Heterologous Expression of a Recombinant Chitinase from *Streptomyces olivaceoviridis* ATCC 11238 in Transgenic Pea (*Pisum sativum* L.)

Von der

Naturwissenschaftlichen Fakultät

Der Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades eines

DOKTORS DER NATUTRWISSENSCHAFTEN

-Dr. rer. nat.-

genehmigte Dissertation

Von

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Geboren am 19.04.1969

in Damaskus-Syrien

2006

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Tag der Prüfung: 08.08.2006

Dedicated to my beloved parents, my wife,
my children, my sister, my brothers
and my friends

Abstract

Heterologous Expression of a Recombinant Chitinase from *Streptomyces olivaceoviridis* ATCC 11238 in Transgenic Pea (*Pisum sativum* L.)

Fathi Hassan

Pea is an important grain legume that gained worldwide economic importance as a source of protein (15.5-39.7 %) for human and animal nutrition. The world pea production exceeded 20 million ton in 2005. In addition, it is well suited as a rotation crop to replenish soil nitrogen levels. Improvement of the resistance to fungal diseases is a major objective in breeding, since fungal diseases can cause in pea a considerable loss to more than 30 %.

The aim of the present study was to enhance the fungal resistance in pea through the heterologous expression of a chitinase gene (*Chit30*) from *Streptomyces olivaceoviridis* ATCC11238. *Chit30* belongs to family 19 chitinases and can hydrolyze chitin, the backbone of the fungal cell wall. Therefore, the bacterial signal peptide was replaced by the signal sequence of an *Arabidopsis thaliana* basic endochitinase gene, which was N-terminally fused to the mature bacterial gene. This chimeric chitinase gene (N-*Chit30*) was cloned via PCR based method into the pGreenII binary basis vector 0229, which contains the selectable marker gene bar under the control of a nos-promoter and nos-terminator. The gene was regulated either by a constitutive double 35S promoter from cauliflower mosaic virus or by the plant inducible *vst* promoter from grape and a 35S terminator. The *bar* gene encodes the enzyme phosphinothricin acetyltransferase (PAT), which confers resistance to transgenic plants against phosphinotricin, the active compound of the total herbicide BASTA®. The chitinase and bar genes were arranged divergently in the binary vector.

Leaf disk explants from *in vitro* growing tobacco plants cv. *Samsun* and embryo axis excised from mature seeds of pea (*Pisum sativum* L.) cv. *Sponsor* were used as explants for *Agrobacterium*-mediated transformation. Different shoots were regenerated after transformation of tobacco and pea, which were healthy growing on media supplemented with PPT concentrations up to 15 mg/l. Shoots from tobacco were rooted and potted into greenhouse, whereas regenerated shoots from pea were grafted to recover whole plants which in turn produced T1 progeny. The total procedure from seed to seed was between 6-8 months until getting transgenic pea seeds with transformation efficiencies varying between 0.31 % to 1.4 % with an average of 0.9 %.

Transgene detection was made by PCR using different primer combinations (chit 555, and

bar447). The results clearly indicated and confirmed the successful integration of T-DNA into genomic DNA of pea and tobacco progenies. Copy numbers and integration patterns were investigated in T0, T1 and T2 generation using Southern blot analysis with different probes (chit 555, bar, and nptI), proving the presence of single copies in most of the pea plants tested, while two copies were also shown in some plants. In transgenic pea no vector backbone sequence were detected, neither by PCR- nor by Southern blot analysis on the presence of the *nptI* gene, which is used for the maintenance of the pGreenII plasmids in the bacteria.

The transcript and accumulation of chitinase in transgenic pea and tobacco plants were confirmed by RT-PCR and Western blot analysis. After immunostaining it was possible to detect two bands corresponding to the mature protein (29 kDa) and non-processed protein (31-32 kDa). Immunostaining of proteins from the apoplast and suspension cell cultures of tobacco showed also two bands for mature and full-length protein.

Leaf paint analysis showed positive results in most tested tobacco and pea clones indicating bar gene expression by BASTA® herbicide detoxification.

Chitinase activity was analyzed using *in-gel* assays, which showed the presence of additional 3 isoform bands compared to the non-transformed pea, whereas between 3 and 5 bands were detected in tobacco. The chitinase activity of tobacco ranged from 0.07 to 0.14 U/ 10 µg total protein, whereas it was between 0.09 and 0.25 U/ 10 µg total protein extract from pea.

Trichoderma harzianum was used to study the *in vitro* antifungal activity of crude extracts from pea and tobacco leaves, clearly showing inhibition of hyphal growth after 8 and 16 h., compared to non-transformed control or the same samples after boiling.

In the present study, the heterologous expression of the bacterial chitinase gene from *Streptomyces olivaceoviridis* ATCC 11238 in stable transformed pea- and tobacco plants was investigated for the first time.

Keywords: *Agrobacterium*, Pea, Chitinase, Heterologous expression, *Streptomyces*, Resistance.

Zusamenfassung

Heterologe Expression einer rekombinanten Chitinase aus *Streptomyces olivaceoviridis* ATCC 11238 in transgenen Erbsen (*Pisum sativum* L.)

Fathi Hassan

Die Erbse hat als proteinreiches (15.5-39.7 %) Nahrungs und Futtermittel für die menschliche und tierische Ernährung weltweit an Bedeutung gewonnen. Die Weltprodukion an Erbsen betrug im Jahre 2005 über 20 Mio. Tonnen. Weiterhin dient die Erbse in geeigneter Fruchtfolge der Stickstoffanreicherung des Bodens. Die Verbesserung der Resistenz gegen Pilzkrankheiten ist ein wichtiges Züchtungsziel, da Pilzkrankheiten in Erbsenkulturen zu einem Ernteverlust von mehr als 30 % führen können.

Das Ziel der vorliegenden Arbeit war es, die Widerstandskraft von Erbsen gegen pathogene Pilze durch die heterologe Expression eines Chitinasegens (*Chit30*) aus *Streptomyces olivaceoviridis* ATCC 11238 zu erhöhen. *Chit30* gehört zu den Chitinasen der Familie 19 und bewirkt den hydrolytischen Abbau von Chitin, dem Grundgerüst der Pilzzellwand. Das bakterielle Signalpeptid wurde durch das pflanzliche Sekretionssignal einer basischen Endochitinase aus *Arabidopsis thaliana* ersetzt, welche N-terminal mit dem bakteriellen Gen fusioniert wurde. Das so entstandene chimäre Chitinase-Gen (N-*Chit30*) wurde mittels einer PCR-basierten Methode in den binären pGreenII Basis-Vektor 0229 kloniert, welcher als Selektionsmarker ein *bar* Gen unter der Kontrolle eines *nos*-Promotors und eines *nos*-Terminators beinhaltet und der transgenen Pflanze somit eine Resistenz gegenüber Phosphinotricin, dem Wirkstoff des Totalherbizid BASTA® vermittelt. Die Genregulation des Chitinasegens erfolgte entweder über den konstitutiven, doppelten 35S-Promotor des Blumenkohl-Mosaik-Virus (CaMV) oder des induzierbaren *vst*1-Promotors aus Wein (*Vitis vinifera* L.) und einem 35S-Terminator. Das Chitinasegen befindet sich in dem binären Vektor in divergenter Leserichtung zum *bar* Gen.

Die Agrobakterium vermittelte Transformation erfolgte an Blattscheiben von *in vitro* gezogenen Tabakpflanzen der Varietät Samsun (*Nicotiana tabacum* L. cv. *Samsun*) und longitudinal geschnittenen Embryoachsen, aus reifen Erbsensamen der Sorte Sponsor (*Pisum sativum* L. cv *Sponsor*). Aus unabhängigen Transformationsereignissen konnten Sprosse sowohl von Tabak als auch Erbse auf Pflanzenmedium mit einer Phosphinotricin-Konzentration von bis zu 15 mg/l regeneriert werden. Die Sprosse der Tabakpflanzen wurden bewurzelt und dann im Gewächshaus bis zur Samenreife kultiviert, während die transgenen Erbsensprosse auf eine nicht transgene Unterlage gepfropft wurden. Die Regenerationsperiode für Erbsen betrug von der Transformation bis zum transgenen

Samen (T1) 6 bis 8 Monate mit einer Transformationseffizienz von durchschnittlich 0,9 % (Variation zwischen 0.31 % und 1,4 %).

Nachgewiesen wurde das Transgen in den Pflanzen durch die PCR-Methode unter der Verwendung verschiedener Primerkombinationen. Die Ergebnisse bestätigten klar den Einbau der T-DNA in die gDNA der Erbsen- und Tabaknachkommenschaft. Die Kopienanzahl und Integrationsmuster wurden in den T0, T1 und T2-Generationen mit Hilfe der Southern-Blot-Analyse bestimmt, dies erfolgte mit Sonden für das Chitinase-, *bar*- und *nptI* Gen, nach einem Verdau der gDNA mit *Eco*RI oder *Xba*I. Dabei wurden überwiegend Einzelkopien in den Erbsenpflanzen nachgewiesen, allerdings trugen einige Erbsen auch zwei Kopien. In den transgenen Erbsen konnten weder mittels PCR noch mit der Southern-Blot-Analyse eine Integration von Vektorsequenzen (Backbone integration) nachgewiesen werden. Dazu wurden Primer bzw. Sonden gegen das *npt*I Gen eingesetzt, welches zur Stabilisierung der pGreenII Plasmide in Bakterien eingesetzt wird.

Das Transkript und die Akkumulation der rekombinanten Chitinase in den transgenen Erbsen- und Tabakpflanzen wurden durch eine RT-PCR und Western-Blot-Analysen bestätigt. Nach der Antikörperdetektion war es möglich zwei Fragmente nachzuweisen: das reife, 29 kDa große Protein und das nicht-prozessierte 31 bis 32 kDa große Protein.

Die Expression des *bar*-Gens wurde durch den Leaf-Paint-Assay getestet, der in den meisten beprobten Tabak- und Erbsenklonen eine Resistenz gegenüber dem BASTA®-Herbizids bewirkte. Die rekombinante Chitinase-Aktivität wurde durch *in-gel-assays* analysiert, diese Assays zeigen die Präsenz von 3 zusätzlichen isoformen Banden, verglichen mit nicht-transgenen Erbsen, beziehungsweise 3 bis 5 Banden bei Tabak. Die Chitinase-Aktivität in Tabak hat eine Bandbreite zwischen 0,07 bis 0,14 U/10 µg Gesamtprotein, während sie bei Erbsen zwischen 0,09 und 0,25 U/10 µg Gesamtprotein lag. Des weiteren wurde eine Kultur von *Trichoderma harzianum* benutzt, um *in-vitro* die antifungale Aktivität von Rohextrakten aus Tabak- und Erbsenblättern zu untersuchen. Dabei wurde die eindeutige Hemmung des Hyphenwachstums nach 8 bzw. 16 Stunden gezeigt, verglichen mit einer nichttransformierten Kontrolle oder denselben Proben, die zuvor durch Kochen abgetötet wurden.

In der vorliegenden Doktorarbeit wurde die heterologe Expression des bakteriellen Chitinase-Gens aus *Streptomyces olivaceoviridis* ATCC 11238 in stabil transformierten Tabak- und Erbsenpflanzen zum ersten Mal untersucht.

Stichworte: Agrobaktrium, Erbse, Chitinase, Heterologe Expression, *Streptomyces*, Resistenz

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Abbreviation

µ1	Micro litre
µM	Micro mole
mM	Milli mole
B5	B5 basal medium
BAP	6-benzyl-amino-purine
bar	BASTA® (bialaphos) resistance gene
CaMV	Cauliflower mosaic virus
g/l	Gram per litre
GOI	Gene of interest
GUS/*gusA*	β-glucuronidase (enzyme/gene)
IBA	Indole butyric acid
LB	Left border
mg/l	Milligram per litre
MS	Murashige and Skoog medium
NAA	1-naphthyl-acetic acid
nptII	Neomycin-phospho-transferase (gene)
PAT	Phosphinothricin acetyltransferase
PCR	Polymerase chain reaction
PPT	Phosphinothricin
RB	Right border
RT-PCR	Reverse transcription polymerase chain reaction
T0, T1, T2, T3	Independently derived transgenic generation
T-DNA	Transferred DNA
Ti-Plasmid	Tumour-inducing plasmid
vir	Virulence
bp	Base pair
kb	Kilo base pair
BSA	Bovine Serum Albumin
CTAB	Cetyl Tri-methyl ammonium bromide
EDTA	Ethylene diamine tetra acetate
SAR	Systemic acquired resistance
PR-proteins	Pathogenesis-related proteins

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1 INTRODUCTION

Protein-rich grain legumes are important crops especially in arid and semi-arid areas. Environmental stress factors impose major limitations on food legume productivity. Biotic and abiotic stresses can reduce legume yields up to 50 %. There is urgency to improve commercial and desired legume crops including pea varieties, which are of economic importance worldwide. In particular, the need to improve these varieties and producing fungal resistant varieties is prominent. Established tissue culture methods are a prerequisite for *in vitro* genetic manipulations, since genetic transformation entirely depends on successful *in vitro* regeneration.

Biotechnology is a tool with an enormous potential for overcoming some of the constraints to increase agricultural production. It adds new methods to accelerate plant improvement. It is a technology aimed at invigoration of national industrial and agricultural sectors, and maintaining or increasing national competitiveness. Genetic engineering will broaden the genetic variability in certain cases where the natural variability within a species is not sufficient. It, in the first instance, addresses improvement of breeding material by introducing special valuable genes from different germplasm or other sources. On the other hand, people have used various conventional breeding techniques to modify plants and animals to improve food production for thousands of years. A traditional form of genetic manipulation is selective breeding, which makes it possible to promote preferred traits, such as higher yields or resistance of crops and animals. Today, these low-tech methods of genetic modification are being supplemented by marker assisted breeding and haploid technologies in breeding programs with mainly cereals.

Sophisticated tools of modern biotechnology depend on established transformation compatible tissue culture methods, which are up to now the bottleneck for the genetic manipulation of leguminous crops. Researchers can now take a single gene from a plant or animal cell and insert it into another species to give that species a desired trial, such as resistance to a destructive pest or disease.

Grain legumes are commonly known as pulses and are cultivated throughout the world in tropics, subtropics and temperate regions. The pulses are amongst the earlier food crops to be cultivated by man and these crops have been treated as one of the most important source of dietary protein, especially in Asia, Latin America and Africa. Pea (*Pisum*

sativum L.), faba bean (*Vicia faba* L.), lentil (*Lens culinaris* Medic) and chickpea (*Cicer arietinum* L.) are the main grain legumes grown as dry seed for human consumption and animal feed or as vegetables.

First of all, the narrow gene pool of these species requires precise marker assisted breeding programs. Genetic engineering is an additional tool for functional gene analysis, which helps to understand the physiological background as well as its contribution for the genetic improvement of desired characters.

Pea is an important grain legume that gained worldwide economic importance as a source of protein for human and animal nutrition just behind soybean [*Glycine max* (L.) Merill], groundnut (*Arachis hypogea* L.) and bean (*Phaseolus vulgaris* L.). The productivity and the value of peas could be greatly increased by the introduction of stably inherited traits such as pest and disease resistance, herbicide resistance and improved protein quality. These traits are not available in natural populations or near relatives of cultivated peas, but current advances in plant genetic engineering provide a potentially powerful tool for achieving these goals by genetic transformation. Because of its relative importance on the global market routine genetic engineering protocols for pea became elaborated in the past decade and could serve as an applicable tool for broadening the gene pool in addition to conventional breeding.

Improvement of the plant resistance to fungal diseases has always been a desired aim of the breeders for long time. However, these efforts were met with a limited success. In this context, plant genetic engineering and molecular breeding provide a chance to solve this problem and improve the resistance of pea to fungal diseases.

Because pea often suffers severe yield loss due to diseases and environmental conditions, emphasis should be put on developing screening procedures for the major biotic and abiotic stresses. Resistance to diseases, drought, cold weather, high protein content, altered amino acid composition and plant architecture are some of the desirable traits that breeders wish to transfer. The techniques of plant tissue culture have been developed as a new and powerful tool for crop improvement. Besides, the genetic transformation technique is becoming a popular tool for improving varieties by transforming desirable gene, which is often not possible by conventional breeding methods. Considering the importance of pea in human nutrition and the potential of tissue culture methods for the genetic improvement of pea, a great effort should be made in this field. Therefore, great importance is given to

the introduction of genetically based tolerance/resistance in pea.

Additionally, pea is a suitable and interesting object for studying different morphological processes. As a leguminous plant, it can be a model for studying nodule formation and symbiotic nitrogen fixation. Pea is an important crop (used as food and forage), rich in protein. Elaboration of pea transformation and regeneration systems could contribute finally to crop improvement (Malysheva et al. 2001).

This research proposes to use biotechnological tools to improve one of the most important grain legume crops, i.e. Pea, against fungal diseases by using a bacterial chitinase gene optimized to plant expression system with the need to sharpen the awareness of what advantages biotechnology can offer to the environment, health care and food security particularly in developing countries. From a bio-safety and acceptance point of view, it is worthwhile to notice that pea is a self-pollinated crop. In addition, expansion of grain legume cultivation, which is now very low in Europe, is highly desirable as grain legumes are superior crops from an agro-ecological point of view: they have the unique capacity to fix nitrogen, contribute to soil fertility, and enhance efficiency in agricultural rotations. They form, therefore, integral components of a more sustainable agricultural system with better water conservation.

2 LITERATURE REVIEW

2.1 Importance of pulses and legumes

The *Leguminosae* family comprises almost 700 genera and 1800 species, which make it the largest family of flowering plants (Polhill and Raven, 1981). Legumes and pulses (edible dry seeds of leguminous plants) are one of the most nutritionally and economically important food crop families for humans (e.g., soybean, common bean, pea, peanut, lentil, chickpea), edible oils (peanut, soybean), or animal fodder and forage (alfalfa, clover). Archaeological evidence suggests that the legumes have always been an important component of the human diet, and still are, especially in the developing countries where pulses account for 90 % of global consumption.

Today's agriculture continues to depend on legume crops because they all have high energy and high protein production for human and animal nutrition as well as amino acid profiles complementary to those of other crops, like cereals.

The unique symbiotic ability of legumes to use atmospheric nitrogen for plant growth makes them preferable crops for sustainable agriculture by reducing the dependence on expensive nitrogen fertilizer by 40 % when legumes are used in rotation with other crops and improving soil structure. In addition, legumes are also diverse in both their adaptations to most of the world's agricultural and natural habitats (Oram and Agocaoili, 1994; ICARDA, 1998 and 2000; Wheeler, 2000).

2.2 Pea (*Pisum sativum* L.)

2.2.1 Importance, origin, and taxonomy

Pea (*P. sativum,* 2n=14, genome size is 4400 Mbp) is an annual growing plant and considered as one of the most important legumes for human, animal and environment. It is widely spread due to its many uses as fresh green peas, dry peas (whole, split, or made into flour), tender green pods, green foliage and leaves (vegetables and herbs), in the canning and freezing industry, ripened seed (snack and oil) (Duke, 1981; Davies et al., 1985; Kay, 1979). Pea is among the four important cultivated legumes next to soybean, groundnut, and beans. It is the most widely grown of the cool season pulses in subtropics before chickpea and lentil (Table 1), and has the highest average grain yields (1,757 kg/ha) after soybeans. Canada is the leading country in pea production with more than 3 million metric tons in 2005 followed by France, China, Russian Federation and USA (Table 2).

Table 1. World pea yield and production in comparison with other important crops in 2005.

Crop	Yield (kg/ha)	Production (Mt)
Cereals	3.255	2,219,357,500
Maize	4.707	692,034,184
Wheat	2.898	626,466,585
Rice	4.004	614,654,895
Soybeans	2.292	209,531,558
Groundnut	1.447	36,492,147
Beans	0.709	25,419,286
Peas	1.757	20,721,735
Chickpea	0.818	9,172,530
Green corn	8.708	8,887,136
Broad beans	1.731	5,641,642
Pulses nes	0.854	4,169,741
Lentil	1.007	4,031,837
Cow peas	0.354	3,689,386
Pigeon Peas	0.714	3,277,995
Vetches	1.191	1,164,561
Lupines	1.019	1,107,018

Source: FAO statistical data, FAOSTAT database (http://faostat.fao.org/faostat/form?collection=Production.Crops.Primary&Domain=Production&servlet=1&hasbulk=&version=ext&language=EN)

Table 2. Main pea producing countries in 2005.

Peas, Dry	*Production (Mt)*	*Peas, Green*	*Production (Mt)*
Belgium	4,443	Australia	29,471
Bolivia	5,17	Austria	4,942
Canada	3,169,900	Bolivia	25,85
China	1,200,000	Canada	63,47
Ecuador	2,938	China	2,208,700
France	1,332,000	Cyprus	900
Germany	464	Ecuador	11,692
Greece	500	France	428
Guyana	500	India	3,200,000
Hungary	18,531	Jordan	4,022
India	800	Madagascar	580
Italy	33,378	Malawi	400
Russian Federation	1,290,000	Morocco	145
Rwanda	18,854	Pakistan	78,2
Slovakia	31,364	Romania	23,303
Slovenia	800	Reunion	500
South Africa	1,032	Serbia	29,7
Spain	119,7	Slovenia	200
Syrian	3,001	South Africa	23,129
Ukraine	600	Spain	96,7
United Kingdom	200	United Kingdom	322
USA	666,55	USA	884,7
WORLD	**11,565,006**	**WORLD**	**9,156,729**

Source: FAO statistical data, FAOSTAT database (http://faostat.fao.org/faostat/form?collection=Production.Crops.Primary&Domain=Production&servlet=1&hasbulk=&version=ext&language=EN)

The protein concentration of peas range from 15.5-39.7 % (Davies et al., 1985; Bressani and Elias, 1988). The amino acid profile and different components of pea are summarized in Table 3.

Table 3. Chemistry and amino acids composition of pea (Duke, 1981; Hulse, 1994; Huisman and van der Poel, 1994; Bressani and Elias, 1988)

100 g seeds	**Green peas**	**Dry peas**
Energy	44 calories	
Water	75.6 %	10.9 %
Protein	6.2 g	22.9 %
Fat	0.4 g	1.4 g
Carbohydrate	16.9 g	60.7 g
Crude fiber	2.4 g	1.4 g
Ash	0.9 g	2.7 g
Ca P Fe Na K β-carotene Thiamine Riboflavin Niacin Ascorbic acid	32 mg 102 mg 1.2 mg 06 mg 350 mg 405 µg 0.28 mg 0.11 mg 2.8 mg 27 mg	

Amino acids	**100 g protein**
Lysine	6.9-8.2 g
Methionine	1.4-2.7 g
Threonine	3.9 g
Tryptophan	0.9 g
Cystine	0.8-1.7 g

Kay (1979) proposed that the primary centers of origin of pea are in southwestern Asia (India, Pakistan, former USSR and Afghanistan) since 10000 years ago (Fig. 1), and thereafter spread to the temperate zones of Europe. Four centers of origins, i.e., Central Asia, the Near East, Abyssinia and the Mediterranean have been recognized based on genetic diversity (Gritton, 1980). However, wild pea species like *Pisum formosum* (Stev.), *Pisum fulvum* (Sibth. Et Sm.) and *Pisum syriacum* (Berger Lehm.) found in Middle East suggest that Northwest Asia is the origin of pea and from there it distributed to the west, north and east (Makasheva, 1983). Peas were reported to be originally cultivated as a winter annual crop in the Mediterranean region (Smart, 1990).

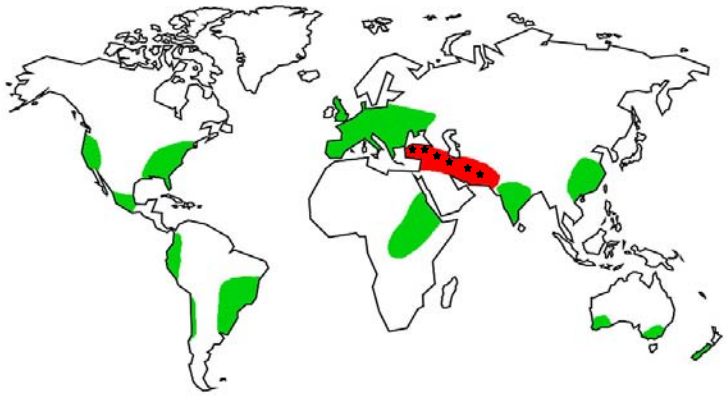


Fig. 1. World map showing the center of origin (in red with stars) and cultivated area (in green) of pea. (http://www.biologie.uni-hamburg.de/b-online/schaugarten/Pisumsativum L/Gertenerbse.html#20)

According to Duke (1981), garden peas are treated as *P. sativum* ssp. *hortense* Asch. & Graebn., field peas as *P. sativum* ssp. *arvense* (L.) Poir., edible podded peas as *P. sativum* ssp. *macrocarpon;* and early dwarf pea as *P. sativum* var *humile*. While Smart (1990) classified pea into *Pisum sativum* and *P. fulvum* Sibeth. & Smith.

Peas require a cool, relatively humid climate and are grown at higher altitudes in tropics with temperatures from 7 to 30 °C (Duke, 1981; Davies, 1985). The optimum temperature levels for the vegetative and reproductive periods of peas were reported to be 21 and 16 °C, and 16 and 10 °C (day and night), respectively. Growing seasons vary from 80-100 days in semi-arid regions and up to 150 days in humid and temperate areas.

2.2.2 Biotic and abiotic stress

When comparing the pea production between developed countries and developing countries we can see a clear difference in the yield (for example Syria 1328 kg/ha *vs.* Canada 2321 kg/ha). This is mainly due to different biotic and abiotic stresses constraining the production of pea since the yield is still lower than the potential yield. These unpredictable stresses affect the cultivation of legumes in developing countries resulting in reducing cultivation despite the increased demand for legumes.

Constraints affecting pea production are divided into biotic stresses caused mainly by different microorganisms (Table 4) and abiotic stresses.

Table 4. Main biotic constrains of pea. (Johansen et al., 1994; Slinkhard et al., 1994; Monti et al., 1994; Muehlbauer et al., 1983; Davies et al., 1985; van Emden et al., 1988).

Constraint	***Causal organism***
Seed, seedling and root rot	
Aphanomyces root rot	*Aphanomyces euteiches*
Fusarium root rot	*Fusarium solani*
Wilt	*Fusarium oxysporum*
Damping-off	*Phythium ultimum*
Seedling rot	*Rhizoctonia solani*
Bacteria	
bacterial blight	*Pseudomonas pisi*
Fungi	
Ascochyta blight	*Ascochyta fabae, A. pisi, A. piodella*
Gray mold	*Botrytis cinerea*
Powdery mildew	*Erysiphe polygoni, E. pisi*
Downy mildew	*Peronospora pisi, P. viciae*
Sclerotina white mold	*Sclerotina sclerotiorum*
Virus	
	Pea Enation Mosaic virus (PEMV)
	Pea Early Browning Virus (PEBV)
	Pea Mosaic Virus (PMV)
	Pea top yellows (PTY)
	Pea seed-borne Mosaic Virus (PsbMV)
	Pea Streak Virus (PSV)
Nematodes	
Pea cyst nematode	*Heterodera goettengiana*
Root-knot nematode	*Meloidogyne hapla*
Root-lesion nematode	*Pratylenchus penetrans*
Stem nematode	*Ditylenchus dipsaci*
Insects	
Pea aphid	*Acyrthosiphon pisum*
Pea thrips	*Kakothrips robus*tus
Pea seed beetle	*Sitona lineatus*
Pea weevil	*Bruchus pisorum*
Pea midge	*Contarinia pisi*
Weed	
Broom rape	*Orobanche spp.*

In Asia and Africa, powdery mildew, *Ascochyta*, and *Fusarium* are the major yield reducers, while in the Western hemisphere soil borne diseases, *Ascochyta* and bacterial blight are the yield reducers. In Europe, however, powdery and downy mildew, insects (aphid), and weeds cause about 25 % of overall yield losses. The pea weevil is one of the worldwide major pests of the field pea and is responsible for losses of up to 40 % in seed

yield (Smith, 1990).

Abiotic stresses affecting pea production are mainly cold, frost, water-logging, drought, heat, soil pH, salinity, sodicity and boron toxicity. Among cool season pulses, pea can tolerate cold (-2 °C) while chickpea responds in the opposite way. High temperatures (30-35 °C), causes increased yield losses in pea through flower abortion, early pod abortion and abortion of the seeds within pod (Duke, 1981; Davies, 1985).

Pea is considered sensitive to drought with yield losses varying from 21 to 54 % depending on the developmental stage and environment. Water-logging can cause yield losses of up to 50 % and in some cases up to 100 % by affecting root growth depending on the variety, stage of growth, duration, and the extent of root zone affected by water-logging. pH plays an important role in pea growth and nutrient availability, the optimal pH is 5.5-6.5, in the suboptimal pH (pH > 8.0), pea could suffer from water stress and nutrient deficiency. pH can also cause toxicity of Al, Fe and Mn when it is lower than 4.5. Salinity affects pea, the symptoms are necrosis, and color change of old leaves to yellow causing it to die. However, pea can tolerate salinity better than other pulses.

2.2.3 Germplasm

Germplasm collections are important for all scientists interested in improving and studying any crop from genetic, physiological or pathological aspects. Genetic resources and diversity of cultivated and wild relatives of the crop have to be available on request for different research projects like screening and evaluation for biotic and abiotic stress resistant trails. There are different pea germplasm collections all over the world of which Muehlbauer and Kaiser (1994a) sited 14 major collections. Currently the USDA-ARS *Pisum* germplasm collection located at Pullman, Washington, USA contains 3918 accessions (Coyne et al., 2005) and the John Innes Institute *Pisum* Collection, Norwich Research Park-Norwich, UK maintains 3030 accessions. (http://www.jic.ac.uk/GERM-PLAS /pisum/index.htm 27th April, 2005).

2.2.4 Biotechnology

Although there are comparably many accessions, it is still difficult to find all desired trails in the available primary, secondary and tertiary gene pools of a species or related genera or collections to control different stresses. Although it is possible to use agrochemicals, which address other economical and environmental concerns, or using integrated pest

management systems, host-plant resistance and improved agricultural practices are key. The actual need however, is to develop varieties, which tolerate or resist different stresses and at the same time give large and stable yields. Breeding against multiple stress resistance is not easy, although examples of cultivars resistant to three stresses have been reported (Nene, 1988; Muehlbauer and Kaise, 1994b).

Mutagenesis and hybridization are one option to increase variation in the primary gene pool, which could be used to start a breeding program. However, plant breeding has limitations due to crossability barriers. Therefore, new techniques were used like embryo rescue through in vitro culture to overcome embryo abortion after wide crosses. Biotechnology opened new horizons and ways to control different stresses and to improve crop quality and quantity by enabling rapid transfer of specific genes from different organisms to overcome the crossing barriers and resulting in extension of the variability and gene pools, which can be integrated in breeding programs much faster than with normal breeding strategies. The advantage of genetic transformation is the introduction of genes from unrelated species, wild relatives, or completely different organisms like bacteria, fungi and even human or viruses. Its use has the potential to benefit human and protecting the environment, increasing the yield through controlling weeds and different diseases. It also improves the poor communities' life by increasing the amino acids compounds or reducing malnutrition by genetically enriching vitamins as in the case of Golden Rice.

As has been mentioned before, fungal diseases are considered a severe problem resulting in yield losses of up to 100 % and quality losses because of mycotoxin contamination of the grains in many parts of the world. It was reported that, in some cases, it is possible to control the disease by agricultural practices and using resistant varieties and agrochemicals. However, this is not always found to be effective since the breeding programs take long time to develop resistant varieties and new types of pathogen might develop faster. In addition, fungicides are expensive. For some regions, there are needs to avoid cultivation of infected fields for years and often agricultural rotation in fields infected with root rot is not possible.

One of the promising methods is to use biotechnology and modern techniques of plant genetic engineering, which could provide a powerful tool for achieving these goals and overcoming most of these problems. This technology enhances the plant defense system

against fungal pathogens through producing high levels of antifungal compounds. It has been shown that plants already have defense systems which involve pathogen-related proteins, e.g. chitinase (Legrand et al., 1987; Shinshi et al., 1990; Yamamoto et al., 2000), stilbenes (Wiese et al., 1994; Hain et al., 1993), *β*-1,3-glucanase (Kombrink et al., 1988) or polygalacturonase-inhibiting proteins (Faize et al., 2003; Agüero et al., 2005).

2.2.5 Strategies for the development of fungus-resistant transgenic plants

Different steps were achieved to develop fungal resistance in plants like hypersensitive response (HR), production of pathogenesis-related (PR) proteins and systemic acquired resistance (SAR).

According to review of Grover and Gowthaman (2003), these strategies could be classified into two categories, either production of transgenic plants with antifungal molecules like proteins and toxins or by using R genes to activate hypersensitive response and systemic acquired resistance pathway (Fig. 2).

Antifungal proteins from different sources showed *in vitro* activity by inhibiting hyphal and fungal growth as reported by many scientists (Asao et al., 1997; Bolar et al., 2000; Rajasekaran et al., 2000; Boller, 1993). Antifungal proteins consist of pathogenesis-related (PR) proteins, ribosome-inactivating proteins (RIPs) where plant RIPs inactivate foreign ribosomes by removing an adenine residue from 28S rRNA so the 60S ribosomal subunit cannot bind to elongation factor 2 resulting in protein elongation inhibition, small cystein-rich proteins such as chitin-binding proteins, plant defensins, hevein and thionins, lipid transfer proteins (LTPs). Storage albumins have a dual role in storage and defence, polygalacturonase inhibitor proteins (PGIPs), antiviral proteins, and non-plant antifungal proteins (Faize et al., 2003; Gao, 2000).

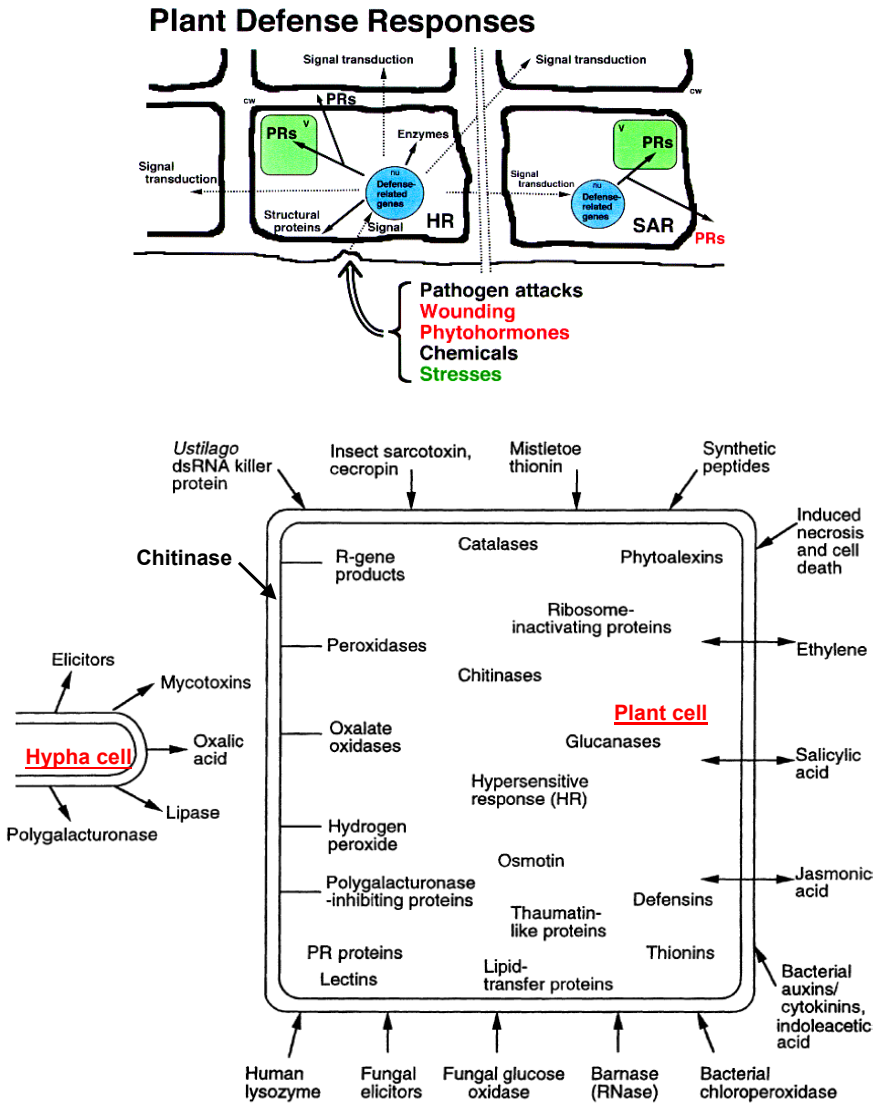


Fig. 2. Higher plants defense response (up), Genes and its products (down) used to produce transgenic plants with enhanced fungal disease resistance by using fungal virulence products (hypha cells), expression of plant-derived gene products (in plant cell) or gene products from non-plant sources (outside plant cell). Adapted and modified from Punja (2001).

2.2.5.1 Pathogen related (PR) proteins

PR proteins are large group of proteins with different biochemical and enzymatic activities. They were first described as a consequence of pathogen attack and abiotic stresses, but later it was shown that they are induced by different ways like wounding, fungal cell wall elicitors, ethylene, salicylic acid, UV light, and heavy metals. PR proteins are also constitutively expressed at different developmental stages. Induction of PR proteins during hypersensitive response and SAR indicate its role in natural defense of plants to protect themselves against pathogen infection. PR proteins have been classified into 17-PR protein families based on primary structure, serological relatedness and

enzymatic and biological activities. The functions and source of these different families are summarized in Table 5.

Table 5. Pathogen related proteins families and their function (Gachomo et al., 2003; Kasprzewska, 2003; Hoffmann-Sommergruber, 2002; Okushima et al., 2000; Çaliskan, 2000; Gao, 2000; Narváez-Vásquez, 1992; Wei et al., 1998).

Family	***MW (kDa)***	***Designation***	***Function and target***
PR-1	14-17	PR protein 1 precursor	Membrane
PR-2	25-35	1,3-ß-glucanase	Cell wall glucan
PR-3	25-35	Endochitinase (classes I, II, IV, VI and VII)	Cell wall chitin
PR-4	13-19	Endochitinase (prohevein)	Cell wall chitin
PR-5	22-26	Osmotin and thaumatin-like proteins	Membrane
PR-6	6-13	Proteinase inhibitor	Proteinase
PR-7	69	Proteinase	Not defined
PR-8	28	Endochitinase (class III)	Cell wall chitin
PR-9	39-40	Peroxidase	Indirect activity
PR-10	17-18	RNase	Pathogen-RNA
PR-11	41-43	Endochitinase (class V)	Cell wall chitin
PR-12	5.6	Defensin	Membrane
PR-13	14	Thionin	Membrane
PR-14	7-12	LTPs	Lipid
PR-15	22-26	Oxalate-oxidase	Cell membrane
PR-16	22-26	Oxalate-oxidase-like proteins	Cell membrane
PR-17	27	Unknown	-

One widely used method to improve disease resistance of economically important crops is to express PR proteins or to enhance and over-express their endogenous forms. Of these proteins, chitinases are considered as one important class of enzymes and prime candidate for further improvement of plant defense against fungal diseases.

2.2.5.2 Chitinase

Chitinases (EC 3.2.1.14, also called chitodextrinase; 1,4-*β*-poly-*N*-acetylglucosaminidase; poly-*β*-glucosaminidase; *β*-1,4-poly-*N*-acetyl glucosamidinase) are glycosyl hydrolases which catalyses the hydrolytic cleavage of the *β*-1,4-linked polymer of N-acetylglucosamine (GlcNAc) of chitin. Chitin [$(C_8H_{13}NO_5)_n$] is one of the most abundant natural polymers next to cellulose [$(C_6H_{10}NO_5)_n$], chitin consist of linear chains of *β*-1,4-linked sugar residues (Fig. 3). It is found in the cell walls of fungi (20 %), exoskeleton of arthropods (30 %), the shells of crustaceans such as crabs, lobsters, and shrimp (70 %), nematodes and insects (37 %). Chitin has many applications in medicine and the pharmaceutical industry, water and waste-water management, food-, feed-, textile-, cosmetic industries as well as in agriculture.

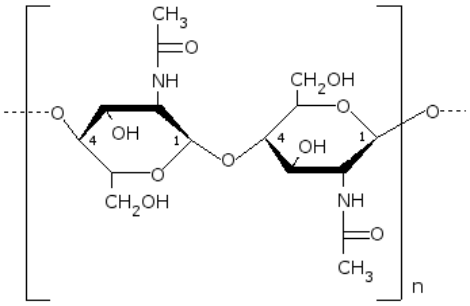


Fig. 3. Chitin chemical structure of poly N-acetylglucosamine. (http://en.wikipedia.org/wiki/Chitin).

Chitinase is found in a broad range of organisms including organisms, which do not contain chitin such as insects, bacteria, higher plants, human and even viruses. It has different developmental, morphological and physiological roles like degrading the old cuticle of insects and crustaceans, pathogen related defense in higher plants, nutritional and parasitism roles in bacteria and fungi, pathogen defense in humans and daughter cell separation in yeast (Renkema, 1995; Carstens et al., 2003; Escott and Adams, 1995).

Chitinases are classified according to mode of action into two classes:

- Endochitinase (EC 3.2.1.14) which cleave and hydrolyse chitin randomly at internal sites of *β*-1,4-glycoside bonds producing chitotetraose, chitotriose, and diacetylchitobiose.

- Exochitinase which has activity on the non-reducing end of the chitin chain and has two subclasses of chitobiosidases (EC 3.2.1.29) and *β*-(1,4) N-acetyl glucosaminidases (EC 3.2.1.30, which now includes EC 3.2.1.52 as b-L-N-acetylhexosaminidase) cleaving the oligomeric products of endochitinases and chitobiosidases and thus generating monomers

of GlcNAc. There is another pathway where chitin deacetylase converts chitin to chitosan, which is degraded by chitosanase (EC 3.2.1.132) to glucosamine residues as demonstrated in Fig. 4 (Davis and Eveleigh, 1984; Dahiya, 2005; Li, 2000).

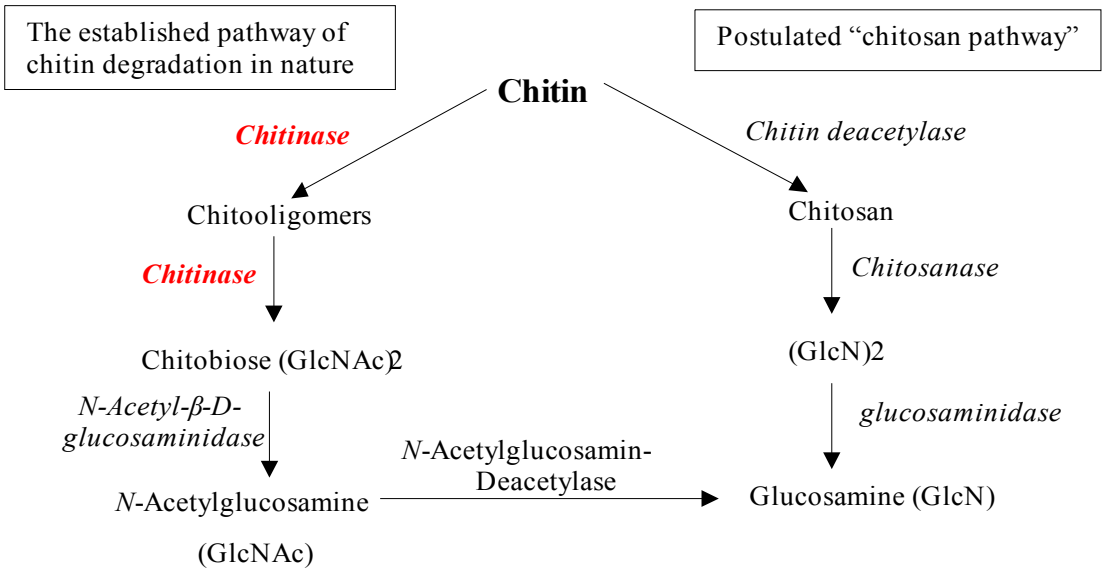


Fig. 4. Chitin degradation pathways in nature (Li, 2001; Davis and Eveleigh, 1984)

Chitinases are also classified according to amino acid sequences and similarity of catalytic domains into three families, 18, 19 and 20. These families are different in the 3D structure. Family 18 contains endochitinase and exochitinase from viruses, bacteria, fungi, insects, animals and some plant chitinases (classes III and V). Family 19 mainly contains plant chitinases (classes I, II and IV) and *Streptomyces* chitinase. Family 20 contains chitinase from human, *Dictyostelium discoideum* and *Vibrio harveyi*. Most chitinases consist of the following main domains: signal peptides, catalytic domain, chitin-binding domain and fibronectin type III domain. Class I is plant chitinase with a cysteine-rich domain (chitin binding domain) at N-terminus and found in rice, tobacco and potato. Class II is found in plants (*Arabidopsis*, barly and tobacco), fungi, and bacteria, and has a similar structure to class I but missing the cysteine-rich domain at N-terminus (Fukamizo, 2001; Patil, 2000; Neuhaus, 1999; Melchers et al., 1994). Class III has similar regions to prokaryotic chitinases and found in cucumber, *Arabidposis*, tobacco and chickpea. Class IV has same structure as class I but smaller due to deletions in cystein-rich domain and catalytic domain and found in bean, sugar beet and rape. Class V is similar to bacterial chitinase and found mainly in tobacco (Nagasaki et al., 1997). Some chitinases have two chitin-binding domain like *Brassica juncea* chitinase (Chye, 2005; Van den Bergh et al., 2004) and *Aeromonas* sp. No. 10S-24 family 19 chitinase (Kojima et al., 2005).

As chitinases are one of the most important protein families showing antifungal activity *in vitro*, many reports, which are available on successful cloning of different plant chitinases as, summarized in Table 6.

Table 6. Examples of plant chitinases successfully expressed in different crops.

Target crop	***Chitinase gene source***	***Reference***
Canola (*Brassica napus* L.)	Bean	Grison et al., 1996
Cotton (*Gossypium hirsutum*)	Bean	Tohidfar et al., 2005
Tobacco (*N. tabacum* L.)	Bean	Broglie et al., 1989 and 1991
Peanut (*Arachis hypogaea*)	Tobacco	Rohini and Rao, 2001
Cucumber (*Cucumis sativus* L.)	Tobacco	Punja and Raharjo, 1996
Tobacco (*N. tabacum* L.)	Tobacco	Neuhaus et al., 1991
Tobacco (*N. tabacum* L.)	*Brassica juncea*	Fung et al., 2002
Potato (*Solanum tuberosum*)	*Brassica juncea*	Chye et al., 2005
Italian ryrgrass (*Lolium multiflorum*)	Rice chitinase	Takahashi et al., 2005
Chrysanthemum (*Dendranthema grandiflorum* (Ramat.)	Rice chitinase	Takatsu et al., 1999
Cucumber (*Cucumis sativus* L.)	Rice chitinase	Tabei et al., 1998; Kishimoto et al., 2002
Grape (*Vitis vinifera* L.)	Rice chitinase	Yamamoto et al., 2000
Rose (Rosa hybrida L.)	Rice chitinase	Marchant et al., 1998
Strawberry (*Fragaria sp.* L.)	Rice chitinase	Asao et al., 1997
Japonica rice (*Oryza sativa)*	Rice chitinase	Nishizawa et al., 1999
Wheat (*Triticum aestivum* L.)	Rice chitinase	Chen et al., 1998
Silver birches (*Betula pendula* R.)	Sugar beet	Pasonen et al., 2004; Pappinen et al., 2002
Tobacco (*N. tabacum* L.)	Peanut	Kellmann et al., 1996
Wheat (*Triticum aestivum* L.)	barley	Bliffeld et al., 1999; Oldach et al., 2001
Grape (*Vitis vinifera* L.)	barley	Bornhoff et al., 2005

Chitinases from other sources than plants were also cloned and investigated from fungus such as *Trichoderma harzianum* endochitinase (Hayes et al., 1994) which introduced into apple (Wong et al., 1999 and Bolar et al., 2000), potato (*Solanum tuberosum* L.), broccoli (Mora and Earle, 2001), tobacco (Lorito et al., 1998) and grape (Kikkert et al., 2000). *Rhizopus oligosporus* chitinase was introduced into tobacco (Terakawa et al., 1997). Mycorrhiza (*Glomus mosseae)* chitinase expressed into pea (Slezack et al., 2001), yeast (*Saccharomyces cerevisiae)* chitinase was expressed into tobacco (Carstens et al., 2003). Insect chitinase from hornworm (*Manduca sexta*) was expressed into tobacco and showed reduction in growth of budworm (*Heliothis virescens*) larvae (Ding et al., 1998). Also, baculovirus chitinase was expressed in tobacco (Shi et al., 2000).

Itoh et al. (2003) reported, for the first time, the introduction of family 19 chitinase gene into plant, where they transformed the *ChiC* gene of *Streptomyces griseus* HUT6037 into rice using the *Agrobacterium* strain EHA101. They found a clear antifungal activity by inhibiting the hyphal growth of *Trichoderma reesei*. They found that *ChiC* is accumulated intercellularly in rice plants and possess an activity against glycol chitin.

A large group of bacterial chitinases were isolated, cloned and characterized, but a few of them were expressed in plants. Lund et al. (1989), Jach et al. (1992), and Howie et al. (1994) investigated the expression of *ChitA* gene from *Serratia marcescens* in tobacco. Table 7 summarizes some examples of the cloned bacterial chitinases so far.

Table 7. Examples of cloned and characterized bacterial chitinases

Organism/plant	***Enzyme (MW) kDa***	***Gene***	***Family***	***Target***	***Reference***
Streptomyces olivaceo-viridis ATCC11238	Chit30 (30)	*Chit30*	19	Pea	Present study
Streptomyces olivaceo-viridis ATCC11238	Chit30 (30)	*Chit30*	19	Tobacco	Present study
Streptomyces griseus HUT6037	ChiC (33)	*ChiC*	19	Rice	Itoh et al., 2003
Aeromonas hydrophila	85 kDa chitinase	ND.		*E. coli*	Chen et al., 1991
Aeromonas sp. No10s-24	Chitinase II (53) Chitinase III(55)	Chit II, III ORF-1-4			Ueda et al., 1994; Shiro et al., 1996
Alteromonas sp. Strain O-7	Chi-85 (85) Chi-78 (78)	*Chi-85*			Tsujibo et al., 1993
Bacillus cereus 28-9	ChiCW (70), ChiCH (37)	*ChiCW, ChiCH*	18	*E. coli*	Huang et al., 2005
Clostridium paraputrificum	ChiB (87)	*chiB*			Morimoto et al., 1997
Enterobacter agglomerans IC 1270	Chia_Entag (59) ChiA (60)	*chiA*			Chernin et al., 1995, Park et al., 1997
Janthinobacterium lividum	Chi69 (69), Chi56 (56)	*Chi69, chi56*			Gleave et al., 1995
Serratia liquefaciens	ChiA, ChiB	*chiA, chiB*	18		Joshi et al., 1988
Serratia marcescens QMB1466	ChiA (58), ChiB (54)	*chiA, chiB*	18		Jones et al., 1986; Harpster and Dunsmuir, 1989
Serratia marcescens	Chitinase A	*chiA*	18	Tobacco	Lund et al., 1989, Howie et al., 1994, Jach et al., 1992
Serratia marcescens BJL200	Chit A (62) Chit B (55)	*chiA, chiB*	18		Brurberg et al., 1994 and 1995
Serratia marcescens 2170	Chitinase A (57), B (52)	*chiA, chiB*	18		Watanabe et al., 1997
Serratia marcescens 2170	Chitinases C1 and C2 (36)	*chic*	18		Suzuki et al., 1999
Serratia marcescens KCTC2172	54 and 22 chitinases	Not designated	18		Gal et al., 1997
Streptomyces griseus HUT6037	ChiC (33), C-1, C-2 (27)	*Chic*	19		Ohno et al., 1996; Mitsutomi et al., 1995
Streptomyces lividans 66	Chitinase A (36), C (65), D (41), B (46)	*chiA, chiC, chiB*			Miyashita et al., 1991& 1997; Miyashita & Fujii, 1993; Fujii & Miyashita, 1993
Streptomyces olivaceoviridis ATCC 11238	Exochitinase (59 and 47)	*Exo-chiO1*			Blaak et al., 1993; Blaak and Schrempf, 1995
Streptomyces olivaceoviridis ATCC 11238	Chi30 (30), Chi92 (92)	*Chi30, chi92*	19		Li, 2001
Streptomyces plicatus	Cht 63 (63)	*chtA*			Robbins et al., 1988 & 1992
Streptomyces thermo-violaceus OPC-520	Chi40 (40)	*Chi40*			Tsujibo et al., 2001
Streptomyces specis Bacillus chitinolyticus	Chitinase (32)	*chIS*	19		Hoster et al., 2005
Steptomyces sp. J-13-3	Chitinase (32)		19	*E.coli*	Yamashita and Okazaki, 2004
Stenotrophomonas maltophilia strain *C3*	Chitinase (32)				Zhang et al., 2000

2.2.6 Pea improvement via *Agrobacterium*-mediated genetic transformation

It was mentioned that the economical, nutritional and environmental importance of pea is affected by the different stresses constraining the production of this crop leading to heavy losses in quality and quantity of the product mainly due to fungal diseases.

Genetic transformation has potential impacts for crop improvement through alleviation of specific production constraints. These techniques include vector based method using *Agrobacterium tumefaciens* or *A. rhizogenes* transformation and direct gene delivery methods such as electroporation (Chowrira et al., 1998), PEG-mediated gene transfer (Böhmer et al., 1995; Maccarrone et al., 1995) and particle gene bombardment (Öktem et al., 1999; Masood et al., 1996). For each method, there are advantages and disadvantages. For *Agrobacterium tumefaciens,* there are many advantages in comparison to other systems like the respective simplicity without need for highly sophisticated equipments, predictable integration pattern of the transgene, possibility to transfer large fragments of T-DNA and relatively stable transformation events. The disadvantage is that not all species were susceptible to *Agrobacterium* infection, but now there are different articles on monocot transformation using *Agrobacterium* such as rice, wheat, maize and barley. Protoplasts can also be used for species, which cannot be transformed with Agrobacterium, but clones, which are defined as single event transformants takes long time to become regenerated to plants. Particle bombardment could be targeted to any explant or tissue and used for transient gene expression but the disadvantage is unpredictable gene integration and high risks for gene rearrangement and silencing. The method of choice therefore is to use the natural system of *Agrobacterium*-mediated transformation since many legumes are susceptible for *Agrobacterium* infection. There are some successful achievements in pea, chickpea and soybean showing stable transformation, but still challenges for other legumes like bean and lentil, which are considered to be more recalcitrant for regeneration and transformation in comparison to other crops (De Kathen and Jacobsen, 1990). The prerequisites for any successful transformation protocol are: (I) the development of a reproducible regeneration system accompanied with (II) efficient transformation and selection protocol to increase the transformation efficiency.

Since the regeneration is the critical step for any transformation success, there are different techniques used for regeneration of pea from callus through organogenesis from protoplast

(Böhmer et al., 1995) or somatic embryogenesis which was first reported by Jacobsen and Kysely (1984), from leaf-derived callus using Picloram, and from immature zygotic embryos or from shoot apices using 2,4-D or Picloram (Lehminger-Mertens and Jacobsen, 1989; Kysely and Jacobsen, 1990; Kysely et al., 1987). Puonti-Kaerlas and Eriksson (1988) reported regeneration of pea shoots from protoplast culture from 10 different cultivars. Epicotyls were the source of protoplasts, shoots could be observed after nine months and regeneration frequency of shoots was 1 % in two cultivars. Nielsen et al. (1991) used hypocotyl segments for regeneration of the pea cultivar *Fjord*, then four cultivars were tested using the same protocol using 10 µM IAA and then 5 µM Zeatin. The regeneration frequency obtained was 10 % and plants could be obtained within three months. Özcan et al. (1993) described pea regeneration using adventitious shoot regeneration and somatic embryogenesis from immature cotyledon explants of two pea cultivars, i.e. *Orb* and *Consort*, where regeneration was affected with cotyledon size and orientation on the medium. Kosturkova et al. (1997) reported high efficiency (50-10 %) regeneration of 10 Bulgarian pea genotypes from embryonic axes of immature embryos through direct and indirect organogenesis induction on MS medium supplemented with 10 mM BA+1 mM NAA or 0.2 mM 2,4-D, respectively. Griga (1998 and 2002) described direct somatic embryogenesis from shoot apical meristems of pea (without callus intervention) from meristematic tissues grown on a medium supplemented with 2.5 µM Picloram and then TDZ achieving 78 % germination of harvested somatic embryos. Loiseau et al. (1995) described somatic embryogenesis from shoot apices of pea with 95-100 % explant forming embryos from genotype C1 830. Ochatt et al. (2000) used hypocotyl sections lacking pre-existing meristems to induce callus or somatic embryogenesis where they could regenerate a whole plant via organogenesis. Tzitzikas et al. (2004) described a procedure of pea regeneration from nodal tissue in 4 cultivars using TDZ or BAP for induction of bud-containing tissue and multiplication of these buds, then different combinations of GA3, NAA and BAP were used for shoot formation, while IAA or IBA or NAA were used for root induction. They used a cyclic multiplication of bud-containing tissue.

Legume transformation was reported by different groups. Köhler et al. (1987) described PEG method of direct transformation of moth bean (*Vigna aconitifolia*) which is highly dependent on the cultivar used. Russell et al. (1993) described *Phaseolus vulgaris* transformation via electric discharge mediated particle acceleration. Ikea et al. (2003)

transformed for the first time cowpea (*Vigna unguiculata* L. walp.) using particle bombardment method, where the transformation efficiency was 1.26 %. Examples of other legumes, which were transformed via Agrobacterium-mediated transformation, are listed in the Table 8.

Table 8. Few selected publications on different legumes transformed via *Agrobacterium*

Crop	***Agrobacterium strain***	***Explant***	***Transformation efficiency/genes used***	***References***
Soybean (*Glycine max*)	A281, C58, ACH5 and EHA105	cotyledonary node (for EHA105)	0.4 % (for EHA105)/ GUS	Donaldson and Simmonds (2000)
Soybean (*Glycine max*)	EHA105	immature zygotic cotyledons	0.03%, GUS, *Hpt*	Yan et al. (2000)
Azuki bean (*Vigna angularis*)	LBA4404, EHA105 and AGL1	elongated epicotyls of etiolated seedlings	2 %, *npt*II, GUS or GFP	Yamada et al. (2001)
Chickpea (*Cicer arietinum* L.)	EHA101	cotyledonary node	/bar and vst genes	Kiesecker (2000)
Pigeon pea (*Cajanus cajan* L.).	EHA105	embryonic axes and cotyledonary nodes	51-67%, *npt*II and hemagglutinin gene	Satyavathi et al. (2003
Faba bean (*Vicia faba* L.)	EHA105	embryo axes	sulphur rich sunflower albumin	Hanafy et al. (2005)
Medicago truncatula	LBA4404, C58 and AGL1	young leaflets	*npt*II, GFP and GUS	Chabaud et al. (2003)
Desi and Kabuli chickpeas	AGL1	germinated seedlings	5.1 %	Senthil et al. (2004)
Chickpea	LBA4404	embryo axes	4 %	Fontana et al. (1993)
Grasspea (*Lathyrus sativus* L.)	EHA105 and LBA4404	epicotyl segments	*npt*II and GUS	Barik et al. (2005)

Puonti-Kaerlas et al. (1989) had tested different *Agrobacterium* strains, i.e. A281, B6S3, C58, GV3101(pGV2298), GV3101(pGV3851) and GV3101(pGV3304) for susceptibility of five cultivars of pea i.e. *Bello, Filby, Petra, Stivo* and *Vreta.* As explants for the transformation with *Agrobacterium* strain GV3101 they used epicotyl segments and cut stems and leaf pieces from shoot culture. They found that the response in pea is influenced more by the bacterial strain and the limited factor in pea transformation is regeneration *in vitro*.

Puonti-Kaerlas et al. (1990) reported transformation of pea plants of three cultivars, i.e. *Filby*, *Puget* and *Stivo* using axenic shoot culture and epicotyl co-cultured with *Agrobacterium* strain GV3101, where the best results of callus formation in all three cultivars were in media supplemented with BA and 2,4-D. The efficiency of callus regenerating shoots was 15 % but it took 6 months for shoot induction on regeneration

medium. 28 transgenic plants were transferred into the greenhouse where they flowered but without raising any seeds, apparently the use of *npt*II gene lead to sterility. They also reported the effects of cultivars and selectable marker on the callus production. Puonti-Kaerlas et al. (1992a) transformed two cultivars of pea protoplast by direct gene transformation of GUS as marker gene using electroporation with transformation efficiency of 1-2.2 %.

De Kathen and Jacobsen (1990) co-cultivated epicotyl segments and node explants from etiolated seedlings of pea with wild-type *Agrobacterium* strains C58C1, A281 and 8683 harboring binary vectors GV 2260 (p35S GUS INT) and GV 3850 HPT carrying either a neomycin- or hygromycin phosphotransferase-gene as selectable markers, where they could recover around 5 % of plantlets showing GUS and NPTII activity but without raising any seed. They found that transformation frequency was influenced by explant source, *Agrobacterium* strain, pea genotype and duration of co-cultivation.

Davies et al. (1993) used *Agrobacterium* strain C58/3 for transformation of the pea cultivar *Puget*. The explants were prepared from germinating seeds after removing the shooting and rooting part of the cotyledonary lateral buds, which were used for transformation by injection of the bacteria, suspension harboring a binary vector containing *npt*II and *GUS* genes. Transformation efficiency was 1.44 %, with four months time mentioned to produce and transfer the transgenic plants into greenhouse.

Schroeder et al. (1993) developed transgenic peas through organogenesis using longitudinal slices embryogenic axis of immature seeds of cultivars *Greenfeast* and *Rondo* and the explants were incubated with *Agrobacterium* strain AGL1 harboring binary vector containing *npt*II and *bar* genes. PPT at concentration of 15 mg/l was used as a selecting agent. They reported nine months from inoculation of explants with *Agrobacterium* suspension until producing a whole plant. Transformation efficiencies were between 1.5 and 2.5 % of starting explants. They found that the explant has an important role on successful transformation, in addition to the bacterial strain, selectable marker and hormones used during co-cultivation.

Grant et al. (1995) developed a transformation system for four pea cultivars i.e., *Bolero*, *Trounce*, *Bohatyr* and *Huka* using immature cotyledon explants, the *Agrobacterium* strain used was AGL1 harboring a binary vector containing *npt*II and *bar* genes. Transgenic plants were recovered from all four cultivars and a first generation was obtained after

seven months. Transformants were selected on 10 mg/l PPT and the transformation efficiency was 1.47 % for all cultivars. Grant et al. (1998) used four pea cultivars i.e. *Bolero, Hadlee, Crown* and *Courier* and two breeding lines *94-A26* and *89T46.UK* for *Agrobacterium* transformation strain AGL1 harboring four different binary vectors. Selecting transformants happened on a kanamycin medium, transformation efficiencies ranged from 0.8 % (cv. *Hadlee*) to 3.4 % (*89T46.UK*) depending on the cultivar used.

Bean et al. (1997) transformed the pea cultivar *Puget* with the hypervirulent *Agrobacterium* strain EHA105 harboring a binary vector containing the bar gene, the rooting and shooting parts of the germinating pea seeds were excised and lateral cotyledonary meristems were inoculated with bacterial suspension, 10 mg/l PPT was used for selection. They used the grafting techniques to achieve rooting since direct rooting was slow and takes between 6-12 weeks. They found that grafting efficiency was over 95 % and overall transformation efficiency was 1.1±0.43 %.

Chowrira et al. (1998) demonstrated *in planta* pea transformation by decapitating the apical part followed by injection and electroporation of axillary meristems with a plant expression vector pPCP4-5 harboring PEMV coat protein. The buds were injected with the vector then electroporated at 100 V, and afterwards plants were placed in greenhouse to collect the seeds for further analysis where they found resistance to infection by PEMV in different generations. They proved PEMV coat protein integration in R0 by PCR and Southern blot, they also found high chimera in R0 and R1 plants.

Schroeder et al. (1995) and Shade et al. (1994) introduced a bean α-amylase inhibitor gene into pea in order to increase the resistance to pea weevil. They also used the *Agrobacterium*-mediated transformation system. In addition, Morton et al. (2000) continued the previous work of Schroeder et al. (1995) and introduced two bean α-amylase inhibitor genes (αAI-1 and αAI-2) into the pea cultivar *Laura*. They tested the performance under field condition and found that αAI-1 could inhibit more than 80 % of the larval amylase providing a total protection against pea weevil damage. αAI-2 was less effective comparing to αAI-1[1].

Nadolska-Orczyk and Orczyk (2000) studied the factors affecting pea transformation efficiency using the three *Agrobacterium* strains LBA4404, C58C1 and EHA105

1 Bean α-amylase has altered composition when expressed in pea due to glycosylation leading to immunogenicity in mice (Prescott et al., 2005).

harboring binary vectors containing the *β*-glucuronidase reporter gene (*uidA*) and one of the four (*npt*II, *hpt*, *dhfr*, *bar*) plant selectable genes. They used cotyledonary nodes of cultivars *Laser* and *Heiga* for transformation. The transformation efficiency was 4.2 % and 3.6 % when kanamycin and PPT respectively were used as selecting agent. No transformants could be recovered when hygromycin or methotrexate were used for selection. They also noticed the influence of different *Agrobacterium* strains on transformation efficiencies where the highest rate was 8.2 % for EHA105 followed by 2.2 % for C58C1 and then by 1 % for LBA4404.

Polowick et al. (2000) studied the effect of genotype on transformation efficiency by using slices of embryogenic axis from one cultivar and seven pea breeding lines adapted to western Canadian condition and inoculated with *Agrobacterium* strain EHA105 harboring a binary vector containing the reporter gene *uidA* coding for *β*-glucuronidase (GUS) with different selection genes *npt*II or *pat*. It took around six months until getting transgenic plants in greenhouse; the transformation efficiency was 0.6 %.

Grant et al. (2003) compared two *Agrobacterium* strains, AGL 1 and KYRT1, for pea transformation using the same protocols of Grant et al. (1995) with two different cultivars, i.e. *Bolero* (Selgen) and *Lincoln* and one breeding line *97-B19*. Two binary vectors were used harboring a *GUS* gene and a *β*-1,3-glucanase gene from pea. They found that *Agrobacterium* strain KYRT1 was at least fourfold superior to AGL1 in pea transformation.

Wu and VanEtten (2004) used *Agrobacterium rhizogenes*-mediated transformation of pea to inactivate synthesis of pisatin (an isoflavonoid phytoalexin synthesized by pea) by using two senses or antisense constructs containing three different genes *Ifa, Pda* and *Hmm* involved in the biosynthesis of pisatin. Stem tissue was used as explant for transformation. They found that the reduced amount of pisatin is due to reduced transcript of *Hmm.* Their results support the hypothesis that phytoalexin production is a disease resistance mechanism.

Pniewski and Kapusta (2005) reported high transformation efficiency of 4.1 % when pea was transformed with *Agrobacterium* strain AGL0 in comparison with AGL1 and EHA105; they used slices of immature embryos as explants for transformation.

Švàbovà et al. (2005) compared six pea cultivars, i.e. *Adept, Komet, Lantra, Olivin, Oskar* and *Tyrkys* transformed with *Agrobacterium* strain EHA105 harboring pBIN19 containing

*npt*II and *uid*A genes using *in vitro* (axillary buds) and *in vivo* (imbibed germinating seeds) based regeneration methods by using sonication and vacuum infiltration treatments to facilitate the penetration of *Agrobacterium*. The time needed until getting mature seeds was 5-6 months using the *in vitro* system while it was only 3-4 months with *in vivo* system.

From all previously cited references, it can be concluded that a number of different strategies were used by different groups in order to improve the transformation efficiency and reduce the period needed for the production of transgenic plant in the greenhouse. Different explants and *Agrobacterium* strains were tested together with different selection agents. All systems were based on *Agrobacterium* -mediated transformation, even though there was one report on electroporation (Chowrira et al., 1998) and one *in vivo* transformation (Švàbovà et al., 2005) was reported. *Agrobacterium tumefaciens* was used in most experiments whereas *A. rhizogenes* was used only in few experiments (Wu and VanEtten, 2004).

The previous investigations were mainly focused on the improvement of the pea transformation, as pea was considered to be a recalcitrant crop for regeneration and transformation. Only a few reports have used economically important genes of interest (Schroeder et al., 1995; Morton et al., 2000; Grant et al., 2003 and Wu and VanEtten, 2004) while others introduced reporter genes or selectable markers.

Pea is also becoming more important for the production of high-value recombinant molecules in molecular farming due to its high protein content in the seeds. scFV antibodies and β-interferon genes (Perrin et al., 2000; Saalbach et al., 2001 and Kiesecker personal communication) have been introduced in pea.

2.3 Tobacco (*Nicotiana tabacum* L.) the top model plant

Tobacco plants were used in this study as a model for testing the functionality of the constructs developed in order to save time. Tobacco is also used as experimental system for studies of other basic phenomena in plants (Helgeson, 1979). Tobacco is used in different disciplines i.e. physiology, genetics, tissue culture and botany due to its easy handling, simplicity of controlled pollination, large seed production and huge amount of leaves and green tissue. Tobacco played an important role in the development of plant tissue culture methods and media (Murashige and Skoog, 1962). Genetic transformation systems as well as tissue and cell culture are much advanced in tobacco plants compared

to other economical important plants or even with other model plants. It is also used for secondary metabolite production in cell cultures which makes it a useful biotechnological tool for the production of natural products instead of using industrial microorganisms (Sommer et al., 1998).

3 OBJECTIVES OF THIS STUDY

This research proposes to improve one of the most important grain legume crops, i.e. pea, against fungal diseases using a chitinase gene with the need to sharpen the awareness of what advantages biotechnology can offer to the environment, health care and food security particularly in developing countries.

The overall goal of this study is to enhance the resistance to fungal disease in pea through the heterologous expression of a wild type bacterial chitinases gene (*Chit30*) from *Streptomyces olivaceoviridis* ATCC 11238 and a modified gene (N-*Chit30*) where the coding region of the functional-undefined and catalytic domains of the bacterial gene was N-terminally fused to the secretory leader peptide from an *Arabidopsis thaliana* basic endochitinase gene.

To achieve this goal, first the transformation of tobacco was performed in order to test the functional integrity of the new constructs before the more time-consuming transformation of pea. The study focuses on the following objectives:

- Cloning of chitinase gene into a binary vector.

- Transformation of tobacco and pea via *Agrobacterium*- mediated system.

- Molecular characterization of the transformants.

- Testing of the antifungal properties of the heterologously expressed chitinase.

- Evaluation of the genetically modified plants for their fungal resistance

To achieve this aim, the plant binary vector of pGreenII series was used and the chitinase gene was driven either by a 35S cauliflower mosaic virus (CaMV) constitutive promoter or by the plant inducible *vst* promoter from grape. Subsequently the constructs would be transformed to *Agrobacterium tumefaciens* strain EHA105. A modified method adapted from Schroeder et al. (1993) and Bean et al. (1997) was used for pea transformation (cultivar *Sponsor*).

Transgenic plants were subjected to various molecular and cellular characterizations to study and prove stable introduction and inheritance of the gene of interest to the following generations. The ability of transformed pea and tobacco plants to express the heterologous chitinase to a level, which protect the plants from fungal invasion, was also analysed.

4 MATERIALS AND METHODS

4.1 Chemicals

4.1.1 Growth media

Substance	Molecular weight	Company
MS basal salts mixture		DUCHEFA
B5 vitamins		DUCHEFA
Plant agar		DUCHEFA
D(+) saccharose	342.3	ROTH
MES (2-[N-morpholino] ethane sulfonic acid)	213.85	BIOMOL
Potato dextrose agar (PDA)		Difco

4.1.2 Plant hormones and additives

Substance	MW	Company	Solvent
2,4-D	221.6	DUCHEFA	KOH
IBA	203.2	DUCHEFA	KOH
NAA	186.2	DUCHEFA	KOH
Kin	215.2	DUCHEFA	KOH
BAP	225.3	DUCHEFA	KOH
TDZ	220.2	DUCHEFA	KOH
GA_3	346.4	DUCHEFA	KOH
Acetosyringone	196.2	ROTH	DMSO
Glufosinate-ammonium (PPT)	198.16	Riedel DeHaen	dd H_2O
BASTA® (200g/l)		Aventis GmbH	
Zeatin	219.25	DUCHEFA	NaOH
IPTG (isopropyl-β-D-thiogalstopyranoside	238.3	Applichem	

4.1.3 Antibiotics

Substance	Molecular weight	Company	Solvent
Combactam		Pfitzer	dd H_2O
Kanamycin	582.6	DUCHEFA	dd H_2O
Ticarcillin	428.4	DUCHEFA	dd H_2O
Ampicilin	371.39	ROTH	dd H_2O

4.1.4 GUS-assay buffer

100 mM sodium phosphate buffer (pH 7.0),

0.5 mM potassium ferrocyanide,

10 mM EDTA

1 mM (0.5 mg/ml) X-*GLUC* (dissolved in DMSO before adding it to Gus buffer)

4.1.5 Restriction enzymes and buffers

Enzyme	10x Buffer	Company
*Eco*RI	10x O^+ (orange)	MBI Fermentas
*Hind*III	10x R^+ (red)	MBI Fermentas
*Eco*RI/ *Hind*III	10xY^+/$T_{ANqo™}$ (yellow)	MBI Fermentas
*Xho*I	10x R^+ (red)	MBI Fermentas
*Bam*HI	10x Bam HI^+	MBI Fermentas
RNaseA		QIAGEN
Shrimp alkaline phosphatase (SAP)	10x SAP buffer	MBI Fermantas
T4 ligase	10x ligation buffer	MBI Fermantas

4.1.6 DNA markers

DNA marker	Concentration	Company
Gene Ruler™ λ Mix DNA marker	0.5 mg/ml	MBI Fermentas
Gene Ruler™ 100 bp DNA ladder	0.5 mg/ml	MBI Fermentas
Gene Ruler™ 1 kbp DNA ladder	0.5 mg/ml	MBI Fermentas
DIG labeled DNA II & III marker *	5 µg/µl	Roche
DNA MB grade fish sperm	10 mg/ml	Roche

* Dig II marker: 125, 564, 2027, 2322, 4361, 6557, 9416, 23130 bp.

Dig III: 125, 564, 831, 947, 1375, 1584, 1904, 2027, 3530, 4268, 4973, 5148, 21226 bp.

4.1.7 Primers

Primer	Sequence	Product	Company
StrepChit For. StrepChit Rev.	5'-TCCATGGATCCATGCCGAGGCGTC GCACATCCGCCCTGCT-3' 5'-CGGACTCTAGATCAGCAGTAGAG TTGCCGCCCGGGAGA-3'	900 bp	MWG Biotech
pGII 297-F pGII 303-R	5'-GTTGGGTAACGCCAGGG-3' 5'-GGAGCTCGCCTGCTGGTCACTGG-3'	~1300 bp[2] ~2200 bp[3]	MWG Biotech
Chit 555-F Chit 555-R	5'-GGTGACATCGTCCGCTACAC-3' 5'-GGTGTTCCAGTACCACAGCG-3'	555 bp	MWG Biotech
bar447-F bar447-R	5'-GATTTCGGTGACGGGCAGGA-3' 5'-TGCGGCTCGGTACGGAAGTT-3'	447 bp	MWG Biotech
bar-F bar-R	5'-GCAGGAACCGCAGGAGTGGA-3' 5'-AGCCCGATGACAGCGACCAC-3'	260 bp	MWG Biotech
NptI-F NptI-R	5'-GAAAAACTCATCGAGCATCA-3' 5'-TTGTCCTTTTAACAGCGATC-3'	400 bp	MWG Biotech
Chit-NcoI-F Chit-XbaI-R	5'-GATCCATGAAGACTAATCTT-3' 5'-CTAGATCAGCAGTAGAGGTT-3'	870 bp	MWG Biotech
Chit-2783-R	5'-CCTACCCCGGCTTCGCGAAC-3'	Sequencing	MWG
Chit-2787-F	5'-GGCAGCGACACCACCAAGAA-3'	Sequencing	MWG
Chit-3233-R	5'-TCCAGAGCCGCGTGAACAAC-3'	Sequencing	MWG
Chit-2394-F	5'-GGATCCATGCCGAGGCGTCG-3'	Sequencing	MWG
Chit-A-leader- F Chit-XbaI-R	5'-TTTGGATCCATGAAGACTAATCTT TTTCTCTTTCCATCTTTTCACTTCTCC TATCATTATCCTCGGCCGCGGCCTGT TCGAGCTACC-3' 5'-GACTCTAGATCAGCAGTAGAGGTT GCCGC-3'	900 bp	MWG Biotech
iPCR-pGII-F iPCR-pGII-R	5'-GGGAGAGGCGGTTTGCGTAT-3' 5'-ACATAGATGACACCGCGCGC-3'	2770 bp	MWG Biotech
HMG-F HMG-R	5'-ATGGCAACAAGAGAGGTTAA-3' 5'-TGGTGCATTAGGATCCTTAG-3'	570 bp [4] 350 bp [5]	MWG Biotech

4.1.7 Solvents and sterilizes

Substance	Company
Dimethyl sulfoxide (DMSO) KOH	SERVA Roth
NaOCl	Riedel de Haen

2 Plasmid without insert,
3 Plasmid with insert
4 PCR product with intron
5 PCR products without intron

4.1.8 Equipment

Equipment	**Manufacturer**
Autoclave	Tuttnauer systec
Balances	Sartorius
Cold centrifuge	Sigma 302K
Deep freezer –80 ºC	Lozone
Dry oven	Memmert
Electrophoresis chamber	Bio-RAD
Electrophoresis power supply	Bio-RAD
Film	Kodak
Ice machine	ZIEGRA
Incubator	JURGENS
Lab centrifuge	Eppendorf 5415C
Magnetic stirrer	Heidolph
Microwave	Thomson
Nylon membrane	Roche
PH meter	HANNA
Pipette	Gilson, Eppendorf
Refrigerator 4 ºC	LIEBHERR
Rinsed water station	MILLIPORE
Sonicator	SonoRex RK255S
Spectrophotometer	Pharmacia Biotech
Stereomicroscope	Leica Wild M3Z
Thermocycler PCR	Biometra®
Thermostat shaker	Heidolph Unimax 1010
UV-Transilluminator	Vilber Lourmat
Vacuum pump (~100 mbar)	ABM
Vacuum resistance container	Duran
Vortex	Heidolph
Water bath	GFL®
Scalpel blade	AESCULAB® No.11
Stock solution vessel	NALGENE® CRYOWARE™

Sterilization filter	MILLEX®-GS 0.22µM
Parafilm Filter paper	NESCOfilm Schleicher & Schuell
Disposable plastic wares: - 2ml microtube-centrifuge - 1.5 ml and 2 ml Eppendorf-caps - Petri dishes - pipette tips	

4.2 Plasmid construction and cloning

4.2.1 Reagents

Sterile Luria Broth (LB) media
100 mM $CaCl_2$, at 4 °C
86 % and 10 % sterile glycerol
37 °C and 28 °C shaker
Centrifuge and centrifuge bottles

4.2.1.1 Media

SOC 20 g/l tryptone
5 g/l yeast extract
10 mM NaCl
2.5 mM KCl
10 mM $MgSO_4$ x 7 H_2O
2.033 g/l $MgCl_2$ x 6 H_2O
20 mM glucose (filter sterilized added before using)

LB (Lauria Bertoni) (Sambrook et al. 1989)
10 g/l tryptone
5 g/l yeast extract
8 g/l NaCl
pH 7

YEP (Yeast Extract Peptone)
10 g/l tryptone
10 g/l yeast extract
5 g/l NaCl
pH 7

LB and YEP media were solidified by addition of 15 g/l Agar Agar to prepare solid media.

4.2.2 Preparation of competent *E. coli* cells for heat shock transformation (Nakata et al., 1997 and Tang et al., 1994)

The required *E. coli* strains (NM522, DH5α, GM6233 and Top10) were grown overnight in 1-5 ml of LB medium at 37 °C (without antibiotics) to stationary phase. The overnight

culture was diluted in fresh LB 1:50 and grown at 37 °C until $O.D_{600}$ reached ~0.4. The cells were harvested by centrifugation at 4 °C, 4400 rpm, and re-suspended in 1/2 volume ice-cold 100 mM $CaCl_2$ and centrifuged again. The supernatant was discarded and the pellet was resuspended in 1/2 volume ice-cold 100 mM $CaCl_2$. Pellet cells were re-suspended in 1/10 volume cold 100 mM $CaCl_2$ and incubated on ice for 1 hour and used immediately for heat shock transformation. Alternatively, 86 % sterile glycerol was added to a final concentration of 15 % and then aliquots of 100 µl in 1.5 ml tubes, which were put immediately in liquid nitrogen and stored at -80 °C for long-term storage.

4.2.3 Heat shock/Calcium chloride method for *E. coli* transformation

Competent *E.coli* cells were taken from the -80 °C freezer and kept on ice to avoid melting, 50 ng (1-5 µl) of ligation mixture (or ready plasmids) were added to a 1.5 ml tube (Eppendorf or similar) and gently mixed with 50 µl competent cells. The tube was incubated on ice for 20 min and then placed in a water bath without shaking at 42 °C for 30 seconds, returned back immediately onto ice for 2 minutes. 950 µl of pre-cooled SOC medium without antibiotics were added to develop antibiotic resistance and to reduce damage of *E.coli* cells. Finally, the tubes were incubated on a shaker at 250 rpm for 90 min at 37 °C.

100 µl of the resulting culture was spread on LB plates with the appropriate antibiotic and grown overnight at 37 °C. The colonies were picked about 12-16 hours later.

4.2.4 Preparation of *Agrobacterium tumefaciens* EHA105pSoup competent cells for electroporation

The hypervirulent *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993) was co-transformed with the pSoup helper plasmid according to the pGreenII system (pGreen website, Hellens et al., 2000). An overnight seed culture of 25 ml YEP supplemented with 5 mg/l tetracycline was incubated with 250 µl of glycerol stock of EHA105pSoup at 28 °C on a shaker. 2 ml of bacterial suspension (overnight seed culture) were added to 50 ml YEP supplemented with antibiotic and grown for 2-5 h until $O.D._{600}$ reached ~0.4-0.5 was reached.

Bacteria were pelleted by centrifugation at 4400 rpm and 4 °C for 10 min, re-suspended twice in 25 ml ice-cold 10 % glycerol. The pellet was then re-suspended twice in 2.5 ml ice-cold 10 % glycerol after centrifugation at 4400 rpm at 4 °C for 10 min. Finally, the pellet was re-suspended in 1 ml ice-cold 10 % glycerol. Aliquots of 200 µl were split in 2

ml Eppendorf tubes and transferred immediately into liquid nitrogen and stored at -80 °C.

4.2.5 *Agrobacterium* transformation through electroporation

Competent *Agrobacterium* (EHA105-pSoup) were taken out from -80 °C freezer and kept on ice to avoid melting. 50 ng (1-5 µl) of a plasmid solution was gently mixed with 50 µl competent cells in a 1.5 ml tube (Eppendorf or similar).

The mix was transferred to a pre-cooled cuvette (gap 0.2 cm) and electroporated in a BioRad electroporator at: 25 µF capacitor, 200 Ω (ohm) resistance and 2.5 KV.

The field strength was between 6,25 – 12 kV/cm for 4-8 msec. 500-1000 µl of pre-cooled SOC medium (with no antibiotic) were added immediately afterwards, then the mixture was transferred to a new 2 ml tube and incubated for 3 hours at 28 °C with shaking (250 rpm). 100 µl of the resulting culture was spread on YEP plates (with the appropriate antibiotic-Kanamycin) and grown overnight at 28 °C. The colonies were picked about 24-48 hours later.

4.2.6 *Agrobacterium* inoculation and harvest

25 ml YEP medium in 100 ml Erlenmeyer flask including appropriate antibiotics for the respective plasmid (50 mg/l kanamycin for pGII35Schit and pGIIvstchit) were inoculated with 250 µl glycerol stock of *Agrobacterium tumefaciens*. The medium was inoculated and placed on a shaker at 250 rpm, at 28 °C in the dark for 15 h.

Bacteria were harvested by centrifugation at 4400 rpm, the supernatant was discarded and then the pellet was re-dissolved in liquid B5-i medium (see 4.8.1.1) supplemented with 3.24 µM BAP or 5 µM TDZ. $O.D_{600}$ was measured using a spectrophotometer and adjusted to 1-1.3. Acetosyringone was added at a concentration of 100 µM (filter sterilized).

4.2.7 Preparation of glycerol stocks of bacteria

Glycerol stocks of bacteria were prepared in a ratio of 1:3, where one colony was picked from the master plate, dissolved in 2 ml YEP or LB medium and inoculated for 2-3 hours on a shaker at 250 rpm, then transferred to 25 ml YEP or LB medium containing the necessary amounts of antibiotics and incubated on a shaker at 250 rpm, 28 ºC or 37 °C in the dark for 15 h. The stock solution was prepared using 500 µl glycerol (86 %) and 1000 µl of growing bacterial-suspension in 2 ml cryogenic vials (Cryoware-Nalgene, Rochester, USA) which were stored at -80 °C for further use.

4.2.8 Maintenance of the plasmid and *Agrobacterium* properties

Since legume transformation is highly labor and time consuming, it is advisable to check the correct insertion of the plasmid by restriction digest or sequencing and from time to time preparing stocks from checked colonies. Plasmid isolation was performed according to Birnboim and Doly (1979) as will be explained below (4.3.6.1).

4.2.9 Binary vectors

The dual-binary vector system pGreenII/pSoup was used in the present study (Hellens et al., 2000). pGreenII vector has advantage over the other vectors due to its smaller size, easier handling, multiple cloning sites, high copy number and improved stability in *E. coli* while under non-selective condition the number of *Agrobacterium* colonies containing pGreen plasmid reduced by 50 % after one day which enhance the safety used of this vector (Hellens, 2000). Since pGreen system is dual and need presence of pSoup in *Agrobacterium* which act in *trans* providing replication of pGreen, the system give another advantage of using pSoup for co-transformation to produce marker-free transgenic plants by second T-DNA containing the marker gene in pSoup and the gene of interest in pGreen (Vain et al., 2003; Afolabi et al., 2005). The prerequisite for this technique is high efficient transformation protocol, which serves high numbers of different transgene localization of the two T-DNAs.

The T-DNA contains the *bar* gene fused between the *nos* promoter and terminator sequences of *Agrobacterium tumefaciens*. The *bar* gene encodes a phosphinothricin acetyltransferase (PAT) enzyme which confers resistance to bialaphos and the related compounds phosphinothricin (PPT), the active ingredient of the herbicide BASTA® and glufosinate ammonium through acetylation (Fig. 5).

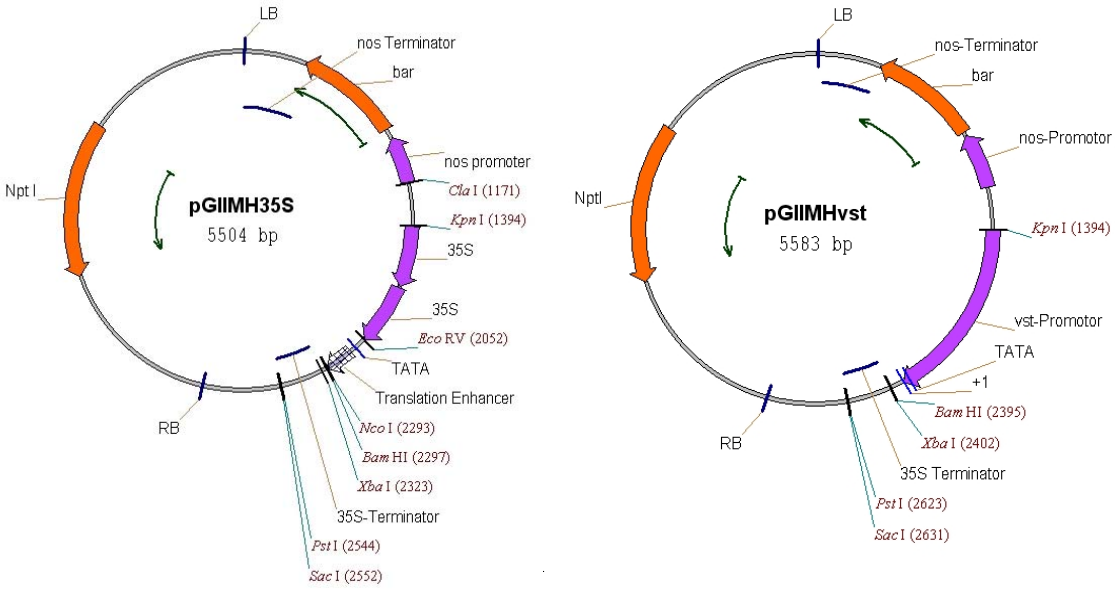


Fig. 5. Functional maps of the pGII35S and pGIIvst vectors used in the cloning work.

The chitinase gene was cloned into the Ti-plasmid using PCR; the chitinase gene was amplified using two cloning primers StrepChit forward:

5'-TCCATGGATCCATGCCGAGGCGTCGCACATCCGCCCTGCT-3' and StrepChit reverse 5'-CGGACTCTAGATCAGCAGTAGAG GTTGCCGCCCGGGAGA-3' flanking *Bam*HI and *Xba*I restriction sites, respectively (underlined) to the PCR product using proof reading Combi*Zyme* DNA polymerase (Invitek GmbH, Germany). The PCR mixture was prepared according to the manufacturer protocol as follows:

PCR reaction mixture:

Compound and concentration	**Amount per reaction**
Double distilled water	27 µl
10x PCR buffer	5 µl
50 mM $MgCl_2$	2.5 µl
5 mM nucleotides mixture (dNTPs)	2 µl
5X OptiZyme Enhancer	10 µl
10 pmole forward primer	1 µl
10 pmole reverse primer	1 µl
20-50 ng plasmid DNA	1 µl
Combi*Zyme* DNA polymerase (4 U/µl)	0.5 µl
Total volume	50 µl

PCR program:

PCR step	Temperature (°C)	Time (s)	No. of cycles
Initial denaturation	94	300	1
Denaturation	94	30	30
Annealing	55	30	
Extension	72	30	
Final extension	72	300	1
Cooling down	4	∞	

The template for the PCR was pUChi30, which is a derivative of pUC18, and was provided by Dr. Jochen Meens group from Microbiology Institute-Hannover University (Haiming 2001, accession gi: 4456813, AJ133186 NCBI database, Fig. 6). It was fused to either a constitutive double 35S promoter of cauliflower mosaic virus or the plant inducible *vst* promoter from grape (Wiese et al. 1994).

The PCR product was purified directly using PCR rapid purification kit from Invitek GmbH (Germany) or from gel using Easy Pure kit from BioZyme GmbH (Germany).

For modification and replacing the bacterial signal peptide sequence with one of plant origin, an *Arabidopsis thaliana* encoded basic endochitinase (accession number gi:30682210, NM112085, NCBI database, Fig. 7, Haseloff et al. 1997) with a different set of primers was used: forward primer include *Bam*HI site (underlined) and *Arabidopsis* signal peptide sequence (bold) designed as chit-A-leader-For. 5'-TTTGGATCC**ATG AAGACTAATCTTTTTCTCTTTCCATCTTTTCACTTCTCCTATCATTATCCTC GGCC**GCGGCCTGTTCGAGCTACC-3' and Chit-*Xba*I-Rev. 5'-GACTCT AGATCAGC AGTAGAGGTTGC CGC-3' which contains *Xba*I site (underlined).

```
LOCUS       SOL133186         1199 bp    DNA     linear   BCT 15-APR-2005
DEFINITION  streptomyces olivaceoviridis ATCC11238 chi30 gene.
ACCESSION   AJ133186  GI:4456813
KEYWORDS    chi30 gene; chitinase.
SOURCE      Streptomyces olivaceoviridis
     gene       gene="chi30"
     CDS        /gene="chi30"
                /translation="MPRRRTSALLAALVISTAAPVLLPAAPAAAAACSSYPSWVAGRSYAA
                GDIVRYTDGKAYIAEHANPGYDPTISTWYWEPYACDGGSGTPVGTFVVTEAQFNQMFPNRN
                SFYSYSGLTAALSAYPGFANTGSDTTKKQEAAAFLANVSHETGGLVHVVEQNQANYPHYCD
                WSRPYGCPAGQAAYYGRGPIQLSWNFNYKAAGDALGIDLLNSPWLVERDSAVAWKTALWYW
                NTQTGPGTMTPHNAMVNGAGFGQTIRSINGSLECDGKNPAQVQSRVNNYQRFTQILGVSPGGNLYC"
```

Fig. 6. Mature protein of chitinase 30 from NCBI database (signal peptide sequence is in red).

```
LOCUS       NM_112085    1107 bp    mRNA    linear   PLN 04-NOV-2005
DEFINITION  Arabidopsis thaliana ATHCHIB (BASIC CHITINASE); chitinase AT3G12500
(ATHCHIB) mRNA, complete cds.
ACCESSION   NM_112085  VERSION     NM_112085.2  GI:30682210

     gene
          /gene="ATHCHIB" /note="synonym: B-CHI, BASIC CHITINASE, CHI-B,PATHOGENESIS-
          RELATED 3, PR-3, PR3" /function="encodes a basic chitinase involved in
          ethylene/jasmonic acid mediated signalling pathway during systemic acquired
          resistance based on expression analyses."
     CDS
         /product="ATHCHIB (BASIC CHITINASE); chitinase"
         /translation="MKTNLFLFLIFSLLLSLSSAEQCGRQAGGALCPNGLCCSEFGWCGNTEPY
          CKQPGCQSQCTPGGTPPGPTGDLSGIISSSQFDDMLKHRNDAACPARGFYTYNAFIT
          AAKSFPGFGTTGDTATRKKEVAAFFGQTSHETTGGWATAPDGPYSWGYCFKQEQ......."
```

Fig. 7. *Arabidopsis thaliana* basic endochitinase signal peptide sequence (in red color).

4.3 Molecular biological methods

4.3.1 Agarose gel electrophoresis

4.3.1.1 6x loading buffer

50 mM EDTA
0.25 % bromophenol blue
0.25 % xylene cyanol FF
25 % Ficoll40 (type 400, Pharmacia)

4.3.1.2 TAE buffer

40 mM Tris-acetate
20 mM glacial acetic acid
1 mM EDTA
pH 7.5

4.3.1.3 Ethidium bromide EtBr (stock 10 mg/ml, Roth)

Electrophoresis is used to separate molecules (DNA and RNA) based on their size. DNA has a negative charge in solution, so it will migrate to the positive pole in an electric field. In agarose gel electrophoresis, the DNA is forced to move through a sieve of molecular

pores made by agarose. Large fragments of DNA move slower than small fragments of DNA. So the concentration of the gel depends on the fragment lengths to be separated.
0.8-1 % (w/v) agarose gel was prepared in 1x TAE buffer, where it melts in a microwave oven until the agarose was totally dissolved. Then the agarose solution was cooled down until it reached 50 °C and ethidium bromide (0.5 µg/ml) was added and the solution was casted into a gel mold to solidify. A suitable comb was used to make slots. The gel was transferred to the electrophoresis chamber containing running buffer (1x TAE buffer). Samples were mixed with 6x loading buffer and loaded together with molecular weight marker onto the wells for electrophoresis at a voltage of 60-100 V for 30-40 min after which the DNA fragments were observed and photographed under UV-light.

4.3.2 Digestion of DNA by restriction endonucleases

DNA was digested using different restriction endonucleases with respective buffers as recommended by the supplier. When two enzymes had to be used for digest, the buffer was selected to be suitable for both enzymes; otherwise it was done one after the other. Digestion was done at 37 °C for 2 h or overnight, and then enzymes were heat-inactivated for 15-20 min at 65 or 85 °C, depending on the enzyme.

4.3.3 Purification of PCR product and DNA fragments

4.3.3.1 Purification of PCR product (Invitek)

130 µl buffer P were added to the PCR product, mixed and transferred into a spin filter and incubated for 1 min at room temperature, then centrifuged for 30 sec. at 10000 rpm. The spin filter was washed with 700 µl washing buffer and centrifuged for 30 sec., followed by 3 min centrifugation to remove the ethanol residual. Finally, the DNA was eluted using elution buffer and centrifugation for 1 min.

4.3.3.2 Easy Pure® DNA purification from agarose gel (Biozyme)

Agarose gel band slice weight was determined in a 1.5 or 2.0 ml tube, 3 volumes of SALT-Solution were added and mixed well and incubated for 5 min at 55 °C to melt the agarose with occasionally mixing by shaking thoroughly, then 5 µl plus 1 µl of DNA BIND-Solution per µg DNA was added and incubated for 5 minutes at room temperature, then centrifuged for 30 seconds in a lab centrifuge. The pellet was re-suspended in 1 ml of WASH-Solution by vortexing for 5-10 seconds, then again centrifuged for 30 seconds. The pellet was re-suspended in water or TE buffer (10mM Tris, 0.1mM EDTA). Samples

were incubated for 5 min at room temperature and centrifuged for 1 min, and then the supernatant was removed immediately and transferred to a new tube.

4.3.4 Dephosphorylation of 5'-ends of digested vector DNA

Shrimp alkaline phosphatase (SAP) was used for dephosphorylation of the 5'-ends of the digested vector to prevent re-ligation of the vector with the excised fragment as adapter. Dephosphorylation was done according to the manufacturer's protocol at 37 °C for 1 h., and then the enzyme was heat-inactivated at 65 °C for 15 min.

4.3.5 Ligation

DNA ligases catalyze the phosphodiester binding between a free 5'-phosphate group and a free 3'-hydroxyl group of the same strands of a dsDNA. Intramolecular ligation results in a circularization of the DNA molecule. If an insertion is planned, self-circularization and oligomerization has to be prevented by dephosphorylation or eluting the fragment from the gel. Ligation of cohesive ends and the vector was done at a molar ratio of 3:1 in 5x ligation buffer, so 150 ng insert and 50 ng vector were mixed and 2 U of T4 DNA ligase were added. The reaction was incubated at 22 °C overnight, and then the ligase was heat-inactivated at 65 °C for 15 min. Afterwards the ligation product was monitored by running on a gel to check the efficiency of ligation and then used for *E.coli* transformation (4.2.3).

4.3.6 DNA preparation

4.3.6.1 Isolation of g-DNA from plant tissue by CTAB-based extraction method (Doyle and Doyle 1990)

Genomic DNA isolation is one of the basic requirements for the characterization of transgenic plants. The purity and the amount of isolated DNA are important for the detection of the transgene. In the present work, total genomic DNA was isolated according to the CTAB method of Doyle and Doyle (1990). First, for the PCR screening, a small (100-200 mg leaf material) scale DNA isolation was performed and in the connection with that large scale (1-2 g leaf materials) DNA isolation was carried out. Both protocols are shown below.

Buffers and Solutions

CTAB-buffer

3 % CTAB (added after autoclaving and stirred overnight)

1.4 M NaCl

0.2 % β-Mercaptoethanol (added directly before using)
20 mM EDTA
100 mM Tris-HCl pH 8.0 (base)
0.5 % PVP-40 polyvinyl pyrolidone (soluble)

24:1 CI-Mix

23 part Chloroform
1 part Isoamylalcohol

TE-buffer + RNAse A

10 mM Tris-HCl, pH 8.0
1 mM EDTA
10 µg/ml RNAse A

Wash buffer (WB)

76 % $Ethanol_{Abs.}$
10 mM Ammonium acetate

7.5 M NH_4-Acetate

0.5 M EDTA (pH 8)

RNAse A

10 µg/µl Stock sol. in ddH_2O

4.3.6.1.1 Mini-Isolation of genomic DNA (gDNA) for PCR

150-200 mg leaf material (either already frozen or fresh from greenhouse) was harvested in liquid nitrogen. The leaves crumbled to powder using pre-cold mortar and pistils and transferred to 2 ml reaction tubes where 800 µl of preheated (60 °C) CTAB-buffer was added followed by vigorous vortexing under a fume hood and incubation for 30 min at 60 °C. After that, 800 µl CI-Mix was added and tubes were gently mixed to avoid shearing of genomic DNA by inverting the tube for 4-5 times. Centrifugation was done at room temperature for 10 min at 10000 xg and the aqueous phase (800 µl) was transferred into a fresh 1.5 ml tube (to obtain a clear sample, the step was repeated). 2/3 volume (550 µl) of pre-cooled (-20 °C) isopropanol was added and gently mixed to allow precipitation of gDNA. gDNA was pelleted by centrifugation for 10 min (full-speed), the supernatant was discarded and the pellet was washed in 200 µl WB until the pellet floats, then the washing-buffer was carefully removed and the pellet was re-suspended in 200 µl TE buffer supplemented with RNAse A and incubated for 30 min at 37 °C. Afterwards, 100 µl 7.5 M NH_4-acetate and 750 µl $EtOH_{abs.}$ was added and gently mixed, centrifuged (full-speed) for 10 min at room-temperature. Supernatant was completely discarded and the pellet dried for 40-50 min at 37 °C, then re-suspended in 100-250 µl dd H_2O or 100 µl TE buffer (for better solving and storing) and kept overnight at 4 °C to allow complete dissolving.

4.3.6.1.2 Max-Isolation of genomic DNA for Southern blot

1-2 g of leaf material (either already frozen or fresh from the greenhouse) were harvested in liquid nitrogen, then transferred into pre-cooled mortar and pulverized. The resulting

powder was transferred to a 50 ml tube, and 3-5 ml of preheated (60 °C) CTAB-buffer was added with vortex under the fume hood, and incubated for 30 min at 60 °C. and then 1 vol. of CI-Mix (3-5 ml) was added and gently mixed by inverting the tubes to avoid shearing of genomic DNA.

Centrifugation was done for 10 min (6400 rpm) at room-temperature and the aqueous clear phase was transferred into a fresh tube (3-5 ml); for precipitation of the gDNA, 2/3 volume of pre cooled (-20 °C) isopropanol (2-3 ml) was added and gently mixed, until when the nucleic acids began to precipitate. Precipitated DNA was collected by centrifugation for 10 min at 4000 rpm at RT; the resulting pellet was washed with 1-2 ml WB until pellet floats.

The washing-buffer was carefully removed and the pellet was re-suspended in 0.5-1 ml TE buffer supplemented with RNAse A and incubated for 30 min at 37 °C. 1/2 vol 7.5 M NH_4-acetate and 2.5 vol. $EtOH_{abs.}$ were added and gently mixed then, centrifuged for 10 min at 4600 rpm at room-temperature. The supernatant was completely discarded and the pellet dried at 37 °C for 60 min, re-suspended in 200-400 µl TE buffer at 4 °C overnight to allow dissolving the gDNA. Samples were heated for 5 min at 60-65 °C before checking on agarose gel.

4.3.6.2 Leaf disk PCR

A quick method for fast transgenes analysis by PCR was used. Although the DNA quality was much lower than the CTAB method but it was enough to perform PCR in comparably short time. The method was reproducible and the suitability of resulting DNA for PCR was checked using HMG gene (see 4.3.6.4) and chitinase gene primers together in PCR mixture, resulting in two different bands at 570 bp and 755 bp corresponding to HMG and chitinase genes respectively (Fig. 8).

Extraction buffer:

200 mM Tris-HCl pH 7.5
250 mM NaCl
25 mM EDTA
0.5 % SDS

Procedure:

One leaf was harvested in liquid nitrogen and then it was macerated in Eppendorf tube at RT. 400 µl extraction buffer was added and vortexed for 5-20 sec. Tubes were incubated at RT until finishing all samples, then samples were centrifuged for 1 min at 13000 rpm.

300 µl of supernatants were transferred to new tube and 300 µl isopropanol was added. Samples were mixed well and incubated for 2 min at RT. And then DNA was pelleted by centrifugation for 5 min at 13000 rpm. Pellets were dried and then after dissolved in 100 µl TE buffer and 3-4 µl was used for PCR.

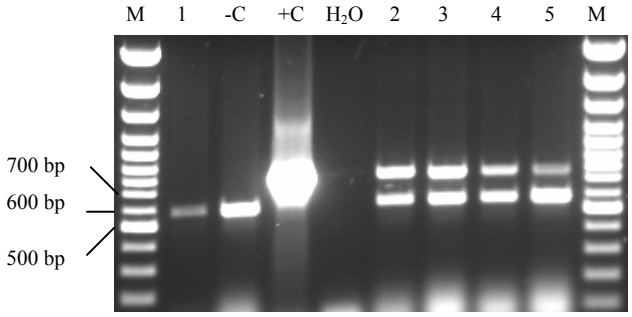


Fig. 8. Leaf disk PCR result for quick screening of transgenes using two different primers set in the same PCR reaction mix one for chitinase gene and the second for HMG gene. Lane 1, non-transformed plant, Lanes 2, 3, 4, and 5 different transformed pea plants showing 2 bands; -C, non-transformed negative control pea showing one band only corresponding to HMG gene; +C, plasmid DNA used as positive control showing one band corresponding to chitinase gene; M, 100 bp DNA ladder molecular weight marker.

4.3.6.3 Mini-preparation of plasmid DNA (modified after Birnboim and Doly 1979)

4.3.6.3.1 Buffers and Solutions for Plasmid Isolation

Sol. A. 15 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM Glucose, 2 mg/ml fresh lysozyme.

Sol. B. 0.2 M NaOH, 1 % SDS.

Sol. C. 3 M NaOAc, pH 4.8.

Sol. D. 0.1 M NaOAc, pH 7.0, 0.05 M Tris-HCl pH 8.0.

4.3.6.3.2 Procedure

2ml of bacteria suspension were centrifuged at 12000 rpm for 5 min and the supernatant was quantitatively removed. The step was repeated using 1 ml of bacteria suspension. The pellet was carefully re-suspended in 200 µl of sol. A, and incubated for 15 min at RT. Then 400 µl of sol. B and 300 µl of sol. C were added and mixed gently, followed by incubation for 15 min on ice. The mixture was centrifuged twice for 10 min and the clear supernatant (800 µl) was transferred into a new 1.5 ml Eppendorf-cap's and spin down for another 10 min. Then 600 µl cold isopropanol (-20 °C) were added and gently mixed till the DNA started precipitating. Centrifugation was done for 10 min and the supernatant was quantitatively discarded. The DNA pellet was re-dissolved in 200 µl of sol. D, and incubated for 5 min at RT. Then 400 µl $EtOH_{abs.}$ was added and mixed, centrifuged for 10 min. Then the pellet was washed in 200 µl 70 % EtOH, then centrifuged again for 10 min. The pellet was dried for 30-60 min at RT. The pellet (plasmid DNA) was dissolved in 20-

50 µl of sterile deionized H_2O + 1 µl RNaseA (1 mg/ml) or 50 µl TE buffer + 1 µl RNaseA and, the DNA quantity (10-20 µg for *E. coli*) was estimated.

4.3.6.4 DNA quality measurement

The DNA measurement using a spectrophotometer is based on the fact that OD at 260 nm is twice that at 280 nm if the solution contains pure DNA. The absorbance (*A*) of the DNA preparations was determined at 260 nm and 280 nm where *A260* = 1 is equivalent to about 50 µg / ml double-stranded DNA. The quotient *A260* / *A280* gives the level of DNA purity. If there is contaminating protein the OD ratio between 260 and 280 nm decreases. Pure DNA has an OD-260/OD280 between 1.8 and 2.0. If this quotient is below 1.8, it indicates a contamination.

To check the suitability of isolated genomic pea DNA for PCR, a single-copy gene encoding high-mobility group protein (HMG-I/Y, NCBI accession No. X99373) primers were used to amplify 570 bp fragment of HMG gene (Fig. 9A). These primers also used together with chitinase primers resulting of 2 different bands at 570 bp and 755 bp corresponding to HMG and chitinase genes subsequently.

In addition to spectrophotometer, DNA concentration was also estimated in agarose gels. Sample DNA was applied and in parallel fish sperm DNA dilutions (stock 10 mg/ml) was also applied in order to enable an estimation of the DNA-quantity in the gel. This method proved to be a quite dependable method with regard to uniformly loaded DNA digested with any restriction enzyme for Southern blot (Fig. 9B).

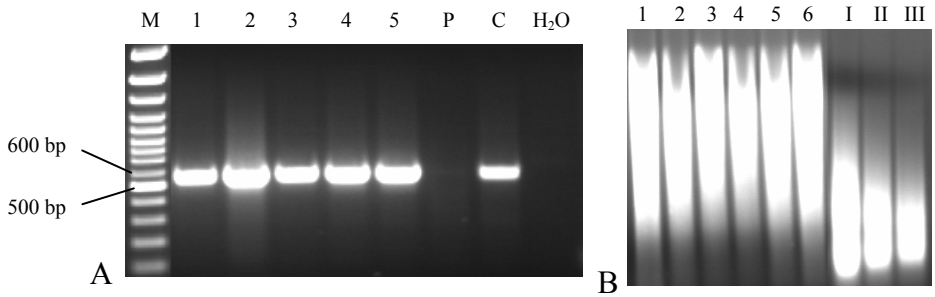


Fig. 9. A, PCR of pea gDNA using the house keeping gene (HMG) to control the quality of isolated DNA; lanes 1 to 5 different transgenics; P, plasmid DNA; C, gDNA of non-transformed pea; M, 100 bp DNA ladder molecular weight marker; B, Control Southern blot gel; lanes 1-6 different samples of g-DNA digested with *Eco*RI and separated on 0.8 % agarose gel; lanes I, II, and III are 20 µg, 10 µg and 5 µg fish sperm DNA, respectively.

4.3.6.5 PCR, colony PCR

The process of Polymerase Chain Reaction (PCR) was first described by Mullis et al. (1986). PCR is a relatively simple process by which virtually unlimited copies of selected

DNA fragments using known sequence fragment (primers) can be generated and amplified *in vitro* in a short period.

Primers are short oligonucleotides (typically 18-22 bases in length) that are necessary to start the extension reaction in a specific manner. The reaction is carried out by a heat-stable Taq-DNA polymerase from thermophilic bacteria like *Thermus aquaticus* (Foolad et al., 1995).

4.3.6.5.1 PCR reaction mixture:

Compound and concentration	**Amount per reaction**
Double distilled water	18.3 µl
10x PCR buffer with 50 mM $MgCl_2$	2.5 µl
5 mM nucleotides mixture (dNTPs)	1 µl
10 pmole forward primer	1 µl
10 pmole reverse primer	1 µl
20-50 ng template DNA (plasmid- or gDNA)	1 µl
1-2 U Taq DNA polymerase*	0.2 µl
Total volume	25 µl

* BioTherm Red Taq (10 U/µl) from Natutec.

4.3.6.5.2 PCR program:

PCR step	**Temperature (°C)**	**Time (s)**	**No. of cycles**
Initial denaturation	94	300	1
Denaturation	94	30	30
Annealing	Specific for primer	30	
Extension	72	30	
Final extension	72	300	1
Cooling down	4	∞	

Colony PCR was used during cloning work as a rapid screening method for positive colonies, where the same PCR reaction mixture was used and a few cells from a single colony were picked using sterile pipette tips and mixed with PCR reaction mixture in PCR caps.

4.3.7 RT-PCR (Reverse Transcription-Polymerase Chain Reaction)

RT-PCR was applied to study the transcription of the introduced genes. Total RNA was isolated from transformed and non-transformed plants and the cDNA was synthesized by reverse transcriptase (MMLV-RT). Then normal PCR was performed with the cDNA as template. In the case of the 35S promoter, total RNA was isolated directly from leaf explants, whereas plants with the vst promoter required induction with UV light (254 nm) for 5 min then explants were incubated overnight in growth room.

4.3.7.1 Isolation of RNA

RNA was isolated from young leaves using NucleoSpin RNA plant kit (Machery-Nagel, Germany) following the manufacturer's instructions or using Plant RNA Reagent (Invitrogen, Canada) using the following protocol:

100 mg plant material was harvested in liquid nitrogen and pulverized using cold mortar and pestle. The plant powder was transferred to 1.5 ml caps and 500 µl cooled (4 °C) Plant RNA Reagent was added and mixed by vortexing. The mixture was incubated for 5 min at room-temperature then centrifuged for 2 min at 12000 rpm at RT. Supernatants were transferred to fresh tubes, 100 µl 5 M NaCl was added and mixed by inverting the tubes. Finally, 300 µl chloroform was added and mixed by inverting the tubes, followed by centrifugation for 10 min at 12000 rpm at 4 °C. The upper phase was transferred to a fresh tube (~ 500 µl) and the same volume of isopropanol was added and mixed by inverting the tubes. A pellet was formed by centrifugation for 10 min at 12000 rpm at 4 °C. The supernatant was discarded and the pellet was washed with 1000 µl Ethanol $_{abs.}$ Followed by centrifugation for 1 min at full speed, the supernatant was discarded quantitatively using a pipette. Pellet was redissolved in 30 µl RNAse free water (DEPC-water) and the RNA concentration was measured by spectrophotometer.

4.3.7.2 Measuring RNA concentration

RNA was diluted 1:200 (199 µl H_2O + 1 µl RNA), then the RNA concentration was measured using a spectrophotometer. The respective RNA-concentration was calculated as follows:

$$\text{RNA concentration } \mu g/ml = (OD260 \times \text{Dilution factor} \times 40)$$

The purity of the RNA was determined using the ratio of OD260:OD280, which should be between 1.9 and 2.0 for pure RNA.

4.3.7.3 cDNA synthesis (reverse transcriptase)

5 µg total RNA was used for synthesizing cDNA in a final volume of 12 µl RNAse free water. 1 µl oligo dT primer (18 mers) was added and the mixture was incubated for 10 min at 70 °C, followed by a reverse transcriptase reaction (MBI Fermentas, Germany) prepared on ice as follows:

4 µl RT 5x buffer
1 µl RNAse inhibitor (40 U/µl)
2 µl dNTPs (5mM)

The mixture was incubated for 5 min at 37 °C in a thermo block, and then 1 µl M-MuLV Reverse Transcriptase polymerase (200 U/µl) was added and incubated for 1 hour at 42 °C followed by 10 min inactivation at 70 °C. cDNA was used directly or stored at -20 °C while RNA was stored at -80 °C.

4.3.7.4 Quantification of RNA using agarose gel electrophoresis in MOPS buffer

Agarose gel was used to quantify the isolated RNA. 5 µl RNA were mixed with 5 µl of sample buffer and incubated for 15 min at 65 °C, then 2 µl of loading buffer was added and RNA was separated by electrophoresis for 30 min at 100 v.

Sample buffer:

100 µl formamid
38 µl formaldehyde
20 µl 10x MOPS buffer
42 µl DEPC H_2O

Loading buffer:

100 µl 6x loading buffer
1 µl EtBr (10 mg/ml)

Running buffer:

1x MOPS buffer in DEPC H_2O

DEPC H_2O

0.01 % DEPC in water, autoclaved and stirred overnight at RT.

Agarose gel:

0.36 g agarose
21 ml DEPC H_2O
boil to solve agarose then cool down to 60 °C and add
6 ml formaldehyde
3 ml 10x MOPS buffer

10x MOPS buffer

200 mM MOPS
80 mM Na-acetate
10 mM EDTA
pH 7.0

4.3.8 Southern blot using non-radioactive probe

The blot was performed according to Southern (1975) to confirm integration patterns of T-DNA and to determine the copy number of the integrated transgenes using the genomic DNA of transformed plants. Genomic DNA was prepared from transgenic and non-

transgenic plants by large scale DNA preparation. Non radioactive detection methods were used and DIG labelled PCR products for the different genes were prepared as probe (as described below).

4.3.8.1 Buffers and solutions

Pre-hybridization solution: Dig Easy Hyb. (Roche Diagnostics, Mannheim, Germany)

Hybridization solution: 45 µl probe + 33 ml Dig Easy Hyb.

Blocking Solution: 1 % blocking solution (Roche) in maleic acid buffer.

Antibody solution (Anti-Digoxigenin-alkaline phosphatase conjugate Fab Fragments) (Roche Diagnostics) 1:20000 in blocking solution.

Depurinizing solution

0.25 M HCl

Neutralization Solution pH 7.5

0.5 M Tris-HCl

3 M NaCl

10 % SDS (Filter sterilized)

Maleic acid buffer pH 7.5 (autoclaved)

0.1 M maleic acid

0.15 M NaCl

Detection buffer pH 9.5

100 mM Tris-HCl

100 mM NaCl

DEA buffer pH 9.8

0.1 M DEA

1m M $MgCl_2$

Denaturation Solution

0.5 N NaOH

1.5 M NaCl

20x SSC buffer pH 7

3 M NaCl

0.3 M sodium citrate

Washing buffer

Maleic acid buffer

0.3 % Tween 20 (do not autoclave)

Stripping buffer

0.2 M NaOH

0.1 % SDS

Developing solution

1: 3.5 dil. of Roentogen developer (Tetental Photowerk GmbH, Norderstedt, Germany)

Fixation solution

1:4 dilution of Roentogen Superfix (Tetental Photowerk GmbH, Norderstedt, Germany).

4.3.8.2 DIG labeling probe preparation by PCR

4.3.8.2.1 PCR mixture

Compound and concentration	Probe	Control
Double distilled water	35.75 µl	18.3 µl
10X buffer with 50 mM $MgCl_2$	5 µl	2.5 µl
5 mM dNTPs	0.25 µl	1 µl
10 pmol For. Primer	2 µl	1 µl
10 pmol Rev. primer	2 µl	1 µl
Plasmid DNA	1 µl	1 µl
DIG labeling mix	2 µl	-
Taq polymerase (10 U/µl)*	0.5 µl	0.2 µl
Total volume	50 µl	25 µl

* Either from BioTherm (Natutec) or CombiZyme (4 U/µl, Invitek GmbH)

Different probes were prepared to detect the gene of interest (chitinase), selectable marker gene (*bar*) and backbone sequence (*npt*I gene).

4.3.8.2.2 PCR program

PCR step	Temperature (°C)	Time (s)	No. of cycles
Initial denaturation	94	300	1
Denaturation	94	60	39
Annealing	Specific for primer	90 *AS* 1 sec.	
Extension	72	90	
Final extension	72	300	1
Cooling down	4	∞	

The quality of the probes was controlled by running 5 µl on a 1 % agarose gel and compared with the control sample (25 µl). The probe should be a little heavier than control due to incorporation of the Dig label as shown in Fig. 10.

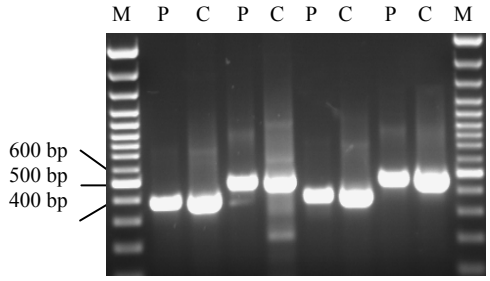


Fig. 10. 1 % agarose gel to control and check the quality of probes prepared with different primer combinations; P, for probe; C, for control without Dig labeling; M, 100 bp DNA ladder molecular weight marker.

4.3.9 Restriction digest of gDNA for Southern blot

About 20-30 µg of gDNA was digested by *Eco*RI or *Xba*I in the respective buffer at 37 °C overnight. Another amount of the enzyme was added and incubated for further 3-4 h. to ensure complete digest, followed by heat inactivation for 10-15 min at 65 °C.

4.3.9.1 Precipitation of the digest

In order to precipitate the digest, 1 volume of 7.5 M NH_4-acetate (100 µl) and 7.5 vol. $EtOH_{abs.}$ (750 µl) were added and gently mixed, followed by centrifugation at full-speed (13000 rpm) in a lab centrifuge for 10 min at room-temperature. Supernatants were discarded completely and the pellet was re-dissolved in 100µl TE buffer, then the digest was precipitated by adding 100 µl $EtOH_{abs}$ to remove salts. Ethanol was removed by a centrifugation step, then the pellet was dried for 1 h. at 37 °C and finally re-dissolved in 40 µl TE buffer at 4 °C overnight.

4.3.9.2 Electrophoresis

8 µl of 6x loading buffer were added to the restriction digest (40 µl), mixed and briefly centrifuged. Then samples and DIG-labelled-DNA Molecular Weight Marker II (Roche) were loaded on a 0.8 % agarose gel containing 0.5 µg/ml EtBr in 1x TAE buffer. The gel was run overnight at 0.6 V/cm (20-30 V) and 1-2 µl positive control (PCR product or plasmid DNA) were added the next day. The gel was monitored under a UV-transilluminator and then rinsed in ddH_2O, followed by submerging in 250 ml of depurinizing sol. (0.25 M HCl) for 10 min to nicks the DNA and thereby facilitating the transfer of large fragments. After that, the gel was rinsed in ddH_2O to remove the acid followed by submerging in denaturation sol. for 2 x 15 min at RT on a shaker. The gel was rinsed in ddH_2O and then it was neutralized in neutralization sol. for 2 x 15 min at RT.

4.3.9.3 Capillary Southern-transfer (over night)

20x SSC solutions were placed in a tray where filter paper bridges were built on a glass plate, and 3 filter papers were soaked in 20x SSC solution, then placed on the top of the bridge (avoiding any air bubbles under the paper). A plastic wrap was placed in between to prevent by-pass between filter papers that were placed on top of the gel and the filter papers under the gel. A piece of positively charged nylon membrane (Roche) was first wetted in ddH_2O, then in 20X SSC and placed on top of the gel. Another 3 filter papers were soaked in 20 x SSC solution, then placed on the membrane to avoid air bubbles. Tissue papers stacks were loaded onto the filter papers and a glass plate centered on top of the paper towels. 500 ml bottle full of water was placed in the center of the glass plate to distribute the weight evenly across the gel, papers and membrane. Transfer by capillary form would take place over night. When the transfer was completed, the membrane was rinsed 3x in 2X SSC and then air dried. The membrane was either UV exposed (254 nm) for 10-15 min for covalently cross-linking the DNA to the membrane or placed between two filter papers for 30 min at 120 °C in the oven, then covered with foil and stored at RT.

4.3.9.4 Pre-hybridization and hybridization

The dry blot was placed in an autoclaved hybridization tube and 20 ml of pre-hybridization solution was added and incubated for 3 h at 42 °C, and then a preheated (68 °C) probe was added and incubated overnight at 42 °C.

The membrane was washed as follow: 2X5 min in 2 X SSC + 0.1 % SDS at 42°C, then 1X15 min in preheated (65 °C) 0.5 X SSC + 0.1 % SDS at 65 °C followed by 1X15 min in 0.1 X SSC + 0.1 % SDS at 65 °C, 1 min in maleic acid buffer at RT, then the membrane incubated in blocking solution for 30 min followed by incubation with antibody solution for 30 min.

Afterwards the blot was rinsed in washing buffer for 2X 15 min at RT. and equilibrated for 2 min in detection buffer.

4.3.9.5 Non-radioactive detection

The substrate was prepared by mixing 5 µl CDP star (Roche) and 500 µl DEA buffer and the mixture was dropped by pipetting on a warp foil. The membrane was removed from the detection buffer and transferred immediately onto the substrate and incubated for 5 min at RT. The excess substrate was removed and Biomax-Luminescence-film (Kodak) was laid on the membrane and incubated for 30-60 min and then it was developed.

4.3.9.6 Stripping of the membrane

After usage, the membrane can be stored in 2x SSC buffer for a second hybridization. The membrane was rinsed in sterile H_2O and incubated twice for 15 min in stripping buffer at 37 °C in the hybridization tube followed by rinsing in ddH_2O. The membrane could be stored in 2x SSC buffer without SDS at 4 °C.

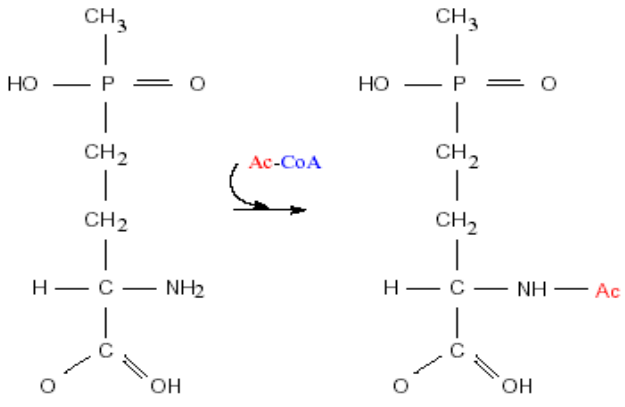
4.3.10 Leaf paint analysis

In the constructs used for transformation (Fig.1) the *bar* gene was used as selectable marker gene. It encodes the enzyme phosphinothricin acetyltransferase (PAT), isolated from *Streptomyces hygroscopicus*. It is analogous to *pat* gene isolated from *S. viridochromogenes* (Murakami et al., 1986; Thompson et al., 1987; Strauch et al., 1988), which confers resistance to bialaphos and the related compounds phosphinothricin (PPT), the active ingredient of herbicide BASTA® , Liberty® and glufosinate ammonium.

Phosphinotricin inhibits Glutamine Synthetase (GS), the enzyme that incorporates NH3 into amino acids. When glutamine synthetase is blocked, the plants run out of amino acids and pH of the cell rises causing the plant/tissue death due to accumulation of NH_3.

Transgenic plants expressing *bar* gene confers resistance to BASTA® through PAT enzyme by covalently linking an acetyl group to PPT to inactive and detoxified compound of acetyl-PPT (De Block et al., 1987; Murakami et al., 1986) (Fig. 11).

BASTA® is non-selective herbicide with no residual activity and has been regarded as environmentally safe (Nap and Metz, 1996) and the bar gene offer an efficient cheap selection system since all plants not containing or expressing bar will die.



PPT (MW: 181.13) acetyl-PPT (MW: 224.17)

Fig. 11. Detoxification and inactivation of PPT by acetylation. (Droege et al., 1992).

BASTA® (Aventis GmbH, Germany) at a dilution of 600 mg/l (stock 200 g/l) was sprayed on germinating tobacco seeds to select transformants. It was also applied to the upper surface of one marked tobacco leaf or to one leaflet of each pea leaf pair using a small paintbrush while the opposite leaflet was not treated and left as control. Transgenic plants and control plants were treated in the same way, and BASTA® effect was controlled after one week.

4.3.11 DNA sequencing and sequencing results

DNA (plasmid DNA and cDNA) was sequenced using different primers by MWG Biotech Company (Ebersberg, Germany). The sequencing results were compared with the original sequence of chitinase 30 using AlignX and ContigExpress options of Vector NTI software (Invitrogen Corporation) and Blast from NCBI website.

4.4 Biochemical and biotechnological methods

4.4.1 Protein extraction from *E. coli*

4.4.1.1 Induction of E. coli containing expression vector PUC19-Chit30

A single colony from a LB plate supplemented with 100 mg/l ampicillin and 1 M glucose was grown in LB liquid medium overnight at 37 °C. 2 ml from an overnight seed culture were centrifuged for 10 min at 8000 rpm. Then the pellet was washed with LB liquid medium and centrifuged once again and the pellet was resuspended in 5 ml liquid LB supplemented with antibiotic (ampicillin) for two different treatments: one part was supplemented with 0.5 % (w/v) glucose for suppression, while the other part was supplemented with 1 mM IPTG for induction.

4.4.1.2 Extraction

The bacteria were grown for 3 hours at 37 °C, centrifuged for 10 min at 8000 rpm and 4 °C. The cells were washed twice with 2 ml TE-PMSF buffer (1 mM EDTA, 0.1 M Tris, 14 µM PMSF, pH 7.5), then the pellet was re-suspended in 3 ml TE-PMSF buffer and disrupted using an ultrasonic cell disrupter equipped with a microprobe for 5 x15 sec. with cooling on ice. The cell lysate was centrifuged for 30 min, 15300 rpm, 4 °C. The supernatant was collected and the protein concentration was determined with Amersham Quant kit according to the manufacturer's manual. Aliquots of 100 µg were dried using a SpeedVac and stored at - 20 °C until use. Prior to separation in the first dimension (IEF)

the proteins were resuspended in 50 µl lysis buffer and vortexed for 5 min at RT. Samples were cleared by centrifugation twice for 30 min, 17000 g at 18 °C, then the clear supernatant was stored at -80 °C.

4.4.2 Extraction of proteins from plant tissue

The same treatment was used as in the case of isolation of RNA, where the plants with 35S promoter were used directly for extraction of total proteins, while the plants with the *vst* promoter needed to be induced with UV light (254 nm) for 5 min and then explants were incubated in growth room for 24-48 h.

4.4.2.1 Extraction buffer

4.4.2.1.1 For crude extract

100 mM Na-acetate pH 5, or 25 mM Na-acetate with 1 M NaCl pH 5.

4.4.2.1.2 For 2-D gel (Usuda and Shimogawara, 1995)

50 mM Tris-HCl pH 8.3
5 mM EDTA
100 mM KCl
50 mM DTT
700 mM sucrose
5 % PVPP
1 tablet protease inhibitor cocktail (Roche)
Adjust the volume to 10 ml with Millipore water.

Lysis buffer

7 M Urea
2 M Thiourea
4 % CHAPS
0.8 % IPG buffer pI 3-10 or 4-7 according to the strips used.
1 % DTT

1 % Bromophenol Blue

100 mg Bromophenol Blue
50 mM Tris-base
Adjust to 10 ml with Millipore water.

4.4.2.2 Crude extract of protein

Fresh leaves were harvested and grounded to a fine powder in liquid nitrogen with a mortar and pestle. The powder were suspended in the extraction buffer in a ratio of 1:3 and mixed by vortexing. The samples were incubated for 2 h at 4 °C on a shaker. After centrifugation at 12000 rpm for 10 min the supernatant was transferred to a new tube and designated as crude extract.

4.4.2.3 Protein extraction from plant tissue for 2-D gel electrophoresis

About 100-150 mg of fresh tissue was ground in a pre-cooled mortar with a pestle in liquid nitrogen, then transferred to a 1.5 ml tube and 330 µl extraction buffer were added and the mixture vortexed briefly. 330 µl Tris buffered phenol was added and vortexed for 10 min at 4 °C. The samples were centrifuged for 10 min, 13000 rpm at 4 °C, then the phenolic phase was collected. Proteins were re-extracted by adding 330 µl extraction buffer, centrifuged for 10 min, and 13000 rpm at 4 °C. Then the phenolic phase was transferred into a new tube. The proteins were precipitated overnight with 5 volumes of 100 mM ammonium acetate in methanol at -20 °C. Samples were centrifuged for 60 min, 17000 xg at 4 °C. Supernatant was discarded and the pellet was rinsed with 1.5 ml rinsing solution (cold acetone /0.2 % DTT), centrifuged again for 15 min. The supernatant was discarded and 1.5 ml rinsing solution was added. Samples were left in rinsing solution for 20 min at -20 °C, then centrifuged again for 30 min at 17000 xg, and 4 °C. The supernatant was discarded and the pellet was dried under vacuum for 5 min. The pellet was suspended in 50 µl lysis buffer and vortexed for 5 min at RT. Samples were cleared by centrifugation twice for 30 min, 17000 xg at 18 °C, then the clear supernatant was stored in aliquots at -80 °C. Samples were quantified using Amersham 2D Quant kit.

4.4.2.4 Extraction of proteins from apoplast of tobacco plants/centrifugation technique

According to Hogue and Asselin (1987) with slight modifications, the leaves were harvested and submerged in PBS buffer in a plastic box, and covered with a metal sieve. Infiltration in PBS buffer was done for 4 min, and then leaves were dried between tissue papers and inserted into home-made holder as illustrated in Fig. 12. Centrifugation was done for 5 min at 100 xg in an HS-4 rotor (Sorvall) to remove the excess liquid. Subsequently, the samples were collected by centrifugation at 4 °C for 20 min at 700 xg. The obtained apoplastic washing fluid was stored at -20 °C.

Fig. 12. Home-made holder for apoplastic fluid isolation from leaves; A, holder without plant leaves; B, holder with plant leaves before fixing; C, fixed plant leaves within the holder; D, holder with plant leaves, ready for centrifugation.

4.4.3 Determination of protein content

4.4.3.1 Method according to Bradford (1976)

4.4.3.1.1 Equipment

Spectrophotometer (595 nm), plastic cuvettes, vortex, pipettes, falcon tubes.

4.4.3.1.2 Reagents

Coomassie Brilliant Blue G250 (Serva), Bovine serum albumin (BSA) stock 20 mg/ml (MBI Fermentas), 98 % ethanol, 85 % phosphoric acid.

4.4.3.1.3 Bradford stock solution

100 mg CBR G250

50 ml 98 % ethanol

100 ml 85 % phosphoric acid

4.4.3.1.4 Bradford working solution

15 % (v/v) of stock solution in distilled water.

4.4.3.1.5 Assay

A standard curve was prepared using BSA at different concentrations of 0, 10, 20, 50, 75, 100, and 150 µg/ml in 100 mM Na-acetate buffer. 100 µl from different concentrations

was mixed by vortex with 4.9 ml of working solution in falcon tubes and incubated for 5 min at RT. Absorbance was measured at 595 nm and the standard curve was drawn. Protein samples were diluted 1:100 in 100 mM Na-acetate buffer, then 100 µl from the diluted samples were mixed with 4.9 ml working solution, vortexed, and incubated for 5 min at RT, and then the protein content was measured at A_{595} and calculated.

4.4.3.2 Method according to Amersham

Quantification was done using Amersham Quant kit according to the manufacturer's manual against BSA standard.

The measurement was done in Tecan multiplate reader (Amersham) at 492 nm. The optimal protein concentration for IEF focusing using 7 cm IPG strips was adjusted to 2-4 µg for silver staining and 10-60 µg for Coomassie staining in 125 µl rehydration buffer, whereas using 24 cm strips the protein concentration was adjusted to 20-40 µg for silver staining and 100-600 µg for Coomassie staining in 450 µl rehydration buffer.

4.4.3.3 Absorbance at 280 nm (A_{280})

A rapid method was used to determine whether samples contain protein was applied (Wetlaufer, 1962). The correction for protein concentration can be done according to Schleif and Wensik (1981) when nucleic acid is present where the absorbance was measured at 280 nm and 260 nm, and then the protein content was calculated using the following formula:

$$\text{Protein (mg/ml)} = 1.55\ A_{280} - 0.76\ A_{260}$$

4.4.4 Preparation of substrate for *in-gel* assay (Molano et al. 1979)

Glycol chitin was prepared by acetylation of glycol chitosan, where 1g of glycol chitosan (G-7753, Sigma, USA) was dissolved in 20 ml of 10 % acetic acid and the viscous solution was mixed overnight at room temperature, then 90 ml methanol were added and mixed continuously. The solution was filtered through Whatman filter paper (No.3) under vacuum. The resulting filtrate was mixed with 1.5 ml acetate anhydride. The formed gel was kept at RT for 30 min, the excessive liquid was poured off, and the gel was cut into small slices and homogenized by homogenizer. The homogenate was centrifuged at RT at 27000 xg for 15 min; the pellet was re-suspended in 100 ml of double distilled water so that the final concentration of this stock is 1 %.

4.4.5 Gel diffusion assay for chitinase activity

This method was used to measure and quantify chitinase activity in gel following the method of Zou et al. (2002) and Velasquez and Hammerschmidt (2004). The method is based on corporation of glycol chitin as a substrate for chitinase in agarose gel, after incubation where the reaction start in the gel by catalyzing the substrate by chitinase through diffusion, then the gel is stained with Fluorescent Brightener 28 (Calcofluor White M2R) which has affinity to chitin and bind to uncatalyzed chitin only resulting in dark area on a florescent background when monitored under UV light as a result of chitinase activity.

4.4.5.1 Gel-plate preparation

1 % or 1.6 % (w/v) agarose was prepared in 100 mM Na-acetate buffer pH=5, and heated in microwave to dissolve the agarose then cooled down (50-60 °C) and 0.5 % or 1 % glycol chitin was added gradually by stirring the mixture well. 40-50 ml of the mixture was poured into plastic petri dishes at RT, and afterwards 2-mm wells were made in the solid gel using cork borer (Fig. 13).

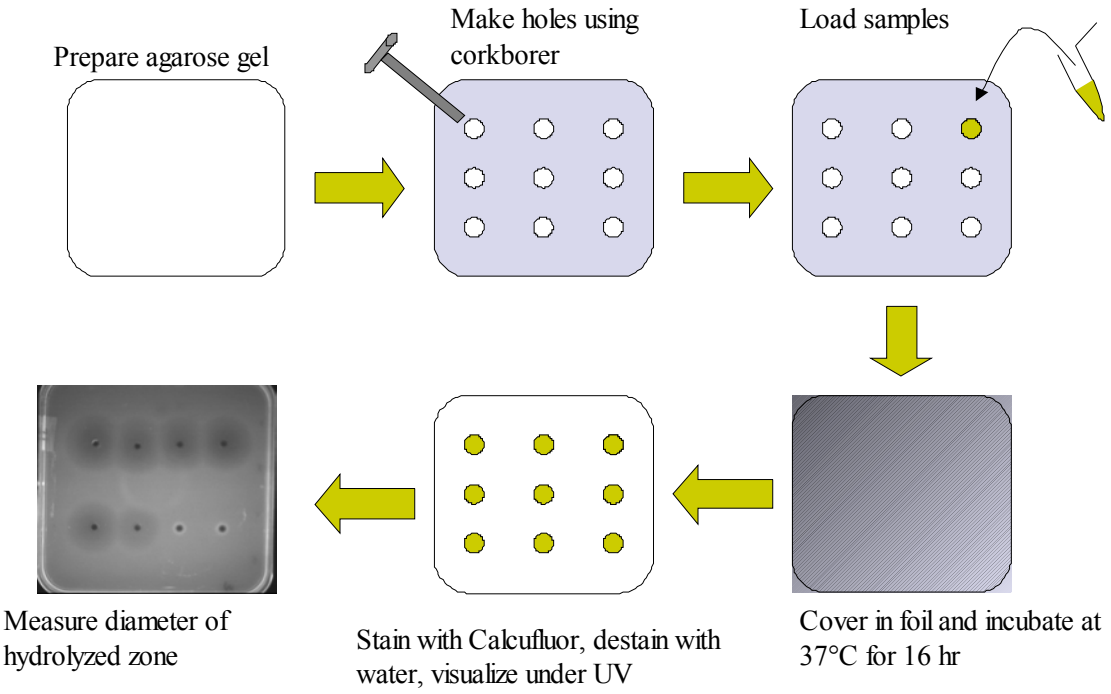


Fig. 13. Sketch for the method of agarose diffusion assay.

4.4.5.2 Assay

Standard chitinase from *Streptomyces griseus* (C-6137, Sigma Chemical Company, St. Louis, USA) was diluted in Na-acetate buffer containing 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, 0.01, and 0.001 unit. Different samples from transgenic and non-transgenic negative control plant were also prepared to contain equal amount of 10 µg total protein in a final volume of 20 µl. Then standard and samples were pipetted into wells in the gel plate followed by incubation at 28 °C or 37 °C for 16 h. or ON.

4.4.5.3 Visualization and quantification of chitinase activity

Fresh staining solution was prepared by dissolving 0.01 % (w/v) Fluorescent Brightener 28 in 500 mM Tris-HCl, pH 9 buffer, then the gel was stained for 10 min. After discarding the satining solution, the gel was de-stained in distilled water for several times to overnight at room temperature in an orbital shaker. The chitinolytic effect was visualized under UV light (254 nm) as a dark areas around wells with a bright background as a result of chitinase activity. The dark circular zones were measured using electronic digital caliper (Omnilab GmbH, Germany) and by comparing with the standard, the activity was calculated from regression equation of the hydrolyzed area diameter versus the logarithm of the standard activity.

4.4.6 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)/electro blotting

Electrophoresis of protein using SDS-PAGE was according to Reinard and Jacobsen (1995) and Laemmli (1970), using a mini-80 mm x 80 mm x 1 mm- vertical gel system (Biometra GmbH, Goettingen, Germany).

4.4.6.1 Buffers and solutions

SDS-Running buffer

25 mM Tris-HCl, pH=8.3
192 mM Glycine
0.1 % SDS

Blocking solution

5 % instant milk powder in PBST buffer.

Sample buffer

0.5 M Tris-HCl pH 6.8
10 % SDS
15 % sucrose
With or without reducing agent (DTT or 2-mercaptoethanol)
1 % Bromophenol Blue

DBD buffer

0.05 % BSA

0.025 % Tween 20

0.5 % FCS

0.01 % NaN_3 (Serva 30175)

Phosphate buffered saline-Tween (PBST)

1x PBS pH 7.4

0.05 %-0.1 % Tween 20 (Fluka 93773)

Substrate buffer pH 9.5

100 mM Tris-Hcl

0.5 mM $MgCl_2$

Washing solution Phosphate buffered saline (10X PBS, PH 7.4)

2.56 g $NaH_2PO_4xH_2O$ (Applichem A1047)

14.90 g $Na2HPO_4x2H_2O$ (Roth 4984.1) OR 22.5g $Na2HOP_4x7H_2O$

87.66 g NaCl (Roth 9265.1)

Adjust pH and add ddH2O up to 1L. then autoclave.

First antibody solution

1:1000 dilution of α-chit 30 from rabbit in DBD buffer.

Second antibody goat anti rabbit alkaline phosphatase (GARAP) IgG

1:1000 dilution of GARAP in DBD buffer.

NBT (Applichem A1243): 30 mg/ml 70 % Dimethylformamid (DMF)

BCIP (Applichem A1117): 10 mg/ml 100 % Dimethylformamid (DMF)

4.4.6.2 SDS-PAGE gel

Compounds	*12 % Resolving Gel*	*12 % Stacking Gel*
30 % acrylamide/0.8 % bis-acrylamide	2.5 ml	680 µl
1.5 M Tris-HCl, pH= 8.8	1.6 ml	-
1 M Tris-HCl, pH=6.8	-	540 µl
10 % SDS	62.5 µl	40 µl
ddH_2O	2.13 ml	2.7 ml
10 % APS (ammonium persulphate)	62.5 µl	30 µl
TEMED	2.5 µl	8 µl

Resolving gel mixture was poured in the gel mold and overlaid with ddH_2O to ensure level interface. Polymerization happened within 30-40 min, then H_2O was removed and the interior of the glass was dried with filter paper.

The stacking gel mixture was poured over the resolving gel and the comb was inserted and

allowed for polymerization. The comb was removed and excess gel was washed with dH_2O. The tank was filled with 1 x SDS-PAGE running buffer.

Samples were prepared by mixing with sample buffer (1:1) and boiled for 5 min at 100 °C, then used directly or stored at -20 °C until use. Samples were loaded onto slots and run at 10-12 mA/gel afterwards when the protein reached the resolving gel the power was increased to 14-16 mA/gel. The gel was stopped when the tracking dye reached the end (about 3 h). Then the gel was used directly for staining or blotting.

4.4.7 Protein staining after SDS-PAGE

4.4.7.1 Coomassie staining

The SDS-PAGE gel was stained in Coomassie staining (25 % methanol, 10 % acetic acid and 0.1 % Coomassie Brilliant Blue R-250) for 20 min, and then de-stained in de-staining solution (10 % acetic acid) overnight.

4.4.7.2 Silver staining according to Blum et al. 1987

The silver staining method is about 10-100 times more sensitive than various Coomassie Blue staining techniques.

4.4.7.2.1 Solution

Stop solution

500 ml methanol
120 ml acetic acid
Add 1l ddH_2O

Silver staining solution

0.5 ml of 20 % $AgNO_3$
37.5 µl 37 % formaldehyde
Add 50 ml ddH_2O

Fixation solution

50 ml stop solution
25 µl 37 % formaldehyde

Sensitizer sodium thiosulfate solution

10 mg $Na_2S_2O_3$ x $5H_2O$
Add 50 ml ddH_2O

Developing solution

3 g Na_2CO_3
25 µl 37 % formaldehyde
1 ml of Na-thiosulfate solution
Add 50 ml ddH_2O

4.4.7.2.2 Procedure

The SDS-PAGE was incubated in fixation solution for 1 h or overnight at RT, then it was submerged 3x 20 min, followed by 1 min incubation in Na-thiosulfate solution and then washed 3x 20 sec. in ddH_2O. The gel was stained in silver staining solution for 20 min, followed by 2x 20 sec. washing in ddH_2O and then developed in developing solution for

1-10 min until the bands become clear, washed 2x 2 min by ddH_2O. The reaction was stopped by incubating the gel in stopping solution for 10 min.

4.4.7.3 Fluorescent activity staining of chitinase after SDS-PAGE (Trudel and Asselin 1989 and 1990)

4.4.7.3.1 Solutions

Re-naturation buffer

0.05 M acidic HEPES and 1 % v/v Triton X-100 were dissolved in ddH_2O. The solution was stirred until Triton was completely dissolved. Then pH was adjusted to 7 with 3 M KOH.

Fluorescent staining solution

0.01 % (w/v) Fluorescent Brightener 28 (Calcofluor White M2R) (F-3397, Sigma, USA) was totally dissolved in 0.5 M Tris-HCl pH 9 buffer.

4.4.7.3.2 SDS-PAGE gel with substrate

12 % SDS-PAGE was prepared with 1 % of glycol chitin. After electrophoresis the gel was incubated in re-naturation buffer at 37 °C at 40 rpm overnight.

4.4.7.3.3 Detection

The gel was washed twice in 0.5 M Tris-HCl pH 9 buffer, followed by staining in fresh fluorescent staining solution for 2 min. Subsequently, the gel was rinsed in deionized water several times and the chitinolytic effect was visualized under UV light as dark bands with a bright background as a result of chitinase activity.

4.4.7.4 Western blot (modified Towbin et al., 1979)

Semi-dry Blotting and detection

Semi-dry blotting was done using semi-dry trans-electroblotter (BioRad, Germany) to transfer the separated protein from the SDS-PAGE gel to a membrane using transfer buffer (1x running buffer without SDS). Three pieces of filter paper were soaked in transfer buffer and placed on the anode (+), while avoiding any air bubbles, then a PVDF membrane (0.45 µM pore size, Roche) was activated for 5 min in methanol, and then put on the filter papers. The gel was laid on the membrane, and then 4 soaked filter papers were placed on top avoiding any air bubbles, the cathode (-) electrode plate was added on top.

The power was set at 0.77 limit, 20 volt and 0.8 time (around 45 min). Afterwards, the membrane was stained using Ponceau staining solution (stock 10 g Ponceau in 50 ml 30 % TCA, from this stock a 1:50 dilution was prepared) for 10 min on a shaker, then the marker was cut and the remaining part of the membrane was blocked in blocking solution for 30 min on a shaker (or stored overnight at 4 °C after adding 0.02 % NaN_3 to prevent

bacterial growth). The membrane was washed briefly then incubated for 5 min in washing solution (1x PBS), then the washing solution was removed and the first antibody solution (α-chit 30) was added for 1 h on shaker. After washing with PBS buffer the second antibody (GARAP) was added for 1 h on a shaker. Afterwards, the membrane was washed in PBS buffer then it was incubated in NBT-BCIP substrate buffer for 5 min. The membrane was incubated in substrate solution (20 ml NBT-BCIP substrate buffer + 200 µl NBT + 200 µl BCIP) for 2-20 min without shaking until color development become visible. The reaction was stopped by ddH_2O and the membrane was dried with filter paper.

4.4.8 Proteomics and 2-D gel electrophoresis

Proteomics and two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) were used to study proteins, particularly their structures and functions. This method facilitates the separation of complex mixture of proteins so that individual proteins are more easily analysed with other techniques like mass spectrometry. It is used to identify the relative mass of a protein and its isoelectric point.

In this method, proteins are separated according to charge (pI) by isoelectric focusing (IEF) in the first dimension and according to size (Mr) by SDS-PAGE in the second dimension, which can be horizontal or vertical (O'Farrell 1975). It is also used to identify the relative mass of a protein and its isoelectric point by comparing with known standards.

The aim of using this technology was to differentiate between endogenous and recombinant chitinases in transgenic plants of tobacco and pea, where the candidate spots will be identified using silver stained gels and then excised from Coomassie Blue stained gels and then analyzed using **M**atrix **A**ssisted **L**aser **D**esorption **I**onization **T**ime-**O**f-**F**light (**MALDI-TOF)** Mass Spectroscopy Analysis.

4.4.8.1 Solutions

Rehydration buffer

7 M Urea
2 M Thiourea
30 mM Tris-HCl pH 8.5
4 % CHAPS
10 % glycerol
0.002 % bromophenol blue
2 % IPG buffer pI 3-10
0.28 % DTT

Fixing solution:

40 % ethanol
10 % acetic acid

Sealing solution:

0.5 % agarose
0.002 % bromophenol blue
Dissolve in 1x running buffer

Silver staining solution:

0.625 g silver nitrate
0.1 ml formaldehyde
Fill up to 250 ml with ddH_2O

Stopping solution:

3.65 g Na-EDTAx2 H_2O
Fill up to 250 ml with ddH_2O

SDS equilibration buffer:

50 mM Tris-HCl pH 8.8
6 M Urea
30 % glycerol
2 % SDS
1 % DTT
1 % Iodoacetamide

Running buffer

250 mM Tris base
1.9 M glycine
1 % SDS

Sensitizing solution:

75 ml ethanol 95 %
1.25 ml 25 % glutaraldehyde
10 ml 5 % sodium thiosulfate
28.17 g sodium acetate
Fill up to 250 ml with ddH_2O

Developing solution:

6.25 g sodium carbonate
0.05 ml formaldehyde
Fill up to 250 ml with ddH_2O

4.4.8.2 IEF focusing (first dimension)

IPG strips (pH 4-7, 7 cm and 24 cm Immobiline^{TM} DryStrip from Amersham Biosciences AB, Sweden) were rehydrated in rehydration buffer containing the sample protein (4 µg protein for silver staining and 100 µg for Coomassie and Western blot analysis of 7 cm strips) in the reswelling tray (gel side facing the buffer), strips were covered with Drystrip cover fluid (Amersham), and incubated overnight at RT.

Rehydrated strips were rinsed with deionized water for a few seconds and slightly blotted to remove excess water. The cooling device was set to 20 °C, 15 ml Drystrip cover fluid was poured onto the cooling plate of the multiphore unit and the IEF unit was placed onto

the cooling plate avoiding air bubbles under the IEF unit. Power was connected, and 15 ml Drystrip cover fluid was poured onto the IEF unit. Then the plastic support was placed onto cover fluid in the IEF unit (avoid air bubbles). Rehydrated IPG strips were placed onto the depressions of the plastic support where the gel side was upwards and the acidic ends towards the anode. Two electrode pads (filter paper) were moistened with 150 µl deionised water and applied on the surface of IPG gel at the anodic and cathodic ends of the IPG strip. The movable electrodes were positioned above the electrode filter paper pads and gently the electrodes were pressed on the filter paper. IPG strips were overlaid with Drystrip cover fluid and IEF focusing was run according to the program below (for 7 cm IPG strips) in gradient mode:

1. 200 V	0:01 min	5 Watt	0.5 mA/strip
2. 3500 V	1:30 min	5 Watt	0.5 mA/strip
3. 3500 V	1:30 min	5 Watt	0.5 mA/strip

The total volt hours should be less than 50 kVh.

After IEF focusing the IPG strips were stored in test tubes at -80 °C until the second dimension was run.

4.4.8.3 Equilibration of IPG strip

The IPG gel strips were equilibrated twice for 15 min in 2x10 ml equilibration buffer on a shaker. The first equilibration step contained 1 % DTT, and the 2nd equilibration step contained 4.5 % iodoacetamide. The equilibrated IPG gel strips were slightly rinsed and blotted to remove excess equilibration buffer and then applied onto the second dimension SDS gel.

4.4.8.4 SDS-PAGE (second dimension)

4.4.8.4.1 Horizontal gel electrophoresis (ready-made)

SDS EcxelGel was assembled for the second dimension: It was removed from its foil package. 15 ml of cover fluid were pipetted on the cooling plate of the multiphor unit (cooling device was set at 15 °C). Protective cover was removed from the top of the EcxelGel and placed on the cooling plate, avoiding air bubbles trapped between the gel and the cooling plate. The cathodic SDS buffer strip and anodic buffer strip were placed on the gel, then the equilibrated IPG strip was placed on the surface of the EcxelGel, 1 mm apart from the cathodic buffer strip, the electrodes were positioned on the buffer strips. Following program was used:

Homogenous gel 12.5 % acrylamide:

1. 120 V	20 mA	30 W	40 min
2. 600 V	50 mA	30 W	70 min

Gradient gel 12-14 %:

1. 200 V	20 mA	20 W	40 min
2. 800 V	40 mA	40 W	2:40 h.

After the first step, the IPG strip was removed from the gel surface and the cathodic buffer strip was moved to the position where the IPG strip was placed before. Continuing to the second step at the end of the program, the gel was stained with silver nitrate.

4.4.8.4.2 Vertical gel electrophoresis

An IPG strip was placed on top of a 12 % SDS-PAGE gel and overlaid with 2 ml of 2 % hot agarose solution in running buffer. Electrophoresis was started at 100 V. and 400 mA for 15 min. Then power was increased to 180 V and 50 mA.

The electrophoresis was terminated when the Bromophenol Blue tracking dye has migrated off the lower end of the gel and then it was used for staining or blotting.

4.4.8.4.3 Silver staining after Amersham

Incubate the gel for 30 min in fixing solution (or over night)

Incubate the gel for 30 min in sensitizing solution

Wash the gel 3 x 5 min in distilled water

Incubate the gel for 20 min in silver staining solution

Wash the gel 2 x 1 min in distilled water

Incubate the gel for 2-5 min in developing solution or until seeing the spots

Incubate the gel for 30 min stopping solution

Wash the gel 3 x 5 min in distilled water

4.4.8.4.4 Storage of the gels

Horizontal gels with plastic supports were incubated at least for 20 min in 87 % glycerol diluted 1:10 by water, and then stored sealed in plastic foil at 4 °C.

Vertical gels without support were incubated twice for 30 min in 250 ml distilled water containing 75 ml ethanol (95 %) and 11.5 ml glycerol (87 %) solution, then stored sealed in plastic foil at 4 °C.

4.4.8.4.5 Coomassie Blue staining

Since the development of highly sensitive micro-sequencing techniques, it is also possible to gain N-terminal or even internal amino acid sequence information of proteins blotted onto PVDF membranes. Coomassie Blue stained spots are excised from the immobilizing PVDF membrane and directly applied into an automated protein sequencer. Usually, a

single well-stained spot yields sufficient protein (1-10 µg) to obtain an N-terminal amino acid sequence. For mass spectrometry, lower amounts of protein are sufficient.

Reagent solutions

Staining solution:

0.1 % Coomassie Blue R-250 in water/methanol/acetic acid (45/45/10). To prepare 500 ml, 500 mg Coomassie Brilliant Blue R-250 (or Serva Blue R) were dissolved in 225 ml of methanol. The resultant solution was stirred for 30-60 min, and then 225 ml of deionized water and 50 ml acetic acid was added, stirred again and filtered.

Destaining solution: 45 % methanol, 45 % deionized water and 10 % glacial acetic acid.

4.4.9 Quantitative assay of Chitinase enzyme activity using CM-chitin-RBV according to Stephan and Wolf (1990)

A solubilized, dye-labeled form of chitin, carboxymethyl-chitin-Remazol Brilliant Violet (CM-chitin-RBV, Loewe, Germany) was used as a substrate for colorimetric chitinase activity assay. Chitinase activity assay was performed according to Stephan and Wolf (1990) in triplicate, where aliquots of 200 µl CM-chitin-RBV (stock 2 mg/ml) were mixed with 100 µl 200 mM sodium acetate buffer, pH 5 and 100 µl enzyme solution and incubated for 2 hours at 37 °C. The reaction was terminated by the addition of 100 µl HCl (1.0 N) on ice and incubated for 10 min to facilitate precipitation of the non-degraded substrate, then centrifuged at 14500g for 5 min. Subsequently, 350 µl of the supernatants were transferred to the wells of a microtiter plate (350 µl cavities). The absorbance was measured spectrophotometrically at 550 nm against a blank, which was prepared similarly, but the enzyme was added onto pre-incubated substrate and stopped directly with HCl. The difference between the values (OD) of blank and samples indicated the enzyme activity.

4.5 Bioinformatic and statistical programs

Different programs and bioinformatic websites were used such as vector NTI (Invitrogen corporation, http://www.invitrogen.com), NCBI-Blast (http://www.ncbi.org), EXPASy serve (http://www.expasy.org), BIOINFX (http://studwww.ugent.be/~mdgroeve/bioinfx/) and *Sig-Pred* program from the bioinformatics group of Leeds University (http://bioinformatics.leeds.ac.uk/prot_analysis/Signal.html).

For statistical analysis SPSS software version 11 were used with Student-Newman-Keuts test.

Sig-Pred program from the Bioinformatic group of Leeds University was used to study the prediction of the cleavage site of the *Streptomyces* chitinase signal peptide sequence in prokaryotes and eukaryotes. The results are shown in the Fig. 14 and Fig. 15 respectively.

Sequence origin

Gram-positive bacterium

Possible cleavage positions

Position	Site	Score
28 | 29	STAAPVLLPAAPA | AA	7.7
30 | 31	AAPVLLPAAPAAA | AA	11.5
31 | 32	APVLLPAAPAAAA | AC	10.2
32 | 33	PVLLPAAPAAAAA | CS	8.1
33 | 34	VLLPAAPAAAAAC | SS	7.0

Most likely signal peptide and its passenger peptide

1 MPRRRTSALLA 11 ALVISTAAPV 21 LLPAAPAAA | A 31 ACSSYPSWVA 41 GRSYAAGDIV
51 RYTDGKAYIA 61 EHANPGYDPT 71 ISTWYWEPYA 81 CDGGSGTPVG 91 TFVVTEAQFN 101
QMFPNRNSFY 111 SYSGLTAALS 121 AYPGFANTGS 131 DTTKKQEAAA 141 FLANVSHETG 151
GLVHVVEQNQ 161 ANYPHYCDWS 171 RPYGCPAGQA 181 AYYGRGPIQL 191 SWNFNYKAAG 201
DALGIDLLNS 211 PWLVERDSAV 221 AWKTALWYWN 231 TQTGPGTMTP 241 HNAMVNGAGF 251
GQTIRSINGS 261 LECDGKNPAQ 271 VQSRVNNYQR 281 FTQILGVSPG 291 GNLYC

Fig. 14. Prediction of the cleavage site of the *Streptomyces* chitinase signal peptide sequence using prokaryotic sequencing origin.

Sequence origin

Eukaryote

Possible cleavage positions

Position	Site	Score
18 | 19	TSALLAALVISTA | AP	8.9 ←
19 | 20	SALLAALVISTAA | PV	7.4
30 | 31	AAPVLLPAAPAAA | AA	7.4

Most likely signal peptide and its passenger peptide

1 MPRRRTSALLA 11 ALVISTA | APV 21 LLPAAPAAAA 31 ACSSYPSWVA 41 GRSYAAGDIV 51 RYTDGKAYIA
61 EHANPGYDPT 71 ISTWYWEPYA 81 CDGGSGTPVG 91 TFVVTEAQFN 101 QMFPNRNSFY 111 SYSGLTAALS
121 AYPGFANTGS 131 DTTKKQEAAA 141 FLANVSHETG 151 GLVHVVEQNQ 161 ANYPHYCDWS 171 RPYGCPAGQA
181 AYYGRGPIQL 191 SWNFNYKAAG 201 DALGIDLLNS 211 PWLVERDSAV 221 AWKTALWYWN 231 TQTGPGTMTP
241 HNAMVNGAGF 251 GQTIRSINGS 261 LECDGKNPAQ 271 VQSRVNNYQR 281 FTQILGVSPG 291 GNLYC

Fig. 15. Prediction of the cleavage site of *Streptomyces* chitinase signal peptide sequence using eukaryotic sequencing origin, showing highest score for position different from the correct position at 30:31 (arrow).

The prediction of the cleavage site of the *Arabidopsis* signal peptide sequence fused to the coding region of *Streptomyces* chitinase 30 are shown in Fig. 16.

Sequence origin

Eukaryote

Possible cleavage positions

Position	Site	Score
18 | 19	FLFLIFSLLLSLS | SA	8.2
19 | 20	LFLIFSLLLSLSS | AA	8.3
20 | 21	FLIFSLLLSLSSA | AA	11.2
22 | 23	IFSLLLSLSSAAA | CS	9.7
23 | 24	FSLLLSLSSAAAC | SS	9.6
24 | 25	SLLLSLSSAAACS | SY	8.2
25 | 26	LLLSLSSAAACSS | YP	10.8

Most likely signal peptide and its passenger peptide

1 MKTNLFLFLIF **11** SLLLSLSSA | A **21** ACSSYPSWVA **31** GRSYAAGDIV **41** RYTDGKAYIA **51**
EHANPGYDPT **61** ISTWYWEPYA **71** CDGGSGTPVG **81** TFVVTEAQFN **91** QMFPNRNSFY **101**
SYSGLTAALS **111** AYPGFANTGS **121** DTTKKQEAAA **131** FLANVSHETG **141** GLVHVVEQNQ **151**
ANYPHYCDWS **161** RPYGCPAGQA **171** AYYGRGPIQL **181** SWNFNYKAAG **191** DALGIDLLNS **201**
PWLVERDSAV **211** AWKTALWYWN **221** TQTGPGTMTP **231** HNAMVNGAGF **241** GQTIRSINGS **251**
LECDGKNPAQ **261** VQSRVNNYQR **271** FTQILGVSPG **281** GNLYC

Fig. 16. Prediction of the cleavage site of *Arabidopsis* signal peptide sequence, showing the correct cleavage site with the mature protein of chitinase 30 (in red).

4.6 *In vitro* bio-assays

In vitro bio-assay test was used following the methods of Schlumbaum et al. (1986) and Chye et al. (2005) in order to investigate the effect of recombinant chitinase on inhibiting fungus hyphal growth.

The following fungi were used:

- Subculture of *Trichoderma harzianum* isolate T12 (fungi collection of the Institute of Plant Diseases and Plant Protection (IPP), University of Hannover, Germany, originally obtained as isolate T000 from the Institute of Phytopathology and Applied Zoology (IPAZ), Justus-Liebig-University Gießen, German).
- *Ascochyta rabiei* (DSMZ collection, Braunschweig, Germany)
- *Colletotrichum* (DSMZ collection, Braunschweig, Germany).

A plug of growing fungi cultured on PDA medium was transferred to the centre of a fresh PDA plate. Following incubation at 25 °C for 24 h (*T. harzianum*) or 48 h (*Ascochyta* and *Colletotrichum*), during which the hyphae grew outwards from the centre, wells (0.3 mm) were bored on the outer surface of the PDA, in equal distance from the plug. Crude protein extract from transformed and untransformed plants of tobacco and pea as well as buffer

were added to each well (30-50 µg) and the plate was further incubated in the dark at 25 °C. Growth inhibition was observed and photographed after 8 h, 16 h and 24 h. The assay was repeated two times.

4.7 Plant Material

4.7.1 Pea seeds

4.7.1.1 Surface sterilization of the seeds

Pea seeds cultivar *Sponsor* were surface sterilized by soaking in 70 % ethanol (EtOH) (v/v) for 1 min followed by 6 % sodium hypochlorite (NaOCl) for 5-10 min, with agitation. Seeds were washed for 5-6 times with sterile deionised water and imbibed in water overnight.

4.7.1.2 Preparation of explants

Pea transformation was done according to the modified protocol of Schroeder et al. (1993) and Bean et al. (1997). Seeds were split open, root tips were removed and the remaining embryo axis was sliced longitudinally into three to five segments (Fig. 2A) with a scalpel blade that was dipped in the *Agrobacterium* suspension before each cut.

These explants were inoculated with *Agrobacterium* suspension supplemented with 100 µM acetosyringon and 5 µM TDZ for 60-90 min, then explants were blotted dry on sterile filter paper and plated for three days in the dark at 22±2 °C in growth-room on B5hT co-cultivation medium.

After co-cultivation, explants (white and white greenish color) were washed several times in sterile distilled water until the wash out water become clear, the final wash was supplemented with 100 mg/l Ticarcillin and incubated for 15 min on a shaker to remove the *Agrobacteria* then the explants were blotted dry on sterile filter paper and cultured on shoot regeneration MST medium for 10 days under dim light, then subcultured to MST medium for another 10 days in light. Thereafter, the explants were sub-cultured on selection medium P2 and the healthy green shoots were sub-cultured every three weeks to P2 fresh medium with increased concentrations of PPT to 2.5 mg/l, 5 mg/l, 7.5 mg/l, 10 mg/l, 12.5 mg/l, and 15 mg/l. Briefly the pea transformation was done using the following scheme:

- Explants preparation from mature embryo.
- Inoculation with *Agrobacterium* suspension.

- Co-culture for three days in the dark (B5hT medium+TDZ+Kin.).
- First subculture for one week in semi-dark condition (MST+TDZ+NAA).
- Second subculture for one week in light (MST+TDZ+NAA).
- First selection for three weeks (P2+BAP+NAA+2 mg/l PPT).
- Second subculture after three-four weeks (P2+BAP+NAA+5 mg/l PPT).
- Further subcultures to fresh media in three to four weeks interval for selection and multiplication.
- In vitro grafting of shoots surviving the selection at 15 mg/l PPT.
- Successful grafted shoots (after 8-15 days) transferred to greenhouse.
- T0 seeds harvested after 30-45 days post grafting.

4.7.2 Tobacco plant

In vitro growing tobacco plants (*Nicotiana tabacum* L.) cv. *Samsun* were the source for the explants used in transformation procedure as described by Moebius (2000). The young leaves were cut into 0.5 ~ 1 cm square and inoculated with *Agrobacterium* suspension for 15-30 min. The explants were blotted dry on sterile filter paper and co-cultivated adaxial face down on Petri dishes containing MSZ medium for three days in the dark at 22±2 °C. The explants were washed with sterile distilled water for at least 5 times, and then were dried on sterile filter papers. Explants were plated on MSZP selection medium supplemented with 100 mg/l Ticarcillin, 100 mg/l Combactam and 2.5 mg/l PPT. The explants were sub-cultured every three weeks to fresh medium with increasing concentration of PPT from 5 mg/l, 7.5 mg/l until 15 mg/l, respectively. Regenerated shoots from the edge of explants were removed and sub-cultured to fresh medium. When shoots were big enough they were sub-cultured on rooting medium. The rooted shoots were potted, acclimated and then transferred to the greenhouse for flowering and setting seeds through self-pollination.

4.7.3 Selection agent

Glufosinate-ammonium was used as selective agent in vitro to select the transgenic shoots (see 3.3.10), healthy green shoots were sub-cultured every three weeks to P2 fresh medium with increased concentrations of PPT to 2.5 mg/l, 5 mg/l, 7.5 mg/l, 10 mg/l, 12.5 mg/l and 15 mg/l.

4.8 Media

4.8.1 Media for pea transformation

4.8.1.1 B5-i re-suspension medium

B5 basal micro- and macro salts (Gamborg et al., 1968)
10 g/l glucose
10 g/l sucrose
2 g/l MES
pH was adjusted to 5.6 with 1N KOH/1N HCl

4.8.1.2 B5hT Co-cultivation medium

B5 basal micro- and macro salts	0.8 g/l glutamine
B5 vitamin mixture	10 mg/l glutathione
30 g/l sucrose	1 mg/l adenine
0.88 g/l $CaCl_2$, $2H_2O$	2.0 g/l MES
0.5 g/l KNO3	0.2 mg/l kinetin (1 µM)
0.5 g/l $MgSO_4$, $7H_2O$	1.1 mg/l TDZ (5 µM)

pH was adjusted to 5.5 and the medium was solidified by adding 4.5 g/l GelRite.

4.8.1.3 MST regeneration medium

MS macro- and micro salt's (Murashige and Skoog, 1962)
B5 vitamin mixture
30 g/l sucrose
1 g/l MES
1.1 mg/l TDZ (5 µM)
0.002 mg/l NAA (0.01 µM)

pH was adjusted to 5.8 and the medium was solidified by adding 7.5 g/l Plant Agar. Post autoclaving and cooling to 60 °C the medium was supplemented with 100 mg/l Ticarcillin and 100 mg/l combactam.

4.8.1.4 P2 selection medium

MS basic micro- and macro salts
B5 vitamin mixture
30 g/l sucrose
1 g/l MES
4.5 mg/l BAP (14.58 µM)
0.02 mg/l NAA (0.1 µM)

pH was adjusted to 5.8 and the medium was solidified by adding 7.5 g/l Plant Agar, and post autoclaving the medium was supplemented with 100 mg/l Ticarcillin, 100 mg/l combactam and 2.5 mg/l PPT.

4.8.2 Media for tobacco transformation

4.8.2.1 MSZ co-cultivation medium

MS basal media with vitamins
3 % sucrose
1 mg/l Zeatin (4.5 µM)
1 g/l MES

pH was adjusted to 5.8 and the medium was solidified by adding 0.3 % GelRite.

4.8.2.2 MSZP selection medium

MSZ medium
1 g/l MES
100 mg/l Ticarcillin
100 mg/l combactam
2.5 mg/l PPT.

pH was adjusted to 5.8 and the medium was solidified by adding 7.5 g/l Plant Agar.

4.8.2.3 Rooting medium

½ MS (half strength of MS macro and micro salts)
100 mg/l Ticarcillin
100 mg/l combactam
15 mg/l PPT.

pH was adjusted to 5.8 and the medium was solidified by adding 7.5 g/l Plant Agar.

4.9 Designation of the transformation experiments

In order to easily handle different transformation experiments and analyze different transgenic clones, we used a code or ID to differentiate between different clones and generations, the code used is: XX-YY-E-S, T1, T2, T3,...

Where:

XX stands for the transformation experiment

YY stands for the year of transformation

E stands for the explant

S stands for the shoots regenerated from same explant E

T1 stands for the first transgenic generation

T2 stands for the second transgenic generation

T3 for third generation and so on.

For example, the following code 03-04-3-2, 1, 2, 3 is explained as follow: 03 is

transformation experiment number 3 in the year 2004, -2 for the shoot number two regenerated from explant -3. The number after comma indicates different generations where, 3 is seed number three from plant growing from seed number ,2 which was grown from plant from seed number 1.

The primary transformant 03-04-3-2 is called T0 generation and the seeds growing from these plants is T1 generation (03-04-3-2, 1) and the following T2 generation is 03-04-3-2, 1, 2 and so on where each generation is separated with comma.

4.10 Segregation and homozygousity analysis

Segregation analysis was tested using Chi-squared test at 1 % and 5 % significance for chitinase gene obtained from PCR data, also for establishing the homozygous lines. The homozygousity of the parental plant was calculated from its progeny (K), the homozygous plants will not be segregating any more.

Homozygousity was calculated using the following formula adapted from Kiesecker (2000):

$$P = \frac{1}{1+2\,(3/4)^{K}}$$

Where P: probability at 5 %.

K: number of transgenic progeny.

4.11 Tobacco cell culture

Plant cell cultures are important tool for elucidation of biosynthetical pathways, production of secondary metabolites by large-scale growth of plant cells in liquid culture, screening systems for pharmacy and agrochemistry.

4.11.1 Callus induction media (4X medium)

B5 basal micro- and macro salts + vitamins	3,16 g/l
Saccharose	20 g/l
Nz-Amin (Caseinhydrolysat)	2 g/l
2,4 D	2 mg/l
NAA	0,5 mg/l
IAA	0,5 mg/l
Kinetin	0,2 mg/l

pH was adjusted to 5.6 and solidified with 0.8 % Plant Agar (for solid medium).

4.11.2 Procedure

Callus culture was induced by culturing 0.5-1 cm^2 tissue from shoots or roots on 4X medium for 4 weeks, explants were subcultured every 4 weeks into fresh 4X medium, supplemented with PPT for selection, until getting suitable callus phase.

To start suspension culture, small pieces from the resulted callus (4-6 g fresh weight) were placed in 100 ml flask containing enough 4X liquid medium to allow easily movement for the cells and shacked at 100 rpm.

Cultures were monitored every 2 or 3 days; a new liquid media could be added if necessary. After 2-3 weeks, there cultures were transferred to larger flasks (300 ml) containing 80-100 ml 4X liquid media. Cells were then subcultured to fresh medium every week.

5 RESULTS

5.1 Cloning of the chitinase gene with a bacterial signal peptide sequence

The architecture of the constructed plasmids pGIIvst-Chit and pGII35S-Chit was confirmed by restriction digest and sequencing of the coding region including the interface to the vector in *E.coli* derived plasmids (Fig.17). Thereafter the constructs were transferred into the disarmed *Agrobacterium tumefaciens* strain EHA105 harboring the pSoup plasmid from the pGreen II collection. Different glycerol stocks were prepared and stored at -80 °C as deposit for the plant transformation. Again, the plasmid integrity was checked by sequencing of *E. coli* derived plasmids after re-transformation of *E.coli.* The results of BLAST search at NCBI database using the sequencing results of constructed plasmid found 100 % homology to Chit30 gene from *Streptomyces* (Fig. 18).

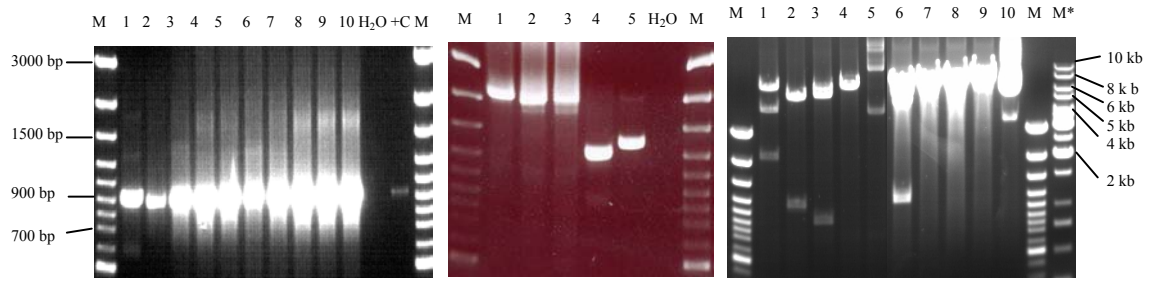


Fig. 17. Results of cloning work, plasmid (lane 1 and 2) and colony PCR of different colonies (3-10) using 900 bp primers (right); PCR using pGII primers 297 & 303, lane 1, pGIIvst-Chit; lane 2 and 3, pGII35S-Chit; lane 4, pGII35S; lane 5, pGIIvst (middle); restriction digest of plasmid pGIIvstchit and pGIIvst (left) using *Sac*I+*Kpn*I (lane 1 and 6), *Bam*HI+*Sac*I (lane 2 and 7), *Bam*HI+*Xba*I (lane 3 and 8), *Nco*I (lane 4 and 9), and control (lane 5 and 10) respectively; +C, positive control; M, 100 bp DNA molecular ladder; M*, 1 Kp DNA molecular ladder.

>gi|4456813|emb|AJ133186.1|SOL133186 *Streptomyces olivaceoviridis*
ATCC11238 chi30 gene Length=1199

```
 Score = 1140 bits (575),  Expect = 0.0
 Identities = 575/575 (100%), Gaps = 0/575 (0%)Strand=Plus/Plus

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Query  199  CCGCTACACGGACGGCAAGGCGTACATCGCCGAGCACGCCAACCCGGGTTACGACCCGAC  258
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  351  CCGCTACACGGACGGCAAGGCGTACATCGCCGAGCACGCCAACCCGGGTTACGACCCGAC  410

Query  259  CATCAGCACCTGGTACTGGGAGCCGTACGCCTGCGACGGCGGGTCCGGGACGCCGGTCGG  318
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Query  319  CACCTTCGTGGTGACCGAGGCCCAGTTCAACCAGATGTTCCCGAACCGGAACTCCTTCTA  378
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  471  CACCTTCGTGGTGACCGAGGCCCAGTTCAACCAGATGTTCCCGAACCGGAACTCCTTCTA  530
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Query  379  CAGCTACAGCGGACTCACCGCCGCGCTCAGCGCCTACCCCGGCTTCGCGAACACCGGCAG  438
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Query  499  CGGCCTGGTGCACGTGGTCGAGCAGAACCAGGCCAACTACCCGCACTACTGCGACTGGAG  558
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Query  559  CCGGCCGTACGGCTGCCCGGCGGGCCAGGCGGCCTACTACGGGCGCGGCCCGATCCAGCT  618
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Sbjct  711  CCGGCCGTACGGCTGCCCGGCGGGCCAGGCGGCCTACTACGGGCGCGGCCCGATCCAGCT  770

Query  619  CAGCTGGAACTTCAACTACAAGGCCGCGGGCGACGCGCTCGGCATCGACCTGCTGAACAG  678
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Sbjct  771  CAGCTGGAACTTCAACTACAAGGCCGCGGGCGACGCGCTCGGCATCGACCTGCTGAACAG  830

Query  679  CCCCTGGCTGGTCGAGCGCGACTCGGCCGTCGCCT  713
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Sbjct  831  CCCCTGGCTGGTCGAGCGCGACTCGGCCGTCGCCT  865

 Score =  133 bits (67),  Expect = 9e-28
 Identities = 67/67 (100%), Gaps = 0/67 (0%)Strand=Plus/Plus

Query  48   TGCCGAGGCGTCGCACATCCGCCCTGCTGGCCGCGCTGGTCATCTCGACCGCAGCGCCGG  107
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Sbjct  200  TGCCGAGGCGTCGCACATCCGCCCTGCTGGCCGCGCTGGTCATCTCGACCGCAGCGCCGG  259

Query  108  TGCTCCT  114
            |||||||
Sbjct  260  TGCTCCT  266
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Fig. 18. BLAST alignment of constructed plasmid sequence of pGII35S-Chit.

The resulted T-DNAs are shown in the Fig. 19.

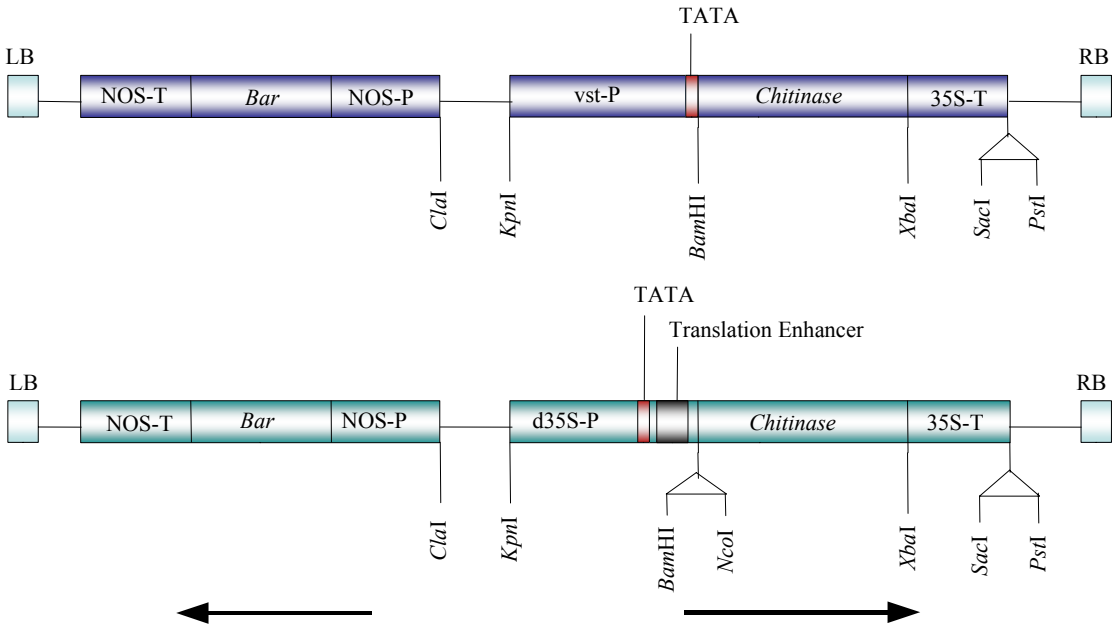

Fig. 19. Schematic diagrams of the T-DNA region of the binary vectors pGreenII constructed with respective restriction sites containing the bacterial chitinase gene with two different promoters (vst-P and double 35S-P), selectable marker gene *bar* inserted between nos-terminator and -promoter, LB and RB are left and right T-DNA border. Chitinase and bar organized divergently. Arrows indicate direction of transcription.

5.2 Construction of a chimeric chitinase gene with an *Arabidopsis* signal peptide

As the cleavage sites for signal peptides differ between bacteria and plants, it was necessary to replace the bacterial signal peptide sequence with a plant signal peptide sequence from an *Arabidopsis* basic endochitinase signal peptide (Fig. 7). Using PCR, the signal peptide was included in a forward dove tail primer (87-mer) flanked by a *Bam*HI restriction site at the 5'-end. The reverse primer contained a *Xba*I restriction site at the 3'-end. The prediction of the favorable cleavage site of the *Arabidopsis* signal peptide sequence with the coding region of the *Streptomyces chit30* gene is shown in Fig. 16.

The PCR products with the modified primers were subcloned as described in 4.1 to get the vector pGII35S-N-Chit and pGIIvst-N-Chit. After *E.coli* transformation, different colonies were checked by restriction digest, PCR and sequencing for successful cloning (Fig. 20). The resulted T-DNAs with modified signal peptide sequence are shown in Fig. 21.

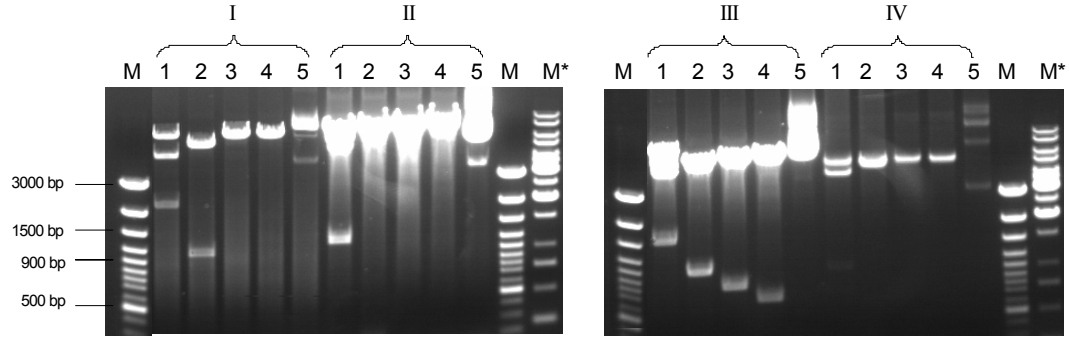

Fig. 20. Restriction digest of plasmids I, pGIIvst-N-chit; II, pGIIvst; III, pGII35S-N-chit; IV, pGII35S; using different enzymes 1, *Kpn*I+*Sac*I; 2, *Bam*HI+*Sac*I; 3, *Bam*HI + *Xba*I; 4, *Nco*I; 5, control; M, 100 bp DNA molecular ladder; M*, 1 Kp DNA molecular ladder.

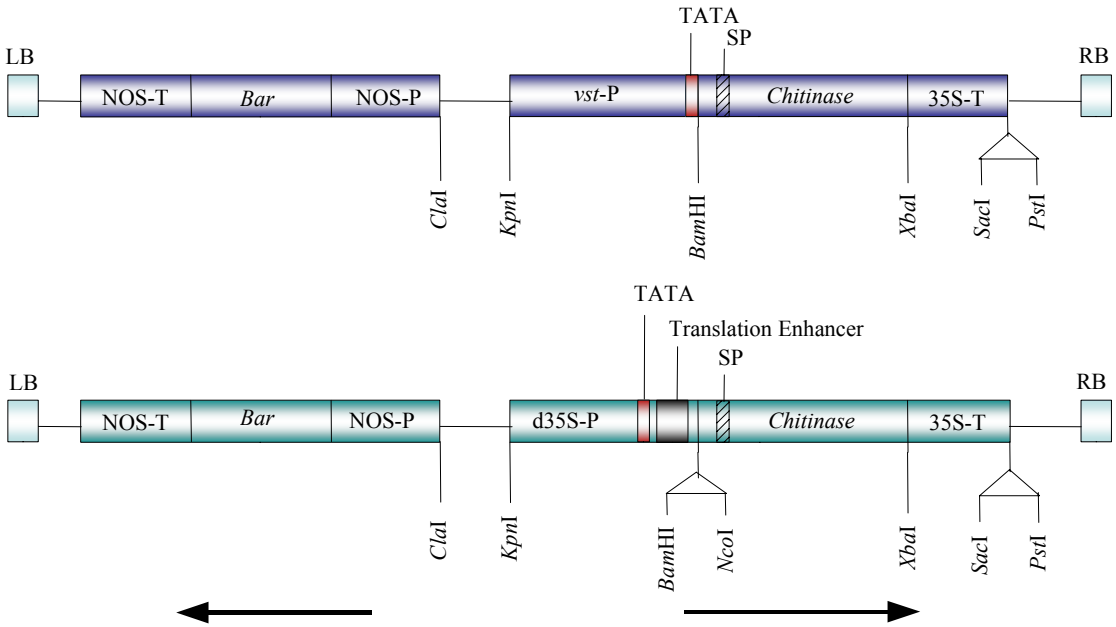

Fig. 21. Schematic diagrams of the T-DNA region of the binary vectors pGreenII constructed with respective restriction sites containing bacterial chitinase gene fused to *Arabidopsis* signal peptide (SP) with two different promoters (vst-P and double 35S-P), selectable marker gene *bar* inserted between nos-terminator and -promoter, LB and RB are left and right T-DNA border. Chitinase and bar organized divergently and arrows indicate direction of transcription.

5.3 Tobacco transformation

In order to check the functionality of the constructed binary vectors and to save the time and the efforts of using it for pea or any important crops, it is wise to use a model plant first to relatively quickly prove the vector and make any necessary modifications that might be needed to improve the function, transformation efficiencies and the putative expression in the target plant. The chitinase gene was therefore introduced into tobacco plants (*Nicotiana tabacum* L.) cv. *Samsun* (Fig. 22) for checking the construct functionality, where 25 clones were selected from each bacterial chitinase construct with either *vst* or 35S promoter (further clones selected are listed in Table 8). T0 seeds were germinated in the greenhouse and 2-3 weeks after germination, the growing seedlings were sprayed with 600 mg/l BASTA® to eliminate the sensitive seedlings, whereas the surviving healthy green seedlings conferring the herbicide resistance were grown to set T1 seeds through self-pollination (Fig. 22).

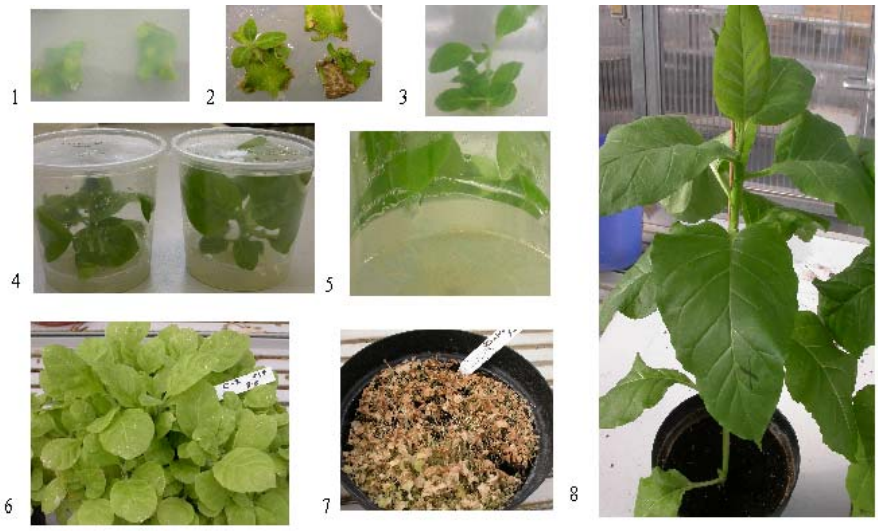

Fig. 22. Tobacco transformation steps using leaf disc method. 1, 2, and 3 leaf explants and regenerated T0 shoots growing on MSZ medium and selection medium supplemented with 2.5 mg/l PPT; 4 and 5 T0 green healthy single shoot separated and cultured on ½ MS medium for rooting with different concentrations of PPT ranging from 7.5 mg/l to 15mg/l; 6, transgenic T0 seeds germinated and sprayed with 600 mg/l BASTA®; 7, non-transformed negative control tobacco sprayed with 600 mg/l BASTA®; 8, well established tobacco plant with normal phenotype growing in the greenhouse to set seeds.

Successful integration of the T-DNA into tobacco genomic DNA was analyzed using different primers for the chitinase and the bar gene in the progeny of T0 and following generations by PCR. Transfer of backbone sequences was also checked using primers including the nptI primer. Fig. 23 clearly shows the successful integration of T-DNA into the tobacco genome. This result also shows the presence of the bar gene and indirectly its products since these plants survived high BASTA® applications (600 mg/l).

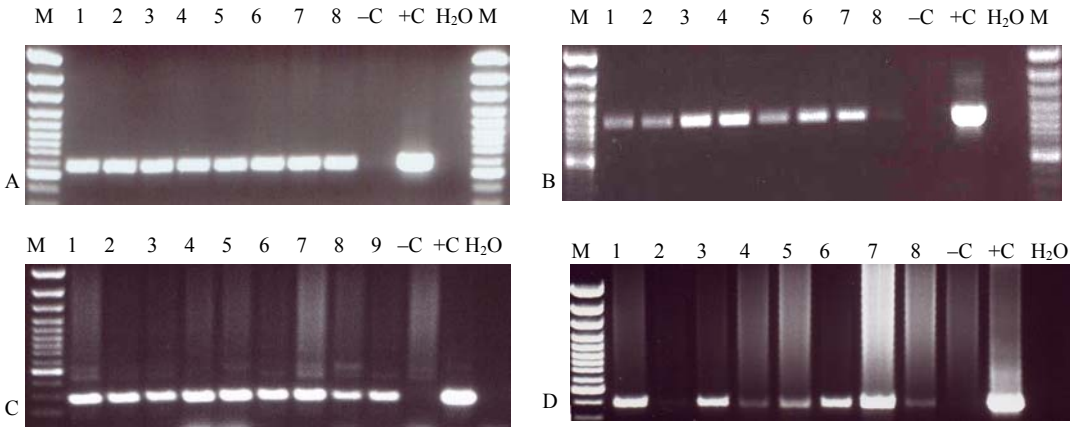

Fig. 23. PCR of different transgenic T0 tobacco clones cv. Samsun using different primers, A, chit555 primer (spanning a 555 bp fragment of the chitinase gene); B, strep-chit primer (PCR product 900 bp); C, bar primers (spanning a 260 bp fragment of the bar gene); D, nptI primer (backbone, PCR product 400 bp); transgenic plants driven by vst promoter (lanes 1-4) and by double 35s promoter (lanes 5-8); -C, untransformed negative control tobacco plant; +C, positive control (plasmid pGIIvst-Chit); H2O, water control; M, 100 bp DNA molecular ladder.

Tobacco plants were grown in the greenhouse for two generations (T1 and T2) and maintained through self-pollination. Seeds of the T2 plants were collected for further analysis to select homozygous plants. Stable inheritance of the T-DNA was confirmed using different molecular characterization methods in different generations (Fig. 24).

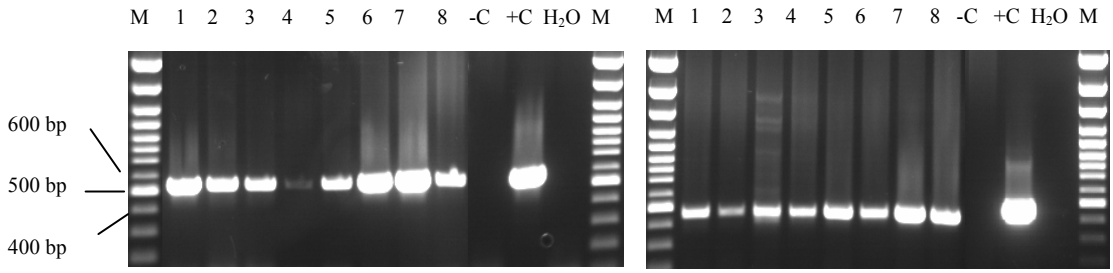

Fig. 24. PCR of different T1 tobacco transformed with pGII35S-Chit and pGIIvst-Chit using chit primer (left) and bar primer (right), M, 100 bp DNA molecular ladder.

At the transcriptional level, the chitinase and bar transcripts were detected using RT-PCR and sequencing of the PCR products derived from cDNAs as templates. Both T0 and T1 plants clearly exhibited the transcription of the transgenes. The *vst* promoter driven transcripts were UV induced (254 nm) before RT-PCR analysis (Fig. 25).

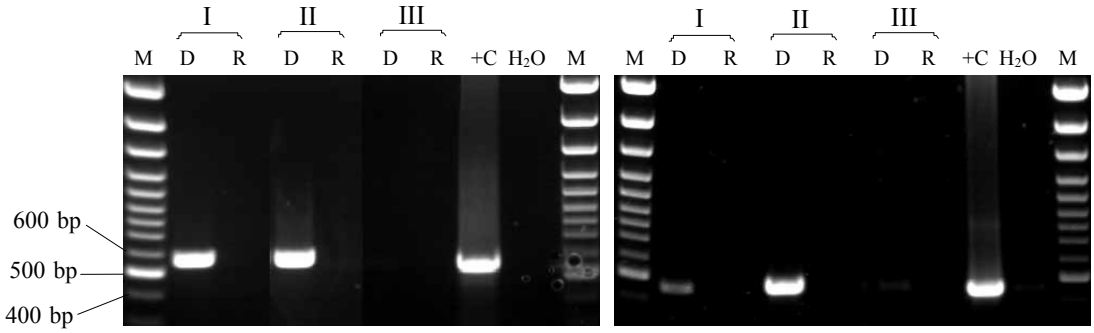

Fig. 25. RT-PCR of T1 tobacco plants transformed with pGIIvst-Chit construct, promoter was UV induced, using Chit555 primer (product size 555 bp, left) and bar447 primer (product size 447 bp, right) where I, clone T-1-2; II, clone H-1-1; III, non-transformed negative control; D, cDNA used as template; R, RNA used as control; +C, positive control (plasmid); M, 100 bp DNA molecular ladder.

Copy numbers and integration patterns were investigated in T0, T1 and T2 respectively, using Southern blot analysis with different probes (chit 555, bar, and nptI). We detected multiple copies in the majority of the tobacco plants tested. The resulting copy numbers were different according to the restriction enzyme used (*Eco*RI which cuts in the genomic DNA or *Xba*I which cuts directly behind the chitinase gene in T-DNA). In both cases,

there were between one and five copies in T0 plants when the chit 555 probe or the bar probe were used, while a single copy was the result of some clones using the backbone nptI probe. The copy number was less in the following progenies of T1 and T2 due to segregation, the copy number in T2 clones of L-15-2-1, L-15-2-2, C-2-2-2, F-1-2-1, H-4-1-2 and H-5-2-1 was same when using the two different restriction enzymes, whereas it was different for the clones R-1-3-2-1, O-1-2-1 and O-1-2-2 which means that the inserts are linked and behave as single copy (*Eco*RI digest) (Fig. 26 and Table 9).

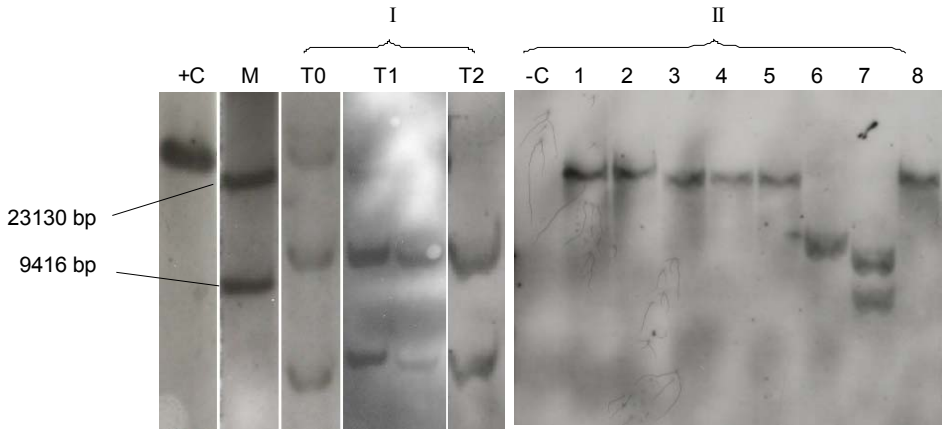

Fig. 26. Southern blot analysis of genomic DNA from tobacco plants transformed with pGIIvst-Chit and pGII35S-Chit digested with *Eco*RI, using Chit555 probe; I, clone F-1, T0, T1 and T2; II, T2 generation of different clones (1, clone O-1-2-1; 2, clone O-1-2-2; 3, clone L-15-2-1; 4, clone L-15-2-2; 5, clone H-5-2-1; 6 clone C-2-2-2; 7, clone H-4-1-2; 8, clone R-1-3-2-1); M, Dig II labeled marker; -C, untransformed negative control tobacco plant; +C, 1:1000 dil. of PCR products as positive control.

Table 9. Southern blot results and copy numbers in different transformants at T0, T1 and T2. Tobacco plants transformed with EHA105-pGII35S-Chit (highlighted) or EHA105-pGIIvst-Chit, DNA was digested with *Eco*RI or *Xba*I and probed with Chit555, bar and nptI probe.

Clones			T0						T1						T2					
			*Eco*RI			*Xba*I			*Eco*RI			*Xba*I			*Eco*RI			*Xba*I		
			Chi	Bar	nptII	Chi	Bar	nptII	Chi	Bar	nptII	Chi	Bar	nptII	Chi	Bar	nptII	Chi	Bar	nptII
T0	**T1**	**T2**																		
- control			-	-	-	-	-	-	-	-	-				-	-	-			
R-1-3	R-1-3-1	R-1-3-1-1	2	2	-	4	3	1	1		-									
	R-1-3-2	R-1-3-2-1							1		-				1	1	1	2	2	1
L-15	L-15-1	L-15-1-1	1	1	1	4	1	1	1		-									
	L-15-2	L-15-2-1							1		-				1	1	-	1	1	-
		L-15-2-2													1	1	-	1	1	-
L-14	L-14-1	L-14-1-1	5	5	2	6	6	7	1		-									
	L-14-2	L-14-2-1							1		1									
O-1	O-1-1	O-1-1-1	-	-	-	2	1	2	1		-									
	O-1-2	O-1-2-1							1		-				1	1	1	2	2	2
		O-1-2-2													1	1	1	3	3	2
C-2	C-2-1	C-2-1-1	3	3	1	2	1	1	3		1									
	C-2-2	C-2-2-1							2		1									
		C-2-2-2													1	1	-	1	1	-
F-1	F-1-1	F-1-1-1	3	2	1	3	2	1	2		1									
	F-1-2	F-1-2-1							2		1				2	2	1	2	2	1
T-1	T-1-1	T-1-1-1	2	1	1	2	2	1	1		1									
	T-1-2	T-1-2-1							1		1									
H-4	H-4-1	H-4-1-1	2	2	-	2	2	1	2		1									
		H-4-1-2													2	2	1	2	2	1
	H-4-2	H-4-2-1							1		1									
H-5	H-5-1	H-5-1-1							1		-									
	H-5-2	H-5-2-1							1		-				1	1	-	1	1	-
H-1	H-1-1		-	-	-	1	1	-	1		-									
	H-1-2								2		1									

5.4 Analysis of chitinase expression in transgenic tobacco

The production of chitinase in transgenic plants of tobacco was analyzed by Western blot and immunostain. Total protein was extracted from leaf explants of transformants with the 35S and the *vst* promoter, respectively. In the case of the 35S promoter, total protein was extracted directly from leaf explants, whereas plants with the *vst* promoter required induction using UV light (254 nm) for 5 min. (see 4.3.7). No expression was detected in

T0 plants of both transformants with pGII35S-Chit and pGIIvst-Chit constructs, but it was clearly detected in the following progenies of T1 and T2 plants (Fig. 27, A and B). In contrary, T0 transformants with the constructs pGII35S-N-Chit and pGIIvst-N-Chit showed positive signals in the Western blot (Fig. 27, C).

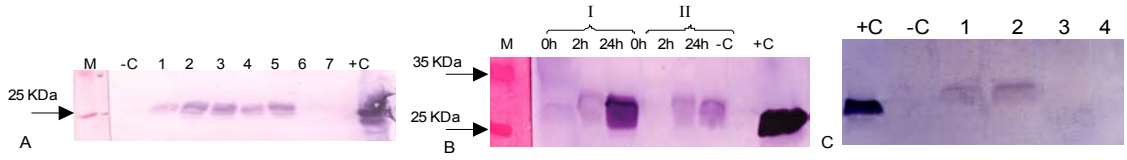

Fig. 27. Western blot analysis of total protein extracted from tobacco cv. *Samsun* hybridized with α-Chit30 antibody; A, T1 plants transformed with pGII35S-Chit (1-7 different transformants); B, T1 transformants with pGIIvst-Chit after UV induction and protein extraction (I, clone H-5-1; II, clone H-4-2; 0h, without induction; 2h, two hours after induction; 24h, one day after induction); C, comparison of T0 transformants of pGII35S-N-Chit (lanes 1 and 2) and pGII35S-Chit (lanes 3 and 4); +C, Chit30 standard from *Streptomyces* used as positive control; -C, non-transformed negative control plant; M, BioRad low protein standard.

Chitinase activity was investigated using a modified method of Trudel and Asselin (1989, 1990), where the protein crude extracts was separated in SDS-PAGE containing glycol chitin as substrate. After staining the gel with fluorescent staining solution, the chitinolytic effect was visualized under UV light as dark bands on a bright background as a result of chitinase activity. Activity was detected in T1 plants transformed with the constructs pGIIvst-Chit and pGII35S-Chit (containing bacterial signal peptide sequence) and T0 plants transformed with the chimeric constructs pGIIvst-N-Chit and pGII35S-N-Chit (containing plant signal peptide sequence). Enzyme activity was also detected in T2 tobacco plants transformed with pGIIvst-Chit and pGII35S-Chit under 35S or *vst* promoter after UV induction; prominent additional high molecular bands only occurred in transgenic plants compared to the non-transformed negative plant (Fig. 28).

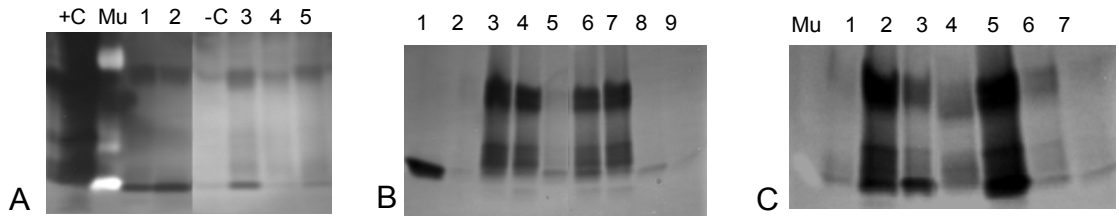

Fig. 28. *In gel* activity of tobacco chitinase; A, T1 tobacco plants: lane 1, 4 and 5 transformants with pGII35SChit; lane 2 and 3 are transformants with pGIIvstChit after UV induction; B, T2 tobacco plants (lane 1 and 2 non-transformed control induced and not induced respectively; lane 3 and 4 clone H-5-1-2 induced and not induced respectively; lane 5 clone L-14-1-1; lane 6 clone L-15-2-1; lane 7 clone R-1-3-1-2; lane 8 and 9 clone O-1-2-2 and O-1-2-1); C, T2 tobacco plants (lane1 clone F-1-2-1; lanes 2, 3, 4 and 5 clones H-1-1-2, T-1-2-2, H-5-2-2, C-2-2-1 respectively; lanes 6 and 7, clones C-2-2-2 induced and not induced respectively); -C, untransformed negative control tobacco; +C, Chit30 from *Streptomyces;* Mu, Mucor.

5.4.1 Tobacco Cell culture

Cell culture was established from different transgenic clones and untransformed tobacco plants in the plant cell culture lab, DSMZ GmbH (German Collection of Microorganisms and Cell Culture).

Stable integration was proved by PCR using chitinase and bar primers (Figure 29, A). Total protein was extracted from the cells and the medium and subjected to PAGE and Western blot analysis and *in-gel* chitinase activity tests. Chitinase was detectable in suspension cells, which were derived from chimeric T0 plant transformed with pGII35SChit (Fig. 29, B and C, lanes 1 and 2). The secretion of the chitinase to the medium was hardly detectable from cells transformed with both the bacterial signal peptide sequence and the plant signal peptide sequence (Fig. 29, B and C, lanes 3 and 4). It was very difficult to detect chitinase activity in the case of pGII35S-N-Chit (Fig. 28, B and C, lanes 5, 6, 7, and 8).

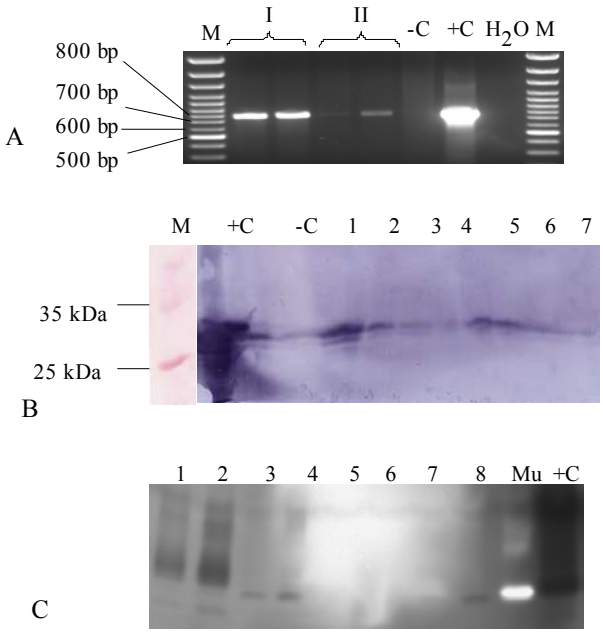

Fig. 29. Tobacco cell culture. A, PCR result using chit primers (product size 750 bp), I, cell culture from tobacco clone L-15 (construct pGII35S-Chit), II, cell culture from tobacco clone 03-04 (construct pGII35S-N-Chit), -C, untransformed negative control ; lane 9, plasmid pGII35S-N-Chit as positive control; M, 100 bp DNA ladder molecular weight marker; B and C, Western blot/immunostain and in-gel assay respectively of total protein extracted from cell culture and medium transformed with pGII35S-Chit, clone L-15 (lanes 1 and 2 from cells; lanes 3 and 4 from medium) and pGII35S-N-Chit clone 03-04-54 (lanes 5 and 6 from cells; lanes 7 and 8 from medium); Mu, protein from Mucor sp.; +C, chit30 standard from Streptomyces used as positive control.

5.4.2 Apoplast protein

In order to study the secretion of chitinase and whether it is correctly targeted to the cell

wall, proteins were extracted from the apoplast and used for Western blot analysis and immunostaining and *in-gel* chitinase assays. The western blot reveals two bands in the range of 28-30 KDa, which may be related to the mature protein and the pre-mature protein. These results prove the presence of the recombinant chitinase in the intracellular space (Fig. 30).

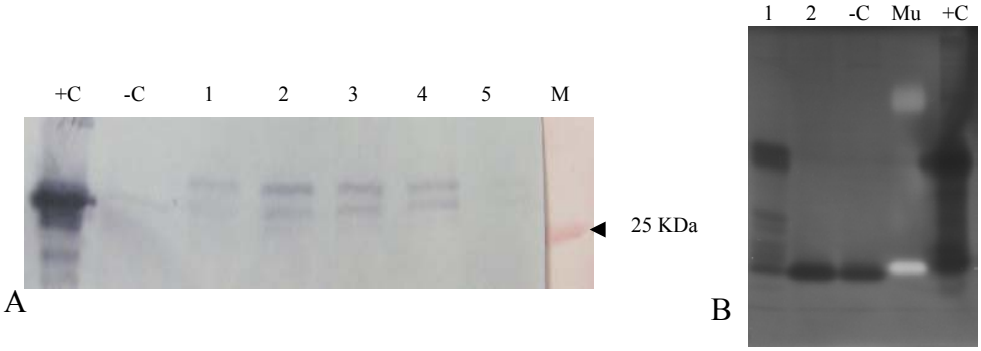

Fig. 30. Apoplast protein extracted from tobacco plants. A, Western blot of tobacco plants transformed with pGII35S-Chit (lanes 1 and 2, clones R-1-3-1 and L-15-2, respectively) and pGIIvstChit construct (lanes 3, 4, and 5, clones H-1-1 UV induced, H-1-1 not induced, C-2-2 UV induced respectively); B, *in-gel* chitinase activity assay of pGII35S-Chit(lane 1) and pGII35S-N-Chit (lane 2); -C, untransformed negative control tobacco plant; +C, chit30 as positive control; M, BioRad low protein marker; Mu, protein from *Mucor* sp.

5.4.3 Chitinase enzyme activity

CM-chitin-RBV (Loewe, Germany) was used as a substrate for colorimetric chitinase activity assays. These assays were performed according to Stephan and Wolf (1990). The difference between the values (OD_{550}) of blank and samples indicated the enzyme activity. (Fig. 31 and Table 10).

Table 10. Results obtained from chitinase enzyme activity measurement.

	L-15-2-1	R1-3-1-2	H-4-2-1	H-4-2-1*	H1-1-2-1	H-1-1-2-1*	H-5-1-2	H-5-1-2*	03.04.54	03.04.30	03.04.24	-control	Standard
Blank1	0,18	0,16	0,13	0,1	0,11	0,12	0,12	0,11	0,1	0,14	0,11	0,1	0,1
Blank2	0,2	0,17	0,12	0,11	0,1	0,13	0,1	0,11	0,11	0,13	0,16	0,1	0,12
Blank3	0,18	0,16	0,13	0,12	0,11	0,12	0,11	0,12	0,12	0,16	0,13	0,11	0,13
Sample1	0,33	0,28	0,28	0,29	0,25	0,29	0,23	0,28	0,29	0,34	0,29	0,22	0,41
Sample2	0,34	0,28	0,27	0,29	0,24	0,28	0,22	0,27	0,29	0,38	0,3	0,2	0,43
Sample3	0,32	0,29	0,27	0,29	0,39	0,27	0,21	0,27	0,28	0,4	0,31	0,21	0,43
Mean blank	0,19	0,16	0,12	0,11	0,11	0,12	0,11	0,11	0,11	0,14	0,13	0,1	0,12
Mean sample	0,33	0,28	0,27	0,29	0,29	0,28	0,22	0,27	0,29	0,37	0,3	0,21	0,42
OD	0,15	0,12	0,15	0,18	0,19	0,16	0,11	0,16	0,18	0,23	0,17	0,11	0,31

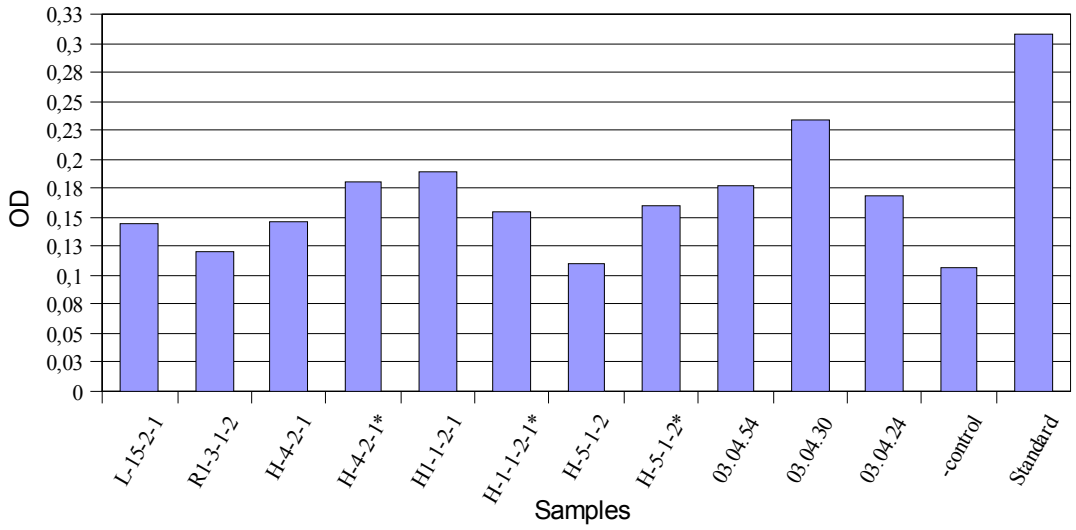

Fig. 31. Colorimetric chitinase activity assay of different tobacco samples using CM-chitin-RBV as substrate at OD_{550}; Standard (100µg), chitinase from *Streptomyces* (Sigma).

5.4.4 Gel diffusion assay

A standard curve for chitinase activity was prepared using dilutions of *Streptomyces griseus* chitinase at different concentrations of 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, 0.01, and 0.001 units. Samples of 10 µg total protein were applied, after visualizing the hydrolysis activity and measuring the diameters of the dark halos, which indicates the degradation of the substrate. The diameters of halos were plotted against logarithmic value of chitinase activity since the activity was not linear (Fig. 32). Then activities of different tobacco samples were calculated through regression analysis (Table 11).

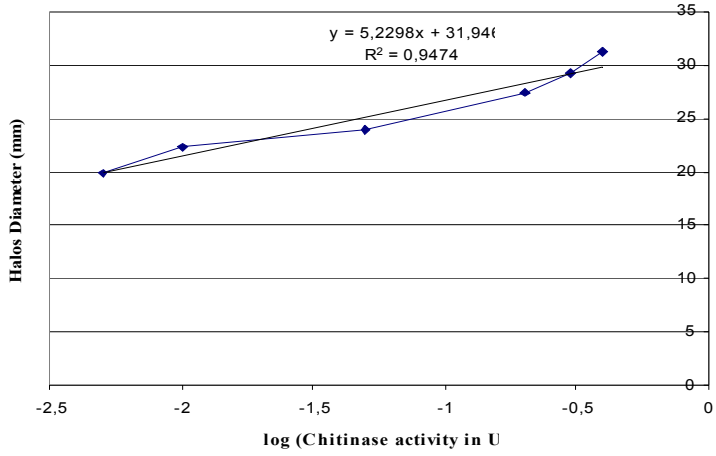

Fig. 32. Regression analysis of chitinase activity of standard in units against halos diameter in mm. The equation for calculating trend line is y= 5.2298x + 31.946.

Table 11. Chitinase activity in unit of different tobacco samples transformed with pGIIvst-Chit construct (highlighted) before UV light induction and after induction (marked with *), and the pGII35S-Chit construct.

Sample	***Halos diameter (mm)***	***Chitinase activity (U)***
T-1-1-2-2	18,92	0,0829
H-4-2-2	19,84	0,0989
H-5-2-2	18,70	0,0795
- Control	18,06	0,0703
* T-1-1-2-2	21,25	0,1294
* H-4-2-2	21,44	0,1341
* H-5-2-2	21,67	0,1402
* H-1-1-2-1	20,77	0,1179
* - Control	18,83	0,0814
03-04-54	20,56	0,1134
L-15-1-1	20,20	0,1059
L-15-2-2	17,83	0,0672
R-1-3-2-2	21,75	0,1423

The results clearly show the effect of *vst* promoter induction on the chitinase activity where the induced samples showed higher activity compared to the same samples without induction. For the non-transformed negative control, after induction the activity was higher than before induction which may be due to an endogenous chitinase activity, which can be induced under different stresses. Plants under control of inducable *vst* promoter were higher in activity than plants under control of 35S promoter. In some cases, the chitinase activity showed unexpected lower activity than negative control (clone L-15-2-2).

Results of tobacco transformation are summarized in Table 12.

Table 12. Summary of tobacco transformation, highlighted clones are transformed with pGII35s-Chit construct, the remaining clones are transformed with pGIIvst-Chit construct (+, positive; -, negative; n.t., not tested)

Clone	***PCR***		***RT-PCR***		***Southern***			***Western blot***	***In gel assay***
	Chit	Bar447	Chit	Bar447	Chit	Bar447	NptI		
- Control	-	-	-	-	-	-	-	-	-
R-1-3 (T0)	+	+	+	+	+	+	+	-	-
(T1)	+	+	+	+	+	n.t.	n.t.	+	+
(T2)	+	+	+	+	+	+	+	n.t.	+
L-15 (T0)	+	+	+	+	+	+	+	-	-
(T1)	+	+	+	+	+	n.t.	-	+	+
(T2)	+	+	+	+	+	+	-	n.t.	+
L-14 (T0)	+	+	n.t.	n.t.	+	+	+	-	-
(T1)	+	+	+	+	+	n.t.	+	+	n.t.
(T2)	+	+	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	+
O-1 (T0)	+	+	+	+	+	+	+	-	-
(T1)	+	+	+	+	+	n.t.	-	-	-
(T2)	+	+	+	+	+	+	+	n.t.	-
C-2 (T0)	+	+	+	+	+	+	+	-	-
(T1)	+	+	+	+	+	n.t.	+	+	n.t.
(T2)	+	+	n.t.	n.t.	+	+	-	n.t.	+
F-1 (T0)	+	+	+	+	+	+	+	-	-
(T1)	+	+	+	+	+	n.t.	+	+	n.t.
(T2)	+	+	n.t.	n.t.	+	+	+	n.t.	-
T-1 (T0)	+	+	n.t.	+	+	+	+	-	-
(T1)	+	+	+	+	+	n.t.	+	+	n.t.
(T2)	+	+	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	+
H-4 (T0)	+	+	n.t.	n.t.	+	+	-	-	-
(T1)	+	+	+	+	+	+	+	+	n.t.
(T2)	+	+	n.t.	n.t.	+	+	+	+	+
H-5 (T0)	+	+	n.t.	n.t.	n.t.	n.t.	n.t.	-	-
(T1)	+	+	+	+	+	n.t.	-	+	n.t.
(T2)	+	+	n.t.	n.t.	+	+	-	n.t.	+
H-1 (T0)	+	+	+	+	-	-	-	-	-
(T1)	+	+	+	+	+	n.t.	+	+	+
(T2)	+	+	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	+

5.5 Pea transformation

5.5.1 Transient GUS expression

Transient GUS assays were used to check the capability of pea cv. *Sponsor* for *Agrobacterium* transformation. 150 embryos were co-cultured with the *Agrobacterium* strain EHA101pIBGUS and compared with control embryos. Based on the GUS assays, explants were found to show 100 % GUS expression in explants transformed with GUS gene, while no activity was detected in control explants (Fig. 33)

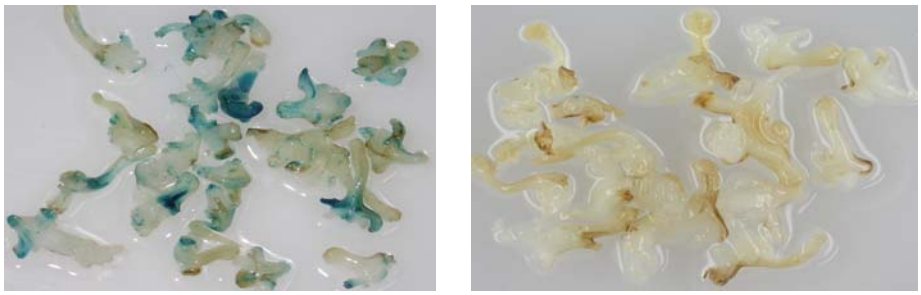

Fig. 33. Transient GUS expression in pea embryos transformed with EHA101pIBGUS (left) and untransformed negative control embryos (right).

5.5.2 Pea transformation with the chitinase gene

Pea transformation (c.v. *Sponsor*) was done according to the modified protocol of Schroeder et al. 1993 and Bean et al. (1997). During co-culture, the explant color was white and white greenish where the color became light to dark green on MST medium although there were explants with green yellowish color. These tissues failed, in most cases, to survive (between 10-20 % of total explants). These explants mostly resulted from longitudinally slicing of the outer part of the embryo, which normally has no meristematic tissue.

In the first and second selection step using 2.5 mg/l and 5 mg/ l, all control explants died, while most transformed explants were green. Between 30-50 % of dead explants were observed and removed subsequently from every subculture. The percentage of dead explants increased with increasing selection pressure to 50 % at 7.5 mg/l PPT and more than 60 % at 10 mg/l PPT. At higher concentrations of PPT (12.5 mg/l and 15 mg/l), only a few clones survived (Table 13) which were still green and growing normally. A good sign for healthy shoots was the green color of the cutting side during subculture. Shoots from these clones were used for in vitro grafting on a rootstock in order to avoid a rooting step, minimize losses during rooting as well as to recover whole plants in a relatively short

time. Therefore, sterile pea seeds of cv. *Sponsor* were germinated on water agar medium (0.4 % plant agar) in dark, the etiolated two-weeks old plantlets were used as rootstock for *in vitro* grafting. The epicotyls were cut horizontally then a vertical cut was done through the stem of the rootstock, which allows insertion of a scion into the cut of rootstock. After making a "V" shape cut on the base of the transgenic scion, it was grafted on the rootstock and the plants were placed in growth room for 10 days until wound callus closed the cutting surface. They were then potted in soil and the pots were sealed with plastic bag to protect them from excessive water loss, the bag was removed gradually to allow for growing of the plant to flower and set seeds. Successfully grafted shoots were potted and covered with a plastic bag and acclimatized gradually. After removing the plastic bag, pots were transferred to the greenhouse to grow normally and set T1 seed (Fig. 34).

Root induction was not successful where there was stagnant growth of the shoots, which became harder without any roots. Nevertheless, when the shoots were subcultured into ½ MS hormone free medium, 27 % of the cultured shoots produced roots within 4-6 weeks. These rooted plants died upon transfer to greenhouse.

The period from inoculation of embryo slices with *Agrobacterium* suspension to get the shoots ready for *in vitro* grafting was between 90-120 days and from grafting to flowering between 45-60 days.

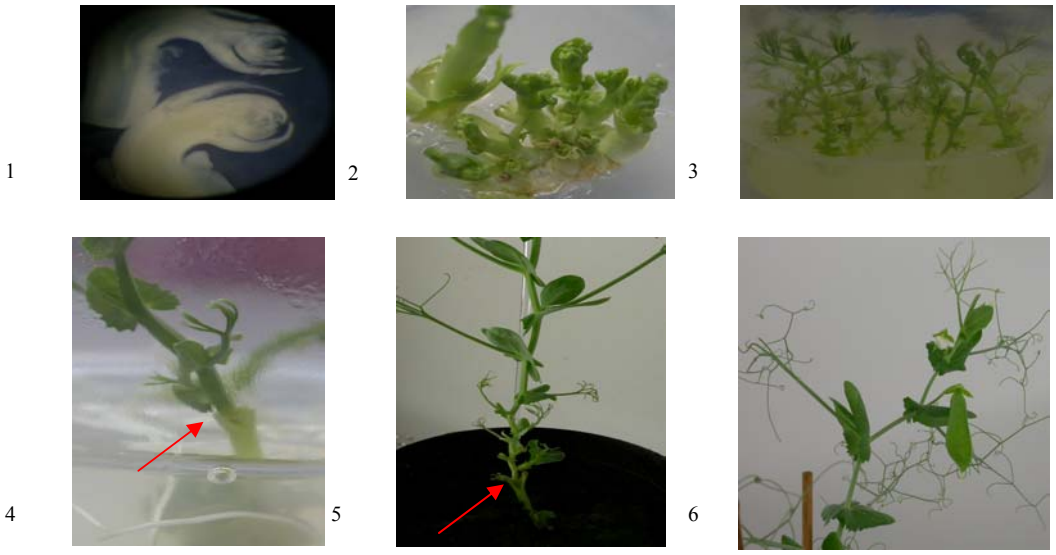

Fig. 34. Procedure used for pea transformation cv. *Sponsor* using *Agrobacterium tumefaciens* EHA105. 1, embryos slices of pea inoculated with *Agrobacterium* suspension; 2 and 3, multiple shoots regenerated on medium supplemented with 5 and 12 mg/l PPT respectively; 4, in vitro grafting of putative transformants on non-transgenic rootstock; 5, grafted plant acclimatized and grown in soil (arrows show the grafting site); 6, grafted shoot showing flower and setting the first pod.

A total of 53 independent transgenic clones were obtained out of 5863 explants from different transformation experiments (Table 12). Out of these, 23 resulted from transformation with the pGII35S-N-Chit plasmid, 19 from transformation with the pGIIvst-N-Chit, 6 were transformed with the pGIIvst-Chit and the remaining 6 were transformed with the pGII35s-Chit plasmid.

Table 13. Results of pea transformation experiments and transformation efficiency.

	Transform-ation code	***Construct***	***No. of explants***	***No. of clones***	***Transformation efficiency %***	
1	02-04	pGII35S-N-Chit	744	7	0.94	*
2	03-04	pGII35S-N-Chit	420	8	1.9	*
3	04-04	pGII35S-N-Chit	400	-	0	*
4	05-04	pGII35S-N-Chit	400	6	1.5	*
5	06-04	pGII35S-N-Chit	631	-	0	+
6	07-04	pGIIvst-N-Chit	400	4	1	*
7	08-04	pGIIvstChit	250	2	0.8	*
8	09-04	pGII35S-N-Chit	550	-	0	+#
11	10-04	pGII35S-Chit	250	-	0	*#
12	11-04	pGIIvst-N-Chit	500	5	1	*
13	12-04	pGIIvstChit	250	-	0	*
14	13-04	pGII35SChit	250	-	0	*
15	14-04	pGIIvst-N-Chit	684	5	0.73	*
16	15-04	pGIIvst-N-Chit	420	4	0.95	+
17	16-04	pGIIvstChit	350	2	0.57	*
18	17-04	pGII35SChit	350	2	0.57	*
19	18-04	pGIIvst-N-Chit	600	-	0	*
20	19-04	pGII35SChit	350	4	1.4	*
21	20-04	pGIIvstChit	350	2	0.57	*
22	11-04N	pGII35S-N-Chit	645	2	0.31	+
Total			8794	53	0.6	
	Sub-total		5863	53	0.9	

***** Experiment done without help.

+ Experiment done with help of students.

Contamination.

5.5.3 Molecular characterization of transformants

5.5.3.1 Detection of T-DNA integration by PCR

Stable integration of T-DNA into genomic DNA of T0 transformants of pea was confirmed by PCR using different primer combinations to detect the chitinase and the *bar* gene. The results clearly indicate and confirm the successful integration of chitinase and *bar* gene into genomic DNA of transformed peas (Fig. 35 A and B). Many clones from different transformation experiments were positive as shown in the Table 14.

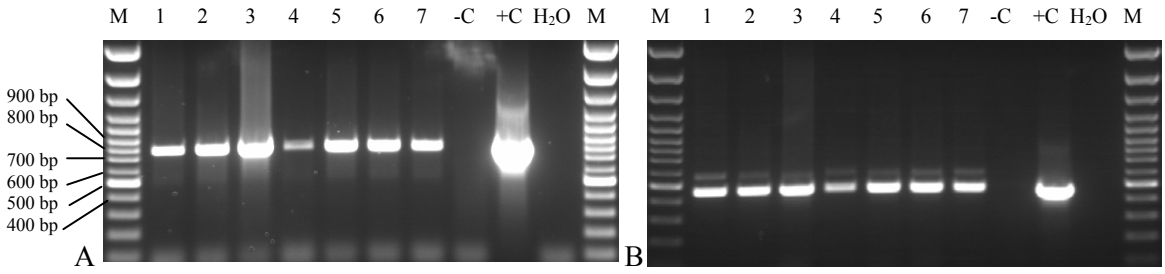

Fig. 35. PCR analysis of T0 transformants from pea. A, using chitinase primers (expected product size 750 bp); B, bar447 primer (expected product size 450 bp); lanes 1, and 2 from transformation 02-04; lanes 3 and 4 from transformation 03-04; lane 5, transformation 07-04; lane 6, transformation 14-04; lane 7, transformation 15-04; -C, untransformed negative control pea; +C, plasmid pGII35s-N-chit as positive control; H_2O, water control; M, 100 bp DNA ladder molecular weight marker.

Table 14. Few selected T0 clones from pea transformation with pGII35S-N-chit and pGIIvst-N-Chit (highlighted).

T0 clone	***Leaf paint***	***PCR***		***T1 seeds***	***Segregation***
		Chitinase	Bar		
02-04-7-1	+	+	+	6	1:1
02-04-7-4	+	+	+	5	4:1
02-04-7-5	+	+	+	3	3:0
02-04-7-7	+	+	+	5	4:1
02-04-7-10	+	+	.	2	1:1
03-04-1-3	+	+	+	45	3:1* (X^2= 0.6)
03-04-1-4	+	+	+	23	3:1* (X^2= 0.36)
07-04-4-1	+	+	+	12	3:1* (X^2= 1.17)
07-04-4-4	+	+	+	8	3:1* (X^2= 0)
14-04-2-1	+	+	+	4	1:1
14-04-2-4	+	+	+	4	1:0
14-04-2-6	+	+	+	3	1:0

*Significant at p = 0.05 and P = 0.01, tabulated value X^2 =3.84 and X^2= 6.64, respectively.

T1 seeds were collected and then germinated in the greenhouse for further characterization and production of the following transgenic progenies. Unfortunately, the number of seeds produced per plant was low (2-5 seeds) due to high temperature in the greenhouse (summer 2005), which limited the segregation analysis. PCR was used to investigate the presence and segregation of chitinase and *bar* genes in T1 progenies (Fig. 36).

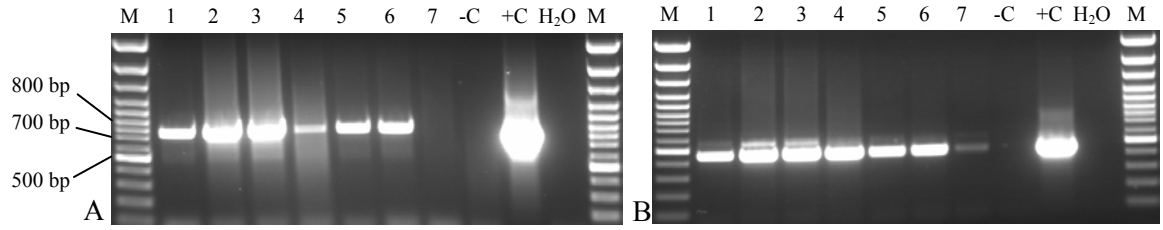

Fig. 36. PCR analysis of T1 transformants from pea. A, using chitinase primers (product size 750 bp); B, bar447 primer (product size 450 bp); lanes 1, and 2 clones 02-04-7-1,1 and 02-04-7-4,1 respectively; lanes 3 and 4 clones 03-04-1-3,30 and 03-04-1-4-,16 respectively; lane 5, clone 07-04-4-1,13; lane 6, clone 14-04-2-4,1; lane 7, clone 15-04-1-1,1; -C, untransformed negative control pea plant; +C, plasmid pGII35S-N-chit as positive control; H_2O, water control; M, 100 bp DNA ladder molecular weight marker.

Transformants from transformation experiments 02-04, 03-04, 07-04 and 14-04 proceeded further to produce the T2 generation. The analyses of T2 transformants using PCR shows presence and stable integration of transgenes in the progenies of positive transformants of T1 plants. Two primer pairs were used to amplify the chitinase gene and internal control (HMG) gene (Fig. 37). No backbone integration was detected using nptI primers. Results of two lines from transformation 02-04 are shown in Table 15.

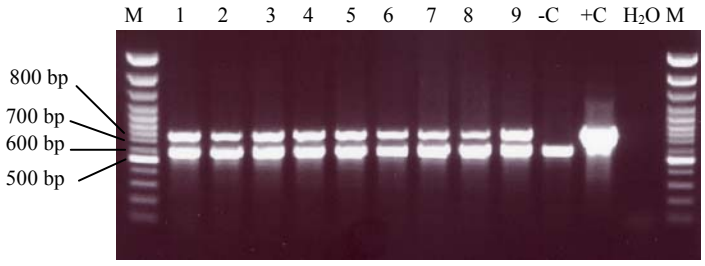

Fig. 37. Confirmation of stable inheritance and integration of chitinase gene (750 bp) in T2 progeny of transformation 14-04 (lanes 1 & 2); 15-04 (lane 3); 02-04 (lanes 4, 5, & 6); 03-04 (lane 7 & 8) and 07-04 (lane 9); -C, untransformed negative control pea plant; +C, plasmid pGII35S-N-chit as positive control; M, 100 bp DNA ladder molecular weight marker.

Table 15. Results of few clones from transformation 02-04 in T2

T1 Clone	*T2 Seeds*	*LB test*	*PCR*		*Segregation*	*Homozygosity %* [6]
			Chit	*Bar*		
02-04-7-1,1	10	10	10	10	0	89.87
02-04-7-1,2	18	18	18	18	0	98.88
02-04-7-1,4	10	8	6	9		73.74
02-04-7-4,1	9	9	9	9	0	86.94
02-04-7-4,2	10	9	9	9	0	86.94
02-04-7-4,3	8	8	8	8	0	83.81

5.5.3.2 Southern blot analysis

Copy number and integration patterns of stable transformants were investigated in T0, T1 and T2 generations using Southern blot analysis where gDNA was digested either with *Eco*RI which cuts in the genomic DNA (not presented in the plasmid vector) or with *Xba*I which cuts once in T-DNA. Results of Southern blot analysis are summarized in the Table 16 and Fig. 38. Single copy insertions were observed in most of the tested pea clones, but two copies were also shown in some clones like 03-04-1-3 and 07-04.

Clone 03-04-1-3 shows two copies of chitinase and bar genes in T0 progeny and in its offspring clone 03-04-1-3,46 whereas the other tested clones 6, 9, 24, and 30 contain one copy only due to segregation. Clones 02-04-7-1,1; 02-04-7-1,2 and 02-04-7-1,4 showed one copy of chitinase and bar while clones 02-04-7-1,3; 02-04-7-1,5 and 02-04-7-1,6 did not show any integration of the chitinase or bar gene. These findings were in agreement with PCR results suggesting a 1:1 segregation in the T1 progeny. No backbone sequence was detected using nptI probe in any clone subjected to Southern blot. It was difficult to test all T0 clones using Southern due to limited plant material suitable for large scale DNA isolation.

6 Calculated from formola shown in 4.10 (Materials and Methods)

Table 16. Transgene copy numbers resulted from Southern blot analysis of transgenic peas.

Clone	*EcoRI*			*XbaI*
	T0	*T1*	*T2*	*T1*
02-04-1-1,1		1		
02-04-7-1,1		1		
02-04-7-1,1,2			1	
02-04-7-1,2		1		
02-04-7-1,2,1			1	
02-04-7-1,4		1		
02-04-7-1,4,9			1	
02-04-7-4,1		1		
03-04-1-3	2			
03-04-1-3,46		2		2
03-04-1-3,30		1		2
03-04-1-3,24		1		
03-04-1-3,6		1		
03-04-1-3,9		1		
03-04-1-4	1			
03-04-1-4,16		1		
03-04-1-1,1		1		
07-04-4-1,7		1		1
07-04-4-2,5		2		2
07-04-2-1,1		2		

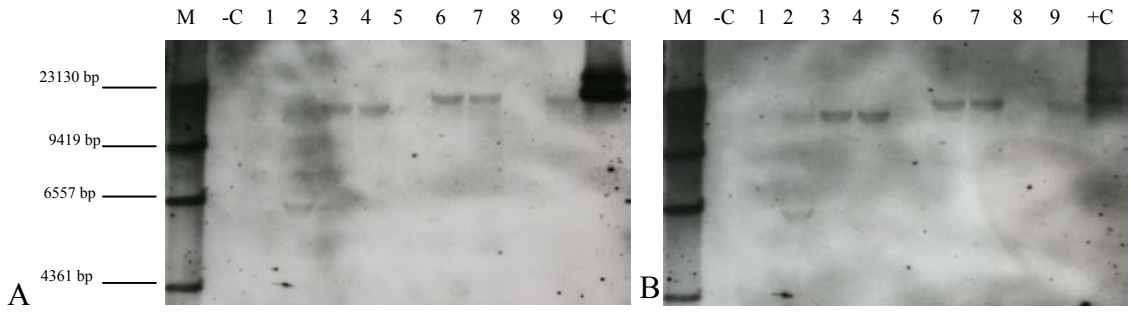

Fig. 38. Southern blot analysis (digested with *Eco*RI) of pea gDNA isolated from T0 and T1 lines. A, blot probed with chitinase probe; B, blot probed with bar 447 probe; +C, positive control; -C, untransformed negative pea plant; lanes 1, clone 03-04-3-1,1; Lane 2, clone 03-04-1-3; lane 3, clone 03-04-1-4; line 4 clone 03-04-1-1,1; lane 5, T0 of transformation 08-04-2-1; lanes 6, 7, 8, and 9, T1 progeny of clones 02-04-7-1; M, Dig Marker.

5.5.3.3 RT-PCR

At the transcription level using, the chitinase and bar transcripts were detected using RT-PCR and sequencing the PCR product. A BLAST search at NCBI database using the sequencing results found 100 % homology to two matches. The first one was 100 % identical to the signal peptide of basic chitinase from *Arabidopsis* (Fig. 39), while the second one was identical to the mature protein of *Streptomyces* chitinase *Chit30* gene (Fig.40).

```
>gi|30682210|ref|NM_112085.2|  Arabidopsis thaliana ATHCHIB (BASIC
CHITINASE); chitinase AT3G12500
(ATHCHIB) mRNA, complete cds Length=1107

 Score =  121 bits (61),  Expect = 4e-24  Identities = 61/61 (100%), Gaps = 0/61
(0%)  Strand=Plus/Plus

Query  8 ATGAAGACTAATCTTTTTCTCTTTCTCATCTTTTCACTTCTCCTATCATTATCCTCGGCCG 68
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 20 ATGAAGACTAATCTTTTTCTCTTTCTCATCTTTTCACTTCTCCTATCATTATCCTCGGCCG 80
```

Fig. 39. Signal peptide alignment of PCR derived cDNA products sequence in BLAST program of NCBI database.

```
>gi|4456813|emb|AJ133186.1|SOL133186  Streptomyces olivaceoviridis ATCC11238
chi30 gene Length=1199

 Score = 1594 bits (804), Expect = 0.0
 Identities = 804/804 (100%), Gaps = 0/804 (0%) Strand=Plus/Plus

Query   65   GCCGCGGCCTGTTCGAGCTACCCGAGCTGGGTCGCCGGCAGGTCCTACGCGGCCGGTGAC  124
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  286   GCCGCGGCCTGTTCGAGCTACCCGAGCTGGGTCGCCGGCAGGTCCTACGCGGCCGGTGAC  345

Query  125   ATCGTCCGCTACACGGACGGCAAGGCGTACATCGCCGAGCACGCCAACCCGGGTTACGAC  184
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  346   ATCGTCCGCTACACGGACGGCAAGGCGTACATCGCCGAGCACGCCAACCCGGGTTACGAC  405

Query  185   CCGACCATCAGCACCTGGTACTGGGAGCCGTACGCCTGCGACGGCGGGTCCGGGACGCCG  244
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  406   CCGACCATCAGCACCTGGTACTGGGAGCCGTACGCCTGCGACGGCGGGTCCGGGACGCCG  465

Query  245   GTCGGCACCTTCGTGGTGACCGAGGCCCAGTTCAACCAGATGTTCCCGAACCGGAACTCC  304
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  466   GTCGGCACCTTCGTGGTGACCGAGGCCCAGTTCAACCAGATGTTCCCGAACCGGAACTCC  525

Query  305   TTCTACAGCTACAGCGGACTCACCGCCGCGCTCAGCGCCTACCCCGGCTTCGCGAACACC  364
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  526   TTCTACAGCTACAGCGGACTCACCGCCGCGCTCAGCGCCTACCCCGGCTTCGCGAACACC  585

Query  365   GGCAGCGACACCACCAAGAAGCAGGAGGCCGCGGCCTTCCTCGCCAACGTCAGCCACGAG  424
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  586   GGCAGCGACACCACCAAGAAGCAGGAGGCCGCGGCCTTCCTCGCCAACGTCAGCCACGAG  645

Query  425   ACCGGCGGCCTGGTGCACGTGGTCGAGCAGAACCAGGCCAACTACCCGCACTACTGCGAC  484
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  646   ACCGGCGGCCTGGTGCACGTGGTCGAGCAGAACCAGGCCAACTACCCGCACTACTGCGAC  705

Query  485   TGGAGCCGGCCGTACGGCTGCCCGGCGGGCCAGGCGGCCTACTACGGGCGCGGCCCGATC  544
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  706   TGGAGCCGGCCGTACGGCTGCCCGGCGGGCCAGGCGGCCTACTACGGGCGCGGCCCGATC  765
```

```
Query  545   CAGCTCAGCTGGAACTTCAACTACAAGGCCGCGGGCGACGCGCTCGGCATCGACCTGCTG  604
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  766   CAGCTCAGCTGGAACTTCAACTACAAGGCCGCGGGCGACGCGCTCGGCATCGACCTGCTG  825

Query  605   AACAGCCCCTGGCTGGTCGAGCGCGACTCGGCCGTCGCCTGGAAGACCGCGCTGTGGTAC  664
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  826   AACAGCCCCTGGCTGGTCGAGCGCGACTCGGCCGTCGCCTGGAAGACCGCGCTGTGGTAC  885

Query  665   TGGAACACCCAGACCGGCCCCGGCACCATGACCCCCCACAACGCCATGGTCAACGGCGCG  724
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  886   TGGAACACCCAGACCGGCCCCGGCACCATGACCCCCCACAACGCCATGGTCAACGGCGCG  945

Query  725   GGCTTCGGCCAGACCATCCGCTCCATCAACGGCTCCCTGGAGTGCGACGGCAAGAACCCG  784
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  946   GGCTTCGGCCAGACCATCCGCTCCATCAACGGCTCCCTGGAGTGCGACGGCAAGAACCCG  1005

Query  785   GCGCAGGTCCAGAGCCGCGTGAACAACTACCAGCGGTTCACCCAGATCCTCGGAGTCTCC  844
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1006  GCGCAGGTCCAGAGCCGCGTGAACAACTACCAGCGGTTCACCCAGATCCTCGGAGTCTCC  1065

Query  845   CCGGGCGGCAACCTCTACTGCTGA  868
             ||||||||||||||||||||||||
Sbjct  1066  CCGGGCGGCAACCTCTACTGCTGA  1089
```

Fig. 40. Chitinase30 mature protein alignment of PCR derived cDNA products sequence in BLAST program of NCBI database.

5.5.4 Functional analysis (leaf paint assay)

Leaf paint analysis provides evidence whether the level of *bar* expression and PAT enzyme activity is sufficient to confer resistance to BASTA® application. One leaflet of each pair from a transformed and a non-transformed control pea were treated with 600 mg/l BASTA® using a small brush. The effect of treatment can be seen after 2-3 days on non-transformed plants where the treated leaflets start wilting. After one week, it became clear as the non-transformed plants showed necrotic symptoms and the whole treated leaflet turned yellow and died. In contrast, the transgenic plants stayed green, healthy and thus showed tolerance to BASTA® application. There was no difference in case of transgenic plants between treated and untreated leaflet as well as untreated leaflet of non-transformed negative control plant, which were left as internal control for the treatment, since BASTA® is a contact herbicide and it will affect only the treated part, as BASTA® is not translocated throughout the plant (Fig. 41).

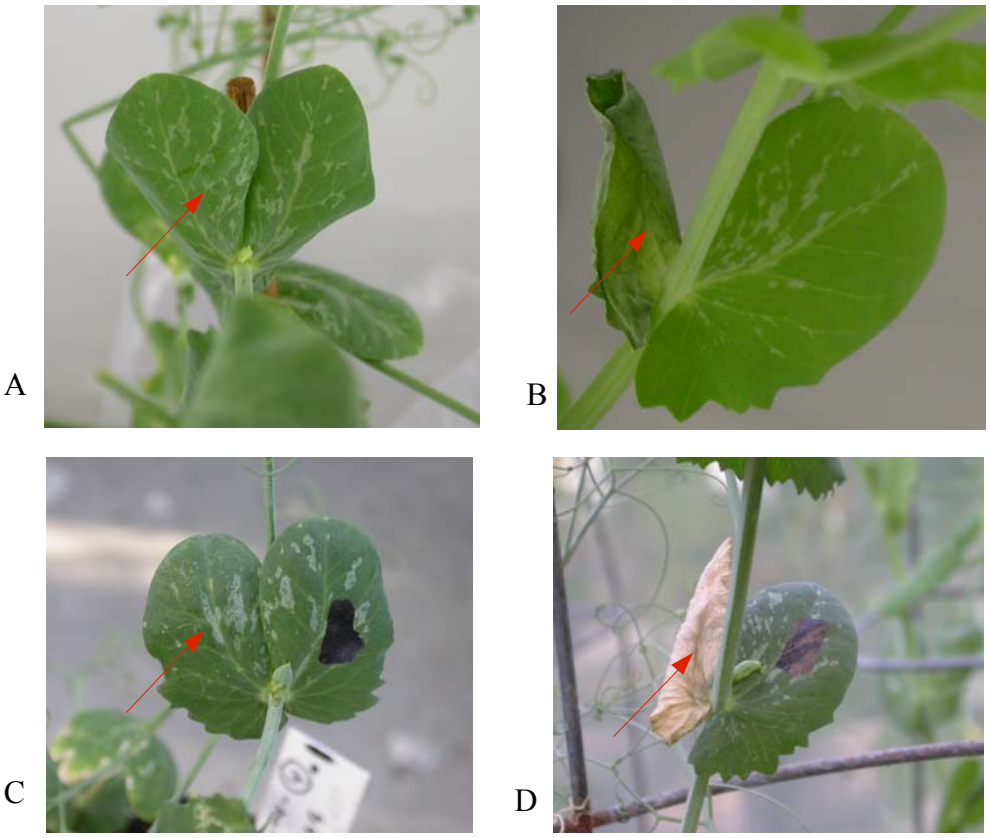

Fig. 41. Leaf paint assay on pea plants cv. *Sponsor* 1 week (A and B) and 4 weeks (C and D) after 600 mg/l BASTA® application. A, transgenic pea plant (+); B, not-transformed negative control pea plant (-) arrow indicates the treated leaflet presenting the treatment effect on the control plant comparing it with the tolerance of transgenic plant expressing PAT.

Although chitinase and bar genes are closely linked on the same transgene, not all clones were PCR positive for both chitinase and bar showed PPT-resistance when leaf paint was applied. For example the progeny of clone 14-04-2-4, which was transformed with pGIIvst-N-Chit construct, proved positive results in PCR using chitinase and bar primers but had a negative leaf paint. Out of 34 plants 17 plants showed negative responses for leaf paint (of these 15 plants were PCR positive for chitinase and bar genes), 5 plants were in between showing a little necroses (they were PCR positive) and 12 plants were leaf paint positive and BASTA® resistant.

5.5.5 Biochemical characterization of transformants

5.5.5.1 Western blot

The accumulation of chitinase in transgenic pea plants was examined by Western blot and immunostain analysis to prove the translation and accumulation of active protein in different clones. Chitinase was clearly detected in the transgenic T0 clones (as seen in the

table below) and no signals could be detected in the untransformed negative control pea plant (Fig. 42).

Experiment	*T0 clones*
02-04	7-2, 7-4, 7-5, 6-1, 3-1
03-04	1-2, 1-3, 3-1
07-04	4-1
14-04	2-6

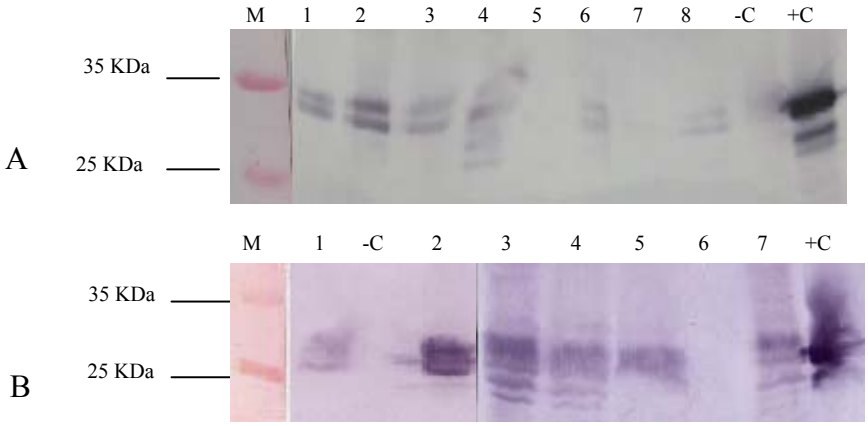

Fig. 42. Western blot analysis of chitinase accumulation in pea transformed with plasmid pGII35S-N-Chit. (A) T0 pea transformants: lanes 1, 2, 3, 4, 5 and 6 different clones of 02-04 (7-5/7-4/7-2/3-1/1-1/6-1 respectively); lane 7, clone 03-04-1-5; lane 8, clone 14-04-2-6; -C, untransformed negative control pea plant; +C, chit30 standard from *Streptomyces* used as positive control; (B) pea T1 clones; lane 1, clone 07-04-4-1,12; lanes 2, 3, and 4, clone 03-04-1-3 ,38/,34/, 46 respectively; lane 5, 02-04-7-10,1; lane 6, blank; lane 7, 02-04-3-1,1; M, Fermentas protein standard.

5.5.5.2 In-gel Chitinase enzyme activity

Chitinase activity was detectable in the T0 transformants of the clones showing positive signals in western blot analysis (Fig. 43 A), whereas there was no chitinolytic activity for the samples which did not exhibit any positive signal in Western blot and immunostain. Different bands, which have chitinase activity, could easily be detected. Three of them were dominant and have strong activity in the range of 30 and 50 KDa compared to untransformed negative controls, which show only one band at ~ 30 KDa (Figure 43 A). The chitinase activity was difficult to detect when reducing agents like 2-mercaptoethanol or DTT were used in protein extraction or sample buffer (Fig. 43 B), and a single band could be detected instead of multiple bands in these cases.

Chitinolytic activity was detected also in T1 and T2 progenies (Fig. 43 C). The

effectiveness of the plant originating inducible promoter (*vst*) from grape was also analysed in driving the chitinase expression following the induction of the promoter with UV light for 5 min and extraction of protein 24-48 h after induction (Fig. 43 D).

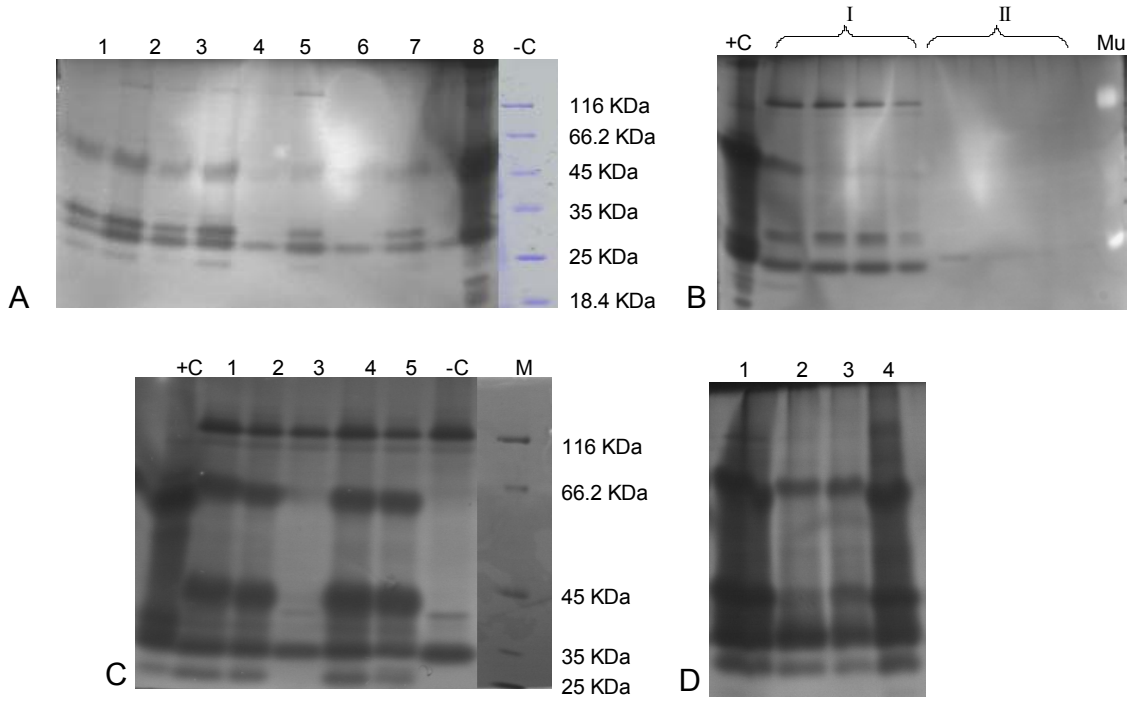

Fig. 43. Detection of chitinolytic activity of chitinase after SDS-PAGE electrophoresis of pea. A, T0 pea transformants: lanes 1, 2, 3, 4, 5 and 6 different clones of 02-04 (7-5/7-4/7-2/3-1/1-1/6-1 respectively); lane 7, clone 03-04-1-5; lane 8, clone 14-04-2-6; -C, untransformed negative control pea plant; +C, chit30 standard from *Streptomyces* used as positive control; M, Fermantas protein standard; B, comparison between non-reducing (II) and reducing (I) conditions of different samples; Mu, protein from *Mucor* sp.; C, T1 pea transformants of different clones; D, T1 pea transformants with vst promoter induced with UV light.

5.5.5.3 Chitinase enzyme activity

Enzyme activity was measured according to Stephan and Wolf (1990) as mentioned earlier (5.4.3). The experiments were carried out in triplicate for the samples and blanks, the difference between sample OD and blank OD gave the activity. Results are shown in Table 17 and Fig. 44.

Table 17. Results obtained from chitinase enzyme activity measurement of crude extracts of pea at OD550. Protein extracts from plants under vst promoter are highlighted and UV induced plants are marked with *.

Sample	14-04-2-4,2,6	14-04-2-6,3,2	14-04-2-4,4,8	15-04-1-2,2	07-04-4-4,8,3	- Control
OD	0,10	0,11	0,13	0,13	0,12	0,12
Sample	07-04-4-2,5,1	14-04-2-4,2,6*	14-04-2-6,3,2*	14-04-2-4,4,8*	15-04-1-2,2*	- Control*
OD	0,12	0,13	0,16	0,23	0,29	0,19
Sample	07-04-4-2,5,1*	03-04-1-3,41,3	02-04-7-5,3,6	02-04-7-4,1,1	03-04-1-3,3,1	03-04-1-3,6,2
OD	0,19	0,13	0,14	0,30	0,13	0,21
Sample	02-04-7-5,1,7					
OD	0,16					

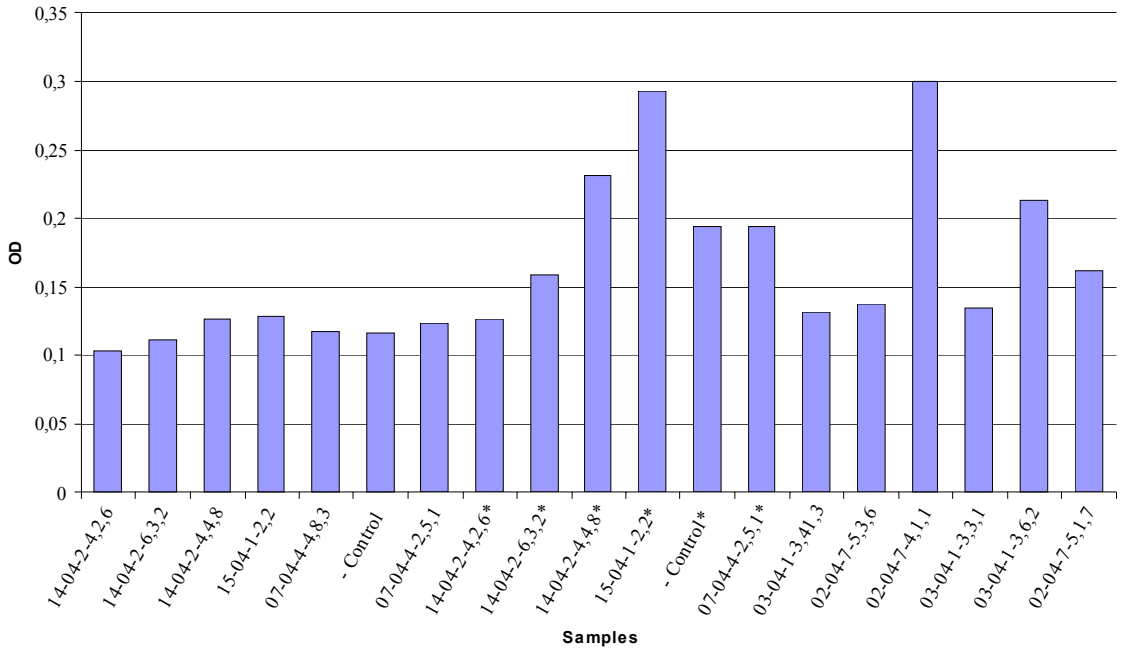

Fig. 44. Colorimetric chitinase activity assay of different pea samples using CM-chitin-RBV as substrate. UV induced plants a re marked with *.

5.5.5.4 Gel diffusion assay

A standard curve for chitinase activity was prepared using dilutions of *Streptomyces griseus* chitinase at different concentrations of 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, 0.01, and 0.001 units. Aliquots of 10 µg total protein were applied (Fig. 45). Afterwards, visualization of the hydrolysis activity was done and the diameter of the dark halos was measured, the dark halos indicated the degradation of the substrate. The diameters of halos were plotted against the logarithmic value of chitinase activity (Fig. 46), and then activities of different samples were calculated through regression analysis (Table 18).

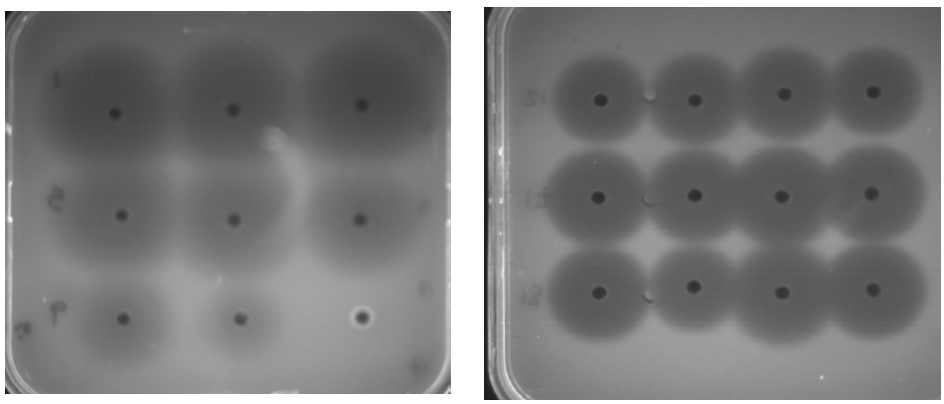

Fig. 45. Gel diffusion assay showing chitinase activity viewed and photographed under UV light. Standard was prepared using dilution of chitinase from *Streptomyces griseus* (left) at concentrations 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, 0.01, 0.001 unit and buffer. Different samples (right).

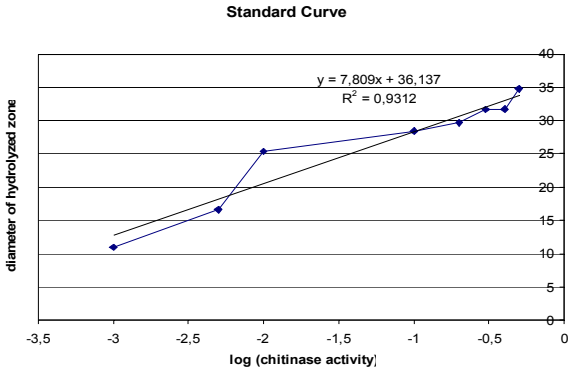

Fig. 46. Regression analysis of chitinase activity of standard in units against halos diameter in mm. The equation for calculating trend line is y= 7.809x + 36.137.

Table 18. Chitinase activity of different pea samples transformed with pGIIvst-N-Chit construct (highlighted) before UV light induction and after induction (marked with *), and pGII35S-N-Chit construct.

Sample	***Diameter of halos (mm)***	***Chitinase activity (U)***	***Significant (5 %) #***
14-04-2-4,2,8	22,30	0,1700	bcde
15-04-1-2,1	17,56	0,0927	i
07-04-4-4,7,1	17,97	0,0976	hi
07-04-4-4,2,3	21,40	0,1514	bcdefg
- Control	20,41	0,1335	bcdefgh
* 14-04-2-4,2,8	23,64	0,2019	abc
* 15-04-1-2,1	25,37	0,2519	a
* 07-04-4-4,7,1	23,93	0,2095	ab
* 07-04-4-4,2,3	23,03	0,1866	bcd
* 14-04-2-1,2,2	23,64	0,2019	abc
* - Control	21,66	0,1567	bcdef
03-04-1-3,35,3	19,72	0,1221	efghi
03-04-1-3,5,2	17,99	0,0978	hi
03-04-1-3,6	21,76	0,1588	bcdef
03-04-1-1,2,19	17,99	0,0978	hi
03-04-3-1,1,11,8	19,45	0,11797	efghi
03-04-1-3,46,8	20,58	0,1364	bcdefgh
02-04-7-4,1,1	18,35	0,1025	ghi
02-04-7-4,3,6	18,94	0,1106	fghi
02-04-7-4,1,7	20,92	0,1425	bcdefgh
02-04-7-5,2,1	20,30	0,1315	cdefgh
02-04-7-4,3,2	21,62	0,1557	bcdef
02-04-7-1,2,2	22,30	0,1701	bcde

Values followed by different letters are significantly different ($\propto$=0.05).

The results clearly show the effect of *vst* promoter induction on the chitinase activity where the induced samples showed higher activity compared to the same samples without induction. For the non-transformed negative control, after induction the activity was higher than before induction which may be due to the endogenous chitinase activity, which can be induced under different stresses. Plants with the chitinase gene under control of induced *vst* promoter were higher in activity than plants under control of 35S promoter. In some cases the chitinase activity showed unexpected lower activity than negative control (such as clones 03-04-1-1,2,19; 03-04-1-3,35,3 and 02-04-7-4,1,1).

5.5.6 *In vitro* bioassay

Trichoderma harzianum (T12 strain) was cultured on PDA medium at 25 °C, and different crude protein extracts were applied in the wells, which were prepared using a 3 mm borer in the region around hyphal growth. The plates were incubated at 25 °C for 24 h, during which the hyphae grew outwards from the centre. Hyphal growth inhibition of *T. harzianum* was observed at 8 h and 24 h after treatment.

5.5.6.1 Tobacco experiments

Chitinase (crude protein extract) from different samples of tobacco could inhibit *T. harzianum* hyphae growth as can be seen in Fig. 47, where the inhibition in wells 1, 2, 3, 4, 6, and 7 could be detected after 8 hours (Fig. 47, A), especially in the area around the well facing hyphal growth. After 24 hours the inhibition became more clear for the same samples (Fig. 47, B), while no inhibition was detected in case of non-transformed negative control tobacco plant (well 8) or in case of samples containing only buffer (well 9). Well 10 contained chitinase from *Streptomyces griseus* (Sigma, C6137), but no activity was detected. After 30 hours, there was no difference among samples, since the hyphae could grow around the wells, in spite of a very clear zone without growth around some wells (1-2 mm). *Vst* promoter efficiency in driving chitinase expression as inducible promoter of plant origin was studied in order to test its induction. Therefore, samples were collected from the greenhouse and induced with UV light for 5 min and then incubated in a growth room for 30-40 h. Crude protein was extracted from induced and non-induced plants. All samples were applied for in vitro hyphae inhibition assay. *Vst* promoter proved remarkable efficient in chitinase expression in induced samples where it could inhibit the hyphal growth (Fig. 48 A 1, 2, 3, 4, 5 and 6) compared to non-induced samples (Fig. 48 B 1, 2, 3 and 4) which showed no effect on the growth of *Trichoderma* as in the negative induced

and non-induced controls (Fig. 48 D and C respectively).

The two different constructs pGII35S-Chit and pGII35S-N-Chit were also compared in the ability to inhibit the fungal growth. Protein extracts from plants growing in greenhouse from both constructs inhibited the hyphal growth and showed no difference (Fig. 47, A).

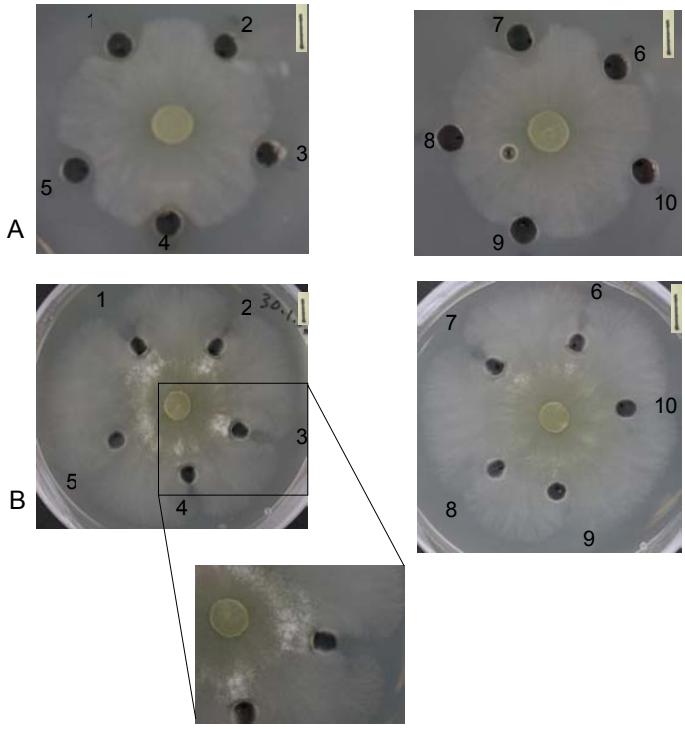

Fig. 47. *In vitro* bioassay of *T. harzianum* hyphae growth inhibition using crude protein extracts of tobacco. A, assay after 8 h from treatment; B, after 24 h; well 1, extract from pGII35SChit; wells 2, 3, and 7, extracts from pGIIvstChit; wells 4, 5, and 6, extracts from pGII35S-N-Chit; well 8, extract from not-transformed control tobacco plant; well 9, Na-act buffer only; well10, chitinase from *Streptomyces* (sigma). Arrows show the region of inhibition which can be detected after over growth of hyphae after 30 h. Bar = 1cm.

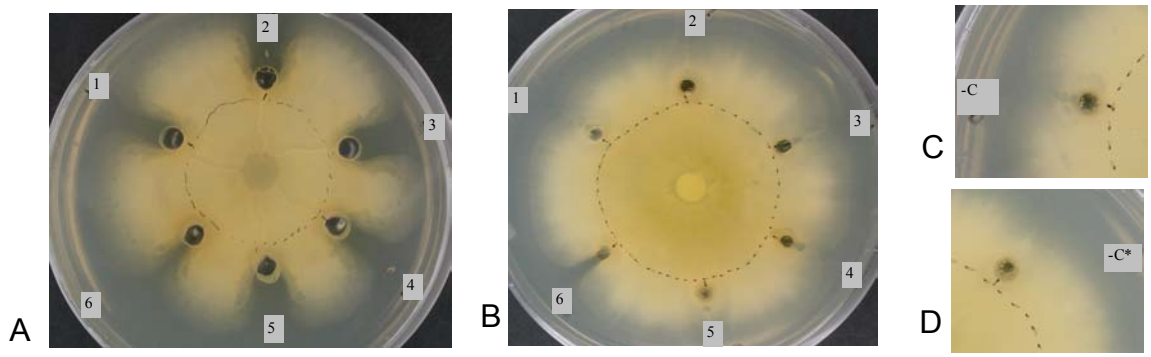

Fig. 48. Comparison of promoter activity on *T. harzianum* hyphal growth *in vitro* bioassay using crude protein extracts of tobacco plants transformed with pGIIvst-Chit construct. A, crude extract from plants induced with UV light for 5 min. to induce vst promoter; B, crude extracts from plants without induction using the same plants as in treatment A. well 1, clone C-2-2-2; well 2, clone H-1-1-2-2; well 3, H-4-2-2; well 4, H-1-1-2-1; C, extract from non-transformed control tobacco plant; D, extract from non-transformed control tobacco plant induced with UV.

5.5.6.2 Tobacco cell culture

Crude extracts from tobacco cell cultures derived from plants transformed with constructs of pGII35S-Chit and pGII35S-N-Chit were prepared from cells and medium and used for the hyphal inhibition bioassay of *T. harzianum*. Strong effect could be detected using crude extracts of the cells, whereas no activity was detected using samples from the medium (Fig. 49).

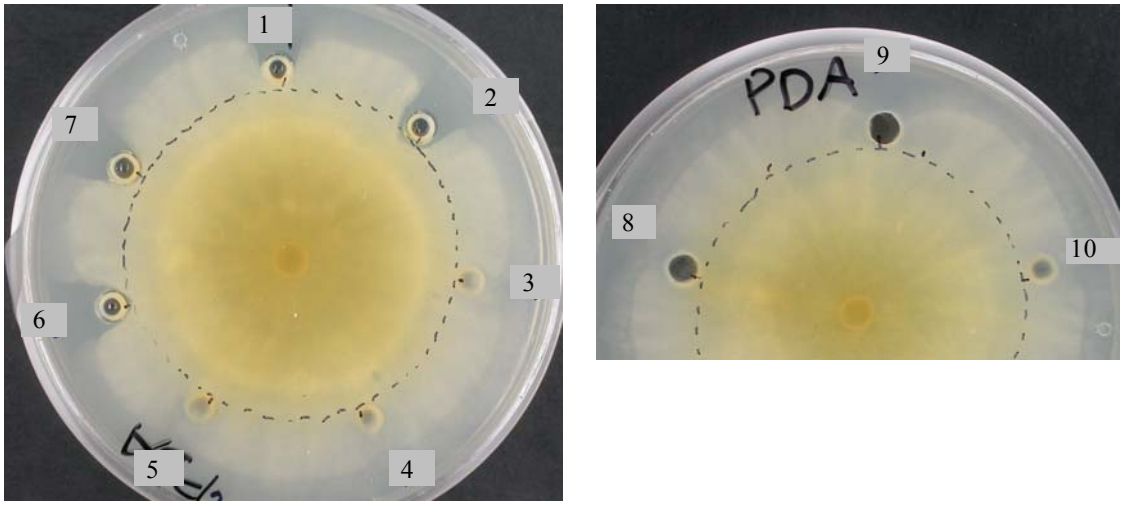

Fig. 49. Tobacco cell culture inhibition effect of crude protein extract of two different constructs pGII35S-Chit (wells 1, 2, and 3) and pGII35S-N-Chit (wells 4, 5, 6 and 7) extracted from cells (wells 1, 2, 6, 7 and 8) and from medium (wells 3, 4, 5 and 10) on *T. harzianum* hyphae growth inhibition. Wells 8 and 10 contains crude extracts from non-transformed control tobacco plant, well 9, contains buffer.

5.5.6.3 Pea experiments

Crude extracts from different pea transformants harboring constructs pGII35S-N-Chit and pGIIvst-N-Chit were tested for their inhibiting activity compared to non-transformed plant crude extracts. Extracts containing the recombinant chitinase could inhibit the extension of fungal mycelium (Fig. 50).

The applied samples are: 1. (03-04-1-5,1); 2. (02-04-7-6,1); 3. (02-04-7-6,3); 4. (14-04-2-3,1); 5. (03-04-3-3,1); 6. (02-04-7-1,5,4); 7. (15-04-1-1,1); 8. (03-04-1-2,1,5,1); 9. (03-04-1-2,1,5,2); 10. (02-04-7-1,2,2); 11. (03-04-1-3,6); 12. (03-04-1-4,8); 13. (02-04-7-1,1,1); 14. (11-04-1-1,2,17); 15. (non-transformed – control); 16. (Na-acet. buffer).

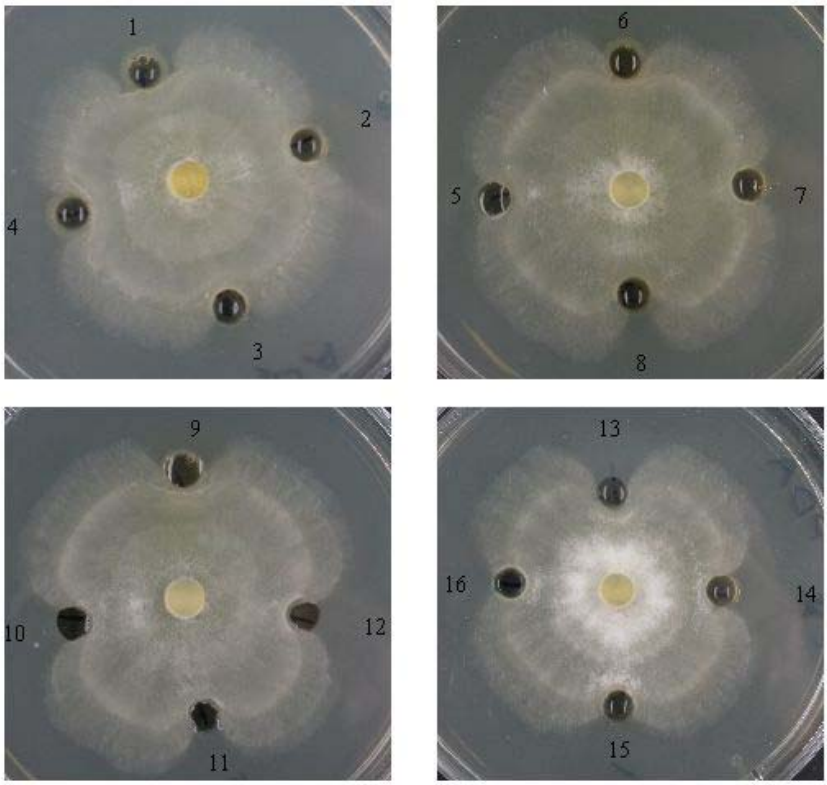

Fig. 50. In vitro assay of *T. harzianum* hyphae growth inhibition using crude protein extracts from different transformants of pea (for sample number see the text above).

In order to study the effect of crude extract on hyphae extension, the spores of *Trichoderma harzianum* were collected in water and the concentration of spores was adjusted to 10^5 spores/ml. 10 µl spore suspension was mixed with 10 µl protein crude extract and incubated overnight at RT. The effect of crude extracts on spore germination was examined under a light microscope.

Crude extract of non-transformed negative control did not show any effect on the germination of spores, which could produce normal hyphae, whereas crude extracts from transformed plants showed inhibition and lowering the germination of the spores, where the spores became enlarged and the elongation of germination hyphae was shorter (Fig. 51).

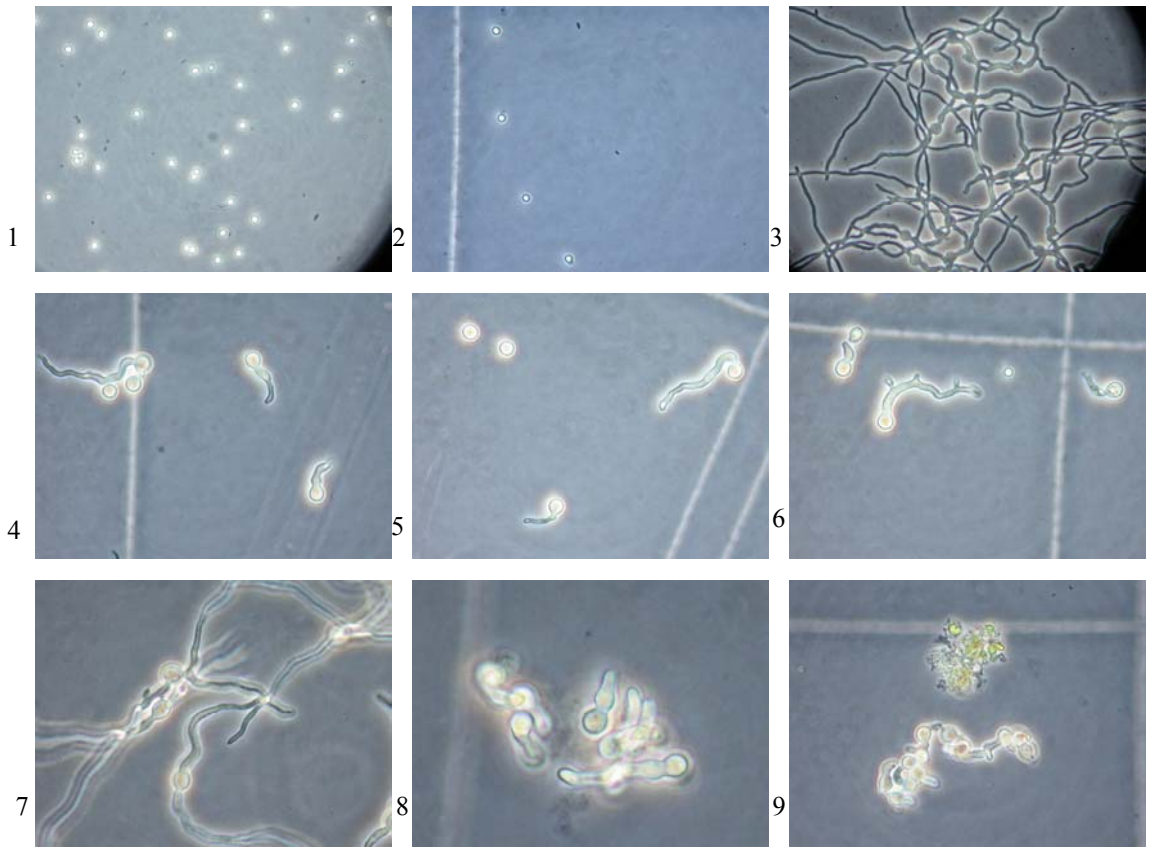

Fig. 51. Effect of different crude extracts on spore germination of *T. harzianum* under the light microscope (x40). 1, spores after isolation without treatment; 2, sporse mixed with *Streptomyces griseus* chitinase; 3, extract from non-transformed tobacco plant; 4, extract from transformed tobacco plant with pGIIvst-Chit; 5 and 6, extract from transformed tobacco with pGII35S-Chit; 7, extract from non-transformed pea plant; 8 and 9 extracts from transformed pea plants.

5.5.7 2-D gel electrophoresis

2-D gel electrophoresis is used to separate protein mixture according to their isoelectric point in a first dimension and the molecular weight in the second dimension. This technique was mainly used to distinguish between endogenous chitinases and recombinant enzyme using silver and Coomassie blue staining applied after the second dimension. It was very difficult to identify the spots correlated with chitinase in transformed and untransformed plant as a huge amount of spots was present in both gels due to the presence of many up-regulated and down-regulated genes.

Chitinase could be detected only after western blot analysis and immunostain, as antibody detection is much more sensitive. First, we used protein from induced and non-induced *E. coli* cells harboring the expression vector pUChit30 (a derivative of pUC18) in order to study the isoforms of chitinase. Four spots were detected at 30 KDa according to isoelectric focusing (IEF) and one smaller spot with a molecular weight around 28 KDa

which could be the mature protein without signal peptide (Fig. 52, A). No spots could be detected in non-induced *E. coli.*

Protein preparations from tobacco and pea were also used for western blot analysis. Proteins were electroblotted from acrylamide gel to PVDF membrane after the second dimension separation. After detection, using antibody raised against chitinase 30, we could detect mainly six spots in two rows as can be seen in Fig. 52, B, C, and D.

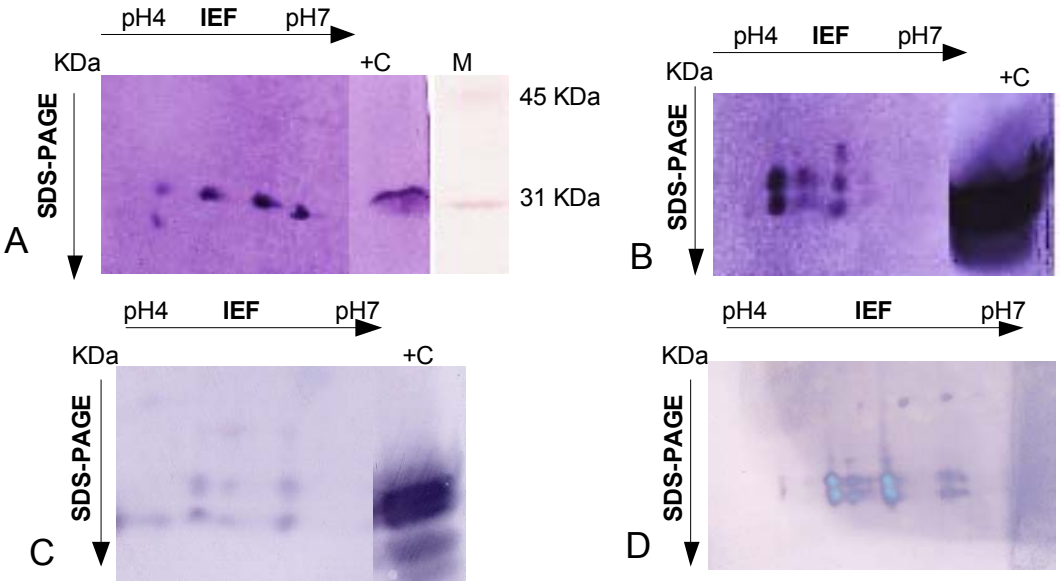

Fig. 52. Western blot analysis after 2-D PAGE, total protein extract was subjected to First-dimension isoelectric focusing (IEF) on 7 cm IPG strip, pH 4-7, followed by separation on 12% SDS-PAGE and transferred to a PVDF membrane. The blot was probed with Chit30 antibody followed by goat anti-rabbit IgG. A, Induced *E. coli* cells harboring pUChit30 expression vector; B, Total protein from tobacco (clone 03-04); C, Total protein from pea (clone 02-04 with 35S promoter); D, Total protein from pea (clone 14-04 with vst promoter); +C, positive control; M, BioRad low molecular weight protein marker.

6 DISCUSSION

Biotechnological techniques, including plant transformation, are considered to amend conventional plant breeding since these technologies offer novel possibilities for gene transfer between different species. A look on the recent increase of GMO cropping areas will illustrate the success but also different public acceptance of this new technology. The global area of transgenic crops was 1.7 million hectares in 1996 and increased to 52.6 million hectares in 2001 (3000 %). An increase of 20 % was reported between 2003 and 2004 and 11 % between 2004 and 2005. This resulted in a total cropping area of about 90 million hectares (Fig. 53). The most important transgenic crops produced were soybean, maize, cotton and canola. The introduced transgenes were herbicide tolerance (77 % in 2001) and insect resistance genes (15 % in 2001). The market value of biotech crops was around $ 4.7 billion (Clive, 2003, 2004 and 2005).

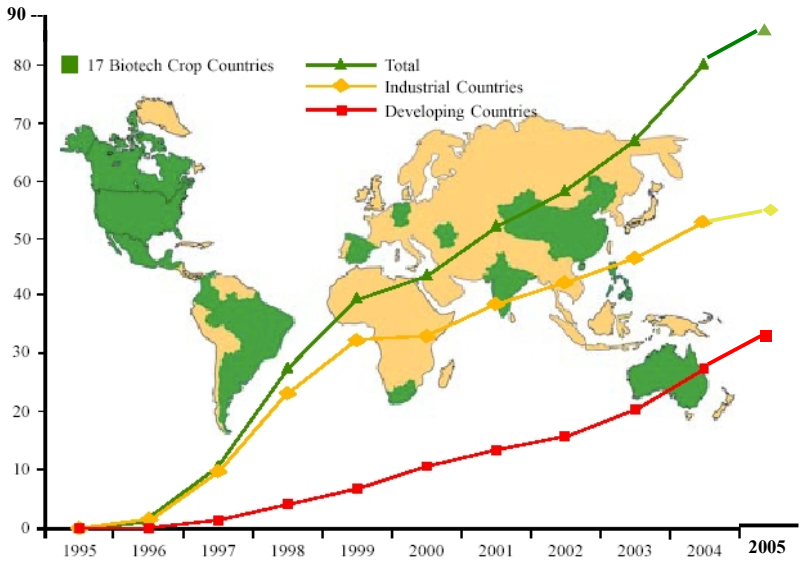

Fig. 53. Global area of genetically modified crops, in million hectares from 1996-2005. (Adapted from Clive James, 2004 and 2005).

Most of the other crops did not find the same interest by multinational companies, particularly grain legumes, which play a role in developing countries. Fungal disease resistance took less attention although it is an important factor as yield reducer in both developed and developing countries. Therefore, producing such resistant plants would greatly affect the sustainability of production of these staple foods, which in turn can have a great socioeconomic effect. In addition, fungi resistant plants require less or no fungicides and have fewer problems with mycotoxines. One way to increase antifungal

resistance level in plants is to over express of pathogenesis-related proteins which has important roles in plant defense system such as chitinase which believed to play such a role in plant through different classes and isoformes found during fungal attack.

6.1 Cloning of chitinase gene

The unique advantage of biotechnology is the principle cross kingdom usage of genes. The recent most competitive transgenic plants harbor transgenes providing highly effective monogenetic resistances against herbicides and insect pests, characters that are not available in wild type pea cultivars. So the successful cloning and vector construction depend on the correct procedure to identify the gene of interest, cloning the gene and transformation into *E. coli* cells and then into *Agrobacterium* for plant transformation. Different molecular tools were used to check and characterize the cloned genes, like PCR, restriction digest and sequencing of the binary vector and blast the sequencing results with the original sequence to check if there are any errors, deletion or substitution of any base pair which may affect the open reading frame of the gene and the final product of the gene as functional protein. Since it was difficult to check the vector in *Agrobacterium* using restriction digest and sequencing, re-transformation of *E. coli* was done with plasmids isolated from *Agrobacterium* and then isolated plasmids from *E. coli* were confirmed.

6.1.1 *Streptomyces* chitinase gene *Chit30*

In this study, the chitinase gene *Chit30* from *Streptomyces olivaceoviridis* ATCC 11238 (identified by R.M. Kroppenstedt), which has an open reading frame of 888 bp and consists of 296 amino acids with a GTG start codon (it was changed to ATG later) and a total of 70 % GC content with a molecular weight of the mature protein of 28.9 kDa, was used in the present study in order to increase disease resistance of plants against fungal pathogens. The soil-borne Gram-positive mycelia-forming bacterium was used for a fungal cell wall degradation test of viable filamentous ascomycetes (Beyer and Diekmann, 1985 and 1984). They also found that, under certain conditions *Streptomyces* releases high enzyme activities into the culture medium. This chitinase was used for fungal protoplast preparation in good yield (Romaguera et al., 1993) and cellulase production (designed *Actinomyces* sp., QM-B814, Reese et al., 1950). In addition, actinomycetes (including *Streptomyces*) are interesting agents for biological control of soil-borne root diseases of crops, where different reports show that actinomycetes are a promising group of antifungal, root-colonizing microbes and which showed protection from soil-borne fungal

pathogens, making them a promising tool for crops protection (Crawford et al., 1993).

The structure of *Chit30* is similar to other family 19 chitinases. It is composed of a catalytic domain (CAD) and a function-undefined domain (FUD) as shown in Fig. 54 (Li, 2001). The FUD was later assigned as chitin-binding domain (Dr. Jochen Meens, personal communication), which makes it similar to *ChitC* from *Streptomyces griseus* HUT6037 (Watanabe et al., 1999). Family 19 chitinases mainly contain plant chitinases classes I, II and IV and *Streptomyces* chitinase, which have catalytic domain homology to plants and chitin-binding domain at the N-terminaus. Ohno et al. (1996) and Watanabe et al. (1999) explained the relationship between plant and *Streptomyces* chitinases by proposing two hypotheses. The first proposed that the ancestral chitinase was present before plants and bacteria and then evolved independently to them. The second possibility is that *Streptomyces* get the chitinase from plants by horizontal gene transfer.

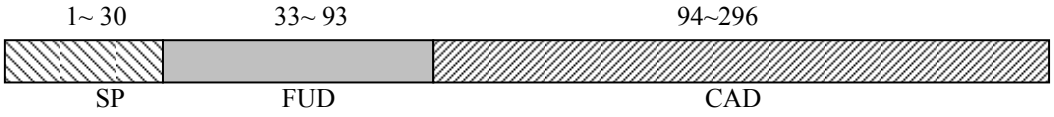

Fig. 54. Primary structure of Chit30 gene. SP, Signal peptide; FUD, functional-undefined domain; CAD, catalytic domain (Li, 2001).

Chit30 (GOI) and *bar* (selectable marker) genes were cloned into the pGreenII vector under control of two different promoters. For chitinase, either the constitutive cauliflower mosaic virus (CaMV) 35S RNA promoter or the inducible *vst* promoter were used, whereas for controlling *bar* gene expression the *Agrobacterium* nopaline synthase (*nos*) promoter was used. The *nos* promoter is considered to be weaker than the 35S promoter and its activity is organ-, position- and developmental-stage dependent, taking into consideration the fact that *nos* promoter activity is differentially expressed in various organs, which indicates potential problems in regenerating transformants, the activity of the *nos* promoter differs between different plant species (An et al., 1987 and 1988). Sanders et al. (1987) compared the CaMV 35S promoter and the *nos* promoter at transcriptional levels in transgenic petunia plants, where they found that 35S promoter was at least 30 times stronger than the *nos* promoter. These results were similar to those obtained by Harpster et al. (1988). The *chitinase* and *bar* genes were arranged in divergent orientation to reduce antisense effects and for efficient expression (Becker et al., 1992). Tobacco plants were used in the present study as model plant for testing the functionality

of the binary vector and cloned chitinase gene, and pea as legume model plant.

6.1.2 Chimeric *Chit30* gene with *Arabidopsis* signal peptide sequence

In addition to the original bacterial gene cassette, the FUD / CAD domains were N-terminally fused to a plant secretion signal peptide, since the highest prediction score for the bacterial gene after heterologous expression in eukaryotic cells shows an aberration of the mature protein. These aberrations would most probably not affect the functionality of the heterologous expressed gene but could result in low efficient secretion of the chitinase. It was reported by Lund and Dunsmuir (1992) and Lund et al. (1989) that the secretion of a *ChiA* gene from *Serratia marcescens* in tobacco plants was more efficient when they replaced the bacterial signal peptide with a plant signal peptide from tobacco PR1b, where they found that *ChiA* was completely secreted. In addition, there was no secretion of *ChiA* when signal peptide sequence was absent. Similar results were reported by Su et al. (2004), using GFP protein fused to *Arabidopsis* basic chitinase gene, which was introduced into a tobacco cell culture. The authors found secreted GFP in the medium of cell culture due to a regulated secretion. The explanation of an improved secretion was that plant signal peptide lead the protein to the secretory pathway. There are contradictory reports on the ability to use signal peptides from different origin. Kaiser et al. (1987) reported that the replacement of an original signal peptide of *Saccharomyces cerevisiae* invertase with random peptide sequences was functioning, whereas Bird at el. (1987) reported that signal peptide sequences of *Saccharomyces cerevisiae* were not functioning in mammalian cells and they concluded that the signal peptide which is functioning in one organism not necessarily functions in another organism.

The signal peptide of *A. thaliana* was fused to the coding region of the chitinase gene (Haseloff et al., 1997). According to the cleavage prediction for an authentic mature protein the secretion signal peptide was adopted. That no major differences were observed regarding the functionality of the chimeric chitinase gene in comparison to the wild type gene could be explained by the fact that the catalytic domain of the chitinase gene is located at the C-terminus (Fig. 54).

6.2 Tobacco transformation

Shoots from different leaf explants were regenerated 6-8 weeks after inoculation with *Agrobacterium* suspension. 80 % of the inoculated explants showed regeneration of shoots. The response of explants to regeneration was affected by genotype and

developmental stage as well as endogenous growth regulators level, since the explants were *in vitro*-derived plants. Shoots were individualized and subcultured on selection medium containing 15 mg/ PPT and were afterwards rooted on MS hormone-free medium. Rooted transformed plants were acclimatized and then transferred to the greenhouse for further analysis and to set seeds. Transgenic tobacco plants were healthy growing, fertile and without any aberrations from the natural phenotype, except one clone (T1-1) from a transformation experiment with the *vst* promoter construct, showing a different leave color phenotype in T0 (Fig. 55). In the following generations, this kind of difference could not be seen any more, which may be due to the segregation and selection after BASTA® spray of the germinating T1 seedlings.

Fig. 55. Phenotype difference of clone T1-1 (left) compared to control plant (right).

Seed grown progenies from herbicide resistant plants were germinated and sprayed with 600 mg/l BASTA®. Different T1 sibling plants were characterized at the molecular level, proving the presence of chitinase and bar genes. The progeny of clone T1-1 was confirmed to be transgenic, showing a normal phenotype.

By applying leaf paint assay, the transgenic plants could be discriminated from non-transgenic plants, by exhibiting the resistance against the total herbicide BASTA® (600 mg/l PPT), whereas non-transgenic plants shows necrosis and the treated part or the whole leaf turned yellow and died. Due to possible gene silencing phenomena, the herbicide sensitive plants are not necessarily non-transgenic and due to this fact the leaf paint assay only allows positive selection.

It is well known that T-DNA transfer to the plant cells occurs in a defined direction, starting from the right border to the left border (Becker et al., 1992; Zambyski, 1992),

where the selectable genes are located to ensure selecting transformants containing complete T-DNA insertions.

Almost all tested transformants according to Southern analysis after *Eco*RI and *Xba*I digest of gDNA showed multiple copy integration. When *Xba*I was used, different integration patterns were detected with copy numbers ranging from 1 to 6 copies in T0 plants. This dramatically reduced in T1 and T2 plants due to segregation or methylation, where transformant like R-1-3 with 4 copies in T0 showed 2 copies in T2, and L-15 which had 4 copies in T0 resulted in only one copy in T2. On the other hand, when *Eco*RI was used in T0, 75 % showed multiple copies, but resulted in a single copy in the T1 and T2, due to segregation whereas single copy insertion was detected in 25 % of the tested plants. Binary vector backbone was detectable in T0 plants, using PCR (with *npt*I primers) and Southern analysis. When the membrane was hybridized with the *npt*I probe, it showed a single insert in most of the tested clones (12 out of 19 clones or 63 %), one clone (L-14) showed two copies. 6 clones out 19 showed no backbone integration. In the T1 generation, most clones that showed single copy in T0 showed again the same copy except clone L-15, which showed no backbone copy any more in T1 and T2. This may be due to a periclinal chimeric phenomenon by integration in different cell layers since germ-line originate from L2-layer (Satina et al., 1940), but the clone L-14 which had two copies in T0 showed single insert in T1. The backbone of pGreen vector was also observed in transgenic rice by Vain et al. (2003). They detected 45 % of the lines with multiple copy insertion with backbone, while only 15-20 % of the lines contained single copy T-DNA integration without backbone and the overall expression did not improve with increasing the copy number of T-DNA. The difference detected using the two different restriction enzymes could be ascribed according to the digestion site, where *EcoR*I was cutting the genomic DNA outside the T-DNA while *Xba*I was cutting in the T-DNA once. This will explain higher copy numbers resulting from the use of *Xba*I compared to *Eco*RI. There is a possibility that the different inserts are somehow linked and behave as one copy while *Xba*I ensures avoidance of this kind of linkage by cutting in the T-DNA.

At the transcriptional level, the expression could be confirmed by using RT-PCR in different clones and subsequent generations. All transformed T0 plants in the greenhouse were tested by using the leaf paint assay. Most plants showed a positive leaf paint test, and some were in between. This indicates either sectorial or periclinal chimeric character. It also resulted in relatively low expression rates of the recombinant chitinase. Post-

transcriptional silencing or methylation is rather unlikely in this stage, but could not be excluded, since the bacterial signal peptide is GC rich (82 %). However, T1and T2 plants harboring the bacterial signal peptide exhibited full functional properties. In contrast, T0 plants of the chimeric chitinase gene with *Arabidopsis* signal peptide (GC content 35 %) did show signal in the Western blot. No cross-reaction was found using the protein extracts from non-transformed negative control plants. In most cases, two bands could be detected at an expected level of 29 kDa and 31-32 kDa depending on the signal peptide used, which may be due to either cleavage of the signal peptide with a molecular weight of 2.9 kDa and 2.1 kDa for the bacterial signal peptide and *Arabidopsis* signal peptide, respectively, or post-translational protein modifications like N-*glycosylation.* Apparently, chitinase contains at least two predicted glycosylation site (from NetNGlyc 1.0 Server - Technical University of Denmark) at position 145 (Asn-V-Ser) and 259 (Asn-G-Ser**)** for chitinase with bacterial signal peptide or position 135 (Asn-V-Ser) and 249 (AsnG-Ser**)** for chitinase with the *Arabidopsis* signal peptide. It is also possible that the post-translational processing is incomplete in a heterologous host. This processing is necessary for the correct folding and targeting. The lower bands, which sometimes appear, could be degradation products of the chitinase as proposed for *ChiA* from *Serratia marcescens* when introduced into tobacco (Lund et al., 1989). The chimeric character of some T0 plants could also be one of the reasons for the BASTA® sensitivity but this is even more an explanation for the negative immuno-blots from T0 material. The expression level proved to be varying between different clones from the same transformant and even between plants from one clone “inter individual differences” (Richter et al., 2005 in press). Linked and unlinked copies of introduced genes and related endogenous genes in plants can be silenced by homology-based mechanisms at transcriptional or post-transcriptional level, through DNA methylation or triggering RNA turnover (Matzke and Matzke, 1998). In addition, the expression level can be affected by adjacent plant DNA and the different sequences flanking the integration sites, which are known as “position effect” (Hobbs et al., 1990; Finnegan and McElory, 1994). The variation in expression can be of several reasons: a different T- DNA copy number in different transgenic plants, *cis*-acting elements such as silencers and enhancers at the T-DNA target site, transcriptional interference of T- DNA and target expression units and the general chromatin structure (Birch, 1997; Warkentin and McHughen, 1991). Some authors concluded positive correlation of copy numbers (Klimaszewska et al., 2003). Hobbs et al. (1990) found that

two allelic copies of T-DNA resulted in doubling the expression, whereas non-allelic copies reduced the expression. This may explain the incomplete co-suppression of the introduced chitinase gene in the T0 generation in this study, since most of the plants derived contained multiple insertions. It may also be due to endogenous auxin and cytokinin levels, which showed suppression of glucanase and chitinase transcription in tobacco tissue (Mohnen et al., 1985; Shinshi et al., 1987).

In T1 and T2 the translation product of the chitinase was detectable using Western blot and immunostaining and *in-gel* assay. Although there were still clones with more than one copy like R-1-3, C-2, F-1, L-14 and H-4, chitinase activity using in-gel assay was lower for the clones L-14 and F-1, which contain more than two copies. Clone H-5-1 (1 copy) and clone H-4-2 (2 copies) showed no difference in immunostaining. The presence of two copies did not improve the expression of chitinase where the two clones showed similar activity using in-*gel* assay 0.134 U and 0.140 U for clone H-4 and H-5 respectively. This was confirmed in the colorimetric assay.

Hobbs et al. (1993) explained that the nature of the T-DNA is much important than copy number which can increase or decrease the level of expression.

6.2.1 Promoter analysis

By comparing the promoters used in this study to drive the expression of the chitinase gene, we found higher levels of expression when the inducible promoter (*vst*) was used and induced by UV light compared to the 35S constitutive promoter, probably because a background activity of endogenous chitinase, which is obviously also UV inducible. The expression kinetics show that the inducible promoter was higher over time (2 h. *vs.* 24 h.), similar to the results observed in lettuce plants transformed with a chimeric *gus* gene and a tobacco pathogenesis-related protein PR1a promoter, where the expression was 3-50 fold higher than with the 35S promoter (Enomoto et al., 1990). A peroxidase promoter (isolated from sweet potato) was also used to drive the expression of a *gus* gene in transgenic tobacco which produced 30 times more GUS activity after induction by hydrogen peroxide, wounding or UV light than the 35S promoter (Fischer et al., 2004). The 35S promoter has properties that make it useful in transgenic crop development because of constitutively rather high levels of gene expression activity in many plant cells. It is one of the best-studied elements controlling gene expression in plants. However, there are some disadvantages of using 35S promoter as it shows morphological, developmental and physiological alterations in the transgenic plants (Fladung et al., 1997). Several other

promoters from viruses have been reported to have activities similar to the 35S promoter. Samac et al. (2004) compared the activity of three constitutive promoters, i.e., the 35S promoter (CaMV), the cassava vein mosaic virus (CsVMV) promoter and the sugarcane bacilliform badnavirus (ScBV) promoter, which each were fused to the *gusA* gene in transgenic alfalfa plants. They found that the highest GUS activity was obtained using the CsVMV promoter followed by the 35S promoter. The activity was approximately 24-fold greater than the activity from the 35S promoter and 38-fold greater than the activity from the ScBV promoter.

In the present study, leaf samples were used for crude protein extraction since the 35S promoter confers high levels of expression in leaves and stems of transgenic tobacco plants and lower expression in flowers and seeds (Malik et al., 2002).

6.2.2 Suspension culture and apoplast

For both constructs, protein extracts from suspension cultured cells showed a stronger signal compared to the proteins extracted from medium, suggesting that the chitinase is not fully secreted to the medium since there were two bands presented in the Western blots which can be ascribed to processing of the chitinase protein. The upper band may represent the pre-protein without cleaving the signal peptide sequence and the lower band the mature protein (about 3 kDa smaller), which needs to be studied more in detail. The two bands shown in Western blots are from a construct containing the bacterial signal peptide sequence. As it could be expected there is cleavage of the signal peptide in a different position than it should be (*Sig-Pred* program results for prediction of signal peptide cleavage site), whereas the construct containing a plant signal peptide sequence showed one band, indicating the correct processing of the mature protein. Similar results were obtained by Liu et al. (2001) using GFP as reporter gene fused to a N-terminal signal peptide and a C-terminal H/KDEL sequence. The authors could detect GFP in the cells and in the medium. The H/KDEL improved the stability and folding of the protein leading to an improved expression since it is not sufficient to retain the protein in ER (Liu et al., 2001).

On the other hand, two bands could be clearly detected from samples extracted from apoplast, one band had the same size as the positive control purified from bacteria, presenting the mature protein and the other band had a higher molecular weight, which could be the full-length protein without cleavage of the signal peptide. These bands were not due to nonspecific binding of the antibody since no signal was detected in non-

transformed negative plants. Unfortunately, we could not test the contamination of apoplast proteins with cytoplasmic protein, which would require an antibody against cytoplasmic proteins. Even though, contamination with cytoplasmic protein due to cell damage could not be excluded even when a gentle treatment was used. Lund at el. (1989) and Botha et al. (1998) proposed that only 0.15 % and less than 2 % respectively of cytoplasmic proteins were found in the apoplast proteins. The weak signal on Western blot was mainly due to the limited amount of total protein (20-50 µg total protein), which could be applied for SDS-PAGE *vs.* 100-200 µg as it was used by Lund et al. From the observation of cell culture and apoplast proteins using Western blot and immunostainig, it can be concluded that the bacterial signal peptide could be recognized in plant cells and enters the secretory pathway. As it was also concluded by Lund et al. (1989), the *ChiA* bacterial chitinase is glycosylated by plant cells. However, the roles of this modification still not clear.

6.3 Pea transformation and regeneration

Regeneration of mature plants with identical phenotype and genotype is a pre-requisite for any successful transformation. Adventitious regeneration can be obtained either by somatic embryogenesis or by shoot organogenesis, and both types of regeneration can be either direct or indirect via a callus phase. The direct regeneration from pre existing meristems is preferred for genetic modification in pea. Callus based regeneration systems have the disadvantage that they have a much higher chance of yielding plants with somaclonal variation than direct regeneration. In the present study, direct shoot organogenesis was used from mature embryos after inoculation with *Agrobacterium tumefaciens* harboring the binary vectors pGIIvst-N-Chit or pGII35S-N-Chit containing a chimeric bacterial chitinase gene fused to an *Arabidopsis* signal peptide sequence. Pea was also transformed with the original constructs pGIIvst-Chit and pGII35S-Chit but so far there are no solid data since the transformants are still in selection medium.

In the present study, TDZ at concentration of 5 µM was used in the first two to three weeks to induce normal shoot regeneration; this concentration was favorable over 10 µM in lentil regeneration (Hassan, 2001). Murthy et al. (1998), applied TDZ to induce a diverse array of cultural responses ranging from induction of callus to formation of somatic embryos. They found that TDZ exhibits the unique property of mimicking both auxin and cytokinin effects on growth and differentiation of cultured explants. A number of physiological and biochemical events in cells are likely to be influenced by TDZ, since

several authors reported that higher TDZ concentrations (20 μM) result in stunted shoots and consequently slow development, elongation and failure in root production (Lu, 1993; Malik and Saxena, 1992).

In the present study, the first transgenic seed could be obtained after 6-8 months depending on the number of repeated subcultures in between, where regenerated shoots were subcultured for 2 or 3 times on selection medium containing 10 mg/l or 15 mg/l PPT to ensure elimination of escape shoots and for multiplication of the clones selected. This period, from transformation to harvesting transgenic seeds looks realistic and comparable to other protocols. For example, Davies et al. (1993) and Grant et al. (1995) reported around 7 months from explant inoculation to getting the seed-bearing primary regenerants. Bean et al. (1997) obtained transgenic shoots in 4 months after inoculation. Polowick et al. (2000) required 6 months to get transgenic plants in the greenhouse, and, Švàbovà et al. (2005) required 5-6 months to get mature transgenic seeds using an *in vitro* system and only 3-4 months using *in vivo* system, whereas Schroeder et al. (1993) and Nadolska-Orczyk and Orczyk (2000) got plants bearing seeds in 9 months. Puonti-Kaerlas et al. (1990) required longer time for getting transgenic plants as they needed 6 months for shoot induction and 15 months for the whole experiment. The time was comparable with other legumes, where azuki bean required 5-7 months (Yamada et al., 2001). Transgenic medicago was obtained within 4-5 months (Chabaud et al., 2003), but regeneration of transgenic pigeonpea was achieved in 3 months only (Dayal et al., 2003). In faba bean primary seeds of T1 were recovered within 9-10 months (Hanafy et al., 2005).

6.3.1 Transformation efficiency

The transformation efficiency obtained in the present study varied from 0.31 % to 1.4 %, with an average of 0.6 % for the whole transformation experiments, but when eliminating the experiments, which did not render any transgenic shoots, the efficiency became 0.9 %. These results correspond to results of other authors taking into account different *Agrobacterium* strains used, different explants and selection procedures applied. When using strain EHA105, the transformation efficiency was 1.1±0.43 % (Bean et al., 1997), 8.2 % (Nadolska-Orczyk and Orczyk, 2000), 0.6 % (Polowick et al., 2000) and 0.6-0.9 % (Pniewski and Kapusta, 2005). On the other hand, when strain AGL1 was used, the transformation efficiency was 1.4-4.1 % (Pniewski and Kapusta, 2005), 0.8-3.4 % (Grant et al., 1995) and 1.5-2.5 % (Schroeder et al., 1993), while for other strains, the rate was 2.2 % (strain C58C1), 0.7-3.3 % for AGL0 (Pniewski and Kapusta, 2005) and 1 % for

LBA4404 (Nadolska-Orczyk and Orczyk, 2000).

6.3.2 Selectable marker

Selectable marker plays an important role on the transformation efficiency of pea. There are two kinds of markers used in all publications, either antibiotic or herbicide resistance. It is noticeable that the higher transformation efficiency was achieved when antibiotics were used. The efficiency were 0.8-3.4 % (Grant et al., 1995), 8.2 % (Nadolska-Orczyk and Orczyk, 2000), 1.44 % (Davies et al., 1993) and 2.5 % (De Kathen and Jacobsen, 1990) when kanamycin was used. When hygromycin was used as selective marker, the transformation efficiency was 4.9 % (De Kathen and Jacobsen, 1990), or up to 12 % (Puonti-Kaerlas et al., 1992). However, there were contradictory results when antibiotics were used, where Puonti-Kaerlas et al. (1992) could not get any transformants when kanamycin was used, whereas, Nadolska-Orczyk and Orczyk (2000) reported the same result when hygromycin was used, in addition fertility problems occurred with antibiotic resistance genes. The alternative for antibiotic was to use a herbicides like phosphinothricin as selectable marker. The transformation efficiency were up to 4.1 % (Pniewski and Kapusta, 2005), 1.5-2.5 % (Schroeder et al., 1993), 1.47 % (Grant et al., 1995), 1.1 % (Bean et al., 1997) and 3.6 % (Nadolska-Orczyk and Orczyk, 2000). For the same groups, when they used different selectable markers, they got different transformation efficiency like 8.2 % for kanamycin *vs* 3.6 % for phosphinothricin (Nadolska-Orczyk and Orczyk, 2000) and 0.8 %-3.4 % for kanamycin *vs* 1.47 % for phosphinothricin (Grant et al., 1995; Grant et al., 1998). Comparing the results of these two groups who used two different markers, it was clear that the transformation efficiency was higher for kanamycin, but this result may be due to the fact that the regenerants on kanamycin were chimeric or not transgenic “escapes”. In addition, kanamycin has long stay and causes abnormal phenotype plants and changes the nuclear DNA content, while phosphinothricin-resistant plants were phenotypically identical (Nadolska-Orczyk and Orczyk, 2000).

In the present study, phosphinothricin was used for selection of the transformants since it is much more stringent in decreasing the rate of escapes and chimerics in comparison to kanamycin. On the other hand, transgenic plants with herbicide resistant genes can be used as dual strategy as selective marker *in vitro* and as weed control in the field. For biosafety aspects in Europe, it might be more accepted by the public and easier to commercialize. In addition, the PAT proteins have been shown to be rapidly degraded by digestion and

heating (Novartis Seeds AG, 1999). No reports indicated the allergenicity or toxicity of the PAT protein or its degradation products (Nap, 1999). From the investigations carried out by Novartis Seeds AG (1999) feeding mice with transgenic maize containing *PAT* gene, they found no toxicological or potential effects on health, especially when the seeds were processed.

6.4 Molecular and biochemical characterization of pea

Molecular analyses indicate successful integration of T-DNA into genomic DNA of T0, T1 and T2 transgenic pea clones as it was confirmed by PCR and Southern blot analysis. Single copy was obtained in most of T1 tested clones but also tow copies was also obtained when using *Eco*RI enzyme, but when *Xba*I was used some of the single copy insert from *Eco*RI digest showed two copies, i.e. clones 03-04-1-3,30 which may be indicate closely linked inserts which behave as single copy. Two clones progeny i.e., 02-04-7-1 and 02-04-7-4 were subjected to homozygosity test to predicate the homozygosity of the parental clones which showed homozygosity ranged between 73.74-98.88 % for the clone 02-04-7-1 and between 83.81-86.94 % for the clone 02-04-7-4, these values depend on the number of tested seeds and as higher the sample size as better the homozygosity test result which give the homozygosity of the parental line.

Chitinase activity assays according to Trudel and Asselin (1989, 1990). In the present study we used a modified protocol by adding the substrate directly to the gel. The assay showed the presence of three additional isoform bands compared to non-transformed pea, which showed one band. This was consistent with the western blot analysis without cross-reaction in the non-transformed plant or non-expressing plants (Fig. 40 A and 41 A; lanes 7 and -C) which even proved positive in PCR. This result is in agreement with the results of Mohammadi and Karr (2002) where they could detect 4 isoformic bands in chitinase activity and western blot analysis. In the present study, we used the SDS-PAGE gels as well as chitin agar plate (Gohel et al. 2005, Chernin et al. 1998) to detect chitinolytic activity only, without showing the different isoforms or bands. The migration in SDS-PAGE containing the glycol chitin as substrate was slower compared with gels without glycol chitin. This is due to the presence of the polysaccharide in the gel. The enzyme denatured in SDS-PAGE could be renatured using renaturation buffer to remove the SDS, while addition of reducing agent caused irreversible denaturation due to the disulfide bond reduction (Hung et al. 2002).

6.5 Functional analysis

6.5.1 Leaf paint assay

Successful expression and functionality of the *bar* gene was confirmed by the leaf-painting assay. A high concentration of BASTA® was used (600 mg/l) compared to others, who used 200 mg/l (Pniewski and Kapusta, 2005; Nadolska-Orczyk and Orczyk, 2000), 5-10 l/ha (Schroeder et al., 1993), 3 mg ml/l (Bean et al., 1997), 3 l/ha (Grant et al., 1995), or 400 mg/l on faba bean (Hanafy et al., 2005). Herbicide tolerance gives another advantage for the transformed plants as they to survive when the same herbicide is used to control weeds.

Despite the plants showing negative leaf paint as in the case of clone 14-04-2-4 (which was positive in PCR). This may be due to gene inactivation, methylation or co-suppression (D' Halluin et al., 1992) 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 gene near the left border. This can explain negative PCR results for *bar* and positive one for chitinase.

6.5.2 *In-gel* and agarose diffusion assays

In this assay, glycol chitin was used as substrate, which is the most suitable substrate to detect the catalytic activity of family 19 chitinase (Itoh et al., 2003). The agarose diffusion assay was originally optimized for muskmelon seeds (Zou et al., 2002) and cucumber (Velasquez and Hammerschmidt, 2004). This assay needs to be optimized for tobacco and pea plants as used in the present study. There are different factors affecting the assay, like pH, substrate concentration, agarose concentration, buffer, incubation time and temperature. In the present study, overnight incubation at 28 °C or 37 °C with 1 % or 1.6 % agarose for gel preparation were used. The results were obtained with 37 °C in 1.6 % agarose overnight. Combining the conditions of the previous two methods. We used the direct measurement of the halo diameter by electronic digital caliper of the photographed plates. This needed to be scaled from photo size to the original plate size. However, Velasquez and Hammerschmidt (2004) used an automated method of image digital analysis by measuring the number of pixels in the activity area, area and diameter of the halos, where they got the best resolution after 4 hrs. of incubation. The results were used to generate regression analysis where there was a difference in the linearity due to enzymatic reaction and possible interference of the reaction product with the reaction

(Algranati, 1963).

In general, the chitinase activity of UV induced pea plants with the *vst* promoter controling chitinase was higher than with 35S promoter. Although the 35S constitutive promoter is highly expressed in most tissues, it can yield high activities. The samples were taken from the middle of the plant between node 4 and 8, which presumably has an effect on the expression level due to the fact that the expression is tissue, developmental-stage- and species-dependant. This may explain the variation of activities between different samples and between samples and negative controls, which ranged from 0.07 to 0.14 U/ 10 µg of total protein in tobacco. The activity was higher in pea where it ranged between 0.9 and 0.25 U/ 10 µg of total protein. Statistically, significant difference could be detected between some *vst* promoter samples before and after induction such as 15-04-1-2,1 and 07-04-4-4,7,1 and between *vst* promoter and 35S promoter plants such as 15-04-1-2,1 and 03-04-1-3,5,2. The unexpected lower chitinase activities of some samples compared to the negative controls in tobacco and pea suggest that the recombinant chitinase can inhibit expression of the endogenous chitinase as similar results were reported for tobacco plants transformed with tobacco class I endochitinase (Neuhaus et al., 1991). This variation in activity between tobacco and pea was similar using *in-gel* activity assay using SDS-PAGE, where crude extracts of pea showed stronger effects and more catalytic response than tobacco. The induction level of total chitinase activity after UV light was around one fold, which was much lower than in other reports. In bean, between two and five fold higher activity was detected upon infection with incompatible fungus and *Fusarium solani* respectively (Lange et al., 1996), while a 30 fold increase was detected after 24 h. after ethylene induction in bean leaves (Boller et al., 1983). In grapevine, wounding and salicylic acid induced total chitinase activity by a factor of 4.9 and 5.5, respectively (Derckel et al., 1996). A 5-fold increase in chitinase activity was detected after 24 h in rice cells treated with fungal elicitors (Velazhahan et al., 2000). It seems that UV light has a lower capacity in chitinase induction compared to other methods used, but this should be studied more by prolonging the exposure time to UV light or by using a different wavelength than we did in this study.

Agarose diffusion has a number of advantages over SDS-PAGE based activity assays (Trudel and Asselin, 1989 and 1990) as it allows quantifying the total chitinase enzyme activity. Interfering chemicals (acrylamide, bis-acrylamide and TEMED) are also avoided. Denaturation of the protein, which needs renaturation after running the gel, can be avoided

since native crude extracts of proteins are used without any SDS or boiling steps, retaining the whole activity. The agarose diffusion method is simple, less error-prone and easy to perform compared to the SDS-PAGE method. On the other hand, the advantages of SDS-PAGE over agarose diffusion are differentiation of different isoforms of chitinase, comparing it with non-transformed control plant and calculation of the molecular weight referring to a protein molecular standard marker. In the original protocol of Trudel and Asselin (1989), glycol chitin was added to a separate acrylamide gel and overlaid with resolving gel. Thereafter, the overlaid gel was stained with florescent staining. In the present study, the substrate was added directly to the resolving gel of SDS-PAGE without using another gel. This method was also used by Van Sluyter et al. (2005).

In-gel assay showed the presence of additional three isoform bands at 28, 32 and 50 kDa compared to non-transformed pea, which shows only one band at 30 kDa. This was consistent with the western blot analysis but without cross-reaction in the non-transformed plant or non-expressing plants (Fig. 40 A and 41 A; lanes 7 and -C). This result is in agreement with the results of Mohammadi and Karr (2002) who could detect four isoforms of chitinase activity in soybean nodules using non-denaturing polyacrylamide gel and silver-stained with molecular weight of 27, 35, 71 and 94 kDa. These isoforms showed cross-reactivity and could be detected using immunoblotting. Van Sluyter et al. (2005) detected up to six chitinase isoforms in grape extracts separated by SDS-PAGE. In wheat, 7 distinct isoforms were detected in healthy plants with induction of one or two new isoforms after injury and ethylene treatment of plants, respectively (Botha et al., 1998).

The reason for different chitinase isoforms found in plants is that chitinase, in addition to its antifungal activity, has different roles in regulation of plant development or regulation of legume response to *rhizobial* node factors (Kaomek et al., 2003). However, little information is known about expression and enhancement of different isoforms using elicitors or pathogens (Botha et al., 1998).

6.5.3 *In vitro* bio-assay

Trichoderma harzianum was used to study the antifungal effects of chitinases and lectins on fungal hyphae growth since its walls contain chitin-glucan (Mirelman et al., 1975; Schlumbaum et al., 1986). Watanabe et al. (1999) used *ChitC* of *Streptomyces griseus* to study the inhibition of hyphal extension of *T. reesei,* Itoh et al. (2003) used protein extracts from rice transformed with *ChitC.* They found that *ChitC* showed antifungal effects by inhibiting the hyphal extension. Tsujibo et al. (2000) proved the antifungal effects of

Chit25 and *Chit35* from *S. thermoviolaceus* in contrast to the non-antifungal activity, which was detected using other bacterial chitinases, which belong to family 18. Fung et al. (2002) tested antifungal activity of tobacco-expressing *BjCHI1* from *Brassica juncea* using *T. viride.* They found that the chitin-binding domain enhances antifungal activity, but an additional domain did not improve the fungal inhibition. Chye et al. (2005) and Iseli et al. (1993) used *T. viride* to study antifungal effects in potato expressing *BjCHI1* and tobacco basic chitinase A, respectively.

In the present study, *T. harzianum* was used to study the antifungal effect of crude extracts from tobacco and pea transformed with *Chit30*, where different samples clearly inhibited the extension of the hyphae. Inhibition could be shown to occur after 8 h. and 16 h. However, in many cases, the fungi could overgrow the inhibition effect after 24-30 h. This was in agreement with results of Mauch et al. (1988) when using *T. viride*. The inhibition varies among different samples where some show strong effects and others less. This can be attributed to the use of total protein extract whereas the other investigators used purified chitinase, especially when they used expression vectors in *E. coli.*

The inhibitory effects of crude extracts of tobacco and pea were much higher for plants transformed with an inducible *vst* promoter after UV induction compared to non-induced plants. To abolish the endogenous chitinase effect, non-transformed negative controls were used before and after induction in the same way as transformed plants, and in both cases protein crude extracts from non-transformed did not show any hyphae extension inhibition. This clearly demonstrates that the effect on *T. harzianum* was due to the recombinant protein expression and not due to an endogenous chitinase. This may indicate that the endogenous chitinase level is lower than the threshold to show an inhibition effect, or that UV light is not sufficient to induce it. It may also be due to the specific interaction between pathogen and host plant and the pathogens ability to produce peptides (inhibitors), which inhibit plant chitinases, whereas, the recombinant protein is incompatible. This may be explained as an evolutionary adaptation of the pathogen to one of the plant's defenses (Mauch et al., 1988).

Mauch et al. (1988) explained why an *in vitro* assay is more sensitive in showing anifungal activity than *in vivo* responses. This may be due to the fact that the fungi do not come in contact with the hydrolysing enzymes which accumulated in vacuoles but need to be secreted to the extracellular space. This occurs through a hypersensitive response. However, the presence of the inhibitors will prevent fungal colonization and will increase

the half-life of *in vivo* generated elicitors leading to resistance of plants to fungal pathogen (Cornelissen and Melchers, 1993).

There are different inducers for the PR-related proteins such as H_2O_2, wounding, elicitors, ethylene, heavy metals as well as pathogens. El Ghaouth et al. (2003) used UV light to induce chitinase in peach fruit where they got two folds of induction after 96 h. However, the level of induction should be sufficient in order to overcome the pathogen, which acts with other defense systems differing due to different inducers and plants responses to induce specific isoforms, either acidic or basic, where certain isoforms of chitinase act unrealistically with glucanase and are able to defend plants against particular pathogens (Sela-Buurlage et al., 1993).

When the crude extract was boiled, there was no inhibitory activity against hyphae extension, which indicates that the chitinase lost its activity during boiling. A similar result was reported by Mauch et al. (1988) where the samples lost their activity against all tested fungi after boiling for 10 min.

Comparing two signal peptides showed no difference in inhibition of hyphae extension and both constructs show similar effects.

The antifungal effects of crude protein from tobacco cell culture of chitinase with a bacterial signal peptide or a plant signal peptide were compared with non-transformant cell culture. Crude extracts of both types of cells could clearly show the inhibition of the hyphae extension, whereas the samples of culture medium could not show clear effects. This may be due to not fully secreting chitinase to the medium or due to the hormonal constitution of the medium (Kunze et al., 1998), which subsequently, was not enriched in the medium. These findings suggest and confirm that recombinant chitinase of *Streptomyces olivaceoviridis* ATCC 11238 inhibits fungal growth *in vitro* which make it a good candidate to produce fungal resistant plants. Still, the targeting of the chitinase may play an important role in plants after pathogen challenge.

Examination of spores mixed with different protein crude extracts, and incubated overnight, showed under light microscope clear effects on spores, which became enlarged and did not germinated completely as compared to crude extract from non-transformed plant which showed fully germination of spores and extension of the hyphae. Similar results were obtained by Huang et al. (2005) when they mixed the conidia of *B. elliptica* with purified chitinase ChiCW from *E. coli*. They got 84 % inhibition on conidia germination.

Different fungus were tested for inhibition assays, but no results could be obtained since they had very slow growth *in vitro* compared to *T. harzianum,* which can grow very fast and the results of the assay could be recorded after a few hours (after 8 and 16 hrs.). Its growth is also inhibited by chitinase alone, while other fungi require a combination of chitinase and glucanase (Mauch et al. 1988).

6.6 2-D-electrophoresis

Proteomics is considered as one of the most promising techniques to identify the proteins in different developmental stages by studying induction, repression, or post-transcriptional modification (Schiltz et al., 2004). In the present study, 2D-electrophoresis was used to study the different patterns of proteins and spots using immunostaining, when total proteins were extracted from *E. coli* induced by IPTG and separated in two dimensions. The result of immunostaining showed the presence of four spots separated in the first dimension according to IEF at 30 kDa, which may present different isoforms and one spot in the second dimension with lower molecular weight, which could be the mature protein after cleaving the signal peptide sequence. No spots could be detected in non-induced *E. coli* cells.

The pattern was different when using total proteins from plants (tobacco and pea) where at least six spots in two rows could be detected with approximate molecular weights of 30 kDa, which may be also present unprocessed and mature proteins. It was worthy to mention that the induced *vst* promoter showed much stronger exression than the 35S promoter, no cross-reaction could be detected when total proteins from non-transformed plants were used. It was not possible to detect any spots after running the second dimension in SDS-PAGE containing glycol chitin as substrate for activity assay, since the activity of protein was lost and could not be retained due to different reducing agents used in protein preparation even after incubating the gels in re-naturation buffer containing Triton X-100.

"A new agriculture, combining genetic modification technology with sustainable farming, is our best hope for the future" Trewavas (1999).

7 OUTLOOK AND FURTHER EXPERIMENTS

In the present study, different transgenic pea clones could be obtained from different binary vectors. Several lines are still *in vitro* culture and need to be grafted and transferred to the greenhouse in order to further analyse them.

It is necessary to continue multiplication of the selected clones in order to establish homozygous lines, which could be used for crossing and gene stacking with the other existing lines developed in the institute. These lines express different antifungal proteins i.e., *PGIP*, *RPGIP*, *Vst* and also *glucanase,* which has a synergistic effect with Chitinase.

Further studies are needed to investigate the differences regarding the protein targeting, secretion efficiency and C-terminal modification.

Since the crude extract could inhibit the *in vitro* hyphal extension of *Trichoderma harzianum,* it will be effective to test the antifungal effect *in vivo* under field conditions with different fungi (pathogen challenging).

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9 ACKNOWLEDGEMENTS

First of all, I would like to express my deep gratitude to Prof. Dr. Hans-Jörg Jacobsen for his supervision of my research study and invaluable help he extended me throughout my study, for his intellectual guidance and follow-up in each and every plan and outcomes of the study, for scientific advises and technical orientation during the present research work. I am also greatly grateful to him for the correction and critical comments in reviewing and revising the manuscript of this thesis and his friendly company and continuous encouragement throughout my study saving no effort that made this research work a fruitful and successful one.

I am deeply grateful to Dr. Heiko Kiesecker, DSMZ, Braunschwieg-Germany, for his valuable scientific guidance, supervision and suggestions throughout this research work, I appreciate his practical and theoretical advices and comments in the preparation and revising of this manuscript.

I am very grateful for the Islamic Development Bank-Merit Scholarship Program (Jeddah, Saudi Arabia) for the financial support provided to make this work a reality.

I am very grateful to Dr. Ahmad Abdulkader, GCASR, Damascus-Syria, for ongoing support, encouragement, reading and revising the manuscript.

My thanks and acknowledgements are also extended to all members of the LG Molecular Genetics for their constant help and assistance especially Dr. Andrea Richter and Dr. Thomas Reinard.

I thank Dr. Schumacher and the members of the Plant Cell Culture Department, DSMZ, for their assistance, support and facilitating the 2D gel electrophoresis.

I thank Prof. Wolf and his Ph.D student T. Ngwenya for their help in chitinase assay.

Finally, I deeply thank my family members and relatives, especially my parents, for their loving and praying, my wife and my children, my sister and my brothers.

Last but not least, I would like to thank my colleagues in Biotechnology Department, GCASR, Damascus-Syria.

Also, I would like to thank my all friends for their kind supports.

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11 STATEMENT

I declare that I wrote this thesis myself. I did not use other auxiliary material than indicated. Other work has been always cited.

I have not tried to get Ph-D degree before.

Hannover, 28.06. 2006
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