Expression of Rice Chitinase Gene in Genetically Engineered Tomato Confers Enhanced Resistance to Fusarium Wilt and Early Blight

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This is the first study reporting the evaluation of transgenic lines of tomato harboring rice chitinase (RCG3) gene for resistance to two important fungal pathogens Fusarium oxysporum f. sp. lycopersici (Fol) causing fusarium wilt and Alternaria solani causing early blight (EB). In this study, three transgenic lines TL1, TL2 and TL3 of tomato Solanum lycopersicum Mill. cv. Rio-grande genetically engineered with rice chitinase (RCG3) gene and their R1 progeny was tested for resistance to Fol by root dip method and A. solani by detached leaf assay. All the R0 transgenic lines were highly resistant to these fungal pathogens compared to non-transgenic control plants. The pattern of segregation of three independent transformant for Fol and A. solani was also studied. Mendelian segregation was observed in transgenic lines 2 and 3 while it was not observed in transgenic line 1. It was concluded that introduction of chitinase gene in susceptible cultivar of tomato not only enhanced the resistance but was stably inherited in transgenic lines 2 and 3.

Keywords: early blight, fusarium wilt, rice chitinase gene, tomato

Fungal diseases are the major constraint to substantial yield in agronomically important crops and impacted the well-being of human’s world wide (Agrios, 1998). The fungal species Alternaria, Aspergillus, Fusarium, Verticillium causes severe damage in crops such as potato, tomato, cotton, caster and chickpea and many other monocotyledonous and dicotyledonous crops. With the advancement in biological techniques in the early 1980’s, a major area of research has been to identify, clone and characterize various genes involved in disease resistance. The identification of disease resistant genes has made it possible to evaluate their specific roles and importance in disease response pathways using transgenic plants developed with genetic engineering. From the last two decades advances have been made in utilizing a broad range of cloned genes (from plants or other organisms) to develop resistance against fungal pathogens in plants.

The pathogenesis related proteins (PR proteins) include hydrolytic enzymes (chitinases and glucanases), antimicrobial proteins (thionin, defensins, lectin), ribosome inactivating protein (RIP), phytoalexins and antifungal proteins osmotin and thumin like. The expressions of PR are directly toxic to pathogens or reduce their growth. Chitinases hydrolyzes poly-β-1,4-N-acetyl glucosamine known as chitin. Cell wall of most of the fungi is composed of chitin as the major component and thus in plants chitinase acts as the one of the important defenses against these fungi (Collinge et al., 1993). Plant chitinase serve as a safe and biodegradable biocontrol agent for use instead of a conventional fungicide (Karasuda et al., 2003).

Rice has a small genome and hence serving as the model for plants genetics and genomics. Several defense related important genes have been cloned from rice (Song and Goodman, 2001). These genes have been engineered into many important dicotyledonous and monocotyledonous crops such as straw berry to develop resistance against powdery mildew (Asao et al., 1997), cucumber and Chrysanthemum kitamura against Botrytis cinerea (Tabei et al., 1998; Tabaeizadeh et al., 1999), Japonica rice against sheath blight (Nishizawa et al., 1999), grape against Elisinoe ampelina (Yamamoto et al., 2000), rice against

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Six weeks old non-transgenic and transgenic tomato plants (Solanum lycopersicum Mill cv. Rio grande harboring rice chitinase (RCG3) were evaluated for resistance to fusarium wilt caused by F. oxysporum and early blight caused by A. solani.

**Materials and Methods**

**Evaluation of the transgenic plants for their resistance to fungal pathogens.** Three transgenic lines of tomato (Jabeen et al., 2009) Solanum lycopersicum Mill cv. Rio grande harboring rice chitinase (RCG3) were evaluated for resistance to fusarium wilt caused by F. oxysporum and early blight caused by A. solani.

**Fusarium wilt test.** Six weeks old non-transgenic and transgenic plants were evaluated to compare the level of tolerance to F. oxysporum. For this purpose plants were dipped in the freshly prepared inoculum of F. oxysporum for one minute. Plant treated with water was used as control. Plants were incubated at 28°C for three weeks.

**Early blight test.** Six weeksold non-transgenic and transgenic plants were evaluated for their resistance to A. solani by detached leaflet assay.

**Detached leaflet assay.** Three leaves from the upper leaflets were detached from each transgenic and non-transgenic plant. These leaves were placed in petri plates containing 0.1% agar. A 50 µl drop of the spore suspension (containing 2,000 conidia) was deposited on the upper surface of each leaf using micropipette (Foolad and Ntahimpera, 2000). Leaves treated with water were used as control. The inoculated leaves were incubated in the dark for 24 h at 22°C then maintained under cool white florescent diurnal light with a 12 h photoperiod. Lesion size (length × perpendicular width) was measured on the 7th day. EB severity on each leaf was recorded on a scale of 0 to 5 (Chaerani et al., 2007; Vakalounakis, 1983) where

0 = No visible lesion on leaf
1 = Up to 10% leaf area affected
2 = 11–25% leaf area affected
3 = 26–50% leaf area affected
4 = 51–75% leaf area affected
5 = More than 75% leaf area affected or leaf abscised

The disease scales were converted into percentage of EB index (PEBI) for each plant using the following formula (Pandey et al., 2003).

\[
\text{Percentage of early blight incidence (PEBI)} = \frac{\text{Sum of all ratings} \times 100}{\text{No of leaves sampled} \times \text{maximum disease scale}}
\]
Determination of inheritance of chitinase gene in T1 seedling. In order to determine the inheritance of chitinase gene in T1 progeny six weeks old seedling was tested for resistance to *F. oxysporum* and *A. solani* by simple inoculation method. The Mendelian segregation in the seedlings was determined to check the inheritance of chitinase gene.

Statistical analysis. All field experiments were carried out in randomized completely block design (RCBD) and lab experiments were carried out in completely randomized design (CRD). Each experiment was replicated thrice. Data was analysed by using Minitab 13, Statistica and MSTAT-C softwares. Significance of data was checked by using least significance (LSD) test.

Results

Resistance of transgenic plants and non-transgenic control plants against fungal pathogens was evaluated by pathogenesity test.

Assay with *Fusarium oxysporum*. Transgenic and non transformed control plants after 4 weeks of their establishment in soil were incubated with *F. oxysporum f. sp. lycopersici*. After two days of infection at lower leaves turned golden yellow in non-transformed control plants while leaves remained fresh and green in transgenic plants. On the fifth day leaves of the whole non-transgenic plant became yellow and finally plant wilted on 7th day of infection while transgenic plant stayed alive in the infected soil for three weeks (Fig. 1).

Leaf assay with *Alternaria solani*. Leaves of the transgenic and non transgenic control plants were also tested for their resistance against *A. solani*. After 14 days of incubation with *A. solani* complete necrosis and chlorosis of the non-transgenic plant was observed because fungus covered the whole lamina while in case of transgenic leaves infection spreaded in media (Fig. 1A, 1B) and fungus could not grow on the leaf lamina. Susceptibility/resistance of the control (treated with water) and transgenic and normal leaves (infected with *A. solani*) was determined in terms of the lesion size and percentage of early blight incidence (PEBI). Comparison of the lesion size of three transgenic lines and non transgenic leaves is summarized in Table 1 and Table 2. Results show (Table 1) that lesion size and PEBI of non-transgenic (treated with *A. solani*) was high compared to transgenic leaves (transgenic infected with *A. solani*). In case of non transgenic PEBI was 100 (HS, score = 5) while in case of transgenic lines it ranged from 6.6 to 20. All the transgenic lines were resistant (moderate or high). Transgenic line 1 was moderately resistant (PEBI = 20, score = 2) and transgenic lines 2 and 3 were resistant (PEBI = 6.66, score = 1). It was concluded that chitinase enhanced the level of resistance in all the transgenic lines.

Transgene inheritance for hygromycin resistance. Fruits from the R0 transgenic plants were harvested and seeds...
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were isolated. These seeds were subjected for germination on medium containing 50 mg/l hygromycin to get the hygromycin resistant R1 progeny. The genetic segregation to hygromycin resistance was observed in R1 progeny seedling. Expected Mendelian 3:1 ratio (Table 3) was observed in transgenic lines 2 and 3 while transgenic line 1 had shown no expected segregation.

**Table 2.** Comparison of the mean lesion size in the leaves of the control (treated with water), non-transgenic and transgenic plants (treated with *A. solani*)

| Plant              | Leisionsize ± S.E (cm) | Percentage of early blight incidence | Disease score | Disease reaction |
|--------------------|------------------------|-------------------------------------|---------------|-----------------|
| Control            | 0.00 ± 0.00           | 0                                   | 0             | Nil             |
| Non-transgenic     | 635.00 ± 7.63         | 100                                 | 5             | Highly susceptible |
| Transgenic line 1  | 5.66 ± 2.02           | 20                                  | 2             | Moderately resistant |
| Transgenic line 2  | 0.33 ± 0.33           | 6.6                                 | 1             | Highly resistant |
| Transgenic line 3  | 0.33 ± 0.33           | 6.6                                 | 1             | Highly resistant |

LSD for plants = 18.78
Values followed by the same letters are not significantly different at α = 0.01
Data is the average of three uppermost leaves from each plant

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**Table 3.** Mendelian segregation in selfed R1 hygromycin resistant progeny

| Transgenic plant | Total number of T1 seedling | Resistant | Susceptible | Segregation of hyg |
|------------------|-----------------------------|-----------|-------------|--------------------|
|                  |                             |           |             | Expected ratio     | $x^2$ | p-value |
| Transgenic line 1| 12                          | 8         | 4           | 2:1               | 0.44  | 0.50    |
| Transgenic line 2| 12                          | 9         | 3           | 3:1               | 0.00  | 1       |
| Transgenic line 3| 13                          | 10        | 3           | 3:1               | 0.00  | 1       |

**Fig. 2.** Leaf assay for the early blight incidence in tomato. (A) Non transgenic leaf on media after 14 days of incubation with *A. solani*. (B) Transgenic leaf on media after 14 days of incubation with *A. solani*.

**Table 4.** Mendelian segregation in selfed T1 (wilt resistant) progeny

| Transgenic plant | Total number of T1 seedling | Resistant | Susceptible | Segregation of chitinase |
|------------------|-----------------------------|-----------|-------------|--------------------------|
|                  |                             |           |             | Expected ratio     | $x^2$ | p-value |
| Transgenic line 1| 13                          | 9         | 4           | 2.2:1               | 0.43  | 0.51    |
| Transgenic line 2| 8                           | 6         | 2           | 3:1                  | 0.00  | 1       |
| Transgenic line 3| 16                          | 12        | 4           | 3:1                  | 0.00  | 1       |

**Discussion**

Three transgenic lines of (TL1, TL2 and TL3) and their R1

**Transgene inheritance for resistance to fungal pathogens.** The resistance of T1, six weeks old hygromycin resistant seedling to early blight and wilt was evaluated by simple inoculations. The pattern of segregation of three independent transformant is listed in Table 4 for *Fol* and Table 5 for *A. solani*. Mendelian segregation was observed in transgenic line 2 and 3 while it was not observed in transgenic line 1. It was concluded that chitinase gene was stably inherited in transgenic lines 2 and 3 only.

**Table 5.** Mendelian segregation in selfed T1 (wilt resistant) progeny

| Transgenic plant | Total number of T1 seedling | Resistant | Susceptible | Segregation of chitinase |
|------------------|-----------------------------|-----------|-------------|--------------------------|
|                  |                             |           |             | Expected ratio     | $x^2$ | p-value |
| Transgenic line 1| 13                          | 9         | 4           | 2.2:1               | 0.43  | 0.51    |
| Transgenic line 2| 8                           | 6         | 2           | 3:1                  | 0.00  | 1       |
| Transgenic line 3| 16                          | 12        | 4           | 3:1                  | 0.00  | 1       |

$L^2$ = Chi-square
3 : 1 ratio indicates integration of transgene at single locus.
Table 5. Mendelian segregation in selfed R₁ (early blight) resistant progeny

| Transgenic plant | Total number of T₁ seedling | EB resistant | EB susceptible | Segregation of chitinase |
|------------------|-----------------------------|--------------|----------------|-------------------------|
| Transgenic line 1| 8                           | 5            | 3              | 2:1                     | $\chi^2$ = 1.77, p = 0.182 |
| Transgenic line 2| 12                          | 9            | 3              | 3:1                     | $\chi^2$ = 0.00, p = 1 |
| Transgenic line 3| 13                          | 10           | 3              | 3:1                     | $\chi^2$ = 0.00, p = 1 |

$\chi^2$ = Chi-square
3:1 ratio indicates integration of transgene at single locus.

progeny harboring rice chitinase gene were evaluated for resistance to fusarium wilt caused by *F. oxysporum lycopersicii* and early blight caused by *A. solani*.

**Assay with Fusarium oxysporum.** Three PCR positive R₀ transgenic plants were tested for resistance to *F. oxysporum* by root dip method. Results indicated that all transgenic plants were highly resistant to *F. oxysporum* and no symptoms of wilt were observed. It was concluded that chitinase gene (RCG3) expressed its antifungal activity in all the transgenic plants tested. (Kumar et al., 2004) produced transgenic pigeonpea plant with rice chitinase gene and induced resistance against wilt caused by *Fusarium* spp. (Hussain et al., 2008) produced transgenic plants harboring rice chitinase gene in potato. These plants were highly resistant to local Pakistani isolate of *F. oxysporum*.

**Assay with Alternaria solani.** These PCR positive T₀ transgenic plants were also examined for resistance to early blight (EB) caused by *A. solani* by detached leaf assay. Detached leaf assay method has already been reported for the evaluation of resistance in transgenic lines against different fungal pathogens in number of crops like cucumber (Tabei et al., 1998) and rice (Nishizawa et al., 1999). Results indicated that chitinase gene induced resistance to *A. solani* in all the transgenic plants (PEBI was less than 20) compared to the control (PEBI was 100).

Rice chitinase gene (RCC2 and RCG3) has already been widely reported for its broad spectrum antifungal activity against number of fungal species in many monocots and dicots (Nishizawa et al., 1999) observed resistance in transgenic rice plants harboring rice chitinase gene against blast (*Magnaporthe grisea*). (Tabei et al., 1998) and (Kishimoto et al., 2002) produced cucumber transgenic lines highly resistant to gray mold caused by fungal pathogen *B. cinerea* (Yamamoto et al., 2000) produced transgenic grapevine plants expressing rice chitinase gene resistant to powdery mildew caused by fungus *Uncinula necator* and anthracnose caused by *Elisineae ampellina*. Maziah et al. (2007) reported resistance against fungal pathogens in banana plants harboring rice chitinase gene. Results of the present study and earlier reports revealed that rice chitinase could be utilized to enhance the resistant against number of fungal pathogens in agronomically important crops.

**Transgene inheritance for hygromycin resistance.** In order to determine the transgene inheritance Seeds were isolated from the three R₀ transgenic lines and hygromycin resistance of R₁ progeny was determined on the MS-medium supplemented with 50 mg/l hygromycin. Hygromycin resistant R₁ seedlings were also evaluated for resistance to *Fol* and *A. solani* by inoculation method. Mendelian segregation ratio 3:1 (resistant: susceptible) was observed in R₁ offsprings of the two resistant transgenic lines. (Ignacimuthu and Cesar, 2012) reported that expression of rice chitinase (RCC2) gene in finger millet resulted in the development of resistance against leaf blast disease. Inheritance of RCC2 was also confirmed in R₁ progenies. The segregation of disease resistance among the progenies was according to predicted Mendalian ratio of 3:1. Parasad et al. (2013) reported the enhanced resistance against soil borne and foliar fungal pathogens in transgenic peanut plants harboring rice chitinase gene. They observed that transgene inheritance was on the pattern of Mendelian inheritance in all R₁ progenies. In our study Mendelian segregation was not followed by the R₁ progenies of the transgenic line 1 although it was moderately resistant. This contradictions in our study and earlier report is may be due to the transient integration of rice chitinase gene in transgenic line 1 developed during the present study. This difference is may be the difference in the varietal response to transgene integration among difference species.

It was concluded that expression of rice chitinase gene in tomato confers enhanced resistance against two major fungal diseases of tomato. If it is stably inherited the offspring can be as strong as their parents. Resistance of these transgenic lines against many other fungal pathogens can also be tested.
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