Research Paper

Nitric oxide synthase-mediated early nitric oxide-burst alleviates drought-induced oxidative damage in ammonium supplied-rice roots

Running title: Early nitric oxide burst in drought tolerance

Cao Xiaochuang[¶1], E-mail: caoxiaochuang@126.com
Zhu Chunquan[¶1], E-mail:situyajie@126.com
Zhong Chu[¶], E-mail:1035305743@qq.com
Zhang Junhua[1], E-mail:Jhzhang316@gmail.com
Zhu Lianfeng[1], E-mail:zlfnj@163.com
Wu Lianghuan, E-mail:21014105@zju.edu.cn
Ma Qingxu[*2], E-mail:592214410@qq.com
JinQianyu[*1], E-mail:11014041@zju.edu.cn
[1]State Key Laboratory of Rice Biology, China National Rice Research Institute, Hangzhou, 310006 China
[2]Ministry of Education Key Laboratory of Environmental Remediation and Ecosystem Health, College of Environmental and Resource Sciences, Zhejiang University, Hangzhou, 310058 China
[¶] These authors contributed equally to this work
[*]Correspondence: Email: 11014041@zju.edu.cn
Phone number: (+86) 0571 63370355
Or Email: 592214410@qq.com
Phone number: (+86) 0571 88982079

Date of submission: 2th Aug, 2018
Number of tables: 0; Number of figures: 7; Number of words: 5221
Number of Manuscript Pages: 28
Number of figures to be in color in the print copy: 7
Specify which figures are to be printed in color: Fig. 1, 2, 3, 5, 7
Specify which figures are to be in color on line-only: Fig. 1, 2, 3, 5, 7
Supplementary data: Fig. S1, S2, S3, S4, Method S1

34 Highlight: NOS-mediated early NO burst plays an important role in alleviating
35 oxidative damage induced by water stress, by enhancing the antioxidant defenses in
36 roots supplemented with NH_4^+

37

Abstract

Ammonium (NH_4^+) can enhance rice drought tolerance in comparison to nitrate (NO_3^-). The mechanism underpinning this relationship was investigated based on the time-dependent nitric oxide (NO) production and its protective role in oxidative stress of NH_4^+-/NO_3^--supplied rice under drought. An early burst of NO was induced by drought 3h after root NH_4^+ treatment but not after NO_3^- treatment. Root oxidative damage induced by drought was significantly higher in NO_3^- than in NH_4^+-treatment due to its reactive oxygen species accumulation. Inducing NO production by applying NO donor 3h after NO_3^- treatment alleviated the oxidative damage, while inhibiting the early NO burst increased root oxidative damage in NH_4^+ treatment. Application of nitric oxide synthase (NOS) inhibitor N(G)-nitro-*L*-arginine methyl ester (L-NAME) completely suppressed NO synthesis in roots 3h after NH_4^+ treatment and aggravated drought-induced oxidative damage, indicating the aggravation of oxidative damage might have resulted from changes in NOS-mediated early NO burst. Drought also increased root antioxidant enzymes activities, which were further induced by NO donor but repressed by NO scavenger and NOS inhibitor in NH_4^+-treated roots. Thus, the NOS-mediated early NO burst plays an important role in alleviating oxidative damage induced by drought by enhancing antioxidant defenses in NH_4^+-supplied rice roots.

Keywords: Ammonium, nitric oxide, nitric oxide synthase, antioxidant enzymes, oxidative damage, drought stress, rice

Abbreviations: ascorbate peroxidase (APX), catalase (CAT), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), N(G)-nitro-*L*-arginine methyl ester (L-NAME), malondialdehyde (MDA), nitric oxide (NO), nitric oxide synthase (NOS), nitrate reductase (NR), peroxynitrite ($ONOO^-$), polyethylene glycol (PEG-6000), peroxidase (POD), reactive nitrogen species (RNS), reactive oxygen species (ROS), sodium nitroprusside (SNP), superoxide dismutase (SOD).

Introduction

As human population and global climate change increase, drought stress is becoming a major abiotic factor limiting crop growth and yield. Plants have evolved several strategies to contend with water stress. These include morphological,

physiological, and molecular adaptations (Bogeat-Triboulot *et al*., 2007; Guo *et al*., 2007; Slewinski, 2012). Nitric oxide (NO) is an important signaling molecule in various physiological functions like seed germination, floral transition, stomatal movement, leaf senescence, and yield development, and it has gained increasing attention since the 1980s (Neill *et al*., 2003; Wilson *et al*., 2007; Simontacchi *et al*., 2015). Certain plant responses and adaptations to abiotic stresses involve NO, and sufficient data indicate that NO mediates plant responses to various stimuli including drought (Mata and Lamattina, 2001), salt (Zhao *et al*., 2007), and metal toxicity (Gonzalez *et al*., 2012) stresses, thereby enhancing plant stress tolerance and survival.

Water deficits significantly increase NO production in plants (Signorelli *et al*., 2013; Planchet *et al*., 2014). As a free radical, NO can form various reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2), dinitrogentrioxide (N_2O_3) and S-nitrosoglutathione (GSNO), which are involved in many physiological functions of plants (del Rio, 2015), indicating that NO and NO-derived molecules take part in inorganic nitrogen (N) metabolism. A combination of transgenic technology and pharmacological analysis have indicated that NO induces antioxidant activity and alleviates water stress in plants in several ways: 1) It limits reactive oxygen species (ROS) accumulation and ROS-induced cytotoxic activity by inhibiting the ROS-producer NADPH oxidase via *S*-nitrosylation (Fan *et al*., 2012); 2) It functions as an antioxidant and reacts with ROS (e.g. O_2^-) to generate transient $ONOO^-$, which is then scavenged by other cellular processes (del Rio, 2015); 3) It induces the expression of genes coding for antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR), and may increase enzyme activity by posttranslational modifications thereby reducing lipid peroxidation under water stress (Farooq *et al*., 2009; Fan and Liu, 2012); 4) It helps maintaining high vacuolar concentrations of osmotically active solutes and amino acids like proline (Verdoy *et al*., 2006); and 5) It acts as a downstream abscisic acid (ABA) signal molecule and participates in "ABA-H_2O_2-NO-MAPK" signal transduction processes. It also increases plant antioxidant ability (Zhang *et al*., 2007). The accumulation of ROS in water-stressed plants impairs the function of biochemical processes, damages organelles, and ultimately results in cell death (Jiang and Zhang, 2002). Therefore, endogenous NO production may enhance plant antioxidant capacity and help plant cells survive under various types of stresses.

However, NO also has biphasic properties on plants. The duality of its effects depends on stress duration and severity, and on the cell, tissue, and plants species (Neill, 2007; Santisree *et al*., 2015). At low concentration or early stage of abiotic stress, NO participates in important functions in higher plants through its involvement in physiological and stress-related processes (as described above). Arasimowicz-Jelonek *et al.* (2009a, b) demonstrated that NO synthesis slightly increased in roots subjected to <10 h water deficit, but significantly up-regulated after prolonged (≥17h) drought. Under severe or protracted longtime stress, NO overproduction in plants can shift the cellular stress status from oxidative stress to severe nitrification stress, finally damaging proteins, nucleic acids, and membranes (Groß *et al*., 2013; del Rio 2015). Protein tyrosine nitration is considered a good marker to evaluate the process of nitrosative stress under various abiotic environments (Corpas *et al*., 2007, 2008). Excess NO can also act synergistically with ROS resulting in nitro-oxidative stress and eliciting undesirable toxic effects in plant cells (Signorelli *et al*., 2013). Liao *et al.* (2012) and Sun *et al.* (2014) argued that the ability of endogenous or exogenous NO production in plants to alleviate oxidant damage was dose-dependent. Therefore, determining instantaneous plant NO content under drought stress may not completely reflect the specific role of NO in drought tolerance.

In higher plants, nitrate reductase (NR) and nitric oxide synthase (NOS) are the two key enzymes for NO production (Guo *et al*., 2003; Neill *et al*., 2003). Moreover, NR-dependent NO production occurs in response to pathogen infection (Shi and Li, 2008), drought (Freschi *et al*., 2010), and freezing (Zhao *et al*., 2009). Arasimowicz-Jelonek *et al*. (2009a, b) applied the NO donor sodium nitroprusside (SNP) and GSNO to water-stressed cucumbers and demonstrated that both NR and NOS participated in drought tolerance. Shi *et al.* (2014) reported that rat neuronal NO synthase overexpression in rice plants increased their tolerance to drought stress, thus demonstrating the importance of NOS-mediated NO production in water deficit tolerance. Despite increasing knowledge on NO-mediated plant functions, NO origins and signaling in response to prolonged stress and their regulation in plant drought tolerance remain poorly understood.

Ammonium (NH_4^+) and nitrate (NO_3^-) are the two primary N sources for plants. It is known that the negative effects of drought stress on plant development can be more effectively alleviated by NH_4^+ than NO_3^- supplementation, as evaluated by plant growth, physiological characteristics, and gene expression levels (Guo *et al*., 2007,

Yang *et al.*, 2012; Ding *et al.*, 2015). NO has a key role in plant water stress acclimation and drought tolerance. Nevertheless, information on the dynamic changes in NO production and its role in drought acclimation in plants supplied with NO_3^- or NH_4^+ during the early stages of water stress is scarce. In the present study, variations in endogenous NO production were monitored in roots supplied with this two N nutrition supplements during water stress. The specific role and origin of the endogenous NO produced were investigated using pharmacological methods. The present study revealed that an early NO burst is crucial for alleviating the water stress-induced oxidative damage through enhancement of antioxidant defenses in roots of NH_4^+-supplied plants. Further analyses demonstrated that this early NO burst might be triggered by NOS-like enzymes.

Materials and methods

Plant material and growth conditions

Rice (*Oryza sativa* L. 'Zhongzheyou No. 1' *hybrid indica*) seedlings were grown hydroponically in a greenhouse. Seeds were sterilized in 1% (v/v) sodium hypochlorite solution. After germination, seeds were transferred to a 0.5 mmol L^{-1} $CaCl_2$ solution (pH 5.5). Three days later, the seedlings were transferred to 1.5-L black plastic pots containing a solution with the following composition: NH_4NO_3 (0.5 mM), $NaH_2PO_4{\cdot}2H_2O$ (0.18 mM), KCl (0.18 mM), $CaCl_2$(0.36 mM), $MgSO_4{\cdot}7H_2O$ (0.6 mM), $MnCl_2{\cdot}4H_2O$ (9 μM), $Na_2MoO_4{\cdot}4H_2O$ (0.1 μM), H_3BO_3 (10 μM), $ZnSO_4{\cdot}7H_2O$ (0.7 μM), $CuSO_4$ (0.3 μM), and $FeSO_4{\cdot}7H_2O$-EDTA (20 μM).All experiments were performed in a controlled growth room under the following conditions: 14/10 h light/dark photoperiod, 400 μmol m^{-2} s^{-1} light intensity, 28°C or 23°C during day or night, respectively, and 60% relative humidity. The solution pH was adjusted to 5.5 with 5 mM2-(N-Morpholino)ethanesulfonic acid (MES). The solution was replaced every 3 days.

After 6 days, seedlings of similar size were cultivated under one of the following treatments: 1 mM NO_3^-, 1 mM NO_3^- + 10% polyethylene glycol (PEG-6000), 1 mM NH_4^+, or 1 mM NH_4^+ + 10% PEG-6000. Water stress was induced by adding 10% PEG-6000. Eight treatments were performed in the NO donor (i.e., SNP) experiments: NH_4^+, NH_4^++ SNP, NH_4^++ PEG-6000, NH_4^++ PEG-6000 + SNP, NO_3^-, NO_3^-+ SNP, NO_3^-+ PEG-6000, and NO_3^-+ PEG-6000 + SNP. The final SNP concentration was 20 μM. For each N nutrition experiment, treatments receiving sufficient water were

defined as control (CK) treatments.

For the NO scavenger2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO, 100 μM) experiment, rice seedlings supplied with 1 mM NO_3^- or 1 mM NH_4^+ solution were pretreated with c-PTIO for 3 h and then given sufficient water (CK) or subjected to water stress for 24 h under the same conditions as those described above.

To investigate the effects of the NO biosynthesis inhibitors, rice seedlings supplied with 1 mM NO_3^- or 1 mM NH_4^+ solution were pretreated with the NO scavenger, tungstate NR inhibitor (100 μM), or NOS inhibitor [Nx-Nitro-*L*-arginine methyl ester hydrochloride (L-NAME); 100 μM] for 3 h, and then given sufficient water (CK) or subjected to water stress for 24 h under the same conditions as described above. There were eight treatments for each N nutrition: Tungstate, L-NAME, Tungstate + SNP, PEG-6000 + Tungstate, PEG-6000 + Tungstate + SNP, L-NAME + SNP, PEG-6000 + L-NAME, and PEG-6000 + L-NAME + SNP.

Determination of NO and $ONOO^-$ contents

The 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) probe was used to determine endogenous root NO levels (Sun *et al*., 2014). Root tips (1 cm) were incubated with 10 μM DAF-FM DA in the dark for 30 min, washed 3× with 20 mM HEPES-KOH (pH 7.4) to remove excess fluorescence, and then observed and photographed under a Nikon Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan; EX 460-500, DM 505, BA 510-560). The relative fluorescence intensity was measured with Photoshop v. 7.0 (Adobe Systems, Mountain View, CA, USA).

Root endogenous $ONOO^-$ was determined using the aminophenylfluorescein (APF) probe method. Root tips were incubated with 10 μM APF dissolved in 10 mM Tris-HCl (pH 7.4) in the dark for 60 min, and then washed 3× with 10 mM Tris-HCl. Fluorescence images and relative fluorescence intensities were analyzed as described above for NO.

Histochemical analyses

Lipid peroxidation and root cell death were histochemically detected with Schiff's reagent and Evans blue (Yamamoto *et al*., 2001). Root tips were incubated in Schiff's reagent for 20 min and washed by three consecutive immersions in 0.5% (w/v)

K_2O_3S solution. A red/purple endpoint indicated the presence of aldehydes generated by lipid peroxidation. Roots were also washed by performing three serial immersions in distilled water, then incubated in 0.25% (w/v) Evans blue for 15 min, and finally washed 3× with distilled water. Roots stained with Schiff's reagent and Evans blue were immediately photographed under a Leica S6E stereomicroscope (Leica, Solms, Germany).

The oxidative damage level, specifically expressed as membrane lipid peroxidation and protein oxidative damage, were estimated by measuring the concentrations of malondialdehyde (MDA) and carbonyl group with 2,4-dinitrophenylhydrazine (DNPH) according to the methods described in Velikova *et al*. (2000).

Determination of ROS contents

Root O_2^- content was estimated using the method described in Liu *et al*. (2007) with some modifications: about 0.15 g fresh root was powdered with 2 mL of 65 mM phosphate buffer saline (PBS, pH 7.8) in a pre-cooled mortar, and centrifuged at 5,000 × *g* for 10 min at 4 °C. Then, 0.9 mL of 65 mM PBS (pH 7.8) and 0.1 mL of 10 mM hydroxylammonium chloride were added to 1 mL of the root extract supernatant, thoroughly mixed, and left to react for 25 min. After this period, 1 mL of 1% (w/v) sulfanilamide and 1 mL of 0.02% (w/v) *N*-(1-naphthyl)-ethylenediaminedihydrochloride were added to 1 mL of root extract solution and left to react for 30 min. Absorbance was then measured at 540 nm.

Root H_2O_2 content was determined by the photocolorimetric method: ~0.15 g fresh root was powdered with 2 mL acetone in a pre-cooled mortar, and centrifuged at 5,000 × *g* for 10 min at 4 °C. Then, 0.1 mL of 5% (w/v) $TiSO_4$ and 0.1 mL pre-cooled ammonium hydroxide were added to 1 mL of the root extract supernatant, which was re-centrifuged at 5,000 × *g* for 10 min. The supernatant was discarded and the sediment was re-dissolved in 4 mL of 2 M H_2SO_4. The absorbance of the root extract solution was measured at 415 nm (Wang *et al*., 2010).

Root OH^- was analyzed by the methods described in Liu *et al*. (2010): ~0.1 g fresh root was powdered with 3 mL of 50 mM PBS (pH 7.0) in a mortar, and centrifuged at 10,000 × *g* for 10 min at 4 °C. Then, 1.0 mL of 25 mM PBS (pH 7.0) containing 5 mM 2-deoxy-*D*-ribose and 0.2 mM NADH were added to 1 mL of the root extract supernatant, completely blended, and left to react for 60 min at 35°C in

the dark. Following this incubation, 1 mL of 1% (w/v) thiobarbituric acid and 1 mL glacial acetic acid were added to the filtrate. The mixture was heated to 100°C for 30 min and then placed on ice for 20 min. The absorbance of the root extract solution was then measured at 532 nm, and the OH^- content was inferred from the production of MDA.

Determination of enzyme activities

Fresh rice root samples (0.5 g) were homogenized in 5 mL of 10 mM phosphate buffer (pH 7.0) containing 4% (w/v) polyvinylpyrrolidone and 1 mM ethylenediaminetetraacetic acid. The supernatant was used as crude enzyme solution and collected by centrifugation at 12,000 × *g* for 15 min at 4°C. The activities of SOD, catalase (CAT), APX, and peroxidase (POD) were estimated using the photocolorimetric methods described in Jiang and Zhang (2002) and Sachadyn-Krol *et al*. (2016).

Root NR and NOS activities were assayed using the methods described in Scheible *et al*. (1997) and Lin *et al*. (2012), with some modifications. Briefly, total protein was extracted using a buffer containing 100 mM HEPES-KOH (pH 7.5), 1 mM EDTA, 10% (v/v) glycerol, 5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100 (v/v), 1% PVP, and 20 μM FAD. The supernatant was collected by centrifugation at 12,000 × *g* for 20 min at 4°C, and then used to determine the NR and NOS activities at 520 nm and 340 nm, respectively.

Determination of arginine and citrulline

Arginine and citrulline contents were estimated using the method described in Salazar *et al*. (2012). Briefly, 1.0 g root samples were frozen in liquid N_2 and extracted with 4 mL 80% (v/v) methanol, and then centrifuged at 10,000 × *g* for 5 min at 4 ℃. The supernatant was then used in derivatization and reaction processes. Serial concentrations of amino acid standards were prepared as described above for the derivatizing reagent, and the derivatizing samples were used to determine the arginine and citrulline contents using liquid chromatography/electrospray ionization tandem mass spectroscopy(LC-ESI-MS).

Statistical analyses

All experiments conducted in this study were performed in triplicate, at least. All

data, expressed as means ± standard error (SE), were processed in SPSS v. 13.0 (IBM Corp., Armonk, NY, USA). The Least Significant Difference (LSD) test was used to determine statistical significant differences among the treatments (P<0.05). Figures were drawn in Origin v. 8.0 (OriginLab Corporation, Northampton, MA, USA).

Results

Plant growth and physiological characteristics

Growth- and physiology-related parameters, such as biomass, photosynthesis rate (P_n), and root N uptake rate in rice seedlings supplied with different N sources were negatively and differently influenced by the 21days water stress (Supplementary Fig. S1a-f). While there were significant decreases in the biomass of NO_3^--supplied plants (62.1% and 52.2% reductions in shoot and total biomass, respectively) (Supplementary Fig. S1a, c), biomass accumulation was not significantly affected in NH_4^+-supplied plants, in relation to CK plants. Water stress reduced P_n in the leaves of NO_3^--treated plants by 40.4% (P<0.05) but that of NH_4^+-treated plants was only reduced by 17.3% (Supplementary Fig. S1d) in relation to CK plants. Thus, NH_4^+-supplied rice seedlings can alleviate PEG-induced drought stress more effectively than NO_3^--supplied rice seedlings.

Root endogenous NO production and histochemical analyses of oxidative damage

To investigate whether NO participates in water stress acclimation, endogenous NO levels in the roots were monitored with the NO-specific fluorescent probe DAF-FM DA. Significant differences in endogenous NO production were observed in roots after 48 h of water stress (Fig. 1a). In CK plants, NO production was relatively stable and varied little between the two N treatments. In contrast, water stress significantly induced endogenous NO production 3 h after the roots received NH_4^+. However, endogenous NO gradually increased only after 6 h in the NO_3^- treatment. Relative fluorescence indicated a significant early burst of NO at 3 h of water stress in the NH_4^+ treatment relative to the control. The NO level in the seedlings treated with NH_4^+ was 2.92× higher than that ofNO_3^--treated plants. Nevertheless, NO in the NO_3^--treated seedlings was 2.72×higher than in NH_4^+-treated plants after 24 h of water stress (Fig. 1b).

Histochemical visualization by Schiff's reagent and Evans blue staining showed that water stress caused severe oxidative damage to the plasma membrane and cell

death in the roots of the plants receiving NO_3^-, whereas the damage was far less pronounced in the seedlings given NH_4^+ (Fig. 1c, d). The following analysis of the MDA and carbonyl concentrations also confirmed that water stress induced more severe lipid peroxidation in the roots of NO_3^--treated than in the roots of NH_4^+-treated seedlings.

Effects of the NO donor on root NO production and oxidative damage

To determine the roles of NO in water stress tolerance, the NO donor SNP was used to simulate NO production. Pre-experimentation with various SNP concentrations (0-100 μM) was performed to quantify the efficacy of SNP against root oxidative damage. As shown in Supplementary Fig. S2, root oxidative damage induced by water stress was significantly alleviated by ≤20 μM SNP. However, the remedial effect of SNP on root oxidative damage was reversed at higher application doses (≥40 μM), suggesting that high SNP or NO contents are toxic to root growth. Therefore, 20 μM SNP was used in the NO donor experiments conducted in the present study. After3 h of water stress, SNP application significantly increased root NO fluorescence intensity for both N treatments. At 3 h, the NO production levels were ~2.05× and 3.85× higher in the SNP-treated roots of the seedlings receiving NH_4^+ and NO_3^-, respectively, than in the roots of CK plants (Fig. 2a, b). However, this phenomenon was not observed after 24 h of water stress.

After 3 h of water stress, ROS (O_2^-, H_2O_2, and OH^-) levels were increased in the roots of both the NH_4^+- and NO_3^--treated seedlings in relation to that of CK seedlings. Under water stress, the O_2^-, H_2O_2, and OH^- in the roots given NH_4^+ and NO_3^-increased by 78.1% and 107.3%, 28.3% and 47.8%, and 10.6% and 48.4%, respectively (Fig. 3a-c). After 3 h of water stress, root MDA and carbonyl were ~1.28× and 1.4× higher in the plants receiving NO_3^- than in CK plants, respectively. In turn, MDA and carbonyl levels were significantly higher in the CK than in the plants given NH_4^+ (Fig. 4a, b). Water stress induced higher root $ONOO^-$ in the NH_4^+-treated plants than in the NO_3^--treated seedlings (Fig. 4d), and exogenous NO significantly reduced water stress-induced ROS (O_2^- and H_2O_2) accumulation and oxidative damage (as reflected by MDA and carbonyl) in both N treatments (Figs. 3, 4).

To determine whether the alleviation of water stress-induced oxidative damage by SNP was related to NO production, the NO scavenger c-PTIO was applied to the plants. After pretreatment with 100 μM c-PTIO for 3 h, alleviation of the water

343 stress-induced root oxidative damage by SNP was reversed (Fig. 4). Depletion of
344 endogenous NO by c-PTIO significantly aggravated root oxidative damage in the
345 NH_4^+-treated plants but had no significant effect on the NO_3^--treated plants (Fig. 4), in
346 relation to that observed in CK plants. Therefore, the water stress-induced early NO
347 burst observed in the NH_4^+-treated plants alleviates root oxidative damage by
348 reducing ROS, such as O_2^- and H_2O_2.
349
350 **Source of endogenous NO**
351 Endogenous plant NO production is mostly driven by NR and NOS. Water stress
352 increased NR activity in the NO_3^--treated roots, and this activity was higher at 24 h
353 than it was at 3 h of water stress (Supplementary Fig. S3a). The activity of NOS was
354 also significantly elevated at 3 h of water stress, and significantly higher in the
355 NH_4^+-treated than in the NO_3^--treated roots (Supplementary Fig. S3b). In contrast,
356 water stress suppressed NOS activity in the NO_3^--treated roots at 24 h. Tungstate and
357 L-NAME, which inhibit NR and NOS activities, respectively, were used to identify
358 the origin of the early NO burst in the NH_4^+-treated roots. Although L-NAME
359 significantly inhibited endogenous NO production in the NH_4^+-treated roots under 3 h
360 water stress, it had no significant effect in the NO_3^--treated roots. At 24 h, the
361 tungstate and L-NAME applications suppressed NO production in the NO_3^--treated
362 roots and tungstate had the stronger inhibitory effect. On the other hand, tungstate had
363 no significant effect on NO production in the NH_4^+-treated roots (Fig. 5a, b).
364 The effect of SNP on the alleviation of water stress-induced root oxidative
365 damage was reversed after pretreatment with 100 μM c-PTIO for 3 h. Application of
366 the NOS inhibitor c-PTIO significantly aggravated water stress-induced oxidative
367 damage in the NH_4^+-treated roots, and SNP application reversed the effect of the NOS
368 inhibitor but not that of the NR inhibitor (Fig. 5c, d). For the NO_3^--treated roots, the
369 application of the NR inhibitor or NOS inhibitor had no significant effect on root
370 oxidative damage relative to the PEG (water stress) treatment.
371
372 **Activities of antioxidative enzymes and nitrate/nitrite and arginine/citrulline**
373 **metabolism**
374 Water stress significantly enhanced the activities of root antioxidant enzymes
375 CAT, SOD, APX, and POD by ~107% and 38%, 52% and 36%, 152% and 128%, and
376 45% and 37% in the NH_4^+-treated roots and the NO_3^--treated roots, respectively,

compared to the CK roots (Fig. 6). While SNP application further increased CAT, SOD, and APX activities (Fig. 6a-c), these antioxidant enzymes were inhibited by the application of the NO scavenger c-PTIO and by the NOS inhibitor L-NAME in the NH_4^+-treated roots under water stress.

As NR and NOS activities increased in the NO_3-treated roots, water stress lowered the nitrate level in the NR pathway and the arginine level in the NOS pathway (Supplementary Fig. S4a, b). Similarly, NR inhibitor and NOS inhibitor applications enhanced root nitrate and arginine contents, respectively. In the NH_4^+-treated roots, water stress significantly decreased arginine level, indicating that arginine metabolism was relatively high. In this treatment, the NR inhibitor had no significant effect on root arginine content. On the other hand, the NOS inhibitor suppressed arginine metabolism, and thus the NH_4^+-treated roots had higher arginine levels than CK roots (Supplementary Fig. S4c). These results indicate that the NO production burst in the NH_4^+-treated roots might originate from the NOS pathway.

Discussion

Ample experimental evidence has demonstrated that NO is involved in plant abiotic stress (Neill *et al*., 2003; Santisree *et al*., 2015). However, to our knowledge, no detailed study has been conducted to evaluate the role of NO in drought acclimation in plants supplied with NO_3^- or NH_4^+.In the present study, biomass, root N uptake rate, and leaf photosynthesis were reduced relative to the control treatments after 21days of water stress (Supplementary Fig. S1). However, these reductions were less severe for seedlings receiving NH_4^+ suggesting that NH_4^+ supplementation can enhance drought tolerance in rice seedlings more effectively than NO_3^- supplementation (Guo *et al*., 2007; Li *et al*., 2012). Our study also demonstrated that, in the short term (48 h), endogenous NO production in response to water stress is usually time-dependent, varying according to water stress duration. This finding is consistent with those reported for other stressors (Planchet *et al*., 2014; Sun *et al*., 2014). Early NO bursts were induced at 3 h of water stress in the roots of NH_4^+-treated seedlings but not in the roots of NO_3^--treated seedlings. Thus, there might be significant differences between NH_4^+-/NO_3^--supplied plants in terms of NO signal-mediated drought tolerance. In addition, accumulation of ROS, such as O_2^-, OH^-, and H_2O_2, and root oxidative damage were significantly lower in the NH_4^+-treated than in the NO_3^--treated roots at 3 h of water stress. Because ROS

411 accumulation damages cells and their plasma membranes by inducing lipid
412 peroxidation (oxidative stress) (Jiang and Zhang, 2002), the early NO burst in
413 response to water stress observed in NH_4^+-supplied seedlings might play a crucial role
414 in their antioxidant defense system and drought tolerance.

415 The role of the early NO burst in the water stress tolerance of
416 NH_4^+-/NO_3^--supplied seedlings was confirmed using NO donors and scavengers. Our
417 study demonstrated that NO donors induced NO in the NO_3^--treated roots at 3 h but
418 not at 24 h of water stress. Plant ROS accumulation and MDA and carbonyl levels
419 under water stress were significantly alleviated after the application of the NO donor
420 in both N treatments. Nevertheless, the levels of these substances were higher in the
421 NO_3^--treated roots than in the NH_4^+-treated roots. Therefore, the NO production
422 enhanced at 3 h by the exogenous NO donor can alleviate water stress-induced
423 oxidative damage in the NO_3^--treated roots. On the other hand, elimination of the
424 early NO burst by NO scavengers like c-PTIO significantly aggravated water
425 stress-induced oxidative damage. These results provide direct evidence that the early
426 NO bursts plays a crucial role in drought tolerance in NH_4^+-treated roots. Because
427 theNH_4^+-supplied roots maintained a higher N uptake rate thanNO_3^--supplied roots
428 under water stress (Supplementary Fig. S1f), we hypothesized that the higher NH_4^+
429 uptake rate is beneficial for the NO early burst due to the NO production involved in
430 root N metabolism (Corpas *et al.*, 2008; del Rio, 2015). This NO burst can also be an
431 active adaptation mechanism of plants to abiotic stress as, in addition to drought stress,
432 it has been reported to occur repeatedly in plants challenged by pathogens
433 (Floryszak-Wieczorek *et al.*, 2007), metal toxicity (Gonzalez *et al.*, 2012; Sun *et al.*,
434 2014), and cold stress (Cantrel *et al.*, 2011).

435 Our study demonstrated that an early NO burst improves plant drought tolerance
436 by enhancing the antioxidant defense system of the root. Elevated plant antioxidant
437 enzyme activities and gene expression levels in response to water stress have been
438 widely demonstrated (Jiang and Zhang, 2002; Arasimowicz-Jelonek *et al.*, 2009a; Fan
439 and Liu, 2012). In the present study, the tips of the NO_3^--treated roots presented more
440 serious water stress-induced oxidative damage (due to the excessive production ofO_2^-,
441 OH^-, and H_2O_2) than those of the NH_4^+-treated roots (Figs. 1-3). In contrast,
442 NH_4^+-supplied roots maintained relatively higher antioxidant enzyme (CAT, SOD, and
443 APX) activity levels to catalyze $O_2^{\cdot-}$ and H_2O_2 decomposition (Fig. 3). It has been

444 demonstrated that there is significant crosstalk between NO and ROS in plants. The
445 antioxidant function of NO was explained by its ability to reduce H_2O_2 and lipid
446 peroxidation, and induce antioxidant gene expression and enzyme activity
447 (Bogeat-Triboulot *et al*., 2007; Farooq *et al*., 2009). Our results showed that enhanced
448 NO levels and antioxidant enzymeactivities(CAT and SOD) were significantly and
449 simultaneously increased after NO donor application in NO_3^--treated roots thereby
450 reducing ROS concentration and oxidative damage. The early NO burst observed in
451 NH_4^+-treated roots can enhance antioxidant enzyme activity and ROS accumulation
452 ($O_2^{\cdot-}$, OH^-, and H_2O_2). These results were confirmed by subsequent experimentation in
453 which the application of NO scavenger significantly suppressed SOD and CAT in
454 NH_4^+-treated roots. Thus, drought tolerance in the NH_4^+-treated roots might be
455 associated with the NO induced up-regulation of antioxidant enzymes and
456 down-regulation of ROS accumulation.

457 Nitric oxide can also serve as a source of reactive nitrogen species (RNS). Over
458 accumulation of RNS under abiotic stress can cause tyrosine nitration and inactivate
459 proteins like CAT, manganese-dependent (Mn-)SOD, and GR (Clark *et al*., 2000) as
460 well as the peroxidative activity of cytochrome c (Batthyany *et al*., 2005). Our results
461 show that NO_3^--supplied plants had more severe oxidative damage and accumulated
462 extremely high NO levels after 24 h of water stress. This latent NO production can be
463 partially alleviated by replenishing the early NO burst at 3 h with SNP (Fig. 1). These
464 results indicate that both ROS and RNS metabolism participate in the water stress
465 response. High NO accumulation in the NO_3^--treated roots likely cause the nitrosative
466 stress at 24 h, which also damaged root redox balance. A similar phenomenon was
467 described in plants subjected to cold (Airaki *et al*., 2012), salinity (Tanou *et al*., 2012),
468 and drought (Signorelli *et al*., 2013) stresses. Because NO competes with oxygen for
469 cytochrome c oxidase binding (Complex IV), it affects both the respiratory chain and
470 oxidative phosphorylation (Millar and Day, 1996; Yamasaki *et al*., 2001). Thus, under
471 drought stress, the higher NO production in the NO_3^--treated roots than in the
472 NH_4^+-treated roots could aggravate respiratory inhibition and induce greater oxidative
473 damage.

474 Our investigation suggests that the early NO burst in NH_4^+-treated roots is
475 mainly mediated NOS at the early stages of water stress. Nitrate reductase-mediated
476 NO generation is known to occur under water deficit (Arasimowicz-Jelonek *et al*.,
477 2009b; Yu *et al*., 2014). Drought-induced NO generation by NOS-like enzymes in

plants has also been demonstrated but this NO production pathway varies significantly with species, tissue type, and plant growth conditions (Corpas *et al*., 2009; Liao *et al*., 2012; Shi *et al*., 2014). For the NH_4^+-treated roots, both NOS activity and NO production increased simultaneously at 3h of water stress, whereas the application of the NOS inhibitor completely repressed NO synthesis at this time point. The NOS inhibitor also aggravated water stress-induced membrane lipid peroxidation and oxidative protein damage, indicating that some NOS associated proteins may play an important role in NO-mediated drought protective responses (Guo *et al*., 2003; Zhao *et al*., 2007). In contrast, the NR inhibitor did not significantly affect NO production or membrane lipid peroxidation. The aggravation of lipid peroxidation by L-NAME may have been the result of the alteration of the NOS-mediated early NO burst. In NO_3^--treated roots, water stress enhanced NR activity significantly more than NOS activity at 24 h. However, separate NR inhibitor and NOS inhibitor applications only partially suppressed NO production. The NO produced by the NR pathway might therefore play an important role in later NO production (24 h), consistent with previous reports (Arasimowicz-Jelonek *et al*., 2009a, b). Although several studies support the arginine-dependent NO production model in higher plants, the genes encoding NOS in such plants have not yet been identified (Zemojtel *et al*., 2006). For this reason, the nitrate/nitrite and arginine/citrulline levels in the NR and NOS pathway, respectively, were determined. It was found that water stress significantly increased NOS activity and accelerated the conversion of arginine to citrulline. However, the arginine content was significantly enhanced in the NH_4^+-treated roots after the NOS inhibitor application, in relation to the CK roots. These results provide additional evidence that the early NO burst in NH_4^+-treated roots is mainly mediated by NOS (Fig. 7).

Our study is the first to demonstrate that the early NO burst in NH_4^+-treated rice roots significantly enhanced plant antioxidant defense by reducing ROS accumulation and enhancing the activities of antioxidant enzymes, thereby increasing plants' acclimation to water stress. The early NO burst which occurs in response to water stress may be triggered by NOS-like enzymes in root. Our results provide new insight into how NO-signaling molecules modulate drought tolerance in NH_4^+-supplied rice plants. However, the signaling crosstalk between ROS and RNS in response to water stress merits further investigation and may help elucidate the role of the NO-signaling process in enhancing drought tolerance in NH_4^+-supplied rice.

512

513 **Supplementary data**

514 **Fig. S1.** Responses to water stress in NH_4^+- and NO_3^--supplied rice

515 **Fig. S2.** Effects of exogenous nitric oxide donor on root oxidative damage under
516 water stress

517 **Fig. S3.** Effect of water stress on nitrate reductase and nitric oxide synthase in rice
518 roots

519 **Fig. S4.** Levels of nitric oxide-related compounds under sufficient water or water
520 stress

521 **Method S1.** Determination of leaf photosynthesis, root N uptake rate, root nitrate and
522 nitrite content in rice seedlings after 21days under control or water stress treatments

523

524 **Acknowledgements**

525 This work was supported by the National Key Research and Development Program of
526 China (No. 2017YFD0300100, 2016YFD0101801); the Natural Science Foundation
527 of Zhejiang Province (No. LY18C130005). We would like to thank Editage
528 [www.editage.cn] for English language editing.

References

Airaki M, Leterrier M, Mateos RM, Valderrama R, Chaki M, Barroso JB, del Rıo LA, Palma JM, Corpas FJ. 2012. Metabolism of reactive oxygen species and reactive nitrogen species in pepper (*Capsicum annuum* L.) plants under low-temperature stress. Plant Cell and Environment **35**, 281-295.

Arasimowicz-Jelonek M, Floryszak-Wieczorek J, Kubis J. 2009a. Involvement of nitric oxide in water stress-induced responses of cucumber roots. Plant Science **177**, 682-690.

Arasimowicz-Jelonek M, Floryszak-Wieczorek J, Kubis J. 2009b. Interaction between polyamine and nitric oxide signaling in adaptive responses to drought in cucumber. Journal of Plant Growth Regulation **28**,177-186.

Batthyany C, Souza JM, Duran R, Cassina A, Cervenansky C, Radi R. 2005. Time course and site(s) of cytochrome c tyrosine nitration by peroxynitrite. Biochemistry **44**, 8038-8046.

Bogeat-Triboulot MB, Brosche M, Renaut J, Jouve L, Le Thiec D, Fayyaz P. 2007.Gradual soil water depletion results in reversible changes of gene expression, protein profiles, ecophysiology, and growth performance in *Populuseuphratica*, a poplar growing in arid regions. Plant Physiology **143**, 876-892.

Cantrel C,Vazquez T,Puyaubert J,Rezé N,Lesch M,Kaiser WM,Dutilleul C,Guillas I,Zachowski A,Baudouin E. 2011. Nitric oxide participates in cold-responsive phosphosphingolipid formation and gene expression in *Arabidopsis thaliana*. New Phytologist **189**, 415-427.

Clark D, Durner J, Navarre DA, Klessig DF. 2000. Nitric oxide inhibition of tobacco catalase and ascorbate peroxidase. Molecular Plant-Microbe Interactions **13**, 1380-1384.

Corpas FJ, del Río LA, Barroso JB. 2007. Need of biomarkers of nitrosative stress in plants. Trends in Plant Science **12**, 436-438.

Corpas FJ, Chaki M, Fernández-Ocana A, Valderrama R, Palma JM, Carreras A, Begara-Morales JC, Airaki M, del Río LA, Barroso JB. 2008. Metabolism of reactive nitrogen species in pea plants under abiotic stress conditions, Plant Cell Physiology **49**, 1711-1722.

Corpas FJ, Palma JM, del Rio LA, Barroso JB. 2009. Evidence supporting the

existence of L-arginine-dependent nitric oxide synthase activity in plants. New Phytologist **184**, 9-14.

Del Rio LA. 2015. ROS and RNS in plant physiology: an overview. Journal of Experiment Botany **66**, 2827-2837.

Ding L, Gao C, Li Y, Li Y, Zhu Y, Xu G, Shen Q, Kaldenhoff R, Kai L, Guo S. 2015. The enhanced drought tolerance of rice plants under ammonium is related to aquaporin (AQP). Plant Science **234**, 14-21.

Fan HH, Li TC, Guan L, Li ZP, Guo N, Cai YP, Lin Y.2012. Effects of exogenous nitric oxide on antioxidation and DNA methylation of *Dendrobiumhuoshanense* grown under drought stress. Plant Cell Tissue and Organ Culture **109**, 307-314.

Fan QJ, Liu JH. 2012. Nitric oxide is involved in dehydration/drought tolerance in *Poncirustrifoliata* seedlings through regulation of antioxidant systems and stomatal response. Plant Cell and Reports **31**, 145-154.

Farooq M, Basra SMA, Wahid A, Rehman H. 2009. Exogenously applied nitric oxide enhances the drought tolerance in fine grain aromatic rice (*Oryza sativa* L.). Journal of Agronomy and Crop Science **195**, 254-261.

Floryszak-Wieczorek J, Arasimowicz M, Milczarek G, Jelen H, Jackowiak H. 2007. Only an early nitric oxide burst and the following wave of secondary nitric oxide generation enhanced effective defence responses of pelargonium to a necrotrophic pathogen. New Phytologist **175**, 718-730.

Freschi L, Rodrigues MA, Domingues DS, Purgatto E, Van Sluys MA, Magalhaes JR, Kaiser WM, Mercier H. 2010. Nitric oxide mediates the hormonal control of Crassulacean acid metabolism expression in young pineapple plants. Plant Physiology **152**, 1971-1985.

Gonzalez A, de los Angeles Cabrera M, Henríquez MJ, Contreras RA, Morales B, Moenne A. 2012. Cross talk among calcium, hydrogen peroxide, and nitric oxide and activation of gene expression involving calmodulins and calcium-dependent protein kinases in *Ulvacompressa* exposed to copper excess. Plant Physiology **158**, 1451-1462.

Groß F, Durner J, Gaupels F. 2103. Nitric oxide, antioxidants and prooxidants in plant defence responses. Frontiers in Plant Science **4**,419.

Guo FQ, Okamoto M, Crawford NM. 2003. Identification of a plant nitric oxide synthase gene involved in hormonal signaling. Science **302**, 100-103.

Guo P, Cao Y, Li Z, Zhao B. 2004. Role of an endogenous nitric oxide burst in the resistance of wheat to stripe rust. Plant Cell and Environment **27**, 473-477.

Guo SW, Zhou Y, Shen QR, Zhang FS. 2007. Effect of ammonium and nitrate nutrition on some physiological processes in higher plants-growth, photosynthesis, photorespiration, and water relations. Plant Biology **9**, 21-29.

Jiang MY, Zhang JH. 2002. Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. Journal of Experimental Botany**53**,2401-2410.

Liao WB, Huang GB, Yu JH, Zhang ML. 2012. Nitric oxide and hydrogen peroxide alleviate drought stress in marigold explants and promote its adventitious root development. Plant Physiology and Biochemistry **58**, 6-15.

Lin A, Wang Y, Tang J, Xue P, Li C, Liu L, Hu B, Yang F, Loake GJ, Chu C. 2012. Nitric oxide and protein S-nitrosylation are integral to hydrogen peroxide-induced leaf cell death in rice. Plant Physiology **158**, 451-464.

Liu Y, Wu R, Wan Q, Xie G, Bi Y. 2007. Glucose-6-phosphate dehydrogenase plays a pivotal role in nitric oxide-involved defense against oxidative stress under salt stress in red kidney bean roots. Plant and Cell Physiology **48**, 511-522.

Liu Y, Jiang H, Zhao Z, An L. 2010. Nitric oxide synthase-like activity-dependent nitric oxide production protects against chilling-induced oxidative damage in *Chorisporabungeana* suspension cultured cells. Plant Physiology and Biochemistry **48**, 936-944.

Mata CG, Lamattina L. 2001. Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Physiology **126**, 1196-1204.

Millar AH, Day AD.1996. Nitric oxide inhibits the cytochrome oxidase but not the alternative oxidase of plant mitochondria. FEBS Letters **398**,155-158.

Neill SJ, Desikan R, Hancock JT. 2003. Nitric oxide signalling in plants. New Phytologist **159**,11-35.

Neill S.2007. Interactions between abscisic acid, hydrogen peroxide and nitric oxide mediate survival responses during water stress. New Phytologist **175**, 4-6.

Planchet E, Verdu I, Delahaie J, Cukier C, Girard C, Paven MM, LimamiAM. 2014. Abscisic acid-induced nitric oxide and proline accumulation in independent pathways under water-deficit stress during seedling establishment in *Medicagotruncatula*. Journal of Experimental Botany **65**, 2161-2170.

Sachadyn-Krol M, Materska M, Chilczuk B, Kara M, Jakubczyk A, Perucka I,

Jackowska I. 2016. Ozone-induced changes in the content of bioactive compounds and enzyme activity during storage of pepper fruits. Food Chemistry **211**,59-67.

Salazar C, Armenta JM, Shulaev V. 2012. An UPLC-ESI-MS/MS assay using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization for targeted amino acid analysis: application to screening of *Arabidopsisthaliana* mutants. Metabolites **2,** 398-428.

Santisree P, Bhatnagar-Mathur P, Sharma KK. 2015. NO to drought-multifunctional role of nitric oxide in plant drought: Do we have all the answers? Plant Science **239**, 44-55.

Scheible WR, Lauerer M, Schulze ED, Caboche M, Stitt M. 1997.Accumulation of nitrate in the shoot acts as a signal to regulate shoot-root allocation in tobacco. Plant Journal **11**, 671-691.

Shi FM, Li YZ. 2008. *Verticilliumdahliae* toxins-induced nitric oxideproduction in Arabidopsis is major dependent on nitrate reductase. BMB Reports **41**, 79-85.

Shi H, Ye T, Zhu J, Chan Z. 2014. Constitutive production of nitric oxide leads to enhanced drought stress resistance and extensive transcriptional reprogramming in Arabidopsis. Journal of Experimental Botany **6**, 4119-4413.

Signorelli S, Corpas FJ, Omar Borsani O, Barroso JB, Monza J. 2013. Water stress induces a differential and spatially distributed nitro-oxidative stress response in roots and leaves of *Lotusjaponicus*. Plant Science **201-202**, 137-146.

Simontacchi M, Galatro A, Ramos-Artuso F, Santa-María GE. 2015. Plant survival in a changing environment: the role of nitric oxide in plant responses to abiotic stress. Frontiers in Plant Science **6**, 977.

Slewinski TL. 2012. Non-structural carbohydrate partitioning in grass stems: a target to increase yield stability, stress tolerance, and biofuel production. Journal of Experimental Botany **63**, 4647-4670.

Sun CL, Lu LL, Liu LJ, Liu WJ, Yu Y, Liu XX, Hu Y, Jin CW, Lin XY. 2014. Nitrate reductase-mediated early nitric oxide burst alleviates oxidative damage induced by aluminum through enhancement of antioxidant defenses in roots of wheat (*Triticumaestivum*). New Phytologist **201**, 1240-1250.

Tanou G, Filippou P, Belghazi M, Job D, Diamantidis G, Fotopoulos V, Molassiotis A. 2012. Oxidative and nitrosative-based signaling and associated post-translational modifications orchestrate the acclimation of citrus plants to salinity

stress. Plant Journal **72**, 585-599.

Velikova V, Yordanov I, Edreva A. 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protectiverole of exogenous polyamines. Plant Science **151**, 59-66.

Verdoy D, Coba De La Peña T, Redondo FJ, Lucas MM, Pueyo JJ. 2006. Transgenic *Medicagotruncatula* plants that accumulate proline display nitrogen-fixing activity with enhanced tolerance to osmotic stress. Plant Cell and Environment **29**, 1913-1923.

Wang HH, Huang JJ, Bi YR. 2010. Nitrate reductase-dependent nitric oxide production is involved in aluminum tolerance in red kidney bean roots. Plant Science **179**, 281-288.

Wilson ID, Neill SJ, Hancock JT. 2007. Nitric oxide synthesis and signalling in plants. Plant Cell and Environment **31**, 622-631.

Yamamoto Y, Kobayashi Y, Matsumoto H. 2001. Lipid peroxidation is an early symptom triggered by aluminum, but not the primary cause of elongation inhibition in pea roots, Plant Physiology **125**,199-208.

Yamasaki H, Shimoji H, Ohshiro Y, Sakihama Y.2001. Inhibitory effects of nitric oxide on oxidative phosphorylation in plant mitochondria. Nitric Oxide**5,**261-270.

Yang X, Li Y, Ren B, Ding L, Gao C, Shen Q, Guo S. 2012 Drought-induced root aerenchyma formation restricts water uptake in rice seedlings supplied with nitrate. Plant Cell and Physiology **53**,495-504.

Yu M, Lamattina L, Spoel SH, Loake GJ. 2014. Nitric oxide function in plant biology: a redox cue in deconvolution. New Phytologist **202**, 1142-1156.

Zemojtel T, Frohlich A, Palmieri MC, Kolanczyk M, Mikula I, Wyrwicz LS, Wanker EE, Mundlos S, Vingron M, Martasek P, ***et al.*** 2006. Plant nitric oxide synthase: a never-ending story? Trends in Plant Science **11**, 524-525.

Zhang AY, Jiang MY, Zhang JH, Ding HD, Xu SC, Hu XL, Tan MP. 2007. Nitric oxide induced by hydrogen peroxide mediates abscisic acid-induced activation of the mitogen-activated protein kinase cascade involved in antioxidant defense in maize leaves. New Phytologist **175**,36-50.

Zhao MG, Tian QY, Zhang WH. 2007. Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in Arabidopsis. Plant Physiology **144**, 206-217.

Zhao MG, Chen L, Zhang LL, Zhang WH. 2009. Nitric reductase-dependent nitric

oxide production is involved in cold acclimation and freezing tolerance in Arabidopsis. Plant Physiology **151**,755-767.

Figure legends

Fig. 1. Time-dependent endogenous nitric oxide (NO) production and histochemical detection of oxidative damage in the root apices ofNH_4^+- and NO_3^--supplied rice seedlings under water stress. (a) Detection of NO fluorescence using 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) staining and a fluorescence microscope. NO generation is indicated by green fluorescence. Bar=300 μm. (b) NO production is expressed as relative fluorescence. To detect the NO production time course, seedling roots exposed to 10% polyethylene glycol(PEG) were collected at 0, 3, 6, 12, 24, and 48 h. (c) and (d) Histochemical detection of the aldehydes derived from lipid peroxidation and Evans blue uptake in root apices of rice seedlings under water stress. Rice seedlings were either untreated or subjected to 3 or 24 h of water stress, respectively. Roots were stained with Schiff's reagent (c) and Evans blue (d), and then immediately photographed under a Leica S6E stereomicroscope (Leica, Solms, Germany). Red/purple indicates the presence of lipid peroxidation detected with Schiff's reagent. Bar=1 mm. Endogenous NO concentrations and histochemical detection of oxidative damage in the root are given. In Fig. 2b, the red dotted oval represents the high endogenous NO production in the NH_4^+- and NO_3^--supplied rice, respectively. Values represent means± standard error (SE) (n=10). CK indicates control treatment, i.e., plants receiving sufficient water.

Fig. 2. Responses of endogenous nitric oxide (NO) concentrations to water stress (a, b) and NO donor and NO scavenger (c, d) in root apices. (a) Photographs of NO production after sodium nitroprusside (SNP) application. Bar=300 μm. (b) NO production expressed as relative fluorescence. Rice seedlings were either untreated or treated with SNP under water stress. After 3 h and 24 h of treatment, root tips were loaded with 10 μM 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) and NO fluorescence was imaged after 20 min using a fluorescence microscope. Endogenous NO concentrations in root are displayed. Values represent means± standard error (SE) (n=10). Different letters indicate significant differences at $P<0.05$. CK, control treatment, i.e., plants receiving sufficient water.

Fig. 3. Reactive oxygen species (ROS) and peroxynitrite ($ONOO^-$) accumulation in root apices of rice seedlings treated with NO donor (sodium nitroprusside) and either receiving sufficient water (CK treatment) or subjected to water stress using polyethylene glycol(PEG). After 3 h, O^{2-} (a), H_2O_2 (b), and OH^- (c) levels in rice seedlings roots were measured by spectrophotometry. The accumulation of $ONOO^-$was detected with 10 μM aminophenyl fluorescein. Fluorescence images and relative fluorescence intensity were analyzed as described in Fig. 2 for NO determination.

Fig. 4. Responses of oxidative damage in root apices of rice seedlings to NO donor (sodium nitroprusside) and NO scavenger (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; c-PTIO) in plants either receiving sufficient water (CK treatment) or subjected to water stress using polyethylene glycol (PEG). In the c-PTIO and PEG + c-PTIO treatments, the rice seedlings were pretreated with NO scavenger (c-PTIO) for 3 h followed by sufficient water or water stress. After 3 h, the malondialdehyde (MDA) content was determined. The MDA in rice roots represents lipid peroxidation (a) and carbonyl concentration (b). Values represent means± standard error (SE) (n=6). Different letters indicate significant differences at $P<0.05$ level.

Fig. 5. Effects of a nitrate reductase(NR) inhibitor (tungstate) and a nitric oxide (NO) synthase (NOS) inhibitor (Nx-nitro-*L*-arginine methyl ester hydrochloride; L-NAME) on NO content and oxidative damage in root apices of rice seedlings. Rice seedlings were pretreated with NR inhibitor (100 μM tungstate) or NOS inhibitor (100 μM L-NAME) for 3 h, and then subject to water treatment. (a) NO fluorescence. Bar = 300 μm. (b) NO production expressed as relative fluorescence. (c, d) malondialdehyde (MDA) content representing lipid peroxidation (c) and carbonyl concentration (d) in rice seedling roots measured after 3 h of water treatment following tungstate or L-NAME pretreatment. Values represent means± standard error (SE) (n=6). Different letters indicate significant differences at $P<0.05$ level. CK, control treatment, i.e.,

plants receiving sufficient water.

Fig. 6. Effects of different treatments on antioxidant enzyme changes in rice seedlings under water stress. Roots were collected to assay catalase (CAT) (a), superoxide dismutase (SOD) (b), ascorbate peroxidase (APX) (c), and peroxidase (POD) (d) after 3 h of treatment with sufficient water (CK treatment) or water stress. For the 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), polyethylene glycol (PEG) + c-PTIO, and PEG + Nx-nitro-*L*-arginine methyl ester hydrochloride (L-NAME) treatments, the rice seedlings were pretreated with NO scavenger (c-PTIO) or NOS inhibitor (100 μM L-NAME) for 3 h followed by sufficient water or water stress. Values represent means± standard error (SE) (n=6). Different letters indicate significant differences at $P<0.05$ level.

Fig. 7. Schematic illustration of a proposed model for the different responses of early NO production and its effects on the defense response of rice to water stress. In the roots of NH_4^+-supplied rice, the nitric oxide synthase (NOS)-mediated early nitric oxide (NO) burst (3h) significantly enhanced plant antioxidant defense by reducing reactive oxygen species (ROS) accumulation and enhancing antioxidant enzyme activity; the relative lower NO production after 24 h of water stress in comparison to NO_3^--supplied rice, also helped maintaining the redox balance in root cells, thus enhancing their drought tolerance. In the roots of NO_3^--supplied rice, ROS accumulation and oxidative damage induced by 3h of water stress were significantly higher than that in NH_4^+- supplied rice. High NO accumulation in the NO_3^--treated roots likely caused the nitrosative stress at 24 h of water stress. A combined effect of oxidative and nitrification stresses might have led to the weak resistance to water stress in NO_3^--supplied rice. NR, nitrate reductase. Red arrows represent increase, green arrows represent decrease. Black solid arrows represent defined pathways, dotted arrows represent undefined pathway.

Legends to supplementary figures

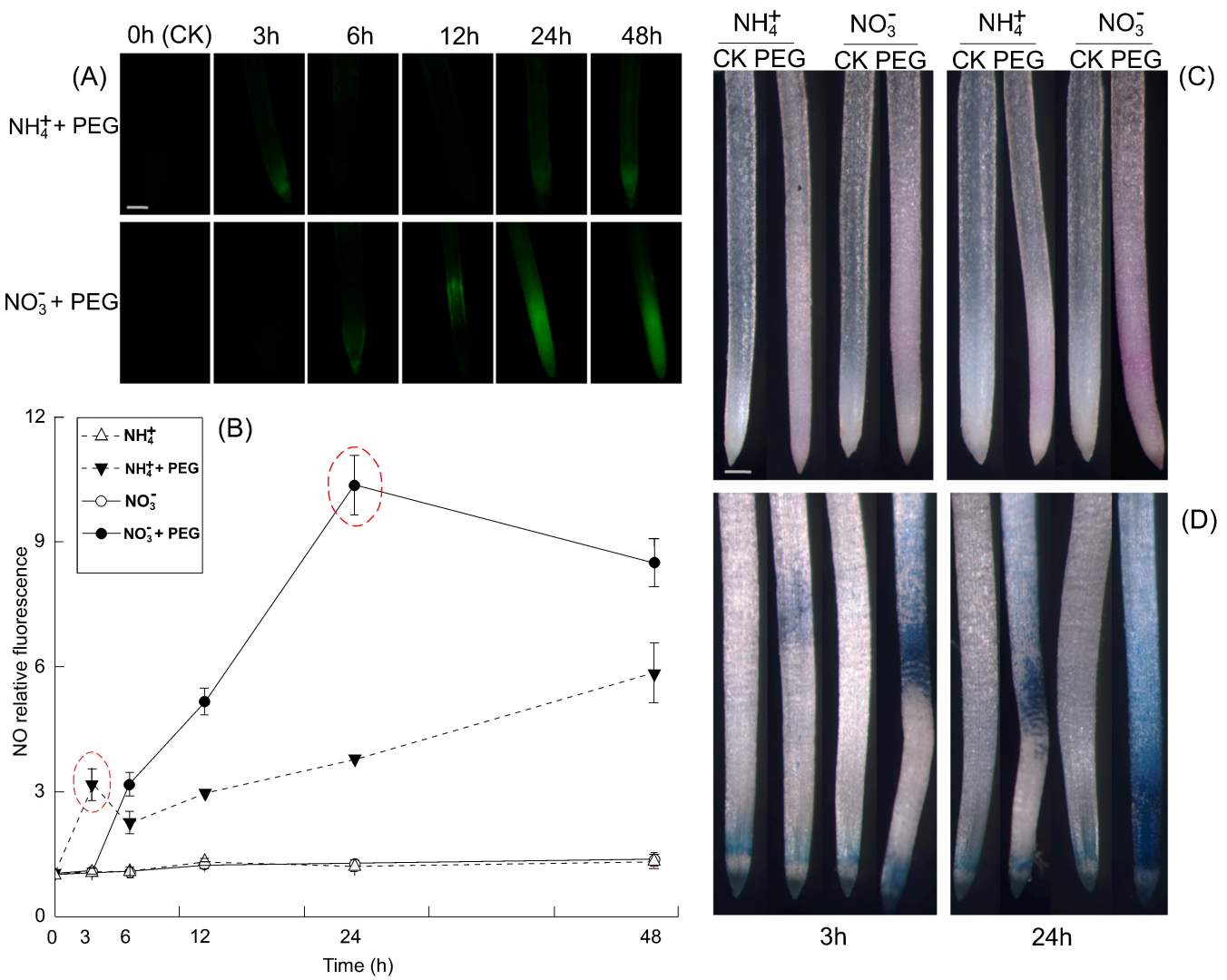
Fig. S1. (a), (b), and (c) response of NH_4^+- and NO_3^--supplied rice agronomic characteristics and biomass to water stress induced by 10% PEG after 21days of treatment. (d) Effects of water stress on leaf photosynthesis in NH_4^+- and NO_3^--supplied rice after 21days of treatment. (e) Effects of water stress on root activity in NH_4^+- and NO_3^--supplied rice after 21days of treatment. (f) Effects of water stress on root ^{15}N-labeled uptake rate in NH_4^+- and NO_3^--supplied rice after 21days of treatment. Rice leaf photosynthesis, root activity, and ^{15}N uptake rate were determined according to Method S1. Values represent means±SE (n=6). Different letters indicate significant differences at $P<0.05$ level. CK, control treatment, i.e., plants receiving sufficient water.

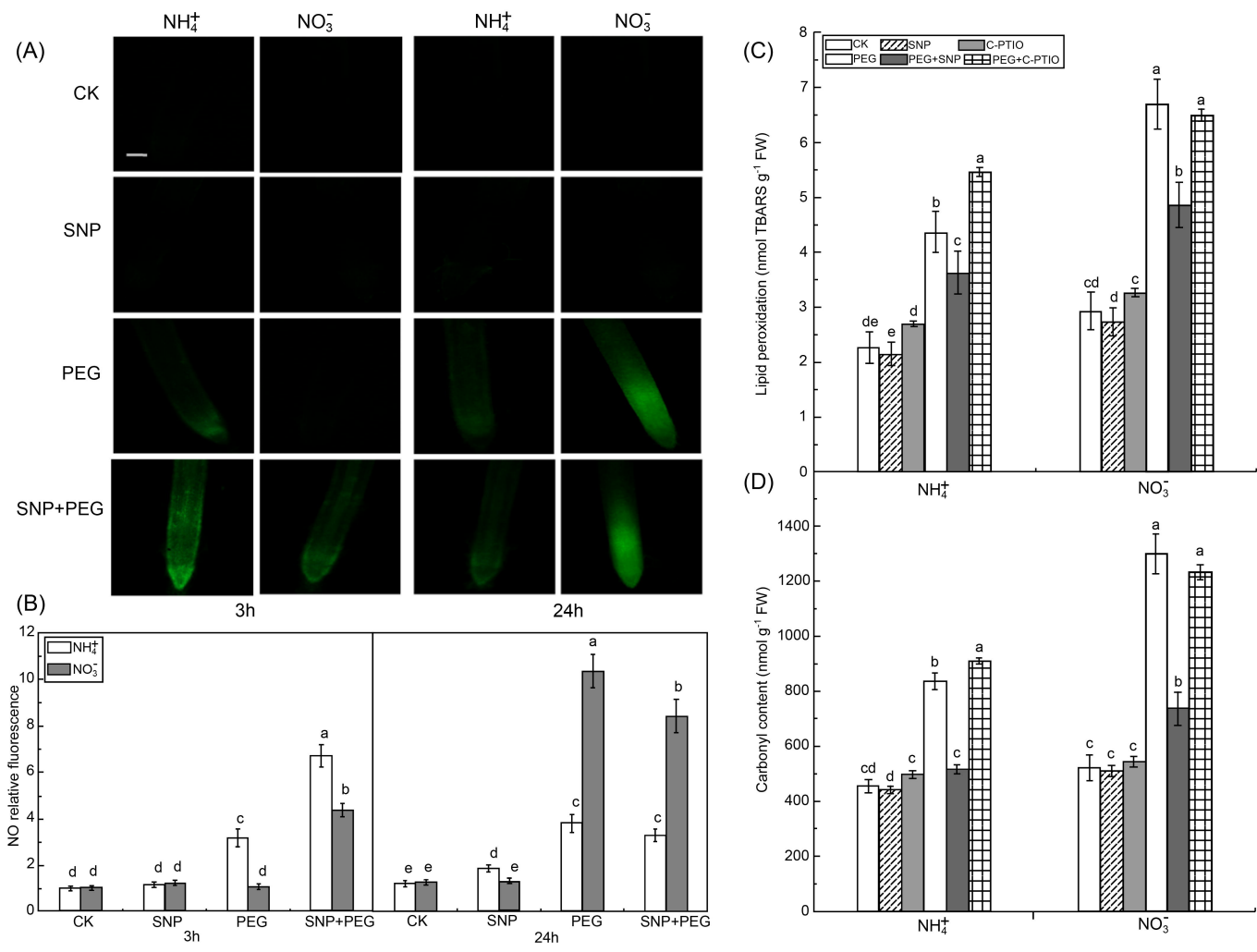
Fig. S2. Effect of exogenous NO donor (SNP) on root oxidative damage under water stress. Rice roots were exposed to mixed N (NH_4^+ + NO_3^-) nutrient solution containing 0 μM, 5 μM, 10 μM, 20 μM, 40 μM, 80 μM, or 100 μM SNP either with or without 10% PEG for 48 h. MDA levels representing lipid peroxidation (a) and carbonyl concentration (b) in rice seedling roots were determined. Values represent means±SE (n=6). Different letters indicate significant differences at $P<0.05$ level. CK, control treatment, i.e., plants receiving sufficient water.

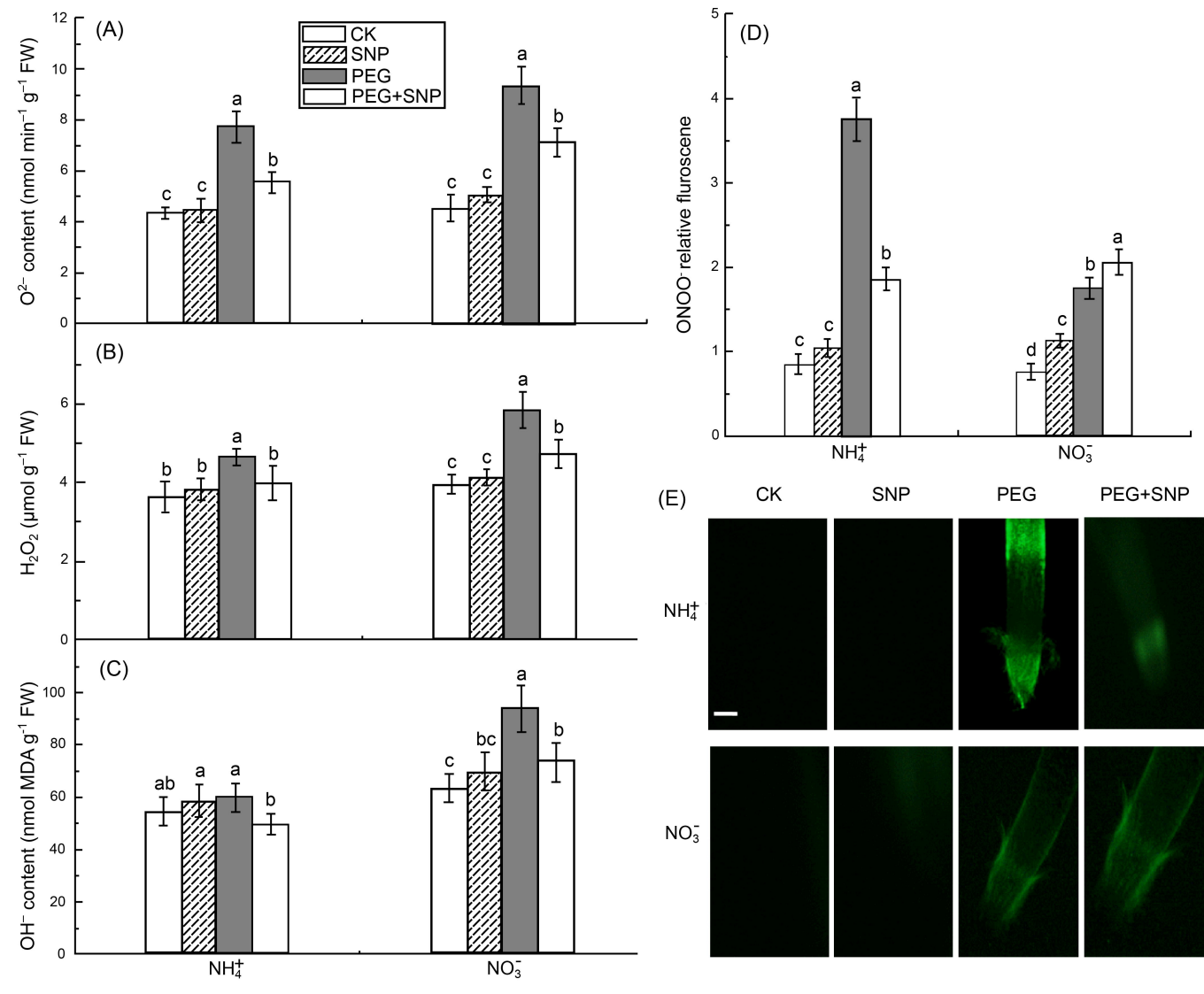
Fig. S3. Effect of water stress on NR (a) and NOS (b) in root apices of rice seedlings. Roots were collected for the NR and NOS assays after 3 h and 24 h of water stress, respectively. Values represent means±SE (n=6). Different letters indicate significant differences at $P<0.05$ level. CK, control treatment, i.e., plants receiving sufficient water.

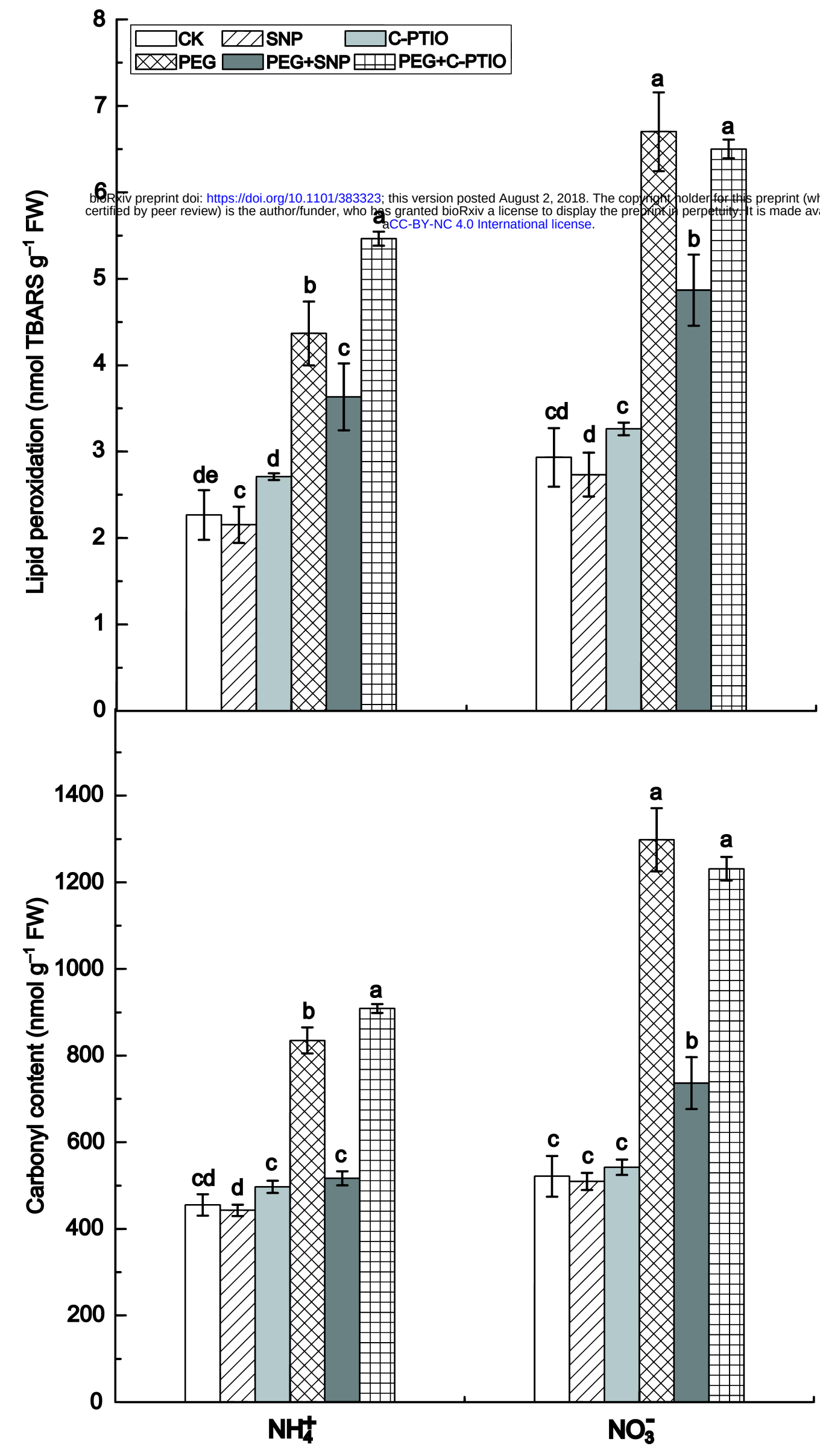
Fig. S4. Related compounds in NR-mediated and NOS-mediated NO pathways in root apices of rice seedlings treated with NR inhibitor (tungstate) and NOS inhibitor (L-NAME) under sufficient water or water stress treatment. (a) Levels of nitrate and nitrite in NO_3^--treated roots. (b) Levels of arginine and citrulline in NO_3^--treated roots.

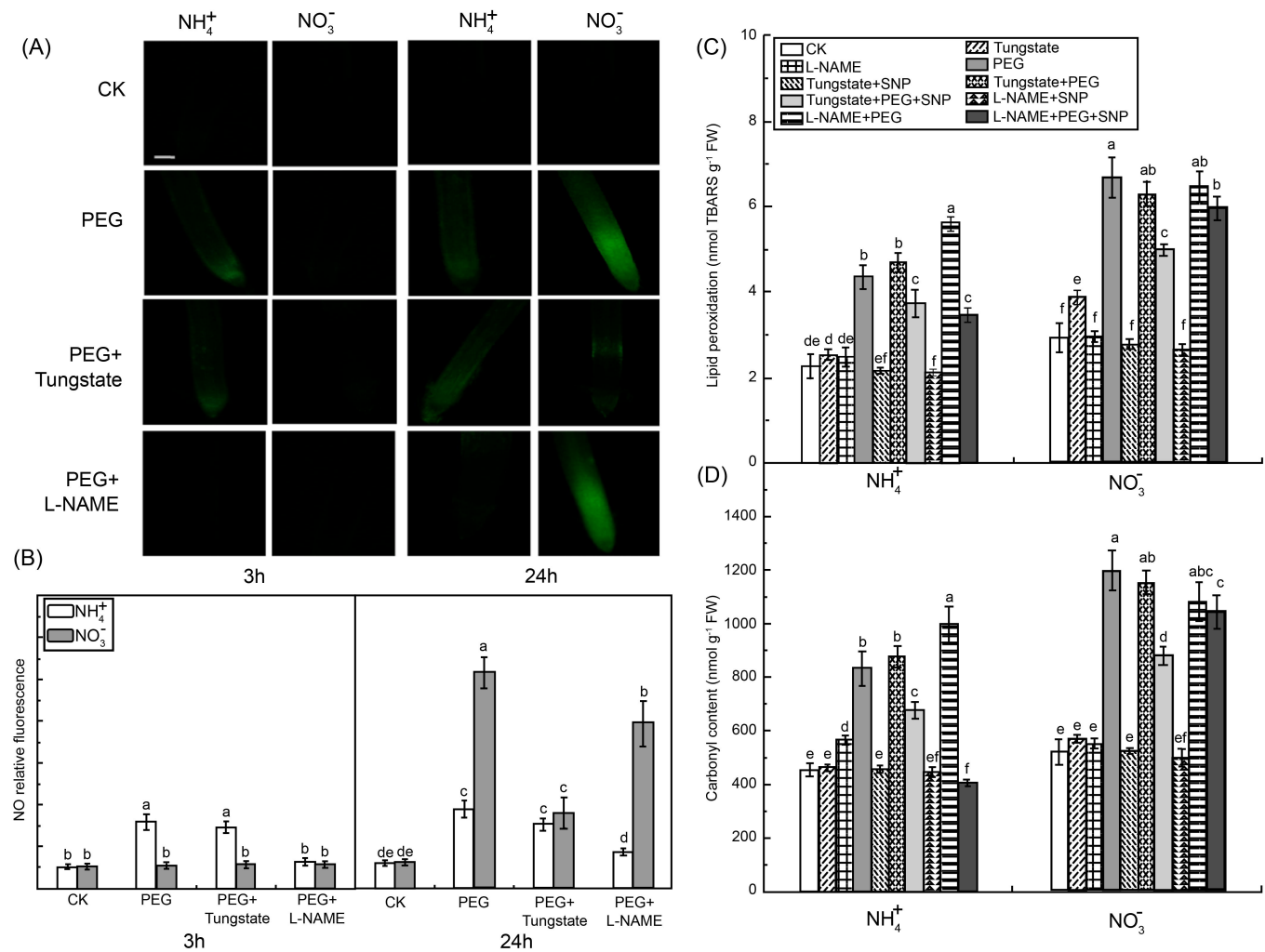
(c) Levels of arginine and citrulline in NH_4^+-treated roots. For the PEG + Tungstate and PEG + L-NAME treatments, the rice seedlings were pretreated with NR inhibitor (100 μM tungstate) or NOS inhibitor (100 μM L-NAME) for 3 h, followed by sufficient water (CK treatment) or water stress treatment. Values represent means±SE (n=6). Different letters indicate significant differences at $P<0.05$ level.

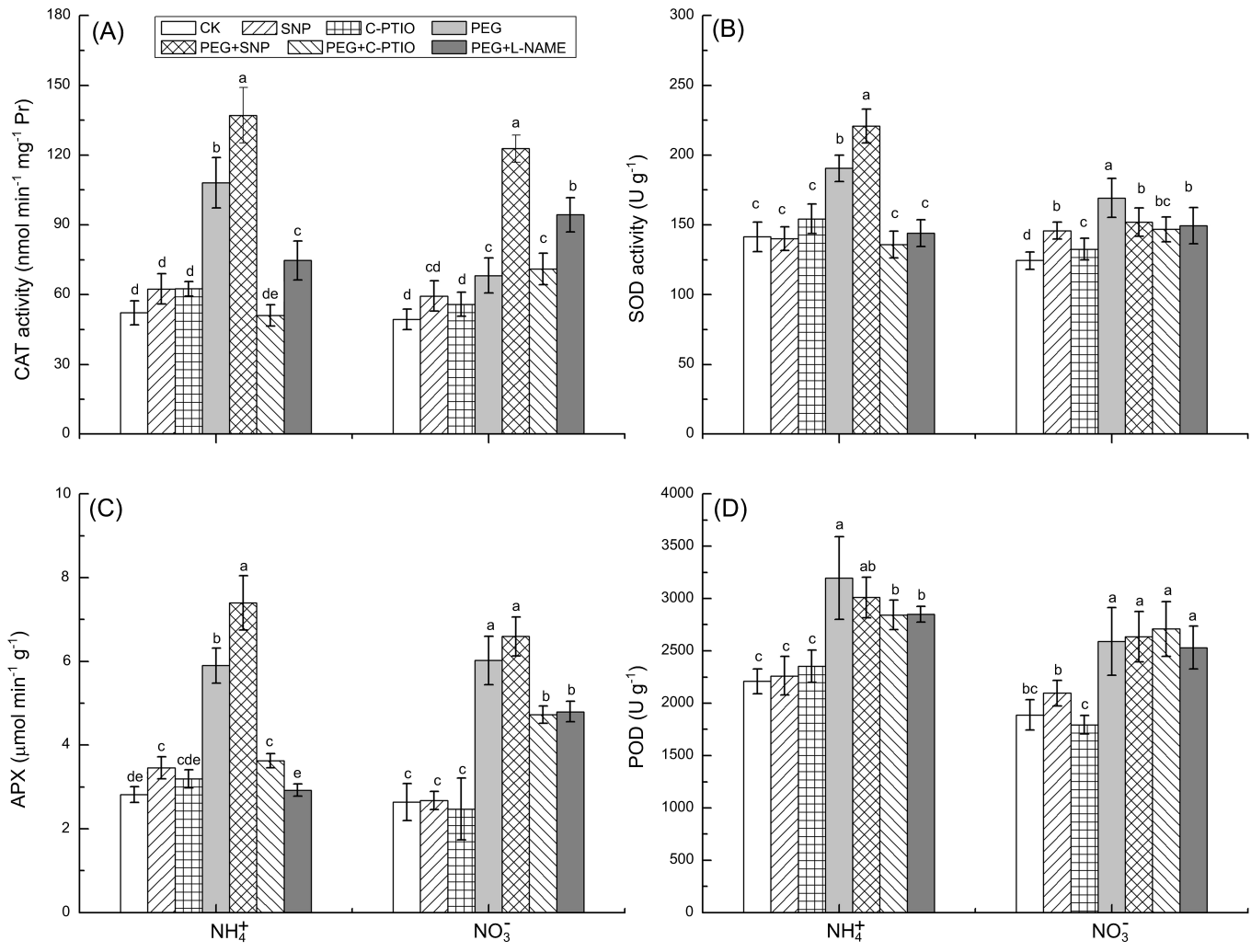

0h (CK)
3h
6h
12h
24h
48h
(A)
NH4+ + PEG
NO3- + PEG
(B)
NH4+
NH4+ + PEG
NO3-
NO3- + PEG
NO relative fluorescence
Time (h)
0
3
6
12
24
48
(C)
NH4+
NO3-
CK PEG
(D)
3h
24h

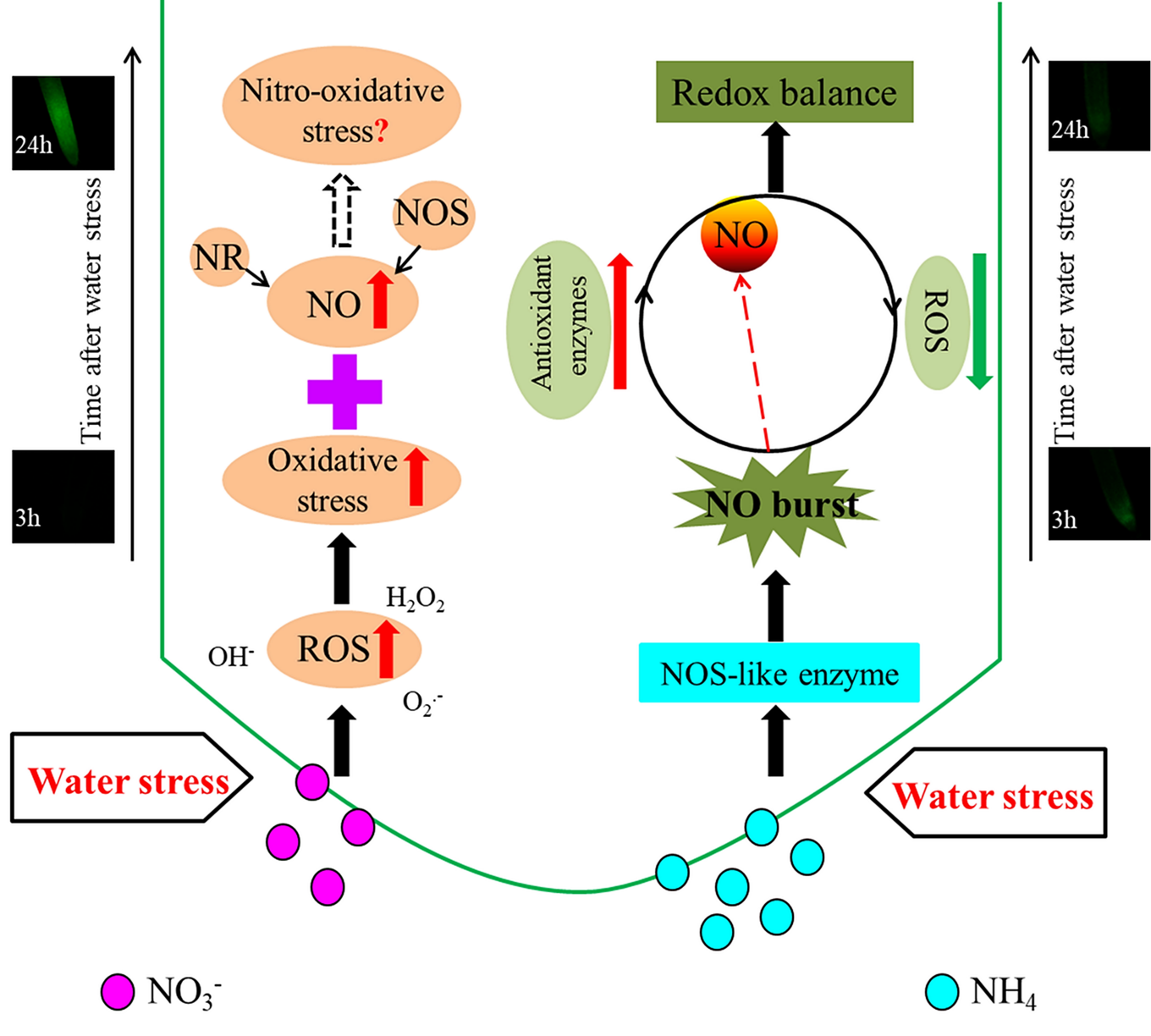

(A)
NH4+
NO3-
NH4+
NO3-
CK
SNP
PEG
SNP+PEG
3h
24h
(B)
NH4+
NO3-
NO relative fluorescence
CK
SNP
PEG
SNP+PEG
CK
SNP
PEG
SNP+PEG
3h
24h
(C)
CK
SNP
C-PTIO
PEG
PEG+SNP
PEG+C-PTIO
Lipid peroxidation (nmol TBARS g-1 FW)
NH4+
NO3-
(D)
Carbonyl content (nmol g-1 FW)
NH4+
NO3-


(A)
CK
SNP
PEG
PEG+SNP
O^{2-} content (nmol min^{-1} g^{-1} FW)
(B)
H_2O_2 (μmol g^{-1} FW)
(C)
OH^- content (nmol MDA g^{-1} FW)
NH_4^+
NO_3^-
(D)
$ONOO^-$ relative fluroscene
(E)
CK
SNP
PEG
PEG+SNP
NH_4^+
NO_3^-




(A)
NH4+
NO3-
NH4+
NO3-
CK
PEG
PEG+ Tungstate
PEG+ L-NAME
(B)
3h
24h
NH4+
NO3-
NO relative fluorescence
CK
PEG
PEG+ Tungstate
PEG+ L-NAME
3h
24h
(C)
CK
L-NAME
Tungstate+SNP
Tungstate+PEG+SNP
L-NAME+PEG
Tungstate
PEG
Tungstate+PEG
L-NAME+SNP
L-NAME+PEG+SNP
Lipid peroxidation (nmol TBARS g-1 FW)
NH4+
NO3-
(D)
Carbonyl content (nmol g-1 FW)
NH4+
NO3-


(A)
CK
SNP
C-PTIO
PEG
PEG+SNP
PEG+C-PTIO
PEG+L-NAME
CAT activity (nmol min-1 mg-1 Pr)
(B)
SOD activity (U g-1)
(C)
APX (μmol min-1 g-1)
(D)
POD (U g-1)
NH4+
NO3-


24h
Time after water stress
3h
Nitro-oxidative stress?
NOS
NR
NO
Oxidative stress
H_2O_2
OH^-
ROS
$O_2^{\cdot-}$
Water stress
NO_3^-
Redox balance
NO
Antioxidant enzymes
ROS
NO burst
NOS-like enzyme
Water stress
NH_4
24h
Time after water stress
3h