Response of *AtNPR1*-expressing cotton plants to *Fusarium oxysporum* f. sp. *vasinfectum* isolates

Sameer G. Joshi¹ · Vinod Kumar¹ · Madhusudhana R. Janga¹ · Alois A. Bell³ · Keerti S. Rathore^{1,2}

Received: 7 November 2016 / Accepted: 27 December 2016 / Published online: 3 January 2017
© Prof. H.S. Srivastava Foundation for Science and Society 2017

Abstract In our earlier investigation, we had demonstrated that transgenic cotton plants expressing *AtNPR1* showed significant tolerance to *Fusarium oxysporum* f. sp. *vasinfectum*, isolate 11 (*Fov11*) and several other pathogens. The current study was designed to further characterize the nature of the protection provided by *AtNPR1* expression and its limitations. Green Fluorescent Protein-expressing *Fov11* was generated and used to study the progression of the disease within the plant. The results show that the spread of the pathogen was slower in the *AtNPR1*-transformants compared to the wild type plants. Transcript analysis in the seedling root and hypocotyl showed that the transgenic lines are capable of launching a stronger defense response when infected with *Fov11*. We further confirmed that *AtNPR1* transformants showed greater degree of tolerance to *Fov11*. However, little or no protection was observed against a related, but more virulent isolate, *Fov43*, and a highly virulent isolate, CA9.

Keywords Cotton · Disease resistance · *Fusarium* wilt · *NPR1* · Transgenic cotton

Electronic supplementary material The online version of this article (doi:10.1007/s12298-016-0411-x) contains supplementary material, which is available to authorized users.

✉ Keerti S. Rathore
rathore@tamu.edu

¹ Institute for Plant Genomics & Biotechnology, Texas A&M University, College Station, TX 77843-2123, USA

² Department of Soil & Crop Sciences, Texas A&M University, College Station, TX 77843-2474, USA

³ USDA-ARS, Southern Plains Agricultural Research Center, College Station, TX 77845, USA

Introduction

Several organisms such as fungi, nematodes and bacteria can cause diseases in cotton resulting in significant yield losses (Hillocks 1992). The average annual cotton production loss due to diseases and nematodes in the United States and other cotton producing countries is approximately 12% (Blasingame and Patel 2001). *Fusarium* wilt, caused by *Fusarium oxysporum* f. sp. *vasinfectum* (*Fov*), reduces cotton production by ~100,000 bales per year in the U.S. (Davis et al. 2006). There are six recognized races of *Fov*, cotton cultivars with resistance to all races do not exist and host resistance is the most effective approach for managing *Fusarium* wilt of cotton (Cianchetta and Davis 2015).

In plants, *NPR1* (Non-expressor of Pathogenesis Related genes-1) is a key regulator of salicylic acid mediated systemic acquired resistance against pathogens (Cao et al. 1994; Shah et al. 1997). Transgenic expression of *NPR1* gene in various plant species has been shown to confer tolerance to a broad spectrum of diseases. Makandar et al. (2006) showed that the expression of Arabidopsis *NPR1* gene in susceptible wheat cultivar conferred resistance to *Fusarium* head blight (FHB) caused by *F. graminearum*. They reported that this resistance against FHB is associated with faster activation of defense response in *AtNPR1*-expressing transgenic wheat. In another study, Malnoy et al. (2007) overexpressed a homologous *NPR1* (*MpNPR1*) in apple. They showed that the transgenic apple plants exhibited increased resistance against apple scab, cedar apple rust and fire blight diseases. Zhang et al. (2010) reported that over-expression of *AtNPR1* in citrus increased resistance against citrus canker, caused by the bacterial pathogen *Xanthomonas citri* subsp. *Citri* (Xcc). A recent study showed enhanced resistance against

Huanglongbing disease in citrus plants expressing the *AtNPR1* gene (Dutt et al. 2015). In our earlier investigations, we found that expression of *AtNPR1* in cotton conferred protection against two necrotrophic fungal pathogens, *R. solani*, (a cause of seedling damping-off) and *A. alternata* (a cause of foliar disease), two wilt diseases, *Fusarium* wilt caused by *Fov* and *Verticillium* wilt caused by nondefoliating pathotype of *Verticillium dahliae*, black root rot caused by *T. basicola*, and reniform nematodes (Parkhi et al. 2010a; Parkhi et al. 2010b; Kumar et al. 2013).

In the current investigation, we examined disease progression and defense response of *AtNPR1* transformants at the molecular level, in response to a challenge with *Fov11* isolate. In addition, we examined whether the *AtNPR1*-mediated protection observed earlier against *Fov11*, could be sustained with a related isolate, *Fov43*, and another highly virulent isolate, CA9.

Materials and methods

Plant material

Transgenic cotton plants (Coker 312) expressing an *AtNPR1* gene under the control of CaMV 35S promoter have shown resistance to multiple fungal pathogens, including *Fov11* (Parkhi et al. 2010a; Kumar et al. 2013). T4 generation plants of a transgenic line, 68L-19, were used in the current investigation.

Fusarium isolates

Two isolates belonging to VCG1a, namely *Fov11* (ATCC 46644) and *Fov43* (obtained from Insect Control & Cotton Disease Research Unit, Southern Plains Agricultural Research Center, USDA-ARS, College Station, TX) and CA-9 (race 4, a highly virulent isolate) were used to study

Fig. 1 The extent of disease progression in **a** wild-type (WT) and **b** *AtNPR1*-expressing transgenic plants, 7 days post-inoculation with *Fov11* by the stem puncture method. The presence of *Fov* was

confirmed by culturing the stem sections on PDA plates. Asterisk indicates that the distal leaf petiole was infected, meaning that the infection had progressed to the distal-most leaf

Fig. 2 Analysis of defense-related gene expression in the roots of the wild-type (WT) and *AtNPR1*-expressing cotton plants, 24 h post-inoculation with *Fov11* by the root dip method

the response of wild type (WT) and transgenic cotton plants.

Generation of GFP-expressing *Fov11* and measurement of disease progression

sGFP expression vector pCT74 was used to transform fungal protoplasts (Lorang et al. 2001). The protoplast preparation and transformation were carried out as described (Hohn and Desjardins 1992; Oren et al. 2003) with minor modifications. A few blocks of *Fov11* from potato dextrose agar culture were inoculated in 100 ml potato dextrose broth and grown for 10 days on a rotary shaker at 25 °C at 150 rpm. The culture was filtered through a sterile miracloth and centrifuged at room temperature at 5000 rpm for 10 min. The pellet was resuspended in 4–5 ml of the supernatant and a spore count of 10^8 was mixed in 100 ml of YEPD broth containing 0.3% Yeast Extract (Difco laboratories, Detroit, MI), 1% BactoTM peptone (Difco Laboratories, Detroit, MI) and 2% Dextrose (D-glucose) broth and grown for 12–14 h at 25 °C at 175 rpm on a rotary shaker. Protoplasts were prepared and regenerated as described by Hohn and Desjardins (1992) with the exception that the fungal cell walls were digested with 500 mg driselase, 1 mg chitinase, and 100 mg lysing enzyme (Sigma Chemical Co., St. Louis) and the regeneration/transformant selection was performed in plates

containing low melting temperature agarose (0.65%) in TB3 (0.3% yeast extract, 0.3% casamino acids and 20% sucrose) supplemented with 50 mg/L ampicillin (Sigma Chemical Co., St. Louis) and 50 mg/L hygromycin B (Sigma Chemical Co., St. Louis). GFP-expressing *Fov11* transformants were selected with the aid of fluorescence microscope (Zeiss Stemi SV11 with GFP filters; excitation: 470 nm and emission: 500–525 nm). Transgenic lines were examined for their virulence and one line was selected to examine disease progression in WT and 68L-19 cotton transformants.

Stem puncture method of inoculation was used to follow the progression of the infection and specifically to measure the spread of *Fov* into the shoot. GFP-expressing *Fov11* was grown on plates containing DifcoTM potato dextrose agar (PDA) medium supplemented with 50 mg/L of tetracycline for 1 week. A section of agar with sporulating mycelia was cut from the PDA plate and transferred to 2 mL sterile distilled water and vortexed for 5 s to prepare a conidial suspension. This conidial suspension was filtered through a sterile paper towel to remove mycelial debris. Conidial count was obtained using a haemocytometer and the final count was adjusted to 10^7 conidia/ml by diluting with sterile distilled water. A 23-gauge needle was used to inoculate 4-week-old cotton plants using the stem puncture method (Bugbee and Presley 1967; Bolek et al. 2005). Distilled water was used in place of the inoculum for mock treatment. Seven days post-inoculation, stem sections cultured on potato dextrose agar (PDA) medium. Internode sections from infected plants were surface sterilized in 10% bleach for 10 min followed by thorough rinsing (5 times) with sterile distilled water. The bark was removed using a sterile scalpel, the first, second, third and fourth internodes were split longitudinally into two halves. Sections from each internode were cultured separately on individual PDA plates and incubated at 25 °C for 4 days. The presence of *Fov11* in an individual internode was confirmed by observing the GFP-expressing mycelia with the aid of a fluorescence microscope (Zeiss M2 BIO Fluorescence Combination Zoom Stereo/Compound microscope; excitation: 470 nm and emission: 525 nm). A schematic diagram showing the strategy used to conduct this assay is shown in Fig. S1.

Fusarium wilt assay

Plants were grown for 2 weeks in sand before inoculating with *Fov* cultures, root-dip inoculation method was followed as described in a previous report (Parkhi et al. 2010a). To check the defense related gene expression analysis samples were harvested 24 h post infection (hpi) of the roots with conidial inoculum of *Fov11*. Controls consisted of mock treatment of the roots with water. To study the performance of cotton plants against high and low virulent isolates, various parameters such as stem

Fig. 3 Analysis of defense related gene expression in the hypocotyl tissue of the wild-type (WT) and *AtNPR1*-expressing cotton plants, 24 h post-inoculation with *Fov11* by the root dip method. M = Mock, I = Infected. Data represent mean $\pm$ SE for three biological replicate samples

height, stem weight, leaf weight and leaf disease index were recorded 2 weeks post-inoculation as described in Parkhi et al. (2010a) where plants were survived.

Defense related gene expression analysis

Gene expression analysis of the defense response was performed by northern blot hybridization in case of root samples. Total RNA was isolated using spectrum plant total RNA kit (Cat # STRN50-1Kt; Sigma, St. Louis, MO, USA). There were three biological replications for each treatment and the three replications were pooled to make one sample. A 6 μ g RNA sample was fractionated on a denaturing agarose gel and transferred to a nylon membrane (Cat #RPN303B, Amersham Pharmacia). Primers were designed based on cotton EST sequences available in the NCBI database (Table S1). These primers were used to amplify target gene sequences from the cDNA prepared from infected root RNA. PCR products were purified, labeled with P^{32} -dCTP, and used as probes in Northern hybridization as previously describe by Kumar et al. (2013). qRT-PCR analysis was performed on hypocotyl samples to study the expression of *GhPR1*, *GhLOX1*, *Ghchitinase* and *GhGlucanase* genes. Three independent biological replicates of seedling hypocotyls were harvested at 24 hpi. An aliquot

of 0.8 μ g total RNA was used to synthesize cDNA using Taqman reverse transcription kit (part #N808-0234; Applied Biosystems, USA). Gene specific primers were used to conduct qRT-PCR analysis are listed in Table S2. All the reactions were carried out with three technical replicates. Transcript levels of the defense genes were normalized to *GhHistone3*. Wild-type mock treatment (WT-Mock) was used as a standard for comparison of expression levels. The induction of defense related genes was calculated by using relative quantification method, i.e. $2^{-\Delta\Delta C_t}$ (Li et al. 2004).

Statistical analysis

In the experiments each treatment consisted of 10 replications unless otherwise stated. The data were analyzed through one-way ANOVA and are presented as mean $\pm$ standard error.

Results

Progression of disease in transgenic and WT plants

GFP-expressing *Fov11* was used to inoculate 4-week-old plants using stem puncture method. The plants were at the

Fig. 4 Disease severity observed in experiment #1 in the wild-type (WT) and *AtNPR1*-expressing cotton plants, 2 weeks post-inoculation with *Fov11* and *Fov43* by the root dip method, under growth chamber conditions

Fig. 5 Performance of wild-type (WT) and *AtNPR1*-expressing line 68L-19 against *Fov11* and *Fov43*. Parameters a. stem height, b. stem weight, c. leaf weight and d. leaf disease index were recorded at

2 weeks following the root dip method of inoculation with the pathogen in experiment #1, conducted in a growth chamber. Data represent mean $\pm$ SE, * $P < 0.05$; $n = 10$

three or four internode stage of growth at the time of termination of the experiment. Individual internodes from each plant were cultured separately. In addition, the leaf that had

fully emerged from the distal end of the uppermost node (3rd or 4th above the cotyledonary node) was also removed and its petiole cultured on a PDA plate to ascertain whether the

Fig. 6 Disease severity observed in experiment #2 in the wild-type (WT) and *AtNPR1*-expressing cotton plants, 2 weeks post-inoculation with *Fov11* and *Fov43* by the root dip method, under growth chamber conditions. **a** *Fov11*, **b** *Fov43*, and **c** mock treatment

pathogen had spread to this most distal tissue. Having a GFP-tagged *Fov11* culture allowed us to disregard other pathogens, if any that survived the surface sterilization process. The results, presented in Fig. 1, show that the disease had progressed in both sets of plants from the point of inoculation, however, the extent of its spread was lower in the transgenic line compared to WT. This is reflected by the number of distal internodes that remained free of the pathogen. The results from the cultured petiole segments (an asterisk on top of the bar indicates that the fungal infection had progressed to the distal most leaf) further support the notion that pathogen spread is inhibited in the transgenic plants.

Analysis of defense-related gene expression in root and hypocotyl tissues

The expression of nine different defense related genes were examined in the root tissue by northern blot hybridization. Results presented in Fig. 2 show that at least four of these genes, pathogenesis related-1 (*GhPR1*), lipoxygenase (*GhLOX1*), chitinase (*GhChitinase*), and glucanase (*GhGlucanase*) are induced at a higher level in the transgenic roots compared to their WT counterparts at 24 hpi in response to *Fov11*. Results from qRT-PCR analysis for the hypocotyl tissues are shown in Fig. 3. With the exception of *GhChitinase*, other three genes do not show any induction in WT hypocotyls in response to infection. In contrast, substantially elevated expression of *GhPR1*, *GhChitinase* and *GhGlucanase* was observed in the transformants. These results indicate that the *AtNPR1* transgenic line is able to launch a stronger defense response compared to their WT counterparts. In the absence of a biotic challenge, the *AtNPR1* transformants do not show increased activity of the defense-related genes with the exception of PR1 that showed slightly elevated expression.

Performance of *AtNPR1* transgenic lines cotton plants against different *Fov* isolates

Wilt assays were conducted using the root-dip inoculation method to determine the response of *AtNPR1* transgenic plants to various *Fov* isolates. In the first experiment *Fov11* and *Fov43* were used to infect the *AtNPR1* expressing and WT plants. Two weeks following the inoculation, plant health parameters were examined. Following inoculation with *Fov11*, the transgenic plants were relatively healthy and showed significantly lesser disease severity compared to their WT counterparts (Figs. 4, 5). Stem height, stem weight and leaf weight were 27, 66, and 80% higher, respectively, in the transformants compared to their WT counterparts. The leaf disease index for the 68L-19 line was 67% lower in comparison to the non-transgenic plants. In contrast, *Fov43* was equally damaging to the WT and the *AtNPR1* transformants (Figs. 4, 5).

This experiment was repeated for the second time using the same conditions. As seen in the first experiment, the *AtNPR1*-expressing transgenic plants were relatively healthy and showed significantly lesser disease severity compared to their WT counterparts following inoculation with *Fov11*. However, little, if any, protection was observed in the transgenic plants against *Fov43*. With the exception of one parameter (stem height), no significant differences were detected between the transgenic and WT plants (Figs. 6, 7). In a separate experiment, CA-9 (race 4, a highly virulent) isolate was used to infect both the

Fig. 7 Performance of wild-type (WT) and *AtNPR1*-expressing line 68L-19 against *Fov11* and *Fov43*. Parameters, **a** stem height, **b** stem weight, **c** leaf weight, and **d** leaf disease index were recorded at

2 weeks following the root dip method of inoculation with the pathogen in experiment #2. Data represent mean $\pm$ SE, * P < 0.05; n = 10

AtNPR1 and WT seedlings. Following the infection, both sets of plants were killed within a week.

Discussion

Constitutive expression of homologous and heterologous *NPR1* gene in several plant species has been shown to confer a broad-spectrum resistance to a variety of pathogens. In our earlier investigations with *AtNPR1* expressing cotton lines, we found that the transformants exhibited significant tolerance to *V. dahliae* (non-defoliating isolates TS2 and EZ2) *Fov11*, *R. solani*, *T. basicola*, *A. alternata*, and also reniform nematodes (Parkhi et al. 2010a; Kumar et al. 2013). In this study we examined the disease progression using GFP-expressing *Fov11* culture, which provided us with a reliable assay to examine the spread of the disease. The results presented in this study show clearly that while the transgenic plants are not immune to the disease, its progression is substantially inhibited. We conducted Northern blot analysis on root tissues and qRT-PCR analysis on hypocotyl segments following inoculation of plants with *Fov11*. Results obtained in this study confirm that several defense-related

genes are induced at higher levels in the *AtNPR1* transformants compared to their non-transgenic counterparts. Similar results, showing higher-level induction of downstream defense genes, have been obtained by others in wheat and apple (Makandar et al. 2006; Chen et al. 2012). It is interesting that the WT plants show little, if any, induction of these defense-related genes. Also, note that in the absence of a biotic challenge, the basal level activities of most of these genes are not elevated in the *AtNPR1* transformants. Thus, in this regard we have further confirmed the results of our earlier studies (Parkhi et al. 2010a; Kumar et al. 2013).

Based on the promising results against a broad spectrum of pathogens (Parkhi et al. 2010a; Kumar et al. 2013), we wondered if the tolerance would extend to other *Fov* isolates and especially, those known to be more virulent compared to *Fov11*. In two comparative experiments in the current investigation, while significant tolerance was observed in the *AtNPR1* transformants against *Fov11*, no such protection was seen against the more virulent isolate, *Fov43*. Next, we tested *AtNPR1* transformants against a highly virulent isolate CA-9 that belongs to race 4. Both WT and transgenic plants died within a week following inoculation.

In conclusion, while *AtNPR1* expression confers significant tolerance to the milder isolate *Fov11*, it is unable to provide any defense against the more virulent *Fov* isolates. Similar limitations were observed in the *AtNPR1* transformants against *Verticillium* wilt, where protection was observed only against the non-defoliating isolates of *V. dahliae* and not the defoliating isolates (Parkhi et al. 2010b). Nevertheless, there are a large number of reports that show that *NPR1* overexpression confers a strong protection against a variety of diseases (Lin et al. 2004; Makandar et al. 2006; Malnoy et al. 2007; Wally et al. 2009; Zhang et al. 2010; Dutt et al. 2015; Silva et al. 2015). Thus, controlled expression of *NPR1* gene may still prove to be a useful means to confer tolerance to some pathogens in the field.

Acknowledgements This research was supported by funds from Cotton Inc., Texas Higher Education Coordinating Board-Advanced Research Program (#000517-0005-2006), and Texas AgriLife Research. The pCT74 vector was kindly provided by Dr. Marty Dickman (Texas A & M University, USA).

References

- Blasingame DJ, Patel MV (2001) Cotton Diseases and their causal agents. In: Kirkpatrick TL, Rockroth CS (eds) Compendium of cotton diseases. American Phytopathological Society (APS Press), St. Paul, pp 7–8
- Bolek Y, Bell A, El-Zik K, Thaxton P, Magill C (2005) Reaction of cotton cultivars and an F2 population to stem inoculation with isolates *Verticillium dahliae*. J Phytopathol 153:269–273
- Bugbee WM, Presley JT (1967) A rapid inoculation technique to evaluate the resistance of cotton to *Verticillium albo-atrum*. Phytopathology 57:1264
- Cao H, Bowling SA, Gordon AS, Dong X (1994) Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell 6:1583–1592
- Chen XK, Zhang JY, Zhang Z, Du XL, Du BB, Qu SC (2012) Overexpressing *MhNPR1* in transgenic Fuji apples enhances resistance to apple powdery mildew. Mol Biol Rep 39:8083–8089
- Cianchetta AN, Davis RM (2015) Fusarium wilt of cotton: management strategies. Crop Protect 73:40–44
- Davis RM, Colyer PD, Rothrock CS, Kochman JK (2006) Fusarium wilt of cotton: population diversity and implications for management. Plant Dis 90:692–703
- Dutt M, Barthe G, Irely M, Grosser J (2015) Transgenic citrus expressing an *Arabidopsis* *NPR1* gene exhibit enhanced resistance against huanglongbing (HLB; citrus greening). PLoS ONE 10:e0137134
- Hillocks R 1992 Cotton diseases, CAB International
- Hohn TM, Desjardins AE (1992) Isolation and gene disruption of the *Tox5* gene encoding trichodiene synthase in *Gibberella pulicaris*. Mol Plant-Microbe Interact 5:249–256
- Kumar V, Joshi S, Bell A, Rathore K (2013) Enhanced resistance against *Thielaviopsis basicola* in transgenic cotton plants expressing *Arabidopsis* *NPR1* gene. Transgenic Res 22:359–368
- Li Z, Hansen J, Liu Y, Zemetra R, Berger P (2004) Using real-time PCR to determine transgene copy number in wheat. Plant Mol Biol Rep 22:179–188
- Lin W-C, Lu C-F, Wu J-W, Cheng M-L, Lin Y-M, Yang N-S, Black L, Green SK, Wang J-F, Cheng C-P (2004) Transgenic tomato plants expressing the *Arabidopsis* *NPR1* gene display enhanced resistance to a spectrum of fungal and bacterial diseases. Transgenic Res 13:567–581
- Lorang JM, Tuori RP, Martinez JP, Sawyer TL, Redman RS, Rollins JA, Wolpert TJ, Johnson KB, Rodriguez RJ, Dickman MB, Ciuffetti LM (2001) Green fluorescent protein is lighting up fungal biology. Appl Environ Microbiol 67:1987–1994
- Makandar R, Essig JS, Schapaugh MA, Trick HN, Shah J (2006) Genetically engineered resistance to *Fusarium* head blight in wheat by expression of *Arabidopsis* *NPR1*. Mol Plant Microbe Interact 19:123–129
- Malnoy M, Jin Q, Borejsza-Wysocka EE, He SY, Aldwinckle HS (2007) Overexpression of the apple *MpNPR1* gene confers increased disease resistance in *Malus x domestica*. Mol Plant Microbe Interact 20:1568–1580
- Oren L, Ezrati S, Cohen D, Sharon A (2003) Early events in the *Fusarium verticillioides*-maize interaction characterized by using a green fluorescent protein-expressing transgenic isolate. Appl Environ Microbiol 69:1695–1701
- Parkhi V, Kumar V, Campbell LAM, Bell AA, Rathore KS (2010a) Expression of *Arabidopsis* *NPR1* in transgenic cotton confers resistance to non-defoliating isolates of *Verticillium dahliae* but not the defoliating isolates. J Phytopathol 158:822–825
- Parkhi V, Kumar V, Campbell LM, Bell AA, Shah J, Rathore KS (2010b) Resistance against various fungal pathogens and reniform nematode in transgenic cotton plants expressing *Arabidopsis* *NPR1*. Transgenic Res 19:959–975
- Shah J, Tsui F, Klessig DF (1997) Characterization of a salicylic acid-insensitive mutant (*sai1*) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. Mol Plant Microbe Interact 10:69–78
- Silva KJP, Brunings A, Peres NA, Mou Z, Foltá KM (2015) The *Arabidopsis* *NPR1* gene confers broad-spectrum disease resistance in strawberry. Transgenic Res 24:693–704
- Wally O, Jayaraj J, Punja ZK (2009) Broad-spectrum disease resistance to necrotrophic and biotrophic pathogens in transgenic carrots (*Daucus carota* L.) expressing an *Arabidopsis* *NPR1* gene. Planta 231:131–141
- Zhang X, Francis M, Dawson W, Graham J, Orbović V, Triplett E, Mou Z (2010) Over-expression of the *Arabidopsis* *NPR1* gene in citrus increases resistance to citrus canker. Eur J Plant Pathol 128:91–100