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Enhancing transgenic pea (*Pisum sativum* L.) resistance against fungal diseases through stacking of two antifungal genes (chitinase and glucanase)

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Key words: pea, combined transgenes, crossing, fungal resistance, expression stability

Abbreviations: *bar*, bialaphos resistance gene; PPT, phosphinothricin; PR-protein, pathogenesis related protein

One way of enhancing and broadening resistance of plants to different biotic and abiotic stresses is to combine transgenes expressing several genes into a single line. This can be done using different strategies such as crossing, single vector with multiple genes, co-transformation, sequential transformation and IRES elements. In the present study, conventional crossing method was used. Parental transgenic lines transformed via *Agrobacterium tumefasciens*-mediated gene transformation with pGreenII binary vector harboring a *bar* gene as a selectable marker in combination with the family 19 chitinase gene from *Streptomyces olivaceoviridis* for one line and 1,3-β-glucanase from barley (*Hordeum vulgare*) for the other line were used for crossing. Both chitinase and glucanase genes were cloned into pGreenII vector under the control of the constitutive double 35S-promoter from cauliflower mosaic virus. Progenies expressing the two genes were characterized at the molecular level using PCR, RT-PCR and Southern blot analysis, as well as segregation and stability studies of the respective expression levels. Leaf paint assay was used as a functional test for herbicide resistance genes. Stable inheritance of the antifungal genes in the transgenic plants was demonstrated. The synergistic effect of crossed plants was tested using in vitro assay which shows higher inhibition of spore germination.

Introduction

Diseases are considered the most important cause of high yield losses with fungal diseases being the most important, yield losses can range between 50–75%.[1-4] Constitutive expression of individual PRs in transgenic plants can lead to reduced pathogen growth and symptom development, consistent with a role of PRs in the expression of acquired resistance.[5,6] Dana et al.[7] generated transgenic tobacco (*Nicotiana tabacum*) lines that overexpress the endochitinases CHIT33 and CHIT42 from the mycoparasitic fungus *Trichoderma harzianum* and evaluated their tolerance to biotic and abiotic stresses. Both CHIT33 and CHIT42, individually, conferred broad resistance against fungal and bacterial pathogens as well as tolerance to salinity and heavy metals. Transgenic plants overexpressing 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.[8-10]

Most frequently described antifungal proteins are probably chitinases and β-1,3-glucanases. The expression of glucanase and chitinase genes respectively in plants enhances their resistance against fungal pathogens.[11-13] It has also been demonstrated that these hydrolases act synergistically in both in vitro and in vivo assays, resulting in a very strong antifungal activity.[14-16] A high level expression of these genes has been detected in transgenic rice and barley leading to a high resistance.[8,17] The co-expression of chitinase and glucanase genes in tobacco enhanced resistance against *Cercospora nicotianae*.[8,17-19] In tomato, simultaneous expression of the basic tobacco chitinase PR-3d and glucanase PR-2e provided substantial protection against *Fusarium oxysporum* f.sp. *lycopersici*, whereas transgenic plants expressing either one of these genes were not protected.[20]

Two or more transgenes can be sequentially introduced into a plant by conventional iterative procedures, e.g., a plant containing one transgene is crossed with individuals harboring other transgenes[21] or alternatively, is re-transformed with new genes,[22] co-transformation[23] and dicistronic vector with IRES elements.[24] The crossing techniques have been used at the research level to combine or reinforce existing transgenic traits. It has been demonstrated that crossing plants expressing different *Bacillus thuringiensis* (Bt) toxins provided an effective way of delaying the emergence of Bt-resistant pests. Cao et al.[25] illustrated this in

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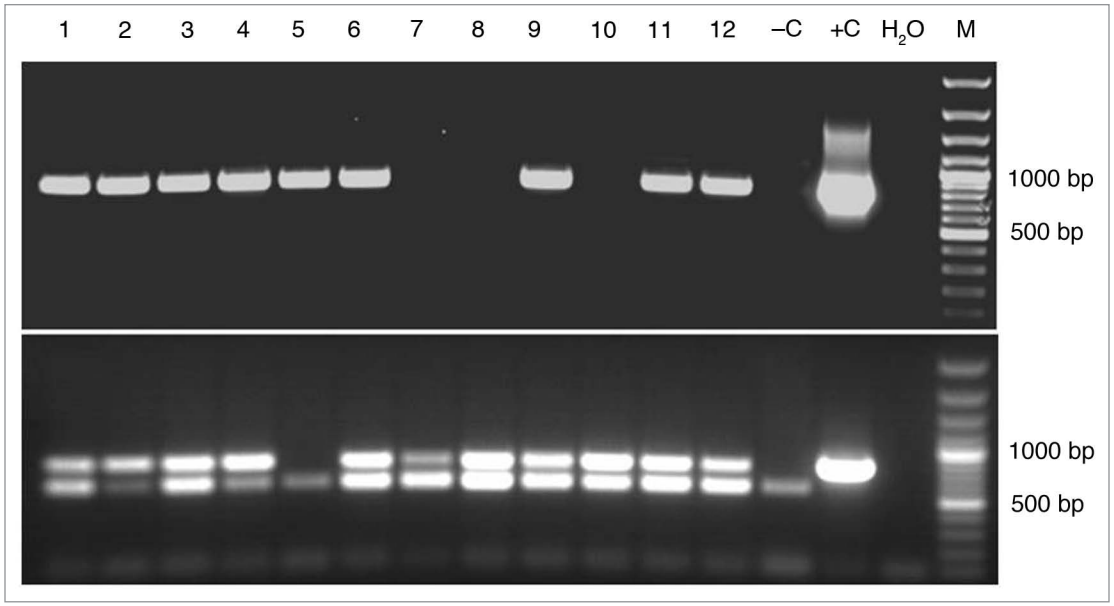

Figure 1. Simple and Multiplex PCR using primers for glucanase with product size of 750 bp (upper photo), chitinase with amplification of 750 bp and HMG presenting product size 570 bp (lower photo) to show segregation in the F_2 plants. Lanes 1–4, 6, 9, 11 and 12 have both genes while segregation is observed in lanes 5, 7, 8 and 10; -C, negative control untransformed plant; +C, positive control (Plasmid pGII-gluc and pGII-chit30) and M, 100 bp DNA molecular marker.

broccoli where pyramided *cry*1Ac and *cry*1C Bt genes controlled diamondback moths resistant to either single protein and significantly delayed the evolution of resistant insects.[26] Similarly, Datta et al.[27] developed disease- and pest-resistant rice by crossing plants expressing the *Xa*21 gene (resistance to bacterial blight) with plants expressing both a Bt fusion gene and a chitinase gene (resistance to yellow stem borer and tolerance to sheath blight, respectively).

The objective of the present study was to enhance the resistance level of pea to fungal diseases by crossing two transgenic pea lines one expressing chitinase gene and the second expressing glucanase gene. The consequence was transgenic pea plants expressing both chitinase and glucanase genes.

Results and Discussion

One of the major problems impeding the advance of plant genetic engineering and biotechnology is the fact that the expression or manipulation of multiple genes in plants is still difficult to achieve. Gene pyramiding is emphasized to obtain many complex biochemical pathways in plants for crop improvement and durable resistance. Approaches can involve conventional sexual crossing, re-transformation, co-transformation and the use of linked transgenes.[27] The level of expression of a transgene is variable and is influenced by various factors, such as the site of integration or position effect.[28] Crossing of transgenic parental lines allows a direct evaluation of protective interaction between the transgenes in the crossed plants with that provided by each transgene alone at the same respective loci in the parental lines.[29] Gene 'pyramiding' or 'stacking' present advantages and offer the potential for providing higher resistance to diseases. Interesting genes in this study were chitinase and β-1,3-glucanase, two of pathogenesis-related proteins, produced in response to microbial infection. These enzymes hydrolyse chitin and glucan, respectively of fungal cell walls.[30]

Successful introduction of the chitinase and glucanase genes into the pea genomic DNA was analysed using specific primers for the chitinase and glucanase in the F_0 and subsequent generations (**Fig. 1**).

Copy numbers and integration patterns were investigated in the crossed transgenic F_1 to F_4 generations respectively using Southern blot analysis with different probes (*chit* and *gluc*). One or two copies were detected in the progenies which reflected the same copy numbers of the parental transgenic lines (**Fig. 2**).

RNA denaturing gel using formaldehyde was used to check the RNA integrity. Transcription of the glucanase and chitinase genes was confirmed using RT-PCR. Most of the crossed transgenic F_1 to F_4 generations clearly exhibited the transcription of the genes as shown in **Figure 3**.

Leaf paint assay was done to verify the expression of the bar gene. One week after application, clear effects of herbicide were observed (**Fig. 4**). The 600 mg/l of BASTA® used in the present study is high in comparison to that used by other groups, for example 200 mg/l on pea[31] or 400 mg/l on faba bean (*Vicia faba*).[32] Recovery of herbicide resistant plants from sensitive parental plants through recombination in meiosis was observed in some lines. **Table 1** shows a summary table of leaf paint results from F_1 to F_4 generations.

During the establishment of homozygous lines most of the transgenic lines which inherited the *bar* gene became sensitive to the herbicide BASTA® in subsequent generations (**Fig. 5**). This result is similar to those observed by Richter et al.[33] In this study,

polygalacturonase inhibiting proteins (*PGIP*) from raspberry and stilbene synthase (*Vst*) from grape were expressed in pea. The *PGIP*, as well as the *bar* gene, exhibited independent expression dynamics in different generations. They observed the silencing of the bar gene in the T1 of some lines.

In some cases, this may be due to gene inactivation or silencing, methylation, co-suppression or due to the physical loss of the gene due to incomplete T-DNA transfer to the plant genome, since the bar gene is located next to the chitinase and glucanase genes near the left border of T-DNA.[34,35]

Crude extracts of different transgenic F_2 and F_3 progenies showed inhibitory effects on spore germination of *Trichoderma harzianum* in contrast to extracts from isogenic transgenic lines, untransformed pea line (negative control) or Na-acetate buffer as shown in **Figure 5**.[36]

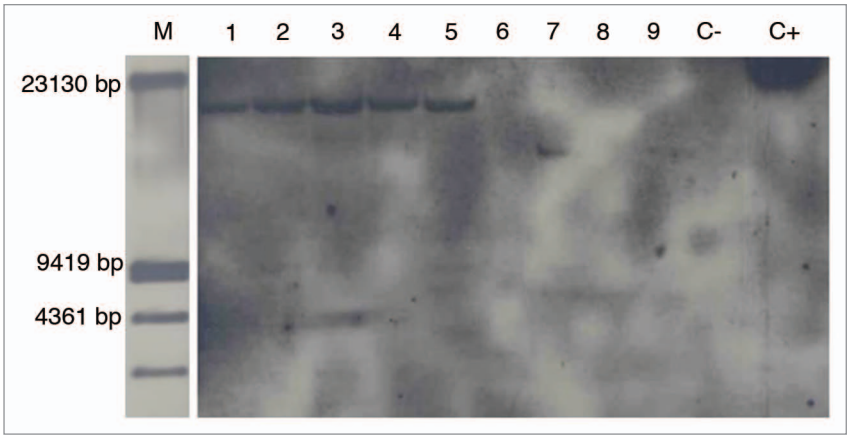

Figure 2. Southern blot results using chitinase probe for F_4 crossed transgenic plants. Lanes 1–9 (07/18-1-2-1-3, 07/18-1-2-2-3, 07/20-2-2-2-1, 07/20-2-2-2-4, 07/38-1-2-3-1, 07/38-1-2-3-3, 07/38-1-2-4-3, 07/38-1-2-2-6, 07/20-2-2-2-2), -C: negative control untransformed plant, + C: plasmid pGII-chit 30 as positive control.

Materials and Methods

Plant material. Parental transgenic pea lines (03-04-1,3,6,1-F and 02-04-7-1,1,2,3,2-F) were carrying a *Chit* 30 gene coding for chitinase from *Streptomyces olivaceoviridis*,[34] as well as (98-49-6,1-1-5-9-3-) containing *gluc* gene coding for 1,3-β-glucanase from barley (*Hordeum vulgare*) [35] in their homozygous state. The chitinase and glucanase genes were inserted in the T-DNA region of the pGreenII binary vector under the control of double 35S-promoter (from cauliflower mosaic virus) and a pA-terminator (**Fig. 6**). A selectable marker, herbicide resistant *bar* gene from *Streptomyces hygroscopicus* was inserted between the NOS-promoter and a Tg7pA-terminator.

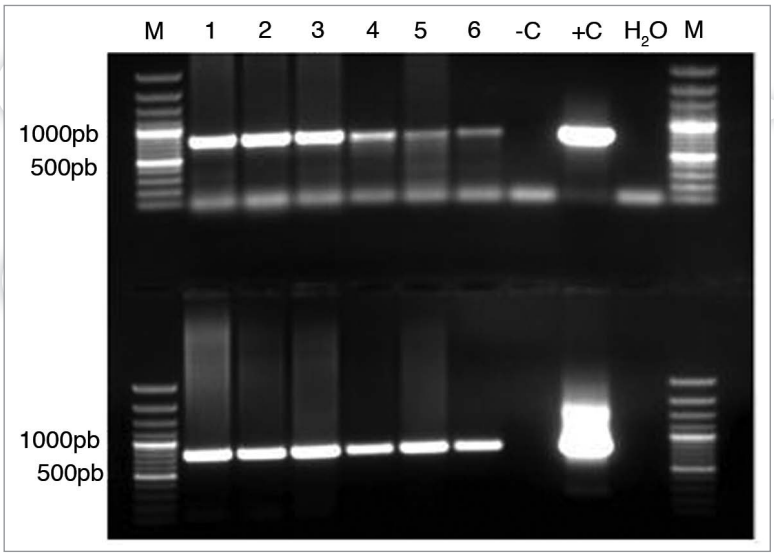

Figure 3. RT-PCR results of F_4 crossed transgenic plants using *chit* primer (upper photo) and *gluc* primers (lower photo). Lanes 1–6 (07/18-1-2-3-3, 07/18-1-2-2-3, 07/20-2-2-2-2, 07/20-2-2-2-4, 07/38-1-2-3-1, 07/38-1-2-3-3), +C: positive controls: plasmid pGII-chit 30 & pGII-gluc; -C: negative control untransformed plant; M: 100 bp DNA molecular marker.

Cross pollination. Transgenic seeds were grown at 17–22°C in a 16/8 h day night interval. At flowering stage, after 6–8 weeks of planting, mature pollen was transferred manually from the donor plants to the stigma of emasculated recipient flower for pollination, the flowers were closed, labelled and let to continue growing until setting the seeds. Mature crossed seeds were harvested and dried before germination in greenhouse for multiplication and further analyses. Detection of integrated genes into transgenic pea plants was done by polymerase chain reaction (PCR).

Detection of transgenic progenies by PCR. Genomic DNA was isolated from transgenic and non-transgenic plants using the CTAB (cetyl-trimethyl ammonium bromide) extraction method after Doyle and Doyle.[37] Specific primers were used to detect *glucanase* gene (forward: 5'-TGC ATG GCG TGT GCT ACG GA-3'/reverse: 5'-TTT CCA CCG ATG CCC CGA AC-3'), *chitinase* gene (forward: 5'-GGT GAC ATC GTC CGC TAC AC-3'/reverse: 5'-CTA GAT CAG CAG TAG AGG TT-3') and HMG (high-mobility group protein, accession X99373) gene as an internal control (forward: 5'-ATG GCA ACA AGA GAG GTT AA-3'/reverse: 5'-TGG TGC ATT AGG ATC CTT AG-3').

PCR was carried out in a volume of 25 µl reactions consisting of 1 µl template DNA (30–50 ng), 5 µl 5x Green GoTaq® Flexi PCR buffer, 2.5 µl $MgCl_2$ (25 mM), 1 µl of dNTPs (10 mM), 1 µl (10 pmol) each primer, 0.2 µl (1 U) GoTaq® polymerase (Promega) and 13.3 µl sterile nuclease-free water. The amplification was carried out in a T3 thermal cycler (Biometra). Positive, negative and water controls were also run along with DNA samples of interest to avoid the detection of false positive or false negative results.

PCR conditions were: initial denaturing at 94°C for 1 min, 30 cycles at [denaturing at 94°C, 45 s; annealing at 60°C,

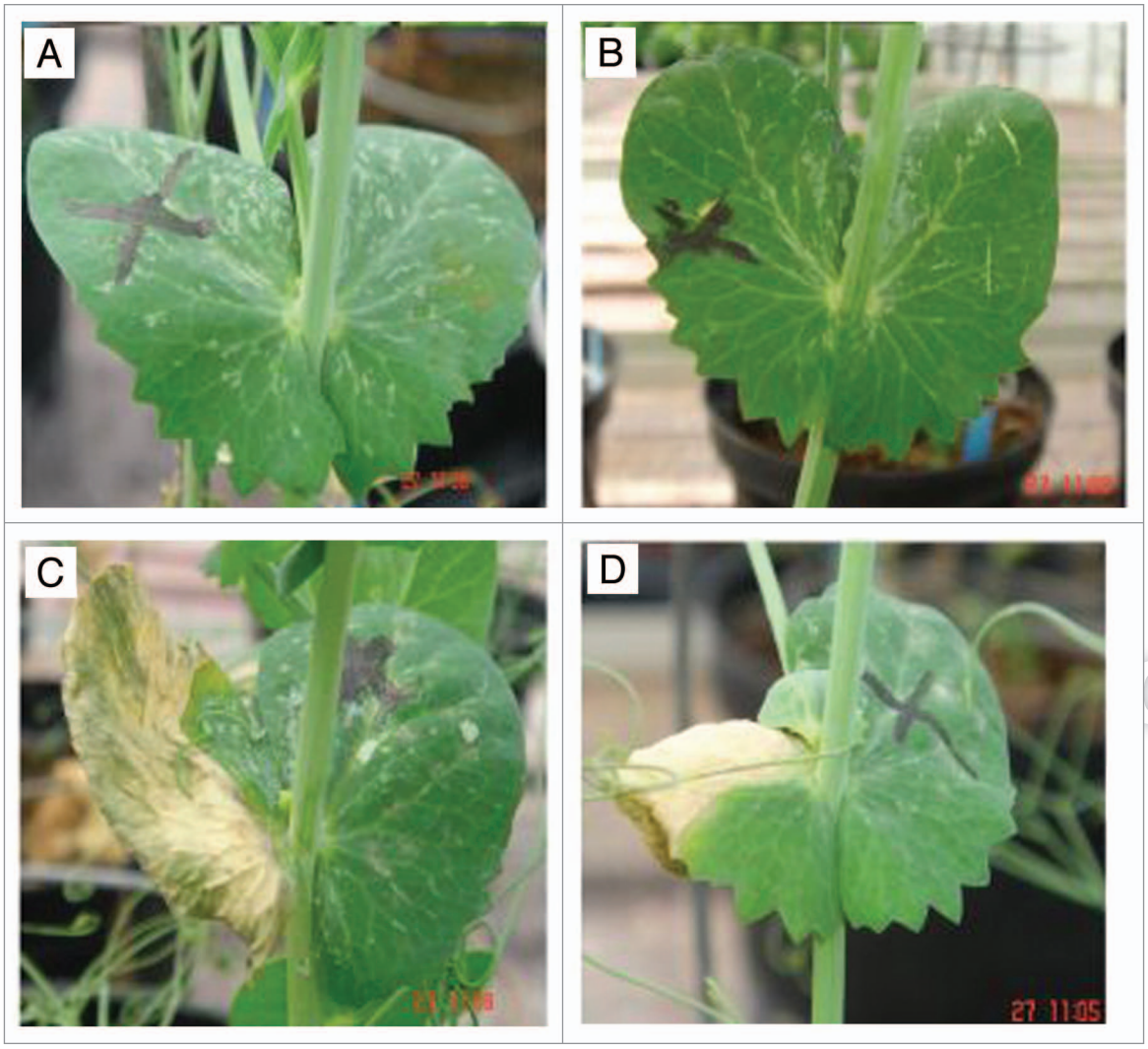

Figure 4. Leaf paint assay after one week of 600 mg/l BASTA® application: (A) crossed transgenic plant; (B) transgenic chitinase plant; (C) trangenic glucanase plant; (D) untransformed control. The unmarked leaflet present the treated leaflet with herbicide while the marked one is control without treatment.

45 s; extension at 72°C; 45 s]; final extension at 72°C, 5 min. The PCR products (20 µl of sample per lane) along with 100 bp DNA molecular weight marker were electrophoresed on 1.0% agarose gel stained with EtBr and visualized by UV transillumination.

Southern blot analysis. To analyse the integration pattern and copy number of introduced genes, Southern blot was performed. About 30 µg of genomic DNA was digested with a restriction enzyme (*Bam*HI), separated by electrophoresis (0.8% agarose), depurinated, denaturated and blotted onto a positively charged nylon membrane (Roche Diagnostics GmbH). Non-radioactive detection method using digoxigenin (DIG) system according to the application manual for filter hybridization (Roche Diagnostics GmbH, 2000) was applied. Filters were prehybridized with DIG Easy Hyb for 3 h at 42°C then hybridized with PCR-DIG-labelled probes over night at 42°C, the probe was prepared according to the manufacturer's manual (Roche Diagnostics GmbH, Mannheim, Germany) briefly to 50 µl final volume the following compounds were added 1 µl template DNA (30–50 ng), 10 µl 5x Green GoTaq® Flexi PCR buffer, 5 µl $MgCl_2$ (25 mM), 0.25 µl of dNTPs (10 mM), 1 µl (10 pmol) each primer, 2 µl PCR Dig labeling mix (Roche) 1 µl (1 U) GoTaq® polymerase. The quality of the probe was checked on 1% agarose gel and compared with control probe without adding PCR Dig labeling mix.

Expression analysis via RT PCR. Total RNA was isolated from very young pea leaves using Trizol reagent (RNAtidy G, AppliChem) according to the manufacturer's protocol and quantified by spectrophotometer, RNA integrity was checked by denatured agarose gel electrophoresis in MOPs buffer. Five micrograms of total RNA was used for cDNA synthesis and RT-PCR according to the manufacturer's manual (MBI Fermentas). cDNA was used as template for the normal PCR as mentioned earlier.

Leaf paint assays. Leaf paint assay was done to verify the expression of the bar gene. The bar gene activity in crossed transgenic plants was assayed according to Schroeder et al.[38] The upper surface of a leaflet was thoroughly wetted by painting with an

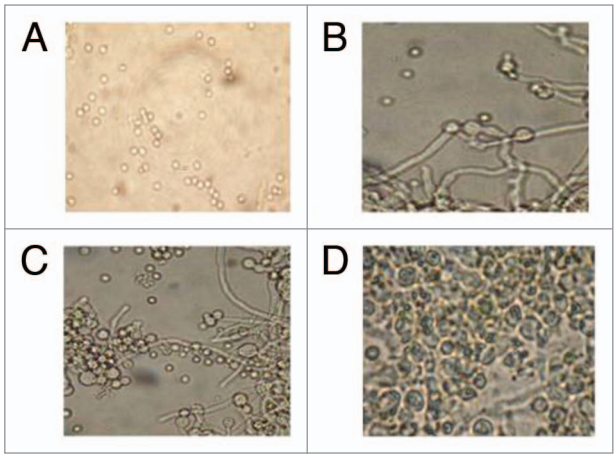

Figure 5. The effect of recombinant protein on the spore germination of *Trichoderma harzianum* under light microscope (x40) (1) Spore suspension on the first day. (2) Spore suspension with untransformed pea crude extract on the second day. (3) Spore germination in protein extract from isogenic parental transgenic pea (Chitinase line 02-04-7-1,1,2) on the second day. (4) Spore germination in protein extract from F_2 crossed transgenic pea on the second day.

Table 1. Leaf paint summary

Generation	Plant type	Total	(+)	(-)
F_1	pyramided progeny	76	63	13
	Chitinase	14	14	0
	Glucanase	9	0	9
	Untransformed	4	0	4
		103		
F_2	Pyramided progeny	147	117	30
	Chitinase	21	21	0
	Glucanase	24	19	5
	Untransformed	28	0	28
		220		
F_3	Pyramided progeny	70	59	11
	Chitinase	15	15	0
	Glucanase	13	5	8
	Untransformed	5	0	5
		103		
F_4	Pyramided progeny	60	51	9
	Chitinase	12	12	0
	Glucanase	12	6	6
	Untransformed	4	0	4
		88		

aqueous solution of herbicide Basta® (Aventis GmbH, Frankfurt, Germany) with a final Phosphinothricin (PPT) concentration of 600 mgl^{-1} and 0.1% Tween20. The opposite leaflet of each pair was left untreated as a control, the herbicide effect was evaluated 1 week later.

In vitro bioassay. In vitro bioassays were performed for testing the synergistic effect of the F_2 and F_3 crossed transgenic plants to inhibit fungal spore germination using *Trichoderma harzianum* (T12 strain),[39] 40 µl spore suspension of *T. harzianum* was mixed with 40 µl protein crude extract and incubated overnight at ambient RT. The effect of crude extracts on spore germination was examined under a light microscope.

Conclusion and Outlook

A successful combination of chitinase and glucanase transgenes in one pea line via conventional crossing was achieved in the present study. However, variation in expression and activity was observed with some lines having a higher activity than the parental transgenic lines. This may possibly be due to the hemizygous state of some of the crossed transgenic lines or due to the

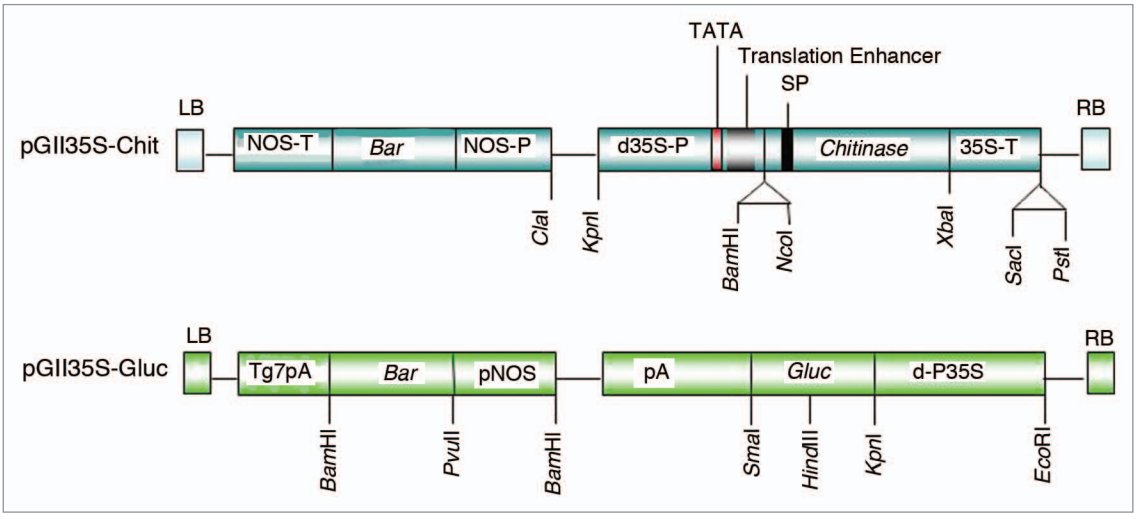

Figure 6. Physical maps of the binary vectors used for pea transformation pGreen II with double 35S promoter (P35S). (d-P35s: double 35S promoter, SP: Arabidopsis signal peptide, T: terminator, P: promoter, NOS: Agrobacterium nopaline synthase gene, Bar: herbicide resistance selectable marker from *Streptomyces hygroscopicus*, RB: right border, LB: left border).

constitutive expression of the 35S promoter. It would be interesting to test the antifungal effects in vivo under field conditions with different fungi. Furthermore, these lines may be used in subsequent studies for stacking of more genes and to understand the inheritance of transgenes since it is different from traditional Mendelian genetics.

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