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# Nitric oxide mediates abscisic acid induced thermotolerance in the calluses from two ecotypes of reed under heat stress

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ABSTRACT

Calluses from two ecotypes of reed (*Phragmites communis* Trin.) plant (dune reed) [DR] and swamp reed [SR]), which show different sensitivity to heat stress, were used to study plant acclimations to heat stress involved with ABA and NO. High temperature induced ion leakage and accumulation of MDA in two calluses, which were significantly alleviated by exogenous ABA and NO application. Pretreatment with fluridone, L-NNA or cPTIO obviously aggravated ion leakage and the accumulation of MDA in DR callus under heat stress whereas had no effect on SR callus. Moreover, endogenous NO and ABA contents and NOS activity increased markedly under heat stress in DR callus while remained stable in SR callus. Further research on two calluses indicated that inhibition of NO accumulation by cPTIO and L-NNA blocked the protective effect of exogenous ABA whereas inhibition of ABA synthesis by fluridone had no influence on the protective effect of exogenous NO. In DR callus, exogenous ABA treatment resulted in further increase of NOS activity and NO release in comparison with that of heat stress alone while fluridone treatment inhibited the increase of NOS activity and NO release under heat treatment. In contrast, both exogenous NO treatment and inhibition of endogenous NO accumulation by cPTIO and L-NNA had no influence on ABA content. In conclusion, NO might act as an intermediate molecular mediating abscisic acid induced thermotolerance under heat stress.



## 1. Introduction

In arid, semiarid, tropical, and subtropical regions of the world, high temperature has been a major limitation to plant growth and crop productivity [1]. High temperature induced accumulation of reactive oxygen species (ROS), including $^1O_2$, $H_2O_2$ and $O_2^-$ and $^{\bullet}OH$ [2], which led to protein denaturation, condensation [3], enzyme inactivation, lipid peroxidation [4], consequent inhibition of the photosynthesis and respiration and plant growth [5,6].

ABA, as an important regulator that coordinates growth and development in plants, is associated with many physiological processes, including seed development, dormancy, germination, reproduction [7]. In addition, ABA mediates responses to environmental stresses such as cold, salt, drought, heat and phosphate deficiency in plants [8–12]. Increase of ABA concentration in response to heat treatments was detected in tobacco [13]. ABA application has been approved to induce thermotolerance in maize and bromegrass, which might be mediated by $Ca^{2+}$ through antioxidant enzymes activation [14]. ABA-insensitive *Arabidopsis* mutant were found to be more susceptive to heat than wild-type plants [8], providing strong genetic evidence that ABA is involved in thermotolerance in plant.

Nitric oxide (NO) is a highly reactive, membrane-permeant free radical with a broad spectrum of regulatory functions in many physiological processes, such as seed germination, leaf expansion, cell senescence, ethylene emission, stomatal closure and programmed cell death, and a signal molecular mediating responses to abiotic and biotic stresses such as drought stress, salinity, UV-B-radiation and heat stress [15–18]. NO may protect plant against stress by acting as an antioxidant directly scavengering the reactive oxygen species (ROS) generated under heat stress or operating as a signal molecule in the cascade of events leading to gene expression [19]. Our earlier work has revealed that NO act as a signal in inducement of thermotolerance in plant by activating active oxygen scavenging enzymes [20]. In addition, Northern blot analysis demonstrated

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that NO protected the chloroplast against oxidative damage under heat stress by inducing expression of gene encoding small heat shock protein 26 (HSP26) [21].

Dune reed (DR), which grows in the desert and sand dune region of northwestern China, is an important ecotype of reed (*Phragmites communis* Trin.). Under a combination of stresses, such as drought, high light, and high temperature, DR vegetates and develops normally and forms some quite large populations [22]. DR had been proved to retain some stable variations of morphological, physiological, and genetic characteristics in response to external stresses [22]. DR is an ideal material for studies on the adaptations of plant to various environmental conditions. Instead, swamp reed (SR), another ecotype of reed, grows in ponds that are full of water all year round [22]. Earlier work has demonstrated that regenerated plantlets from embryogenic DR and SR calluses retained the same genetic characteristics as the wild plants [23].

Using the calluses from DR and SR, this study aimed to explore the role of ABA and NO in heat stress acclimation and the cross talk between them.

## 2. Materials and methods

### 2.1. Plant materials and heat and chemical treatment

Embryogenic calluses, derived from mature seeds of two reed ecotypes of reed (*Phragmites communis* Trin.; DR and SR), were obtained as described by Cui et al. [23]. After 4-month subcultures, 0.65 ± 0.05 g of embryogenic callus was maintained on the 30 mL of Murashige and Skoog solid medium [24]. Different concentrations of SNP, ABA, cPTIO, L-NNA and fluridone were added on the surface of the solid (these reagents were prepared with sterilized water) Murashige and Skoog medium after filter sterilization. Control calluses were treated with sterilized water. After 24 h of pretreatment, the callus was subjected to temperature treatment of 45 °C in the dark while control calluses were exposed to a temperature of 25 °C. After 2 h of heat treatment, the callus was collected, washed for 2 min by distilled water and the excess water was blotted with filter paper. The samples were used immediately.

### 2.2. Membrane permeability (MP) measurement

MP was determined according to Sairam and Srivastava [25] with some modifications. The treated callus was collected, washed for 2 min by distilled water and the excess water was blotted with filter paper. Then the callus (0.5 g) was placed in petri dishes with 10 mL of de-ionized water at 25 °C for 2 h. After the incubation, the conductivity in the bathing solution was determined (C1). Then, the samples were boiled for 15 min, and conductivity was read again in the bathing solution (C2). Electrolyte leakage was expressed as a percentage of the total conductivity after boiling (MP = $C1/C2 \times 100$).

### 2.3. Analysis of lipid peroxidation

Lipid peroxidation was measured in terms of MDA content following the method of Heath and Packer [26] with some modification. Embryogenic cultures (0.5 g) were homogenized with a mortar and pestle in 10% trichloroacetic acid and then the homogenate was centrifuged at 4000 × *g* for 30 min. A 2 mL aliquot of supernatant was mixed with 2 mL of 10% trichloroacetic acid containing 0.5% thiobarbituric acid. The mixture was heated at 100 °C for 30 min. The absorbance of the supernatant was measured at 532 nm, with a reading at 600 nm subtracted from it to account for non-specific turbidity. The amount of malonaldehyde was calculated using an extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$.

### 2.4. Relative growth gate (RGR) determination

For RGR determination, the callus was subjected to 2 h temperature treatment of 45 °C, and then returned to the growth chamber and recovered for 4 days at 25 °C. The RGR of control was defined to be 100% and RGR of samples was calculated accordingly.

### 2.5. ABA determination

ABA was quantified by the method of Qin and Zeevaart [27] with some modification. Callus (4.5 g) was extracted with 80% (v/v) aqueous methanol for 15 h and then the homogenate was centrifuged at 10,000 × *g* for 30 min at 4 °C .The supernatant was extracted three times with petroleum ether to remove pigment and the aqueous phase was collected. The pH of the aqueous phase was adjusted to 3.0 with 0.5 M citric acid and extracted three times with ethyl acetate and the organic phase was collected. The organic solvent was evaporated to dryness under a $N_2$ stream and the sample were dissolved with 1 mL methanol and 3 mL 0.02 M acetic acid buffer (pH 3.5). The sample was subjected to Waters Sep-Pak C18 prepacked column and washed with 40% acetonitrile. The organic phase was reduced to dryness under $N_2$ and subjected to HPLC on a reverse-phase ODS C18 column (250 mm × 4.6 mm). The fractions containing ABA was collected and dried in a centrifugal vacuum concentrator (Jouan, Winchester, VA). After methylation with diazomethane, the fractions were further purified by normal-phase HPLC. The methyl esters of ABA was analyzed on a 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with an automatic injector and electron capture detector. Methyl ABA was analyzed isothermally at 188 °C on an HP-5 capillary column (30-m × 0.32-mm i.d. × 0.25-μm film thickness, Agilent Technologies, Palo Alto, CA). Endrin was used as an internal standard. All values reported in this paper have been corrected for recoveries of added $^3$H-labeled compounds.

### 2.6. NO content determination

NO content was determined as described by Murphy and Noack [28] with some modifications. The method is based on the direct reaction between NO and oxyhaemoglobin ($HbO_2$), which yields methaemoglobin (metHb). Oxyhaemoglobin was prepared by the reduction of 25 mg of methaemoglobin to haemoglobin using 4 mg sodium dithionite in 1 mL of 50 mM phosphate buffer (pH 7.4), followed by oxygenation. The oxyhaemoglobin solution was desalted by passing it through a Sephadex G-25 column eluted with 50 mM phosphate buffer (pH 7.4), and its concentration was estimated spectrophotometrically at 415 nm using an extinction coefficient of 131 $mM^{-1}$ $cm^{-1}$. Fresh calluses (1 g) from two reed ecotypes were incubated with 100 units of catalase and 100 units of superoxide dismutase for 5 min to remove ROS before addition of 3 mL oxyhaemoglobin (5 μM). After a further 2 min incubation, Nitric oxide concentrations were estimated by following the conversion of oxyhaemoglobin ($HbO_2$) to methaemoglobin (metHb) spectrophotometrically at 577 and 591 nm ($A_{577}$ $HbO_2$-$A_{591}$ metHb, $\Delta\varepsilon$ = 11.2 $mM^{-1}$ $cm^{-1}$) [28].

### 2.7. NOS activity determination

NOS activity determination was performed according to Murphy and Noack [28] with some modifications. About 1 g of callus was homogenized in 2 mL of homogenization buffer (50 mM triethanolamine hydrochloride [pH 7.5] containing 0.5 mM EDTA, 1 μM leupeptin, 1 μM pepstatin, 7 mM gluathione, and 0.2 mM phenylmethylsulfonyl fluoride). After centrifuging at 10,000 × *g* for 20 min (4 °C), the supernatant was collected and recentrifuged

at 100,000 × g for 45 min. The supernatant was used for NOS determination. NOS activity was analyzed by haemoglobin assay as previously described [28]. Protein concentration was determined as described by Bradford [29].

### 2.8. Statistical analysis

Each experiment was repeated at least three times. Statistical analysis was performed using ANOVA test.

## 3. Results

### 3.1. Effect of ABA on MPs, MDA and RGR in the calluses from DR and SR under heat stress

As shown in Fig. 1A, 1B, MP increased by 108%, MDA content by 160% in SR callus while MP increased by only 22.4%, MDA content by 22.8% in DR callus under heat stress. Moreover, heat stress resulted in significantly growth suppression in two calluses, with RGR decreasing to 72% and 95.1% of the control in SR and DR callus, respectively (Fig. 1C). Under 10 μM ABA pretreatment, MP and MDA content reduced by 44% and 40%, respectively whereas RGR rose by 16% in comparison with that under heat stress alone in SR callus. In DR callus, MP and MDA content were close to the control level and RGR restored to 97.8% of that of control under 10 μM ABA treatment (Fig. 1A–C). To attribute the role of ABA in heat stress response, fluridone (an inhibitor of ABA biosynthesis) was used alone, which have no effect on both calluses under control condition (data not shown). Results indicated that fluridone treatment had little effect on SR callus whereas resulted in significant increase in MP and MDA content and evidently decrease in RGR in DR callus compared with under heat treatment alone. Moreover, the effect of fluridone on MP, MDA content and RGR in DR callus were reversed by the application of exogenous ABA (Fig. 1).

The effect of ABA is dose-dependent, namely, promotion at low concentration while inhibition at high concentration [30]. The magnitude of cellular response to ABA is determined by ABA level and ABA sensitivity, and both aspects involve complex signal transduction processes. Our results suggested that concentrations between 2.5 and 100 μM ABA were able to alleviate ion leakage, MDA accumulation and growth suppression induced by heat stress in two calluses, with 10 μM ABA being the most effective (results not shown). ABA (10 μM) was used in our experiments.

### 3.2. Effect of NO on MPs, MDA and RGR in the calluses from DR and SR under heat stress

Under SNP (and NO donor) pretreatment, MP and MDA content remarkably decreased by 45.6% and 36.4%, respectively, whereas RGR increased by 16.5% in SR callus compared with under heat stress alone. In DR callus, MP, MDA content and RGR restored to control level in the presence of SNP, respectively. To clarify the physiological role of endogenous NO in thermotolerance under heat stress, cPTIO was used alone. Our work showed that treatment with cPTIO had no impact on SR callus whereas resulted in significant increase in MP and MDA content and evident decrease in RGR in DR callus compared with under heat treatment alone (Fig. 2).

Nitric oxide is itself a reactive nitrogen species and its effects on different types of cells have proved to be either cytoprotective or cytotoxic, depending on its concentration, the plant species, and the developmental stages [31]. Our results showed that increasing concentrations SNP (from 50 to 300 μM) application decreased ion leakage, MDA accumulation and growth suppression induced by heat stress in two calluses, with 200 μM SNP being the most effective (date not shown). SNP (200 μM) was used in our experiments.

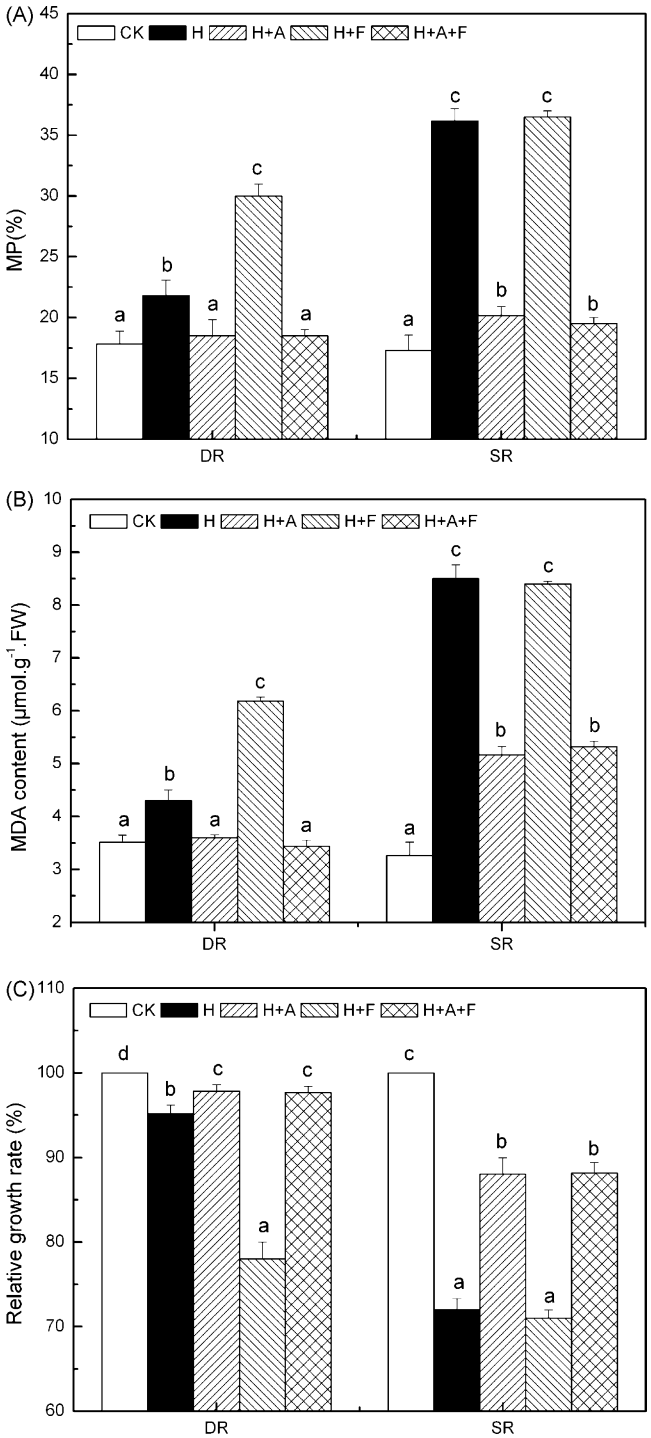


**Fig. 1.** Effect of ABA on MP (A), MDA content (B) and RGR (C) in the calluses from DR and SR under heat stress. The calluses from DR and SR were cultured on the Murashige and Skoog solid medium. One milliliter of 0.01 mM ABA and 0.05 mM fluridone were added to the surface of the medium, respectively. Different treatment represents: control (CK), heat (H), heat + ABA (H + A), heat + fluridone (H + F), heat + ABA + fluridone (H + A + F). After 24 h, the calluses were subjected to temperature treatment of 45 °C. After 2 h of treatment, the callus was collected for determination of MP, MDA and RGR assay. Bars represent the mean ± S.E. of three independent experiments. ANOVA analysis was used for comparisons between the means. Bars with different letters are significantly different at the 0.05 level.

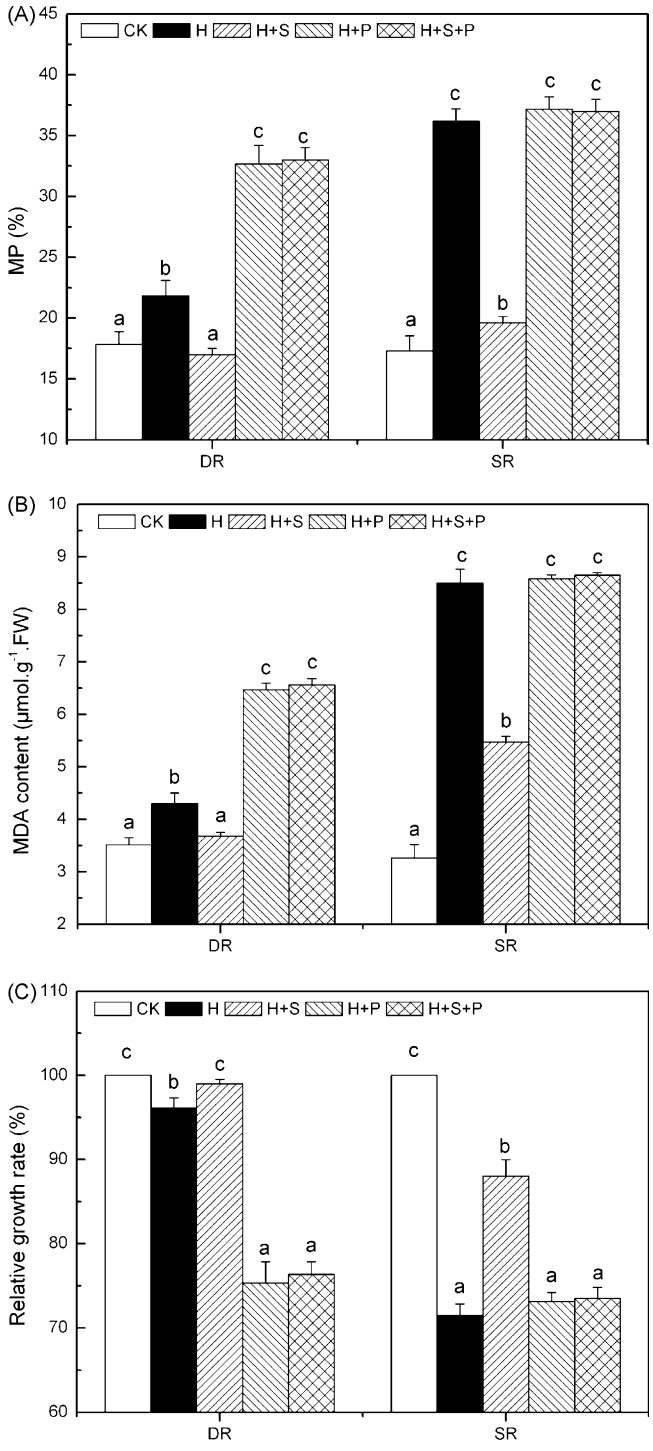


**Fig. 2.** Effect of NO on MP (A), MDA content (B) and RGR (C) in the calluses from DR and SR under heat stress. The calluses from DR and SR were cultured on the Murashige and Skoog solid medium. One milliliter of 0.2 mM SNP and 0.4 mM cPTIO was added to the surface of the medium, respectively. Different treatment represents: control (CK), heat (H), heat + SNP (H + S), heat + cPTIO (H + P), heat + SNP + cPTIO (H + S + P). After 24 h, the calluses were subjected to temperature treatment of 45 °C. After 2 h of treatment, the callus was collected for determination of MP, MDA and RGR assay. Bars represent the mean ± S.E. of three independent experiments. ANOVA analysis was used for comparisons between the means. Bars with different letters are significantly different at the 0.05 level.

### 3.3. Interaction between ABA and NO under heat stress

As shown in Fig. 3A and B, exogenous ABA or SNP significantly alleviated ion leakage and lipid peroxidation induced by heat

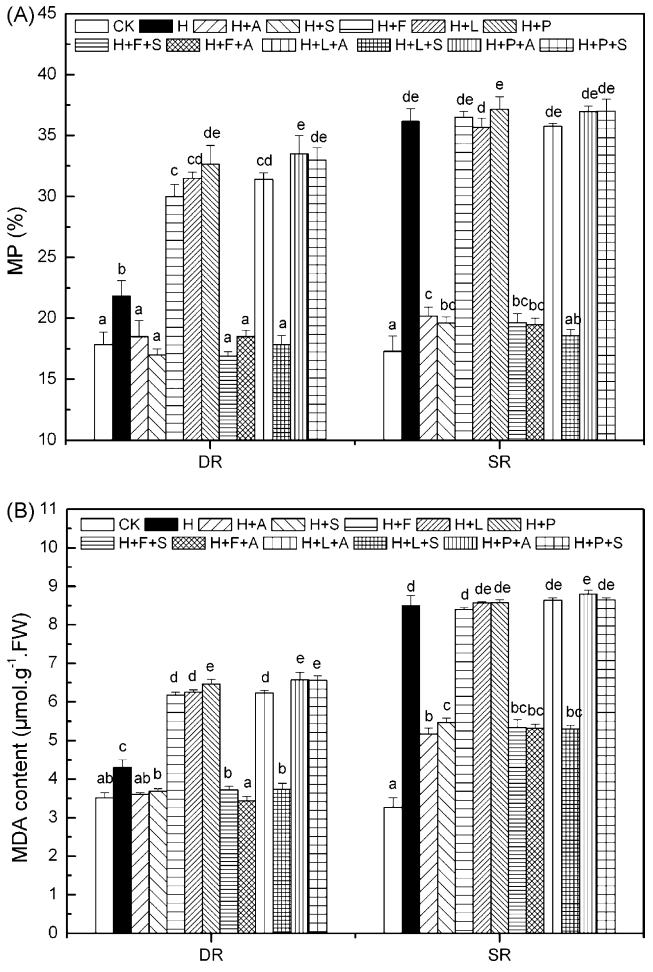


**Fig. 3.** Interaction between ABA and NO in two calluses from DR and SR under heat stress. The calluses from DR and SR were cultured on the Murashige and Skoog solid medium. One milliliters of 0.01 mM ABA, 0.2 mM SNP, 0.05 mM fluridone, 0.4 mM cPTIO and 0.3 mM L-NNA was added to the surface of the medium, respectively. Different treatment represents: control (CK), heat (H), heat + ABA (H + A), heat + SNP (H + S), heat + fluridone (H + F), heat + L-NNA (H + L), heat + cPTIO (H + P), heat + fluridone + SNP (H + F + S), heat + fluridone + ABA (H + F + A), heat + L-NNA + ABA (H + L + A), heat + L-NNA + SNP (H + L + S), heat + cPTIO + ABA (H + P + A), heat + cPTIO + SNP (H + P + S). After 24 h, the calluses were subjected to temperature treatment of 45 °C. After 2 h of treatment, the callus was collected for determination of MP (A) and MDA (B) assay. Bars represent the mean ± S.E. of three independent experiments. ANOVA analysis was used for comparisons between the means. Bars with different letters are significantly different at the 0.05 level.

stress in both calluses, respectively. In the presence of fluridone and SNP, although ABA synthesis was blocked by fluridone, ion leakage and lipid peroxidation induced by heat stress were efficiently alleviated in both calluses, implying that the protective effect of NO is independent of ABA. However, inhibition of NOS activity by L-NNA or removal of NO by cPTIO in the presence of ABA resulted in invalidation of ABA, indicating that NO might be essentially required for the protective effect of ABA. Moreover, L-NNA pretreatment significantly aggravated ion leakage and lipid peroxidation in DR callus compared with that of heat treatment alone whereas had no impact on SR callus (Fig. 3A and B).

### 3.4. Effect of ABA and NO on ABA content in the calluses from DR and SR under heat stress

Measurement of ABA content suggested that ABA content in DR callus is1.96 times higher than that in SR callus under control condition. ABA content increased by 226% in the DR callus under

heat treatment but remained unchanged in the SR callus. Application of exogenous ABA, L-NNA + ABA or cPTIO + ABA significantly increased ABA content in two calluses, respectively. In the presence of fluridone or fluridone + SNP, ABA content evidently decreased compared with that of heat treatment alone in DR callus whereas kept at the level of heat treatment alone in SR callus. SNP, L-NNA, cPTIO, SNP + L-NNA or SNP + cPTIO treatment had no impact on ABA content in two calluses under heat stress, respectively (Fig. 4).

### 3.5. Effect of ABA and NO on NOS activity and NO release in the calluses from DR and SR under heat stress

As shown in Fig. 5, NOS activity and NO level in DR callus increased to 170% and 262% of the control, respectively, under heat stress but remained unchanged in SR callus. With ABA pretreatment, NOS activity and NO level increased further to 193% and 350% of the control, respectively, in DR callus and increased to 174% and 200% of the control, respectively, in SR callus. In the presence of fluridone, NOS activity and NO level decreased to 134% and 150% of the control, respectively, in DR callus while changed little in SR callus. With SNP, fluridone + SNP or L-NNA + SNP treatment, NOS activity markedly decreased, despite elevation of NO release from NO donor, SNP, in DR callus compared with under heat treatment alone. Comparatively, cPTIO, cPTIO + SNP or cPTIO + ABA pretreatment enhanced NOS activity but reduced NO release in DR callus under heat treatment, respectively. L-NNA pretreatment resulted in remarkable decrease of NOS activity and NO release in DR callus compared with under heat treatment alone, respectively, approving that NO production under heat stress in DR callus is NOS-dependent. In addition, we noticed that NO release in DR and SR callus measured by us were different from those reported in other paper, owing to different materials, culture condition and different treatments [32].

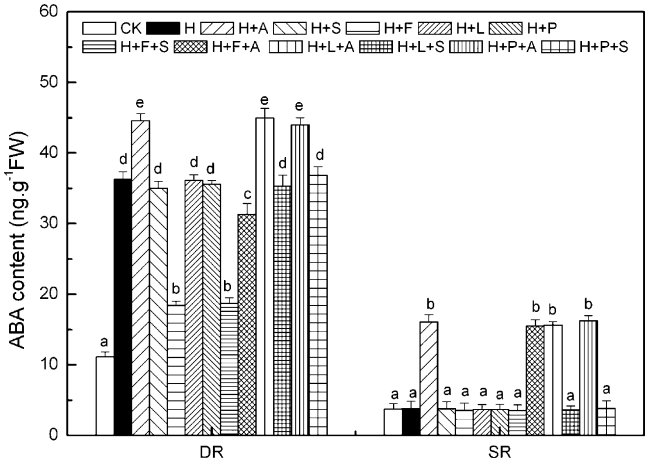


**Fig. 4.** ABA content in the calluses from DR and SR under heat stress. The calluses from DR and SR were cultured on the Murashige and Skoog solid medium. One milliliters of 0.01 mM ABA, 0.2 mM SNP, 0.05 mM fluridone, 0.4 mM cPTIO and 0.3 mM L-NNA was added to the surface of the medium, respectively. Different treatment represents: control (CK), heat (H), heat + ABA (H + A), heat + SNP (H + S), heat + fluridone (H + F), heat + L-NNA (H + L), heat + cPTIO (H + P), heat + fluridone + SNP (H + F + S), heat + fluridone + ABA (H + F + A), heat + L-NNA + ABA (H + L + A), heat + L-NNA + SNP (H + L + S), heat + cPTIO + ABA (H + P + A), heat + cPTIO + SNP (H + P + S). After 24 h, the calluses were subjected to temperature treatment of 45 °C. After 2 h of treatment, the callus was collected for determination of ABA contents. Bars represent the mean ± S.E. of three independent experiments. ANOVA analysis was used for comparisons between the means. Bars with different letters are significantly different at the 0.05 level.

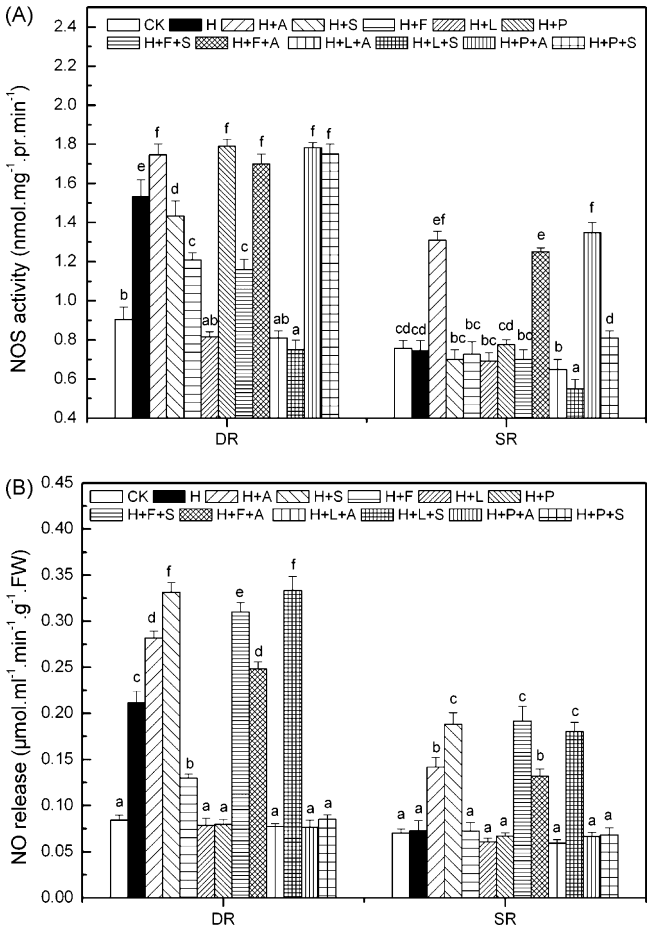


**Fig. 5.** NOS activity (A) and NO production (B) in the calluses from DR and SR under heat stress. The calluses from DR and SR were cultured on the Murashige and Skoog solid medium. One milliliters of 0.01 mM ABA, 0.2 mM SNP, 0.05 mM fluridone, 0.4 mM cPTIO and 0.3 mM L-NNA was added on the surface of the medium, respectively. Different treatment represents: control (CK), heat (H), heat + ABA (H + A), heat + SNP (H + S), heat + fluridone (H + F), heat + L-NNA (H + L), heat + cPTIO (H + P), heat + fluridone + SNP (H + F + S), heat + fluridone + ABA (H + F + A), heat + L-NNA + ABA (H + L + A), heat + L-NNA + SNP (H + L + S), heat + cPTIO + ABA (H + P + A), heat + cPTIO + SNP (H + P + S). After 24 h, the calluses were subjected to temperature treatment of 45 °C. After 2 h of treatment, the callus was collected for determination of NOS activities (A) and NO level (B). Bars represent the mean ± S.E. of three independent experiments. ANOVA analysis was used for comparisons between the means. Bars with different letters are significantly different at the 0.05 level.

## 4. Discussion

Previous studies have indicated that heat stress-induced oxidative stress, which resulted in membrane lipids peroxidation, cellular membranes disruption and growth suppression [4,5]. Membrane thermostability and relative growth rate have been commonly used to distinguish heat-tolerant and heat-sensitive genotypes of many plants [1]. Our work showed that heat stress resulted in severer ion leakage and growth suppression and more MDA accumulation in SR callus than in DR callus, demonstrating DR calluses were relatively thermotolerant in comparison with SR calluses, which is in accordance with our previous results [20].

Our results suggested that exogenous ABA or SNP could elevate thermotolerance in both calluses by alleviating ion leakage, lipid peroxidation and growth suppression induced by heat stress, respectively (Figs. 1 and 2). To elucidate the physiological role of endogenous ABA and SNP in thermotolerance under heat stress, fluridone and cPTIO were used. Results showed that treatment with

fluridone or cPTIO had no impact on SR callus whereas resulted in aggravated membrane damage, lipids peroxidation and growth suppression in DR callus, respectively, compared with under heat stress alone. Because fluridone or cPTIO itself has not any effect on DR callus under control condition, these results might implied that endogenous ABA and NO production increased significantly under heat stress, which might play a physiological role in heat tolerance in DR callus. Measurements of ABA content and NO release indicated that ABA and NO production in DR callus indeed rose remarkably under heat stress, respectively (Figs. 4 and 5). From these results, we concluded that significant increase in ABA or NO level under heat stress was involved in thermotolerance in DR callus. In contrast, in the poor heat-resistant SR callus, ABA and NO production were not induced by heat stress and the callus suffered relatively severer oxidative damage under heat stress. Application of exogenous ABA or SNP to the SR callus significantly reduced ion leakage, lipid peroxidation and growth suppression induced by heat stress, respectively, thus conferring thermotolerance. Therefore, ABA and NO, produced in DR callus under heat stress, might served as signal for the inducement of thermotolerance in DR callus, respectively.

Previous works have provided many evidences for cross talk between NO and ABA. In *Arabidopsis*, NO has been reported to mediate ABA-induced stomata closure [33]. Similarly, NO was also found to be involved in the acquisition of plant adaptation to drought stress by operating downstream of ABA [34]. In contrast, there is some evidence that NO serve as upstream signals of stress-induced ABA accumulation under osmotic stress [35] and salt stress [36]. Interestingly, in wheat seedling leaves, on the one hand, NO might be downstream of the ABA-induced proline accumulation, and on the other, NO activated the synthesis of ABA and might also induce proline accumulation under salt stress via ABA transduction cascade [37], displaying a complex interactions between ABA and NO in plant.

In this study, we found that prevention of NO accumulation by cPTIO or L-NNA could eliminate the protective effect of exogenous ABA whereas inhibition of ABA synthesis by fluridone could not remove the protective effect of exogenous NO, implying that the protective effect of ABA under heat stress might be mediated by NO. More evidence showed that employment of exogenous ABA remarkably activated NOS activity and increased NO release while inhibition of ABA synthesis by fluridone treatment depressed the increase of NOS activity and NO release in DR callus under heat stress. However, neither exogenous SNP nor cPTIO or L-NNA treatment had no influence on ABA content, implicating that ABA-induced thermotolerance in DR callus by promoting NO release via activating NOS. Additionally, in SR callus, exogenous ABA treatment significantly activated NOS activity and elevated NO release, whereas exogenous SNP had no effect on ABA level, which further confirmed that NO might operate downstream of ABA and mediate the protective effect of ABA under heat stress, namely, ABA might act as a signal in inducing NO synthesis under heat stress and thus confer DR callus thermotolerance.

Despite ABA and NO, a wide range of second messengers, including calcium ions, salicylic acid (SA), active oxygen species, and ethylene [8,38,39], have also been proved to be implicated in signaling in response to heat stresses. Addition of exogenous calcium to plant resulted in enhanced survival and reduced oxidative damage imposed by heat treatment in maize [38]. SA, ACC and $H_2O_2$, when added exogenously, all could induce some degree of thermotolerance in plant [8,38]. Increase of endogenous SA, $H_2O_2$ and ethylene have been observed in plant in response to heat stress [38]. Experements using *etr-1* mutant, which is unable to perceive ethylene, and transgenic plants (nahG) expressing the bacterial salicylate hydroxylase gene, which causes break down of SA as soon as it forms, demonstrated that the mutant and transgenic plants all showed increased oxidative damage and reduced survival under heat stress as compared with wild-type plants, providing genetic evidence that ethylene and SA mediate protection against, or repair of heat-induced oxidative damage in plants [38,39]. Recent advance showed that $H_2O_2$ was essential for heat stress-activated heat shock protein gene *Hsp17.6* and *Hsp18.2* and two cytosolic ascorbate peroxidase genes *Apx1* and *Apx2* expression, indicating that $H_2O_2$ is an important component in the heat stress signaling pathway [40]. Recently, 24-epibrassinolide (EBR), a kind of plant polyhydroxy steroids, and glycinebetaine (GB) were found to augment thermotolerance in plants [41,42].

So far, although cross talk involving NO, ABA, $Ca^{2+}$, $H_2O_2$, SA and ethylene under salt, osmotic, and drought stress have been well known [43–46], little is known about the interactions between them and the signal pathway under heat stress. Further investigation into revealing a picture of signal transduction networks and cross talk between these components in response to heat stress will be required.

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