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Effect of abscisic acid on heat stress tolerance in the calli from two ecotypes of *Phragmites communis*

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Abstract

Dune reed (DR) and swamp reed (SR) are two ecotypes of reed (*Phragmites communis* Trin.) that displayed differences in stress tolerance. To uncover the molecular basis for such difference, the effects of heat stress were studied using the calli derived from the two ecotypes. Heat stress caused increased ion leakage, inhibited growth, decreased cell viability, and elevated hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents in the calli of both ecotypes, but DR callus showed better heat tolerance than SR callus. In DR callus, heat stress caused significant increase in the endogenous ABA content but not in SR callus. Application of fluridone (an ABA synthesis inhibitor) aggravated the heat stress damages on the DR callus whereas it had only minimal impact on the SR callus. Exogenous application of ABA alleviated the heat stress symptoms in the calli of both ecotypes. ABA treatment increased the activities of superoxide dismutase, catalase, ascorbate peroxidase and peroxidase, and also decreased H_2O_2 and MDA contents. These results indicate that the ability of ABA synthesis under heat stress is a key factor attributing to the higher heat tolerance of DR than SR.

Additional key words: antioxidant enzymes, dune reed, fluridone, hydrogen peroxide, lipid peroxidation, lipoxygenase, proline, swamp reed, thiol.

Introduction

High temperature, one of the major abiotic stresses, affects plant growth and development, such as tassel initiation and flowering time (Ellis *et al*. 1992), pollen sterility (Saini and Aspinall 1982), and rate and duration of endosperm cell division (Jones *et al*. 1985). The effects of high temperature on plants include many physiological and biochemical changes, including oxidative stress (Larkindale and Knight 2002, Wang *et al.* 2009, Xue *et al.* 2010), protein denaturation and aggregation (Salvucci *et al*. 2001), and cell structural disruptions (Suss and Yordanov 1986).

Abscisic acid (ABA) is an important regulator in the growth and development of plants. It plays an essential role in many physiological processes, including seed development, dormancy, germination and reproduction (Finkelstein *et al*. 2002). In addition, it also plays a pivotal role in abiotic stress tolerance (Leung and Giraudat 1998). ABA mediates responses to environmental stresses such as heat, cold, salt, drought and high irradiance (Taylor *et al*. 2000, Larkindale and Knight 2002, Cousson 2009, Pospíšilová *et al*. 2009). Induced thermotolerance has been reported when plants were pre-treated with ABA (Gong *et al*. 1998, Larkindale and Knight 2002), but the mechanism has yet to be elucidated.

Dune reed (DR), which grows in the desert and sand dune regions of northwestern China, is exposed frequently to various stresses including drought, high irradiance and high temperature. DR is an ideal material for studies on the adaptations of plant to various environmental conditions (Wang *et al*. 1995). On the other hand, swamp reed (SR), another ecotype of reed, grows in ponds that are full of water all year (Wang *et al*. 1995). Therefore, a comparative study of heat stress effects on two ecotypes of reeds can elucidate mechanisms for their different stress tolerance. We suggested that the ability of *de novo* synthesis of ABA can be related to the heat stress tolerance in DR and SR reed.

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Abbreviations: ABA - abscisic acid; APX - ascorbate peroxidase; CAT - catalase; DMAB - *ρ*-dimethylaminobenzaldehyde; DNPH - 2,4-dinitrophenylhydrazine; DR - dune reed; H_2O_2 - hydrogen peroxide; HPLC - high pressure liquid chromatography; LOX - lipoxygenase; MBTH - 3-methyl-2-benzothiazole hydrazone; MDA - malondialdehyde; MP - membrane penetration; NBT - nitroblue tetrazolium chloride; $O_2^{\cdot-}$ - superoxide anion; PM - plasma membrane; POD - peroxidase; PVP - polyvinylpyrrolidone; ROS - reactive oxygen species; SOD - superoxide dismutase; SR - swamp reed.

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Materials and methods

Embryogenic calli were derived from mature seeds of two reed (*Phragmites communis* Trin.) ecotypes, DR and SR, as described by Cui *et al*. (2002). After 4-month subcultures, 0.65 ± 0.05 g of embryogenic callus was plated on the 30 cm^3 of Murashige and Skoog (MS) solid medium. Different concentrations of ABA or fluridone (an inhibitor of ABA biosynthesis) were added on the surface of the solid MS medium after filter sterilization. After 24 h of pretreatment, the callus was subjected to temperature treatment of 45 °C in the dark. Control calli were exposed to a temperature of 25 °C. After 2 h of treatment, the calli were collected, washed for 2 min by distilled water (Heyser and Nabors 1981), and the excess water was blotted with filter paper.

Ion leakage was measured according to Sairam and Srivastava (2002) and expressed as a ratio of conductivity after boiling and initial conductivity (C1/C2) × 100.

Relative cell viability was determined as described by Towill and Mazur (1974).

The callus exposed to 45 °C for 2 h, was returned to the growth chamber and recovered for 4 d at 25 °C. Relative growth rate of control was defined to be 100 % and the relative growth rate of samples was calculated accordingly.

H_2O_2 content was measured according to Veljovic-Jovanovic *et al*. (2002). The cultures were extracted in 1 M trichloroacetic acid with insoluble 5 % polyvinylpyrrolidone (PVP). The reaction mixture consisted of 0.1 M phosphate buffer (pH 6.5), 3.3 mM *ρ*-dimethylamino-benzaldehyde (DMAB), 0.07 mM 3-methyl-2-benzothiazole hydrazone (MBTH) and 0.3 U peroxidase (POX). The absorbance change at 590 nm was monitored at 25 °C.

Lipid peroxidation was determined by measuring the malondialdehyde (MDA) content (Dhindsa *et al*. 1981). The amount of MDA was calculated using coefficient of absorbance of 155 mM^{-1} cm^{-1}.

Plasma membranes were extracted by the method of Yang *et al*. (2004). The calli (5 g) were homogenized in 10 cm^3 homogenization buffer, containing 0.25 M sucrose, 10 % (m/v) glycerol, 0.5 % (m/v) PVP, 3 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 15 mM mercaptoethanol, and 25 mM Tris-MES (pH 7.6). The homogenate was centrifuged at 13 000 *g* for 20 min (4 °C). The supernatant was recentrifuged at 80 000 *g* for 30 min to obtain a microsomal pellet.

Thiol contents were measured according to Jocelyn (1987) and calculated from a standard curve using reduced glutathione.

Carbonyl contents were determined according to the reaction with DNPH (2,4-dinitrophenylhydrazine) as described by Levine *et al*. (1994) and calculated using a molar absorption coefficient for aliphatic hydrazones of 22 000 M^{-1} cm^{-1}.

Superoxide dismutase activity (SOD, EC 1.15.1.1) was assayed by the method of Fridovich and Beauchamp (1971). One unit of SOD was defined as the amount of enzyme required to cause 50 % inhibition of the reduction of nitroblue tetrazolium (NBT) as monitored at 560 nm. Catalase activity (CAT, EC 1.11.1.6) was assayed as described by Durner and Klessing (1996). The activity was determined as a decrease in absorbance at 240 nm (ε = 39.4 mM^{-1} cm^{-1}) for 1 min following the decomposition of H_2O_2. Ascorbate peroxidase activity (APX, EC 1.11.1.11) was measured according to Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). Peroxidase activity (POD, EC 1.11.1.7) was measured according to Hammerschmidt *et al*. (1982) by monitoring the rate of guaiacol oxidation at 470 nm (ε = 26.6 mM^{-1} cm^{-1}). Lipoxygenase activity (LOX, EC 1.13.11.12) was determined according to Page *et al*. (2001) with some modifications. LOX activity was assayed in the citric acid-sodium phosphate buffer (100 mM, pH 4.5) using linoleic acid sodium salt (0.80 mM) as substrate by following the production of conjugated dienes at 234 nm (ε = 25 mM^{-1} cm^{-1}). Protein content was determined according to the method of Bradford (1976) with BSA as standard.

ABA content was quantified by the method of Hansen *et al*. (2000). ABA was identified by retention time, UV absorbance profile and spiking of standard ABA. Quantification was based on a standard curve generated using the standard ABA.

Statistical analysis was done by Student's *t*-test for independent sample. Values were expressed as means ± SE. In all cases, the confidence coefficient was set at $P < 0.05$.

Results

Ion leakage in DR and SR calli was increased by 22.8 and 108.5 %, respectively, after 2 h at 45 °C (Fig. 1*A*). The application of ABA alleviated the increases of ion leakage in both calli under heat stress, and the ion leakage reached a low level in presence of 10 μM ABA in both calli (Fig. 1*A*). So, 10 μM ABA was used in the subsequent experiments.

To further clarify the role of ABA in preventing heat-induced ion leakage, fluridone (an ABA synthesis inhibitor) was used. Under normal growth conditions, fluridone had no effect on ion leakage in both calli (data not shown). Under heat stress, ion leakage in the presence of fluridone was 38.2 % higher than that at heat treatment alone in DR callus, while in SR callus, ion leakage was not affected by the pretreatment of fluridone (Fig. 1*B*).

In DR callus, the relative growth rate under heat stress had no significant difference compared with that in control. However, under heat stress, the relative growth rate in SR

callus was decreased to 71.5 % of the control, and it reached 88.5 % in the presence of 10 μM ABA. The application of fluridone under heat stress had little effect on relative growth rate in SR callus. However, in DR callus, relative growth rate decreased remarkably when DR callus was subjected to heat stress in the presence of fluridone compared with that under heat treatment alone (Fig. 2*B*).

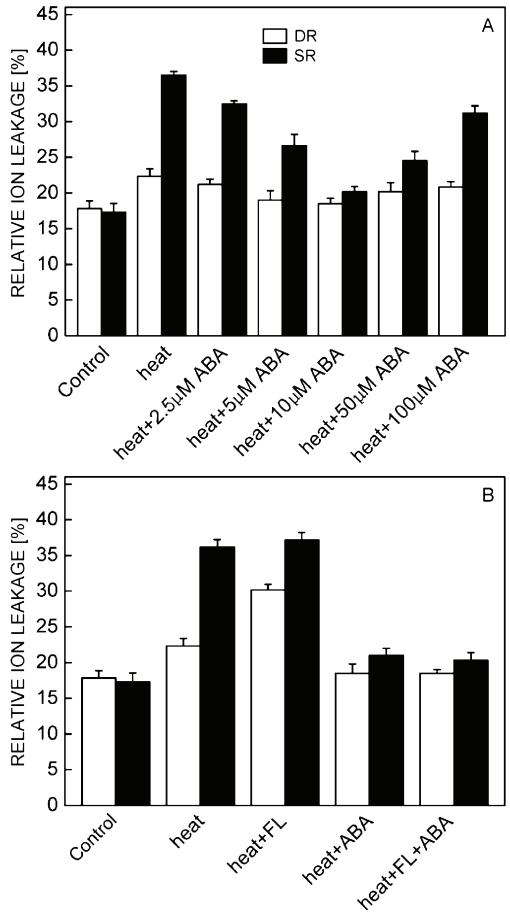

Fig. 1. Effect of ABA on ion leakage in the calli from dune reed (DR) and swamp reed (SR) under heat stress. The calli from DR and SR were cultured on the Murashige and Skoog solid medium. 1 cm^3 of ABA at different concentrations (*A*) or 1 cm^3 of 10 μM ABA or/and 10 μM fluridone (*B*) was added to the surface of the medium. After 24 h, the calli were subjected to high temperature of 45 ° C. After 2 h of treatment, the callus was collected for determination of ion leakage assay. Mean values and SE were calculated from three independent experiments.

Under heat stress, relative cell viability in DR callus was decreased to 88.6 % of the control, whereas it decreased to 70.5 % in SR callus (Fig. 2*C*). Application of ABA increased relative cell viability in both reed calli. There was no pronounced difference in relative cell viability between heat treatment alone and heat + fluridone treatment in SR callus. Comparatively, in DR callus, relative cell viability was lower by 17.0 % in the presence of fluridone than that at heat stress alone (Fig. 2*C*).

H_2O_2 content in SR callus increased to 172.0 % of the control under heat stress, whereas it only reached to 117.7 % in the presence of ABA (Table 1). Fluridone had no significant influence on H_2O_2 content in SR callus under heat stress. In DR callus, H_2O_2 content increased by 22.0 % under heat stress and the treatment with ABA significantly prevented H_2O_2 production. In the presence of fluridone, H_2O_2 content was 17.0 % higher than that of heat treatment alone (Table 1).

Heat treatment caused a significant increase (165.0 %) in MDA content, an indicator of lipid peroxidation, in SR callus in comparison to only slight increase in DR callus (22.8 %, Table 1). Under heat + ABA treatment, MDA

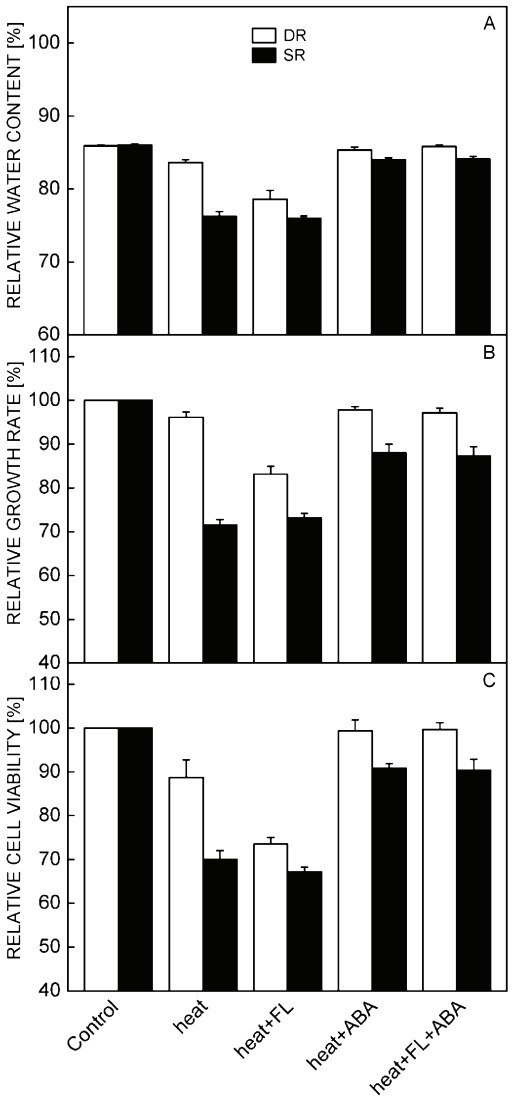

Fig. 2. Effects of ABA on relative water content (*A*), relative growth rate (*B*) and relative cell viability (*C*) in the calli from DR and SR under heat stress. The calli from DR and SR were cultured on the MS medium. 10 μM ABA or/and 10 μM fluridone was added on the surface of the medium. The heat treatment was the same as shown in Fig. 1. Means and SE were calculated from three independent experiments.

Table 1. Effects of ABA on H_2O_2, MDA, thiol and carbonyl contents in the calli from DR and SR under heat stress. The calli from DR and SR were cultured on the MS solid medium and 10 μM ABA or/and 10 μM fluridone was added on the surface of the medium. The heat treatment was the same as shown in Fig. 1. Means and SE were calculated from three independent experiments.

Treatments	H_2O_2 [ng g^{-1}(f.m.)]		MDA [μmol g^{-1}(f.m.)]		Thiol [μmol g^{-1}(prot.)]		Carbonyl [μmol g^{-1}(prot.)]	
	DR	SR	DR	SR	DR	SR	DR	SR
Control	6.2±0.1	6.1±0.2	3.5±0.1	3.3±0.3	116.3±1.5	106.0±2.0	9.6±0.4	9.4±0.3
Heat	7.5±0.3	10.7±0.2	4.3±0.2	8.5±0.3	106.3±2.4	60.0±2.1	10.5±0.4	17.0±0.5
Heat+FL	8.9±0.2	10.2±0.8	6.2±0.2	8.8±0.1	94.8±1.3	59.0±1.3	12.8±0.7	16.9±0.5
Heat+ABA	6.6±0.2	7.2±0.1	3.8±0.3	4.4±0.4	115.0±2.8	86.7±2.5	9.9±0.9	10.8±1.1
Heat+ABA+FL	6.5±0.2	7.3±0.2	3.8±0.1	4.4±0.3	113.5±3.5	87.5±2.5	9.8±0.4	11.0±0.8

content increased 34.5 and 8.4 % in SR and DR callus, respectively. In the presence of fluridone, MDA content in DR callus under heat stress was 44.5 % higher compared with that at heat treatment alone, whereas it was close to that of heat treatment in SR callus (Table 1).

The thiol content under heat stress in DR callus decreased by 8.6 % compared to control, while it decreased by 43.3 % in SR callus (Table 1). The carbonyl content increased by about 9.3 and 81.7 % in DR and SR calli, respectively (Table 1). Application of ABA prevented the decrease of thiol content and the increase of carbonyl content in both calli under heat stress. Fluridone treatment had almost no effect on thiol and carbonyl contents under heat treatment in SR callus. However, in DR callus, the treatment of fluridone accentuated decrease in thiol content and increase in carbonyl content (Table 1).

Proline content in DR callus was 1.75 times higher than that in SR callus in control. The proline content in DR callus increased to 190.5 % of that in control, whereas it remained relatively low in SR callus. Application of ABA or fluridone remarkably elevated proline content in both calli. Moreover, in the presence of fluridone, the proline content in DR callus under heat stress was significantly lower compared with that at heat treatment alone (Fig. 3*A*).

The activities of SOD, CAT, APX and POD increased significantly under heat stress in DR callus (about 73.4, 116.5, 20.5 and 56.2 %, respectively; Fig. 4). In contrast, the activities of SOD and CAT in SR callus increased only about 6.2 and 23.3 %, respectively. Interestingly, APX activity even markedly decreased and POD activity remained almost unchanged in SR callus (Fig. 4). These results indicated that the higher efficiency of ROS scavenging system in DR callus might be an important reason for its better heat tolerance than SR callus.

Under heat stress, pretreatment with ABA alone or ABA + fluridone resulted in a remarkable increase in the activities of SOD, CAT, APX and POD in both calli (Fig. 4). Treatment with fluridone had almost no effect on the activities of antioxidant enzymes tested in SR callus under heat treatment, whereas it induced their marked decline in DR callus (Fig. 4). Our results suggested that ABA, as a signal, participated in the heat-induced activities of antioxidant enzymes in reed calli.

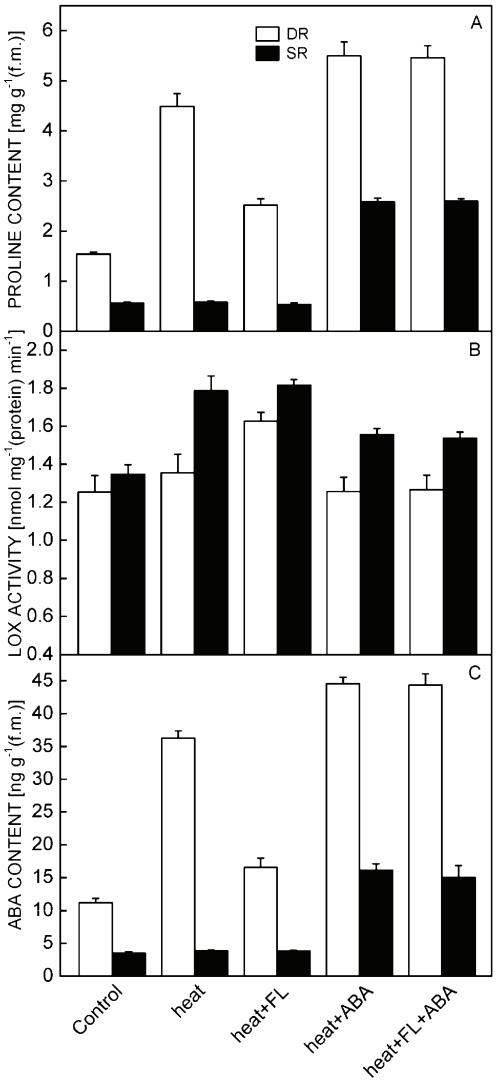

Fig. 3. Effect of ABA on proline content, lipoxygenase (LOX) activity and endogenous ABA content in the calli from DR and SR under heat stress. The calli from DR and SR were cultured on the MS medium and 10 μM ABA or/and 10 μM fluridone was added. The heat treatment was the same as shown in Fig. 1. Means ± SE, $n = 3$.

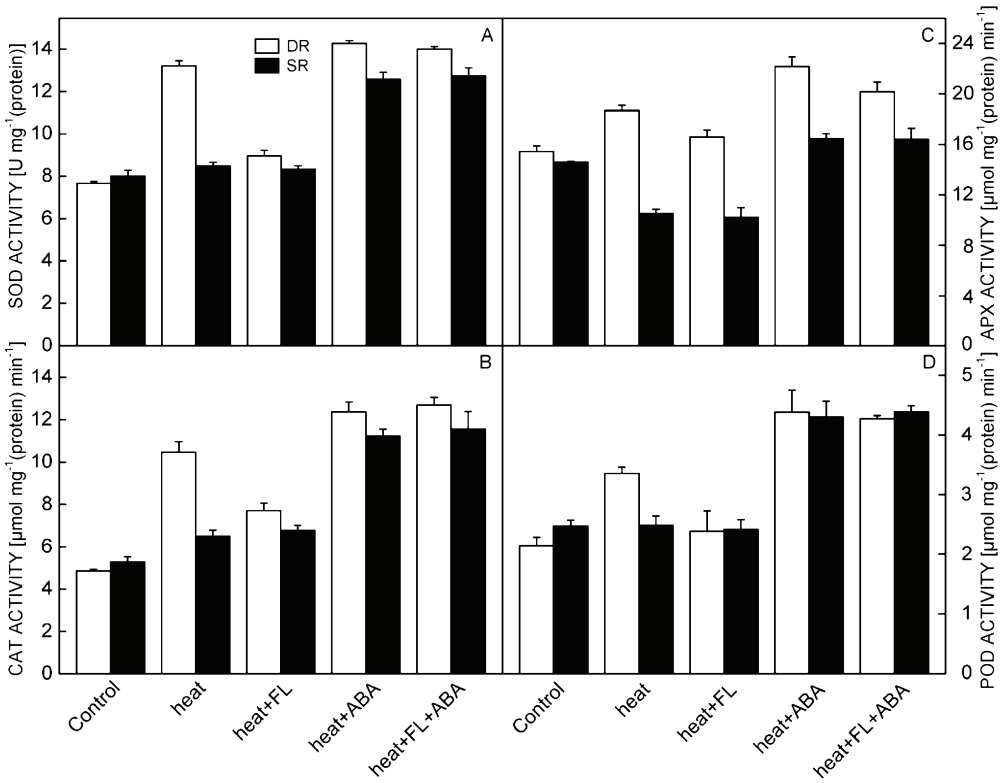

Fig. 4. Effects of ABA on antioxidant enzymes activities [superoxide dismutase (SOD; *A*); catalase (CAT; *B*); ascorbate peroxidase (APX; C); peroxidase (POD; *D*)] in the calli from DR and SR under heat stress. The calli were cultured on the MS medium and 10 μM ABA or/and 10 μM fluridone was added. The heat treatment was the same as shown in Fig. 1. Means ± SE, $n = 3$.

LOX activity in DR and SR callus increased by about 8.5 and 32.8 % under heat stress, respectively, but less after pretreatment with ABA or ABA + fluridone. Treatment with fluridone had almost no effect on LOX activity in SR callus under heat stress, but it resulted in a remarkable increase in LOX activity in DR callus (Fig. 3*B*).

To further elucidate the correlation between ABA accumulation and thermotolerance, ABA content was measured under heat stress (Fig. 3*C*). The result showed that ABA content increased by 226.5 % in DR callus under heat stress, however, it remained stable in SR callus. Under heat stress, exogenous ABA alone or ABA + fluridone treatment markedly enhanced ABA content in both calli. Fluridone obviously counteracted the heat-induced increase in ABA content in DR callus (Fig. 3*C*). The result further confirmed the protective role of ABA under heat stress in DR callus.

Discussion

Previous studies have revealed that heat stress was accompanied by growth suppression (Vierling 1991, Wang *et al.* 2009, Xue *et al.* 2010). Our data showed that growth rate was more reduced in SR callus than that in DR callus, suggesting that DR callus was more resistant to growth suppression imposed by heat stress than SR callus (Fig. 2*B*). Moreover, it has been reported that heat stress induced oxidative stress in plant, which resulted in cellular membrane injuries, solute and electrolyte leakage (Doke *et al*. 1994), the accumulation of H_2O_2 (Doke *et al*. 1994) and MDA (Smirnoff 1995, Wang *et al.* 2009, Xue *et al.* 2010). The relative growth rate measurement, ion leakage and relative cell viability assay have been commonly used to distinguish heat-tolerant and heat-sensitive genotypes of many plant species (Kuo *et al*. 1992). Our results demonstrated that ion leakage, H_2O_2 and MDA contents increased much more significantly in SR callus than that in DR callus, whereas relative cell viability in SR callus was far lower than those in DR callus under heat stress (Figs. 1 and 2, Table 1), further proving that SR callus was more sensitive to heat stress than DR callus.

Oxidative stress contributes to thiol oxidation and carbonyl derivative formation (Yang *et al*. 2003), which resulted in loss of protein function and protein degradation. Our results showed that carbonyl content in SR callus increased more dramatically than that in DR callus, whereas thiol content decreased more in SR callus than in DR callus under heat stress (Table 1). These data indicated that protein injury imposed by heat stress was more severe in SR callus than that in DR callus.

Tolerance to heat stress is often associated with the increase in antioxidant enzyme activities (Kaminaka *et al*. 1999). The activities of antioxidant enzymes in DR callus under heat stress increased remarkably; in contrast, the activities of SOD and CAT in SR callus increased slightly, APX activity decreased and POD activity had almost no response to heat stress (Fig. 4). SOD is a major scavenger of $O_2^{\cdot-}$ and the enzymatic reaction results in the formation of H_2O_2, CAT, APX, POD are all related to the removal of H_2O_2. It was likely that high temperature activated these antioxidant enzymes, and their combined functions contributed to scavenging H_2O_2 generated by oxidation stress and alleviating oxidative injury caused by heat stress in DR callus. LOX is involved in the activation of membrane lipid peroxidation. In our study, LOX activity increased in both calli under heat stress, and it increased more dramatically in SR callus than in DR callus (Fig. 3*B*). It provided other evidence that oxidative stress suffered by SR callus was more severe than by DR callus. Taken together, DR callus might better survive under heat stress in comparison with SR callus by retaining higher activities of antioxidant enzymes and alleviating oxidative damage.

It has been indicated that ABA could mitigate stress damage by up-regulating the activities of antioxidant enzymes (such as SOD, CAT, APX) in maize leaves (Jiang and Zhang 2002). We showed that the application of exogenous ABA significantly alleviated oxidative stress in both calli caused by heat stress by reducing ion leakage, preventing the accumulation of H_2O_2, MDA, carbonyl and the reduction of growth rate, cell viability, and thiol content (Figs. 1 and 2, Table 1). The activities of SOD, CAT, APX, POD in both calli were up-regulated in the presence of ABA under heat stress whereas the activity of LOX seemed to be down-regulated (Figs. 3, 4). Further, the proline was accumulated significantly in the presence of ABA in two calli under heat stress (Fig. 3*A*).These results suggested that exogenous ABA could protect two reeds calli from oxidative damages induced by heat stress and confer thermotolerance to both calli, which is consistent with results from previous reports (Gong *et al*. 1998).

In vegetative tissues, ABA content increases under harsh environmental conditions (Xiong and Zhu 2003). It has been demonstrated that salt, drought, and cold stresses cause the increased biosynthesis and accumulation of ABA, which can be rapidly restored following the relief of stress (Taylor *et al*. 2000). Our data showed that under heat stress ABA content was increased by 226.5 % in DR callus, in contrast to, almost no changes in SR callus (Fig. 3). To further clarify the physiological role of endogenous ABA in thermotolerance under heat stress, an inhibitor of ABA biosynthesis, fluridone, was used. Results indicated that fluridone treatment under heat stress had no impact on SR callus while it resulted in the aggravated oxidative stress in DR callus. In comparison with heat stress alone, increase in ion leakage, MDA and H_2O_2 contents and LOX activity and decrease in SOD, CAT, APX, POD activities were higher in fluridone treated DR callus. These results implied that endogenous ABA production induced by heat stress played an important physiological role in heat tolerance in DR callus since fluridone itself seemed to have almost no effect on DR callus under control condition. In contrast, in the poor heat-resistant SR callus, endogenous ABA production was not induced by heat stress and the callus suffered relatively severer oxidative damage under heat stress. Application of ABA significantly elevated the activities of ROSs scavenging enzymes and eliminated the H_2O_2 overproduction, thus conferring heat resistance of SR callus. From these results, we concluded that endogenous ABA induced by heat stress might serve as a messenger for the inducement of thermotolerance in DR callus by elevating the activities of ROSs scavenging enzymes.

Previous studies showed that ABA could enhance plant stress tolerance by inducing genes encoding enzymes for the biosynthesis of compatible osmolytes and LEA-proteins (Bray 2002). Moreover, the increasing evidences indicated that ABA induced the expression of genes encoding Cu, Zn-SOD, Mn-SOD, Fe-SOD (Kaminaka *et al*. 1999) and CAT (Guan *et al*. 2000). Thus, it is highly possible, that the protective effect of ABA may be mediated by the increased expression of genes encoding these ROSs scavenging enzymes under heat stress.

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