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Functional validation of plant transformation vector with stacked *ech42* and *bgn* from *Trichoderma* in tomato for fungal disease resistance

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Abstract

Transgenic tomato lines (cv. Pusa Ruby) were generated by using *Agrobacterium tumefaciens* strain LBA4404 harboring endochitinase (*ech42*) and endoglucanase (*bgn*) genes stacked in binary vector (pRAGS121). Ten putative transformants in T_0 generation were confirmed by PCR. Progenies of two transgenic tomato lines, CG2 and CG7 showed the presence of transgenes in the T_1 generation. Transgene integration and copy number of transgene was assessed using PCR, Dot blot, Southern hybridization in T_2 generation. Southern hybridization using DIG-labelled *ech42* specific probe revealed the presence of two copies of transgenes. RT-PCR showed expression of *ech42* and *bgn* at transcript level. Chitinase and glucanase assay revealed 4.09 and 3.93 fold higher expression of *ech42* and *bgn* respectively in transgenic plant compared to non-transgenic plant. Bioassay of transgenic plant against *Alternaria solani* showed 2.97 times reduction in the leaf area infection and against *Sclerotium rolfsii* showed significant growth inhibition compared to non-transgenic control.

Key words: Endochitinase, endoglucanase, southern hybridization, tomato, transgenics

Introduction

Tomato (*Solanum lycopersicum*) is the second most important vegetable crop after potato in the world. Tomato is also used as a model plant species for genetic studies related to fruit quality, stress tolerance (biotic and abiotic) and other physiological traits. Despite decades of conventional breeding and selection, still over 200 diseases have been reported to affect tomato (Watterson 1986) which makes its production challenging in various parts of the world. Developing transgene-mediated resistance to fungal, bacterial and other pathogens via genetic engineering is one of the effective methods of combating plant diseases in many important crops including tomato.

Fungal diseases like early blight, late blight, fusarium wilt are very important and cause 30-40 per cent loss in tomato production (Punja 2006). One of the commonly used approaches for imparting fungal disease resistance is the use of genes encoding for chitinases and glucanases. Several studies have reported the synergistic effect of these enzymes in control of phytopathogenic fungi (Sela-Buurlage et al. 1993; Jongedijk et al. 1995; Melander et al. 2006; Awah et al. 2011; Akula and Dinesh 2011; Erika et al. 2013). Therefore, it is desirable to transfer both the genes coding for chitinases and glucanases in order to achieve better resistance to fungal diseases. In this study, the plant transformation vector (pRAGS121) constructed in our laboratory (Sharma 2009) carrying both chitinase and glucanase genes in the same T-DNA was validated in tomato.

Materials and methods

Plant transformation and selection of transformants

The *Agrobacterium tumefaciens* strain LBA4404 with recombinant binary vector pRAGS121 (Fig. 1) carrying stacked endochitinase (*ech42/ chit-g1*) (AM113506.1) and endoglucanase (*bgn*) (EU 149643) genes (cloned in our laboratory from *Trichoderma virens*) along with selection marker gene, *nptII*, was used for

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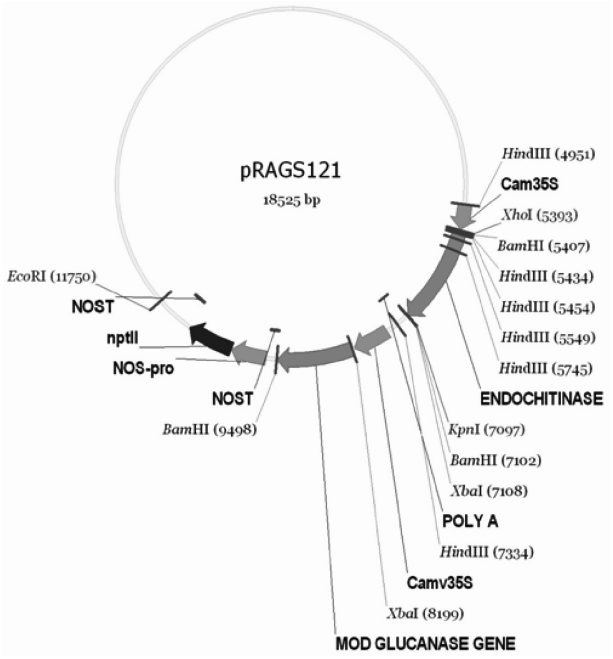

Fig. 1. vector map of pRAGS121

transformation of tomato cv. Pusa Ruby using the protocol given by McCormick (1991) with some modifications. For identification of putative transformants, DNA was isolated using CTAB method and PCR assay was performed for presence of transgene in hardened plants of T_0 generation using selectable marker, *nptII* specific primer. Further confirmation of the presence of transgene was achieved using primers designed for partial length of *ech42* and *bgn*. The primer pairs used and the expected amplicon length are presented Table 1.

Table 1. Primer pairs used for confirmation of putative transgenic tomato plants

Gene	Primer name	Primer seqence	Amplicon length(bp)
nptII	nptII	F-5'-GAGGCTATTC GGCTATGACTG-3' R: 5'-ATCGGCAGGGG CGATACCGTA-3'	700
ech42	chithyb	F: 5'-GGCAAGCACCAT GTCACCCTT-3' R: 5'-TGGGGGAGCTCA GCAGGTTCT-3	516
bgn	modglu5	F: 5'-TTTGCGTGGC TGCCCCAAGAC-3' R:5'- GTGAAGGCGG TCCTGCTGCTGAC- 3'	580

Confirmation for the presence of transgenes in the progenies of putative transformants

Forty seeds each from 10 putative transformants of T_0 generation which were labeled as, CG-2, CG-5, CG-7, CG-12, CG-15, CG-16, CG-18, CG-23, CG-50, CG-52, were sown in nursery trays. DNA isolated from individual T_1 generation plants was used to screen the presence of transgene using specific primers. Further ten seeds from each PCR positive T_1 generation plant were sown in nursery trays. DNA isolated from individual plants was used to screen the T_2 generation using gene specific primers.

Dot blot and Southern blot analysis of T_2 transformants

Dot blot analysis was carried out by using DIG-High Prime DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany, cat. no. 11585614910). DNA isolated from PCR positive T_2 plant along with the non-transgenic control plant and plasmid DNA were loaded on the nylon membrane and rest of the protocol followed was same as that of Southern hybridization except restriction digestion of DNA samples.

Further, complete Southern blot analysis was carried out using the DIG-High Prime DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany, cat. no. 11585614910). High molecular weight genomic DNA was isolated from the young leaves of T_2 PCR-positive tomato plant by CTAB method. Southern blot analysis was carried out using standard protocol (Sambrook and Russell 2001).

Expression analysis of transgenes

RT-PCR

Total RNA was isolated from PCR positive tomato plant and non transgenic control plant using TRIzol reagent (Sigma-Aldrich Pvt. Ltd. USA). The total RNA was treated to remove DNA using Turbo DNA-free^{TM}kit (cat#AM1907 Ambion, USA) as per the manufacturer's instruction. Absence of genomic DNA contamination was subsequently confirmed by PCR with total RNA as template. Single stranded cDNA was prepared by using High Capacity cDNA Reverse Transcription kit (cat#4374966, Ambion, USA) as per the manufacturer's protocol. Prepared cDNA was used as template for PCR amplification by using gene specific primers for *ech42* and *bgn*.

Estimation of chitinase and glucanase activity

Total protein was extracted from transgenic tomato plant CG2-17-6 using the procedure described by Velasquez and Hammerschmidt (2004). The total protein was estimated using Lowry's (1951) method and equal quantity of protein was used for further assays.

Colloidal chitin was prepared following the method of Roberts and Selintrenikoff (1988) with certain modifications. The per cent transmission of the standard and the plant samples was recorded against reagent blank which was adjusted to 100% T at 540 nm and reducing sugars present per gram of the sample were calculated based on the NAG standard graph. Further, for plant samples, 100 µl of crude protein, 100 µl of McILvaine (1920) buffer and 100 µl of colloidal chitin were added and standard protocol (Katany et al. 2000) for chitinase assay was followed. In order to identify the chitinase activity, reducing sugars released by 100 µl of leaf extract in 30 min was converted into pico moles of reducing sugars released per microgram of crude protein per min. Further glucanase enzyme activity was assayed by using colorimetric method described by Katany *et al* (2000).

Bio-efficacy analysis of transgenic tomato for fungal disease resistance

Bioassay against Sclerotium rolfsii

Four different concentrations of crude protein extract from leaves of transgenic and non-transgenic tomato (250 µg, 500 µg, 750 µg, 1000 µg) were used from transgenic and non transgenic plant. First, extract was mixed with PDA and plates were prepared uniformly in four replications and sclerotial bodies were kept in the centre of each plate. Plates were incubated overnight at 25°C and 80-90% humidity in the dark (Harighi et al. 2007). Per cent inhibition of fungal growth was calculated by formula.

$$\text{Per cent inhibition of growth} = \frac{\text{Colony diameter of control - Colony diameter of treatment (Transgenic /Non transgenic)}}{\text{Colony diameter of control}} \times 100$$

Bioassay against Alternaria solani

Detached leaf assay was used for bioassay against foliar pathogen, *Alternaria solani* according to Shah *et al.* (2010).

Results and discussion

Chitinases are thought to play a dual role, both by inhibiting fungal growth by cell wall digestion and by releasing pathogen-borne elicitors that induce further defense reactions in the host. Transgenic plants over expressing chitinases of several origins have been shown to exhibit enhanced levels of resistance to fungal infection and delayed disease symptoms when challenged with fungal pathogens (Lorito et al. 1998; Punja 2006; Jeum and byung 2005). Also there are several reports indicating the synergistic activity of chitinases and glucanases. Further, studies have also indicated the role of chitinase gene in tolerance to various abiotic stresses in tobacco and Arabidopsis (Dana et al. 2006).

Tomato transformation and PCR analysis of putative transgenics

The transformation protocol was repeated in 9 batches and about 1300 explants were infected with recombinant *Agrobacterium tumefaciens* strain LBA4404 carrying pRAGS121 vector. In all, about 81 plants survived in green house condition. The PCR assay of 81 plants with gene specific primers identified 10 transformants for the presence of transgene. These plants showed the amplicon of 701 bp for *nptII,* 516 bp for *ech*42 and 580 bp for *bgn* (Fg. 2).

Confirmation of putative transformants in T_1 and T_2 generations

Per cent germination of T_1 seeds ranged between 55 to 92.5 percent. Progenies of only two plants *viz*., CG -2 and CG-7 showed presence of desired genes in the T_1 generation. In total, out of 296 plants screened, only six plants showed the presence of all the three genes (Table 2). Seeds were collected from all the PCR positive plants and sown to raise next generation plants. Of the progenies of six T_1 generation plants, progenies of only three plants, CG2-2, CG2-17, CG2-18, showed presence of desired genes in the T_2 generation. In total, out of 40 plants screened, only six plants showed the presence of all the three genes (Table 3). Due to severe incidence of white fly, 5 positive plants out of total 6 plants, were infected with

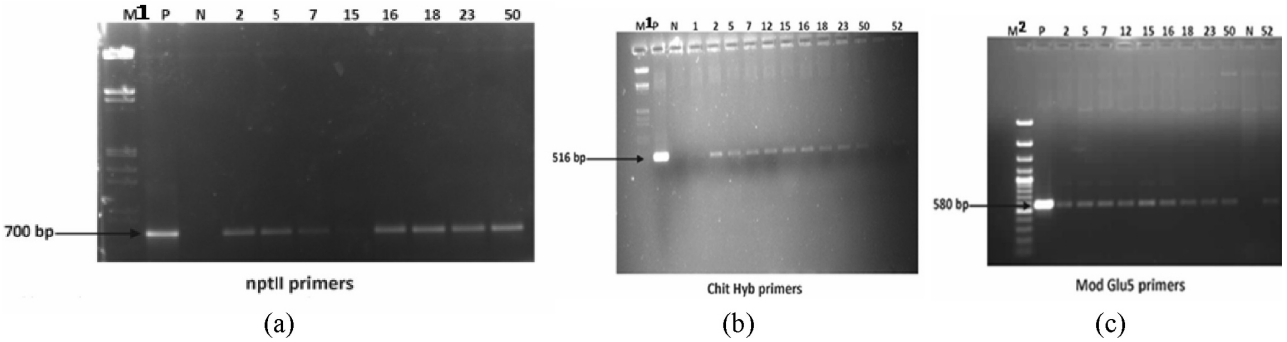

Fig. 2. PCR confirmation of tomato plants transformed with pRAGS121 using (a) *nptII*, (b) chit hyb and (c) mod glu primers. M1- 1Kb DNA ladder , M2- 100 bp DNA ladder, P- Positive control; N- Negative control; 2, 5, 7,12,15,16,18,23,50 52 - Plant DNA samples

Table 2. Screening of T_1 generation tomato transgenics carrying *ech*42 and *bgn*

Plant ID	Seeds sown	No. of seeds germi-nated	Germi-nation (in %)	No. of PCR positive plants	Plant ID given
CG -2	40	25	62.5	5	CG2-2, CG2-17, CG2-18, CG2-19, CG2-22
CG -5	40	31	77.5	-	
CG -7	40	22	55	1	CG7-2
CG -12	40	32	80	-	
CG -15	40	35	87.5	-	
CG -16	40	25	62.5	-	
CG -18	40	31	77.5	-	
CG -23	40	25	62.5	-	
CG -50	40	33	82.5	-	
CG -52	40	37	92.5	-	
Total	400	296	74	6	

Table 3. Screening of T_2 generation tomato transgenics carrying *ech*42 and *bgn*

Plant ID	Seeds sown	No. of seeds germi-nated	Germi-nation (in %)	No. of PCR positive plants	Plant ID given
CG-2-2	10	5	50	1	CG2-2-3
CG-2-17	10	8	80	3	CG2-17-5, CG2-17-4, CG2-17-6
CG-2-18	10	8	80	2	CG2-18-6, CG2-18-7
CG-2-19	10	7	80	-	
CG-2-22	10	6	60	-	
CG-7-2	10	6	60	-	
Total	60	40	66.66	6	

Tomato Leaf Curl Virus (TLCV) and hence could not be included in further studies and bioassays. Only CG2-17-6 was used for further studies. T_1 generation did not show the typical 3:1 segregation. This ratio is expected only in the transgenic events with single copy integration.

Dot blot analysis using *ech42* and *bgn* specific probes quickly helped to confirm the presence of both the genes in the transgenic tomato plant tested. Southern blot analysis revealed the integration of two copies of transgenes within the genome. Two bands of size 10.2kb and 8 kb were observed on the nylon membrane (Fig. 3).

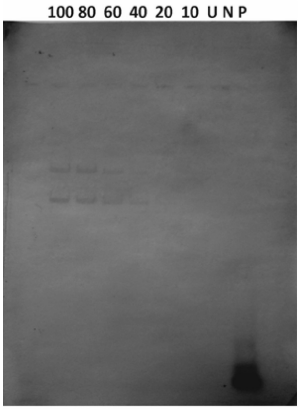

Fig. 3. Southern blot analysis using *ech42* probe. P- Positive control; N- Negative control; U- Uncut DNA 10-10µg to 100µg DNA

Analysis of expression of ech42 and bgn

It is important to express transgenes in the heterologous system without any structural and functional limitations. Many factors are known to play a key role in determining the expression of a transgene

(Butaye et al. 2004; Butaye et al. 2005). RT- PCR using *ech42* and *bgn* specific primers gave the amplicon size of 516 bp and 580 bp respectively and indicated the expression of the transgene at transcript level (Fig. 4a &b). Further it can be noted that, in the PCR reaction with RNA as a template did not give any amplification which ruled out the genomic DNA contamination in the RNA sample.

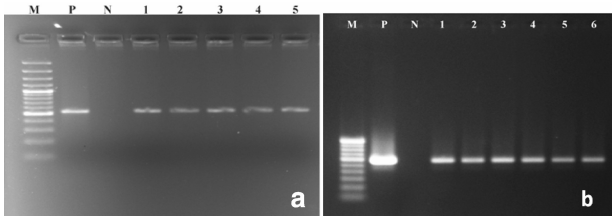

Fig. 4. Confirmation of expression of a) *ech42* and b) *bgn* through RT- PCR, M-100bp DNA ladder; P = Positive control; N = Negative control; 1, 2, 3, 4, & 5 = cDNA samples

The PCR and southern blot positive transgenic plant (CG2-17-6) showed significantly higher (4.09 times) level of chitinase enzyme activity compared to control. The chitinolytic activity observed in non transgenic plant was 75.67 pmol of N-acetyl glucosamine released/µg of crude protein/min, and enzyme activity in transgenic plant observed was 309.89 pmol of N-acetyl glucosamine released/µg of crude protein/min (Table 4). Further the glucanolytic activity observed in non transgenic plant was 95.16 pmol of N-acetyl glucosamine released/µg of crude protein/min, while in transgenic plant it was 374.76 pmol of N-acetyl glucosamine released/µg of crude protein/min (3.93 times higher) (Table 4).

Bioassay of transgenic tomato plants for disease resistance

Per cent growth inhibition of *S. rolfsii* increased with increasing concentration of crude protein and was maximum when 1000 µg of crude protein was added to PDA (83.39 per cent) (Fig. 5a) (Table 5). PDA with 500 µg of crude protein from transgenic tomato, showed

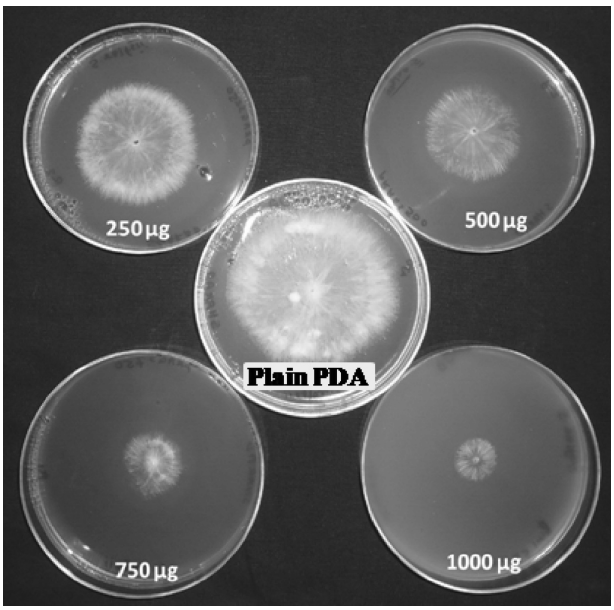

Fig. 5a. Growth inhibition of *Sclerotium rolfsii* on PDA with crude protein extract of transgenic tomato

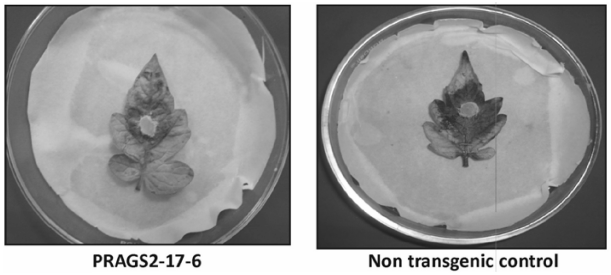

Fig. 5b. Lesion development on tomato leaves inoculated with *Alternaria solani*

65.69 per cent inhibition compared to control. However, Supriya (2012) observed 48.66 per cent inhibition when 500 µg crude protein from transgenic tomato with only *ech42*. This increase in per cent inhibition may be because of synergistic effect of *ech42* and *bgn*. Similar synergistic effects between chitinase and glucanase on fungal pathogens were reported earlier (Melander et al. 2006; Awah et al. 2011; Akula and Dinesh 2011 ; Erika et al. 2013).

Table 4. Chitinolytic and glucanolytic activities (pmol of reducing sugar/µg of total protein/min) in transgenic tomato plants

S.No.	Plant ID	Enzyme activity (pmol/µg/min)			
		Chitinase	Fold increase over control	Glucanase	Fold increase over control
1	CG -17-6	309.8	4.09	374.76	3.93
2	Control	75.67	1	95.16	1

Table 5. Per cent growth inhibition of *Sclerotium rolfsii* on PDA with leaf extract from transgenic tomato

Quantity of crude protein extract in PDA	Percent growth inhibition	
	Non transgenic (control)	Transgenic (CG 2-17-6)
250µg	15.83	54.22
500µg	32.46	65.69
750µg	50.91	78.97
1000µg	54.97	83.39
Control	0	
CV (%)	3.044	
CD at 0.01	2.907	
SEm±	1.54	

Alternaria solani, a foliar pathogen, is the causal agent of early blight of tomato is the most serious disease which causes 5-78 per cent losses worldwide. Detached leaf assay of transgenic plant against *Alternaria solani* showed very slight symptoms, and the symptoms did not spread when the incubation period was extended where as control leaf showed extended chlorosis and decaying (Fig. 5b).

The present study demonstrated the working of pRAGS121 with stacked *ech42* and *bgn* (cloned in our laboratory) in tomato and this vector can be used to impart fungal disease tolerance in other crop plants. Also transgenic tomato plant developed in this study can be crossed with other popular tomato varieties to impart broad spectrum disease tolerance. The transgenic tomato can be studied for their tolerance to some of the abiotic stresses. However, more number of transformants needs to be produced in order to identify a transgenic tomato with relatively higher level of tolerance to fungal diseases.

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