Chitinase gene construct from *Trichoderma harzianum* proved effective against onion purple blotch caused by *Alternaria porri*

Mythili J B*, Chethana B S, Rajeev P R and Girija Ganeshan

1Division of Biotechnology and 2Plant pathology, Indian Institute of Horticultural Research, Bangalore 560089, India
ZARS (AICRP on Rice), VC Farm, Mandy 571405, India

Received 13 July 2015; revised 20 July 2016; accepted 26 July 2016

Purple blotch caused by *Alternaria porri* (Ellis) Ciffis the most devastating disease of onion prevalent in different parts of the country. In the absence of a resistant variety in the gene pool, gene transfer technique becomes appropriate as an alternative tool for genetic improvement. Accordingly, chitinase gene especially from the biocontrol agent *Trichoderma harzianum* Rifai which is used against several fungal pathogens was selected as the candidate gene. Onion transformation is difficult and *Allium* species are recalcitrant to transformation. Hence, before using this gene for transforming onion for conferring resistance/tolerance to *Alternaria porri*, chitinase gene was validated against purple blotch pathogen in a model plant system, tobacco. Tobacco transformants with *T. harzianum* chitinase (*Th-chit*) gene under the control of a strong constitutive CaMV 35S promoter with NPT-II selection marker were generated. The presence and expression of the transgene was confirmed through PCR and RT-PCR analysis, respectively. Bioassay of primary transgenic plants against *A. porri* through *in vitro* detached leaf bioassay revealed significant reduction in lesion size ranging from 73 - 100% as well as mycelial inhibition to the extent of 25 - 65% over the control (untransformed) plants. The results suggest that chitinase gene from *T. harzianum* can be used as a candidate gene for conferring resistance to *A. porri* in onion.

**Keywords:** Chitinase, purple blotch, transgenic plants, tobacco, *Trichoderma harzianum*

**Introduction**

Purple blotch caused by *Alternaria porri* (Ellis) Ciffis one among the serious fungal diseases that affect onion, causing heavy yield loss ranging from 2.5 to 87.8% during *kharif* season. The pathogen is seed, soil and airborne making it difficult to manage the disease. Therefore, an important aim in the genetic improvement of this crop is to breed for resistance to purple blotch. In the absence of a resistant variety in the gene pool, transgenic approach through introduction of pathogen-related (PR) protein genes appears to be the viable alternative for induction of resistance against purple blotch disease of onion. The most widely employed transgenic approach to enhance resistance against fungal diseases has been based on the over-expression of PR proteins, among which chitinases have been the most extensively studied and applied since 1991. Chitinases from different sources such as bacteria, plant and fungi have been used in the development of fungal resistant transgenic plants. For the past one decade, fungi have been identified as better producers of chitinase than bacteria and plants. Among the chitinases from several fungi such as, *Saccharomyces*, *Rhizopus oligosporous*, *Candida albicans* and *Trichoderma harzianum*, the endochitinases from *Trichoderma* has been found to be more effective in controlling fungi than the chitinases found in plants or in other fungi as the enzyme inhibits the spore germination and hyphal elongation of various fungal pathogens in *vitro*. The genome of mycoparasitic fungi, such as, *Trichoderma* spp. has evolved specifically to be capable of using other fungi but not plants as carbon sources and as such represent a potential source of powerful antifungal genes. The *Trichoderma* chitinase genes are capable of producing chitinolytic enzymes, possessing the antifungal activity to the level of chemical fungicides, and extensive testing in *vitro* has shown that there are virtually no chitinous pathogens resistant to *Trichoderma* chitinases. Hence, they have become excellent candidates for reinforcing plant defence hypersensitive reactions. The endochitinase isolated from *Trichoderma* species have been successfully utilized in the production of transgenic tobacco and potato plants with enhanced resistance to *Rhizoctonia solani*, *Alternaria solani*, *A. alternata* and...
Botrytis cinerea\textsuperscript{12}. In view of the effective biocontrol offered by T. harzianum, chitinase gene isolated from T. harzianum was utilized earlier\textsuperscript{13} for developing transgenic tobacco and demonstrating its efficacy in inhibiting the pathogen A. alternata which is the natural causal agent of leaf spot in tobacco. However, efficacy of Trichoderma chitinase gene against Alternaria porri has not been reported so far. As Allium species are recalcitrant to transformation, it was felt that the efficacy of T. harzianum chitinase gene against A. porri needs to be demonstrated in a model crop like tobacco before embarking on transforming a natural host to a difficult plant system. Even though tobacco is not a crop like tobacco before embarking on transforming a control and transgenic plants following CTAB method\textsuperscript{15}. The presence of transgene was analyzed through PCR amplification using CaMV 35S promoter specific forward primer and chitinase specific reverse primer. The PCR was carried out in 25 μl containing 100 ng of sample DNA; 0.5 μl of 10 mM dNTPs mix; 2.5 μl of 10X assay buffer for Taq DNA polymerase containing 15 mM MgCl\textsubscript{2}; 1.0 units (5 μl\textsuperscript{-1}), Taq DNA polymerase; 0.5 μl (10.0 μM) each of forward and reverse primers. Conditions for PCR were as follows: The DNA after initial denaturation at 95°C for 5 min was subjected to 40 cycles at 94°C for 30 sec for denaturation, 55°C for 40 sec for annealing, and 72°C for 1.0 min 30 seconds for extension and 72°C for 10 min for final extension. Amplified DNA fragments were electrophoresed on 1.0% agarose gel and observed under UV light.

### RT-PCR/First Strand cDNA Synthesis

Total RNA was isolated from transformed plants as well as from non-transformed control plants using Trip reagent (Sigma-Aldrich, USA), 100 mg of leaf tissue per 1 ml of Tri reagent was used. RNA from leaves in triplicate of both control and transformed plants were reverse transcribed to obtain cDNA using Thermo Scientific Revert Aid first strand cDNA synthesis kit as per manufacturer’s instructions. The template for the first strand cDNA synthesis reaction was 1.5 μg of DNase (Thermo Scientific) treated total RNA, was primed with oligo (dT) and 20 μl RT reaction was carried out for 1 h 30 min at 42°C. The first strand cDNA synthesized was used directly as template for PCR as indicated above.

### Isolation and Characterization of the Pathogen

Five isolates of Alternaria porri affecting onion was collected from onion growing regions of Andhra Pradesh, Tamil Nadu and Karnataka. Identification of the isolates was done based on morphological (conidial characters i.e. colour, surface, shape, beak, conidial arrangement, size of the conidia) cultural (potato dextrose agar (Difco; PDA) and molecular characteristics (based on ITS region). Fresh cultures of A. alternata isolated from infected tobacco leaves were grown on PDA at 24°C with a photoperiod of 16 h. This culture served as a control to test the efficacy of the test pathogen A. Porri.

### Testing Virulence of the Different Isolates of Alternaria porri Infecting Onion

Alternaria isolates of onion were screened for virulence against onion cv Arka Niketan by detached leaf method. Culture was grown on PDA for seven days at room temperature and the mycelial bits of

### Materials & Methods

Agrobacterium bacterial culture (Agrobacterium strain LBA4404 containing the binary vector pIIHR-Th-Chit with T. harzianum chitinase gene driven by the CaMV 35S promoter while NptII gene served as the plant selectable marker.

#### Transformation of Tobacco

Tobacco transformation protocol reported by Horsch et al (1987)\textsuperscript{14} was used with some modifications. Leaf discs, preconditioned for 2 days, were inoculated for 2 min with the overnight grown Agrobacterium culture diluted to 0.1 OD at A\textsubscript{600}. After the inoculation, the leaf discs were placed on shoot regeneration medium (MS basal medium supplemented with BAP 1 mg L\textsuperscript{-1}). After 2 days of co-cultivation, the explants were washed with half-strength MS medium with cefotaxime (125 mg L\textsuperscript{-1}) before transfer to selection medium (shoot regeneration medium containing 100 mg L\textsuperscript{-1} kanamycin and 500 mg L\textsuperscript{-1} of cefotaxime). The regenerated explants were subcultured on to the same selection medium for shoot elongation. The elongated shoots were then transferred to rooting medium (MS medium supplemented with 0.1 mg L\textsuperscript{-1} IAA, 50 mg L\textsuperscript{-1} kanamycin and 500 mg L\textsuperscript{-1} cefotaxime). The cultures were incubated in culture racks provided with white fluorescent tubes with a light intensity of 30 - 40 μ moles m\textsuperscript{-2} s\textsuperscript{-1} under 16 h photoperiod in a culture room maintained at 25 ± 2°C.

#### Confirmation of Presence of Transgene

**PCR Analysis**

DNA was isolated from leaves of non-transformed control and transgenic plants following CTAB

**Confirmation of Presence of Transgene**

**PCR Analysis**

DNA was isolated from leaves of non-transformed control and transgenic plants following CTAB
5 mm diameter were placed (inoculated) on the onion leaves which was previously pricked by the needle and incubated in humid chamber (80 - 90% RH) for seven days at 25°C. The symptom was observed after four days after inoculation. The lesion size was recorded periodically by measuring the length and width at right angles to each other and the infected area was calculated using the formula of area of ellipse:

$$A = \frac{\pi}{4} \times l \times w,$$

Where A = area of lesion, l = vertical diameter of the lesion in centimeter and w = horizontal diameter of the lesion in centimeter. Among the onion isolates OLA1 was found to be highly virulent which was used in all subsequent experiments.

### Maintenance of the Pathogen Strains for Virulence

All the isolates were maintained on PDA slants. The isolates were sub-cultured every six months and virulence was tested by inoculating the isolates on the host and observed for the necrotic and yellowing symptoms. The pathogen was re-isolated from the infected tissue and maintained on PDA slants. The cultural and morphological characters of the re-isolated culture was again studied and compared with the original culture.

### Functionality or Efficiency of Gene Construct

#### Pathogen Inhibition Assay in Detached Leaves

The T₀ plants were raised in the net-house and the detached leaves from PCR positive (for chitinase gene) plants were surface sterilised using 70% ethanol followed by two washes with sterilized water. The leaves were inoculated with 5 mm fungal discs of *A. porri* from the seven days actively growing culture. Another set of detached leaves were inoculated similarly with *A. alternata*. The leaves were then incubated at 25°C at 80 - 90% RH in a Petri plate of 15 cm diameter double lined moistened filter paper. Observations for symptom development were taken after 1 week of inoculation (at regular intervals). The lesion size was recorded and the lesion area was calculated

$$A = \frac{\pi}{4} \times l \times w,$$

Where A = area of lesion, l = vertical diameter of the lesion in centimeter and w = horizontal diameter of the lesion in centimeter. Among the onion isolates OLA1 was found to be highly virulent which was used in all subsequent experiments.

### Results and Discussion

Tobacco has been used routinely as model system to study the effect of isolated gene in conferring the desired phenotype through its over expression by *Agrobacterium*-mediated transformation. This is especially true in case of difficult to transform systems such as *Allium*. Developing onion (*A. cepa*) resistant to purple blotch is of foremost concern in the breeding of onion. Conventional breeding approaches have failed due to the lack of a resistance source. Under the circumstance, genetic transformation through the introduction of *T. harzianum* chitinase gene seems to be a viable alternative. In such a case, it is essential to confirm the resistant phenotype conferred by the proposed transgene prior to extensive and laborious transformation experiments. The chitinase gene from *T. harzianum* has conferred resistance against a number of fungal pathogens: *R. solani, B. cinerea, A. alternata* and *A. solani* in transgenic tobacco and potato. However, efficacy of *Trichoderma* chitinase gene against *A. porri* has not been reported so far.

Transformation of leaf explants of tobacco with *Agrobacterium* strain LBA4404 harbouring the binary vector (pIIHR-Th-Chit) containing the *T. harzianum* chitinase gene, driven by CuMV 35S promoter, Nos terminator and selectable marker nptII gene, resulted in regeneration of several kanamycin in resistant plants. PCR analysis of 9 randomly chosen kanamycin resistant plants revealed the presence for
full length chitinase gene along with 35S promoter (1.4 kb) in 6 of them and also in case of plasmid isolated from Agrobacterium (positive control) (Fig. 1). RT-PCR revealed the transgene expression in 4/6 plants analyzed (Fig. 2). Polymerase chain reaction (PCR) is the most commonly used technique to screen putative transformants. However, the presence of Agrobacterium in transformed tissues often leads to misleading results. Even a trace amount of plasmid DNA from contaminating Agrobacterium gives spurious results. The use of antibiotics for complete elimination of Agrobacterium during tissue culture is not very successful because most of the antibiotics used are bacteriostatic in nature, and these are used at a concentration that is not harmful to plant tissue. This problem is circumvented either by reverse transcription (RT)-PCR or Southern blot analysis. In the present study, we have used semi-quantitative RT-PCR to prove the authenticity of the obtained transgenic plants. We have also run RT-PCR controls without RT enzyme as DNA contamination would have given the positive results (Fig. 3). Southern blot analysis although successful, involves more processing time, very often requires radioactivity and large amounts of tissue DNA. However, Southern blot has the advantage of determining the copy number of the transgenes. As our study involved only testing the proof of concept, we did not attempt doing Southern as we felt that it would be necessary/mandatory when we are commercializing an event and where gene expression with respect to trait of interest may be linked to the copy number.

Functionality or Efficacy of Gene Construct

Pathogen Inhibition Assay in Detached Leaves (In vitro detached leaf assay)

Pathogen inhibition assay in detached leaves was evaluated on the basis of development of lesions. Before proceeding with evaluation of transgenic lines, the pathogenicity of the test pathogen A. porri was tested on host (onion) and non-host (tobacco) and was compared with virulence of A. alternata on its natural host tobacco. It was observed that A. porri could infect tobacco as effectively as it could infect its natural host onion. However, virulence of A. porri on tobacco was slightly reduced as compared to
*A. alternata* on tobacco but was sufficient enough to test the efficacy of the gene in transgenic lines (Fig. 4). In leaves of tobacco, *A. porri* showed typical necrosis symptoms surrounded by a chlorotic halo as seen with *A. alternata* infection while the symptoms of *A. porri* on onion leaves resulted in necrotic lesion with mycelial growth. In general, there was decline in lesion development in almost all transgenic lines as compared to control when inoculated with *A. porri* or *A. alternata*. Symptoms on transgenics, if present, were a faint chlorosis or a hypersensitive-like necrosis

with little sporulation. Only RT-PCR positive samples were included in the study. The transgenic lines varied in their expression of disease resistance with reduction in lesion size ranging from 73.3 - 100% and 49.3 - 86.7% in comparison to lesion size in control leaves when inoculated with *A. porri* and *A. alternata*, respectively (Fig. 5 & 6.)

**In Vitro Pathogen Inhibition Assay**

*In vitro* pathogen inhibition assay revealed that the transformed plant extract with *T. harzianum* chitinase gene showed 25 - 65% more mycelial growth inhibition of *A. porri* over the control (untransformed) plants as seen in assay of poison food technique. As a control, plant extract of transgenic lines were also tested for mycelial inhibition of *A. alternata*. The reaction exhibited 32 - 62% increased mycelial growth inhibition of the *A. alternata* over the control (untransformed) plants (Figs. 7 & 8). Thus, the inhibition of the mycelial growth of *A. porri* achieved by *T. harzianum* chitinase gene was equivalent to that obtained for *A. alternata*. Variations in pathogen inhibition with respect to both lesion size as well as mycelial inhibition was seen

![Fig. 4 — Virulence/pathogenicity of test pathogen *Alternaria porri* on non-host tobacco (a) host plant onion (b) in comparison with *A. alternata* on tobacco (c).](image)

![Fig. 5 — Percent reduction in lesion size in transgenic lines harbouring the *Trichoderma harzianum* chitinase gene upon inoculation with *Alternaria porri* and *A. Alternata*.](image)

![Fig. 6 — Inhibition of growth of *Alternaria porri* (a) and *A. alternata* (b) in detached leaves of transgenic tobacco harbouring *Trichoderma harzianum* chitinase gene.](image)

![Fig. 7 — Percent inhibition of mycelial growth of *Alternaria porri* and *A. alternata* in transgenic tobacco lines harbouring the *Trichoderma harzianum* chitinase gene.](image)
shown to be effective against A. Alternata, respectively. Such variations in disease resistance has been reported. Variability of expression may be attributable to different sites of integration of the transgene or to differential methylation. Efficacy of T. harzianum chitinase gene in inhibiting growth of A. alternata has been shown earlier as well in our studies. The proposed transgene to be used, viz., chitinase gene from Trichoderma has been successfully used for developing transgenic apple, broccoli, lemon with enhanced resistance to several fungi. The endochitinases from biocontrol agents such as T. harzianum have been found to be the most effective in terms of both antifungal and lytic activities in comparison with other types of chitinolytic enzymes such as those obtained from plant, bacterial or other fungi assayed under the same conditions. In addition to conferring resistance to the several fungal diseases, viz., R. solani, B. cinerea, A. alternata and A. solani in transgenic tobacco and potato, T. harzianum chitinase gene has now been shown to be effective against A. porri. The results of this study will pave way for utilizing T. harzianum chitinase gene for conferring resistance to purple blotch through genetic transformation of onion.

Acknowledgements

The funding from Department of Biotechnology conformant of India (DBT), to carry out part of the work is gratefully acknowledged. Authors also wish to thank Director, IIHR, Bangalore for extending necessary support.

References


Fig. 8—Inhibition of growth of Alternaria porri (a) and A. alternata (b) in potato dextrose agar medium amended with the untransformed (control) tobacco plant extract and the extracts of tobacco plant transformed (Tr) with Trichoderma harzianum chitinase gene.


