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

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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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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).