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

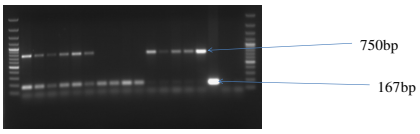
The production of pea is affected by different pests and diseases among which fungal diseases are the most important ones. A major objective in breeding is to improve the resistance of pea to fungal diseases. One way of enhancing or broadening resistance is to combine transgenes expressing several resistance genes into a single line via crossing. This study was carried out to enhance the resistance level of transgenic pea plants expressing chitinase and glucanase individually against fungal diseases.

Materials and method

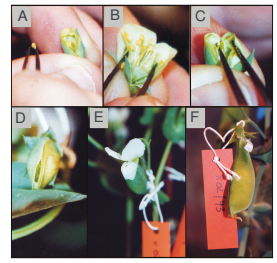
Transgenic pea lines (03-04-1,3,6,1-F and 02-04-7-1,1,2,3,2-F) carrying a *Chit* 30 gene coding for chitinase from *Streptomyces olivaceoviridis* (Hassan, 2006) as well as (98-49-6-1,1-5-9-3) *gluc* gene coding for 1,3-β-glucanase from barley (*Hordeum vulgare*) (Richter, 2005) in their homozygous state were manually crossed. The stability of expression of the transgenic hybrid lines was analysed. Finally, the transgenic hybrids were tested for their resistance against different phytopathogenic fungi.

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.

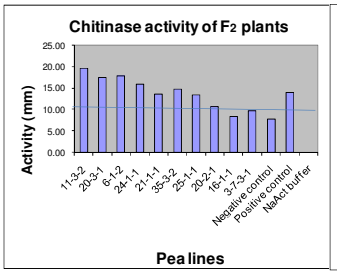


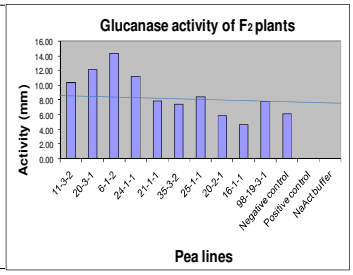
Multiplex-PCR results of F_1 hybrids. Lanes 1-6 (35-3, 11-1,16-1, 33-2, 20-3, 21-2), 7-10 (gluc 1, 2, 3, 4), 12-14 (chit 1, 2, 3, 4), C+&G+(positive controls: plasmid PGII-chit 30 & PGII-gluc),-Negative untransformed plant, H water control & M 100bp DNA molecular marker



Crossing scheme

A: Demasculation of the recipient with a forcep
B: Mature pollen removed with a forcep from a donor plant
C: Pollen from donor plant is transfered to the scars of receptor plant (cross pollination)
D: Flowers after cross pollination
E: Plant tagged and normal development occurs
F: Filled pods after successful cross pollination





Different levels of activity were observed as some activity variation was observed within and between pea lines. The same trend was observed with the photometric readings (data not presented).

Functional analysis

Leaf paint assay

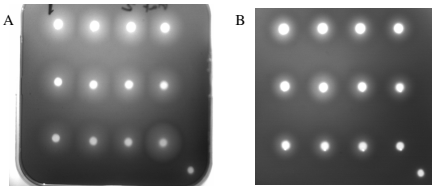
Leaf paint assay was done to verify the expression level of the bar genes and Phosphinotricine Acetyl Transferase (PAT) enzyme activity. One week after painting one leaflet of each pair of hybrid transgenic, isogenic transgenic and untransformed pea with 600 mg/l dilution of BASTA®, clear effects were observed.
Recovery of herbicide resistant plants from sensitive parental plants through recombination in meiosis was observed in some lines (table below).

Generation	Plant type	Total	(+)	(-)
F1	Hybrids	76	63	13
	Chitinase	14	14	0
	Glucanase	9	0	9
	Negative control	4	0	0
		103		
F2	Hybrids	147	117	30
	Chitinase	21	21	0
	Glucanase	24	19	5
	Negative control	28	0	28
		220		

Glucanase and chitinase activity assays

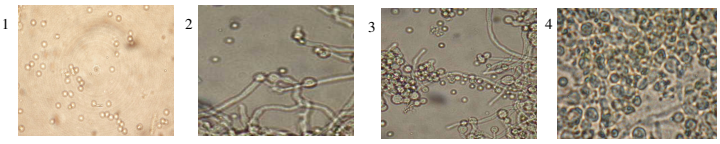
Agarose diffusion and colorimetric assays were carried out to quantify chitinase and glucanase activities. Standardised protein extract was assayed against CM-chitin RBV and CM-curdlan for the quantitative assessment of chitinase and glucanase activities respectively

A: Chitinase activity
B: Glucanase activity

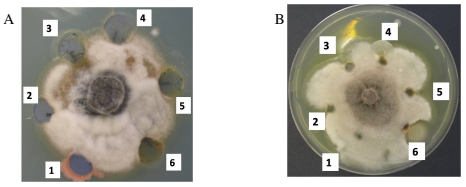


In-vitro bioassays

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



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



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)

Conclusion

A successful combination of chitinase and glucanase transgenes in one pea line via crossing was achieved. However, variation in protein expression and activity was observed. No relationship was found between the chitinase and glucanase activities from colorimetric assay. This may possibly be due to the hemizygous state of some of the hybrid transgenic lines.

References

Hassan, F., (2006). Heterologous expression of a recombinant chitinase from *Streptomyces olivaceoviridis* ATCC 11238 in transgenic Pea (*Pisum sativum* L.). PhD Thesis. University of Hannover, 166p.
Richter, A. (2005). Züchtung mit transgenen Pflanzen: Kombination rekombinanter Pilzresistenzgene mittels Kreuzung transgener Erbsenlinien (*Pisum sativum* L.) PhD Thesis. University of Hannover, 154p.

Acknowledgement

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