Nitric oxide synthase like activity-dependent nitric oxide production protects against chilling-induced oxidative damage in *Chorispora bungeana* suspension cultured cells

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1. Introduction

Low temperature is a major environmental stress that adversely influences the growth and development of plants and significantly constrains the spatial distribution of plants and agricultural productivity. Plants can respond and adapt to low temperature conditions by a series of morphological, physiological, biochemical and molecular changes [1]. Exposure to low temperature induces the overproduction of reactive oxygen species (ROS) such as hydrogen peroxide (\(H_2O_2\)), singlet oxygen (\(^1O_2\)), superoxide radical (\(O_2^-\)), and hydroxyl radical (\(OH^-\)) in plant cells. If not effectively and rapidly removed, the redundant ROS could cause lipid peroxidation, DNA damage and protein denaturation [2]. Numerous studies have demonstrated that oxidative stress may be a significant factor in association with chilling-induced injury [1]. To scavenge ROS and combat oxidative damage, plants have evolved an efficient antioxidant defense system which includes enzymatic scavenging and nonenzymatic scavenging systems. The former involves antioxidant enzymes such as ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6), glutathione reductase (GR, EC 1.6.4.2), peroxidase (POD, EC 1.11.1.7) and superoxide dismutase (SOD, EC 1.15.1.1) and the contents of ascorbic acid (AsA) and reduced glutathione (GSH) increased evidently in the presence of SNP under chilling stress. In addition, under low temperature conditions, treatment with NO scavenger PTIO or mammalian NO synthase (NOS) inhibitor L-NAME remarkably aggravated oxidative damage in the suspension cultures compared with that of chilling treatment alone. Moreover, measurements of NOS activity and NO production showed that both NOS activity and endogenous NO content increased markedly under chilling stress. The accumulation of NO was inhibited by L-NAME in chilling-treated cultures, indicating that most NO production under chilling may be generated from NOS-like activity. Collectively, these results suggest that chilling-induced NO accumulation can effectively protect against oxidative injury and that NOS like activity-dependent NO production might act as an antioxidant directly scavenging ROS or operate as a signal activating antioxidant defense under chilling stress, thus conferring an increased tolerance to chilling in *C. bungeana* suspension cultures.

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as seed germination and dormancy, growth and development of plant tissue, plant cell maturation and senescence, flowering, hormone responses, stomatal closure and programmed cell death [4–6]. Furthermore, NO is suggested to be involved in regulating the multiple plant responses to a variety of abiotic and biotic stresses, including drought, salinity, extreme temperature, UV radiation, mechanical injury, herbicide, heavy metal and pathogen attack [4,7]. NO is itself a reactive nitrogen species and its effects on the multiple plant responses to a variety of abiotic and biotic situations where it is acting [5,6]. NO may act as an antioxidant able to scavenge ROS to protect plant cells from oxidative damage. Several researches have indicated that the protective effect of NO against abiotic stresses is closely related to the NO-mediated reduction of ROS in plants [7,8].

There are several debates on the sources of NO production in plants. A growing body of evidence indicates that NO is formed by mammalian-like nitric oxide synthase (NOS) activity, nitrate reductase (NR), or nonenzymatic sources [4,7,9]. Although NOS-like activity has been detected widely in plants and inhibitors of mammalian NOS such as Nω-nitro-L-Arg (L-NNA) and Nω-monomethyl-L-Arg inhibit NO generation in plants, the molecular identity of NOS (gene, cDNA, or protein) is unknown [9,10]. A unique AtNOS1 gene was isolated from Arabidopsis thaliana, which was indicated to encode a protein with sequence similarity to a protein that is implicated in NO synthesis in the snail Helix pomatia [11]. However, the function of AtNOS1 as an authentic NOS has been recently questioned. The findings that AtNOS1 neither binds nor oxidizes arginine to NO via a mechanism analogous to that of the mammalian NOS demonstrate that the involvement of AtNOS1 in NO biosynthesis and accumulation may be either indirect or regulatory. Therefore, AtNOS1 does not have NOS-like activity; rather, it is likely that the AtNOS1 is a protein associated with biosynthesis of NO. Accordingly, the AtNOS1 was suggested to be renamed as AtNOA1 for NO associated 1 [12,13]. Moreover, AtNOA1 is actually a member of the circularly permuted GTPase family (cGTPase), likely playing general roles in mitochondrial and/or chloroplastic ribosome assembly and subsequent mRNA translation to proteins [13]. Previous studies have shown that AtNOA1-dependent NO synthesis is involved in hormonal signaling and stomatal movement [11].

Of course, the source of NO may be different depending on the plant species, type of tissues or cells, and plant growth conditions. It has been pointed out that the research on elucidation of how NO is made by different plant cells, in different external situations, is clearly a priority [9]. The source of NO production induced by chilling stress in many plants is not very clear. It is still not known whether NO production is mainly from NOS-like activity under low temperature conditions and how this source of NO is associated with the plant responses to chilling. In order to explore and elaborate the roles of NO in plants under chilling conditions, Chorispora bungeana Fisch. and C.A. Mey (C. bungeana), a representative alpine subnival plant which can survive under chilling temperatures, even freezing temperatures and frequent temperature fluctuations, was used in this study. It grows in the freeze-thaw tundra in the borders of glaciers where almost all the other flowering plants have great difficulty for growing and exhibits great resistance to low temperature stress. Previous researches suggest that C. bungeana can retain some stable variations of certain physiological and molecular characteristics in response to cold environment [14]. Therefore, it provides an ideal model system for studying the adaptive mechanisms of plants to chilling conditions. In this work, we used suspension cultured cells from C. bungeana to investigate the function of NO as a second messenger in the chilling stress signaling pathway and the ability of NO to mediate the plant adaptive responses to cope with the chilling conditions.

2. Results

2.1. NO prevents ion leakage, lipid peroxidation and reduction of cell viability induced by chilling stress

Low temperature inevitably induces oxidative stress, which results in cellular membrane injury and membrane lipid peroxidation, thus suppressing cell viability. As shown in Fig. 1, chilling treatment caused the significant increases in ion leakage and MDA content in the suspension cultures. The changes became more evident with decreasing temperatures from 4 °C to 0 °C. For instance, ion leakage increased by 36.8% and 63.6%, MDA content by 19.7% and 37.9% under chilling stress at 4 °C and 0 °C, respectively (Fig. 1A and B). Moreover, chilling stress resulted in obvious cell viability suppression in the suspension cultures, with relative cell viability suppressed by 19.7% and 37.9% under chilling stress at 4 °C and 0 °C, respectively (Fig. 1C). In contrast, the effects of SNP, PTIO and L-NAME were significantly higher than those of L-NNA. A similar trend was observed for MDA content and cell viability, which were significantly reduced by SNP, PTIO and L-NAME treatment.

![Fig. 1. Effects of SNP, PTIO and L-NAME on ion leakage (A), MDA content (B) and relative cell viability (C) in the suspension cultured cells from C. bungeana after 3 days of cultivation at 25 °C, 4 °C or 0 °C. Mean values and SD (standard deviations) were calculated from three independent experiments. Within each set of experiments, different letters indicate significant difference at the 0.05 level according to Duncan's multiple range test.](image)
viability decreasing to 81.4% and 78.4% of the control under 4 °C and 0 °C conditions, respectively (Fig. 1C). It has been well documented that NO could counteract oxidative damage and have protective effect against environmental stresses [4]. To test if NO has capacity to protect C. bungeana suspension cultures from chilling injury, we treated the cells with SNP (a NO donor) under low temperature conditions and measured the above mentioned physiological parameters. Fig. 1 revealed that application of SNP completely prevented or evidently alleviated the increases of ion leakage and MDA content and the decrease of cell viability induced by chilling stress. Additionally, SNP itself had not any effect on the cells growing at 25 °C. To further clarify the physiological roles of endogenous NO in chilling stress responses, the NO scavenger PTIO and mammalian NOS inhibitor L-NAME were used, both of which had no apparent effect on C. bungeana suspension cells under normal temperature conditions. However, under low temperatures, treatment with PTIO or L-NAME resulted in noticeable increases in ion leakage and MDA content and remarkable decrease in cell viability in the suspension cultures compared with those of chilling stress alone, indicating that the reduction of NOS-mediated NO level could be responsible for the observed severe oxidative injury under chilling stress.

2.2. The levels of H2O2, O2•− and OH• are decreased by NO treatment under chilling conditions

As oxidative damage is generated under chilling stress through the formation of ROS in plants, we measured the H2O2, O2•− and OH• levels in C. bungeana suspension cultures under chilling treatment. Low temperatures induced the significant increase in generation of H2O2, especially at 0 °C. Fig. 2A showed that H2O2 content increased by 66.5% and 105.9% in the cells exposed to 4 °C and 0 °C, respectively. In parallel with H2O2 content, chilling treatment also resulted in the increases in O2•− production and OH• content. As shown in Fig. 2B and C, O2•− production rate increased by 84.3% and 114%, OH• content by 45.9% and 53% in the suspension cultures under chilling stress at 4 °C and 0 °C, respectively. SNP treatment evidently reduced the increases of H2O2, O2•− and OH• contents caused by chilling. Thus, increased H2O2, O2•− and OH• productions were strongly suppressed by NO. Moreover, under low temperature conditions, the H2O2, O2•− and OH• levels increased markedly in the presence of PTIO or L-NAME compared with those under only chilling stress. Nevertheless, there were no significant differences in H2O2, O2•− and OH• contents among all the treatments at 25 °C.

2.3. Responses of antioxidant enzymes to chilling and NO treatments

It has been demonstrated that NO could affect the activities of many antioxidant enzymes in plants to alleviate the injury of oxidative stress [15]. Since chilling stress causes oxidative injury and alters the activities of antioxidant enzymes, we next examined the effects of NO on the behavior of antioxidant enzymes. The data in Fig. 3 showed that the activities of APX, GR, POD and SOD increased significantly under chilling stress. However, the activity of CAT decreased by 18.3% and 38.8%, respectively, under chilling treatments at 4 °C and 0 °C. Treatment with SNP, PTIO or L-NAME had no obvious effects on activities of antioxidant enzymes in the suspension cells cultivated at 25 °C. Nevertheless, under chilling conditions, the application of SNP induced the remarkable increases in the activities of APX, CAT, GR, POD and SOD in the cultured cells. With PTIO or L-NAME treatment, the activities of above mentioned enzymes declined markedly in comparison with those under chilling stress alone.

2.4. Effects of NO on AsA and GSH contents

AsA and GSH are not only the important antioxidants against oxidative stress but also the substrates for APX and GR in AsA-GSH cycle [16]. Therefore, the contents of AsA and GSH were determined to test whether water-soluble antioxidants are involved in the defense system against chilling-induced oxidative stress in C. bungeana suspension cells. Chilling stress caused the significant increase in AsA content, especially under 4 °C conditions. Fig. 4A showed that AsA content increased by 44.3% and 21% in the suspension cells exposed to 4 °C and 0 °C, respectively. In parallel with AsA content, chilling treatment also resulted in the dramatic increase in GSH content when the suspension cultures were exposed to 4 °C conditions. As shown in Fig. 4B, GSH content increased by 90.8% under chilling stress at 4 °C. However, chilling stress at 0 °C had only slight effect on GSH content. There were nearly no significant changes of antioxidant contents in the suspension cells among all the treatments at 25 °C. Under low temperature conditions, SNP application caused pronounced increases in the contents of AsA and GSH in the cultured cells. In the
presence of PTIO or L-NAME, the contents of AsA and GSH decreased remarkably compared with those of chilling treatment alone.

2.5. NOS activity and NO production under chilling stress

To further elucidate the possible relationship between NO accumulation and chilling tolerance, we measured NOS activity and NO production in *C. bungeana* suspension cultured cells under chilling stress. As shown in Fig. 5, exposure of the suspension cultures to low temperatures led to the remarkable increases in NOS activity and NO production. The increases became more evident with decreasing temperatures from 4 °C to 0 °C. For example, NOS activity increased by 78.1% and 139.5% in the cells exposed to 4 °C and 0 °C, respectively, and the same treatments resulted in the increases in NO content by 62.2% and 83.1%, respectively. Under chilling stress, NOS activity was obviously inhibited in the presence of SNP, whereas the content of NO increased remarkably in comparison with that of chilling treatment alone. Comparatively, application of PTIO induced significant increase in NOS activity compared with that of chilling stress alone although it inhibited the production of NO. Moreover, NO content decreased distinctly in the presence of L-NAME in combination with chilling treatment because L-NAME inhibited NOS activity. In addition, under normal temperature, the effects of SNP, PTIO or L-NAME on the NOS activity and NO production were similar to these under low temperature conditions.

3. Discussion

Pervious researches have indicated that chilling treatment causes oxidative stress, which leads to cellular membrane disruption, solute and electrolyte leakage and membrane lipid peroxidation, thus suppressing cell viability in plants. Ion leakage assay, lipid peroxidation measurement and relative cell viability assay have been commonly used to evaluate the extent of oxidative injury induced by chilling. In our study, chilling stress resulted in obvious increases in ion leakage and lipid peroxidation in *C. bungeana* suspension cells (Fig. 1A and B). Any such changes are likely to affect normal ion exchange capacity of plasma membrane and all the physiological activities linked to membrane functioning [17]. In addition, relative cell viability declined evidently in the suspension cultures exposed to low temperatures (Fig. 1C).

H$_2$O$_2$, O$_2^.$ and OH$^.-$, as the main kinds of ROS in biological system, accumulate under chilling stress and can be the index of oxidative damage [6]. Our results also showed that H$_2$O$_2$, O$_2^.$ and OH$^.-$ levels increased significantly in the cultures under chilling treatment (Fig. 2), indicating that ROS is involved in chilling-enhanced oxidative injury. To alleviate oxidative stress generated through ROS, plants have evolved an efficient antioxidant defense system. Tolerance to chilling stress is often associated with the enhanced capacity of antioxidant defense system under low temperature conditions [18]. As shown in Figs. 3 and 4, the activities of APX, GR, POD and SOD and contents of AsA and GSH increased remarkably in *C. bungeana* suspension cultures under chilling stress. Among antioxidant defense system, SOD may be central in defending cells against toxic ROS, which detoxifies O$_2^.$ by forming H$_2$O$_2$, while CAT and POD catalyse H$_2$O$_2$ breakdown. APX and GR also play a key role by reducing H$_2$O$_2$ to water through AsA-GSH cycle [16]. Besides those, AsA and GSH can directly scavenge O$_2^.$ and OH$^.-$, and reduce H$_2$O$_2$ to water. It is highly possible that low temperatures activate antioxidant defense system including antioxidant enzymes and antioxidants, whose cooperation with each other is responsible for...
scavenging excessive ROS and alleviating oxidative injury imposed by chilling stress in *C. bungeana* suspension cultured cells. Taken together, *C. bungeana* suspension cells might survive under low temperatures by maintaining efficient antioxidant defense system and alleviating oxidative damage. APX and GR activities as well as the contents of AsA and GSH increased dramatically during chilling treatment, implying that the more active AsA-GSH cycle might play a primary role in development of chilling tolerance in *C. bungeana* suspension cultures.

Recently, a function of NO in protection of plants against oxidative stress under various adverse conditions was reported [15]. The exogenous application of NO to plants has been used as a tool to study its effects in various physiological processes [5]. Our results showed that application of SNP effectively prevented ion leakage increase, lipid peroxidation and cell viability suppression in *C. bungeana* cultured cells under chilling stress (Fig. 1). These findings clearly suggest that exogenous NO could strongly protect plants from oxidative damage induced by chilling, and the mechanisms by which NO might help plants resist oxidative injury were also postulated. First of all, NO might react directly with some ROS to counteract their toxic effects, such as O$_2^\bullet$ to form peroxynitrite, which is less toxic and thus limits cellular damage. Secondly, NO could function as a signaling molecule in the activation of cellular antioxidant defense system [6,19]. In our study, SNP treatment markedly reduced the accumulation of H$_2$O$_2$, O$_2^\bullet$ and OH$^-$ induced by chilling (Fig. 2). Moreover, the activities of APX, CAT, GR, POD and SOD and the contents of AsA and GSH in the presence of SNP under chilling conditions were much higher than those of chilling stress alone (Figs. 3 and 4). In conclusion, NO, as a bioactive antioxidant, can exert a protective function against chilling-induced oxidative damage by scavenging ROS directly or by enhancing these antioxidant enzyme activities and antioxidant contents.

Although the application of exogenous NO appears to be a useful tool to study its physiological effects, such strategy does not take into account the finding that NO is also produced endogenously in plant cells in response to chilling stress [10,20]. To clarify the physiological role of endogenous NO in chilling tolerance, the NO scavenger PTIO and mammalian NOS inhibitor L-NAME were used. Our study showed that under low temperature conditions, treatment with PTIO or L-NAME resulted in significant oxidative injury in *C. bungeana* suspension cultures in comparison with that of chilling stress alone, which means that ion leakage aggravated significantly, MDA and ROS accumulated remarkably, and cell viability, the activities of APX, CAT, GR, POD and SOD and the contents of AsA and GSH decreased obviously (Figs. 1–4). Measurements of NOS activity and NO content indicated that they both increased noticeably under chilling stress (Fig. 5). From these results, we conclude that significant increases in NOS activity and NO level under chilling stress are involved in chilling tolerance in *C. bungeana* suspension cultures. In addition, chilling-triggered NO production was inhibited by specific mammalian NOS inhibitor L-NAME, indicating that NOS-associated protein may play an important role in NO-mediated chilling protective reactions and that chilling-induced NO accumulation is mainly produced from NOS-like activity, which is consistent with the previous report of Corpas et al. [20] in pea (*Pisum sativum*) leaves. However, in contrast to our findings, Zhao et al. [10] found that the increase in NO induced by low temperature is contributed to NR-dependent NO production. The discrepancy in sources of NO release triggered by low temperature, to some extent, may be associated with the differences in plant species (*C. bungeana* versus *Arabidopsis*), type of tissues (suspension cells versus leaves) and culture conditions. In summary, NOS-like activity-dependent endogenous NO production was substantially increased under chilling conditions, and could

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**Fig. 4.** Effects of SNP, PTIO and L-NAME on the contents of AsA (A) and GSH (B) in the suspension cultured cells from *C. bungeana* after 3 days of cultivation at 25°C, 4°C or 0°C.

**Fig. 5.** NOS activity (A) and NO content (B) in the suspension cultured cells from *C. bungeana* under chilling stress at 4°C or 0°C.
serve as a second messenger in the signaling pathway of chilling adaptive responses.

In the present study, both NOS activity and NO production increased remarkably under chilling stress. Interestingly, NO scavenger PTIO treatment enhanced NOS activity but reduced NO content, while the application of NO donor SNP suppressed the NOS activity, despite elevation of NO release from SNP (Fig. 5). These results indicate that NO production mediates the responses to chilling stress and its cellular level has a feedback effect on the NOS activity of C. bungeana suspension cultures. Like the previous reports in maize leaves under water stress or UV-B radiation [9,21], our results confirm that the decreased NOS activity may be achieved by negative feedback regulation of NOS by NO [9]. The modulation of NOS activity is probably determined by the concentration of NO. In detail, under low temperature conditions in the absence of SNP, chilling stress induces the increase of NOS activity to produce an amount of NO which is unable to inhibit the NOS activity; whereas in the presence of SNP, the NO generated by chilling treatment and the addition of exogenous SNP can become an inhibitor of NOS to suppress NOS activity through a feedback mechanism.

Microarrays, northern blots, as well as cDNA-amplification fragment length polymorphism analyses of NO-responsive transcripts reveal that NO governs the regulation of expression of numerous genes. These genes encode proteins related to defense responses, metabolism, cellular detoxification, transport, ion homeostasis, signaling, flowering and lignin biosynthesis [5]. In A. thaliana roots, SNP reportedly modulates (both up and down) a variety of genes that are commonly involved in biotic and abiotic stresses including ROS formation and scavenging, cellular defense and death, and metabolism and only a few are stress-specific in nature [22]. Moreover, some antioxidant enzyme genes including APX and CAT were also found to be induced by NO in Arabidopsis suspension cells [19]. In our study, depletion of endogenous NO with PTIO or L-NAME resulted in aggravated oxidative stress accompanied by pronounced decreases in the activities of APX, CAT, GR, POD and SOD and the contents of AsA and GSH under chilling stress. In accordance, treatment with exogenous NO remarkably elevated activities of APX, CAT, GR, POD and SOD and contents of AsA and GSH, hence alleviating oxidative injury induced by chilling in C. bungeana suspension cultures. Thus, it is highly possible that the protective effect of NO may be mediated through the increased levels of transcription and/or translation of specific genes encoding these antioxidant enzymes as well as the key enzymes involved in antioxidant biosynthesis under chilling stress. In addition, Wu et al. [14,23] found that GSH accumulation was induced by low temperature and the transcript level of γ-glutamylcysteine synthetase (CbyECS), the key enzyme of GSH synthesis, increased rapidly when C. bungeana was treated with chilling. Moreover, under low temperature conditions, application of SNP obviously increased the accumulation of GSH whereas treatment with PTIO or L-NAME caused pronounced decrease in GSH content compared with that of chilling treatment alone. Therefore, it is likely that the effect of NO on GSH content may be associated with NO-mediated expression of CbyECS in C. bungeana under chilling stress, which is in accordance with the finding of Innocenti et al. [24] in Medicago truncatula roots. Further research is needed to elucidate the molecular details regarding the exact cascade of changes that occur and genes or proteins induced/expressed in response to NO signaling in enhancing antioxidant defense under chilling stress.

Plants respond to chilling stress by activating a large variety of defense responses. Besides NO, a wide range of other messengers, including abscisic acid (ABA), calcium ion (Ca2+), salicylic acid (SA), ROS and ethylene, have also been proved to be involved in the chilling signal transduction networks. SA pretreatment could directly or indirectly change cold tolerance and cellular antioxidant enzyme activities during chilling stress, which had an ability to alleviate chilling-induced injury [1]. Increases of endogenous ABA, H2O2, Ca2+ and ethylene have been observed in plants in response to chilling stress [25]. Chilling-induced intracellular Ca2+ overload triggered the enhancement of ROS production in maize cells, implying that the inter-relationship between Ca2+ and ROS is a key component in plant responses to chilling stress [2]. Wang and Li [26] suggested that SA-induced cold tolerance in grape leaves is through Ca2+ homeostasis and a higher activity of antioxidant systems that is correlated to the cytoplasmic Ca2+ increase in cells under cold stress. In addition, ABA and H2O2 have shown a relationship to cold tolerance in many species [26]. Moreover, the previous research demonstrated that the difference in Ca2+ distribution was a response of C. bungeana in adapting itself to the alpine subnival environment and the accumulation of Ca2+ could play an important role in its chilling tolerance [27]. Collectively, these findings imply that the complex relationship between NO and other signal molecules may exist in C. bungeana under chilling stress.

In conclusion, our results demonstrate that NO is involved in the chilling signaling pathway and may function as a second messenger to mediate the adaptive responses to chilling stress. The chilling-induced NO accumulation may be associated with the NOS-like enzyme. The role of NO in alleviating chilling-induced oxidative damages is, at least in part, attributed to its ability to detoxify ROS directly and activate antioxidant defense. These findings will no doubt help us in gaining further insights into the chilling signaling pathway in plants. However, little is known about the complex molecular networks operating during chilling treatment. The cross talk between NO and other signaling molecules in response to chilling stress requires further investigation.

4. Materials and methods

4.1. Plant material

A wild species of C. bungeana was obtained as described by Fu et al. [27]. The leaves of C. bungeana without serrate margins were selected for callus induction. The sterile leaves were cut into 10 mm segments, with margins finely cut off, and cultured on Murashige and Skoog (MS) basal medium containing 4.0 mg L−1 gibberellic acid (GA3), 0.2 mg L−1 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.2 mg L−1 α-naphthalene acetic acid (NAA) with a 16-h photoperiod at 80 μmol m−2 s−1 photosynthetically active radiation under 25 °C. The medium was refreshed every 2 weeks. Suspension cultured cells of C. bungeana were cultivated in liquid MS basal medium containing 0.2 mg L−1 2,4-D, 0.2 mg L−1 NAA, 0.2 mg L−1 6-benzylaminopurine (6-BA), and 0.2 mg L−1 kinetin (KT) on a continuous shaking at 120 rpm. The liquid medium was also refreshed at weekly intervals. After five or more subcultures, the synchronous steady cultures were collected and used in the following experiments.

4.2. Chemicals and experimental treatments

Sodium nitroprusside (SNP) was used as a NO donor, and the compound 2-phenyl-4,4,5,5-tetramethyl-imidazole-1-oxyl-3-oxide (PTIO) was used as a specific NO scavenger. N-nitro-L-Arg-methyl ester (L-NAME) was used as the inhibitor of NOS. The 7-day-old stock cultures were transferred into the different liquid MS mediums containing the following chemicals: (1) sterilized water; (2) 0.1 mM SNP; (3) 0.1 mM PTIO; and (4) 0.3 mM L-NAME. Subsequently, all the treated suspension cultures in same medium were divided into three groups. One was cultured under 25 °C and the other two were exposed to 4 °C
or 0 °C for 3 days. Control cells were treated with sterilized water and exposed to a temperature of 25 °C. Following the treatment, the suspension cultured cells were harvested, washed with sterilized distilled water to remove medium residues, and the excess water was blotted with filter paper. The fresh cells were used for ion leakage and relative cell viability assays immediately and the others were frozen under liquid N₂ and then stored at −80 °C for further analysis.

4.3. Ion leakage measurement

Ion leakage was determined according to Song et al. [5] with some modifications. The suspension cultures (0.2 g) were washed in deionized water and placed with 16 mL of deionized water at 25 °C. After 2 h, the conductivity was measured (C₁) and the samples were boiled for 30 min to achieve 100% electrolyte leakage (C₂). Relative ion leakage was expressed as a percentage of the total conductivity after boiling (Relative ion leakage % = C₁/C₂ × 100).

4.4. Analysis of lipid peroxidation

Lipid peroxidation was determined by measuring the content of malondialdehyde (MDA) according to the method of Guo et al. [28]. The samples were homogenized with a mortar and pestle in 5% trichloroacetic acid (TCA) and then centrifuged at 1,000 g for 10 min. The supernatant was mixed with the equal volume of 5% TCA containing 0.67% thiobarbituric acid (TBA). The mixture was heated at 100 °C for 30 min and quickly cooled to room temperature. Absorbance was read at 450, 532, and 600 nm after centrifugation at 1,000 g for 10 min again. MDA content was calculated using the following formula: MDA (μM/mL) = [(A₅₃₂ - A₆₀₀)/0.56OD₄₅₀] * (1/0.1).

4.5. Relative cell viability assay

Chilling injury was also determined by assessing viability of suspension cells, which was estimated by the 2,3,5-triphenyltetrazolium chloride (TTC) reduction as described by Guo et al. [28]. Fresh cells (0.2 g) were incubated for 13 h in the dark with 2.5 mL of 65 mM potassium phosphate buffer (pH 7.8) and 0.3 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 15,000 g for 10 min and then 0.5 mL of supernatant was mixed with 0.5 mL of 50 mM sodium phosphate buffer (pH 7.0) and 1 mL of 25 mM sodium phosphate buffer (pH 7.0) containing 2.5 mM 2-deoxyribose. The reduction was developed at 35 °C in the dark for 1 h. After adding 1 mL of 1% TBA in 0.05 M NaOH and 1 mL of acetic acid, the mixture was boiled for 30 min and immediately cooled for 10 min in ice. The production of OH⁻ was followed by measurement of absorbance at 532 nm and the OH⁻ content was expressed as absorbance units (absorbance × 1000) per gram sample fresh weight (FW).

4.7. Antioxidative enzyme activity assay

The cultured cells were homogenized with a chilled mortar and pestle in 50 mM potassium phosphate buffer (pH 7.8) that contained 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 15,000 g for 20 min at 4 °C and the supernatant was used for the following enzyme assays. Protein concentration was determined following the method of Bradford [31] using bovine serum albumin (BSA) as the standard.

The APX activity was measured by monitoring the rate of ascorbate oxidation at 290 nm (ε = 2.8 mM⁻¹ cm⁻¹), according to the method of Nakano and Asada [32]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA–N₃, 9 mM H₂O₂, 0.3 mM ascorbic acid (AsA) and enzyme extract. CAT activity was assayed by following the consumption of H₂O₂ (ε = 39.4 mM⁻¹ cm⁻¹) at 240 nm according to the method of Aebi [33]. GR activity was determined by following the rate of NADPH oxidation as measured by the decrease in the absorbance at 340 nm (ε = 6.2 mM⁻¹ cm⁻¹), according to the method of Foyer and Halliwell [34]. The 0.1 mL of enzyme extract was added to 1 mL of reaction solution containing 50 mM potassium phosphate buffer (pH 7.8), 5 mM MgCl₂ and 0.5 mM oxidized glutathione (GSSG). The assay was initiated by adding 0.1 mL of 1.2 mM NADPH at 25 °C. POD activity was measured by the increase in absorbance at 470 nm due to formation of tetraguaiacol (ε = 26.6 mM⁻¹ cm⁻¹) from guaiacol in the presence of H₂O₂, according to the method of Chance and Maehly [35]. SOD activity was measured according to the method of Beauchamp and Fridovich [36]. One unit of SOD activity was defined as the amount of enzyme required for inhibition of photochemical reduction of nitroblue tetrazolium (NBT) by 50% at 560 nm.

4.8. Measurement of antioxidant content

The samples (0.2 g) were ground with a mortar and pestle in 2 mL of 0.5 mM EDTA solution containing 3% TCA at 4 °C. The homogenate was centrifuged at 15,000 g for 10 min. The supernatant was used for estimations of AsA and reduced glutathione (GSH) contents. The AsA concentration was measured by using 2,6-dichlorophenol-indophenol (DCPIP) photometric method of Parida et al. [37]. GSH content was measured according to the method of Ellman [38]. The supernatant (0.2 mL) was added to 1.5 mL of 50 mM potassium phosphate buffer (pH 7.8) and 0.3 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.2 mM 5,5’-dithio-bis
(2-nitrobenzoic) (DTNB). The mixture was incubated at 30 °C for 2 min. Absorbance was determined at 412 nm and the GSH concentration was calculated by comparison to a standard curve.

4.9. NOS activity and NO content determinations

NOS activity assay was performed according to Hevel and Marletta [39] with slight modifications. Briefly, about 1 g of cultured cells was homogenized in 2 mL of homogenization buffer (50 mM triethanolamine hydrochloride, pH 7.5, containing 1 mM EDTA, 1 μM leupeptin, 1 μM pepstatin, 7 mM glutathione, and 0.2 mM phenylmethylsulfonyl fluoride). After centrifuging at 10,000×g for 20 min at 4 °C, the supernatant was collected and recentrifuged at 100,000×g for 45 min. The supernatant was used to measure NOS activity by hemoglobin assay as previously described [39]. The reaction mixture (total volume of 1 mL) contained 200 μL of crude enzyme extract, 6 μL oxihemoglobin, 1 mM L-arginine, 1 mM magnesium diacetate, 1 mM CaCl2, 1 mM EGTA, 100 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) (pH 7.5). This was incubated at 37 °C for 2 min. Absorbance was determined at 412 nm and the GSH concentration was calculated by comparison to a standard curve. The data in all analyses were obtained from three independent experiments. Treatment means were compared by one-way analysis of variance (ANOVA) using the SPSS software (13.0) and Dunnett’s multiple range test at 0.05 level of significance.

Acknowledgments

This research was supported by the National Major Project of Cultivating New Varieties of Transgenic Organisms (No. 2009ZX08009-029B), the National Outstanding Youth Foundation of China (No. 30625008), the National High Technology Research and Development Program of China (863 Program) (No. 2007AA021401), the National Basic Research Program of China (973 Program) (No. 2007CB108902) and the National Science Foundation of China (No. 30600040).

References


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