to improved survival. This suggests that a flux of Ca^{2+} ion is required to switch on some mechanisms by which plants prevent or repair oxidative damage caused by heating and thus a Ca^{2+} flux is required for the plant to germinate under elevated temperature.

Ca^{2+} channel blocker, Ca^{2+} chelator and calmodulin inhibitor pretreatment before imposition of heat stress further substantiate the fact that a flux of Ca^{2+} is required to switch on some mechanism to mitigate oxidative damage of the germinating tissue. The significantly reduced accumulation of $O_2^{\cdot-}$ and H_2O_2 along with elevated activities of antioxidative enzymes (APOX, GR and CAT) and water soluble total thiol level in Ca^{2+} pretreated and heat shock raised *Amaranthus* seedlings further corroborate the involvement of Ca^{2+} in mitigating the oxidative stress. This Ca^{2+}-dependent pathway is presumed to act through calmodulin, as calmodulin inhibitor significantly increased the level of oxidative stress after heating. A role for calmodulin is supported by the fact that higher content of calmodulin has been found in heat-tolerant maize cells than in heat-sensitive (Gong *et al.* 1997a). Higher calmodulin content has also been linked to lower level of heat induced membrane damage in maize (Gong *et al*. 1997a) and in *Arabidopsis*

(Larkindale and Knight 2002). Price *et al.* (1996) reported links between Ca^{2+} signaling and oxidative stress in tobacco.

The work therefore supports the hypothesis that Ca^{2+}-signaling plays significant role in limiting heat induced oxidative membrane damage during the recovery phase in *Amaranthus lividus*.

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