Journal of Plant Physiology 168 (2011) 739–745

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Nitric oxide production is associated with response to brown planthopper infestation in rice

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ARTICLE INFO

Article history:
Received 7 January 2010
Received in revised form
16 September 2010
Accepted 16 September 2010

Keywords:
Nitrate reductase
Nitric oxide synthase

ABSTRACT

Nilaparvata lugens Stål, the brown planthopper (BPH), is one of the most destructive phloem-feeding insects of rice (*Oryza sativa* L.) throughout Asia. Here, we show that BPH feeding increases the level of endogenous nitric oxide (NO) in the leaf and sheath tissue of both resistant and susceptible rice cultivars. However, in the roots, the NO level increased in the resistant cultivar, but decreased in the susceptible one. A burst of NO production occurred in the sheath within 1 h of infestation with BPH. The production of NO in response to BPH feeding appears to be dependent primarily on the activity of nitric oxide synthase. The application of exogenous NO reduced plant water loss by its effect on both stomatal opening and root architecture. It also stimulated the expression of certain drought stress-related genes, reduced plant height and delayed leaf senescence. Over the short term, NO supplementation reduced the seedling mortality caused by BPH feeding. This suggests that NO signaling plays a role in the rice tolerance response to BPH feeding.

1. Introduction

Nilaparvata lugens Stål, the brown planthopper (BPH), is one of the most destructive phloem-feeding insect pests of rice (*Oryza sativa* L.) throughout Asia. It attaches preferentially to the stem, from which it penetrates the phloem through its stylet. BPH feeding interferes with the translocation of assimilate, thereby damaging plant growth and development. When a large number of BPH individuals feed on a single plant leaf desiccation and stem wilting are common outcomes, a condition called hopper-burn (Hao et al., 2008). BPH is also a carrier of two viruses, one responsible for the disease rice grassy stun, and the other rugged stunt. Plants have evolved a variety of defense mechanisms against insect herbivores and microbial pathogens. Jasmonic acid (JA), salicylic acid (SA), H_2O_2 and ethylene are all important signaling molecules in this process. With respect to the cellular response of plants to insect herbivores, most research to date has concentrated on chewing, rather than sucking insects. Phloem-feeding insects are capable of stimulating responses associated with both pathogen infection, wounding and water deficit.

Nitric oxide (NO) is used by plants as a signaling molecule, and is involved in many key physiological processes, including seed germination, plant growth and development, maturation and senescence, root organogenesis, suppression of floral transition, and stomatal movement. It has also been implicated in plant response to abiotic and biotic stress, apoptosis (Mata and Lamattina, 2001; Zaninotto et al., 2006) and wounding (Huang et al., 2004).

Two distinct enzymatic pathways are implicated in NO generation in plants (Guo et al., 2003). One of these involves the reduction of nitrate via nitrite to NO, catalyzed by nitrate reductase (NR); whereas in the other, L-arginine, in the presence of oxygen and NADPH, is converted to NO citrulline by the action of a putative NO synthase (NOS) (Crawford et al., 2006; Guo, 2006). In the *Arabidopsis thaliana* At*nos1* mutant, NO production is impaired, and *in vivo* NOS activity is reduced to approximately 25% of the wild type level (Guo et al., 2003). Furthermore the At*NOS1* gene product shares sequence similarity with a protein involved in NO synthesis in the snail *Helix pomatia*. However, Crawford et al. (2006) questioned whether At*NOS1* is an authentic NOS, on the basis that the recombinant AtNOS1 protein lacks NOS activity *in vitro*. As a result, the gene was renamed NO-associated protein 1 (At*NOA1*) (Crawford et al., 2006). Therefore, At*NOS*/At*NOA1* may function as a cGTPase rather

Abbreviations: FW, fresh weight; JA, jasmonic acid; PBS, phosphate buffered saline; RWC, relative water content; SA, salicylic acid; SNP, sodium nitroprusside.

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doi:10.1016/j.jplph.2010.09.018

than as a NOS (Magali et al., 2008). Regardless of its biochemical function, it is clear that At*NOA1*-dependent NO synthesis is involved in hormonal signaling, wounding, stomatal movement, flowering, pathogen defense, and oxidative stress (Guo et al., 2003; Huang et al., 2004; Zeidler et al., 2004). A recent report indicates that expression of rice gene Os*NOS1*/*NOA1* re-establishes nitric oxide synthesis and stress-related gene expression for salt tolerance in *Arabidopsis* nitric oxide-associated 1 mutant *Atnoa1*. It showed that Os*NOA1* may play similar roles to At*NOA1* (Qiao et al., 2009). In addition to the NOS and NR pathways, there is also evidence that plants, in some situations, generate NO non-enzymatically (Bethke et al., 2004).

Since NO participates in responses to both disease and water stress, it is possible that it also plays a signaling role in response to BPH feeding in rice. Here, we set out to establish whether BPH feeding affects the endogenous level of NO in rice. The rationale for these experiments was based on the idea that enhanced levels of NO may improve the tolerance of rice to BPH infestation by reducing the rate of water loss through an effect on stomatal opening and root architecture.

2. Materials and methods

2.1. Plants and insects

The two rice cultivars used were DV85, which has resistance to BPH (Su et al., 2005) due to the presence of gene *QBPH11*, and TN1, a BPH susceptible genotype. Seedlings were grown in glass cups containing Hoagland's solution (Hoagland and Arnon, 1950), and were maintained at 25 °C under a 10 h photoperiod and 40–50% relative humidity. BPH were maintained on TN1 plants at the Rice Institute, Nanjing Agricultural University.

2.2. Measurement of NO content

Each 14-day-old seedling was infested with ten BPH for a period of 0 h, 1 h, 3 h, 6 h, 12 h, or 24 h. Plant material was harvested, ground under liquid nitrogen and extracted in 4 ml 40 mM HEPES buffer (pH 7.2) per g of plant tissue and centrifuged at 27,000 × *g* for 20 min. Filtration of the supernatant through a 0.45 μm filter was undertaken to increase the ultrafiltration rate. The NO content of the supernatant was measured using the Total Nitric Oxide Assay Kit (Beyotime, China). Three independent experiments were carried out.

NO was visualized *in planta* by means of the specific fluorescent probe DAF-FM DA (Invitrogen, USA) (Arnaud et al., 2006). Leaf, root and sheath segments of 14-day-old seedlings were incubated in the dark at 37 °C in the presence, or absence, of 0.5 mM cPTIO (Sigma–Aldrich, USA), 20 mM HEPES–NaOH, and pH 7.5for 30 min, then 10 μM DAF-FM DA was added in the dark at 37 °C and maintained for 30 min. The segments were washed three times in HEPES–NaOH buffer (15 min per wash) and mounted for confocal laser scanning microscopy using an excitation wavelength of 495 nm and an emission wavelength of 515 nm. Images were processed and analyzed using Olympus FV5000 software. The experiments were repeated three times (six samples per replicate).

2.3. Evaluation of BPH response

To ensure that all seedlings had reached the same growth stage before being exposed to BPH infestation, the seeds were pre-germinated. About 25 seedlings were planted per 10 cm diameter pot, and thinned to 20 per pot after seven days. At the third leaf stage, the seedlings were sprayed with H_2O, 100 μM or 200 μM of the NO donor sodium nitroprusside (SNP, Sigma–Aldrich) in the presence or absence of 100 μM or 200 μM cPTIO, before placing ten third instar BPH nymphs on each plant. Seedling mortalities were recorded on days seven and 10 following BPH infestation. Four replicates of each treatment were carried out.

2.4. Measurement of root elongation, seedling height, root biomass and root number

Seeds were pre-germinated for two days, and then grown in glass cups containing Hoagland's solution supplemented by 0 μM, 25 μM, 50 μM, 100 μM, 150 μM, 200 μM or 300 μM SNP. After 14 days, the lengths and numbers of roots and heights of seedlings were measured. To assess the effect of BPH infestation on root growth, 14-day-old seedlings were infested with 10 BPH for seven days, after which the numbers and lengths of the roots were measured. The dry weights of the root tissues were obtained after holding at 75 °C for at least 24 h. All experiments were repeated at least three times (10 seedlings per replicate).

2.5. Stomatal aperture

Epidermal strips from fully developed leaves were incubated for 2 h in 50 mM PIPES, 50 mM KCl, 1 mM $MgCl_2$ to induce stomatal opening. Following this treatment, the strips were either maintained in the pre-incubation buffer (as control), or the buffer was replaced by 100 μM SNP for 2 h. An assessment of stomatal opening was made by inspection under a 100× optical microscope, with pore width calculated with the help of Motic image advance v3.2 image analysis software. Each data point represented the mean of at least 100 stomates from at least three epidermal strips taken from different leaves.

2.6. Measurements of transpiration and stomatal conductance

Stomatal conductances and transpiration rates were measured at the three leaf stage 24 h after infestation with 10 BPH per plant using a portable photosynthesis measurement system (LI-6400, LI-COR, Inc.). At least 10 individuals were measured for each of three independent experiments.

2.7. Relative water contents (RWCs)

Relative water contents were determined on 14-day-old seedlings as described elsewhere (Mata and Lamattina, 2001). The water deficit treatment involved placing the seedlings on dry white paper under light at 25 °C. Measurements were taken after 3-h of water deficit, and after 24 h of infestation with ten BPH per plant. The RWC (%) was calculated as (TW − DW)/(FW − DW) × 100, where the fresh weight (FW) was measured at the beginning of the treatment, the treatment weight (TW) was determined after water deficit or BPH infestation, and the dry weight (DW) after oven drying the samples. At least ten individual plants were measured for each of three independent experiments.

2.8. Nitric oxide synthase assay

Fourteen day-old seedlings infested with 10 BPH for periods of 0 h, 3 h, 6 h, 12 h, or 24 h were harvested, and immediately ground under liquid nitrogen and extracted in 5 ml 100 mM PBS buffer (pH7.4) per g of plant tissue and centrifuged at 10,000 × *g* for 20 min; the supernatant was then ultracentrifuged at 100,000 × *g* for 15 min. Filtration of the 100,000 × *g* supernatant through a 0.45 μm filter will increase the ultrafiltration rate. The filters were rinsed with HPLC-grade water prior to ultrafiltration. A nitric oxide synthase assay was measured using a Nitric Oxide Synthase Detection System, Fluorimetric (Sigma-Aldrich, USA).

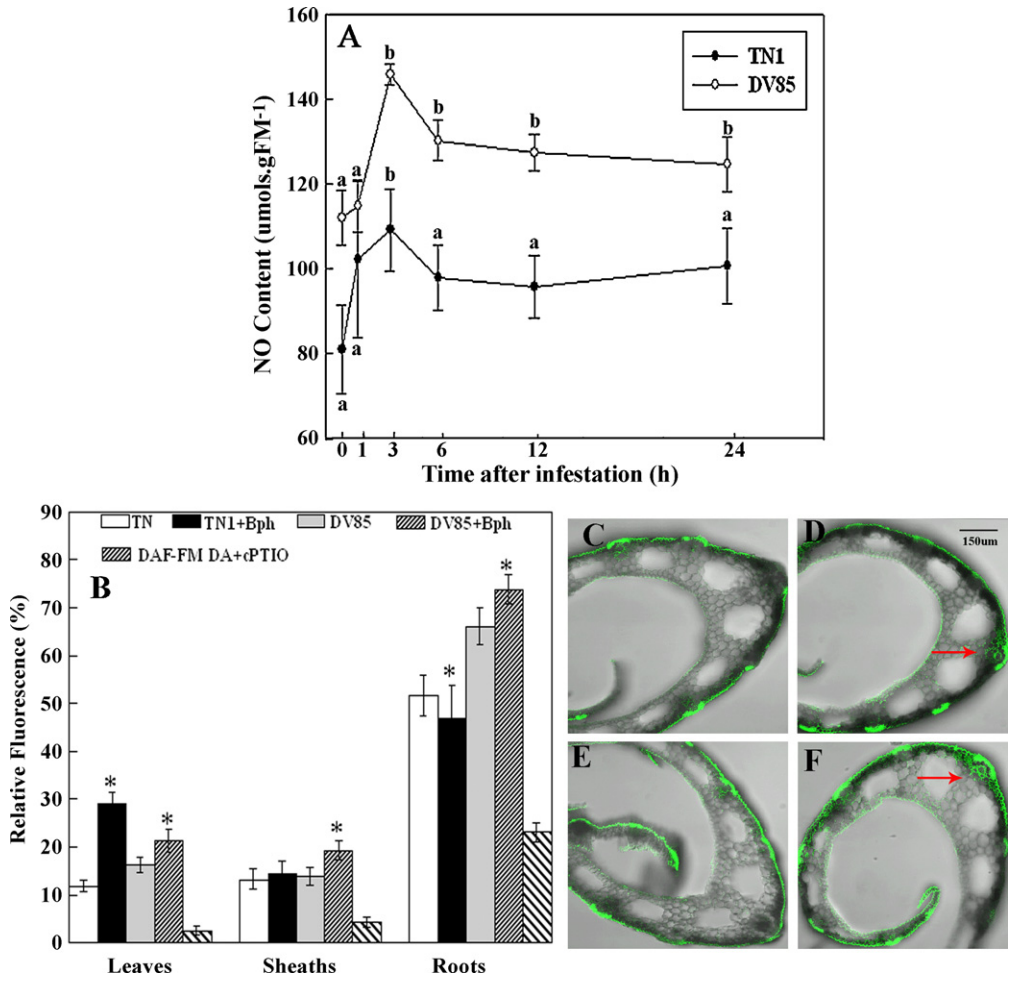

Fig. 1. Effect of BPH feeding on NO level. (A) NO concentrations in resistant (DV85) and susceptible (TN1) rice cultivars, following infestation with BPH. (B) NO levels in leaves, sheaths and roots. (C–F) Endogenous NO levels in the leaf sheath 24 h after BPH feeding. (C) Non-infested TN1. (D) TN1 infested with BPH. (E) Non-infested DV85. (F) DV85 infested with BPH. Red arrows indicate the phloem. Letters and asterisks indicate significant differences (*t*-test, $P < 0.05$) from the control treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.9. Nitrate reductase assay

Fourteen-day-old seedling infested with 10 BPH for a period of 0 h, 3 h, 6 h, 12 h, or 24 h were harvested and ground under liquid nitrogen. The buffer used for preparation of crude extracts contained potassium phosphate (100 mM, pH 7.5), magnesium acetate (5 mM), glycerol (10%, v/v), polyvinylpyrollidone (10%, w/v), Triton X-100 (0.1%, v/v), EDTA (1 mM), DTT (1 mM), PMSF (1 mM), benzamidine (prepared fresh) (1 mM) and 6-aminocaproic acid (1 mM). The leaf tissue (0.25 g) was ground into a fine powder using a mortar and pestle. The extraction buffer was added after the liquid nitrogen had evaporated, and before thawing. The tissue to buffer ratio was 1:3 (w/v) and the mixture was thoroughly homogenized. The extract was filtered through a nylon net (80 mm) and centrifuged at 14,000 rpm for 15 min. The clear supernatant was used immediately for measurement of enzyme activities. The assay was performed as described by Hageman and Hucklesby (1971). Each experiment was repeated three times and mean data were plotted as relative specific activities (%) along with standard errors.

2.10. RNA extraction and RT-PCR

Total RNA was isolated using Tri Reagent (Invitrogen, USA). For first strand DNA synthesis, 2 μg total RNA were reverse transcribed in a 25 μL reaction volume, containing 10 ng oligo (dT) 18 primer, 2.5 mM dNTP and 200 U M-MLV reverse transcriptase (Promega, Madison, WI, USA). PCRs were performed in 25 μL volumes, each containing 1 μL cDNA, 0.2 μM primer, 10 mM dNTP and 1 U rTaq DNA polymerase. The PCR consisted of an initial 5 min denaturation at 94 °C, followed by 21–35 cycles of 94 °C/45 s, 60 °C/30 s and 72 °C/60 s, with a final incubation of 10 min at 7 °C. The amplicons (10 μL) were separated electrophoretically through 1.2% agarose gels. The primer sequences are given in Supplementary Table S1.

3. Results

3.1. BPH feeding induces NO production partially dependent on NOS activity

The level of endogenous NO in the plant tissue increased with BPH feeding on both DV85 (resistant) and TN1 (susceptible) plants, and peaked 3 h after infestation. It remained higher than the constitutive level even 24 h after infestation. The level of NO was higher in DV85 than in TN1 throughout the experiments (Fig. 1A). The *in planta* measurement of NO content showed that the root contained more NO than either the leaf or the sheath (Fig. 1B and Supplementary Fig. S1). In the leaf and sheath tissues of both DV85 and TN1, NO content increased in response to BPH feeding, but the increase in the sheath of TN1 was non-significant. Following infes-

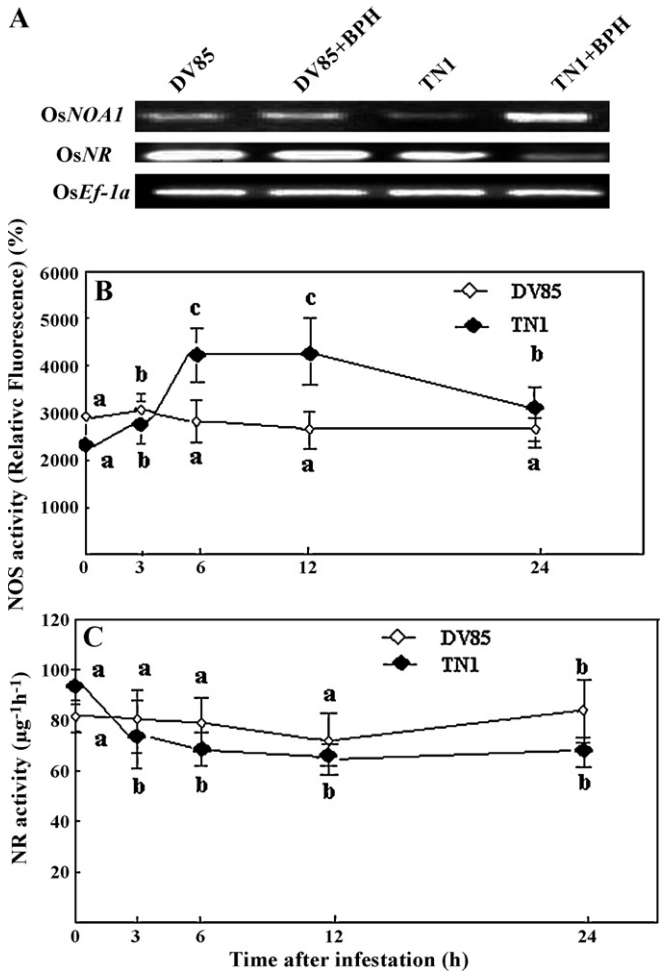

Fig. 2. Effect of BPH feeding (24 h) on expression and activity of the NO-synthesis related gene. (A) NO-synthesis related gene in BPH infested plants, as assessed by RT-PCR. (B and C) Activities of NOS and NR in whole plants following infestation with BPH. Letters indicate significant differences (*t*-test, $P < 0.05$) from the control treatment.

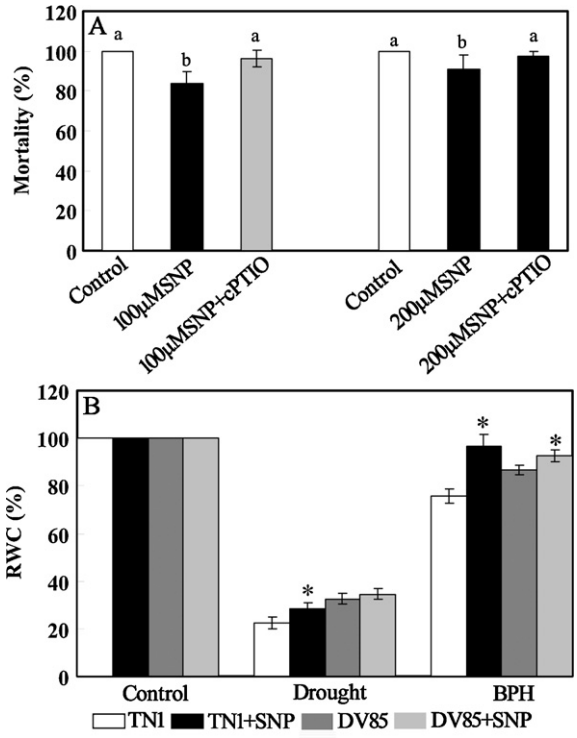

Fig. 3. Effect of NO on the survival rate and RWC of rice seedlings infested with BPH. (A) TN1 seedlings were treated with H_2O, 100 μM or 200 μM SNP in the presence or absence of cPTIO, and survival rates were measured seven and 10 days after infestation. (B) Effects of NO on RWC of 14-day-old TN1 and DV85 rice seedlings subjected to either drought or BPH feeding. RWC was determined after 3 h of drought and after 24 h BPH feeding. Means and standard errors were calculated from four independent experiments. Asterisks indicate significant differences (*t*-test, $P < 0.05$) from the control treatment.

tation, the roots of the two cultivars responded differently, with the NO level increasing in DV85, but decreasing in TN1 (Fig. 1B and Supplementary Fig. S1). BPH probing is always around the periphery of the leaf sheath with a preference for the thick segment of the outer leaf sheath (Wang et al., 2008). A NO burst in the leaf sheath was also detected in the thick segment (Fig. 1C).

The expression of Os*NOA1* was increased in TN1 24 h after infestation, but was unchanged in DV85. In contrast, although the expression of Os*NR* Choi et al. (1989) in DV85 was unaffected by BPH feeding, its level was substantially reduced in TN1 in response to BPH feeding (Fig. 2A). The NOS and NR activities were assayed at various time intervals after infection. The activity of NOS increased with BPH feeding in TN1, but was significant increased only 3 h after infestation in DV85 (Fig. 2B). Whereas the NOS activity was enhanced by BPH feeding, the trend in activity was partially consistent with NO level. On the other hand, the activity of NR in DV85 was similar to Os*NR* expression and was apparently not affected by BPH feeding, compared to the significant reduction in TN1 (Fig. 2C). These results implied that production of NO in response to BPH feeding was partially dependent on the activity of nitric oxide synthase, and possibly not related with nitrate reductase, suggesting that another pathway may also participate in the release of NO. The data also indicate that BPH feeding disturbed normal nitrogen metabolism in TN1.

3.2. Enhanced NO levels reduced water loss caused by water deficit and BPH feeding, via its effect on stomatal opening and root architecture

All TN1 seedlings died at seven days after infestation. The level of tolerance was increased by treatment with 50–200 μM sodium nitroprusside (SNP). This increased tolerance to BPH caused by SNP could be reduced by NO scavenger cPTIO. However, this tolerance was only short-term, as by 10 days after infestation, no benefit remained (Fig. 3A and Supplementary Fig. S2). There was no difference in resistant DV85 seedlings; with or without SNP treatment, no seedlings were dead (data not shown). Under drought stress, relative water content (RWC) was reduced by 13.3% (DV85) and 24.3% (TN1) following BPH feeding compared to the controls, while the RWC reduced to 5.8% and 20.7%, respectively, after SNP treatment (Fig. 3B). Treatment with 100 μM SNP reduced stomatal opening by 50% (Supplementary Fig. S3). Notwithstanding difficulties in directly measuring stomatal opening in infested and non-infested plants, we measured the stomatal conductances and transpiration rates in infested and non-infested plants. Compared to the non-infested controls, both stomatal conductance and transpiration rate were significantly reduced in both DV85 and TN1 following BPH feeding (Fig. 4). This showed that stomatal opening was decreased by BPH feeding, and since stomatal opening is important in delaying plant death in rice, these results suggested that NO levels are increased by BPH feeding and that increasing the supply of NO helps to reduce the rate of water loss, thereby decreasing the mortality caused by BPH feeding.

Although NO played a role in root morphogenesis (Maria et al., 2008), there was no clear morphological effect on lateral roots

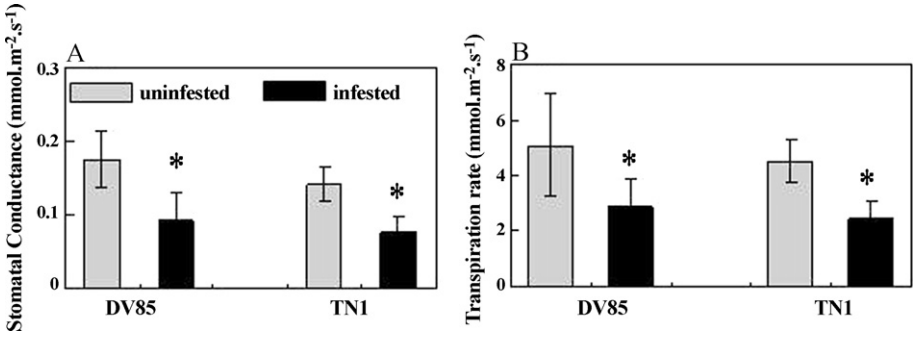

Fig. 4. Effects of BPH infestation on stomatal conductance and transpiration rate. (A) Stomatal conductance and (B) transpiration rate; measured on 14-day-old seedlings infested or non-infested with BPH for 24 h. Means and standard errors were calculated from three independent experiments. Asterisks indicate significant differences (*t*-test, $P < 0.05$) from the control.

with a treatment of 25 μM SNP. However, the lateral root number did respond to treatment levels of >50 μM. At the same time, the 25 μM SNP treatment increased the length of the primary root, but concentrations of >50 μM SNP noticeably inhibited primary root elongation. Lateral root elongation was unaffected by both 25 μM and 50 μM SNP, but was inhibited by levels >100 μM (Fig. 5A and C and Supplementary Fig. S4). Thus, supplemental NO clearly influences root architecture in rice.

Root development of DV85 and TN1 was affected by BPH feeding. Specifically, root biomass and elongation were reduced in both TN1 and DV85 by infestation, but in DV85, the reduction was not significant (Fig. 5D–F). BPH feeding also affected lateral root growth differentially, inducing a decreased lateral root number in TN1 and a slight increase in DV85. The addition of NO reduced the plant height (Fig. 5B) and delayed senescence of the first leaf (Supplementary Fig. S5).

3.3. *Expression of genes associated with the drought stress response*

The OsLea3-1 and OsP5CS1 proteins have been associated with drought response in rice (Hur et al., 2004; Xiao et al., 2007). The expression patterns for genes corresponding to these proteins were similar in the two rice cultivars; they were induced in response to SNP, drought stress and BPH infestation, and were enhanced under drought stress and BPH infestation by the supply of exogenous NO (Fig. 6).

4. Discussion

Nitric oxide signaling represents part of the plant's response to microbial attack and wounding. Here, we addressed the question of whether NO is also involved in wounding response to phloem-

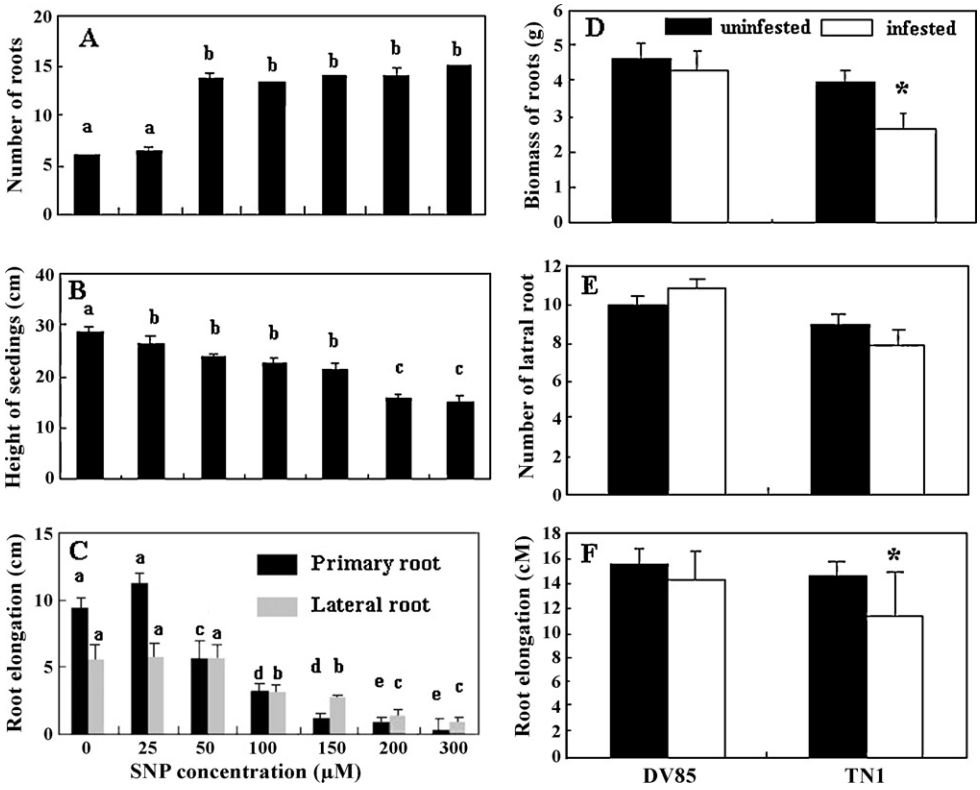

Fig. 5. Effects of NO and BPH infestation on root architecture and plant height. (A–C) Lateral root number, plant height and root length of 14-day-old seedlings after treatment with H_2O, 25 μM, 50 μM, 100 μM, 150 μM, 200 μM, or 300 μM SNP. (D–F) Effects of BPH infestation on root architecture, measured on 14-day-old seedlings infested or non-infested with BPH for seven days. Means and standard errors were calculated from three independent experiments. Asterisks indicate significant differences (*t*-test, $P < 0.05$) from the control treatment.

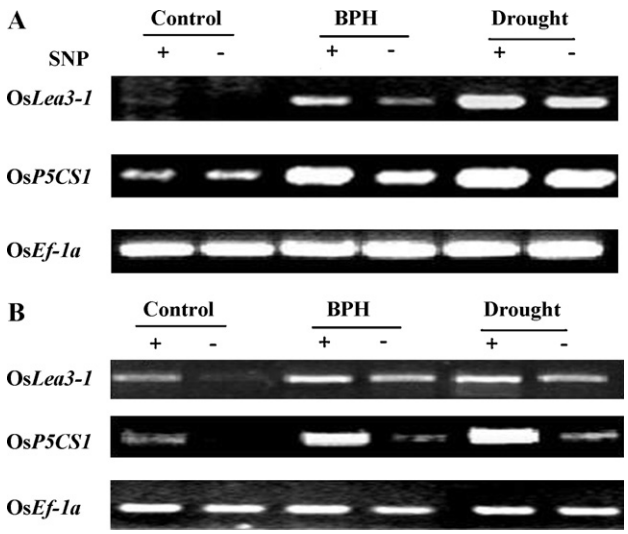

Fig. 6. Accumulation of Os*Lea3-1* and Os*P5CS1* transcripts in water deficit- and BPH-challenged seedlings. Bottom panel: constitutively expressed Os*Ef-1α* control. Samples were collected before treatment C (Control), after 3 h of water deficit (drought), and after 24 h of BPH feeding (BPH). (A) TN1 and (B) DV85.

feeding insect BPH. Within minutes of mechanical wounding, a strong burst of NO release is triggered (Huang et al., 2004). Similarly, a NO burst was observed within 1 h of infestation with BPH in both resistant and the susceptible genotypes (Fig. 1A). This burst was localized to phloem cells of the sheath (Fig. 1C), the preferred site of BPH feeding (Wang et al., 2008). Thus, the presence of BPH appears to induce the release of NO at the feeding site. The role of NO in wound healing in animals is well documented (Lee et al., 2001), but it remains unclear whether NO accumulated at BPH feeding sits in rice plays a similar role to that occurring at wound sites in animals. Although BPH do not feed on the leaf or the root, the NO levels in these organs also rose in response to feeding on the resistant cultivar (Fig. 1B), suggesting that BPH feeding generates a systemic signal to which the plant responds by increasing its rate of NO synthesis.

The production of NO in wounded plant tissue is thought to rely mainly on synthesis via the NOS pathway (Huang et al., 2004). The expression of Os*NOA1* was induced in response to BPH infestation, but because it is not clear whether Os*NOA1* expression leads to increased NOS activity, the activities of NOS were also measured. Although the activity of NOS was also induced by BPH infestation, the trend was only partially consistent with that of NO level. Intriguingly, the expression of Os*NR* was unaffected by BPH feeding in DV85, but was markedly down-regulated in TN1 (Fig. 2A), and confirmed by the activity assay (Fig. 2C). Thus BPH feeding was clearly disruptive to the normal nitrogen metabolism of TN1. Some studies have demonstrated that the photosynthetic rate of leaf blades was suppressed by BPH feeding, and leaf nitrogen concentration in infested plants was also lower than that in control plants (Watanabe and Kitagawa, 2000); over 30 BPH nymphs per tiller significantly reduced N uptake by roots (Qiu et al., 2004). However, these studies did not identify the cause of the reduced leaf nitrogen concentration and uptake of N. According to our findings this might be due to decreasing expression of Os*NR*. A possible reason for this is decreased leaf photosynthesis caused by the BPH infestation, which limits activity of the root system because the translocation of assimilates from leaf photosynthesis to the root system is blocked in susceptible genotypes. These results suggested that the production of NO in response to BPH feeding may not be dependent on the activity of the NR pathway, and only partially dependent on the NOS pathway. Moreover, other pathways may participate in the release of NO. NO can also be produced non-enzymatically from the oxidation of nitrite under acidic conditions prevalent in the plant apoplast (Bethke et al., 2004). The possibility of NO induced by BPH in rice being generated non-enzymatically needs to be studied further.

The salivary sheath is made of solid saliva that is secreted during BPH probing. The amount of probing and number of salivary sheaths is significantly greater in the phloem of resistant cultivars than that in susceptible genotypes (Zhang et al., 2004; Hao et al., 2008) indicating that piercing and sucking per se are not the major cause of seedling death. Rather the loss of phloem sap or water is likely to be the critical factor. The symptoms of BPH feeding are reminiscent of those associated with drought stress (Hao et al., 2008), and this was reflected in the effect of BPH feeding on hydration of the plant tissue, which was reduced more noticeably in TN1 than in DV85. The presence of a NO donor attenuated this effect by decreasing the mortality rate caused by BPH feeding (Fig. 3A). A large number of genes are up-regulated by drought stress; two of these (Os*Lea3-1* Os*P5CS1*) were also induced by BPH feeding, and this induction was enhanced by the supply of exogenous NO (Fig. 6). Thus it appears that rice is able to limit water loss and enhance its tolerance to BPH infestation via the same mechanism, and NO signaling likely plays an important role in this process.

The regulation of stomatal aperture through evapotranspiration and root architecture is the most effective means for plant to minimize water loss. NO acts in concert with ABA to control stomatal opening and closure (Neill et al., 2002), and is a player in the determination of drought tolerance, at least in *Arabidopsis thaliana* (Mata and Lamattina, 2001). Elevation of the level of exogenous NO also reduces stomatal opening in rice (Supplementary Fig. S2) and the reduction of stomatal opening also occurred following BPH feeding (Fig. 4). Increased NO level in leaves caused by BPH feeding suggests its regulation of stomatal aperture may be one of numerous ways of responding to BPH. The root system is responsible for the provision of water, mineral nutrients and physical support to the plant. The length of the primary root and the density of lateral roots determine the architecture of the root system, and this plays a major role in determining whether a plant can prosper in a particular environment (Malamy and Benfey, 1997). The number of lateral roots is not pre-determined genetically; rather it is determined by the prevailing soil conditions (Malamy and Ryan, 2001). In order to survive under water stress conditions plants usually reinforce their water absorption ability by regulating root development (Malamy and Benfey, 1997). Root architecture is clearly influenced by BPH feeding, with TN1 changing with respect to root length, biomass and lateral root number in the presence of BPH (Fig. 5D–F). Root length and biomass in DV85 were reduced by BPH feeding, but the differences were not statistically significant; however, lateral root numbers were slightly increased. Phenotypic response to drought stress shares some features with BPH feeding, but also some differences. Elongation of the primary root is a particularly important adaptation to drought, since it improves access to water deeper in the soil profile (Ludlow and Muchow, 1990). BPH attack is not generally associated with periods of moisture deficit, so the plant response relies more on lateral root proliferation and less on primary root elongation. The addition of exogenous NO inhibits root elongation, but increases lateral root number (Fig. 5A and C). Thus, endogenous levels of NO in roots may help explain observed differences in root length and lateral root number following BPH feeding in susceptible TN1 and resistant DV85 rice cultivars. The results show that the effects of NO on root architecture may be part of the mechanism of rice tolerance to BPH.

Apart from changes in stomatal aperture and root architecture, other early morphological responses to drought and BPH feeding include a reduction in plant shoot height. Adjustments in plant growth are an important adaptive mechanism used by plants to

minimize damage when water is limiting (Xiao et al., 2008). A reduction in the growth could be part of an overall defense strategy against insect herbivory, as reduced growth limits nutrition available to the feeding insects (Hermsmeier et al., 2001). Because the reallocation of energy resources towards resistance reduces the pool of energy available for growth, resistance is believed to be energetically costly (Heil and Baldwin, 2002). One phenotypic effect of both BPH feeding and exogenously supplied NO was decreased plant height. Adjustment of plant growth may also be an important part of the resistance mechanism to BPH attack in rice. Treatment with either 100 μM or 200 μM SNP effectively delayed the senescence of the first leaf (Supplementary Fig. S5). Rosa et al. (2007) showed that the suppression of drought-induced leaf senescence resulted in outstanding drought tolerance and transgenic plants in their study maintained high water contents and retained photosynthetic activity during drought conditions. Suppression of leaf senescence could therefore enable plants to mount vigorous acclimation responses that could result in enhanced drought and BPH tolerance.

We have demonstrated here that induction (or exogenous supply) of NO enhances tolerance of rice to BPH feeding by reducing the extent of water loss from the plant. This effect is exerted through both an influence on stomatal opening and on a re-patterning of root architecture. Co-evolution of the plant and insects that feed on it has produced an array of constitutive and induced defense mechanisms. We believe that plants have exploited the signaling properties of NO to generate one of the numerous components of this overall network of plant defense.

Acknowledgments

This research was supported by National Key Transform Program (2009ZX08009-046B, 2008ZX08001-001, 2008ZX08001-002), NSFC (31000697), Natural Science Foundation of Jiangsu Province of China (BK2010442), Jiangsu Science and Technology Development Program (BE2009301-3), and 111 project (B08025).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2010.09.018.

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