Trichoderma asperelloides enhances local (LAR) and systemic acquired resistance (SAR) response under low nitrate nutrition in *Arabidopsis*

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Summary

- Nitrogen (N) is essential for growth, development and defense but how low N effects defense response is not known.

- Low nitrate fed Arabidopsis plants displayed reduced growth and compromised local and systemic acquired resistance (LAR & SAR) response when infected with avirulent and virulent *Pseudomonas syringae* DC3000. Growth and defense responses were enhanced in the presence of *Trichoderma asperelloides*.

- The mechanism of increased LAR and SAR involve increased N uptake and enhanced protein levels via modulation of various low and high affinity nitrate transporter genes (*CLC-A*, *NPF1.2*, *NRT2.1*, *NRT2.2* and *NRT2.4)*. *nrt2.1* mutant is compromised in LAR and SAR response suggesting a link between enhanced N transport and defense.

- Enhanced N uptake was mediated by Trichoderma elicited nitric oxide (NO). Low NO producing mutants *nia1,2*, *nsHb*$^{+}$ were unable to induce nitrate transporters. These mutants displayed reduced protein levels and *PR1* gene expression in the presence of Trichoderma under low N suggesting a signaling role of NO elicited by *Trichoderma*.

- *Trichoderma* also induced SA levels and defense gene expression under low N. Salicylate hydroxylase mutant (*NahG*) and Non-expresser of Pathogenesis Related gene1 mutant (*npr1*) were compromised in LAR and SAR response in the presence of Trichoderma.

- The mechanism of enhanced plant defense under low N mediated by Trichoderma involve NO, ROS production, *NRT* gene induction, production of SA and induction of defense, SAR marker genes.

Key words: Nitric oxide, nitrate transporters, *Pseudomonas syringae,* SAR, Trichoderma

Introduction

Nitrogen (N) is essential for normal growth and development of plants. It is a crucial component in the chlorophyll structure, nucleic acids and amino acids. N plays an important role in operation of primary and secondary metabolism and also involved in protection of plants from biotic and abiotic stresses (O'Brien *et al.* 2016; Mur *et al.* 2017). N deficiency occurs in soil due to slow mineralization, lack of sufficient organic matter, leaching due to heavy rainfalls and increased activities of denitrifying bacteria. Low N can retard the growth of plants and cause severe physiological and morphological defects (Walker *et al.* 2001; Landrein *et al*. 2018). To cope with this, plants have evolved N uptake systems to support their survival under N deficiency (Li *et al.* 2017). These uptake systems are based on their affinity with nitrates (NO_3^-); mediated by the family of nitrate transporter proteins in plants. First is low affinity nitrate transporters (LATs), which operate under optimum N conditions (>50 mM), second is high affinity nitrate transporters (HATs), which can operate under very low N concentrations (<1 mM) (Tsay *et al.* 2007; Li *et al.* 2017).

N plays a very significant role in plant defense (Gupta *et al.* 2013; Mur *et al.* 2017). Hence, operation of efficient N transport system help plants to defend against pathogens. This defense response involve activation of innate immune responses comprising of two unified branches termed PTI (PAMP Triggered Immunity) and ETI (Effector-Triggered Immunity) that initiate extensive transcriptional reprogramming (Alves *et al.* 2014; Jones and Dangl 2006). These processes require N. Moreover, the host mobilizes salicylic acid (SA) for the plant defense (Oliva and Quibod 2017, Mur *et al.* 2017). This pathway itself requires N for synthesis of various intermediates leading to SA production. The defense mechanism for invading pathogen importantly includes rapid programmed cell death known as the hypersensitive response (HR), which develops during incompatible plant-pathogen (*R-avr*) interactions (Delledonne *et al.* 1998). An early characteristic of HR is the rapid generation of superoxide ($O_2^{\cdot-}$), nitric oxide (NO) and accumulation of H_2O_2 following perception of avirulent pathogen signals (Lamb and Dixon, 1997). Moreover, SA produced during HR, plays an important role in plant defense (Mur *et al.* 2000, 2008; Gupta *et al.* 2013). Thus, N not only improves the nutritional status of the plant, but, also its concentration plays a role in defense. Moreover, the form of N nutrition can greatly influence the HR-mediated resistance in plants (Gupta *et al.* 2013).

Nitrate (NO_3^-) nutrition greatly influences programmed cell death (HR) via the production of NO which is a regulatory signal in plant defense (Delledonne *et al.* 1998) and is involved in the development of cell death (Gupta *et al.* 2011; Delledonne *et al.* 1998). NO production depends on NO_3^-. Therefore, N deficiency can also lead to reduced levels of NO. SA is known to be induced by NO, hence, low N leads to low NO and reduced SA levels. Since NO production also requires NO_3^-, it may also play a role in systemic acquired resistance (SAR) which results in broad-spectrum disease resistance against secondary infections after primary infection (Cameron *et al.* 1994, 1999). SAR develops either as a consequence of HR where NO has its proven role (Delledonne *et al.* 1998), or in the course of a compatible interaction resulting in disease development (Gupta *et al.* 2014). SAR is dependent on SA or its derivatives (Park *et al.* 2007, Metraux *et al.* 1990) and other metabolic pathways such as increased phenylalanine ammonia-lyase (PAL) activity (Smith-Becker *et al.*. 1998; Kim and Hwang 2014) and *PR* gene expression (Ryals *et al.* 1996). Previously, it was shown that NO plays a role in nitrate assimilation/uptake during stress by modulating nitrate transporters (Frungillo *et al.* 2013). Thus nitrate, and defense pathways those dependent on N, play multifaceted roles in plant defense.

Therefore, any mechanism that can increase N uptake may assist in plant defense under low NO_3^-. Many plant symbiotic microbes such as mycorrhiza, *Trichoderma*, PGPR are known to increase nutrient uptake. However, the operation of plant defense under low N and the effect of these microbes in increasing plant defense under N deficiency is not known.

Several species of *Trichoderma* play an important role in plant growth promotion and resistance against various biotic and abiotic stresses. They confer resistance to plants via various mechanisms such as mycoparasitism, activation of plant defense responses via induced systemic resistance response (ISR) and increasing basal resistance. Previously, it was demonstrated that roots colonized by *Trichoderma* species can activate defense responses very rapidly and intensively via priming in *Arabidopsis* (Brotman *et al.* 2012). This occurs via modulation of transcription factors and key metabolites of plant defense (Shoresh 2010). Upon pathogen attack, *Trichoderma* treated plants show accumulated transcript levels of

PAL (Yedidia *et al.* 2003) and increased levels of defense-related plant enzymes (peroxidases, chitinases, and β-1, 3-glucanases; Shoresh *et al.* 2005).

If *Trichoderma* enhances N uptake, it can have positive effect on plant defense via LAR and SAR responses altogether. So far is it not known whether *Trichoderma* can increase plant defense under low N. Therefore, in the present study, we have assessed, the systemic defense response of low N fed *Arabidopsis thaliana* plants to the phytopathogen; *Pseudomonas syringae* p.v. tomato DC3000 induced by the beneficial fungus *Trichoderma asperelloides* (T203) under low and optimum nitrate concentrations. Here, we describe that T203 enhances SAR response in Arabidopsis grown under low N via modulating nitrate transporters and this mechanism involves both NO and SA.

Material and Methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* ecotype Columbia 0 (*Col0*; WT) was sown in plastic pots (10 cm diameter) containing soilrite: agropeat (1:1) mix (mix contains zero N) and stratified at 4°C in dark for 48 h. Then, the pots were kept in growth room under short day conditions (8h-light, 16h-dark), 22/18°C (day/ night) temperatures, relative humidity of 60% and 200 µE m^{-2} s^{-1} light intensity. Initially, plants were bottom irrigated for a week, once with half strength Hoagland solution and once with water. Then, 0.1 mM and 3 mM N concentrations were given to the growing plants weekly. The nitrate nutrient solution in Hoagland's media contained either 0.1 mM or 3 mM KNO_3, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM K_2HPO_4, 1 mM KH_2PO_4, 15 µM H_3BO_3, 3 µM $MnCl_2.4H_2O$, 0.25 µM $ZnSO_4.7H_2O$, 0.1 µM $CuSO_4.5H_2O$, 0.04 µM Na_2MoO_4 and 25 µM NaFe-EDTA. 30-36 day old plants with fully developed rosette were used for the experiments. The seeds of *nrt2.1* (SALK_035429C) were procured from ABRC, and only homozygous T-DNA insertion lines were used for the experiments.

Trichoderma supplementation

T. asperelloides (T203 strain) was grown on potato dextrose agar (PDA; Himedia) plates for 15 days under low light conditions until sporulation. Conidia were harvested by gently scraping the petridish and poured with 10 ml of sterile water. The spores were evaluated up to 1×10^9 spores/ml. It was thoroughly mixed into the

soilrite mix and distributed into the individual plastic pots.

Bacterial strain and growth conditions

Pseudomonas syringae pv. tomato DC3000 (*Pst*DC3000; *avrRpm1,* avirulent) was used as the non-host pathogen strain on *A. thaliana*. Bacteria were grown in King's B (KB) medium containing 50 µg ml^{-1} rifampicin. The culture was revived in 5 ml of King's B medium (Himedia, India) and incubated at 28°C with continuous shaking at 180 rpm for 12h. Upon reaching OD600 of 0.2, bacterial cells were harvested by centrifugation at 5000 rpm for 15 min. The pellet containing bacterial cells was washed thrice with water and re-suspended in 10 mM $MgCl_2$. The OD600 of the bacterial suspension was measured and diluted to OD600 of 0.005 (1×10^6 CFU ml^{-1}). The OD600 was equated to OD 0.1= 2×10^7 CFU ml^{-1}. Similarly, virulent *P. syringae* was cultured and used at OD of 0.005 (2×10^6 CFU ml^{-1}) for challenge inoculation.

Bacterial infiltration in leaves

Mock infiltration (control) was performed with 10 mM $MgCl_2$. *Pst* inoculations (1×10^6 CFU ml^{-1}) were made by syringe infiltration on the abaxial side of the leaves manually. There were total four groups of plants; Group 1: 0.1 mM + $MgCl_2$ plants, Group 2: 3 mM + $MgCl_2$ plants, Group 3: 0.1 mM + *Pst* plants and Group 4: 3 mM + *Pst* plants. Each group consisted of 20-25 plants. SAR experiment initiated with primary inoculation (a*vrRpm1 Pst* DC3000) on one leaf per plant, and the secondary challenge inoculation with (virulent *P. syringae*) on 4 other (distant) leaves per plant, leaving 5-6 healthy un-inoculated leaves per plant (Cameron *et al*. 1999) after 2 days of primary inoculation. The Inoculated (I) and un-inoculated (U) leaves from each treatment were harvested (Fig. S1) for RNA extraction and other experiments post challenge inoculation. Leaves were analyzed for 0, 6, 24 and 48 hours post inoculation (hpi) or challenge (hpc) and the symptoms was determined periodically.

Electrolyte leakage

Leaf discs were taken and electrolyte leakage was monitored by conductivity meter (Lab Man Scientific Instruments, India) at different time points from inoculated and un-inoculated leaves and electrolyte leakage was calculated as described in (Gupta *et al.* 2013).

In planta bacterial number quantification assay

Bacterial multiplication in leaves from *Pst* treated plants was assessed after primary as well as challenge inoculation. Each infected leaf disc (5 mm in diameter) was surface sterilized with 0.02% H_2O_2 for 8 minutes, and was homogenized in 1 ml of sterile 10 mM $MgCl_2$. Upon further serial dilution in 10 mM $MgCl_2$, it was plated on KB agar medium supplemented with rifampicin (50 µg ml^{-1}). For all the bacterial treatments, *in planta* bacterial number was assayed up to 2 days at 24h interval. Bacterial number was calculated as per Gupta *et al.* (2016).

Expression profiling by qRT-PCR

The inoculated and uninoculated leaves were collected at specific time points and were immediately frozen in liquid nitrogen and stored at -80°C. RNA extraction, cDNA synthesis and qPCR was performed according to Wany *et al.* (2017; 2018). The synthesized cDNAs were used as templates in qRT-PCRs performed using primers (Table S1). Fold change in the target genes was normalized to *Arabidopsis* ubiquitin, *18sRNA* (GQ380689) and *YSL8* gene (X69885.1). Fold expression relative to control treatment was determined by ΔΔCT values. Three biological experiments (with three independent replicates for each experiment) were performed for each treatment. The comparisons of the gene expression between the different treatments to control was performed by student's t-test. Differences between the treatments with $p<0.05$, $p<0.01$ and $p<0.001$ were considered significant.

Ex- vivo NO estimation

For this experiment, NO was measured from the following five different combinations in roots; 1. WT; 2. WT + T203 (*Trichoderma*); 3. *nia1,2* double mutants +T203; 4. WT + cPTIO; 5. WT + cPTIO + T203. WT and *nia1, 2* plants were grown for one week in plates containing 0.1 mM and 3 mM nitrogen concentrations. The spore suspension of *Trichoderma* was poured over these one week old plants and incubated for 2 minutes, 10 minutes and 24 hours. Then, the roots were incubated in 10 µM DAF-FM DA (4-amino-5-methylamino- 2′,7′-difluorofluorescein diacetate) in 100 mM HEPES buffer (pH 7.2) placed in a 1.5 ml tube and incubated for 15 minutes in dark. Then, the roots were washed three to four times using same buffer and transferred onto the slide and mounted with a drop of 10% glycerol with a cover-slip.

Each sample was photographed using fluorescence microscope (Nikon*80i*, Japan) with a CCD camera (DXM1200C) equipped with Image software (NIS elements ver3.2) at 495 nm excitation and 515 nm emission wavelength.

Determination of ROS levels

Production of hydrogen peroxide (H_2O_2) in inoculated and un-inoculated leaves was detected by Diaminobenzidine tetrahydrochloride (DAB) staining as per Daudi *et al*. (2012) with slight modifications. The superoxide levels were measured by *in vivo* staining with Nitroblue tetrazolium chloride (NBT, SA, USA) (Jambunathan 2010).

Histochemical detection of HR

Hypersensitive cell death in inoculated leaves was visualized by trypan blue staining method according to Fernández-Bautista *et al.* (2002).

SA levels

The SA levels were measured by HPLC according to the protocol described in Singh *et al.* (2013). The inoculated and uninoculated leaves of both WT and WT + T203 plants (0.1 mM and 3 mM) were harvested at 0, 6, 24 and 48 hpc for SA estimation.

Results

Optimal and low nitrogen effects LAR and SAR differently

Nitrogen plays important role in plant defense, hence, we first tested the effect of low N on plant defense mediated by LAR and SAR response. For this purpose, WT plants were grown under optimum (3 mM) and low (0.1 mM) NO_3^- conditions and challenged with *P. syringae* DC3000 (*avrRpm1*) and observed local HR at different time points. As shown in Fig.1a (lower panel) within 24 hpi, the 3 mM NO_3^--fed plants showed HR (lesion formation) at the inoculation sites and it was further progressed to higher levels till 48 hpi (Fig.1a) whereas 0.1 mM NO_3^- plants showed chlorotic lesions at the site of inoculation at 24 hpi and these spots were enhanced in 48 hpi. Electrolyte leakage (EL) (Fig.1b) was more rapid in 3 mM *Pst* infiltrated plants than

0.1 mM plants which is in accordance with HR response. In order to link HR formation with plant resistance, *in planta* bacterial number were determined (Fig. 1c). A significant increase in CFU count was observed in 0.1 mM grown plants at 48 hpi in comparison to bacterial numbers in 3 mM grown plants. We further found that, the *PR1* (a marker for HR) transcript levels have increased in 3 mM *Pst* treated plants (up to 25 fold till 48hpi), but in 0.1 mM WT plants, this gene was not at all induced at 48 hpi (Fig1d). Taken together these results suggest that low NO_3^- grown plants are compromised with HR linked LAR response.

After observing LAR, we studied SAR (Fig. S1b; Cameron *et al.* 1994 and 1999). Three days post-secondary challenge with virulent *Pst* DC3000, EL, CFU and *PR1* gene expression were assessed. During the primary inoculation, symptoms were visible after 24 and 48 hpi in 3 mM *Pst*-treated leaves, than 0.1 mM *Pst*-treated leaves (Fig. 2a- first panel), which showed enhanced chlorotic and necrotic symptoms. Three days after secondary challenge, disease symptoms were observed from 0 to 48 hpc. 0.1 mM *Pst*-challenged plants show more prominent necrotic and dark yellow lesions spread to both sides of the leaf (Fig. 2a-second panel) confirming susceptible symptoms (yellow specks) where as 3Mm grown plants showed reduced yellowing and necrotic symptoms. This clearly suggests that, 3 mM NO_3^- plants defended better during secondary challenge than 0.1 mM NO_3^- plants suggesting that optimal N is required for SAR development.

Trypan blue images of inoculated and uninoculated leaves are shown in Fig. 2b. It was observed that, after 3 days post challenge, the cell death observed in infiltrated tissues of 0.1 mM NO_3^- plants is profoundly less in comparison to 3 mM NO_3^- plants till 24 hpc. At 48 hpc, there was increased cell death in both 0.1 and 3 mM inoculated leaves but cell death phenotype is different. Interestingly, the uninoculated leaves of 0.1 and 3 mM WT plants, showed small and uniform spread of macrobursts throughout the leaf blades unanimously over the leaf, in all the time points, in exception that, 3 mM leaves displayed slightly larger macrobursts (Fig. 2b). This suggests that during SAR establishment, the mobile signal perceived by the uninoculated leaves results in the occurrence of low frequency microscopic HRs (Cameron *et al.* 1999; Alvarez *et al.* 1998).

Then, we assessed EL (Fig. 2c), we found leakage was significantly higher in 3 mM plants than 0.1 mM plants in both the cases (1° and 2°). But, much higher EL was observed during 2°challenge (Fig. 2c, right panel). Mock inoculated plants

showed moderate EL in both 0.1 mM and 3 mM plants (Fig. S2a). Higher bacterial growth was observed in 0.1 mM *Pst* treated plants as expected till 72 hpc, while there was a significant reduction in the bacterial numbers in 3 mM *Pst* inoculated plants (Fig2d).

Low LAR under low nitrogen is reversed by *Trichoderma*

Several strains of Trichoderma are known to play important role in plant growth, development and stress resistance (Brotman *et al.* 2010). Hence, we investigated whether *Trichoderma asperelloides* plays a role in improving LAR and SAR response under low NO_3^- availability. Interestingly, we found that under 0.1 mM NO_3^-, *Trichoderma* treated plants displayed healthy phenotype than untreated plants (absence of Trichoderma) (Fig. S1a), this enhanced growth was linked to increased leaf number, fresh weight and total chlorophyll content (Fig. S2b, c and d). During LAR, Trichoderma grown 0.1 mM NO_3^- plants did not show any severe disease symptoms, rather they mimicked the HR phenotype of 3 mM plants and showed resistance (Fig. 3a). This suggests that Trichoderma provides resistance under low N stress (Compare Fig. 3a with Fig. 1a). EL was significantly higher in both 3 mM and 0.1 mM Trichoderma treated plants (Fig. 3b) in comparison to untreated plants (Compare Fig. 3b with Fig. 1b). As shown in Fig. 3c, bacteria multiplied to a high level in both 0.1 mM and 3 mM *Pst* inoculated plants till 8 hpi, but strikingly, there was a significant reduction in the bacterial count in 0.1 mM *Pst* plants at 24 hpi, suggesting that Trichoderma enhances LAR response under low N. The higher accumulation of *PR1* transcripts in both 0.1 mM (~9 folds at 48 hpi) and 3 mM (~28 folds at 48 hpi) in Trichoderma grown *Pst* treated plants (Compare Fig 3d with Fig1d) again providing evidence that Trichoderma can enhance LAR under low N.

***Trichoderma* can induce SAR under low N**

During SAR assay, the T203 treated 0.1 mM plants have shown a drastic change in resistance where clear resistant symptoms were evident at 24 and 48 hpc (Compare Fig. 4a left panel with Fig. 2a left panel). During secondary challenge also, there was a clear reduction in yellowing in T203 grown 0.1 mM plants in comparison to untreated plants (Compare Fig. 4a right panel with Fig. 2a right panel). In response to T203, 0.1 mM NO_3^- plants showed more Trypan blue spots during secondary challenge. Upon infection, cell death spots were more widely spread in

these leaves (Compare Fig. 4b with Fig. 2b and Fig. S3 a&b). EL was significantly higher (~4 fold) in both 0.1 and 3 mM T203 treated plants during 2° challenge, in comparison to 1° inoculation at 24 and 48 hpc (Fig. 4c). This suggests that T203 treatment primes the defense responses in the uninoculated leaves immediately at 0 hpi and after 2° challenge. Bacterial count was in accordance with EL that, the *Pst* population decreased several folds in T203 treated low N-fed plants, suggesting that T203 imparts SAR response to the NO_3^- stressed plants (compare Fig. 4d with Fig 2d).

Trichoderma activates nitrate transporters, facilitates N uptake and promotes SAR in low N-fed plants

Increased LAR and SAR response under low N in the presence of *Trichoderma* is probably due to increased nutrient uptake facilitated by *Trichoderma* as these responses are absent in *Trichoderma* untreated plants. Increased nitrate transport is probably the reason for resistance hence, we checked the expression of both LATs (*CLC-A*, *NPF1.2*) and HATs (*NRT2.1*, *NRT2.2* and *NRT2.4*).

CLCa (the chloride channel family) is a tonoplast located antiporter channel system which drives nitrate accumulation in the vacuoles (Krapp *et al.* 2014). It was observed that, *CLCa* is significantly upregulated 4 to 6 folds in *Pst* treated leaves of 3 mM plants in 6h-48h; than *Pst* treated 0.1 mM plants (Fig. 5a), which displayed slight induction at 6 hpc (2 folds) and decreased at later time points. This suggest that under low NO_3^-, C*LCa* is less inducible. But, in response to *Pst* treatment T203 grown 3 mM WT plants (Fig. 5b), significantly induced to 4 to 5 folds at 24 and 48 hpc, whereas it was greatly induced to 6 folds in *Pst* treated 0.1 mM plants at 6 hpc, which was stabilized at later time points. The uninoculated leaves of 0.1 mM T203 grown WT plants showed slight induction of *CLCa* in comparision to non-T203 grown plants (Compare Fig. 5a & 5b). We further checked the expression of *NPF1.2;* which belongs to NRT1 (peptide transporter) family of nitrate transporters (Fig. 5c) and is involved in the transfer of xylem-borne nitrate to the phloem in the petiole (Krapp *et al.* 2014). It was found that, in *Pst* treated 3 mM WT plants, *NPF1.2* levels were highly induced till 48 hpc, in comparison to 0.1 mM *Pst* inoculated leaves (Fig. 5c). This might be the reason behind resistance of 3 mM WT plants and susceptibility of 0.1 mM WT plants after pathogen challenge. But, upon T203 pre-treatment, *NPF1.2* transcript levels in *Pst*-inoculated 0.1 mM plants (Fig. 5d), showed significant

induction in comparison to same treatment without T203 (Compare Fig. 5c & 5d). This suggests that, T203 may cause the regulation of *NPF1.2*.

NRT2 transporters (*NRT2.1*, *NRT2.2* and *NRT2.4*) are HATS and gets activated at low N concentrations (<1 mM). Under optimum N conditions, *NRT2.1* expression levels in *Pst* treated 3 mM plants were highly induced from 2.5 to 6 fold post challenge. Previously, it was shown that *NRT2.1* is active only under N starving conditions (Dechorgnat *et al.* 2012). We found similar results that *NRT2.1* transcript was induced under 0.1 mM *Pst* treated plants (Fig. 6a). Likewise, the uninoculated leaves of 3 mM WT plants showed slight induction at 6 hpc, which significantly down-regulated at later time points. Whereas, in 0.1 mM uninoculated leaves of WT plants, *NRT2.1* showed sharp induction of 4.2 folds at 0 hpc, 6.3 folds at 6 hpc, which drastically reduced at later time points (Fig. 6a). This suggests that during the early initiation phase of SAR, i.e., upto 6 hpc, low N-fed WT plants tried to initiate the immunization potential by maintaining the N supply by displaying higher *NRT2.1* transcripts. The T203 pre-treatment displayed reprogrammed transcript levels of *NRT2.1* in *Pst* treated 3 mM WT plants, which showed extremely rapid induction up to 10 folds at 0 hpc, 5 folds at 6 and 24 hpc which got stabilized at >11 folds at 48 hpc. In line with this, *Pst* treated 0.1 mM T203 grown WT plants showed 14 fold up regulation at 24 hpc, which stabilized at 10 folds at 48 hpc (Fig. 6a). The SAR establishment stage represented by uninoculated leaves showed dynamic and hiked up-regulation of *NRT2.1* transcripts up to 26 folds in 24 hpc and 28 folds in 48 hpc, whereas, there was <5 folds induced level of *NRT2.1* expression in all the time points in 3 mM plants. This revealed that, T203 colonization benefits the plant by facilitating the N supply even in low NO_3^- conditions, after 48 hpc and more, thereby showing improved plant defense mediating SAR.

Further, we checked *NRT2.2* expression levels (Fig. 6b), it was observed that, in *Pst* treated 3 mM plants, there was an induction of this transcript from 1.6 to 3.2 fold at various time points. In comparison with 3 mM plants, 0.1 mM *Pst* treated plants showed very strong induction from 4 to 6 fold at 6 to 24hpc. This suggests that, the *NRT2.2* expression is highly induced in low N-fed plants facilitating their NO_3^- uptake and is also highly correlated to *Pst* treatment. The systemic (U) leaves of 3 mM plants, showed a stable and uniform *NRT2.2* transcript accumulation with at 2-4 fold at 24 and 48 hpc respectively, whereas, 0.1 mM systemic leaves showed

highest of 5.3 fold change at 6 hpc. In Fig. 6b, T203 treated *Pst* infiltrated 3 mM plants showed constant induction from 2.5 fold at 0 hpc, 10 fold at 6 hpc, and 5- 6 fold at 24 and 48 hpc respectively. In 0.1 mM *Pst* treated plants, there was very strong induction of 6 fold at 0 hpc, which hiked to 25 fold at 6 hpc, later came down to 9 fold and 7 fold in 24 and 48 hpc respectively (Fig. 6b). The uninoculated distal leaves showed remarkable 12 fold induction at 6 hpc, 6 and 3.2 fold change at 24 and 48 hpc in 3 mM plants. In 0.1 mM uninoculated WT + T203 leaves, there was rapid accumulation upto 23 folds of *NRT2.2* transcripts, 11 folds in 24 hpc and >12 folds at 48 hpc. This again revealed that faster N uptake by T203 treated low NO_3^- plants can help to defend better.

Consequently, we checked *NRT2.4* expression levels in both set of treatments. It was found that in *Pst* treated 3 mM WT plants, there was consistent induction of *NRT2.4* expression from 0 hpc (1.5 fold) to 48 hpc (<4 fold), whereas in *Pst* treated 0.1 mM plants, there was induction of 6 fold at 6 hpc, which drastically reduced to <0.5 fold in 24 and 48 hpc (Fig. 6c) was observed. The uninoculated leaves of 3 mM plants showed induced expression only at 0 hpc, but later got declined in consecutive time points to <0.1 fold, conversely, 0.1 mM plants showed higher induction of 2.5 fold at 0 hpc till 5.5 fold hike at 6 hpc. Upon T203 treatment (Fig. 6c), 3 mM *Pst* treated plants showed consistent and induced expression pattern from 2.9, 2.8, 6.5 and 4.5 fold at 0, 6, 24 and 48 hpc, whereas, 0.1 mM plants showed initial induction till 6 hpc (6 fold), but, later declined. There was only slight difference in the expression levels of 3 mM and 0.1 mM uninoculated leaves of T203 treated and untreated WT plants.

Taken together, these results suggests that Trichoderma induces HATs and LATs to facilitate N uptake under low NO_3^- conditions. To confirm the role of HATs in increasing plant defense via N uptake, we checked the *PR1* expression in WT and *nrt2.1* mutants (Fig 6d). The expression of *PR1* gene in *Trichoderma* inoculated plants increased 12-13 folds in 24 h but in the case of *nrt2.1* mutant (Fig. 6d), *PR1* gene was not at all induced suggesting that *NRT2.1* plays an important role in increasing plant defense under low nitrogen mediated by Trichoderma. Then, we further found that in *nrt2.1* mutant, the expression of *NRT2.2* gene also suppressed in both control and Trichoderma treatment (Fig. 6e) suggesting that *NRT2.1* is mainly responsible for increasing uptake of N under low NO_3^- facilitated by *Trichoderma*.

Then, we checked the protein levels in WT and *nrt2.1* mutant in the presence or absence of Trichoderma under low N. In response to Trichoderma, protein levels have increased in WT, whereas in *nrt2.1* mutant protein levels were less than WT but upon T203 treatment only slight increase (Fig. 6f) was observed suggesting T203 application leads to increase nitrate transport hence increase protein levels.

Trichoderma elicits NO production during early stages of inoculation which is required for induction of HATs and PR gene expression

Previously, it was shown that Trichoderma elicits NO at early stages (Gupta *et al.* 2014). This NO could have a role in induction of transporter genes. Hence, we checked NO production in WT, *nia1,2* mutants and WT seedlings grown on NO scavenger cPTIO inoculated with Trichoderma. Control roots in 0.1 and 3 mM NO_3^- produced reduced levels of NO. Within 2 min of T203 application, WT plants showed much increased levels of NO in both 0.1 and 3 mM nitrate (Fig. 7) but the increase was slightly higher in 0.1 mM than 3 mM. But, in 10 minutes and 24 h of T203 incubation, the WT plants showed extremely low fluorescence (Fig. 7). This suggests T203 greatly induces NO in low NO_3^- grown plants within 2 min of Trichoderma application. Thus, Trichoderma induced NO probably plays a role in priming and induction of LATs and HATs. Assessment of NO in *nia1,2* mutant revealed that NR is responsible for NO production. C PTIO grown plants showed reduced levels of NO.

Then, we further checked the importance of Trichoderma elicited early stages of NO in the induction of HATs, total protein levels and expression of *PR1* gene in low NO producing phytoglobin over-expressing *nsHb*$^+$ line, *nia1,2* and *nrt2.1* mutants. Expression of *NRT2.1* and *2.2* levels have increased in WT in response to Trichoderma, whereas in Hb$^+$, *nia1,2* and *nrt2.1* mutants these two HATs were not induced (Fig 8a & 8b) suggesting that Trichoderma elicited NO is responsible induction of HATs. Further reduced protein levels were observed in in *nia1,2*, Hb$^+$ plants suggesting that induction of transporters mediated by *Trichoderma* elicited NO plays a role in increased N uptake under low N (Fig. 8c), further, we found that *nia1,2* and Hb$^+$ were unable to induce *PR1* expression under these conditions. Taken together, these results suggests that *Trichoderma* elicited NO plays a role in overall increase in defense response under low N.

Further, we investigated the role of NO in LAR and SAR development in the presence of Trichoderma. Under low NO_3^-, WT plants performed better LAR response than Hb^+ and *nia1,2* mutant. Reduced bacterial growth and increased *PR1* gene expression was observed in WT than Hb^+ and *nia1,2* mutant (Fig. 9 a,b&c). Similarly, SAR response was also compromised in than Hb+ and *nia1,2* like LAR, a similar reduced bacterial number and increased *PR1* gene expression was observed in WT in comparison to Hb^+ and *nia1,2* mutant (Fig. 9 d,e&f).

ROS is a component of *Trichoderma* induced increased resistance via SAR

Both NO and ROS are involved in plant resistance response hence, we investigated the role of ROS. The distal un-inoculated leaves of 0.1 mM NO_3^- WT+T plants showed H_2O_2 production as concentrated patches in comparison to 0.1 mM un-inoculated leaves of WT plants (Fig. 10a-upper panel). Also, the level of H_2O_2 was intense and distributed in 3 mM un-inoculated leaves of WT+T plants in comparison to untreated plants (Fig. 10a-lower panel). This is probably due to suppression of catalase activity by SA. This suggests that T203 inoculation can enhance H_2O_2 pattern in *Pst* uninoculated leaves during SAR, whereas, both the 3 mM *Pst* inoculated leaves from WT and WT+T203 plants showed higher H_2O_2 production in the infiltrated area which is visible as a dark brown patch at 24 and 48 hpc (Fig. 10a-lower panel). This clearly suggests that T203 plays a role in inducing H_2O_2 in low NO_3^- fed WT plants during SAR.

In order to investigate that, $O_2^{-\cdot}$ are key players in cell death, we measured their levels during SAR response (Fig. 10b). It was observed that, there was increased formazan formation (dark blue spots) in inoculated leaves of both 0.1 and 3 mM fed WT and T203 treated WT plants, but increase was much higher in T203 treated WT plants than Trichoderma untreated plants (compare Fig. 10a-lower left and 10b-right panel). This suggests that there was a rapid oxidative burst (HR) which offers resistance to the plants in response to T203 treatment. These results also suggests that, T203 treatment can enhance $O_2^{-\cdot}$ levels of 0.1 mM NO_3^- grown plants to contribute towards H_2O_2 production to enhance resistance. Taken together, the coordinated interplay of H_2O_2, $O_2^{-\cdot}$ and NO leads to the HR associated cell death that generously improvises the LAR and SAR responses in low N-stress plants.

Trichoderma induces defense genes during SAR in low N-fed plants

Next, we checked the expression profiles of defense related genes (*PAL1, PR1, PR2* and *PR5*) during SAR. In both 3 mM and 0.1 mM *Pst* inoculated WT plants, *PAL1* transcripts were highly induced in all time points, but, the uninoculated leaves showed *PAL1* induction only till 6 hpc, but drastically declined at later time points (Fig. 11a-I). Trichoderma grown WT plants under 3 mM and 0.1 mM, showed even more enhanced levels of *PAL1* transcripts (Fig. 11a-II) in inoculated as well as uninoculated leaves till 48 hpc. Similar trend was observed in *PR1* (Fig. 11b), *PR2* (Fig. 11c) and *PR5* (Fig. 11d) expression profiles, which got elevated after Trichoderma application. Out of all the four defense genes examined, *PR1* gene (SA marker) displayed the extremely higher expression levels in Trichoderma grown plants suggesting that T203 might induce SA levels to a greater extent during SAR.

Trichoderma enhances expression of SAR mediated regulatory genes

Further, we checked whether Trichoderma can induce SAR response via induction of regulatory genes such as *DIR1, NPR1, SARD1 and TGA3*. The lipid transfer protein, DEFECTIVE IN INDUCED RESISTANCE1 (*DIR1*) is a key mobile component of SAR response (Maldonaldo *et al.* 2002) involved in long-distance translocation from local to distant leaves (Carella *et al.* 2015, Champigny *et al.* 2013). It was found that, DIR1 induction took place only in the initial time points i.e. 0 and 6 hpc in both 0.1 and 3 mM inoculated and uninoculated WT plants (Fig. 12a-I). But, in T203 grown plants, irrespective of early time points, a slight increased *DIR1* expression was observed at 24 and 48 hpc (Fig. 12a-II). Upon perception of SAR mobile signals, SAR master regulator, Non-Expresser of Pathogenesis-Related Genes1 (*NPR1*) is required to activate defense in challenged plants (Cao *et al.* 1997). We found a similar trend in the *NPR1* expression profile like *DIR1* in WT plants (Fig. 12b-I). Moreover, upon the *Trichoderma* treatment, the levels of *NPR1* gradually increased in 24 and 48 hpc (~10 fold) in both 0.1 and 3 mM inoculated and uninoculated challenged leaves (Fig. 12b-II).

Next, we checked the expression of SAR DEFICIENT 1 (*SARD1*) a pathogen-induced transcription factor (Zhang *et al.* 2010) and a key regulator for Isochorismate Synthase 1 (*ICS1*) and SA synthesis (Wang *et al.* 2011). A remarkably stronger induction of *SARD1* expression levels in T203 treated 0.1 mM uninoculated leaves at

6 hpc (~82 fold) revealed that Trichoderma is a potential inducer of SA biosynthesis (Fig. 12c-II), whereas the other treatments at different time points remained less than 15 fold in both the nitrate conditions (Fig.12c). Another important regulatory gene, TGA3, is an *NPR1*-interacting protein (NIPs) and is a critical component in the SA signaling mechanism regulating the expression of SA-induced genes such as *PR1* (Zhou *et al.* 2000). Upon T203 treatment, it was found that, the TGA3 levels were also re-programmed in all the treatments (Fig. 12d) suggesting that Trichoderma might act as channel in SA signaling pathways.

nrt2.1 and npr1 mutants are compromised in LAR and SAR

Further we checked LAR in *nrt2.1* (nitrate transporter mutant) and *npr1* (SA master regulator mutant) in response to virulent *Pst*DC3000. In 0.1 mM N-fed *nrt2.1* and *npr1* mutants developed more severe symptoms than 0.1 mM WT plants (Fig. 13a- 0.1 mM panel). Whereas the 3 mM NO_3^--fed WT plants showed less disease symptoms as compared to 0.1 mM NO_3^- plants (Compare Fig 1a and Fig. 13a- 3 mM panel). In order to check plant resistance, *in planta* bacterial number were determined (Fig 13b). A continuous increase in CFU count was observed in 0.1 mM grown WT, *nrt2.1* and *npr1* at 24 and 48 hpi in comparison to bacterial numbers in 3 mM NO_3^- grown plants. This suggests that optimum N concentration plays an important role in resistance response towards virulent *Pst* DC3000.

Similarly, we studied the SAR response in the WT, *nrt2.1* and *npr1* plants. 0.1 mM Pst-challenged WT plants show more prominent necrotic and discolored lesions spread to half of the leaf (Fig. 2a) confirming susceptible symptoms (yellow specks). On the other hand, the mutant's *nrt2.1* and *npr1* showed even more disease symptoms (extensive chlorosis and necrosis) (Fig. 13c) under low NO_3^- conditions. Results suggests (Fig. 13c) that, 3 mM NO_3^- plants defended better during secondary challenge than 0.1 mM NO_3^- plants. Bacterial populations also increased in *nrt2.1* and *npr1* mutants (Fig. 13d) suggesting that *NRT2.1* and *NPR1* plays role in increasing defense mediated by Trichoderma.

Salicylic acid pathway is part of enhanced plant resistance under low N mediated by *Trichoderma*

Examination of SA levels revealed that Trichoderma presence accelerated total SA levels in 0.1 mM uninoculated distal leaves, in comparison to 3 mM uninoculated leaves, whereas there was no significant increase in SA levels observed in inoculated leaves of 0.1 mM Trichoderma grown plants, while in 3 mM Trichoderma grown plants a significant increase in SA levels were observed (Fig. 14a). We sought to further confirm role of SA in Trichoderma increased SAR, hence *NahG* plants were challenged in the presence or absence of Trichoderma. An intense chlorotic lesion was evident in both 0.1 and 3 mM grown *NahG* in response to challenge inoculation while *Trichoderma* grown plants when challenged they defended much better which can be seen in the terms of decreased chlorotic lesions and decreased bacterial numbers (Fig. 14b& c). Surprisingly, in *Trichoderma* grown *NahG* plants showed slightly enhanced *PR1* transcript levels were observed (Fig. 14d).

Discussion

Nitrogen availability and supply can severely impact growth and development of plants (Walker *et al.* 2001; Landrein *et al.* 2018). N deficiency can cause chlorosis, which can impact photosynthesis and overall energy demand for growth and defense. Since N is also important for synthesis of various secondary metabolites, severe depletion of N can impact defense related pathways. Hence, plants may not be able to activate defense pathways for tolerance or resistance (Snoeijers *et al.* 2000). Plant take up N in the form of NH_4^+ or NO_3^- or combination of both. Different N forms differentially effect various free radicals such as NO and ROS (Wany *et al.* 2018; Gupta *et al.* 2013). Ammonium uptake and assimilation is less costly to the plants in comparison to NO_3^- but excess of NH_4^+ can cause toxic effects to the plants (Boudsocq *et al.* 2012; Liu *et al.* 2017), hence, many plants use NO_3^- as the preferable N source. Moreover, NO_3^- can help in better charge balance (Boudsocq *et al.* 2012). Nitrate nutrition can also enhance plant defence via increased generation of NO, polyamines and SA (Gupta *et al.* 2013; Fagard *et al.* 2014). Hence, we preferred to check the plant defense response under NO_3^- nutrition rather than under NH_4^+.

Plants which are grown under 0.1 mM NO_3^- showed reduced growth (Fig. S1) and reduced leaf number, chlorophyll (Fig. S2), suggesting the supplied 0.1 mM NO_3^-

is not sufficient for the plants for growth, under these conditions plants become more susceptible. In our experiments, 0.1 mM NO_3^- grown plants were compromised in both LAR and SAR (Fig. 1&2) response, suggesting that N is required for better defense. Plants grown on low N produced less SA (Fig. 14a), further supporting that N is needed for SA biosynthesis. Since plants need N for growth and disease resistance, increasing N use efficiency can help them to defend. However, very less information is available whether increasing N uptake can enhance plant resistance or not. Some group of Trichoderma help in nutrient absorption leading to increased growth and enhanced plant defense (Brotman *et al.* 2010). Previously, it was shown that the supplementation of *Trichoderma asperelloides* enhances plant growth (Brotman et *al.* 2012), and protects against abiotic and biotic stressors, moreover, it induces systemic resistance responses (Contreras-Cornejo *et al.* 2016; Brotman *et al.* 2012). Trichoderma induced increased growth attributed to auxin and ethylene (Garnica-Vergara *et al.* 2016) and increased expression of genes involved in carbon and nitrogen metabolism (Domínguez *et al.* 2016). However, there are hardly any reports on investigating the mechanism of improved plant growth and defense under N starvation mediated by Trichoderma. This study we unraveled the mechanism of Trichoderma induced plant growth and defense under low N. Hence, in this current work, we studied the impact of Trichoderma on enhancing N uptake and support in LAR & SAR response under low NO_3^- stress.

The low NO_3^- fed plants which were grown in the presence of Trichoderma, showed increased growth, increased leaf number, fresh weight and enhanced chlorophyll content (Fig. S2) suggesting that Trichoderma can enhance growth under low NO_3^- (Fig. S1a). We found that this enhanced growth is partly due to increase nitrogen uptake which was evidenced by increased expression of *NRT2.1, NRT2.2, NRT 2.4* nitrate transporters (Fig. 6a, b, c, d, e &f) and increased protein levels (Fig. 6i). Since low NO_3^- fed plants becomes susceptible (Snoeijers *et al.* 2000), it is logical to assume that Trichoderma can increase N uptake and enhance resistance. Hence, in further experiments, we focused on LAR and SAR response under low and high NO_3^- in the presence or absence of Trichoderma. During local *Pst* infection, the plants display LAR response, and the systemic/distant uninoculated leaves induces SAR response. Both are important for plants to defend against pathogens.

During SAR, after primary inoculation, within 4–6 hours, in the inoculated site, where, localized cell death (HR) occurs, plant displays hyponastic response. 3 mM N-fed WT plants showed clear hyponasty after primary inoculation, whereas 0.1 mM did not display such response (Fig. S1c). Hyponastic response is accompanied by SA release, ethylene burst and other auxin transporters present in the leaf lamina and leaf blade to let the petiole elongate (Shah and Zeier 2013). Examination of symptoms in response to primary and secondary challenge (Fig. 2a; Fig. S1b) in 3 mM and 0.1 mM N-fed plants revealed that in 3 mM WT plants the defense response after secondary challenge was more rapid, robust and even long-lasting till 5 days post challenge (data not shown) whereas, 0.1 mM WT plants showed

chlorotic and necrotic lesions, disease symptoms accompanied with reduced electrolyte leakage and increased CFU suggesting that NO_3^- concentration plays a role in LAR and SAR development. Mock infiltrated WT plants (primary and secondary inoculation-both 10 mM $MgCl_2$) does not show any symptoms of wound induced cell death in the treatments (Fig. S2a) during SAR.

In our experiments, roots were treated with *Trichoderma* hence, we examined high and low affinity transporters to see whether *Trichoderma* modulates these transporters to enhance N uptake under low nitrate. HATs operates at <1 mM N concentrations hence, the expression of these transporters were examined in the presence or absence of *Trichoderma*. *NRT2.1* is the main HAT, localized at the plasma membrane. Previously, it was shown that these transporters becomes active during N starvation and is severely inhibited when reduced nitrate sources such as glutamine or ammonium are provided (Dechorgnat *et al.* 2012). Similarly, *NRT2* also induced under low N (Dechorgnat *et al.* (2012). In response to *Trichoderma* treatment, a rapid re-programmed *NRT2.1, NRT2.2* transcripts in both 0.1 and 3 mM was observed during *Pst* inoculation. There was extremely high levels of *NRT2.1* expression in 0.1 mM NO_3^- in both inoculated and uninoculated leaves (Fig. 6b), suggesting that *Trichoderma* can induce HATs at very high levels under low NO_3^-. This feature is very important since plants are NO_3^- starved. *NRT2.1* involvement was further evidenced by the fact that *nrt2.1* mutant produced less protein under low N and *Trichoderma* was unable to increase protein content in this mutant (Fig. 6f). This mutant become very much susceptible to LAR and SAR (Fig. 13).

LATs operates relatively under high N concentrations >50 mM. In our study we examined *CLCa* and *NPF1.2*. In both cases, we found slightly enhanced expression of these genes in response to Trichoderma growth. The expression levels have increased further in inoculated and uninoculated leaves suggesting that Trichoderma can induce expression of LATs even when grown under low NO_3^- suggesting active participation of this opportunistic fungus in activating nitrate transporters proteins irrespective of their affinity (Fig. 5) to nitrate.

We further unraveled the mechanism behind increased expression of LATs and HATs mediated by *Trichoderma*. One of the features of *Trichoderma* is the induction of short-term NO and ROS. These molecules play important role in induction of plant defense responses (Gupta *et al.* 2014). We suspected the role of NO in activation genes of these transporters. *Trichoderma* induced increase expression of *NRTs* are most likely mediated by short term increase in NO upon *Trichoderma* inoculation. An increased NO was observed in WT but not in *nia1,2* mutant suggesting that the NO production is mediated by nitrate reductase. The NO elicited by *Trichoderma* probably responsible for the increased expression of HATs as our experiments revealed that in *nia1,2* and Hb^+, despite of growing in the presence of *Trichoderma* were unable to induce HATs they showed decline in protein levels (Fig. 8c). *Trichoderma* triggers SA dependent SAR pathway (Pieterse *et al.* 2014). *Trichoderma* spp. induced ROS is the plant's resistance response against many biotic stressors (Asmawati *et al.* 2017). It also plays an important role in hypersensitive cell death together with NO (Durner and Klessig 1999, Dorey *et al.* 1999). Among ROS, H_2O_2 is the most stable form of ROIs that play an important role as signal transducer in the plant cell death process (Pieterse *et al.* 2014), and acts as key modulators of NO in triggering cell death. As shown in Fig. 10a, it is observed that, T203 treated low N fed plants displayed increased H_2O_2 levels (Fig. S4 a&b), thus enabling the SAR initiation and the establishment stage. Superoxides ($O_2^{-\cdot}$) are mainly produced via mitochondrial electron transport and NADPH oxidase chain during stress. Pathogens usually employ this mitochondrial disturbance as a strategy to suppress host immunity and thus, increased ROS may contribute some resistance to plants against pathogen attack (Torres *et al.* 2002, 2005). Earlier, it was shown that the extracellular elicitors isolated from *Trichoderma viridae* also induces the $O_2^{-\cdot}$ levels (Calderon *et al.* 1994). Here, we also found that T203 grown, 0.1 mM NO_3^-,

Pst inoculated plants show higher $O_2^{-\cdot}$ levels than untreated ones (Fig. 10b, Fig. S4c), suggesting that Trichoderma can enhance $O_2^{-\cdot}$ production during infection which can aid in defense.

Consequently, the expression of defense marker genes such as *PR1*, *PR2*, *PR5* and *PAL1* (Fig. 11) were also highly induced in the presence of *Trichoderma* in low N-fed plants suggesting that Trichoderma mediated ROS, NO along with increased N probably responsible for higher induction of these genes. Martinez-Medina *et al.* (2013), reported several Trichoderma strains are known to induce systemic responses by acting as a "short circuit" in plant defense signaling. *PAL1* is an important marker gene in SA mediated defense (Kim and Hwang 2014) and accumulates in cells undergoing HR (Dorey *et al.* 1997) and to be essential for local and systemic resistance (Delaney *et al.* 1994). Apart from showing defense, *PAL1* is also an important precursor in phenylpropanoid pathway, which is induced under nutrient stress (Lea *et al.* 2007). Upon T203 pre-treatment to low N fed plants, *PAL1* expression levels were maintained in the inoculated as well as uninoculated systemic leaves which revealed that T203 induces systemic responses in uninoculated organs by mobilizing *PAL1* transcripts (Brotman *et al.* 2012; Shoresh and Harman 2008; Yedidia *et al.* 2003). SAR response is associated with a specific set of SAR genes, including genes encoding pathogenesis related (PR) proteins (Pieterse *et al.* 1996). *PR* gene activates and accumulates during SAR (Fig. 11b,c, and d) which correlated with findings of Brotman *et al.* (2012) and Pieterse *et al.* (1996).

In our study, SAR regulatory genes *DIR1*, *NPR1* and *TGA3* are induced in the presence of Trichoderma under low N stress. The *npr1* mutant compromised in LAR and SAR in the presence of *Trichoderma* suggesting the role of this gene in enhancing defense. In *nahg* mutant, defense responses are slightly enhanced in the presence of Trichoderma despite of reduced SA levels, suggesting that apart from SA other factors such as increased NO, ROS probably responsible for slight enhancement of defense in this mutant. Overall this study proved optimum N is required for LAR and SAR. Trichoderma can enhance N uptake via modulating N transporters via eliciting short term NO under nitrate nutrition. The enhanced N uptake plays a role in enhancing SA levels and defense gene expression in local and distal levels to increase overall plant defense. These defense responses are not

activated in *npr1, nahg, nrt2.1, Hb*$^+$ and *nia1,2* mutant providing strong evidence that Trichoderma mediated enhanced resistance under low nitrate involves, NO, ROS, SA.

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Figure legends

Fig. 1: **LAR response elicited by *Pst*DC3000/*avrRpm1* is compromised in low nitrate grown WT plants**

(a) *Pst*DC3000-*avrRpm1* mediated HR observed in 3mM and 0.1 mM NO_3^- grown WT plants at different time points. An early HR response observed in 3 mM *Pst* treated WT plants immediately after 4 hpi whereas 0.1 mM *Pst* treated WT plants showed extensive leaf discoloration and necrotic lesions shown by red arrows at 24 and 48 hpi. **(b)** Electrolyte leakage from leaf areas of 0.1 mM and 3 mM NO_3^- plants infiltrated with *Pst* and 10 mM $MgCl_2$. Data are mean values ± SE. A significant difference between all treatments is analyzed by t-test at $p< 0.001$ (***), $p<0.01$ (**) and $p<0.05$ (*) with 3 mM $MgCl_2$ as control. **(c)** Bacterial number in terms of colony forming units (CFU) in *Pst* infiltrated leaves of 0.1 mM and 3 mM NO_3^- plants

immediately after infiltration (0 hpi). Data are mean values ± SE. A significant difference between all treatments is analyzed by t-test at p<0.001 (***), p<0.01 (**) and p<0.05 (*) with 3 mM nitrate as control. **(d)** Relative *PR1* transcript levels from 0.1 and 3 mM *Pst* treated leaves at different time points. Data are mean values ± SE. Asterisks indicate statistical significance between 0.1 mM and 3 mM NO_3^- plants is analyzed by t-test at p<0.001 (***), p<0.01 (**). All the results obtained are representative of three independent experiments. Representative images of leaves after *Pst* infiltration taken at respective time points is shown here.

Fig. 2: **Low nitrate grown WT plants show susceptible systemic responses during SAR**

(a) Lesion development post primary inoculation (first panel) and secondary challenge (second panel) in inoculated and uninoculated leaves of 0.1 mM and 3 mM NO_3^- plants. A characteristic lesion pattern in inoculated leaves of both 0.1 and 3 mM NO_3^- plants is noted **(b)** Histochemical staining for the detection of HR- mediated cell death post secondary challenge in inoculated and uninoculated leaves of 0.1 mM and 3 mM NO_3^- plants. Scale bar- 1mm **(c)** Electrolyte leakage observed post primary (first panel) inoculation and secondary challenge (second panel) in inoculated and uninoculated leaves of 0.1 mM and 3 mM NO_3^- plants. A significant difference between all treatments is analyzed by t-test at p<0.05 (*) with 3 mM WT $MgCl_2$ as control **(d)** Bacterial number in log CFU observed post secondary challenge in inoculated leaves of 0.1 mM and 3 mM NO_3^- plants. A significant difference between all treatments is analyzed by t-test at p<0.001 (***), p<0.01 (**) and p<0.05 (*).

Fig. 3: **Trichoderma supplementation enhances LAR response in low nitrate grown WT plants**

(a) *Pst*DC3000-*avrRpm1* induced HR in 0.1 and 3 mM WT plants at different time points after Trichoderma (T203) supplementation. The images are representative of three independent experiments. First panel shows the mock infiltrated leaves i.e. control leaves treated with 10 mM $MgCl_2$ and second panel shows *Pst* infiltrated leaves of 0.1 and 3 mM NO_3^- concentrations **(b)** Electrolyte leakage from 0.1 mM and 3 mM WT + T203 treated plants infiltrated with *Pst* and 10 mM $MgCl_2$. Data are mean values ± SE. A significant difference between all treatments is analyzed by t-

test at p<0.001 (***) and p<0.01 (**) with 3 mM WT-$MgCl_2$ as control **(c)** Bacterial number in terms of log CFU in *Pst* infiltrated leaves of T203 treated 0.1 mM and 3 mM NO_3^- WT plants. Data are mean values ± SE. A significant difference between all treatments is analyzed by t-test at p<0.001 (***), p<0.01 (**) and p<0.05 (*) **(d)** Relative *PR1* transcript levels from 0.1 and 3 mM *Pst* treated leaves of T203 treated WT plants at different time points (hpi). Data are mean values ± SE. Asterisks indicate statistical significance between 0.1 mM and 3 mM plants is analyzed by t-test p<0.001 (***) and p<0.01 (**), by taking 3 mM plants as control.

Fig. 4: **Trichoderma enhances SAR response in low nitrate plants**

(a) HR development post primary inoculation (first panel and secondary challenge (second panel) in inoculated and uninoculated leaves of 0.1 mM and 3 mM T203 treated WT plants. Note a characteristic lesion pattern in inoculated leaves of both 0.1 and 3 mM plants

(b) Histochemical staining for the detection of HR-mediated cell death post secondary challenge in inoculated and uninoculated leaves of 0.1 mM and 3 mM NO_3^- , T203 treated WT plants. Scale bar- 1 mm **(c)** Electrolyte leakage observed post primary (first panel) inoculation and secondary challenge (second panel) in inoculated and uninoculated leaves of 0.1 mM and 3 mM T203 treated WT plants. A significant difference between all treatments is analyzed by t-test at p<0.001 (***), p<0.01 (**) and p<0.05 (*) with 3 mM WT $MgCl_2$ as control **(d)** Bacterial number in log CFU observed post secondary challenge in inoculated leaves of 0.1 mM and 3 mM, T203 treated WT plants. A significant difference between 0.1 and 3 mM NO_3^- is analyzed by t-test at p<0.001 (***), p<0.01 (**) and p<0.05 (*).

Fig. 5: **Expression profile of low affinity nitrate transporters genes (LATs) during SAR**

(a) Relative expression of *CLCa* gene in WT shown in (I panel) and WT+T203 shown in (II panel) grown under 0.1 mM and 3 mM NO_3^- concentration, post secondary challenge in both inoculated and un-inoculated leaves **(b)** Relative expression of *NPF1.2* gene in WT shown in (I panel) and WT+T203 shown in (II panel) grown under 0.1 mM and 3 mM NO_3^- concentration post secondary challenge in both inoculated and un-inoculated leaves. For all the target genes, fold expression values are means (n=3) ± SE. A significant difference between all treatments is analyzed by

t-test at p<0.001 (***), p<0.01 (**) and p<0.05 (*) with 3 mM un-inoculated leaves as control in each gene.

Fig. 6: Expression profile of high affinity nitrate transporters genes (HATs) during SAR and response of genotypes (WT and *nrt2.1* mutant) on priming effect of 24 h-Trichoderma pre-treatment w.r.t *PR1* and *NRT2.2* expression on low nitrate WT plants

(a) Relative expression of *NRT2.1* in WT shown in (I panel) and WT+T203 shown in (II panel) grown under 0.1 mM and 3 mM NO_3^- concentration post secondary challenge in both inoculated and uninoculated leaves **(b)** Relative expression of *NRT2.2* in WT shown in (I panel) and WT+T203 shown in (II panel) grown under 0.1 mM and 3 mM NO_3^- concentration post secondary challenge in both inoculated and uninoculated leaves. **(c)** Relative expression of *NRT2.4* in WT shown in (I panel) and WT+T203 shown in (II panel) grown under 0.1 mM and 3 mM NO_3^- concentration post secondary challenge in both inoculated and un-inoculated leaves. For all the target genes, fold expression values are means (n=3) ± SE. A significant difference between all treatments is analyzed by t-test at p<0.001 (***), p<0.01 (**) and p<0.05 (*) with 3 mM un-inoculated leaves as control in each gene.

(d) Relative *PR1* expression in roots of WT and *nrt2.1* plants grown under low (0.1 mM) nitrate concentration (with and without T203 treatment; 24 h). A significant difference between with and without T203 treatment is analyzed by t-test at p<0.01 (**) and p<0.05 (*) **(e)** Relative *NRT2.2* expression in roots of WT and *nrt2.1* plants grown under 0.1 mM NO_3^- concentration (with and without T203 treatment; 24 h). **(f)** Protein levels in WT and *nrt2.1* plants grown under 0.1 mM NO_3^- concentration in the presence or absence of T-203. A significant difference between with and without T203 treatment is analyzed by t-test at p<0.01 (**) and p<0.05 (*).

Fig. 7: Visualization of nitric oxide by diaminofluorescein (DAF) fluorescence

Nitric oxide estimation by diaminofluorescein (DAF-FM) fluorescence under 0.1 and 3 mM NO_3^- concentrations in I. WT, II. *nia1,2* and III. cPTIO (100 µM) grown WT plants during Trichoderma supplementation The experiment was performed three times independently with similar results.

Fig. 8: Response of the genotypes during early stages of Trichoderma inoculation on *NRT2.1, 2.2* expression, protein levels and *PR1* expression

(a) Relative *NRT2.1* expression in roots of WT, Hb+, *nia1,2* and *nrt2.1* plants grown under 0.1 mM and 3 mM NO_3^- concentrations, with and without T203 treatment, given for 5 minutes and 24 h to the plants. A significant difference between with and without T203 treatment is analyzed by t-test at p<0.01 (**) and p<0.05 (*) **(b)** Relative *NRT2.2* expression in roots of WT, Hb^+, *nia1,2* and *nrt2.1* plants grown under 0.1 mM and 3 mM NO_3^-concentrations, with and without T203 treatment, given for 5 minutes and 24 h to the plants. A significant difference between with and without T203 treatment is analyzed by t-test at p<0.01 (**) and p<0.05 (*) **(c)** Protein levels measured in WT, Hb^+ and *nia1,2* seedlings grown under 0.1 mM NO_3^- for 15 days in vertical plates using Bradford's assay. T203 treatment was given for 24 h to the plants. A significant difference between with and without T203 treatment is analyzed by t-test at p<0.01 (**) and p<0.05 (*) **(d)** *PR1* gene expression in WT, Hb+, *nia1,2* and *nrt2.1* under 0.1 mM NO_3^- plants, with and without *Trichoderma* treatment, given for 5 minutes and 24 h to the plants. A significant difference between with and without T203 treatment is analyzed by t-test at p<0.01 (**) and p<0.05 (*).

Fig. 9: Response of WT and NO mutants (Hb^+ and *nia1,2*) grown under 0.1 mM NO_3^- during LAR and SAR

In LAR response **(a)** HR phenotype in inoculated leaves of WT, Hb^+ and *nia1,2* plants grown under 0.1 mM NO_3^- concentration at 0, 6, 24 and 48 hpi **(b)** *In planta* bacterial growth (Log CFU) at 6, 24 and 48 hpi, asterisks indicate a significant difference (p < 0.01; Student's t test) taking WT as control **(c)** Relative *PR1* expression in infiltrated leaves during localized infiltration. Values are means (n=3) ± SE. Asterisks indicate a significant difference (p < 0.01; student's t-test) taking 0 hpi as control. In SAR response **(d)** HR phenotype in inoculated and uninoculated leaves of WT, Hb^+ and *nia1,2* plants grown under 0.1 mM NO_3^- concentration post secondary challenge **(e)** Bacterial population represented by CFU per leaf disc at 6, 24 and 48 hpc, asterisks indicate a significant difference (p < 0.01; Student's t test) taking 3 mM WT as control **(f)** Relative *PR1* gene expression in infiltrated leaves

post secondary challenge. Values are means (n=3) ± SE. Asterisks indicate a significant difference ($p < 0.01$; Student's t test) taking 0 hpc as control.

Fig. 10: Detection of ROS by measuring H_2O_2 and $O_2^{-\cdot}$ in 0.1 mM and 3 mM NO_3^- WT and Trichoderma grown WT plants during SAR response

(a) DAB staining used to measure H_2O_2 levels in the inoculated leaves and uninoculated leaves after 3 days of challenge inoculation at 0, 6, 24 and 48 hpc. Representative leaves are shown, and the experiment was repeated three times with 10 leaves each. Scale bar =1 mm.

(b) NBT staining used to measure $O_2^{-\cdot}$ levels in the inoculated leaves and uninoculated leaves post challenge inoculation at 0, 6, 24 and 48 hpc. Representative leaves are shown, and the experiment was repeated three times with 10 leaves each. Scale bar =1 mm.

Fig. 11: **Expression profiles of defense related genes during SAR**

Relative expression of defense related genes in wild-type (WT) shown in (I panel) and Trichoderma treated wild-type plants (WT+T203) shown in (II panel) grown under low (0.1 mM) and optimum (3 mM) NO_3^- concentration post secondary challenge of *Pst*DC3000 in both inoculated and uninoculated leaves **(a)** Relative expression of *PAL1* **(b)** Relative expression of *PR1* **(c)** Relative expression of *PR2* **(d)** Relative expression of *PR5*. For all the target genes, fold expression values are means (n=3) ± SE. A significant difference between all treatments is analyzed by t-test at $p<0.001$ (***), $p<0.01$ (**) and $p<0.05$ (*) with 3mM uninoculated leaves as control in each gene.

Fig. 12: **Expression profiles of regulatory SAR genes**

Relative expression of defense genes in WT shown in (I panel) and WT+T203 shown in (II panel) grown under low (0.1 mM) and optimum (3 mM) NO_3^- concentration post secondary challenge of *Pst*DC3000 in both inoculated and uninoculated leaves **(a)** Relative expression of *DIR1* **(b)** Relative expression of *NPR1* **(c)** Relative expression of *SARD1* **(d)** Relative expression of *TGA3*. For all the target genes, fold expression values are means (n=3) ± SE. A significant difference between all treatments is analyzed by t-test at $p<0.001$ (***), $p<0.01$ (**) and $p<0.05$ (*) with 3mM uninoculated leaves as control in each gene.

Fig 13: (a) LAR disease symptoms in *nrt2.1* and *npr1* mutant in 0.1 and 3 mM nitrate (b) Bacterial growth in WT, *nrt2.1* and *npr1* mutant (c) SAR response in *nrt2.1* and *npr1* mutant in 0.1 and 3 mM nitrate (d) Bacterial growth in WT, *nrt2.1* and *npr1* mutant during SAR. A significant difference between with and without T203 treatment is analyzed by t-test at $p<0.01$ (**) and $p<0.05$ (*).

Fig. 14: **SA Accumulation during SAR, and response of *nahg* mutant in the presence and absence of Trichoderma during SAR**

(a) Total (free glucose conjugate) SA levels (µg g^{-1} fresh weight) were determined post challenge inoculation (24 hpc) in inoculated and uninoculated leaves of 0.1 mM and 3 mM NO_3^- fed WT plants, with and without Trichoderma. Two samples for each treatment were collected and the range of the mean is represented by the error bars. Asterisks indicate mean values that are significantly different according to ($p < 0.01$; Student's t test) with 3 mM uninoculated leaves as control (b) HR phenotype in inoculated and un-inoculated distal leaves of *nahg* mutants grown under 0.1 and 3 mM NO_3^- concentration post secondary challenge at 6, 24 and 48 hpc **(c)** Bacterial number represented by Log CFU per leaf disc at 6, 24 and 48 hpc from the inoculated leaves post secondary challenge **(f)** *PR1* gene expression in inoculated and uninoculated leaves of *nahg* mutants grown under 0.1 and 3 mM NO_3^- ,with and without *Trichoderma* treatment, post secondary challenge. Values are means (n=3) ± SE. A significant difference between with and without T203 treatment is analyzed by t-test at $p<0.01$ (**) and $p<0.05$ (*).

Supplementary Information

Supplementary Fig 1: (a) The growth pattern and phenotype of WT and T203 treated WT plants in 3 mM and 0.1 mM NO_3^- nutrition; (b). Effect of post secondary challenge on plant phenotype, 1°- primary inoculation, 2°-secondary challenge and U- uninoculated leaves; (c) Hyponastic response (petiole elongation) shown by 0.1 mM and 3 mM NO_3^- plants after primary inoculation.

Supplementary Fig 2: (a) Electrolyte leakage from mock infiltrated plants in SAR; Morphological growth parameters observed in WT and T203 treated WT plants; (b) Leaf number; (c) Biomass measured as fresh weight (FW); (d) Total chlorophyll content in *Pst* treated and untreated plants.

Supplementary Fig 3: **(a)** Enlarged microscopic view of the *Pst* inoculated leaves showing macrobursts of H_2O_2 shown by red arrows after DAB staining. Scale bar =

500 µm. **(b)** Quantification of DAB staining as intensity per stained area from ten leaves per treatment measured using ImageJ in arbitrary units with the mean values ± SE. **(c)** Quantification of NBT staining as intensity per stained area from ten leaves per treatment measured using ImageJ in arbitrary units with the mean values ± SE.

Supplementary Fig 4: **(a)** Enlarged microscopic view of the necrotic areas of only *Pst* treated 0.1 and 3 mM WT and T203 treated WT plants after trypan blue staining. Scale bar- 500µm **(b)** Asterisks indicate statistically significant differences between treatments and the time points using Tukey's post-hoc test for pairwise comparisons (α = 0.05) in only *Pst* treated leaves. Results are presented as necrotized leaf area compared with the total surface of leaf analyzed by ImageJ (ver 3.2) and represent means ± SE of 6 leaves per treatment.

Supplementary Table 1: List of primers

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Fig 1

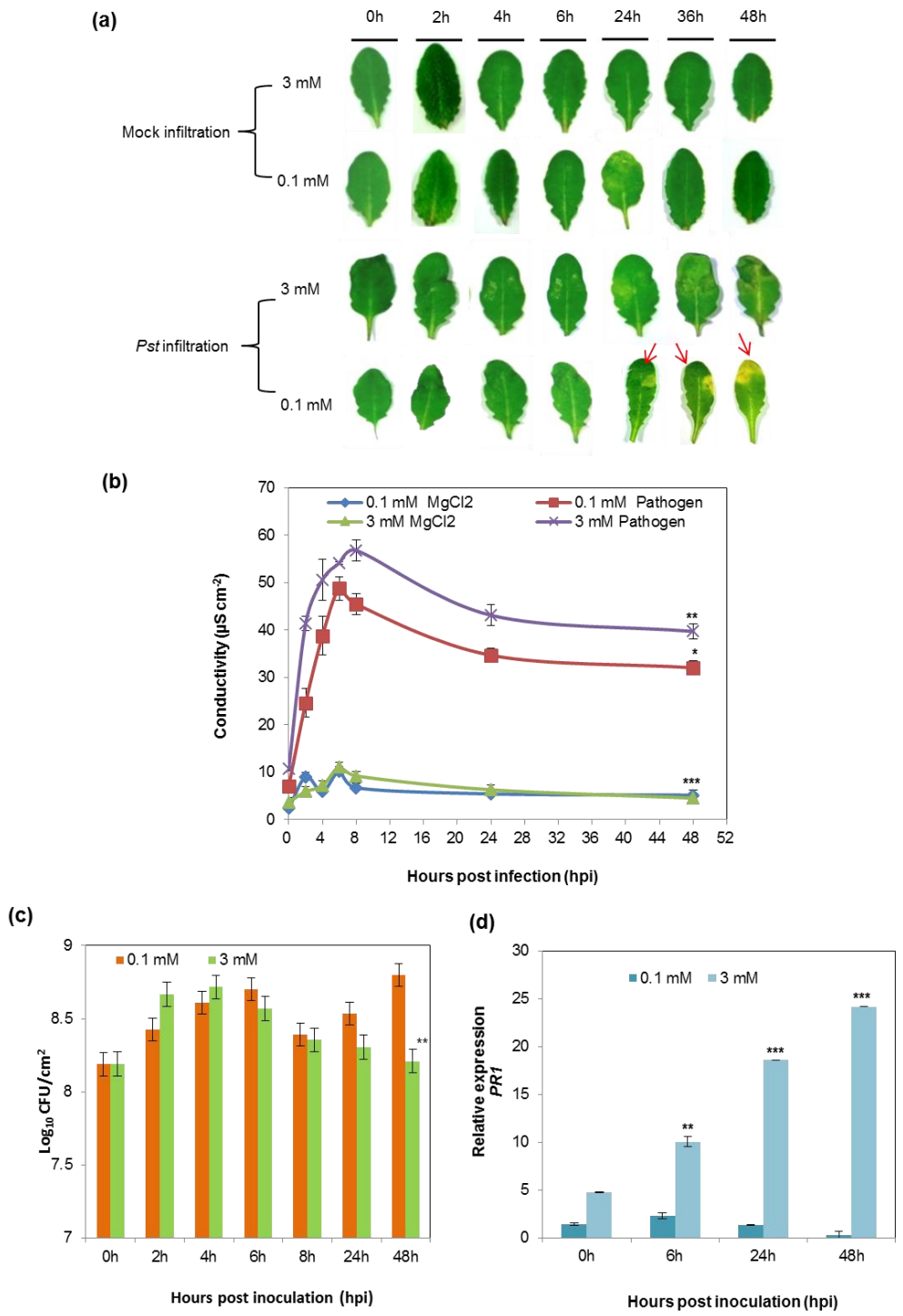

(a)
0h
2h
4h
6h
24h
36h
48h
Mock infiltration
3 mM
0.1 mM
Pst infiltration
3 mM
0.1 mM
(b)
0.1 mM MgCl2
0.1 mM Pathogen
3 mM MgCl2
3 mM Pathogen
Conductivity (µS cm-2)
Hours post infection (hpi)
(c)
0.1 mM
3 mM
Log10 CFU/cm2
Hours post inoculation (hpi)
(d)
0.1 mM
3 mM
Relative expression PR1
Hours post inoculation (hpi)

Fig. 2

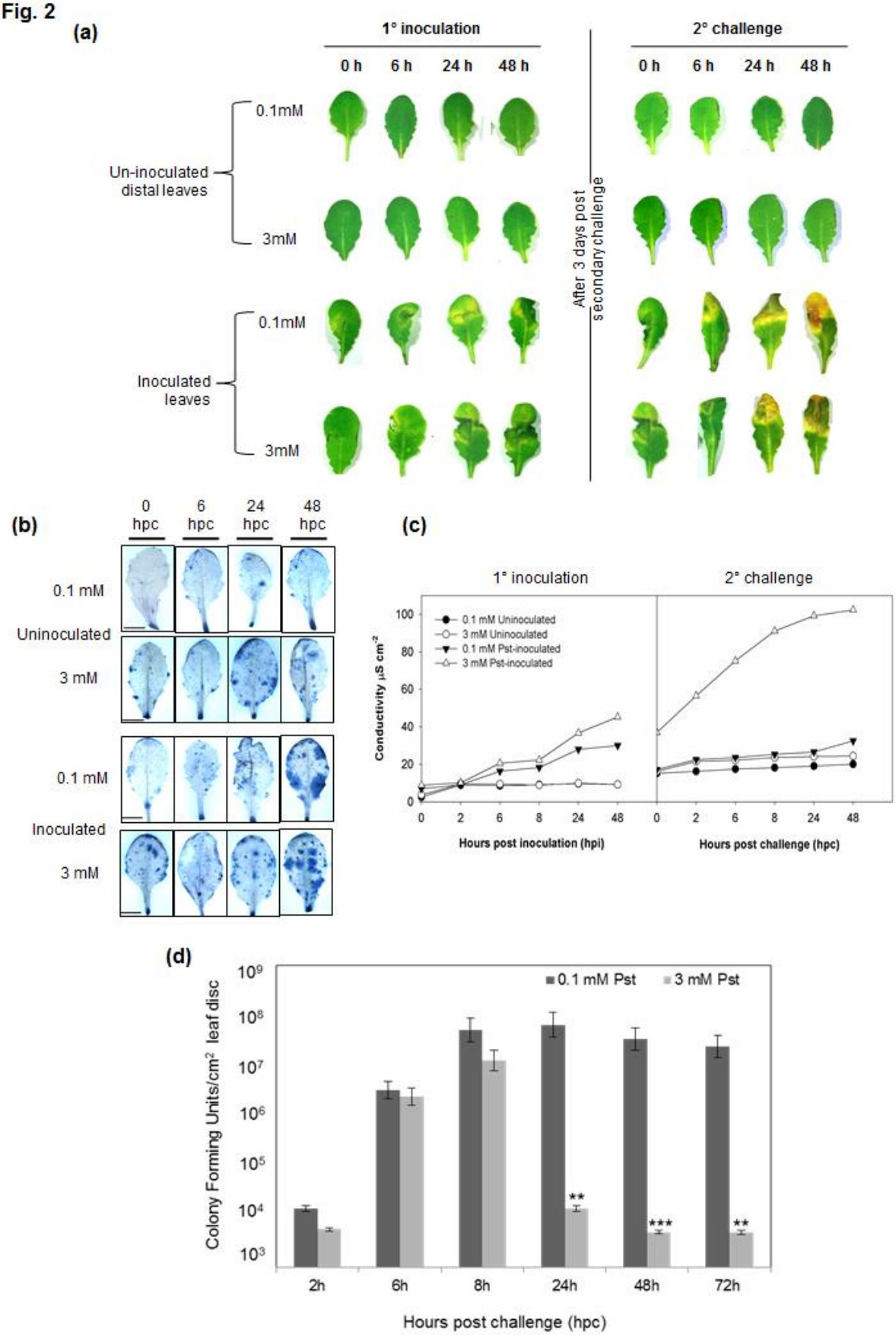

(a)
1° inoculation
2° challenge
0 h
6 h
24 h
48 h
Un-inoculated distal leaves
0.1mM
3mM
Inoculated leaves
0.1mM
3mM
After 3 days post secondary challenge
(b)
0 hpc
6 hpc
24 hpc
48 hpc
0.1 mM
Uninoculated
3 mM
0.1 mM
Inoculated
3 mM
(c)
1° inoculation
2° challenge
0.1 mM Uninoculated
3 mM Uninoculated
0.1 mM Pst-inoculated
3 mM Pst-inoculated
Conductivity µS cm⁻²
Hours post inoculation (hpi)
Hours post challenge (hpc)
(d)
0.1 mM Pst
3 mM Pst
Colony Forming Units/cm² leaf disc
2h
6h
8h
24h
48h
72h
Hours post challenge (hpc)

Fig. 3

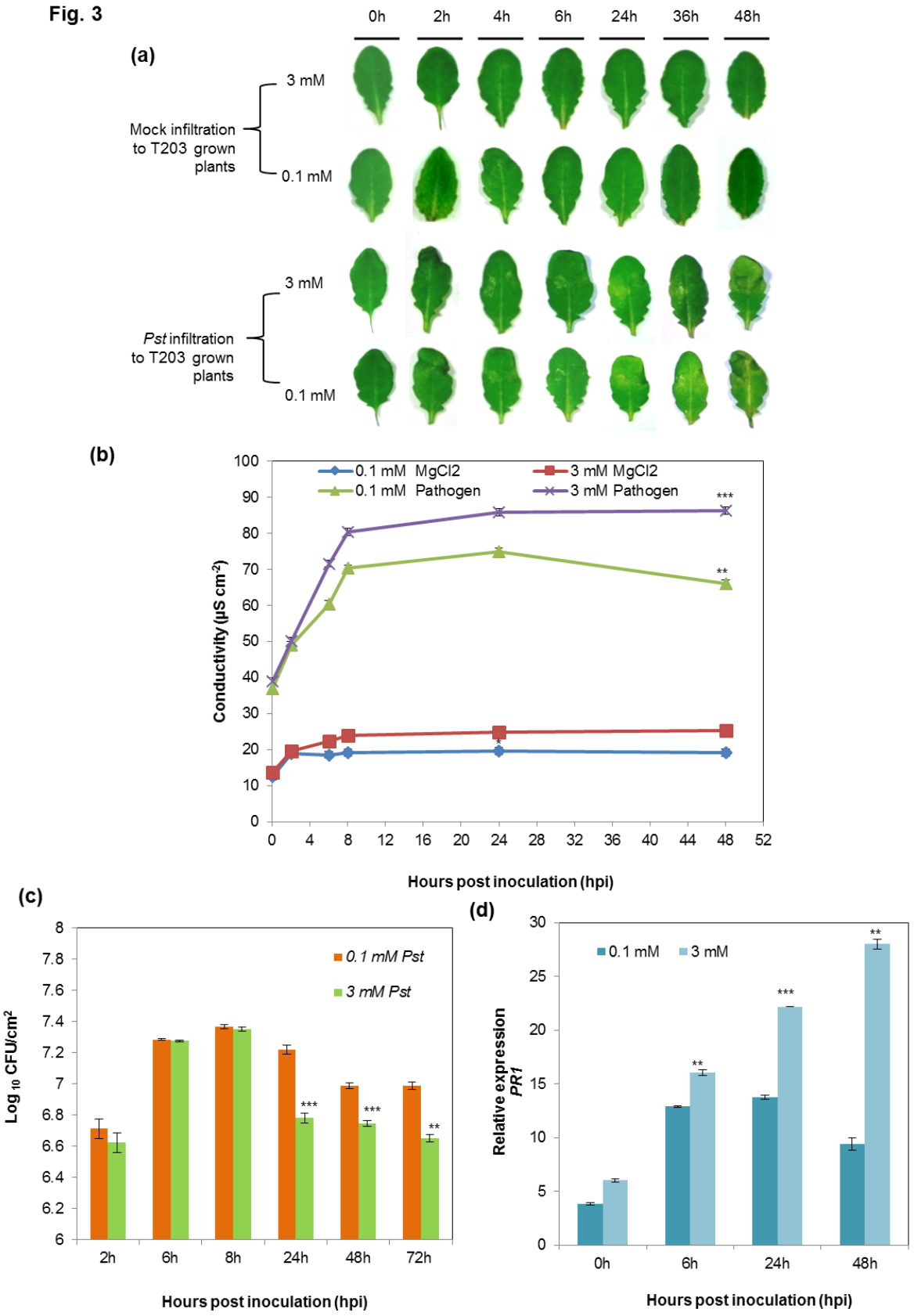

(a)
0h
2h
4h
6h
24h
36h
48h
3 mM
Mock infiltration to T203 grown plants
0.1 mM
3 mM
Pst infiltration to T203 grown plants
0.1 mM
(b)
0.1 mM MgCl2
3 mM MgCl2
0.1 mM Pathogen
3 mM Pathogen
Conductivity (µS cm-2)
Hours post inoculation (hpi)
(c)
0.1 mM Pst
3 mM Pst
Log 10 CFU/cm2
2h
6h
8h
24h
48h
72h
Hours post inoculation (hpi)
(d)
0.1 mM
3 mM
Relative expression PR1
0h
6h
24h
48h
Hours post inoculation (hpi)

Fig. 4

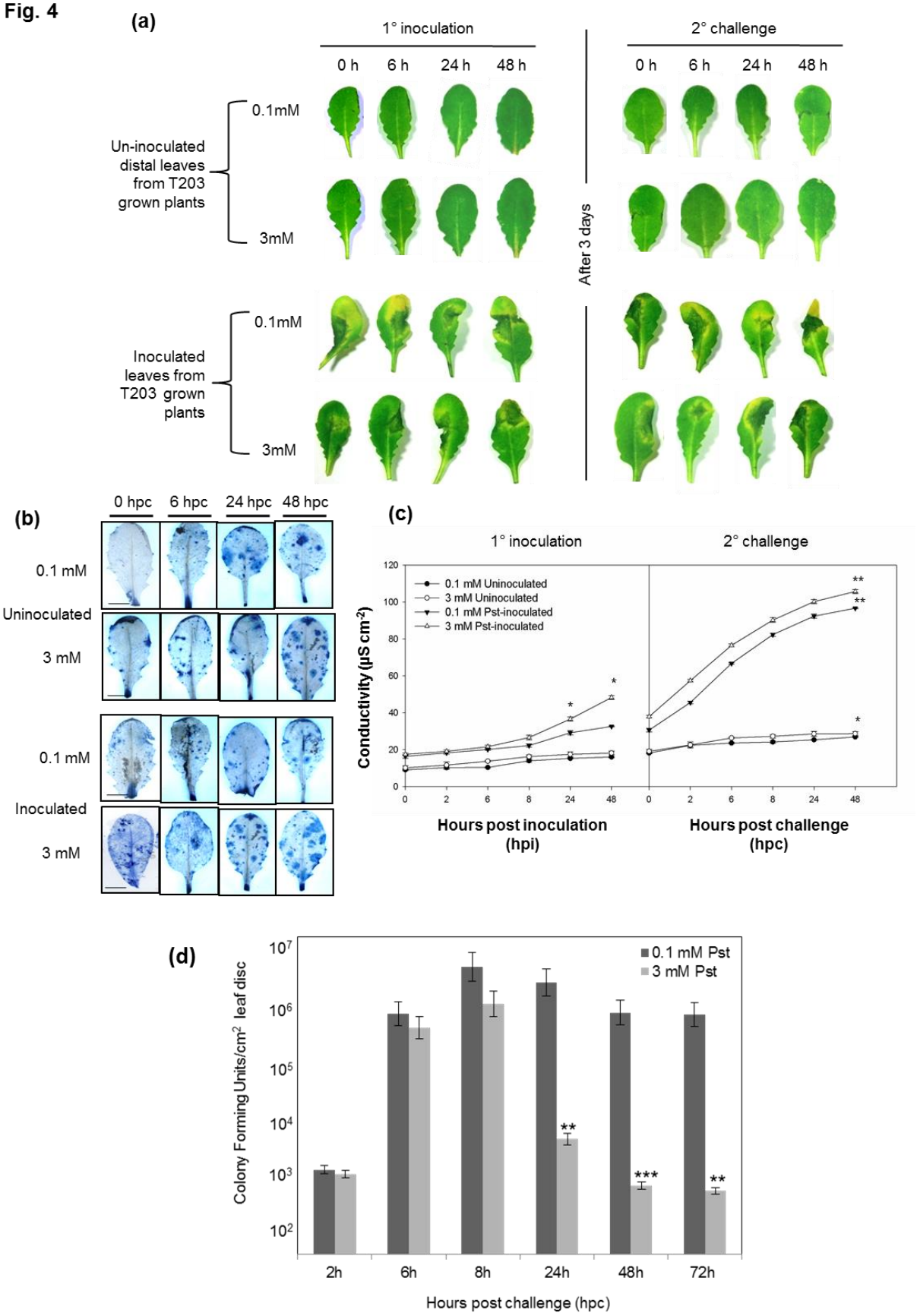

(a)
1° inoculation
2° challenge
0 h
6 h
24 h
48 h
0.1mM
3mM
Un-inoculated distal leaves from T203 grown plants
Inoculated leaves from T203 grown plants
After 3 days
(b)
0 hpc
6 hpc
24 hpc
48 hpc
0.1 mM
Uninoculated
3 mM
Inoculated
(c)
0.1 mM Uninoculated
3 mM Uninoculated
0.1 mM Pst-inoculated
3 mM Pst-inoculated
Conductivity (µS cm-2)
Hours post inoculation (hpi)
Hours post challenge (hpc)
(d)
0.1 mM Pst
3 mM Pst
Colony Forming Units/cm2 leaf disc
2h
6h
8h
24h
48h
72h
Hours post challenge (hpc)

Fig 5

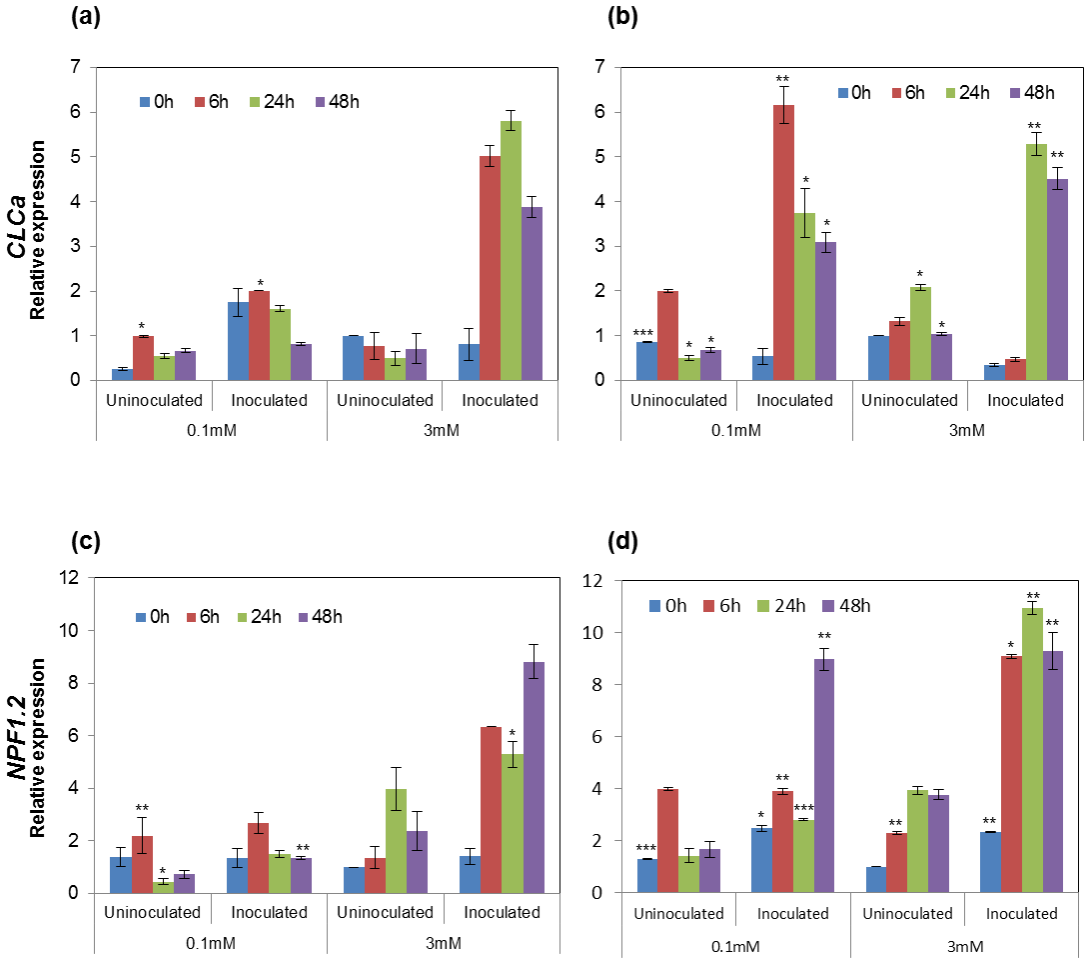

(a)
(b)
(c)
(d)
CLCa
NPF1.2
Relative expression
0h
6h
24h
48h
Uninoculated
Inoculated
0.1mM
3mM

Fig 6

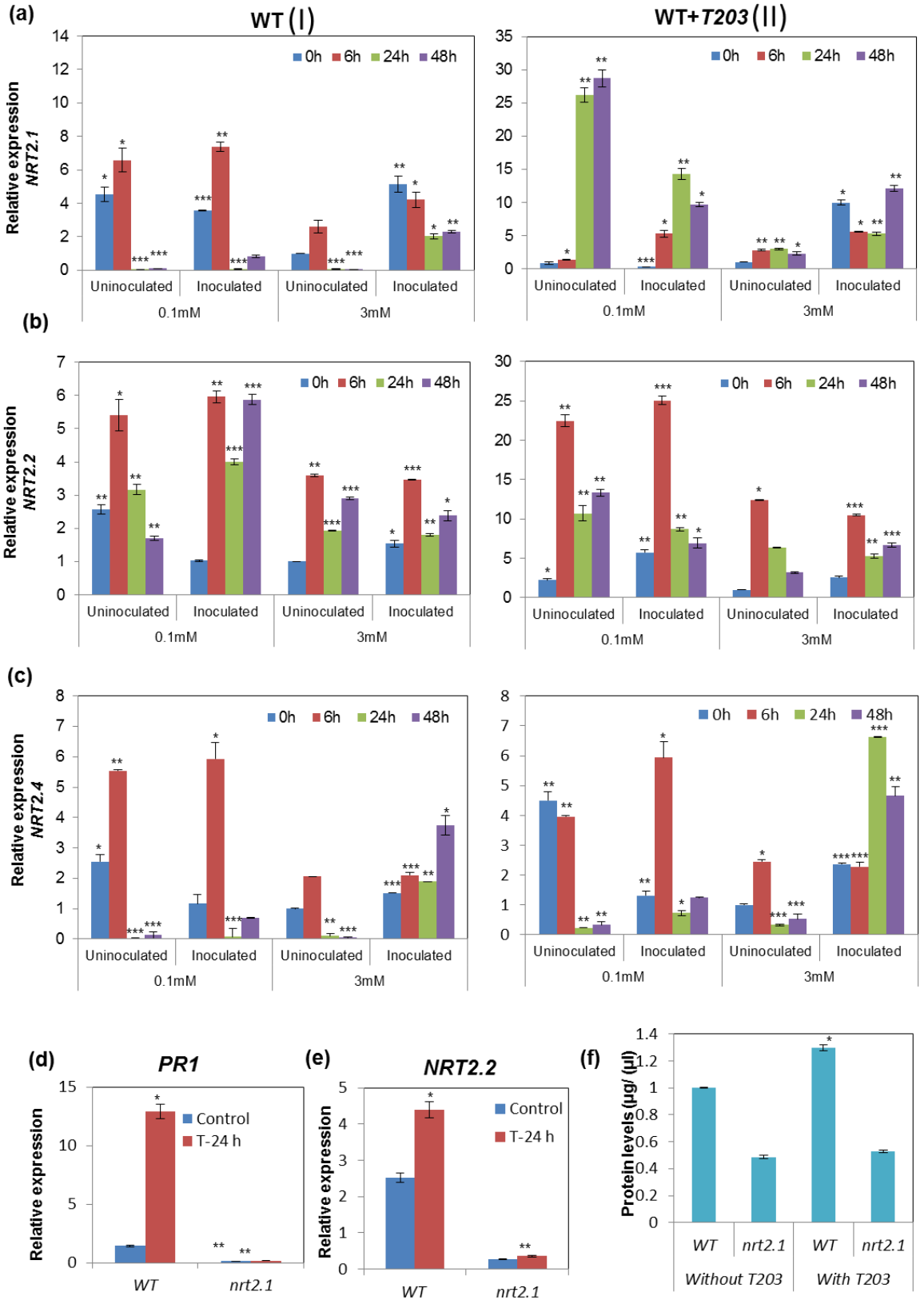

(a)
WT (I)
WT+T203 (II)
Relative expression NRT2.1
0h 6h 24h 48h
Uninoculated
Inoculated
0.1mM
3mM
(b)
Relative expression NRT2.2
(c)
Relative expression NRT2.4
(d)
PR1
Control
T-24 h
Relative expression
WT
nrt2.1
(e)
NRT2.2
(f)
Protein levels (µg/ (µl)
Without T203
With T203

Fig7

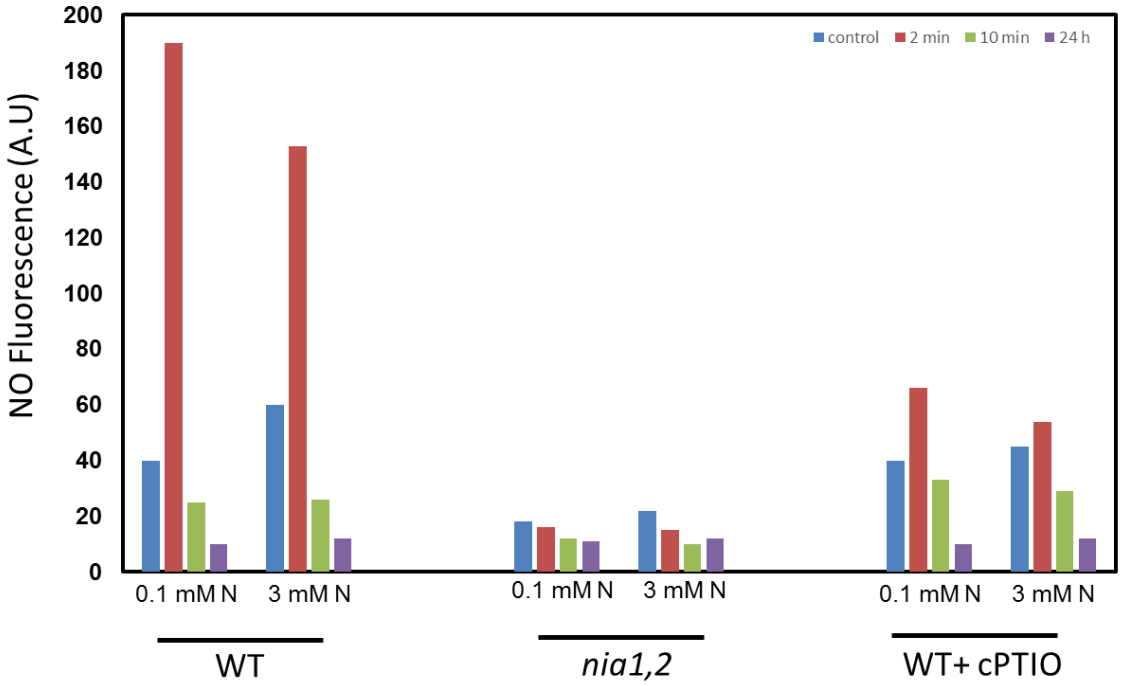

NO Fluorescence (A.U)
200
180
160
140
120
100
80
60
40
20
0
control
2 min
10 min
24 h
0.1 mM N
3 mM N
0.1 mM N
3 mM N
0.1 mM N
3 mM N
WT
nia1,2
WT+ cPTIO

Fig. 8

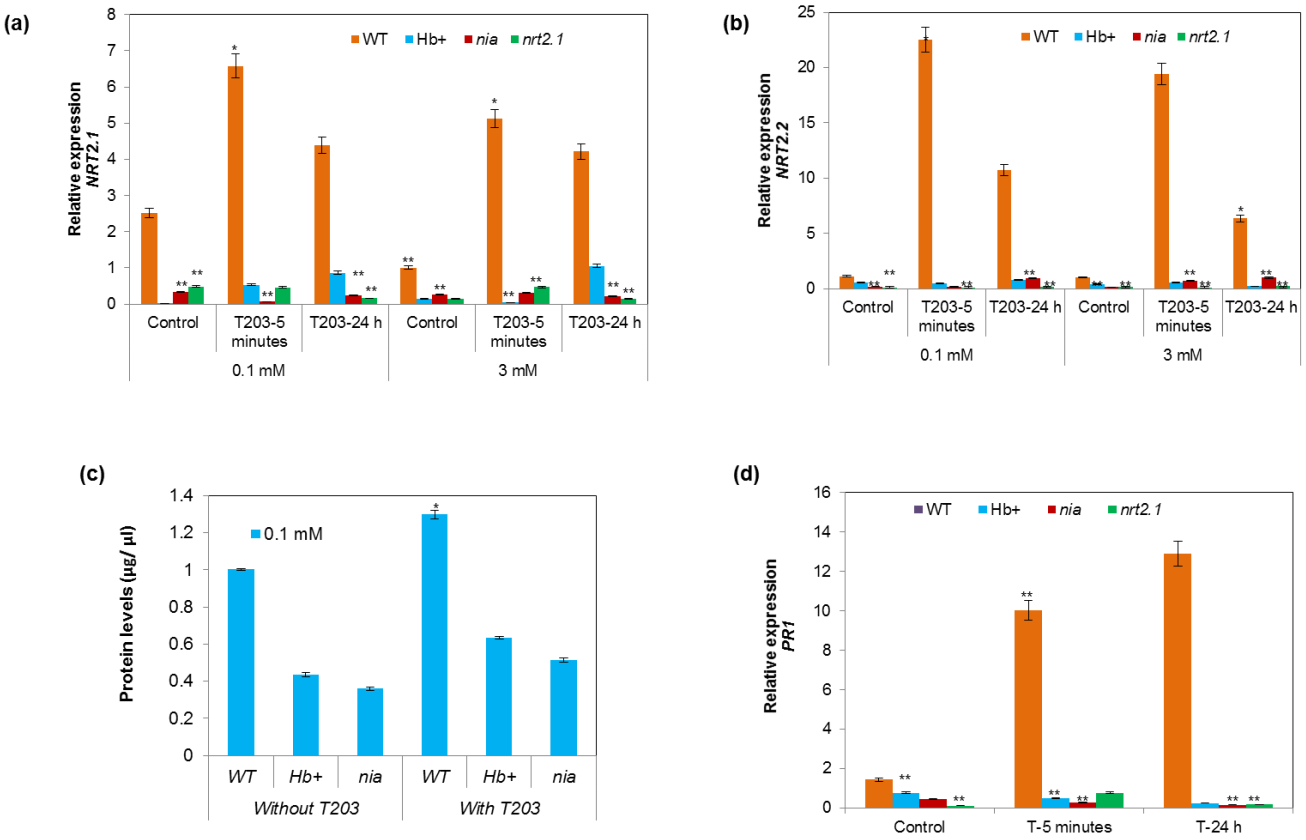

(a)
Relative expression NRT2.1
WT
Hb+
nia
nrt2.1
Control
T203-5 minutes
T203-24 h
0.1 mM
3 mM
(b)
Relative expression NRT2.2
(c)
Protein levels (µg/ µl)
0.1 mM
WT
Hb+
nia
Without T203
With T203
(d)
Relative expression PR1
Control
T-5 minutes
T-24 h

Fig. 9

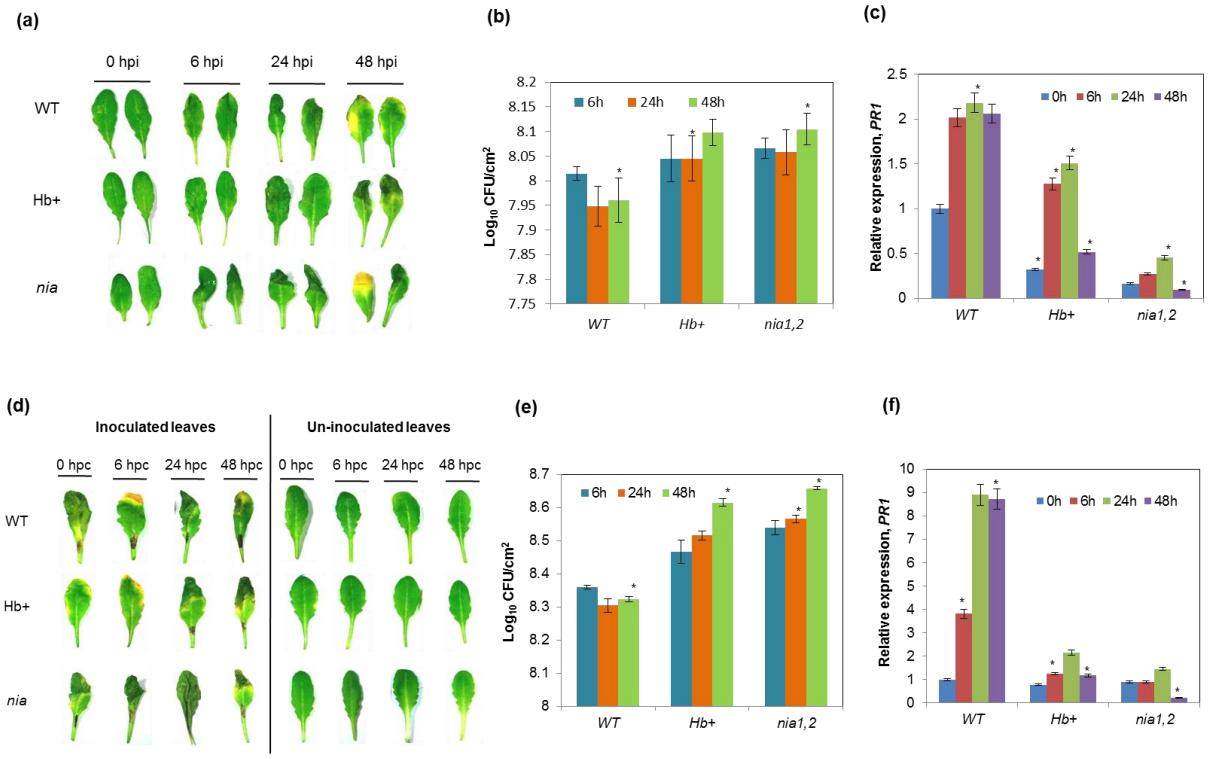

(a)
0 hpi
6 hpi
24 hpi
48 hpi
WT
Hb+
nia
(b)
6h
24h
48h
Log10 CFU/cm2
WT
Hb+
nia1,2
(c)
0h
6h
24h
48h
Relative expression, PR1
WT
Hb+
nia1,2
(d)
Inoculated leaves
Un-inoculated leaves
0 hpc
6 hpc
24 hpc
48 hpc
WT
Hb+
nia
(e)
6h
24h
48h
Log10 CFU/cm2
WT
Hb+
nia1,2
(f)
0h
6h
24h
48h
Relative expression, PR1
WT
Hb+
nia1,2

Fig. 10

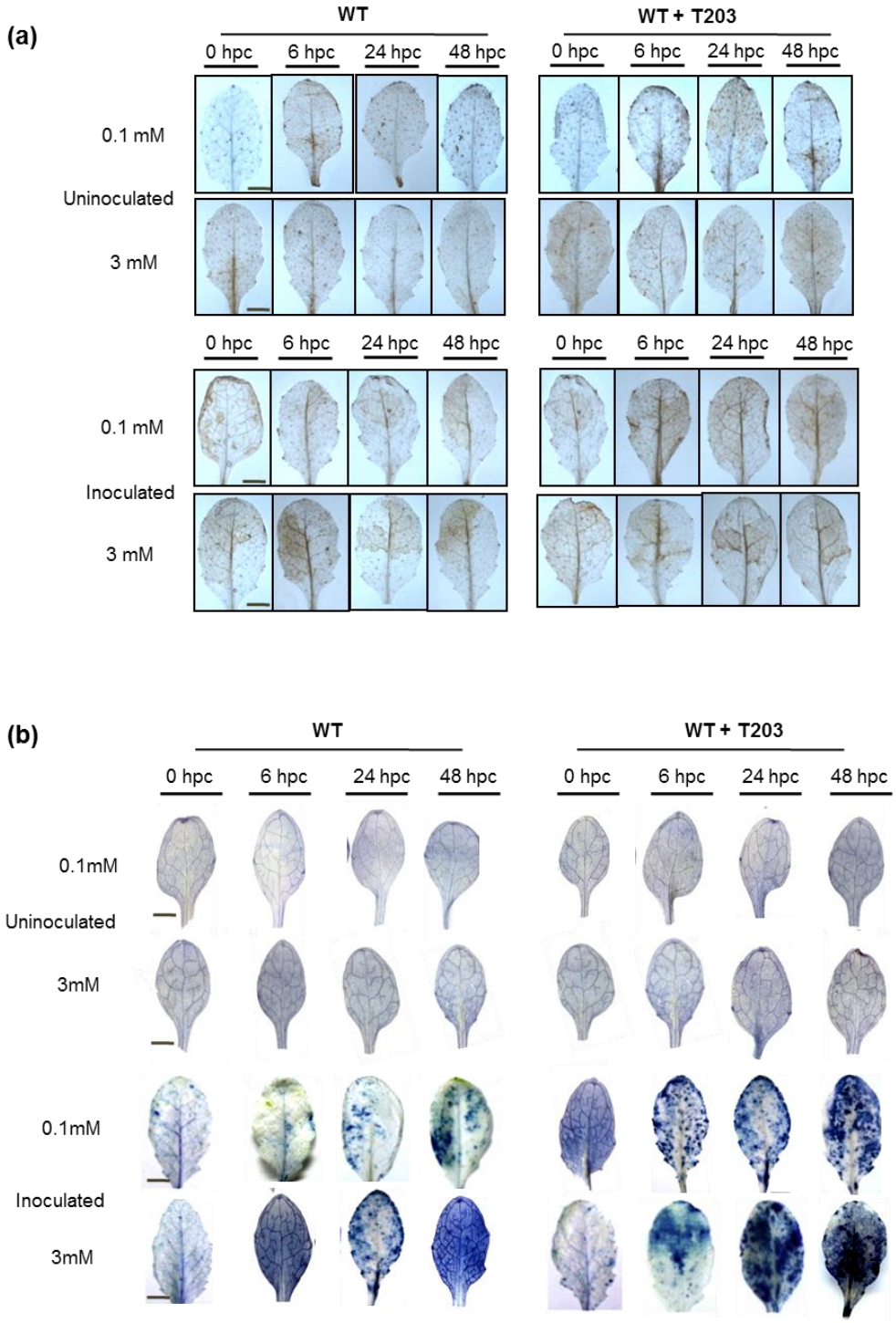

(a)
WT
WT + T203
0 hpc
6 hpc
24 hpc
48 hpc
0.1 mM
Uninoculated
3 mM
Inoculated
(b)
0.1mM
3mM

Fig. 11

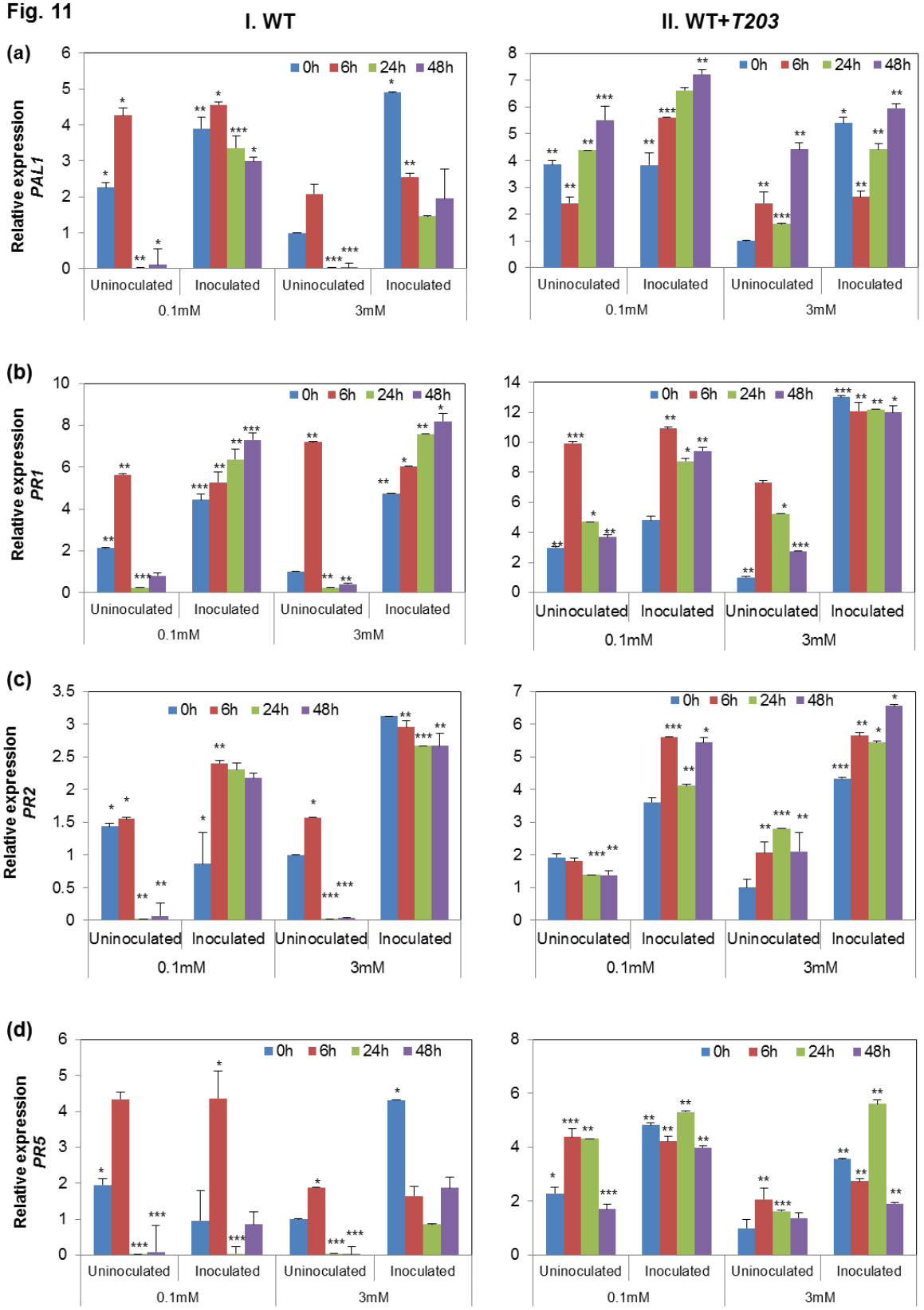

I. WT
II. WT+T203
(a)
Relative expression PAL1
(b)
Relative expression PR1
(c)
Relative expression PR2
(d)
Relative expression PR5
0h 6h 24h 48h
Uninoculated
Inoculated
0.1mM
3mM

Fig. 12

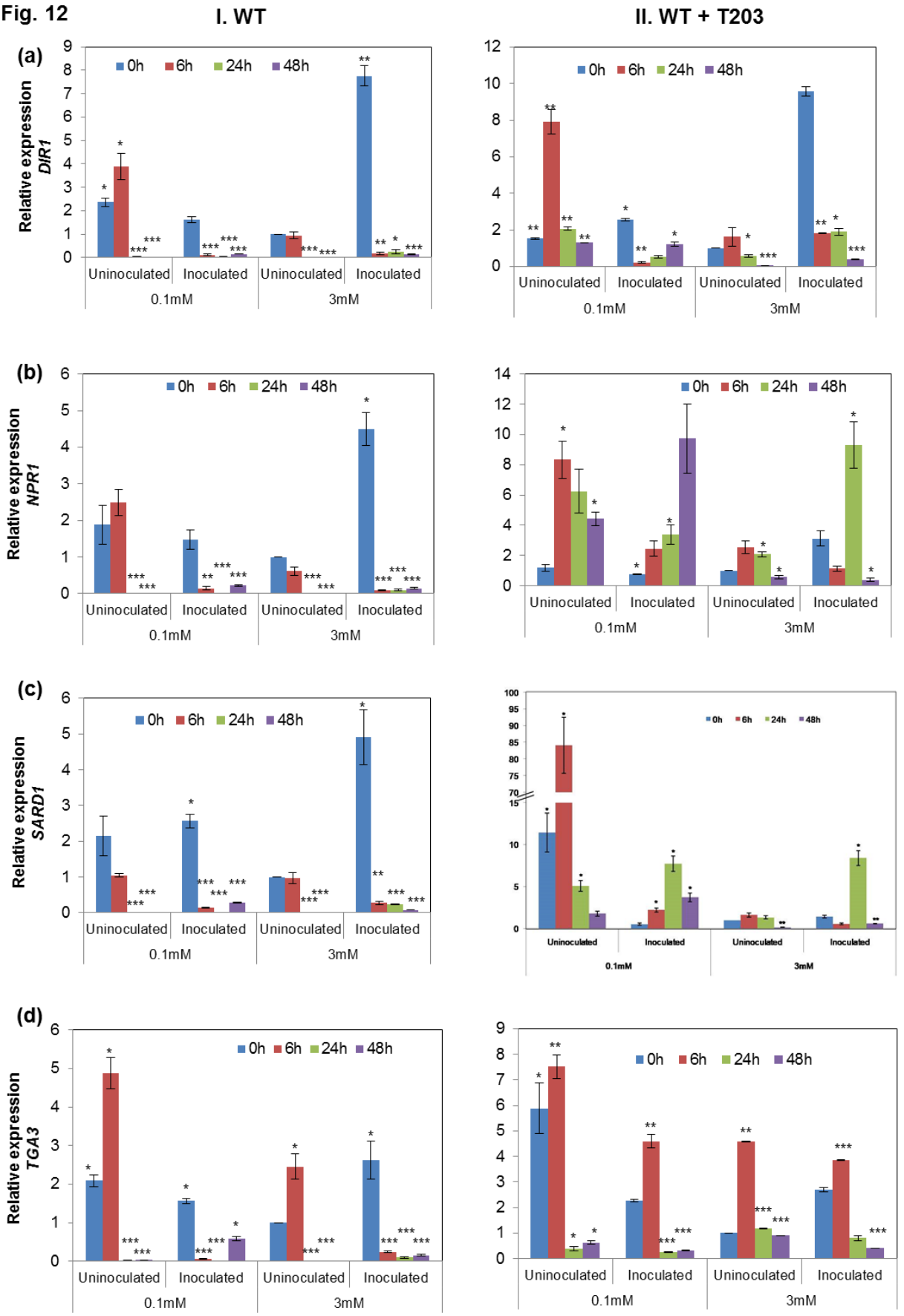

I. WT
II. WT + T203
(a)
Relative expression DIR1
(b)
Relative expression NPR1
(c)
Relative expression SARD1
(d)
Relative expression TGA3
0h 6h 24h 48h
Uninoculated
Inoculated
0.1mM
3mM

Fig. 13

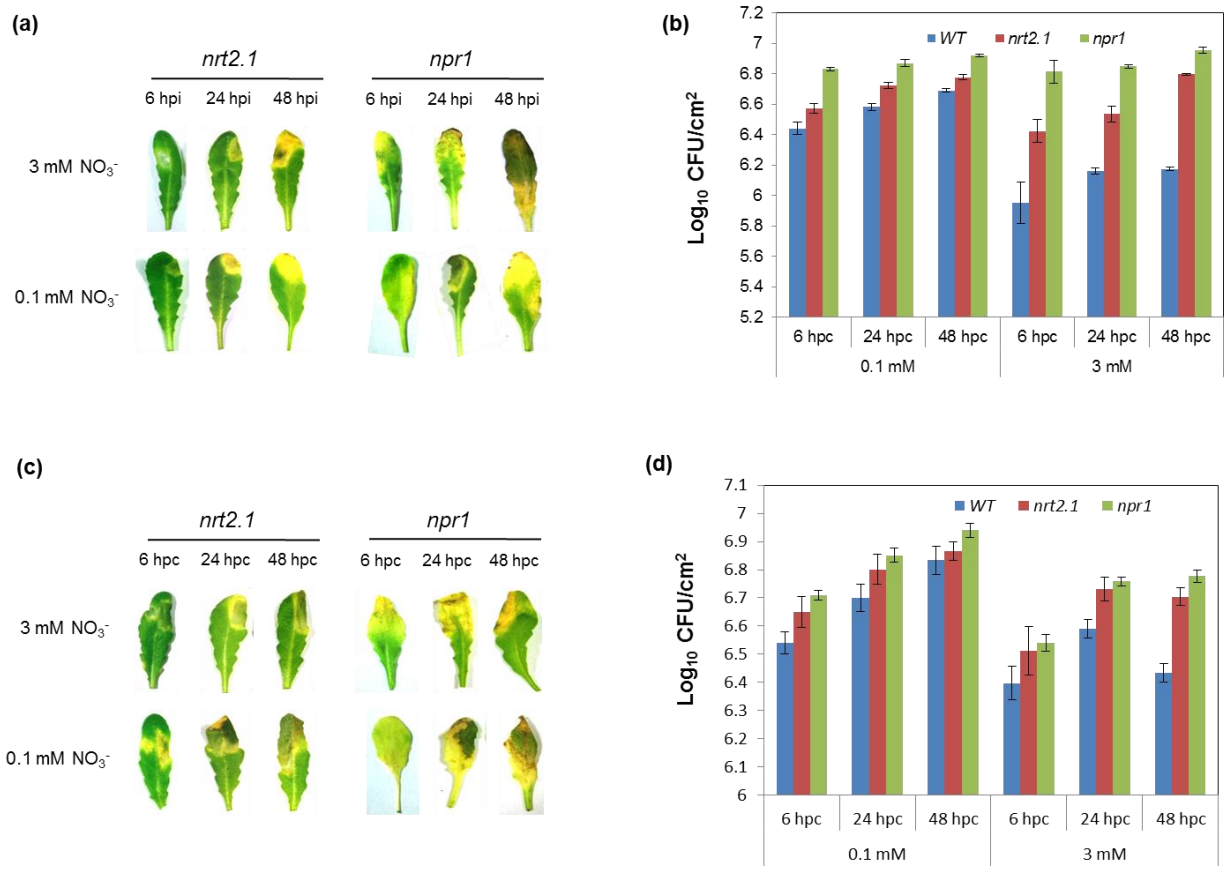
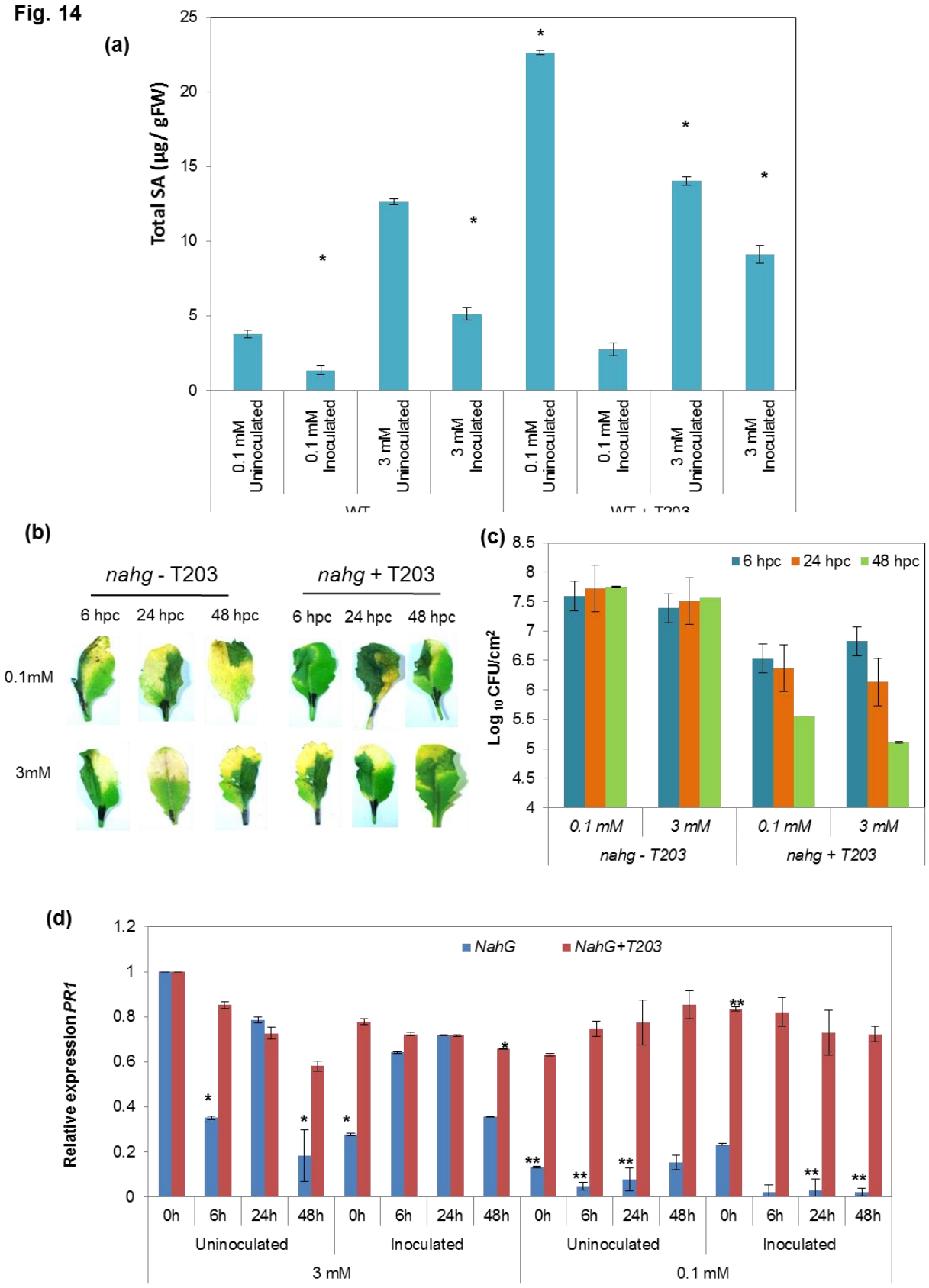

(a)
nrt2.1
npr1
6 hpi
24 hpi
48 hpi
6 hpi
24 hpi
48 hpi
3 mM NO3-
0.1 mM NO3-
(b)
WT
nrt2.1
npr1
Log10 CFU/cm2
7.2
7
6.8
6.6
6.4
6.2
6
5.8
5.6
5.4
5.2
6 hpc
24 hpc
48 hpc
6 hpc
24 hpc
48 hpc
0.1 mM
3 mM
(c)
nrt2.1
npr1
6 hpc
24 hpc
48 hpc
6 hpc
24 hpc
48 hpc
3 mM NO3-
0.1 mM NO3-
(d)
WT
nrt2.1
npr1
Log10 CFU/cm2
7.1
7
6.9
6.8
6.7
6.6
6.5
6.4
6.3
6.2
6.1
6
6 hpc
24 hpc
48 hpc
6 hpc
24 hpc
48 hpc
0.1 mM
3 mM

Fig. 14


(a)
Total SA (µg/ gFW)
25
20
15
10
5
0
0.1 mM Uninoculated
0.1 mM Inoculated
3 mM Uninoculated
3 mM Inoculated
0.1 mM Uninoculated
0.1 mM Inoculated
3 mM Uninoculated
3 mM Inoculated
(b)
nahg - T203
nahg + T203
6 hpc
24 hpc
48 hpc
6 hpc
24 hpc
48 hpc
0.1mM
3mM
(c)
Log 10 CFU/cm2
6 hpc
24 hpc
48 hpc
0.1 mM
3 mM
0.1 mM
3 mM
nahg - T203
nahg + T203
(d)
Relative expression PR1
NahG
NahG+T203
0h
6h
24h
48h
Uninoculated
Inoculated
3 mM
0.1 mM

1160