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Combination of Antifungal Genes (chitinase and glucanase) to Increase the Resistance Level of Transgenic Pea (*Pisum sativum* L.) against Fungal Diseases

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Introduction

Pea (*Pisum sativum* L.) is an important grain legume worldwide, used both as a source of dietary protein for human and animal nutrition. The protein concentration of pea ranges from 15.5 to 39.7 % (Davies et al., 1985). Its production, however, is affected by different pests and diseases among which fungal diseases are the most important ones. A major objective in pea breeding is therefore to improve the resistance of pea to fungal diseases, which are known to cause considerable loss of more than 30 %. In addition, most phytopathogenic fungi leave mycotoxin residues in the crop, which also should be avoided. In order to control fungal infections, pea transgenic lines with a putative enhanced resistance to fungal diseases through heterologous expression of chitinase and glucanase genes have been established.

One way of broadening resistance is to combine transgenes expressing different resistance principles in a single line via conventional crossing (Halpin, 2005). The transgenic lines expressing either chitinase or glucanase were crossed, their progenies characterised at the molecular level as well as segregation and stability of the repective expression levels. Finally, the transgenic hybrids were further tested for their resistance against different phytopathogenic fungi using *in vitro* resistance assays.

Material and Methods

Transgenic pea lines (03-04-1,3,6,1-F and 02-04-7-1,1,2,3,2-F) homozygous for the *Chit* 30 gene from *Streptomyces olivaceoviridis* ATCC11238 (Hassan, 2006) as well as for the *gluc* gene (98-49-6-1,1-5-9-3) coding for 1,3-β-glucanase from barley (*Hordeum vulgare*) (Richter, 2005) were grown at 17-22°C in a 16/8 h day night interval. Manual crossing was carried out at flowering, 6–8 weeks after planting. Detection of integrated genes into transgenic pea plants was done by PCR.

Leaf paint assays were performed to verify the expression level of the bar gene and PAT-enzyme activity, respectively. The *bar* gene activity in hybrid transgenic plants was assayed according to Schroeder et al. (1993). The upper surface of a leaflet was thoroughly wetted by painting with an aqueous solution of total herbicide Basta® (Aventis GmbH) with a final concentration of 600 mg l^{-1} and 0.1% Tween 20. The opposite leaflet of each pair was left untreated. Leaves were evaluated one week later.

Agarose diffusion and colorimetric assays were carried out to quantify chitinase and glucanase activities. Standardized protein extracts were assayed against CM-curdlan (Loewe) [substrate was prepared using 20 ml 0.1 M Na-acetate buffer, 20 ml CM- curdlan (4 mg/ml) and 0.8 % agarose] and CM-chitin RBV (Loewe) [substrate was prepared using 25 ml 0.1 M Na-acetate buffer, 25 ml

CM-chitin RBV (2mg/ml) and 1% agarose]. These were individually heated up to dissolve the agarose, cooled and poured in 12 x 12 cm Petri dishes. Holes (space 6 mm) were made in the solid surface using a cork borer. 25 µg total protein was filled into the holes and incubated overnight at 37 ^oC for 16-24 hours. The reaction was stopped using 1N HCl and the halo diameters were measured.

In vitro bioassays were performed for preliminary testing of the ability of the F_2 and F_3 transgenic plants to inhibit fungal spore germination or hyphal extension using *Trichoderma harzianum* (T12 strain), *Colletotrichum acutatum, Ascochyta pisi* and *Botrytis cinerea. T. harzianum* (40 µl) spore suspension 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. The method of Schlumbaum et al. (1986) was used to test the effect of hybrid transgenic plants on inhibiting fungal hyphal extension. *Botrytis cinerea* was grown on PDA media at ambient room temperature. Crude protein extracts from F_3 hybrid transgenic plants were applied in the wells, which were prepared using a 3 mm borer in the region around hyphal growth. The plates were incubated at ambient room temperature for 72 h, during which the hyphae grew outwards from the centre. Hyphal inhibition was observed at 0 h and 72 h after treatment.

Results and Discussion

Successful introduction of the chitinase and glucanase genes into the pea genomic DNA was analysed using primers for the chitinase and glucanase in the F_0 and subsequent generations.

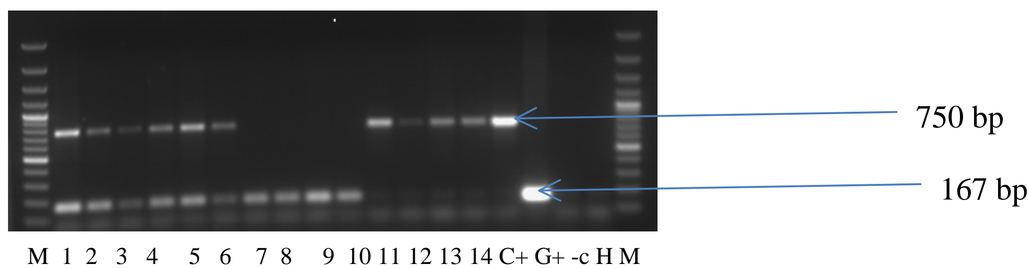

Figure 1: Multiplex –PCR results of F_1 hybrids. Lanes 1-6 (35-3, 11-1, 16-1, 33-2, 20-3, 21-2), 7-10 (gluc 1, 2, 3, 4), 12-14 (chit 1, 2, 3, 4), C+&G+(positive controls: plasmid PGII-chit 30 & PGII-gluc),-c, Negative untransformed plant, H water control & M 100bp DNA molecular marker

One week after painting the transgenic, isogenic transgenic and untransformed pea with BASTA®, clear effects were observed. Recovery of herbicide resistant plants from sensitive parental plants through recombination in meiosis was observed in some lines (table 1).

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		

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. This result is similar to those observed by Richter et al. (2006). 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. This hypothesis could be demonstrated by PCR or Southern blot.
In the case of agarose diffusion chitinase and glucanase assays, different levels of activity were observed within and between pea lines. The same trend was observed with the colorimetric assay (data not presented).

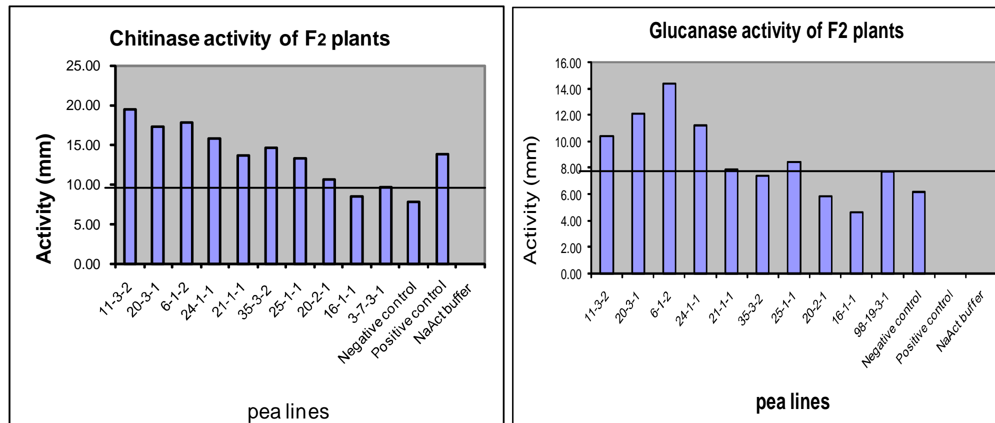

Figure 2: Histograms of halo wideness from agarose diffusion assays of different F_2 transgenic plants using respectively (1) CM-chitin RBV and (2) CM- curdlan as substrates

Hassan, (2006), also obtained similar variation in tobacco and pea transformed with the bacterial chitinase. This may be due to the expression of the 35S constitutive promoter. It is highly expressed in most tissues but can yield higher activities in some. The samples were taken from the middle of the plant between node 4 and 10 which presumably has an effect on the expression level due to the fact that the expression is tissue, developmental-stage- and species-dependant. It is not known also if the regulation of the 35S constitutive promoter is circadian or diurnally regulated. The observed variation might be due to yet unknown epigenetic effects which refer to changes in gene expression that do not involve changes to the underlying DNA sequence.
Crude extracts of different transgenic F_2 & F_3 hybrids showed inhibitory effects on spore germination of *Trichoderma harzianum* and hyphal growth on *Botrytis cinerea* in contrast to extracts from isogenic transgenic lines, untransformed pea line (negative control) or Na-acetate buffer. The same trend was observed with *Colletotrichum acutatum* in the case of spore germination and *Ascochyta pisi* in the case of hyphal growth.

Observing spores after overnight incubation under light microscope showed clear preliminary effects of protein crude extracts from parental transgenic lines became enlarged and did not germinate completely as compared to crude extract from non-transformed plant which showed full germination of spores. In the case of the hybrid transgenic lines, spores were all destroyed hence a 100 % inhibition of spore germination was observed (Mauch et al. 1988).

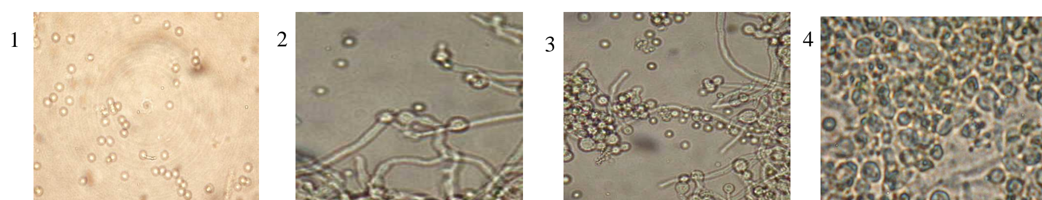

Figure 3: The effect of recombinant protein on the spore germination of *Trichoderma Harzianum* under light microscope (X40) 1: Spore suspension on the first day. 2: spore suspension with untransformed pea crude extract on the second day, 3: Spore germination in protein extract from isogenic parental transgenic pea on the second day, 4: Spore germination in protein extract from F_2 hybrid transgenic pea on the second day

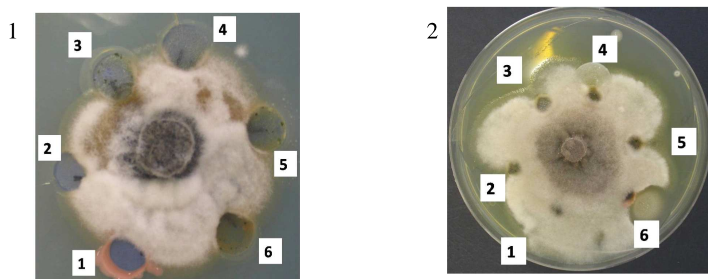

Figure 4: *In-vitro* bioassay of *Botrytis cinerea* on hyphal growth inhibition by using crude extracts from different F_3 generations of transgenic hybrid pea. 1: Na-acetate buffer, 2: untransformed control plant, 3: parental transgenic chitinase, 4: 07/20-3-2-5 (F_3 hybrid transgenic pea) 5: parental transgenic glucanase, 6: 38-1-2-2 (F_3 hybrid transgenic pea)

In the present study, crude protein extract from F_2 and F_3 transgenic plants inhibited *T. harzianum, A. pisi* and *B. cinerea* hyphae growths. Visually, it could be seen that the inhibition rate of the crude extracts from hybrid transgenic peas inhibited hyphal extension more than the transgenic chitinase and glucanase plants (Fig. 4). This was estimated by the wideness of the inhibition area on the plates. These results were in agreement with those of Mauch et al. (1988), who showed that the *in vitro* combination of chitinase with β-1,3-glucanase strongly inhibits fungal growth.

Conclusion and outlook

A successful combination of chitinase and glucanase transgenes in one pea line via crossing was achieved. Inhibition of different fungal spore germination and hyphal extension was observed in *in-vitro* bioassays. However, variation in protein expression and activity was observed. This may possibly be due to the hemizygous state of some of the hybrid transgenic lines or due to the expression of the 35S constitutive promoter. However, for the lines that showed negative leaf paint results, a Southern blot analysis would be done to determine the copy number of the chitinase and β-1,3-glucanase genes. RT-PCR will be of interest to see what happens at the transcription level. Finally, it would be interesting to test the antifungal effects *in vivo* under field conditions with different fungi.

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