

Resistance against various fungal pathogens and reniform nematode in transgenic cotton plants expressing *Arabidopsis NPR1*

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Abstract Cotton is an economically important crop worldwide that suffers severe losses due to a wide range of fungal/bacterial pathogens and nematodes. Given its susceptibility to various pathogens, it is important to obtain a broad-spectrum resistance in cotton. Resistance to several fungal and bacterial diseases has been obtained by overexpressing the Non-expressor of Pathogenesis-Related genes-1 (*NPR1*) in various plant species with apparently minimal or no pleiotropic effects. We examined the efficacy of this approach in cotton by constitutive expression of the *Arabidopsis (Arabidopsis thaliana) NPR1* gene. The

results show that *NPR1*-expressing lines exhibited significant resistance to *Verticillium dahliae* isolate TS2, *Fusarium oxysporum* f. sp. *vasinfectum*, *Rhizoctonia solani*, and *Alternaria alternata*. Interestingly, the transformants also showed significant resistance to reniform nematodes. Analysis of defense-related, biochemical and molecular responses suggest that when challenged with pathogens or certain systemic acquired resistance-inducing chemicals, the transgenic lines respond to a greater degree compared to the wild-type plants. Importantly, the basal activities of the defense-related genes and enzymes in uninduced transformants were no different than those in their non-transgenic counterparts. The results provide additional evidence supporting the role of *NPR1* as an important part of the plant defense system and suggest a means to achieve broad-spectrum resistance to pathogens via genetic engineering.

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Introduction

Systemic acquired resistance (SAR) is a long lasting defense response that is induced in plants by localized infection and provides subsequent protection against a broad spectrum of pathogens (Ryals et al. 1994; Ryals et al. 1996; Grant and Lamb 2006). SAR

induction is associated with localized and systemic elevation of salicylic acid (SA). Application of SA or its synthetic analogs 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester (BTH) have been shown to induce SAR (Ward et al. 1991; Uknes et al. 1992; Cao et al. 1994; Gorlach et al. 1996).

An *Arabidopsis* (*Arabidopsis thaliana*) mutant, *npr1* (Non-expressor of Pathogenesis-Related genes-1, also called NIM1 or SAI1), failed to generate SAR in response to SA, INA, BTH or pathogen treatments, indicating that NPR1 plays a critical role in SAR (Cao et al. 1994; Delaney et al. 1995; Shah et al. 1997). However, NPR1-independent induction of disease resistance pathways culminating in the expression of PR genes has also been reported (Kachroo et al. 2000; Shah et al. 2001; Rairdan and Delaney 2002; Shah 2003). In an uninduced state, NPR1 has been shown to be present in the cytosol in its oligomeric form (Mou et al. 2003). SAR-induction triggers changes in the redox status of the cell that is believed to reduce NPR1 to its monomeric form (Mou et al. 2003). The oligomer to monomer reduction of NPR1 can be catalyzed by thioredoxins (Tada et al. 2008). The monomeric form of NPR1 is able to translocate into the nucleus where it activates the expression of PR genes (Kinkema et al. 2000; Mou et al. 2003; Tada et al. 2008) through its interaction with various TGA transcription factors (Fan and Dong 2002; Zhou et al. 2000; Despres et al. 2000; Kesarwani et al. 2007).

Validation for the key role played by NPR1 in various defense networks has been obtained by a number of overexpression studies. Constitutive expression of *AtNPR1* in *Arabidopsis* and other heterologous species conferred a broad-spectrum resistance to various fungal and bacterial pathogens. Transgenic *Arabidopsis* plants overexpressing *NPR1/NIM1* showed resistance to a bacterial pathogen, *Pseudomonas syringae* and several fungal pathogens, including *Hyaloperonospora arabidopsidis*, *Peronospora parasitica*, and *Erysiphe cichoracearum* (Cao et al. 1998; Friedrich et al. 2001). This gene also conferred resistance to bacterial pathogens, *Xanthomonas oryzae* and *Erwinia chrysanthemi* and, to two fungal pathogens, *Magnaporthe oryzae* and *Fusarium verticillioides* in rice (Chern et al. 2001; Quilis et al. 2008); to *F. oxysporum*, *Stemphylium solani*, *Ralstonia solanacearum* and *X. campestris* in tomato (Lin et al. 2004); to *F. graminearum* in wheat (Makandar

et al. 2006); and to *Botrytis cinerea*, *Alternaria radicina*, *Sclerotinia sclerotiorum*, *Alternaria radicina*, *Erysiphe heraclei*, and *Xanthomonas hortorum* in carrot (Wally et al. 2009). Transgenic apple, overexpressing a homologous *NPR1* gene (Mp*NPR1*), showed increased resistance to the bacterium, *E. amylovora* and to two fungal pathogens, *Venturia inaequalis* and *Gymnosporangium juniperi-verginiana* (Malnoy et al. 2007). Constitutive expression of *AtNPR1* or *Brassica napus*-*NPR1* (Bn*NPR1*) in *B. napus* conferred resistance to the bacterial pathogen, *P. syringae* (Potlakayala et al. 2007). With the exception of rice, other species in the aforementioned studies did not show any adverse growth or developmental effects as a result of *NPR1*-overexpression. Results from these studies suggest that NPR1-mediated defense response mechanism is evolutionarily conserved and that overexpression of *NPR1* can confer a broad-spectrum resistance to pathogens in other important crop species.

Cotton is a crop of great economic importance worldwide. Cotton lint continues to be the most important source of natural fiber for textile industry, while, the seeds are used to produce edible oil and as a feed for cattle. There is a large array of diseases that inflict the cotton plant and cause significant yield losses. In 2007, various pathogens and nematodes caused a 9% loss in the yield of this crop in the U.S. (Blasingame et al. 2008). While transgenes are widely used in cotton for insect control and herbicide resistance, efforts to confer disease resistance to cotton plant through transgenic means are in their infancy, and there are few published reports showing transgene-mediated disease resistance in cotton. Overexpression of *Talaromyces flavus* glucose oxidase gene in transgenic cotton showed some protection against *Verticillium dahliae*, but did not provide protection against *F. oxysporum* (Murray et al. 1999). Overexpression of an endochitinase gene from *Trichoderma virens* showed enhanced resistance to *Rhizoctonia solani* and *Alternaria alternata*, but failed to provide any protection against *F. oxysporum*, *V. dahliae* and *Thielaviopsis basicola* (Emani et al. 2003). Constitutive expression of an anti-fungal protein from *Gastrodia* in cotton has been reported to confer resistance to *V. dahliae* (Wang et al. 2004). In another study, resistance to *T. basicola* (which causes root rot disease) was observed in transgenic cotton that expressed a gene encoding a synthetic peptide,

D4E1 (Rajasekaran et al. 2005). In most of these studies, the protection obtained was a result of the direct effect of the transgene product on the pathogen, thus limiting their efficacy to a narrow spectrum of diseases. In contrast, NPR1/NIM1-mediated protection appears to be due to the activation of plant's own defense machinery and therefore, the resistance obtained in the transformants was more broad-spectrum (Cao et al. 1994; Cao et al. 1998; Delaney et al. 1995; Lin et al. 2004). The current investigation was undertaken to examine if constitutive expression of *AtNPR1* gene can confer a broad-spectrum resistance to diseases in the cotton plant. The results obtained demonstrate that *AtNPR1*-transformants were resistant to four important fungal diseases of cotton caused by *F. oxysporum* f. sp. *vasinfectum*, *V. dahliae* isolate TS2, *R. solani* and *A. alternata*. This study also provides evidence that expression of *AtNPR1* in cotton plant confers some protection against the nematode, *Rotylenchulus reniformis*. In addition to the evidence for disease resistance, we provide results on the transcript levels of some PR genes and the biochemical alterations observed in chemically- or pathogen-induced transgenic plants.

Materials and methods

Vector construction, cotton transformation, and molecular analysis

A pCAMBIA2300 based vector containing the GUS expression cassette from pFF19G (Timmermans et al. 1990) constructed earlier in our laboratory was used to assemble the transformation vector for this study. The *gusA* gene was removed from this vector and replaced with *NPR1* cDNA obtained from *A. thaliana* ecotype Dijon-17 (Zhou et al. 2000), thus placing it under the control of CaMV 35S promoter with the duplicated enhancer element and CaMV 35S polyadenylation signal from plasmid pFF19G (Timmermans et al. 1990).

Gossypium hirsutum cv. Coker 312 was transformed according to the method described by Sunilkumar and Rathore (2001) and Rathore et al. (2006). The primary transformants generated were screened for *AtNPR1* transcript accumulation in the leaf tissue by RT-PCR. The PCR primer pair, *AtNPR1F*: 5'-GAGGACACACTGGTTATACTC-3' and *AtNPR1R*: 5'-CCAGAT

CGAGCAGCGTCATCTT-3' was used to amplify a 690 bp-size fragment of *AtNPR1*. Selected transgenic lines identified from this screen were analyzed by Southern hybridization to confirm transgene integration.

Chitinase assay

Endochitinase activity was measured according to the protocol described by Emani et al. (2003) using 4-methylumbelliferyl- β -D-N,N',N''-triacetylchitotri-*oside* [4-MU- β -(GlucNAc)₃] (Sigma, St. Louis, MO, USA) as the substrate. Enzyme activity was examined in total soluble protein (TSP) obtained from the first leaf of a cotton plant with or without *F. oxysporum* f. sp. *vasinfectum* infection. The endochitinase specific activity is presented as pmole 4-MU/h/ μ g TSP.

Glucanase assay

The method published by Abeles and Forrence (1970) was used to estimate glucanase activity in TSP obtained from the first leaf of a cotton plant with or without *F. oxysporum* f. sp. *vasinfectum* infection. In this assay, laminarin was used as the substrate and dinitrosalicylic reagent was used to measure the reducing sugars produced in the enzymatic reaction. The enzyme activity is expressed as glucose equivalents, mg/h/mg TSP.

Estimation of total terpenoid aldehydes

The method described by Bell (1967) and Bianchini et al. (1999) was followed to extract and measure terpenoids from the stele tissue at the 5th internode of a cotton plant, and concentrations are presented as 'gossypol equivalents'. Stele tissue, 2 cm in length, was weighed, chopped into small pieces and then incubated in 3 ml of 95% acetone containing 1% ascorbic acid. Following 10 s of vortex treatment, the samples were kept at 4°C for 2 h. A 500 μ l extract was then mixed with 250 μ l of 5% phloroglucinol and 500 μ l of concentrated HCl and incubated at room temperature for 30 min. The absorbance was measured spectrophotometrically at 550 nm. A standard curve prepared with pure gossypol was used to estimate terpenoids in each sample, which are presented as μ g gossypol equivalents/g FW of stele tissue.

Treatment of cotton seedlings with SA

The method of Djonovic et al. (2006) was adapted for the application of SA to cotton seedlings. Cotton-seeds, soaked overnight, were germinated vertically within a rolled-up wet filter paper for 2 days at 28°C. The germinated seedling was placed on a filter paper wick support in a test tube (17 × 100 mm, VWR International, LLC, Cat. #60818-667) containing 10 ml of ½ strength MS medium in such a manner that the radicle remained submerged in the medium while the hypocotyl remained above the liquid as the seedling grew. Ten such tube assemblies were kept in a 2-litre glass beaker covered with a loose-fitting plastic Petri dish (150 × 15 mm), sealed with parafilm, and placed under light at 25°C in an incubator. After 24 h, the medium surrounding the root was replaced with 3 mM SA and the seedling was returned to the incubator. After 12, 24 and 48 h of SA treatment, cotyledons were analyzed for chitinase and glucanase activities.

BTH treatment of the cotton plants

Twenty-three-day-old cotton plants were sprayed with aqueous solution of 100 µM BTH (Actiguard, containing 50% active ingredient, Syngenta) in 0.01% silvet till run-off. Cotton plants sprayed with 0.01% silvet (mock-treated) were used as controls. Leaf samples were harvested after 6, 12, 24 h of BTH treatment for the isolation of total RNA.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The Spectrum Plant Total RNA Kit (Cat # STRN50-1KT, Sigma, St. Louis, MO, USA) was used to extract total RNA from a young healthy leaf of a BTH (100 µM) treated, 3-week-old- or *F. oxysporum* f. sp. *vasinfectum* infected, 2-week-old cotton plants. Following DNase treatment (RNase-free DNase Kit, Cat # 79254, Quiagen, USA), Taqman Reverse transcription (RT) reagents kit (Applied Biosystems, Cat # N808-0234) was used to synthesize the complementary-DNA (cDNA) from 400 ng of total RNA. One microliter of the reaction product was used for PCR amplification using a set of gene specific primers (Supplementary Table 1). The PCR conditions were as follows: 94°C for 4 min, initial

denaturation, 28 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 60 s for *Chitinase*, *Glucanase*, and *Osmotin*; 94°C for 4 min, initial denaturation, 22 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s for *PRI*; 94°C for 4 min, initial denaturation, 28 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 60 s for *AtNPR1* and a final extension at 72°C for 10 min. In the duplex RT-PCR, cotton *Histone3* gene was used as an internal control. PCR products were electrophoresed on 1.2% agarose gel.

Rhizoctonia solani infection assay

These assays with *R. solani* (strain J1) were conducted following the protocol described in a recent paper from our laboratory (Kumar et al. 2009). Seeds were rolled in a wet filter paper (VWR, Cat. #28298-020) and placed vertically in a beaker, covered with a polythene bag and incubated for 2 days at 28°C. Two-day-old, pre-germinated seedlings were transferred to soil (with or without the fungal inoculum) in a plastic tray. The tray was covered with a clear, plastic Humidome (Acrodome, Agroplastics Ltd, Canada) to maintain humidity and placed in a growth chamber for 6 days at 25°C under a 14:10 h photoperiod to allow disease development.

Fusarium wilt assay

Fusarium oxysporum f. sp. *vasinfectum* isolate *Fov11* (race 1 on cotton; ATCC #46644) was grown on plates containing Difco™ potato dextrose agar (PDA) medium supplemented with chloramphenicol 200 mg/l and tetracycline 20 mg/l for 1 week. A section of agar with sporulating mycelia measuring 1 cm² was cut from the PDA plate and transferred to 2 ml sterile distilled water and vortexed for 5 s to prepare a conidial suspension. Five drops of this suspension were added to 50 ml Difco™ potato dextrose broth and incubated on a shaker (140 rpm) for 7 days at 23°C. This culture was filtered through paper towel to eliminate mycelial clumps and obtain microconidial suspension. Microconidial count was performed using a hemocytometer and the final count was adjusted to 10⁷ conidia/ml by diluting with sterile distilled water.

Cotton seedlings were grown on washed, sand-loamy clay (75:25) mixture in a greenhouse. The plants were fertilized with 6 g/l of water-soluble,

9-45-15 N-P-K fertilizer (Peters professional® water soluble fertilizer, Scotts-Sierra Horticultural Product Company, Ohio, USA). After 2 weeks, seedlings were removed gently from the soil and roots were washed thoroughly under running tap water. The root portion was immersed in the conidial suspension for 5 min for inoculation and then the seedling was transplanted to a 500 ml plastic cup (with three holes, covered with a plastic screen, at the bottom) containing fresh sand-clay mixture irrigated with 3 g/l of water-soluble 9-45-15 N-P-K fertilizer. Control seedlings were mock-treated with tap water before transfer to the sand-clay mixture. Infected and control seedlings were grown for 3 weeks in a growth chamber at 25/20°C (day/night) temperature, and a 13 h photoperiod. In addition to regular watering, each plant was fertilized once a week with 50 ml of 3 g/l water-soluble 20-20-20 N-P-K fertilizer. Disease severity was scored 3 weeks after inoculation. Leaf disease index (LDI) and total shoot weight were used as the parameters to assess infection severity. LDI was calculated based on the following formula:

$$\text{LDI (\%)} = \left[\frac{\text{(Number of dead leaves)} + \frac{1}{2} \text{ number of blighted leaves}}{\text{total number of leaves}} \right] \times 100.$$

Verticillium wilt assay

Verticillium wilt assay and the scoring of results were performed as described by Bolek et al. (2005). *V. dahliae* Kleb. Isolate TS2 (race 2 on tomato; VCG2A) was grown on Difco™ PDA medium supplemented with chloramphenicol 200 mg/l and tetracycline 20 mg/l and allowed to grow for 1 week at 23°C. An agar block with sporulating mycelia, measuring ~1 cm², was transferred to a tube containing 3 ml sterile distilled water and vortexed for 5 s to prepare a conidial suspension. Conidial count was performed with a hemocytometer and was adjusted to 10⁷/ml by diluting the suspension with sterile distilled water. Cotton plants were grown for 6 weeks in 500 ml-size plastic cups (with holes in the bottom) containing Metromix® 700 (SUN GRO Horticulture Ltd, Bellevue, WA, USA) plant growth medium supplemented with 6 g/l of water-soluble 9-45-15 N-P-K. A 20 µl drop of conidial suspension

was placed on either side of the cotyledonary node of the stem. A small puncture was made into the stem by passing a sterile needle (28.5G Becton-Dickinson & Co., NJ, USA) through the spore suspension droplet. Microwounding in this manner facilitates the movement of spore suspension into the stem that is under negative pressure. Mock-treated plants underwent the same procedure with sterile distilled water. Infected and uninfected plants were grown for 3 weeks in a growth chamber at 27/22°C (day/night) temperature, with a 13 h photoperiod. During this period each plant was fertilized once a week with 50 ml of 3 g/l of water-soluble 20-20-20 N-P-K. Leaf/stem weight ratio, plant height, and total shoot weight at 3 weeks following infection, were used as the parameters to assess disease-severity.

Alternaria alternata infection assay

The method described by Emani et al. (2003) was used to perform *A. alternata* infection of a detached leaf. First, fully developed leaf from an 18-day-old cotton plant was removed and placed on a wet filter paper soaked with 3 ml of sterile distilled water in a Petri plate. An agar plug containing mycelial mat was placed on each side of the midrib on adaxial surface. The plates were sealed with parafilm and kept in dark at 28°C. Twelve days following inoculation, the leaves were photographed and infected area from the digital image was measured by using AlphaEase v5.5 software (Alpha Innotech, USA).

Reniform nematode resistance assay

An assay that is based on a count of motile vermiform stages of the nematode in the soil was used to evaluate the resistance of transgenic plants (Robinson et al. 2007). Cotton plants were grown in a medium favorable for growth of cotton and reniform nematodes. The mixture was prepared by mixing 48 l sandi-loam soil, 8 l vermiculite (SUN GRO Horticulture Ltd, Bellevue, WA, USA), 300 g fine dolomite (Carlpool® Products, Gladewater, TX, USA) 100 g Hoe-down™ gypsum soil conditioner (Earl Ricketts Co., FL, USA) and 50 g Esmigran, (micro-nutrients in sustained released form; Scotts-Sierra Horticultural Product Company, OH, USA) in a cement mixer for 30 min. The soil mixture and washed river sand were pasteurized for 6 h at 70°C.

Fifty milliliter of sand was placed in the bottom of a 500 ml plastic cup (with three holes, covered with plastic screen, at the bottom) and the rest filled with the soil mixture described earlier. The soil in each cup was fertilized with 50 ml of 9-45-15 N-P-K (6 g/l) before planting two-day-old pre-germinated cotton seedlings. Nematode inoculum was introduced in the soil around each plant following 2 weeks of growth in a chamber at 28/22°C (day/night) temperature, with a 13 h photoperiod. Baermann funnel extraction method was used to isolate nematodes, consisting of mixed stages of vermiform nematodes, from 100 g of infested soil (Robinson and Heald 1991). A liquid suspension containing 6,000 motile nematodes was injected into the soil around a plant at a distance of two cm from the stem. After inoculation, cotton plants were fertilized once a week with 50 ml of 3 g/l 20-20-20 N-P-K for 5 weeks and then every 2 weeks up to the 10th week. At this time, three soil samples totaling 30 g were removed from each cup using a 12 mm wide cork borer and the nematodes were extracted as described earlier. Nematodes were counted under a microscope ('Wild Heerbrug M420 Makroskop', a non-stereo zoom, 1,25×) and the results are presented as reniform nematodes/g infested soil. In addition to the nematode count, data for yield parameters such as the number of bolls and boll fresh weight (FW) per plant was noted.

Statistical analysis

Treatments were analyzed for significant differences using one-way ANOVA.

Results

Generation of transgenic cotton lines and their molecular analysis

Using the *Agrobacterium*-mediated transformation method, we generated 144 primary transgenic cotton plants. Ninety-three primary transformants were analyzed by Reverse transcriptase-polymerase chain reaction (RT-PCR) for *AtNPR1* transcripts and 68 were found to express the transgene. These *AtNPR1*-expressing cotton plants appeared normal in their growth and development. Based on consistent transcript levels over several generations, transgenic lines

68L-19 and 68L-20 were selected for detailed analyses (Supplementary Figure 1). Southern blot analysis showed that the transgenic line 68L-19 had a single and line 68L-20 had three transgene copies integrated into their respective genomes (Supplementary Figure 2). T2 or T3 generations obtained from a homozygous parent for both lines were used for disease resistance and other analyses.

Systemic induction of chitinase and glucanase activities in the leaves of transgenic plants infected with *F. oxysporum* f. sp. *vasinfectum*

As a measure of the defense response, chitinase and glucanase enzyme activities were examined at various time points in the leaves of plants whose roots were infected with *F. oxysporum* f. sp. *vasinfectum*. Without infection, there were no differences in the enzyme activities between transformant, 68L-19 and the wild-type plants confirming that *NPR1* expression by itself does not affect the defense response machinery of the plant (Fig. 1a). A small increase in chitinase activity was evident in both sets of plants at 12 h post-inoculation (hpi) with the transgenic plants showing a small but significant induction ($P < 0.01$) in chitinase activity compared to the mock-treated transformants. Between 12 and 24 hpi, the chitinase activity increased in both sets of infected plants, however, the magnitude of induction was significantly greater in the transformants. At 48 hpi, chitinase activity had decreased in both sets of plants from its peak value, but remained higher in the *NPR1*-expressing plants compared to the WT. By 72 hpi, the chitinase activity had markedly declined in *F. oxysporum* f. sp. *vasinfectum*-infected transgenic plants and was similar to that of infected WT plants (Fig. 1a). Thus, compared to the WT plants, the transgenic line 68L-19 showed a greater increase in chitinase activity in response to *F. oxysporum* f. sp. *vasinfectum* infection.

Glucanase activity remained at the basal level up to 12 h following *F. oxysporum* f. sp. *vasinfectum* inoculation. Between 12 and 24 hpi, the glucanase activity increased in both sets of infected plants with the transformants showing a small but significantly higher level at 24 hpi. After this time point, the rate of increase was slightly higher in the WT plants (Fig. 1b). Thus, the transgenic plants do show an early induction of glucanase activity in response to

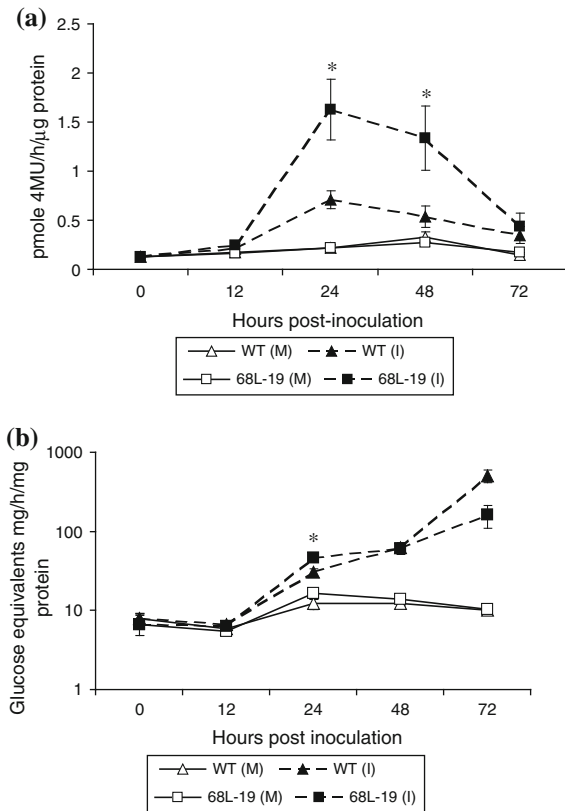


Fig. 1 Activities of PR enzymes in the leaves (first true leaf) of the transgenic cotton line, 68L-19 and untransformed WT plant following inoculation of roots with *Fusarium oxysporum* f. sp. *vasinfectum* isolate Fov11. **a** Chitinase activity, and **b** Glucanase activity. *I*: Roots were treated with *F. oxysporum* conidial suspension; *M*: Roots were mock-treated with tap water. Data represent mean \pm SE, $n = 10$; the induced enzyme activity value for the transgenic line is significantly higher than that of the WT value at * $P < 0.05$

F. oxysporum f. sp. *vasinfectum*-infection; however, it is overtaken by the WT plants after 48 hpi.

Accumulation of gossypol-related terpenoids in stele tissue of WT and transgenic cotton plants in response to *V. dahliae*

Gossypol and related terpenoids are known to be induced in cotton plants in response to *V. dahliae* (Bell 1969; Mace 1983; Mace et al. 1989). Therefore, we were interested in knowing if the terpenoid-based defense mechanism responded differently in the transformants. WT as well as transgenic plants were inoculated with spore suspension of *V. dahliae* isolate TS2 and the total terpenoids were estimated as

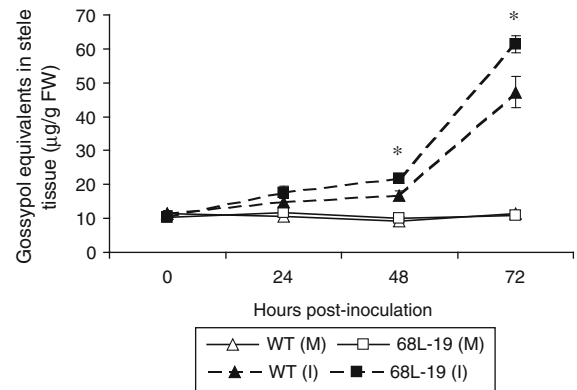


Fig. 2 Induction of terpenoids (gossypol equivalents) at the 5th internode in cotton plants infected with *Verticillium dahliae* isolate TS2. *I*: inoculated with *V. dahliae* conidial suspension; *M*: treated with sterile distilled water as a mock treatment. The stele tissue obtained from the internode was used to extract terpenoids. Data represent mean \pm SE, * $P < 0.05$; $n = 5$

gossypol equivalents at various time intervals. The terpenoid levels in the stele tissue of transformants and WT were similar prior to infection. Compared to WT, transgenic tissues accumulated slightly higher levels of terpenoids at 24 hpi. At 48 and 72 hpi, the transgenic plants had 30.1 and 29.8% more terpenoids, respectively, than the WT plants (Fig. 2). The differences were statistically significant ($P < 0.05$). The terpenoid levels in the mock-treated transgenic as well as WT plants remained at low levels at all time points. The more rapid induction of terpenoids in response to *V. dahliae* isolate TS2 could be one of the factors that provide added protection to the transgenic plants against this disease.

SA-induced systemic induction of chitinase and glucanase activities in the cotyledons of transgenic plants

SA has been shown to be an inducer of SAR and its associated PR genes in various plant species, including cotton (Ward et al. 1991; Uknes et al. 1992; Hudspeth et al. 1996; Djonovic et al. 2006). Therefore, we were keen to examine the influence of SA on *AtNPR1*-expressing cotton lines. After 12, 24, 48 h of treatment of the seedling root with SA, the activities of chitinase and glucanase were determined in the cotyledons of line 68L-19. SA-treatment of the roots induced chitinase and glucanase activities in the

cotyledons of both WT and the transgenic plants. However, the induction in the *AtNPR1*-transformants was faster and significantly higher compared to the untransformed plants (Supplementary Figure 3a, b). Similar inductions were observed in transgenic line 68L-20 at 24 h time point (Supplementary Figure 4a, b). Again, the induced enzyme activities in this line were significantly higher than those observed in WT seedlings in response to SA treatment. Thus, *AtNPR1*-expressing cotton plants exhibit higher levels of defense-related enzyme activities in response to SA.

BTH- and *F. oxysporum*-induced expression of PR genes in transgenic lines

We were interested to know whether BTH, a biologically active analog of salicylic acid (Friedrich et al. 1996; Kohler et al. 2002; Makandar et al. 2006), is capable of inducing PR genes in cotton plants, and if so, do the transgenic plants respond differently. Sequences of *PR1* (Accession no. CK988133.1), *Osmotin* (Accession no. CF932065), *Glucanase* (Accession no. CD486342) and *Chitinase* (Accession no. CD485880) genes, isolated from cotton, were available. Their expression was monitored at 6, 12 and 24 h post-treatment (hpt) of the leaves with BTH, in WT and the transgenic lines, 68L-19 and 68L-20. At 6 hpt, an induction in the transcripts for *PR1*, *Osmotin* and *Glucanase* was evident in the transgenic lines, 68L-19 and 68L-20 (Fig. 3a). The higher level of transcripts is maintained in line 68L-19 even at 24 hpt. Either a small or no induction in the activities of these genes was observed in the WT plants. The activity of the *chitinase* gene was induced in WT as well as in the transgenic lines. Overall, the results suggest that *AtNPR1*-expressing cotton transformants are primed to respond more rapidly and strongly to factors that induce SAR.

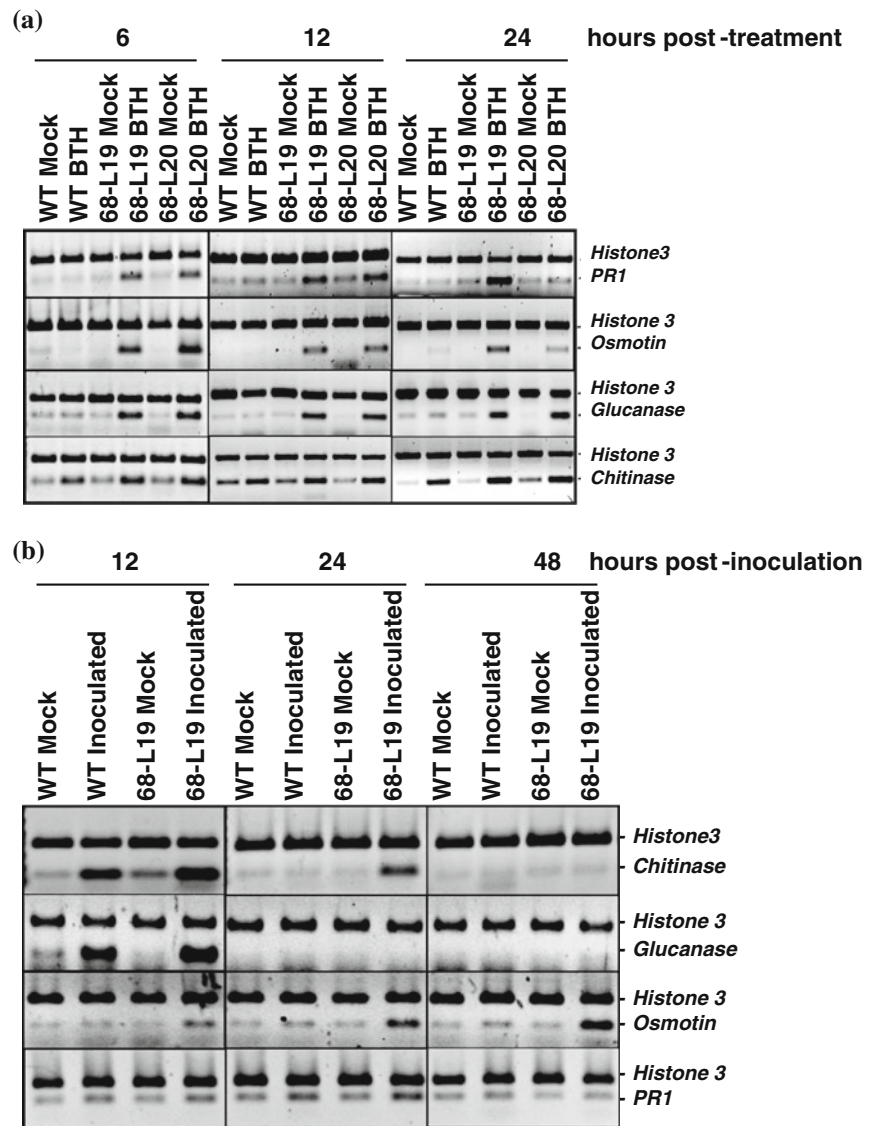
Fusarium oxysporum f. sp. *vasinfectum* can induce SAR related genes in Arabidopsis (Mauch-Mani and Slusarenko 1994). We therefore studied expression of PR genes (*Chitinase*, *Glucanase*, *Osmotin* and *PR1*) in the leaves of transgenic and WT plants following infection of the roots with *F. oxysporum* f. sp. *vasinfectum*. Induction of *Chitinase* gene activity was observed in both the transgenic line and WT at 12 hpi, but the level appears to be slightly higher in the former than the latter (Fig. 3b). By 24 hpi, the

induction had completely subsided in the WT plants while the transgenic line showed transcripts, but at a lower level than at 12 hpi. Transcripts diminished to a very low level at 48 h (Fig. 3b). The results showed that *Chitinase* gene induction in transgenic plants is greater and the heightened activity lasts a little longer in comparison to the WT plants. *F. oxysporum*-induced expression of *Glucanase* gene was observed only at 12 hpi in both sets of plants but the induction was stronger in the transgenic plants. Although induction of *Osmotin* gene was observed in both transgenic plants and WT plants, the level of transcripts appears to be higher in the transformants. In response to *F. oxysporum* f. sp. *vasinfectum* exposure, a progressive increase in transcript levels for this gene was observed in the case of transgenic line (Fig. 3b). No apparent induction of the tested *PR1* gene was observed in WT or the transgenic line 68L-19 (Fig. 3b). Plants usually have several members in the *PR1* gene family, and once the sequences of the remaining cotton *PR1* genes and other PR genes become available, it will be interesting to study their expression in *AtNPR1*-cotton plants during their interaction with *F. oxysporum* f. sp. *vasinfectum*. Taken together, the results suggest that the *AtNPR1* transformants express certain PR genes at higher levels compared to their WT counterparts during their interaction with *F. oxysporum* f. sp. *vasinfectum*.

AtNPR1-mediated resistance to fungal pathogens in transgenic cotton

Rhizoctonia solani is the most common causative agent of post-emergence damping off and is also responsible for pre-emergence death under cool conditions (Hillocks 1992b). Because of its importance as a cotton pathogen, we were interested in examining the resistance of *AtNPR1*-expressing cotton lines to *R. solani*. Disease index, a measure of infection severity, was 79.7% lower ($P < 0.001$) in transgenic line 68L-20 compared to that of WT. Line 68L-19 also showed enhanced resistance to *R. solani* ($P < 0.05$), but at a lower level (Fig. 4a). The collar region in *R. solani*-infected, WT seedlings was necrotized, while the transgenic seedlings were mostly healthy with very few, if any necrotic spots (Fig. 5a). The results show that overexpression of *AtNPR1* gene in cotton confers resistance to *R. solani*.

Fig. 3 a PR gene (*PR1*, *Osmotin*, *Glucanase*, and *Chitinase*)-expression in WT and *AtNPR1*-transgenic cotton lines, 68L-19 and 68L-20, after 6, 12 and 24 h of treatment with 100 μM BTH. A young leaf from five plants each were pooled, frozen in liquid N₂ and used to extract total RNA. **b** PR gene (*Chitinase*, *Glucanase*, *Osmotin* and *PR1*)-expression in the leaves of WT and transgenic line, 68L-19, at 12, 24, 48 hpi with *Fusarium oxysporum* f. sp. *vasinfectum* isolate *Fov11*. A young leaf from ten plants each were pooled together for each time point, frozen in liquid N₂ and were used to extract total RNA



Under certain conditions, such as injury created by pest attack, adverse weather conditions, moisture, or nutrient stress, *A. alternata* can infect cotton and cause leaf spots resulting in significant yield losses in cotton (Hillocks 1992a). Transgenic lines were tested for resistance against this foliar pathogen in a detached leaf assay and the results are shown in Fig. 4b. Twelve days following the placement of fungal inoculum, the leaves from transgenic plants were largely unaffected while those of WT showed infection lesions and chlorosis (Fig. 5b). The infected leaf area in lines 68L-19 and 68L-20 were 18 and 37% lower compared to that in the WT plants

(Fig. 4b). The transgenic lines were clearly more resistant than the WT against *A. alternata*.

Wilt diseases are also a serious problem in the production of cotton. *Fusarium* wilt caused by *F. oxysporum* f. sp. *vasinfectum* can affect cotton at any stage of development resulting in dark brown discoloration of the vascular system, wilting, chlorosis and necrosis of leaves, and plant death (Hillocks 1992b; Davis et al. 2006). *AtNPR1*-expressing lines were examined for their resistance to *F. oxysporum* f. sp. *vasinfectum* isolate *Fov11*. Two parameters were used to measure the degree of disease severity, leaf disease index (LDI) and shoot fresh weight. Both the

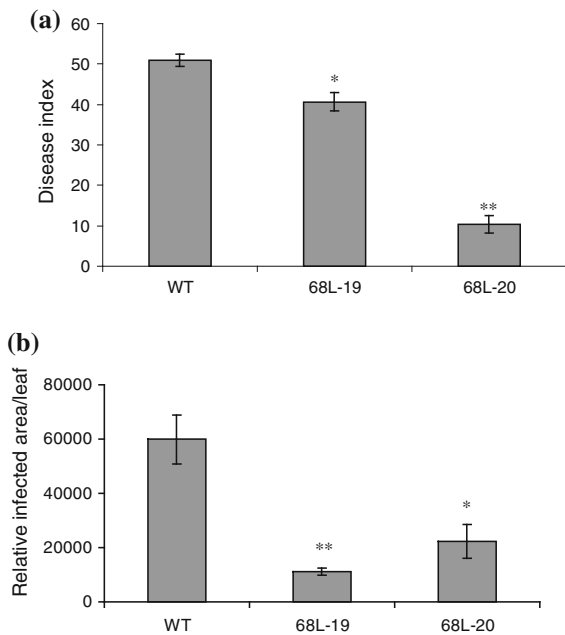


Fig. 4 **a** Resistance to *Rhizoctonia solani* in transgenic cotton lines expressing *AtNPR1*. Disease indices for WT and transgenic cotton lines infected with *R. solani* scored after 6 days in a soil-based assay (data represent mean \pm SE; mean obtained by averaging results from three sets of seedlings tested with each set consisting of 12 seedlings). The value for the transgenic line is significantly different from the control value at * $P < 0.05$; ** $P < 0.001$. **b** Resistance to *Alternaria alternata* in transgenic cotton lines expressing *AtNPR1*. Disease severity was scored on the 12th day following inoculation of detached leaves with the pathogen. Data represent mean \pm SE, * $P < 0.05$; ** $P < 0.001$, $n = 10$

transgenic lines showed lower disease severity in terms of LDI compared to the WT. The LDI for transgenic lines 68L-19 and 68L-20 were 62% ($P < 0.001$) and 26.6% lower, respectively, than the WT values (Fig. 6a). The mean shoot fresh weight for transgenic lines 68L-19 and 68L-20 were 2.73 and 1.76 g, respectively, compared to 0.82 g for WT (Fig. 6b). Three weeks following the inoculation of the roots, most of the leaves of WT plants were either senesced/wilted or had dropped, while the transgenic lines showed significantly higher retention of leaves which appeared healthy (Fig. 5c). In fact, line 68L-19 showed a higher degree of resistance to *F. oxysporum* f. sp. *vasinfectum* than a commercial cotton variety, DP 444 (Supplementary Figure 5a–c). This variety had shown the highest level of resistance to Fusarium wilt in a comparative study involving 50 different genotypes (Bell, unpublished results). The results

from current investigation provide strong evidence that *AtNPR1* expression in cotton confers resistance to *F. oxysporum* f. sp. *vasinfectum*.

Verticillium wilt caused by *V. dahliae* and *V. albo-atrum* is reported in more than two hundred dicotyledonous plant species and affects the production of various economically important crops, including cotton (Fradin and Thomma 2006). Decreased photosynthetic capacity, brown vascular discoloration, wilting and stunting are some of the symptoms of this disease in cotton (Hampton et al. 1990; Fradin and Thomma 2006). We examined the resistance of the *AtNPR1*-expressing transformants against *V. dahliae* isolate TS2. Three weeks following the infection, plants were examined for disease severity by measuring three parameters: shoot weight, leaf/stem weight ratio, and plant height. Fresh shoot weights of *V. dahliae*-infected transgenic lines 68L-19 and 68L-20 were significantly higher at 48.7 g ($P < 0.001$) and 40.6 g ($P < 0.05$), respectively, than that of WT at 16.2 g (Fig. 7a). Infected WT plants showed extensive defoliation and the leaves that did remain on the plant were wilted (Fig. 5d). In comparison to the leaf/stem weight ratio of 0.58 for the WT, these values for transgenic lines 68L-19 and 68L-20 were 1.4 ($P < 0.001$) and 1.11, respectively (Fig. 7b). WT plants were severely stunted in their growth showing an average reduction of 40% in their height as a result of Verticillium infection. The average heights of 68L-19 and 68L-20 plants were 73.8 and 66.3 cm, respectively, significantly higher than the 53.1 cm for the WT plants (Figs. 5d, 7c). Progression of the disease in plants was also monitored by cutting longitudinal sections of the internodes. Severe vascular browning was observed in the stem of Verticillium-infected WT plants at the 5th internode, while this section from the transgenic plants did not show vascular browning indicating suppression of infection (Supplementary Figure 6). In summary, the transgenic lines exhibited strong resistance to *V. dahliae* isolate TS2.

AtNPR1-mediated resistance to reniform nematodes in transgenic cotton

A recent report indicated a possible role for NPR1 in SA-mediated inhibition of parasitism by cyst nematodes (*Heterodera schachtii*) in Arabidopsis (Wubben et al. 2008). On the basis of this report and

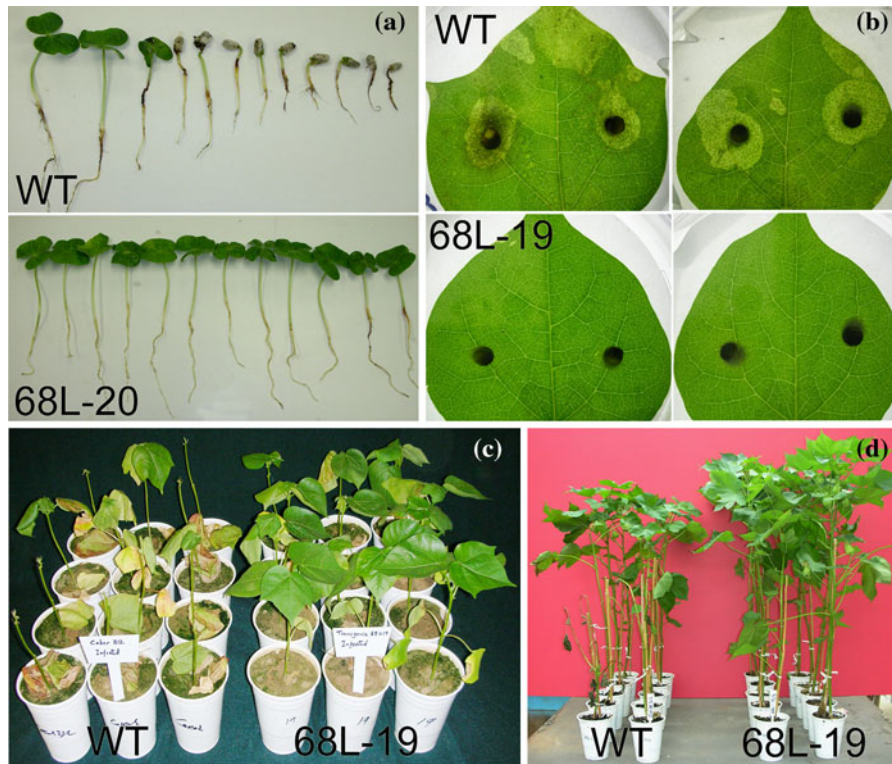


Fig. 5 Broad-spectrum resistance to fungal diseases in transgenic cotton lines expressing *AtNPR1*. Images of transgenic seedlings/plants/leaves on the day disease severity was scored:

a *Rhizoctonia solani*; **b** *Alternaria alternata*; **c** *Fusarium oxysporum* f. sp. *vasinfectum* isolate *Fov11* and **d** *Verticillium dahliae* isolate TS2

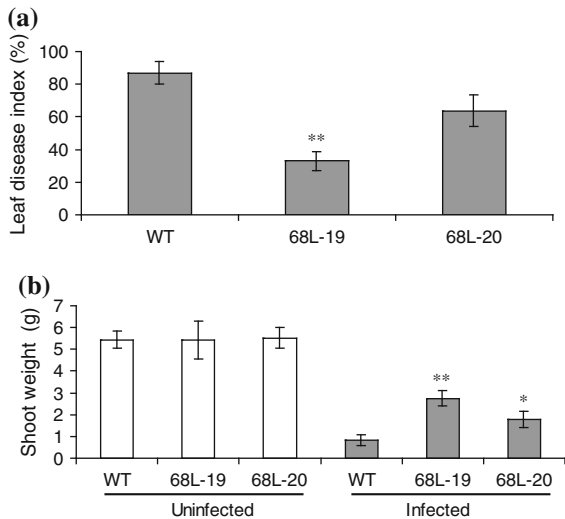
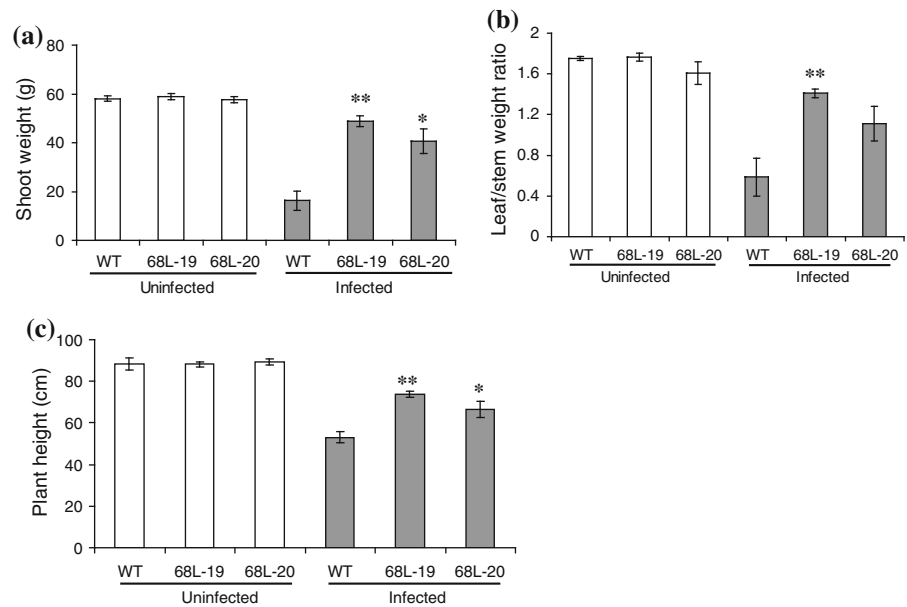


Fig. 6 Resistance to *Fusarium oxysporum* f. sp. *vasinfectum* isolate *Fov11* in transgenic cotton lines expressing *AtNPR1*. **a** Leaf disease index, and **b** shoot weight were used to score disease-severity, 3 weeks following the inoculation of roots. Data represent mean \pm SE, * $P < 0.05$; ** $P < 0.001$, $n = 12$

encouraged by the broad-spectrum resistance of the *AtNPR1*-cotton transformants to various fungal pathogens, we examined these plants for their resistance to reniform nematode, an economically important pest of cotton. A preliminary experiment with these nematodes had provided some encouraging results showing better performance of the transformants and significantly lower nematode count in the soil supporting these plants (Supplementary Figures 7a–d, 8). A second experiment was conducted to confirm these results with the same transgenic lines, 68L-19 and 68L-20. Ten weeks after inoculation, the vermiform nematode count for the untransformed WT was 77.9 per g of infested soil. This number is seven- and two-fold higher compared to the counts obtained for the transgenic lines 68L-19 and 68L-20, respectively (Fig. 8a). Cotton plants growing in nematode infested soil under field conditions, suffer from nutritional deficiencies, fruit abortion, and abnormal maturation of the crop (Koenning et al. 2004). Even under controlled environmental conditions in a

Fig. 7 Resistance to *Verticillium dahliae* isolate TS2 in transgenic cotton lines expressing *AtNPR1*. **a** Shoot weight, **b** leaf/stem weight ratio, and **c** plant height were used as parameters to score disease-severity, 3 weeks following stem inoculation with the pathogen. Data represent mean \pm SE, * $P < 0.05$; ** $P < 0.001$, $n = 10$



growth chamber, nutritional deficiencies and the loss of bolls was evident (Fig. 8b–d, Supplementary Figure 8). In addition to the number of bolls per plant, relative position of the bolls on the plant is a good indicator of the health of the plant and provides an indirect measure of the ability of the plant to retain bolls when facing nematode pressure. The data on boll-retention and -position shown in Fig. 8b–d indicates relatively better health of the transgenic plants growing in nematode-infested soil. Interestingly, line 68L-19 demonstrated a stronger resistance to reniform nematodes compared to the line 68L-20 that showed moderate level resistance. Although the degree of resistance was not as high, we observed a similar trend with a different line (68L-16) with respect to vermiform nematode count and boll number/weight (data not shown). Taken together, the results suggest that the *AtNPR1*-expressing cotton plants are more resistant than WT to the nematode, *R. reniformis*.

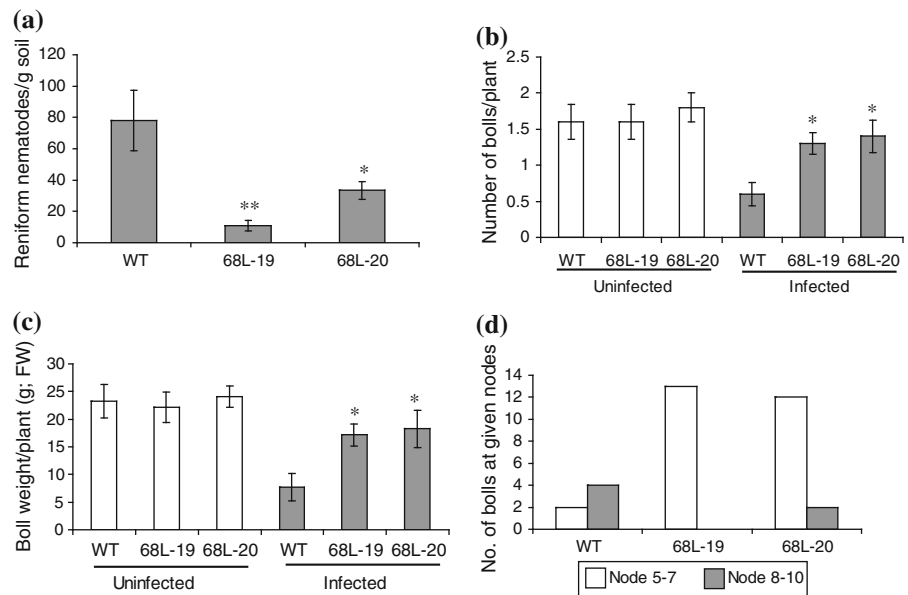
Discussion

Our results show that the expression of *AtNPR1* in cotton can confer increased resistance to two necrotrophic fungal pathogens of cotton, *R. solani*, (a cause of seedling damping-off) and *A. alternata* (a cause of foliar disease). Two wilt diseases in cotton, Fusarium

wilt caused by *F. oxysporum* f. sp. *vasinfectum* and Verticillium wilt caused by *V. dahliae*, together account for significant losses in yield ranging from 0.5 to 5% during 1952–2008 (National Cotton Council of America—Disease Data Base). In the current study, we show increased resistance to *F. oxysporum* f. sp. *vasinfectum* in *AtNPR1*-expressing transgenic lines compared to WT. Transgene-mediated conferral of resistance to *V. dahliae* in cotton has been reported. However, only a small reduction in disease severity due to *V. dahliae* was observed in transgenic cotton plants expressing *T. flavus* glucose oxidase (Murray et al. 1999) and *Gastrodia* anti-fungal protein (Wang et al. 2004). In this report, we provide detailed evidence for resistance obtained against a non-defoliating pathovar of *V. dahliae* isolate TS2 in cotton plants overexpressing the *AtNPR1* gene. Thus, results showing protection against four different fungal diseases demonstrate the broad-spectrum nature of the resistance in transgenic cotton plants overexpressing the *AtNPR1* gene.

Nematodes are a growing threat to cotton farming in the US and elsewhere. In the U.S., the estimated yield loss due to nematodes was 1–2% in 1950, which increased to 4.78% in 2007. The reniform nematode alone contributed to 2.04% of the loss in total yield in 2007. The lack of resistant cultivars is one of the reasons responsible for the increasing yield losses due

Fig. 8 Resistance to reniform nematode *Rotylenchulus reniformis* in transgenic cotton lines expressing *AtNPR1*. **a** Nematode count in soil after 10 weeks of inoculation. **b** Number of bolls per plant. **c** Boll weight per plant. **d** Boll position (number of bolls at given nodes in 10 plants). Data represent mean \pm SE, * $P < 0.05$; ** $P < 0.01$, $n = 10$



to the nematodes (Robinson et al. 1999; Koenning et al. 2004; Blasingame et al. 2008). Thus, there is an urgent need for measures to control losses caused by these parasites. Some studies have shown a reduction in the reproduction of root-knot and reniform nematodes in crop plants such as cowpea, soybean and pineapple by the application of SAR inducer, BTH (Chinnasri et al. 2003, 2006). Nandi et al. (2003) showed that foliar application of SA to okra and cowpea 24 h before the inoculation of roots with *M. incognita*, induced *PRI* and *PAL* in roots and reduced infestation. Recently, Wubben et al. (2008) have demonstrated inhibition of cyst nematode (*H. schachtii*) parasitism in Arabidopsis by SA that was shown to be mediated by NPR1. In the current investigation, we examined the role of NPR1 directly, and provide some evidence that constitutive expression of *AtNPR1* can enhance resistance to nematode, *R. reniformis* in the transgenic cotton plants. The protection provided to the transformants by *NPR1* overexpression is not only reflected in the lower number of nematodes but is also supported by higher boll retention, boll weight and overall health of the plants.

Earlier studies on overexpression of *NPR1* in various plant species including Arabidopsis (Cao et al. 1998), tomato (Lin et al. 2004), wheat (Makandar et al. 2006), *B. napus* (Potlakayala et al.

2007), apple (Malnoy et al. 2007) did not report any abnormalities in the growth and development of transgenic plants under normal growth conditions. In agreement with above-mentioned studies, we did not observe any obvious abnormal growth behavior in cotton transformants expressing *AtNPR1*. Importantly, our visual observations on the phenotypic normalcy of the *AtNPR1*-transformants are also supported by some of the biochemical and molecular data presented in this report showing no significant differences between transgenic and WT control plants in the absence of a challenge from a pathogen or chemical inducer. Besides providing resistance to *Xanthomonas oryzae* pv. *oryzae*, *NPR1*-overexpression in rice resulted in the development of lesion-mimic spots on the leaves under low-light condition of a growth chamber (Fitzgerald et al. 2004; Chern et al. 2005). We did not observe this type of sensitivity in *AtNPR1* cotton transformants that were grown under various light levels ranging from 12 to 950 $\mu\text{mol m}^{-2} \text{s}^{-1}$. In a recent study, *AtNPR1* expression in rice, while conferring resistance to fungal and bacterial diseases, had a negative effect on virus infections and the ability to tolerate salt- and drought stress (Quilis et al. 2008). It is important to note that until now, any negative effects of *NPR1*-overexpression have been observed only in rice, which has very high basal SA level compared to

tobacco, Arabidopsis, and cotton (Yang et al. 2004; Raskin et al. 1990; Nawrath and Metraux 1999; Martinez et al. 2000; Silverman et al. 1995). Thus, endogenous, basal SA levels may be an important consideration in selecting a crop species for the development of *NPR1*-mediated disease resistance.

SA and its synthetic analog, BTH are known to induce SAR and SAR associated genes in various plant species (Ward et al. 1991; Uknes et al. 1992; Hudspeth et al. 1996; Djonovic et al. 2006; Gorlach et al. 1996; Friedrich et al. 1996; Nawrath and Metraux 1999; Colson-Hanks and Deverall 2000; Kohler et al. 2002; Zhu et al. 2003). In some of these studies, SAR was induced by direct bathing of the root or soil-drenching with the chemical. In our investigation, 6 h after treatment with BTH, a clear and strong induction of *PR1*, glucanase and osmotin genes was observed only in the transgenic cotton lines, 68L-19 and 68L-20. The activities of chitinase and glucanase enzymes were determined in the cotyledons of cotton seedlings following treatment of their roots with SA. Compared to WT, significant elevation in the activities of both the enzymes were observed in the transgenic lines, 68L-19 and 68L-20, following SA treatment. Djonovic et al. (2006) have also reported systemic induction of various defense-related genes in cotton seedlings (cv. Deltapine 50) in response to SA treatment of roots. Taken together, the results suggest that the *AtNPR1*-expressing cotton plants are primed to respond to SAR-inducers and the levels of gene/enzyme induction is higher in these plants. These factors may be the basis for observed resistance to various fungal pathogens.

In a number of plant species overexpressing *NPR1* gene, a greater and faster activation of various PR genes in response to pathogen-challenge has been reported (Cao et al. 1998; Makandar et al. 2006; Malnoy et al. 2007; Quilis et al. 2008). In the current investigation, we studied the expression of various PR genes in transgenic cotton line, 68L-19, following a challenge with *F. oxysporum* f. sp. *vasinfectum*. This line had shown a strong resistance to this pathogen. A stronger and longer-lasting induction of *Chitinase* expression was observed in *F. oxysporum*-challenged transgenic line, 68L-19 compared to the WT plants (Fig. 3b). A clear and progressive induction of *Osmotin* was observed only in *F. oxysporum*-inoculated transgenic line. Our investigation into PR gene activities is limited by of the availability of

cotton gene sequences. As additional information on cotton genes that are involved in defense function becomes available, expression of these genes will be examined in the *NPR1* transformants in the future.

As a follow up to the PR gene expression study, we determined the activities of chitinase and glucanase in *F. oxysporum* f. sp. *vasinfectum* challenged transgenic plants. A sharp induction in the activity of both glucanase and chitinase was observed at 24 hpi in *F. oxysporum*-inoculated transgenic line 68L-19, which is significantly higher than the values in similarly treated WT. The stronger and early induction of PR genes and enzyme activities in pathogen-challenged transgenic line, 68L-19 suggests an active role of SAR associated PR genes in *AtNPR1*-mediated resistance to *F. oxysporum* f. sp. *vasinfectum* in transgenic cotton.

Induction of gossypol and related terpenoids is believed to be a part of the defense mechanism in cotton plants in response to pathogen infection (Stipanovic et al. 1999; Eldon and Hillocks 1996). In particular, there are several reports showing the involvement of terpenoids in the defense mechanism against *Verticillium* spp. in cotton. For example, cotton cultivar, Seabrook Sea Island 12B2 (SBSI), which shows resistance to *V. dahliae* and *V. albo-atrum*, produces terpenoid aldehydes faster and at higher levels upon infection (Mace 1983; Bianchini et al. 1999; Bell 1969). The higher induction of phytoalexins in stem tissue of cotton during the early phase of infection with *V. albo-atrum* is positively correlated with resistance in SBSI. In contrast, the susceptible cotton cultivars show a slower induction of terpenoids when challenged with *Verticillium* (Bell 1969). The *NPR1*-expressing transgenic lines had shown high degree of resistance to *V. dahliae* isolate TS2, therefore, we were interested in examining the levels of these compounds in the transformants following a challenge with the pathogen. At 48 and 72 hpi, transgenic line 68L-19 (which shows high level of resistance to *V. dahliae*) had significantly higher levels of terpenoids compared to the untransformed WT plants. It is possible that the higher terpenoid levels form part of the overall defense response that protects the *NPR1*-transformants from the disease. Although there are no published reports showing the involvement of *NPR1* in the induction of gossypol and related terpenoids in cotton, it stands to reason that activation of the plant's basal defenses

will also involve this important resistance mechanism found in the tribe Gossypaeae (Stipanovic et al. 1999).

The data presented in this report show clearly that expression of *AtNPR1* in cotton confers resistance to four different fungal pathogens and the reniform nematode. The transformants appeared normal in their growth and development. These results suggest that NPR1 may provide an effective tool to obtain resistance to diverse pathogens by transgenic means with minimal metabolic cost to the plant. Thus, although the current study and several others reported herein show promise in the use of *NPR1* gene as a transgenic tool to obtain broad-spectrum disease resistance in important crops, additional research is needed to ensure the overall efficacy of such an approach under field conditions where the plants are exposed to a variety of biotic and abiotic stresses. This is because in addition to being an important mediator of SAR and ISR, cytosolic NPR1 plays some role in modulating the antagonistic cross-talk between SA and jasmonic acid signaling (Dong 2004; Spoel et al. 2003, 2007; Pieterse and Van Loon 2004). The complexity of the role of NPR1 in various signaling networks has been further heightened by a recent study on *Arabidopsis* showing that ethylene modulates the NPR1 dependency of SA-jasmonic acid antagonism (Leon-Reyes et al. 2009). Regardless, the results presented in this report provide strong evidence supporting the role of NPR1 as a critical component in the defense mechanism that protects plants from certain biotic challenges.

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