ENHANCEMENT OF *Trichoderma* ENDOCHITINASE SECRETION IN TOBACCO CELL CULTURE USING *α-amylase* SIGNAL PEPTIDE

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1. INTRODUCTION

Plant protection is a major challenge to agriculture worldwide, with fungi being one of the main causes of significant yield losses. It is conservatively estimated that diseases, insects and weeds together annually destroy 31-42% crop loss worldwide of which 14.1% is due to diseases (Agrios, 2004). The distribution of several phytopathogenic fungi, such as *Phythium*, *Phytophthora*, *Botrytis*, *Rhizoctonia* and *Fusarium*, has spread during the last few years due to changes introduced in farming, with detrimental effects on crops of economic importance. Presently, control of disease is mainly based on the application of protective agrochemicals, crop husbandry, such as crop rotation and, the use of resistant varieties. The control of fungal diseases in modern agriculture is mainly achieved by the extensive use of chemical fungicides. However, growing concern about the environment and the high cost of chemicals has encouraged farmers and researchers to look for substitutes, such as the use of biocontrol agents and fungus-resistant crop cultivars.

The genetic approach of breeding to produce crops which are resistant to fungal diseases has proven successful; however, this is time-consuming process, and makes it difficult to react adequately to the evolution of new virulent fungal races. Newly developed technology for the identification, isolation and transfer of specific genes, currently in use for plant breeding, has enabled the insertion of traits for resistance without interfering with the intrinsic properties of the acceptor plant. Therefore, much effort is being put into identifying and isolating genes that, upon transfer, may render target plants resistant to fungi. Some of these efforts are being focused on resistance genes, known from conventional breeding programs. Genes encoding toxic compounds and enzymes involved in direct inhibitory effects on fungi are another direction for intensive research (Schickler and Chet, 1997).

Antagonists of phytopathogenic fungi have been used to control plant diseases, and 90% of such applications have been carried out with different strains of the fungus. The most common BCAs of the *Trichoderma* genus are strains of *T. harzianum, T. virens*, and *T. viride*. The success of *Trichoderma* strains as BCAs is due to their high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi, and efficiency in promoting plant growth and defense mechanisms. This process is further supported by the secretion of extracellular enzymes such as chitinases (Cherif and Benhamou, 1990; Kulling *et al.,* 2000), ß-glucanases (Schirmböck *et al*., 1994) and proteinases (Shakeri and Foster, 2007) as well as secondary metabolites (Ghisalberti and Sivasithamparam, 1991; Lorito *et al.,* 1994).

Chitin is a structural component of the cell wall of many fungi, as well as insects and nematodes, which are major pathogens and pests of crop plants (Collinge *et al*., 1993). Chitinases (E.C. 3.2.1.14) are ubiquitously distributed in bacteria, fungi, animals and plants. They hydrolyze the β-1, 4-linkage between N-acetylglucosamine residues of chitin.

Genes encoding cell wall degrading enzyme (CWDE) from biocontrol fungi belonging to the genus *Trichoderma* have been demonstrated to encode proteins with high antifungal activity against a wide range of plant pathogenic fungi. Chitinases have been implicated in plant resistance against fungal pathogens because of their inducible nature and antifungal activities *in vitro* (Taira *et al*. 2002). Therefore, efforts were made to clone and transfer genes coding for endochitinases from *Trichoderma* into several crop plants to impart resistance to several fungal plant pathogens (Lorito *et al.,* 1998; Bolar *et al.,* 2000; Faize *et al.,* 2003; Liu *et al.,* 2004; Gentile *et al.,* 2007). An extracellular deposition of chitinase has the advantage of allowing an early contact between the antifungal enzymes and the pathogen (Mauch and Staehelin, 1989). Acidic chitinases are located extracellularly, and they may be involved in the recognition process during pathogen infection upon making contact with the pathogen, recognize the cell wall component as chitinolytic or lysozyme activity, and release oligosaccharides as elicitors, and thus trigger the plant defense system (Graham and Sticklen, 1994). The extracellular matrix is believed to be the site where signals originate to elicit defense responses in plants (Bowles, 1990). Signal peptides are short amino acid sequences (motifs) that are located at the N-terminal region of an amino acid sequence which direct protein localization (Tesfaye *et al*., 2005). Recombinant protein accumulation in plants can be enhanced by constructing vectors that contain signal peptides for targeting the recombinant protein to a particular organelle (Conrad and Fiedler, 1998; Stoger *et al*., 2002).

It has been previously reported that the rice α-amylases were found to localize in both cell walls and amyloplasts (Chen *et al*., 1994). Alpha amylase3 signal peptide (αAmy3 SP) was found to be competent in targeting a cargo protein to both plastids and cell walls. Liu (2006) compared different signal peptides for secretion of GFP in rice suspension culture; found that αAmylase3 is the better signal peptide for secreting the GFP out. Chitinase gene from *Trichoderma* has its own signal peptide for secretion into intercellular spaces. In order to compare endochitinase signal peptide with plant signal peptide for better secretion into intercellular spaces in plants, the present investigation was carried out with the following objectives.

1. Fusion of nucleotide sequence of plant signal peptide- α-amylase (PSPα-amy3) to endochitinase gene (*ech*42) cloned from *Trichoderma virens* and its insertion into plant transformation vector.
2. Transformation of tobacco with *ech*42 gene having fungal (*ech*42*SP*) / plant signal peptide (PSP*α-amyech*42).
3. Analysis of transgenic tobacco with *ech*42*SP*/ PSP*α-amyech*42.

2. REVIEW OF LITERATURE

Tobacco is widely used as a model plant system in transgenic research for several reasons: its molecular genetics is well understood, its genomic mapping is almost complete, genetic transformation can be readily achieved, tobacco plants survive well *in vitro* and under greenhouse conditions, and tobacco produces large amounts of biomass. Tobacco is also regarded as one of the best systems for chloroplast transformation, which further enhances the effectiveness of tobacco for the expression of recombinant proteins. Tobacco plants can be used as living factories to produce proteins and enzymes, which can be extracted, purified, and used for the manufacturing of pharmaceuticals and other valuable industrial compounds such as biopolymers. Transgenic tobacco plants are also ideal model organisms for the study of basic biological functions, such as plant-pathogen interactions, environmental responses, growth regulation, and senescence. Tobacco is the most commonly used plant for expression of transgenes from a variety of organisms (Jube and Borthakur, 2007).

Fungal diseases have been one of the principal causes of crop losses ever since humans started to cultivate plants. An estimated 37 per cent crop loss is due to pests, of which 12 per cent is due to pathogens such as fungi, bacteria and viruses (Sarah and Paul, 2005). To date, the epidemic spread of fungal diseases is controlled by (a) various crop husbandry techniques, such as crop rotation, avoiding the spread of infested soil and pathogen-carrying plant materials, (b) breeding of fungus-resistant cultivars of crops, and (c) the application of agrochemicals. Management includes chemical application, or soil cultivation that is expensive to implement and may cause undesirable side effects such as soil erosion (Leong, 2004). Some diseases are only able to be kept in check by the repeated applications of fungicides. Environmental pollution and death of many non target organisms, chemical residues in the food and health issues resulting from high and repeated exposure to these chemicals are of considerable concern (Faize *et al.,* 2003; Lucas *et al.,* 1992). Although breeders have succeeded in producing cultivars resistant to fungal diseases, the time-consuming processes of making crosses and back-crosses and the selection of progenies for the presence of resistance traits make it difficult to react adequately to the evolution of new virulent fungal races. Therefore, farmers often have to use chemicals. The growing concern about the environment, together with a strong motivation to lower production costs, encourages the development of cultivars of crops that require few chemicals.

Newly developed technologies in plant breeding such as molecular marker techniques and gene transfer methods can be used to develop these cultivars. In contrast to conventional breeding, this latter technology allows the transfer of traits from one species into the genomes of plants of other species, with the preservation of the intrinsic properties of the acceptor plant. Currently, much energy is being applied to identifying and isolating genes that upon transfer may render target plants resistant to fungi. Some of these efforts are being focused on resistance genes known from conventional breeding programs. Furthermore, there is an extensive search for genes that encode enzymes involved in the synthesis of compounds toxic to fungi and for genes that encode proteins with a direct inhibitory effect on the growth of fungi.

2.1 Fungal cell wall

Chitin, a β-(1,4)-linked polymer of N-acetyl D-glucosamine (GlcNAc), is widely distributed in nature, particularly as a structural polysaccharide in fungal cell walls in the exoskeleton of arthropods, the outer shell of crustaceans, nematodes, *etc.* (Dahiya *et al*., 2006). In fungal cell walls, chitin is arranged in regularly ordered layers, parallel to each other in the β-form and antiparallel in the α-form, whilst laminarin fibrils are arranged in an amorphic manner (Cohen-Kupiec *et al.,* 1999). Since chitin is the major component of most fungal cell walls, a principle role has been attributed to chitinases in the control of a wide range of phytopathogens (Collinge *et al*., 1993; Gokul *et al*., 2000).

2.2 Pathogenesis 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. However, some PR proteins are also developmentally regulated (Linthorst, 1991). PR proteins are thought to play a major role in plant defense. Some PR proteins have been characterized and their biochemical functions are known. For example, PR-2 proteins are β1, 3-glucanases and PR-3 proteins are chitinases (Van Loon *et al*., 1994). PR proteins have been classified into 17-PR protein families based on primary structure, serological relatedness and enzymatic and biological activities. 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. Chitinases are PR proteins most widely used in gene engineering, because of their well-known mechanism of action and a wide range of fungi whose growth can be inhibited by biotechnological use of chitinases.

2.3 Chitinases

Chitinases are classified into two main categories, endo- and exochitinases. Endochitinases (EC 3.2.1.14) cleave chitin at random inner sites, producing low-molecular-weight multimers of N-acetylglucosamine, such as chitotriose, chitotetraose, and diacetylchitobios dimer. Exochitinases are divided into two subcategories: chitobiosidases (EC 3.2.1.29) catalyzing release of diacetylchitobioses from the nondeoxydizing end of the chitin microfibril, and β-1, 4-N-acetylglucoseaminidase (EC 3.2.1.30), cleaving oligomer products of endochitinases and chitobiosidases with the production of monomer GlcNAc (Cohen- Kupiec *et al*., 1999). Chitinases catalyze the hydrolosis of 3-l, 4N-acetylglucosamine linkages of chitin polymers. Chitinases, which hydrolyze chitin, occur in a wide range of organisms including viruses, bacteria, fungi, insects, higher plants, and animals (Park *et al*., 1997). 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). The findings in the catalytic and substrate-binding mechanisms of chitinolytic enzymes as well as their sequence homology and applications to plant protection against fungal pathogens and insect pests were reviewed in detail by Fukamizo, (2000). Chitinases are thought to play a dual role, both by inhibiting fungal growth by cell wall digestion and by releasing pathogen-borne elicitors that induce further defense reactions in the host. Transgenic plants over expressing chitinases of several origins have been shown to exhibit enhanced levels of resistance to fungal infection and delayed disease symptoms when challenged with fungal pathogens (Jach *et al*., 1995; Lorito *et al*., 1998; Hong and Hwang, 2006 Dahiya *et al*., 2005; Karasuda *et al*., 2003; Bolar *et al*., 2000; Mora and Earle, 2001; Vaidya *et al*., 2001; DeMarco *et al*., 2000; Chang *et al*. 2003; Dana *et al.,* 2006; Emani *et al.,* 2003)

Chitinases have been isolated from fungi (Kang *et al*., 1999; Mathivanan *et al*., 1998), plants (tobacco [Melchers *et al*., 1994], cucumber, beans [Ye *et al*., 2000], peas, grains [Huynh *et al*.,1992], and many others [Esake and Teramoto, 1998; Lee *et al*., 1999; Mathivanan *et al*., 1998; Monzingo *et al*., 1996; Sakurada *et al*., 1996; Yamagami *et al*., 1998]), and bacteria (Chernin *et al*., 1997) and have potent antifungal activity against a wide variety of human and plant pathogens, including *Trichoderma reesei, Alternaria solani, A. radicina, Fusarium oxysporum, R. solani, Guignardia bidwellii, Botrytis cinerea*, and *Coprinus comatus* (Jach *et al*., 1995).

2.3.1 Plant Chitinases

Plants do not contain chitin in their cell walls, whereas major agricultural pests such as most fungi (ascomycetes, basidiomycetes, and deuteromycetes) and insects do (Collinge *et al*., 1993), leading to the assumption that plant chitinases are involved in defence mechanism against pathogens either directly through their antifungal properties or indirectly through the release of chitin oligomers capable of eliciting plant defensive responses (Collinge *et al*., 1993; Suarez *et al*., 2001; Gomez *et al*., 2002). The expression of a number of chitinase genes appeared to be induced upon fungal infection and they were shown to accumulate around hyphal walls of infection sites *in planta* (Wubben *et al*., 1992). Moreover,

plants over expressing chitinases showed decreased susceptibility to infection by some fungi that have chitin-containing cell walls (Broglie *et al*., 1991; Zhu *et al*., 1994; Jongedijk *et al*., 1995; Jach *et al*., 1995).

The first successful employment of a transgenic chitinase in order to increase plant resistance dates back to 1991: Broglie *et al*. (1991) showed constitutive production of a vacuolar isoforms of bean chitinase PR-3(I) in tobacco and rapeseed resulted in a decrease in susceptibility to *Rhizoctonia solani*. The transgene expression was manifested in a reduction of the number of dead plants among the infected *R. solani* by 20% as compared to control (from 50 to 30%), whereas infection by the oomycetes *Pythium aphanidermatum* , whose cell wall lacks chitin, did not show differences between the control and the transgenic genotypes. This experiment not only demonstrated protective properties of chitinase, but also was the first successful experience of constructing plants with high resistance to fungal pathogens.

Likewise, Salehi *et al*. (2005) reported that chitinase BCH from bean *Phaseolus vulgaris* inhibited the growth of the fungus *R. solani* upon expression in soybean (*Glycine max*) plants. In other studies, the capability of constitutively expressed chitinases to suppress the growth of chitin containing pathogens was confirmed. For instance, chitinase PR-3(I) from rice increased resistance against *R. solani* and *Botrytis cinerea* in transgenic rice and cucumber plants (Lin *et al*., 1995; Tabei *et al*., 1998). Bean chitinase ch5B increased resistance against *B. cinerea* in transgenic strawberry but did not result in higher resistance to *Colletotrichum acutatum* (Vellicce *et al*., 2006). The expression of a class IV chitinase from sugar beet in transgenic birch *Betula pendula* showed somewhat higher resistance to pathogen *Pyrenopeziza betulicola* in field tests, but a side effect of the transgenes was higher susceptibility to the leaf rust *Melampsoridium betulinum* (Pasonen *et al*., 2004). The over expression of the own chitinase under the CaMV-35S promoter in *Brassica juncea* plants reduced the growth of the pathogen *Alternaria brassicae* by 12–56% over the control level (Mondal *et al*., 2003). The rapeseed plants transformed by a chimeric endochitinase showed increased resistance to fungal pathogens *Cylindrosporium concentricum, Phoma lingam*, and *Sclerotinia sclerotiorum* (Grison *et al*., 1996).

2.3.2 *Trichoderma* chitinases

Most of the purified plant chitinases tested has lysozyme activity, and some enzymes under optimal conditions hydrolyze bacterial peptidoglycan faster than chitin. In view of low antifungal activity of plant chitinases, using chitinases from alternative sources, in particular of the bacterial and fungal origin, is considered more promising. Their chitinolytic activity has an antifungal effect whose ED50 is comparable to a number of chemical fungicides; this effect is expressed for a wide range of pathogens (Lorito *et al*, 1993; 1994). Such chitinases can degrade both comparatively fragile hyphal ends and more coarse structures of mature hyphae, conidia, chlamodospores, and sclerotia. Their enzymatic, and, consequently, antifungal activity may by orders of magnitude exceed the plant chitinase activity in relation to the plant.

Chitinases from other sources 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). In 1992, De La Cruz *et al.* were the first to isolate, purify, and characterize chitinases of the mycoparasitic fungus *T. harzianum*, used as a means of biocontrol.

Trichoderma spp. commercially represent one-third of all fungal biocontrol agents of soil-borne fungal phytopathogens (Chernin and Chet, 2002), and their prevalence as biocontrol agents has stimulated much research into the mechanisms underlying biocontrol, of which mycoparasitism is considered a major component. *Trichoderma* spp. is biocontrol agents of many economically important pathogens, such as species of *Botrytis, Rhizoctonia* and *Sclerotinia.* Control of these diseases by chemical pesticides is losing effectiveness due to enhanced degradation by soil microorganisms and growing resistance within pathogen populations (Jones and Stewart, 2000). A single *Trichoderma* chitinase expressed in tobacco and potato conferred tolerance or complete resistance to some phytopathogenic fungi (Lorito *et al.,* 1998).

In vitro experiments demonstrate that there are no chitin-containing phytopathogens that would be resistant to chitinases of *Trichoderma* (Lorito *et al*., 1993; 1994). Introduction of a single fungal gene into a cultured plant is expected to produce a transgene with increased resistance to a broad range of pathogenic fungi (multiple introductions are required if plant genes are involved). The gene *ech*42 from *T. harzianum*, (Garcia *et al*., 1994; Hayes *et al*., 1994), codes for a chitinase with significantly higher activity against a broad range of phytopathogenic fungi than other chitinolytic enzymes.

Lorito *et al*. (1998) reported that the endochitinase gene (*chit 42*) of *T. harzianum* P1 and CECT 2413 has been transferred into tobacco and potato, resulting in a high level of constitutive expression in various tissues, which has no detectable effect on the plant growth and development. The transgenic strains thus obtained are highly or completely resistant to the leaf pathogens *Alternaria alternaria, A. solani*, and *B. cinerea* and the soil phytopathogen *R. solani*. The high level and broad spectrum of the resistance acquired with the endochitinase gene from *Trichoderma sp*. surpasses the efficiency of transgenic expression of plant or bacterial chitinases in plants. These results demonstrate that fungi suppressing the development of phytopathogens constitute a rich source of genes appropriate for creating plants with a high resistance to these phytopathogens.

Bolar *et al*. (2000) obtained several lines of very susceptible 'McIntosh' apple with varying levels of *ech*42 expression. Some of them exhibited increased tolerance to *V. inaequalis*. The transgenic cucumber and carrot plants expressing chitinase genes were found to be less susceptible to infection by pathogens such as *Alternaria cucumerina, Botrytis cinerea, Colletotrichum lagenarium* and *Rhizoctonia solani* though not to the same degree (Punja and Raharjo, 1996).

Kikkert *et al.* (2000) reported that chitinases from the biocontrol fungus *Trichoderma harzianum* inhibit spore germination and hyphal elongation of the grapevine pathogens *Botrytis cinerea* (the causal agent of bunch rot) and *Uncinula necator* (the causal agent of powdery mildew) in *in vitro* assays. Embryogenic cultures of *Vitis vinifera* L. cultivars 'Merlot' and 'Chardonnay' were biolistically transformed with the *Trichoderma* endochitinase gene ThEn42 under the control of a double 35S CaMV promoter and Alfalfa Mosaic Virus leader sequence. The selectable marker gene *neomycin phosphotransferase II* (*nptII*) driven by the nopaline synthase promoter was also used. A total of 101 'Merlot' and 93 'Chardonnay' putatively transformed plants were regenerated and evaluated for expression of chitinase using a fluorometric assay. About 41% of the 'Merlot' and 55% of the 'Chardonnay' selections had 10 to 100-fold higher chitinase activity than non-transformed control plants.

Emani *et al.,* (2003) reported that in both tobacco and cotton, transgenic endochitinase activity was only observed in the plants that were transformed with the *Tv-ech1* cDNA construct and the plants transformed with the other two cDNA constructs (*Tv-ech2, Tv-ech3*) and the genomic clone construct (*Tv-ech1 g*) showed endochitinase activity levels that were similar to control values. Reasons for the differential expression of these genes were not known.

2.4 Localization of transgene products

Efficient targeting to appropriate cell organelles is one of the bottlenecks for the production of recombinant proteins in plant systems. A common practice is to use the native secretory signal peptide of the heterologous protein to be produced. Though general features of secretion signals are conserved between plants and animals, the broad sequence variability among signal peptides suggests differing efficiency of signal peptide recognition. Techniques have been developed to optimise expression levels and facilitate protein targeting to particular compartments of the plant cell, preferably in a controlled manner using inducible promoters (Stoger *et al*., 2004). These successes have shown that plants are attractive alternative production systems mainly because of their low cost, high level of safety, ease of scale-up and storage properties (Schillberg *et al*., 2003; Twyman *et al*., 2003; Stoger *et al*., 2004).

2.4.1 The secretory pathway

In the 1960s, key experiments revealed that secreted proteins first entered the endoplasmic reticulum (ER) and passed through membranous structures called

endomembrane system prior to secretion (Pallade, 1975). Experiments carried out in the early 1970s showed that the endomembrane system was composed of the endoplasmic reticulum, Golgi apparatus (or Golgi complex), secretory vesicles, plasma membrane and vacuoles (Morré and Mollenhauer, 1974). Secreted proteins, as well as those normally resident in the vacuole, ER and Golgi apparatus, require a specific leader peptide (also known as the signal peptide) allowing the nascent proteins to enter the ER lumen during translation.

Some experiments demonstrated that the addition of a leader peptide to the N-terminus of cytosolic proteins was sufficient to target such proteins into the ER lumen (Dore *et al*., 1989; Iturriaga *et al*., 1989). Denecke and co-workers established the concept of a *default* secretory pathway, which was based on a model system involving the transient expression of heterologous proteins in tobacco protoplasts (Denecke *et al*., 1990; De Loose *et al*., 1991). This model was supported by the finding that over-expression of vacuolar proteins led to their partial secretion (Stevens *et al*., 1986). This mistargeting to the intercellular space might be due to saturation of the receptors responsible for vacuolar transport, consequently resulting in secretion “by default”. These insights into protein transport in plant cells have led to the view that, in the absence of any specific targeting signals, a protein entering the endomembrane system will follow the default secretory pathway and will be secreted to the cell exterior. While passing through the endomembrane system, chaperone-assisted folding, assembly and post-translational modification of proteins take place.

The leader peptide is usually cleaved off before translation is completed. The removal of the leader peptide appears to be essential for the correct folding of the N-terminal domain of the growing protein. A cascade of interactions takes place between the newly synthesized protein and chaperones that bind to the proteins and retain them in the ER until they are correctly folded and assembled (Hartl, 1996 and Fink, 1999).

2.4.2 Role of Signal Peptide

During *et al.* (1990) constructed chimeric genes containing a plant signal peptide and the structural genes for expression and assembly of the immunoglobulin subunits. A similar approach was used for secretion of a chimeric T4 lysozyme in transgenic plants (During *et al*., 1990; Hippe *et al*., 1989). The barley α-amylase signal peptide effected transport to the intercellular space. Using the same signal peptide they expected to find the immunoglobulin chains within the ER lumen. Comparable experiments in *E. coli* and yeast have shown that assembly of an active antibody is possible in both organisms only in the presence of signal peptides in the foreign precursors (Better *et al*., 1983, Horwitz *et al*., 1988, Skerra and Pluckthun, 1988, Wood *et al*., 1985). Data provide evidence that light and heavy chain is transported to the ER and that the subunits assemble within its lumen.

Enhanced tolerance to a wide range of soil-borne and foliar fungal pathogens was exhibited by tobacco *pschit*42 plants (*chit42* fused with plant signal peptide) over expressing the *Trichoderma* endochitinase *chit42* (Garcia *et al*., 1994; Lorito *et al*., 1998). To ensure its correct processing and secretion to the apoplast, the fungal hydrolase was modified by substituting its native signal peptide with a signal peptide of plant origin (Tornero *et al*., 1994). From the wide array of hydrolases produced by *Trichoderma* during mycoparasitic interactions, CHIT33 shows significant sequence homology with some defense-related class III plant chitinases. CHIT33 exhibited antifungal activity and synergistic lytic properties with CHIT42 in *in vitro* assays (De la Cruz *et al*., 1992; Dana *et al*., 2001) and its overexpression increases the mycoparasitic activity of transgenic *T. harzianum* strains (Limón *et al*., 1999). The *chit33* gene was modified in the same way described previously for *chit*42 (Lorito *et al*., 1998) and transgenic *pschit33* tobacco lines were generated. Five independent F_3 homozygous *pschit33* lines were tested and showed significantly enhanced resistance to both fungal (*R. solani*) and bacterial (*P. syringae*) pathogens. Transgenic homozygous *pschit42* lines also exhibited improved resistance to both pathogens.

Chen *et al*. (1994) analyzed the subcellular localization of α-amylases in cultured rice (*Oryza sativa*) suspension cells and revealed that α-amylases are localized in cell walls as well as in starch granules within amyloplasts. The dual localization of α-amylases disagrees with the general belief that the translocation of proteins to chloroplasts or amyloplasts and the extracellular compartments is carried out by different targeting signals and via different pathways (Verner and Schatz, 1988). Import into chloroplasts of a nuclear-encoded protein from the cytoplasm requires an N-terminal transit peptide as a targeting signal (Schmidt and

Mishkind, 1986; Keegstra, 1989). However, the deduced N-terminal amino acid sequences of nine rice α-amylases all contain typical signal peptides (SPs) characteristic for translocation of proteins across the endoplasmic reticulum (ER) membrane (Chen *et al*., 1994). Previously, they also showed in an assay using transformed rice suspension cells, that the SP of a rice α-amylase isozyme, αAmy8, directed the translocation of β-glucuronidase (GUS) to ER, with subsequent secretion into the culture medium (Chan *et al.,* 1994). Alpha amylase3 signal peptide (αAmy3 SP) was found to be competent in targeting a cargo protein to both plastids and cell walls.

Chan *et al*. (1994) conducted an experiment to determine whether other α-amylase SPs are also capable of directing a cargo protein into plastids. Leaves of a transgenic tobacco line transformed with pAG8 containing the rice αAmy8 promoter and its SP plus GUS coding region, was also examined. GUS expression with the αAmy8 SP was detected in chloroplasts and cell walls by anti-GUS antibodies. Through gain-of-function analyses in transgenic tobacco and rice, they demonstrated that the αAmy3 SP is sufficient for directing GUS to plastids and extracellular compartments and GFP to plastids. SPs are known to carry proteins across the ER membrane prior to transport extracellularly. However, the expression of recombinant proteins fused with SPs, leading to dual targeting of the proteins to plastids and cell walls, is being increasingly observed. For example, assembled antibodies were found in chloroplasts, besides being detected in the ER, when mature light and heavy chains of a monoclonal antibody fused to a barley α-amylase SP were expressed in transgenic tobacco (During *et al*., 1990). αAmy8 SP was shown to direct extracellular translocation of GUS in transgenic tobacco, potato (*Solanum tuberosum*), and rice suspension cells (Chan *et al*., 1994). They also observed two bacterial derived enzymes, amylopullulanase and phytase, when fused to SPs derived from a rice glutelin and αAmy8, respectively, localized in both plastids and cell walls of transgenic rice endosperms and suspension cells (Chiang *et al*., 2005).

Park *et al.* (1997) reported that the gene encoding rice alpha-amylase in *Oryza sativa* was expressed in the yeast *Yarrowia lipolytica*, which is a potential host system for heterologous protein expression. For efficient secretion, the strong and inducible XPR2 promoter was used in the construction of four kinds of expression vectors with the following configurations between the XPR2 promoter and terminator: 1) XPR2 prepro-region-rice alpha-amylase coding sequence, 2) rice alpha-amylase signal peptide-rice alpha-amylase coding sequence, 3) XPR2 signal peptide-rice alpha-amylase coding sequence, and 4) XPR2 signal peptide-dipeptide stretch-rice alpha-amylase coding sequence. Secretion of active recombinant rice α-amylase into the culture medium was achieved only in the first two cases, demonstrating that the XPR2 signal peptide is not sufficient to direct the secretion of heterologous protein. Furthermore, this study shows that the XPR2 prepro-region causes imprecise processing (after Pro150-Ala151 or Val135-Leu136 instead of Lys156-Arg157) and leads to N-terminal amino acid sequences that differ from that of native rice α-amylase. In contrast, utilizing the rice α-amylase signal peptide was sufficient in directing secretion of recombinant protein with the expected N-terminal sequence, indicating that the signal peptide of rice α-amylase was effectively recognized and processed by the *Y. lipolytica* secretory pathway. Instead, the heterologous signal peptide of rice α-amylase was efficiently recognized and processed by the *Y. lipolytica* endoplasmic reticulum translocation machinery, and it conducted foreign protein secretion in this yeast. This result demonstrates that the signal peptide of rice α-amylase can be used for achieving heterologous protein secretion in *Y. lipolytica.*

Yu *et al*. (2001) showed that the 1.2-kb 5' region of αAmy8 contains a putative 25-amino-acid signal peptide sequence which is shown to facilitate targeting of GUS outside the transgenic rice, tobacco or potato cells. Evidence for translocation of GUS to the cell membrane via the ER of the plant cells is based on the observation that cells transformed with the αAmy8/GUS chimeric gene secreted an 85 kD GUS into the culture medium and activity of the secreted GUS was significantly reduced. Treatment of these cells with TM caused a decrease of molecular mass (70 kD) and increase of activity of GUS secreted into the culture medium, suggesting that GUS is transported to the ER, glycosylated in the lumen of the ER, and secreted outside the cells. Therefore, the signal peptide sequence of αAmy8 allows secretion of the expressed passenger proteins which need to be modified and assembled into active molecules in the ER. The GUS activity in the suspension cells of tobacco or rice was slightly increased, indicating that some intracellular GUS was

glycosylated, however, which was not detectable by the Western blot analysis. In conjunction with the signal peptide sequence of α-amylase, this expression cassette can also be used for production of secreted recombinant proteins. This expression system offers new possibilities for basic research in plant biology as well as for large-scale production of genetic engineered proteins.

Altpeter *et al*. (2005) attempted epidermis-specific expression of a PR protein in transgenic wheat. Expression and accumulation of antimicrobial proteins at extra-cellular space where the target fungi invade is another strategy to improve resistance. The crucial role of the ER in safeguarding the correct folding and assembly of proteins has become clearer in the past two decades with the discovery of the ER molecular chaperone machinery and the associated quality control mechanisms. These safeguards not only optimize folding and assembly of newly synthesized secretory proteins but also dispose of defective ones (Haas and Wabl, 1983; Munro and Pelham, 1986; Hammond and Helenius, 1994; Wiertz *et al*., 1996; Pedrazzini *et al*., 1997).

Tesfaye *et al*. (2005) characterized a secreted acid phosphatase (APase) enzyme (EC 3.1.3.2) from roots of white lupin (*Lupinus albus* L.) that contains a signal peptide sequence of 31-amino acids at the N-terminal region of the mature protein (Miller *et al*., 2001). Copious amounts of this APase are exuded from phosphorus deficient roots (Miller *et al*., 2001). A similar coding sequence is also involved in the secretion of APase in Indian mustard (*Brassica juncea* L.) (Haran *et al*., 2000).

Asatsuma *et al*. (2005) determined the role of α-amylase isoform I-1 in the degradation of starch in rice leaf chloroplasts by generating a series of transgenic rice plants with suppressed expression or overexpression of α-amylase I-1. In the lines with suppressed expression of α-amylase I-1 at both the mRNA and protein levels, seed germination and seedling growth were markedly delayed in comparison with those in the wild-type plants. However, the growth retardation was overcome by supplementation of sugars. Interestingly, a significant increase of starch accumulation in the young leaf tissues was observed under a sugar-supplemented condition. In contrast, the starch content of leaves was reduced in the plants overexpressing α-amylase I-1. In immunocytochemical analysis with specific anti-α-amylase I-1 antiserum, immuno-gold particles deposited in the chloroplasts and extracellular space in young leaf cells. They further examined the expression and targeting of α-amylase I-1 fused with the green fluorescent protein in re-differentiated green cells, and showed that the fluorescence of the expressed fusion protein co-localized with the chlorophyll autofluorescence in the transgenic cells. In addition, mature protein species of α-amylase I-1 bearing an oligosaccharide side chain were detected in the isolated chloroplasts. Based on these results, they concluded that α-amylase I-1 targets the chloroplasts through the endoplasmic reticulum–Golgi system and plays a significant role in the starch degradation in rice leaves

Yu *et al*. (2005) reported that the *Arabidopsis thaliana* genome encodes three α-amylase-like proteins (AtAMY1, AtAMY2, and AtAMY3). Only AtAMY3 has a predicted N-terminal transit peptide for plastidial localization. AtAMY3 is an unusually large α-amylase (93.5 kDa) with the C-terminal half showing similarity to other known α-amylases. When expressed in *Escherichia coli*, both the whole AtAMY3 protein and the C-terminal half alone show α-amylase activity. They showed that AtAMY3 is localized in chloroplasts. The starch-excess mutant of *Arabidopsis sex4*, previously shown to have reduced plastidial α-amylase activity, is deficient in AtAMY3 protein. AtAMY3 is unusual among α-amylases not only in having a plastid-targeting signal but also because it is 93 kDa, whereas other α-amylases are typically about half this mass. The C-terminal half of the protein contains the α-amylase domain and is sufficient to give the protein α-amylase activity. The function of the N-terminal region is unknown, but other plant species contain genes encoding α-amylases that are conserved in this region.

Harighi *et al*. (2006) reported that the cDNA from the mycoparasitic fungus *Trichoderma atroviride* PTCC5220 encoding a 42 kDa chitinase (*chit*42) was isolated. The nucleotide sequence of the cDNA fragment as having a 1263 bp open reading frame that encodes a 421 amino acid polypeptide, and a high homology was found with other reported *Chit*42 belonging to the *Trichoderma sp*. The 22 amino acid N-terminal sequence is a putative signal peptide for the possible secretion of the protein. The protein has been expressed and secreted as a mature form in *Escherichia coli* BL21 (DE3) using the pelB leader sequence.

The *E. coli* strain expressed *chit*42 in an active form and secreted the protein into the medium. This recombinant chitinase has been shown to have inhibitory activity on mycelial growth and also, lytic activity on the cell wall of *Rhizoctonia solani* (AG2-2), causal agent of root rots in sugar beet in vitro. Expressed chitinase was optimally active at pH 5 and at 40°C. It is thermally stable at 60°C for more than 120 min at pH 5.

Juge *et al*. (2006) reported that efficient production of recombinant barley α-amylase has been achieved in *A. niger*. The cDNA encoding α-amylase isozyme 1 (AMY1) and its signal peptide were placed under the control of the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter and the *A. nidulans trpC* gene terminator. Secretion yields up to 60 mg/l were obtained in media optimised for α-amylase activity and low protease activity. The recombinant AMY1 (reAMY1), was purified to homogeneity and found to be identical to native barley AMY1 with respect to size, p*I* and immunoreactivity. N-terminal sequence analysis of the recombinant protein indicated that the endogenous plant signal peptide is correctly processed in *A. niger*.

Liu (2006) compared different signal peptides for secretion of GFP in rice suspension culture; found that αAmylase3 is the better signal peptide for secreting the GFP out. To examine whether the supremacy of the αAmylase3 signal is specific for GFP, they analyze the secretion efficiency of mGM-CSF and intend to develop a high efficiency secretion system in rice suspension cells.

2.5 Antifungal protein secreted in liquid suspension media

Considering various forms of *in vitro* plant tissue cultures, cell suspension culture is most amenable to large-scale production of natural compounds, owing primarily to its superior culture homogeneity. This fact has already been demonstrated in several large scale applications, including the commercial shikonin process. Transgenic plant cell cultures provide a convenient method for recovery of a transgenic protein, provided that an extracellular targeting system is available. The signal peptide shows promise for this purpose. The system could be used for the large-scale production of fungal endochitinase or other transgenic proteins of interest. It might also provide a model for studying the processing and secretion of heterologous proteins in plants.

Fakuda *et al*. (1991) constructed a chimeric gene consisting of 5' flanking DNA of the gene fused to the coding sequence of fl-glucuronidase (GUS) (Jefferson *et al*., 1986) as a reporter gene to investigate its mode of expression in suspension cultured tobacco cells. An increase of chitinase mRNA in suspension-cultured tobacco cells BY2 was observed at late logarithmic growth phase. In case of the suspension-cultured cells, chitinase mRNA levels may be controlled by endogenous stress signals, *i.e.* oligo galacturonide fragments of cell walls (Tong *et al*., 1986) or endogenous ethylene that accumulates during cell aging (Felix and Meins, 1987).

Esaka *et al*. (1990) established suspension cultures by inoculating callus cells, which had been subcultured at about 4-week intervals for more than 3 years, into a 200 ml Erlenmeyer flask containing 50 ml of Murashige and Skoog's, (1962) basal medium with 1.0 mg/L 2,4-D, 0.1 mg/L kinetin and 3% (w/v) sucrose. These cultures were agitated on a rotary shaker at 100 rpm at 25 °C, and maintained by transferring about 1 g of the cells to fresh liquid medium at about 4-week intervals. SP32 was purified from culture medium of the cultures grown in the medium containing 40 mm $CaCl_2$. The amino acid composition of SP32 was determined as well as the amino acid sequence of the amino terminal part (14 residues). The amino-terminal amino acid sequence of SP32 was similar to that of chitinase isolated from tobacco (Shinshi *et al.,* 1987) or bean (Hendrick *et al.,* 1988), although it was quite different from that of chitinase isolated from cucumber (Metraux *et al.,* 1989). There was an 80% identity (8 of 10 residues) between the amino-terminal amino acid sequence of SP32 and that of chitinase isolated from tobacco, and was a 71% identity (10 of 14 residues) between that of SP32 and that of chitinase from bean. Thus, a significant homology in amino terminal amino acid sequence was found between SP32 and chitinase from tobacco or bean. Furthermore, SP32 was shown to have chitinase activity. These results suggest that SP32 in pumpkin cell suspension cultures is a chitinase.

Lund and Dunsmuir, (1992) described experiments directed towards improving secretion of ChiA by plant cells. They tested whether secretion depends upon the presence of

an N-terminal signal sequence and if replacement of the signal sequence of ChiA with that of the tobacco PRIb protein increases secretion of the ChiA protein by plant cells. The secretion of mutated forms of ChiA lacking the consensus sequence for N-linked glycosylation was also investigated. They also assayed the level of secretion of ChiA from these different transgenic plant cells by analyzing the media from plant cell suspension cultures and by comparing the profiles of protein extracts from leaf protoplasts and corresponding whole leaves. These data from the analysis of suspension culture media suggest that in the absence of any signal sequence (pChiA-M) the ChiA which is expressed is not secreted. In the presence of a signal sequence, either the ChiA signal (pChiA), or the PRIb signal (pPRSSChiA), ChiA protein is glycosylated and secreted.

Arie *et al*. (2000) reported that a basic chitinase was secreted into culture medium of pumpkin cell suspension cultures. The chitinase was purified from the culture medium. A cDNA encoding the pumpkin chitinase was cloned by reverse transcription (RT)-PCR and rapid amplification of cDNA ends (RACE) methods. The chitinase gene was strongly expressed in pumpkin callus cells, but little or not at all in mature leaf, young leaf, cotyledon, stem, hypocotyl and root of pumpkin. No chitinase mRNA was detected in intact pumpkin fruit tissues. However, chitinase was induced during callus formation from sliced pumpkin fruit tissues. Induction also occurred in the absence of 2, 4-D, a chemical causing callus formation, suggesting that it may be independent of the presence of 2, 4-D. Perhaps, induction is caused by osmotic or wounding stress. Levels of chitinase mRNA markedly increased at 4 h after transfer of pumpkin callus cells into fresh culture liquid medium. They were also high at later stages of cell suspension culture. In transgenic tobacco BY-2 cells, into which the pumpkin chitinase cDNA was introduced, the recombinant pumpkin chitinase was expressed and secreted into the culture medium, suggesting that the signal peptide of pumpkin chitinase also functions for secretion from tobacco BY-2 cells

Brants and Earle, (2001) reported the induction of callus and suspension cultures expressing high levels of endochitinase activity from tobacco plants transformed with *T. harzianum* endochitinase cDNA. In these cultures the fungal endochitinase is secreted from cells and accumulates in the culture medium. Concentrated culture medium can inhibit germination of *Penicillium digitatum* spores. Calli from four primary transformants had high levels of endochitinase activity, like the plants from which they were derived. Endochitinase activity was also detected in the medium surrounding the calli and in the medium from transgenic cell suspensions. Western blots demonstrated the presence of the expected 40-kDa *T. harzianum* protein in transgenic samples but not in controls. These results indicate that the fungal enzyme is secreted and that the fungal signal peptide in the cDNA constructs functions in plant cells. A cell suspension medium in which the protein concentration was increased up to 34-fold by ammonium sulfate precipitation inhibited germination of *Penicillium digitatum* spores.

Hosseini and Mulligan, (2002) employed cell suspension cultures of *Oryza sativa* (L.) *cv* Taipei 309 to demonstrate a correlation between increased single strand preferring nuclease (SSPN) activity with the onset of cell senescence *in vitro*. Taipei 309 cell suspension cultures were initiated from embryogenic calli induced from mature rice seed scutella (Finch, 1991). Cells were maintained in 100 ml conical flasks in modified AA2 medium, based on the formulation of Müller and Grafe, (1978) as modified by Abdullah *et al*. (1986). Suspensions were incubated on a rotary shaker (120 rpm) at 28 ±□2ºC in the dark. The suspension cultures were subcultured every 7 d.

2.6 Chitinase assay

2.6.1 Estimation of Reducing Sugars

A number of assays can be applied to measure both endo- and exochitinase enzyme activity. These include colorimetric, radiochemical and gel electrophoresis based assays (Boller *et al.,* 1983; Molano *et al.,* 1977; O'Brien and Colwell, 1987; Roberts and Selitrennikoff, 1988; Trudel and Asselin, 1989; Wirth and Wolf, 1990).

Endochitinase activity can be measured by using a microtitre plate assay using p-nitrophenyl-β-D-N,N',N''–acetylchitotriose as the substrate (Harman *et al*., 1993; Bielka *et al*., 1984; Heinrikson and Meredith, 1984), and also in some cases p-nitriphenyl-β-N-acetyl-β-D-glucosamine (pNPGlcNAC), p-nitrophenyl-β-D-N-N'-diacetylchitobiose (pNPGlcNAc)2 (Inbar

and Chet, 1991 and Viterbo *et al*., 2002) and 4-methylumbelliferyl β-D-N',N"-diacetylchitotrioside, 4-methylumbelliferyl-N-acetyl-β-D-glucosamide (4-MU-GlcNAc), a fluorogenic analogue of chitin was used as substrate for hydrolysis (Cottrell *et al*., 2002; Haran *et al*., 1995). Some researchers also used 4-Methyl umbelliferone –D-N-N'-N"-triacetyl chitotriose, N-acetyl glucose amine (Kim *et al*., 2002, Lorito *et al*., 1998) as a substrate.

2.6.1.1 Colorimetric assay

Roby *et al*. (1986) measured chitinase activity using chitin as a substrate (Abeles *et al.,* 1971). Chitin was prepared from crab shell chitin (Sigma), according to Tracey (1955). The incubation medium consisted of 0.5 ml of enzymic extract and 1 ml of chitin suspension (12 mg/ml in acetate buffer 0.1 M, pH 5.0). After incubation at 30°C for 45 min or 90 min, the enzymatic hydrolysis was stopped by boiling the whole mixture for 10 min; the unhydrolysed chitin was pelleted by centrifugation and the released N-acetylglucosamine residues were colorimetrically assessed (Reissig *et al*., 1955) on an aliquot of the supernatant. Enzyme and substrate blanks were also carried out. Chitinase activities were stimulated by elicitor or ethylene by a factor of 3 to 11.

Huynh *et al*. (1992) measured chitinase activity by a colorimetric assay. The reaction mixture contained an appropriate amount of the enzyme preparation and 1 mg of chitin in 0.3 ml of 10 mM sodium acetate buffer, pH 4.5. After incubation for 1 h at 37ºC, the reaction mixture was centrifuged and 150 pl of the supernatant was then incubated with 15 pl of 1.0 M potassium phosphate buffer, pH 7.1, and 0.3 mg of snail gut enzyme in 10 mM potassium phosphate buffer, pH 7.1, for 1 h at 37ºC. Released monomeric N-acetylglucosamine was then colorimetrically determined with p- dimethylaminobenzaldehyde following the method of Reissig *et al*. (1955). One unit of enzyme activity was defined as the amount of enzyme which produced 1 pmol of N-acetylglucosamine/min at 37ºC. Specific activity was expressed as units per mg of protein. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard. The results indicated that in contrast to the Chit B, which showed no change in its intensity upon addition of N,N"diacetylchitobiose, a small but significant increase in intensity was observed on addition of N,N"diacetylchitobiose to Chit A.

Katatny *et al*. (2000) assayed chitinase activity using the colorimetric method described by Molano *et al*. (1977) with minor modifications (Ulhoa *et al*., 1992). The assay mixture contained 1 ml of 0.5 % pure chitin (Sigma, suspended in 50 mM acetate buffer pH=5.2) and 1 ml of enzyme solution. The reaction mixture was incubated for 7 h at 37 °C with shaking and was stopped by centrifugation (5000 g/min) for 10 min and the addition of 1 ml of dinitrosalicylate (DNS) reagent. The amount of reducing sugars released was calculated from standard curves for GlcNAc and glucose, and the activities of chitinase were expressed in pkat (pmol/s). Out of 24 fungal isolates the best enzyme producer was T24 identified as *T. harzianum* Rifai which exhibited highest activities of chitinase (29.3 pkat/ml).

Ike *et al*. (2006) used the substrate chitisan 7B and chitosan 10B for the determination of chitinase activity. The reaction mixture contained 1 ml of enzyme in 50mM sodium acetate buffer (pH 5.5) and the substrate, incubated at 30°C for appropriate time. This reaction was terminated by immersing the reaction mixture in boiling water for 10 minutes and reducing sugar released was estimated.

Xiao *et al*. (2007) used 1 g fresh leaf for chitinase assay to prepare intercellular fluids using extract buffer (50mM sodium acetate, pH 5.0). The residual tissues were ground in 6 ml extract buffer, and the slurry was centrifuged at 10,000g for 10 min. The chitinase activities in the supernatants and in the intercellular fluids were determined with colloid chitin as substrate (Bollar *et al*., 1983; Neuhaus *et al*., 1991). Enzyme solutions were incubated with 20 ml colloid chitin in a total volume of 160 ml in phosphate buffer (0.1 M, pH 6.4) at 37°C for 1 h. After pelleting, equal aliquots of 45 ml supernatants were mixed with 5 ml H_2O (for exochitinase activity) or snailase (10%, w/v, for total chitinase activity) and incubated at 37°C for 0.5 h. At this step, the digestion products of endochitinase (chitin oligosaccharides) were hydrolyzed to release GlcNAc by snailase. Reactions were stopped by adding 10 ml 0.8 M K_3BO_3 solutions and boiling them for 10 min. The released GlcNAc was determined colorimetrically by ρ-dimethylaminobenzadehyde test (Reissig *et al*., 1955). One chitinase activity unit was defined as the amount of protein required to release 1 mg GlcNAc by hydrolyzing colloid chitin at 37°C for 1 h. Endochitinase activity was calculated as total activity

minus exochitinase activity. It has been found that the C-terminal extension of class I chitinase is a vacuolar target signal, which is sufficient to sort the mature protein to vacuoles. Mcchit1-overexpressing *N. benthamiana* plants showed high levels of endochitinase activity in the intercellular fluid, suggesting that the Mcchit1 can be secreted into the extracellular space.

2.6.1.2 Radiochemical assay

Roby *et al*. (1986) determined chitinase activity using radiolabeled chitin as a substrate. This substrate was prepared by reacetylation of chitosan with tritiated acetic anhydride (Amersham, 500 mCi/mmol), according to Molano *et al*. (1977); its specific activity (49.7 kBq/mg corresponding to 4.6 Mmol GlcNAceq) was determined by measuring the radioactivity and the N-acetylglucosamine residues (in terms of glucosamine equivalents) released by acid hydrolysis (100°C, 6 h) of an aliquot. The enzymic assay consisted of 0.2 ml of the enzymic extract and 0.5 ml of a suspension of radiolabeled chitin (6 mg/ml in acetate buffer 0.1 M, pH 5.0). After incubation for 30 min at 30°C, the enzymic hydrolysis was stopped by adding 0.4 ml of a 20% TCA solution. The suspension was then centrifuged and the released radioactivity was measured by liquid scintillation counting of a 0.3 ml aliquot of the supernatant added to 5 ml of Ready-Solv MP (Beckman). Since the reaction product formation was nonlinearly related to enzyme concentration, several dilutions of the enzymic extract were performed for each assay, and the activity was determined for an enzyme concentration approaching 0, as described by Boller *et al*. (1983). The chitinase activities which were very low in control plants was 10-fold (radiochemical assay) enhanced upon elicitor treatment.

Williams and Leung (1993) measured chitinase assay radiochemically. Callus tissues were frozen with liquid nitrogen and ground in a mortar and pestle, from which a weighed sample was taken. A measured volume of 50 mM sodium phosphate buffer (pH 6.3) was added and the homogenate centrifuged for 10 min at 18,300 g. The supernatant was assayed immediately. [^{3}H]-chitin was prepared in laboratory from chitosan (Sigma) by acetylation with [^{3}H] acetic anhydride (Amersham). The reaction mixture consisted of enzyme extract, 30 lxl [^{3}H]-chitin, and 30-45 lxl 50 mM sodium phosphate buffer (pH 6.3) in a final volume of 100 lxl. After 30 min of incubation in a shaking water bath (200rpm) at 37°C, the reaction was stopped with 300 ~1 of 10% (w/v) trichloroacetic acid. The suspensions were centrifuged at 7800g for 10min to separate the soluble labelled fragments from the insoluble [^{3}H]-chitin, and 200 p~l of the supernatant was counted on a LKB-WALLAC 1410 scintillation counter after 6 ml ACSII (Amersham) or 10 ml Hi-Safe (LKB) scintillation cocktail has been added. All procedures were carried out at 4 °C. Duplicate assay controls (minus enzyme) were included with each assay and their mean count was subtracted from all readings. Different enzyme dilutions were used for replicate experiments. Chitinase activity is described as the amount of enzyme liberating 1 DPM per second of reaction time (s^{-1}) of radioactive fragments from [^{3}H] labelled chitin under the given assay conditions, at infinite enzyme dilution. Protein content of tissue extracts was determined by the method of Bradford (1976). Callus chitinase was significantly induced to above-basal levels after 72 h incubation on agar solidified medium containing 1 mM and 10 mM salicylic acid, although after 24 h incubation no significant trend of chitinase induction was observed. Similarly, callus chitinase was significantly increased to above-basal levels after 72 h incubation in sealed containers on medium overlaid with 0.05 M ethrel, although no induction trend was observed after 24 h. After 72 h incubation in liquid medium containing 0.1 mM salicylic acid, callus in suspension cultures had a high chitinase specific activity. Chitinase was also detected in the liquid medium, the enzyme induction pattern paralleling that of callus.

Suginta *et al*. (2000) measured chitinase activity by the release of radioactive oligosaccharides from labelled insoluble chitin. This method was found to be more sensitive and convenient than colourimetric or viscometric assays also tested. Chitosan (Sigma practical grade from crab shells) was labelled by acetylation with 1.5ml of acetic anhydride containing 370 MBq of [^{3}H] acetic anhydride essentially according to Cabib (1988). The final specific activity of the labelled chitin was 159 Bq mg^{-1}. For the assay, the labeled chitin was suspended with constant stirring in distilled water to a final concentration of $10mgml^{-1}$. Each assay tube contained 5 µl of 1 $moll^{-1}$ Mes buffer, pH6.0, 15 µl of ^{3}H-chitin suspension and 80 µl of enzyme solution. The tubes were placed in a shaking water bath at 30ºC for 1 h. Trichloroacetic acid (0.3ml of 10% w/v) was added to stop the reaction. Samples (0.2 ml) were centrifuged at 12 000 g for 5 min, then 0.1ml of supernatant was taken for liquid

scintillation counting. One unit of enzyme activity corresponds to 1 mmol of N-[^{3}H]acetylglucosamine released in 1 min at 30ºC. They showed that *V. fischeri* and *V. alginolyticus* 284 (in addition to *V. carchariae* and *V. alginolyticus* 283) secreted high levels of chitinase activity.

2.6.1.3 Gel electrophoretic based assay

Ridout *et al*. (1986) carried Anodic discontinuous PAGE in slabs (160 × 168 × 1.5 mm) or rods (i.d. 2.7 mm) for two-dimensional electrophoresis as described by Davis (1964). Protein concentrates were suspended in 0.062 M-Tris/HCl stacking gel buffer (30 pl) containing glycerol (12.5%, v/v) and aqueous bromophenol blue (12.5%, v/v). Gels were stained with Coomassie blue R250 (0.1 15%, w/v). Three types of PAGE were used.

(i) Homogeneous gel electrophoresis. *T. harzianum* IMI 298372 concentrates were electrophoresed in *5,* 6, 7, 10 and 15% resolving gels at pH 8.8 with a 4% stacking gel at pH 6.8 (Hames, 1981). Samples were electrophoresed at 30 mA until the bromophenol blue reached the bottom of the gel.

(ii) Gradient gel electrophoresis. Gels were prepared (7-20%, w/v) using a gradient mixer (Hames, 1981). Samples were prepared as above and electrophoresed at 30 mA for 2.5 h after the bromophenol blue had reached the bottom of the gel. *M,* markers were also included (Sigma).

(iii) Two-dimensional electrophoresis. *T. harzianum* IMI 298372 concentrate (250 p,g) was electrophoresed in a 6% tube gel (i.d. 2.7 mm) until the bromophenol blue reached the bottom. The gel was laid onto a 7-20% gradient gel. The protein was re-electrophoresed for 2.5 h after the bromophenol blue had reached the bottom of the gel. Unseparated protein and *M,* markers were also included.

When the proteins were analysed by electrophoresis several major bands were present in all strains of *T. harzianum*. However, there were differences in the total number and intensity of bands between strains. The electrophoretic profiles of induced proteins from *T. viride* strains were different from those of *T. harzianum* strains. In general, the number of distinct bands was fewer for *T. viride.* The protein induced by crude cell walls is represented mainly by a single streak after 45 h as shown by electrophoresis. This may correspond to *R. solani* protein being released following the initial lysis of the cell walls, since this feature was absent when purified cell walls were used. After 90 h the electrophoretic profiles were similar from cultures grown on crude or purified cell walls. The strain of *R. solani* used affected the total extracellular protein produced and the corresponding enzyme activity. *R. solani* strain 5316 (AG1) induced the highest total 1, 3-P-D-glucanase activity whereas strain 53 12 (AG4) induced the highest chitinase activity.

Huynh *et al*. (1992) conducted gel electrophoresis based chitinase assay from maize seeds. Maize seeds (2 kg) were ground and homogenized in 2 liters of 10 mM sodium acetate buffer, pH 5.0. After centrifugation, the supernatant fraction was collected and solid ammonium sulfate was added with stirring to 60% of saturation. After stirring overnight, the precipitate was collected by centrifugation, suspended in 50 ml of 10 mM sodium acetate buffer, pH 5.0, and extensively dialyzed against 20 mM sodium bicarbonate buffer, pH 8.4. The small precipitate that formed during dialysis was removed by centrifugation, and the supernatant was incubated with 100 g of chitin in 200 ml of 20 mM sodium bicarbonate buffer, pH 8.4. After 1 h of incubation with constant stirring, the chitin-supernatant mixture was centrifuged and the precipitate which contained chitin-chitinase complexes was washed with 800 ml of 20 mM sodium bicarbonate buffer, pH 8.4, and then with 400 ml of 10 mM sodium acetate buffer, pH 5.0, to remove proteins binding nonspecifically to chitin. Chitinases were separated from chitin by incubation of the chitin-chitinase complexes with 500 ml of 20 mM acetic acid, pH 3.0, for 15 min followed by centrifugation. The supernatant, which contained chitinases, was extensively dialyzed against 10 mM sodium acetate buffer, pH 5.0, and applied to a Mono S column equilibrated with the above buffer. Chitinases were eluted from the column with a linear gradient of 0-0.5 M sodium chloride dissolved in 10 mM sodium acetate buffer, pH 5.0. Purity of the isolated enzymes was confirmed by SDS-polyacrylamide gel electrophoresis with silver staining (Laemmeli, 1970). The molecular weights of the isolated enzymes were determined by SDS-polyacrylamide gel electrophoresis and by gel filtration on a Sephadex G-75 column (2 X 100 cm) equilibrated with 50 mM sodium acetate

buffer, pH 5.0, containing 100 mM NaCI. The purified enzymes showed over 95% homogeneity after this step. The chitin-bound chitinase fraction was further separated by Mono S column chromatography into two fractions, designated Chit A and Chit B, which eluted at 220 mM and 350 mM sodiun chloride, respectively. From 2 kg of maize seeds, approximately 26 mg of Chit A and 30 mg of Chit B were isolated with yields of 20.8 and 6.8%, respectively. The specific activity of Chit B (9.46 units/mg protein) was; however, much lower than that of Chit A (33.26 units/mg protein).

Williams and Leung, (1993) conducted gel elecrophoresis based chitinase assay. Non-denaturing gels were run at pH 8.9. Polyacrylamide (7.5% (w/v)) resolving gels (100 x 140 x 1.5 mm) contained 3.75 ml buffer, 0.1% (w/v) APS and 0.05% (v/v) TEMED. Stacking gels (30 x 140 x 0.75 mm) contained 3.75% (v/v) polyacrylamide, 2.5ml buffer, 0.04% (w/v) APS and 0.15% (v/v) TEMED (Hames 1981). Enzyme extracts were mixed with 15% (w/v) sucrose and 0.01% (w/v) bromophenol blue before analysis. Electrophoresis was carried out at 4℃ for 4h at 20mA. Chitinase activity following electrophoresis was detected using an overlay gel containing 0.01% (w/v) glycol chitin (Trudel and Asselin, 1989). Gels were incubated at 37 ℃ for 1 h in a plastic container under moist conditions. Following incubation, the overlay gel was stained for 5 min in freshly prepared 0.01% (w/v) calcofluor white in 500 mM Tris-HCl (pH 8.9), followed by washing for 1 h at room temperature in distilled water. Gels stained with Calcofluor white were placed on a U.V. Products TM-15 transilluminator to visualize lytic zones. Gels were photographed using a Hoya orange (G) and a Kodak yellow8 filter onto Agfa APX 25 film. Callus extracts and suspension fluid with high chitinase activities from callus suspension cultures incubated with salicylic acid and ethrel for seven days were analysed by nondenaturing PAGE. Electrophoresis at pH 8.9 indicated one major band of activity in control callus and suspension fluid. Treatment of callus suspension cultures with salicylic acid produced a new chitinase activity in both the callus and suspension fluid. Callus extracts and suspension fluid from ethylene-treated cultures contained no new activity

Zou *et al*. (2002) developed a simple, inexpensive, nonradioactive gel-diffusion assay for chitinase that can be used to screen large numbers of samples. In this assay, chitinase diffuses from a small circular well cut in an agarose or agar gel containing the substrate glycol chitin, a soluble, modified form of chitin. Chitinase catalyzes the cleavage of glycol chitin as it diffuses through the gel, leaving a dark, unstained circular zone around the well, because the fluorescent dye calcofluor binds only to undigested chitin. Sample activities can be determined from linear regression of log standard enzyme concentration versus the zone diameter of internal standards on each Petri dish used for a diffusion assay.

2.6.1.4 Fluorometric assay

Mora and Earle, (2001) measured endochitinase (*T. harzianum*) activity of transgenic plants by a fluorometric assay on a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech). The assay is based on fluorescence readings derived from cleavage of a trimeric substrate, 4-methylumbelliferyl *β*-DN, N', N''-triacetyl chitotrioside (4-MU[GlcNAc] (Brants, 1999; Bolar *et al*. 2000; Brants and Earle 2001). Fluorometric readings were normalized according to total protein content of the plant sample (BioRad protein assay). Endochitinase levels of plants maintained *in vitro* or transferred to soil were analyzed. At least two leaves per plant in three replicates were analyzed for each line in each developmental stage. Endochitinase levels in most T1 plants were also lower at the flowering stage than in young plants in soil. However, endochitinase activity of mature T_1 plants relative to controls was higher because the corresponding control plants had lower values.

Tesfaye *et al*. (2005) performed chitinase activity assays of *ech42* from *T. harzianum* using a modification of the method described by Bolar *et al*. (2000). Leaf or root tissues were harvested and kept on ice. Approximately 250 mg fresh plant tissues were ground in 5 ml extraction buffer containing 0.1% SDS (w/v), 10 mM EDTA, 1% (v/v) Tween-20 and 0.07% (v/v) *β*-mercaptoethanol in 100 mM sodium acetate (pH 5), with a mortar and pestle and centrifuged. Aliquots of the supernatant were used for the chitinase activity assay, and detection of endochitinase protein using western blotting. Total protein in tissue extracts was estimated using bovine serum albumin as a standard with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Soluble leaf and root tissue extracts were serially diluted 1:10 and 1:100 with extraction buffer. In a 96-well black-sided plate, 100 *μ*l of tissue extract or concentrated root exudates was mixed with 50 *μ*L substrate and incubated at 37ºC for 30 min. The substrate solution was 0.2 mM 4-methylumbelliferyl *β*-D-N, N-, N-triacetylchitotriside (Sigma,

St. Louis, MO) in 3.2% (v/v) DMSO and 100 mM sodium acetate (pH 5). The reaction was stopped by adding 100 µl of 0.2 M sodium carbonate. The amount of the fluorescent 4-methylumbelliferyl (4-MU) released from the substrate was measured using a BIO-TEK FL600 microplate fluorescence reader at an excitation wavelength of 360 nm and emission at 460 nm (Bolar *et al*., 2000). Microtitre plates were also photographed under a 365 nM UV light. All of the transgenic alfalfa lines tested that expressed the *ech*42 transgene showed significantly higher chitinase activity than control plants.

Dana *et al*. (2006) used the fluorescent-specific substrate [4-MU-(GlNAc) 4] (Sigma) for assaying chitinase activity of *chit33* from *T. harzianum* as described (Limon *et al*., 1995). Assay mixtures (100 ml) containing 1 mg of total protein extract and 250 mM [4-MU-(GlNAc)4] in 100 mM sodium citrate buffer, pH 3.0, were incubated for 15 min at 30°C in the dark. The reactions were stopped with 2.9 ml of 0.5 M Gly-NaOH buffer, pH 10.4, and fluorescence was measured in a Hoefer TK0100 fluorimeter at 350-nm excitation and 440-nm emission wavelengths. The chitinase activity was expressed as picomoles of 4-methylumbelliferone liberated per minute and micrograms of protein. The chitinase activity of each transgenic line correlated with its levels of mRNA *chit33* accumulation and of CHIT33 production, whereas that of control plants was almost undetectable.

Harighi *et al*. (2006) estimated chitinase activity of *Chit42* from *T. atroviride* with 200 µl each of colloidal chitin (5 mg/ml), and recombinant enzyme. The mixture was incubated for 60 min at 40ºC, and the reaction was stopped by adding 1 ml of NaCl (1%) and centrifuged at 6000×*g* for 5 min. The supernatant was boiled with 100 µl of potassium tetra borate buffer for 3 min. 3 ml of DMAB reagent [10 g of Di-methyl amino benzaldehyde in 100 ml of glycial acetic acid (12.5% v/v) and 10N chloridric acid (87.5% v/v)] was added to the reaction and incubated at 40ºC for 20 min, and the amount of N-acetylglucosamine (GLcNAc) produced in the supernatant was determined by the method described by Zeilinger *et al.* (1999) using GLcNAc as a standard. One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 µmol GLcNAc in 60 min at 40ºC. Cytoplasmic and periplasmic fractions were obtained as described by Sashihara *et al.* (1984). About 12.5 % of total chitinase activity was localized in the intracellular fraction and also about 18.5 % of the total enzyme remained in the periplasm, and residual activity of 69 % excreted into the medium.

2.7 Bioassay against pathogen

Utility of the transgenic plants with disease or insect resistance gene depends upon their effectiveness in controlling the respective pathogen or insect. Therefore, it is essential to screen the transgenic plants against target pest. Several studies are available on the bioassay of transgenic plants with fungal disease resistance gene and are reviewed here. Symptoms development and its severity vary significantly between the control and transformants, and again among the transgenics (Emani *et al.,* 2003). Also response may be different for different pathogens.

2.7.1 Bioassay against *Sclerotium*

Sclerotium rolfsii is a fungus that has a wide host range and also is a destructive pathogen of several economic crops (Aycock, 1966; Papavizas and Lewis, 1989). It is a soil-borne fungus that causes blight, root rot; stem rot on more than 500 plant species in tropical and subtropical countries of the world (Aycock, 1966).

Katatny *et al*. (2000) reported inhibition of *S. rolfsii* with *T. harzianum* culture filtrates. Agar plates (PDA; 10 % volume fraction) were prepared with *T. harzianum* culture filtrates (sterilized by filtration) or with water (control). *S. rolfsii* was inoculated in the center of agar plates using 5-mm mycelial discs and incubated at 30 ºC for 3 days. The radial diameter of the colonies was measured at right angles every day, for six replicate plates per treatment, measuring daily growth rate, and percent of inhibition was calculated. Culture filtrates of *T. harzianum* inhibited the growth rate of *S. rolfsii* more than 30 %.

Kucuk and Kivank, (2002) conducted *in vitro* experiment to show inhibition of fungi on agar. A piece of autoclaved sterile cellophane was placed on a 1/4 strength PDA plate in a petri dish and on the sheet, and a 7 mm diameter disc of selected *T. harzianum* isolates cultured on PDA was inoculated. Plates were incubated at 20ºC for 6 days. After the incubation period, the cellophane was removed and each dish was separately inoculated in

the centre with a plug (7 mm in diameter) of a mycelial disc of *S. rolfsii* taken from actively growing colonies. Petri dishes were incubated at 20ºC for a further 6 days. Percentage R I was calculated as follows: R I= 100 x (R2 - R1) / R2 (Watts *et al.,* 1988), where R1 was the distance between the inoculum of the pathogen and the inoculum of the *T. harzianum* isolate, R2 was the colony growth of pathogen measured in the direction of maximum radius and RI was the mean value of 3 replicates per isolate (Grondona *et al*., 1997). 100% inhibition rate for *S. rolfsii* with *T. harzianum* T3 and T19 isolates was observed.

Kareem *et al*. (2006) tested the inhibitory effect of chitin and chitosan (Sigma company) against tomato root rot fungi *in vitro* at four concentrations, *i.e.* 0, 2, 4 and 6 g/l. Chitin or chitosan were added to conical flasks containing sterilized PDA medium before its solidifying and rotated gently then disbanded into sterilized petriplates (9 cm diameter). Plates were individually inoculated at the centre with equal disks (6mm diameter) taken from 10 days old cultures of *S. rolfsii*, then incubated at 25±2℃. Linear growth of tested fungi was measured when the control plates (medium free of Chitin or chitosan) reached full growth and the average growth diameter was calculated. Each treatment was represented by 5 plates as replicates. Chitosan at 6 g/l completely inhibited the linear growth of all tomato root rot fungi. The moderate effect was obtained with chitosan at 4 g/l which reduced the linear growth more than 60.0% for all tested fungi as compared with untreated fungal growth medium. Meanwhile, chitosan at 2 g/l was less effective for this purpose.

Khanzada *et al*. (2006) conducted *in vitro* antifungal assay. Chickpea seed meal agar (CSMA) adjusted at pH 6.0 was prepared, autoclaved for 15 minutes at 15 psi and streptomycin sulphate (1:5000) was added to control the bacterial contamination. Measured quantity of leaf extract was added to CSMA medium after sterilization and leaf extract amended and non-amended (control) media were poured into the Petri plates at the rate of 15 ml in each. Five plates were kept for each treatment. These plates were inoculated with 5 mm diameter agar plugs containing active mycelium (4- 6 days old culture) of *S. rolfsii* in the centre of plates. The inoculated plates were incubated at 25+2ºC and radial mycelial growth was recorded and data were analyzed according to Steel and Torrie, (1980). Sclerotial production was recorded after 25 days of inoculation. The number of sclerotia on each petri plate was counted, average number of sclerotia per disc for each concentration was calculated and similarly the data were also analyzed. Minimum number of sclerotia was formed at 2% leaf extract concentration of all the plant species followed by 1.5% concentration as compared to control.

2.7.2 Bioassay against *Rhizoctonia*:

Rhizoctonia solani is soil-borne pathogen which causes seed decay, seedling blight, and pre-emergence damping off as well as post-emergence damping off and root rot. This pathogen can cause disease under a range of environmental conditions, but it can be devastating in cool, wet soil where it results in pre and post emergence damping off. In general the fungal invasion on seedlings at the soil level produces a brownish, sunken lesion on the hypocotyls due to cortical decay, eventually causing the seedlings to collapse. Disease symptoms appear as brown coloured lesions at the hypocotyl root junction. Infection due to this soil borne pathogens can occur immediately after sowing, resulting in seed decay (Hillocks, 1992a). It is supposed that biocontrol activity of *Trichoderma* sp. toward *R. solani* is based mainly on mycoparasitism, a very complex process that involves recognition, attack, penetration and killing of the host.

Lorito *et al.* (1998) transferred seedlings of one month old endochitinase transgenic tobacco and potato to a water agar (0.7 per cent) plate with the fungal suspension of 0 to 1.0 mg/ml. Here pathogen was highly virulent and killed most of the control plants. Instead, survival rate among transgenic ranged from 65 to 85 per cent. In the other seedlings assays, sterile soil (1 cm deep) was placed in containers and infested by evenly applying a 10ml *R. solani* suspension (0.75 mg/ml) to the surface and then covered with a layer of noninoculated soil (1.5 cm deep). Seedlings grown to emergence of the second set of true leaves (20 plants per line) were transplanted and 10 to 15 per cent of the transgenic lines consistently showed no disease symptoms whereas others had little or no improvement in disease resistance and grew well with larger leaves and with more root growth compare to untransformed control plants. Similarly in transgenic tobacco plants with *chit33* (Dana *et al.,* 2006), the two week old seedlings germinated on Murashige and Skoog (1962) medium were transferred to water

agar plates (0.7 per cent) containing 0.75 g/l of *Rhizoctonia* mycelium. The survival rate of the transgenic *chit33* plants reached 81 per cent, whereas that of control plants was 39.6 per cent.

Rini and Sulochana, (2007) subjected the isolates of *Trichoderma* for *in vitro* screening against *Rhizoctonia solani.* For this, the pathogen was inoculated on sterilized PDA and grown for 7 days. After the establishment of the pathogen in the petriplates, a 5 mm culture disc of the antagonist was inoculated. The plates were kept at room temperature for 9 days. Each set of treatment was replicated thrice. Antagonism expressed as sporulating *Trichoderma* over the pathogen was noted at regular intervals from the fifth day onwards. The culture plates were observed constantly, the radial growth of the pathogen recorded on the sixth day of inoculation, and the percent inhibition was worked out. it was observed that two isolates *viz*., *T. harzianum* TR20 and *T. pseudokoningii* TR17 were more effective against *R. solani* than others whereas *T. viride* TR19 and TR22 were the most promising against *F. oxysporum*. These isolates completely overgrew the pathogens and suppressed it within 7 days of inoculation.

Smolinska *et al*. (2007) have grown the fungus *R. solani* in liquid medium for 7 days. Then, the culture was filtered through filter paper to remove mycelial mats and mixed in a blender. The potground (Klasmann H) was infested with pathogen *R. solani* at the dose of 5g of wet mycellium mats per 1 L of substrate. Immediately the suspension of *T. harzianum* strain PBG was added. *T. harzianum* strain PBG was grown on PDA medium for 7-10 days, afterwards the mycelium with the spores were scraped from Petri plate and mixed with water (20 ml/plate) in a blender. The suspension of the fungus was added to the potground at a dose of 1 Petri plate/1 L of potground. The following treatments were prepared: 1/ control (without fungi); 2/ *R. solani* 3/ *R. solani* + *Trichoderma* strain PBG; 4/ *T. harzianum* strain PBG. After one week of incubation at the room temperature, the potground were placed to the plastic pots (500ml) and the seeds of cucumber cv. Iwa were placed. For each treatment 5 pots with 5 seeds of cucumber were prepared. The experiment was repeated twice. The application of antagonistic fungus *T. harzianum* strain PBG to the potground protected the cucumber plants against root rot caused by *R. solani.* In two out of three experiments *T. harzianum* strain PBG increased the mass of cucumber plants, growing in potground with pathogen, by 19% and 26% compared to infested control. However, when the disease was severe, the protection by *Trichoderma* was unsuccessful.

Kumar *et al*. (2009) performed infection assay against *Rhizoctonia solani* according to the method described by Emani *et al.* (2003), but with Metromix® 300 (SUN GRO Horticulture Ltd., Bellevue, WA, USA) as the plant growth medium. Six liters of Metromix was thoroughly combined with the coarse *R. solani* inoculum (300mg) and moistened with 600 ml tap water in a plastic container. The same procedure was followed for untreated controls, but without the inclusion of fungal inoculum. For germination, the seeds were soaked in water for 12 h, and then subjected to 30s of hot water treatment to obtain synchronized germination. The seeds were rolled in a wet filter paper (VWR, Cat. #28298-020) and placed vertically in a beaker, covered with a polythene bag and incubated for 2 days at 28°C. Two-day-old, pre-germinated seedlings were transferred to soil (with or without the fungal inoculum) in a plastic tray. The tray was covered with a clear, plastic Humidome (Acrodome, Agroplastics Ltd, Canada) to maintain humidity and placed in a growth chamber for 6 days at 25°C with 14:10 h photoperiod to allow disease development. Three independent experiments were conducted and 12 replicate seedlings were used for each treatment in an experiment. Disease index, a measure of infection severity, was calculated based on the following formula (Powell *et al*., 1971). In another plate-based infection assay, inoculum consisting of a single grain of *R. solani*-infested pearl millet, was placed in Petri plate containing phytagel solidified (0.25%) medium with half-strength MS salts and allowed to grow for 24 h. Pre-germinated seedlings were placed on the medium with their roots directly in contact with actively growing mycelia. Roots were harvested at different time points for various analyses. Control roots were obtained from seedlings manipulated in the same manner but without exposure to the pathogen. They also performed leaf disc assay in which the second fully expanded leaf from a 60-day-old plant grown in the greenhouse was collected and placed over phytagel-solidified (0.25%) medium with half-strength MS salts in a petri dish. A 0.5 cm agar plug of *R. solani* from the growing edge of a 2-day-old colony was used as an inoculum. Four plugs of the inoculum were placed on the adaxial surface of each leaf; plates were sealed with parafilm, and incubated for 5 days in dark at 28°C. Transgenic seedlings are protected from infection

by *R. solani*, while the wild-type (WT) seedlings developed severe collar lesions and many were stunted. Even the mycelial growth from the agar plugs was less extensive on the transgenic leaves. After 24 h of incubation of a seedling placed adjacent to the pathogen inoculum, the mycelia were able to grow and spread across the non-transgenic root. Interestingly, fungal mycelia grew only on one side of the root of a transgenic seedling, but did not grow across it

3. MATERIAL AND METHODS

The present investigation was carried out to compare the plant signal peptide (PSPαamy3) with *Trichoderma* signal peptide (*ech*42SP) for efficient secretion of endochitinase (ECH42) in intercellular spaces in tobacco. The materials used and methodology followed are presented in this chapter.

3.1 Designing and synthesis of plant signal peptide (PSPα-amy3)

The plant signal peptide (PSPα-amy3) of 501 bp was taken and *in silico* analysis was done for fusion with *ech*42 sequence and to get correct reading frame using Bioedit software and synthesized by Eurofins MWG Operon, Germany. The sequence (Fig. 1) was cloned via TOPO-TA in vector pCR2.1 and supplied as lyophilized product (Fig. 2).

3.2 Confirmation of alpha amylase plant signal peptide (PSP α-amy3) in pCR2.1 vector

The lyophilized plasmid DNA supplied by Eurofins MWG Operon, Germany was diluted in 10 µl of double distilled water. One µl of plasmid DNA is taken and transferred into *E. coli* DH5α.

3.2.1 *E. coli* transformation

3.2.1.1 Preparation of competent cells

The competent cells of *E. coli* DH5α were prepared following the protocol mentioned by Sambrook and Russell, (2001) with minor modifications.

An isolated colony from *E. coli* DH5α plate was inoculated into 5 ml Luria broth and incubated at 37°C overnight at 200 rpm. The next day, the culture was diluted to 1:100 using Luria broth *i.e.*, 0.5 ml of culture was added to 50 ml of Luria broth. It was incubated for 2-3 hrs till it attained an OD of 0.3 to 0.4 at 600 nm. The culture was chilled in ice for 30 min and 25 ml of culture was dispensed into two centrifuge tubes of capacity 50 ml. The cells were pelleted at 6000 rpm for 5 min. The supernatant was discarded and pellet was suspended in 12.5 ml of ice-cold 0.1 M calcium chloride. The centrifuge tubes were again kept in ice for 45 min and later centrifuged at 4000 rpm for 10 min. The pellet was dispensed in 1 ml of 0.1M $CaCl_2$ and to this 88 µl of dimethyl sulfoxide (DMSO) was added if intended for later use. About 200 µl of cells were distributed to each chilled 1.5 ml micro centrifuge tubes and immediately used.

3.2.1.2 Transformation of *E. coli* DH5 α

About 100µl of freshly prepared competent cells were taken in a chilled centrifuge tube and 1µl of plasmid DNA was added and mixed gently. The mixture was chilled in ice for 45 min and heat shock was given by shifting the chilled mixture to preheated 42°C water bath for exactly 2 minute. It was immediately transferred to ice to chill for 5 min. To this, 800 µl of Luria broth was added and incubated at 37°C at 200 rpm for 45 min to allow bacteria to recover and express the antibiotic marker encoded by the plasmid. The culture was centrifuged at 13,000 rpm for 1 minute and about 700 µl of supernatant was discarded and the pellet was dissolved in remaining supernatant and spread on the plates having Luria agar with Kan_{50}, Nal_{10}, and incubated overnight at 37°C.

3.2.2 Confirmation of plant signal peptide (PSPαamy3)

The clones were streaked on Luria agar with Kan_{50}, Nal_{10}, and incubated overnight at 37°C. Plasmid DNA was isolated using alkaline lysis protocol of Birnboim and Doly, (1979) with certain modifications and further confirmed by restriction analysis to ensure the presence of insert with *Xba*I and *BsrG*I.

Colonies were inoculated to 10 ml Luria broth with kanamycin (100 µg/ml) and incubated over night at 37°C with 175 rpm. Overnight grown culture was centrifuged at 5000 rpm for 2 min at 4°C in 2.0 ml micro centrifuge tubes. The supernatant was removed and pellet was washed with STET (0.25 volume of original culture). It was centrifuged at 5,000

TCTAGAATGGGCAAGCACCATGTCACCCTGTGTTGTGTCGTTTTTGCTGTGC

TCTGCCTGGCGTCCAGCTTAGCACAAGCCCAAGTTCTCTTCCAGGGGTTTAA

CAGCGCTGCAAGCCCTCTAGCTACAGAGGAGCACTCCGTTGAGAAGAGAGC

CAATGGATACGCAAACTCTGTCTACTTCACCAACTGGTAAGTGAAACCAAC

AAGCTTCCATAGATTCACTGCTGACAACACGTCCCTGAAGGGGAATTTACG

AGCGCAACTTCCAGCCTGCCGATTTGGTAGCTTCAGATGTCACTCATGTCAT

CTACTCATTCATGAACCTCCAGGGCAGACGGCACTGTGTAAGTCAGAAACT

CGGAGAGGATATATTCTCTCAAAGTTACTTCCCGGGTACTAACTGTCTTTCA

GTGTCTCTGGCGATACCTACGCCGATTTCGAGAAGCACTATGCCGATGATTG

TAGGGGTTCCTCATCTCTAGCTCCGCTATGTACACAT

■ *Xba* I site ■ α-AMY3e signal peptide sequence

■ chin42 gene sequence ■ *BsrGI* site

Fig.1. Nucleotide sequence of synthesized sequence (α-amylase)

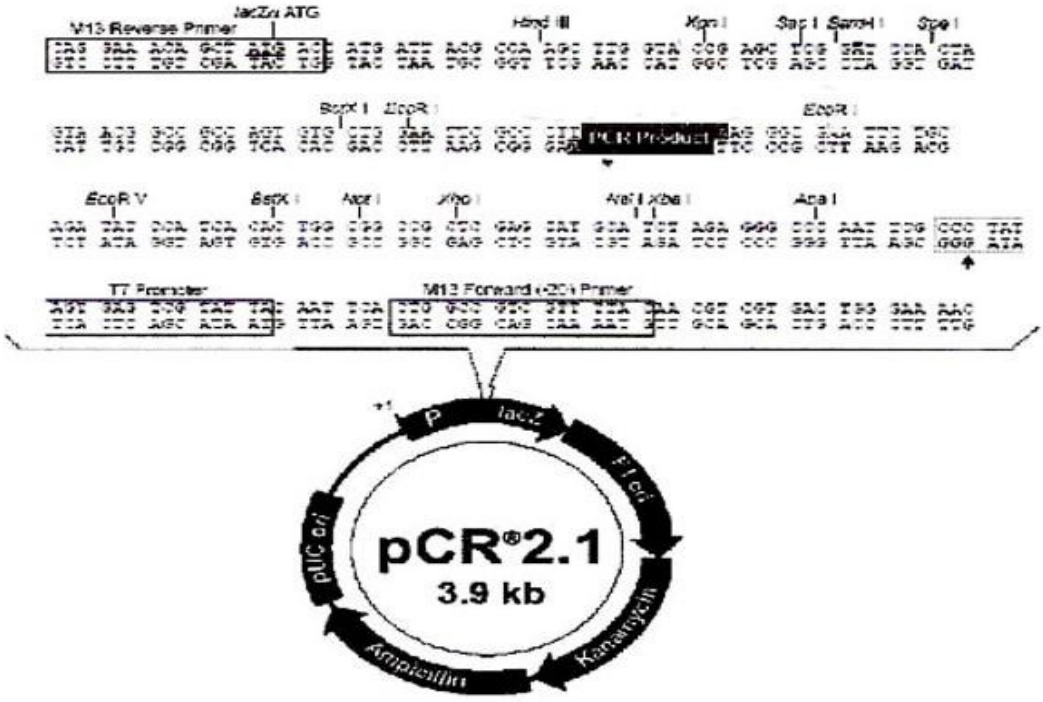


Fig.2. Vector map of pCR2.1

rpm for 2 min. The pellet was resuspended in 200 µl of ice-cold alkaline lysis solution I (Appendix II) by vigorous vortexing. Later, 400 µl of freshly prepared alkaline lysis solution II (Appendix II) was added to each tube and the contents were mixed by inverting the tubes for 4 to 5 times and kept in ice for about 5 min. To this suspension, 300 µl of alkaline lysis solution III (Appendix II) was added and again mixed thoroughly by gently inverting the tubes for 4-5 times. The tubes were stored on ice for 5 min and centrifuged at 13,000 rpm for 8 min. The supernatant was transferred to fresh tubes and equal volume of phenol: chloroform isoamyl alcohol (25:24:1) was added to precipitate proteins and mixed well. It was centrifuged at 13,000 rpm for 10 min at 4°C. The aqueous layer was transferred to a fresh tube and two volumes isopropanol was added. The contents were mixed and allowed to stand for 2 min at room temperature. The solution was later centrifuged at 13,000 rpm for 5 min. The supernatant was discarded and pellet was washed with 70 per cent ethanol and spun for 1 minute at 13,000 rpm to recover the plasmid. The supernatant was discarded, pellet was dried completely and dispensed into 25 µl of $T_{10}E_1$ (pH=8.0) containing 3 µl of RNase A (10 mg/ml). The solution was kept at 50°C for 15 min and then stored at -20°C.

3.3 Cloning of alpha amylase plant signal peptide (PSPα-amy3) from pCR2.1 vector into pTZ57R/T vector

3.3.1 Vector and clone isolation

A pTZ57R/T vector pSUM1 which carries endochitinase gene (*ech*42) from *T. virens* (Upendra, 2006) was used for this purpose. The vector pSUM1 and the pCR2.1-PSPαamy3 were isolated using protocol described in 3.2.2.

.3.3.2 Elution of linearized vector pSUM1

Digestion of pSUM1 was done with restriction enzymes – *Bam*HI and *Bsr*GI. The pSUM1 linearlized vector was eluted and DNA was extracted using Qiagen gel extraction kit as per the protocol given in user's manual.

3.3.3 Elution of inserts (PSPα-amy3) and ligation

The insert was obtained by restricting pCR2.1 vector by *Bam*HI and *Bsr*GI. The plant signal peptide (PSPα-amy3) of 501 bp size was eluted as explained earlier. The purified vector DNA and insert from pCR2.1 were quantified by ethidium bromide spotting method. The ligation reaction was carried out with an optimal molar ratio of 1:3 (vector: insert). The components of the ligation mixture was mixed into a 0.5 ml micro centrifuge tube and incubated at 16°C for 16 hrs.

3.3.4 Transformation of *E. coli* DH5 α

3.2.1. Transformation of *E. coli* DH5 α was carried out as described earlier in the section

3.3.5 Confirmation of clones

The confirmation for the presence of desired DNA fragment in cloning vector was done by signal peptide specific forward PCR primer and gene specific reverse PCR primer and by restriction analysis as follows.

For PCR confirmation of clones the template DNA from plasmids was isolated as described earlier in the section 3.2.2. Confirmation of the presence of cloned fragment was done by PCR amplification of clones with signal peptide specific forward and gene specific reverse primer pairs. The plasmid of cloning vector was used as negative controls. The confirmation was also done through comparative restriction analysis of selected clones and the control vector for the presence of insert with *Bam*HI and *Bsr*GI. The clones are named as pMASG. The endochitinase genes (*ech*42) with plant αamylase signal peptide sequence are named as PSP*α-amyech*42.

3.3.5.1 Primers used for the confirmation of Endochitinase Gene (PSP*α-amyech*42)

The primers specific for endochitinase gene (PSP*α-amyech*42) were designed from NCBI database using the FAST PCR software. The primer sequences designed were:

PSP alpha	FORWARD PRIMER	5'GGCAAGCACCATGTCACCCTGT 3'
	REVERSE PRIMER	5' TGGGGGAGCTCAGCAGGTTCT 3'

The primers used for amplification of template DNA (20ng) were custom synthesized at MWG-Biotech AG, Bangalore and were supplied as lyophilized product of desalted oligos.

3.3.5.2 PCR amplification condition for endochitinase gene (PSP*α-amyech*42)

Stage	Step	Temperature (℃)	Duration (min)	No. of cycles
I	Initial denaturation	94	5	1
II	Denaturation	94	1	35
	Annealing	56	1	
	Extension	72	2	
III	Final Extension	72	20	1
IV	Incubate	4	Forever	

3.3.6 Confirmation of clones by restriction analysis

For confirmation of clones, the plasmid was isolated from the clones by following the protocol mentioned earlier in section 3.2.2. The presence of insert in clones was confirmed by restriction of clones using *Xba*I enzyme and further orientation of clones was confirmed by restriction of clones using *Pst*I enzyme.

3.4 Construction of plant transformation vector carrying (PSP*α-amyech*42) endochitinase gene

3.4.1 Vector and Clone Isolation

A plant transformation vector pBI121 was used for this purpose. The vectors pBI121 and pMASG carrying PSP*α-amyech*42 were isolated using protocol described in 3.2.2.

3.4.2 Elution of linearized pBI121 vector

The vector pBI121 was restricted with *Xba*I and linearlized vector was eluted using Qiagen gel extraction kit as per the protocol given in user's manual.

3.4.3 Elution of insert (PSP*α-amyech*42) and ligation

The insert (PSP*α-amyech*42) from pMASG was restricted with *Xba I*. The insert of 1.6kb size was eluted as explained earlier. The purified pBI121 vector DNA and insert (PSP*α-amyech*42) from pMASG were quantified by ethidium bromide spotting method. Vector DNA was treated with alkaline phosphate. The ligation reaction was carried out with an optimal molar ratio of 1:3 (vector: insert) (Appendix IV). The components of the ligation mixture was mixed into a 0.5 ml micro centrifuge tube and incubated at 22℃ for 16 hrs.

3.4.4 *E. coli* transformation and confirmation

The component cells of *E. coli* DH5α were prepared as described in section 3.2.1.1. The transformation of ligated mixture into *E. coli* DH5α competent cells was done as mentioned in section 3.2.1.2. For confirmation of clones, the plasmid was isolated from the clones by following the protocol mentioned earlier in section 3.2.2. The presence of insert in clones was confirmed by PCR using signal peptide specific forward and gene specific reverse primers. It was further analyzed by complete restriction of clones using *Xba* I enzyme. The clone was named as pMASGK (Fig. 3).

3.4.4.1 PCR amplification condition for *T. virens* endochitinase gene (PSP*α-amyech*42)

Stage	Step	Temperature (°C)	Duration (min)	No. of cycles
I	Initial denaturation	94	5	1
II	Denaturation	94	1	35
	Annealing	59	1	
	Extension	72	2	
III	Final Extension	72	20	1
IV	Incubate	4	Forever	

3.5 *Agrobacterium* transformation

The confirmed clones were further transferred into *Agrobacterium tumefaciens* strain LBA4404 by tri-parental mating technique. The vector pBI121 is capable of replicating in both *E. coli* and *Agrobacterium* and carries unique cloning sites and plant selectable marker between its disarmed T-DNA borders. The chromosomal selection is rifampicin (25 µg/ml) and it contains disarmed Ti-plasmid called pAL4404 which has streptomycin (100 µg/ml) as selection pressure.

The recombinant plasmid with *T. virens* endochitinase (*PSPαamyech*42) *i.e.*, pMASGK was propagated in *E. coli* DH5α cells and grown in Luria broth containing 50 µg/ml of kanamycin overnight at 37°C. The *A. tumefaciens* LBA4404 was grown for 16-22 hours at 28°C in Yeast Extract Mannitol Agar containing rifampicin (25 µg/ml) and streptomycin (100 µg/ml). The *E. coli* helper strain containing pRK2013 vector was grown overnight in LB containing kanamycin (50 µg/ml).

The overnight grown cultures were centrifuged at 13000 rpm for 1 min. The supernatant was discarded and the pellet was washed with 0.01 $MgSO_4$ for 2-3 times to remove traces of antibiotics. It was again centrifuged at 13,000 rpm for 1 min and pellet was dispensed in 50 µl of 0.01 M $MgSO_4$. *A. tumefaciens* LBA4404, *E. coli* DH5α (pRK2013) and *E. coli* containing pMASGK were mixed in 1:1:1 ratio in a separate centrifuge tube. The thoroughly mixed mixture was spotted on plain LA medium and incubated overnight at 28°C. The spotted culture was scraped and dissolved in 200 µl of 0.01 M $MgSO_4$ and were spotted on YEMA medium (Appendix VII) containing streptomycin (100 µg/ml), rifampicin (25 µg/ml) and kanamycin (50 µg/ml) along with *A. tumefaciens* LBA4404, *E. coli* helper strain and *E. coli* with recombinant vectors as negative controls.

The plasmid was isolated from the clones by following the protocol mentioned earlier in section 3.2.2 and the presence of recombinant plasmid in the *Agrobacterium* was confirmed by PCR amplification with plant signal peptide specific forward and gene specific reverse primers and further analyzed for correct orientation by restricting the clones with enzyme *Sac*I.

3.6 Tobacco transformation

The *Agrobacterium* containing recombinant plasmid pMASGK carrying endochitinase gene (PSP*α-amyech*42) and the *Agrobacterium* containing recombinant plasmid pSUM1C which has endochitinase gene (*ech*42*SP*) from *T. virens* with its own signal peptide (Upendra, 2006) was used for tobacco transformation by using protocol mentioned by Hooykaas *et al.* (1992) with some modification.

Both the *Agrobacterium tumefaciens* clones were grown in YEMA (Yeast Extract Mannitol Agar) with streptomycin (100 µg/ml), rifampicin (25 µg/ml) and kanamycin (50 µg/ml) as selection at 28°C for 24 hrs. Tobacco leaves from 5-6 week old plants from variety White Burley were surface sterilized by rinsing them for 1 min in 0.1 per cent mercuric chloride. The sterilized leaves were given 3-4 times water wash, to remove the trace of the mercuric

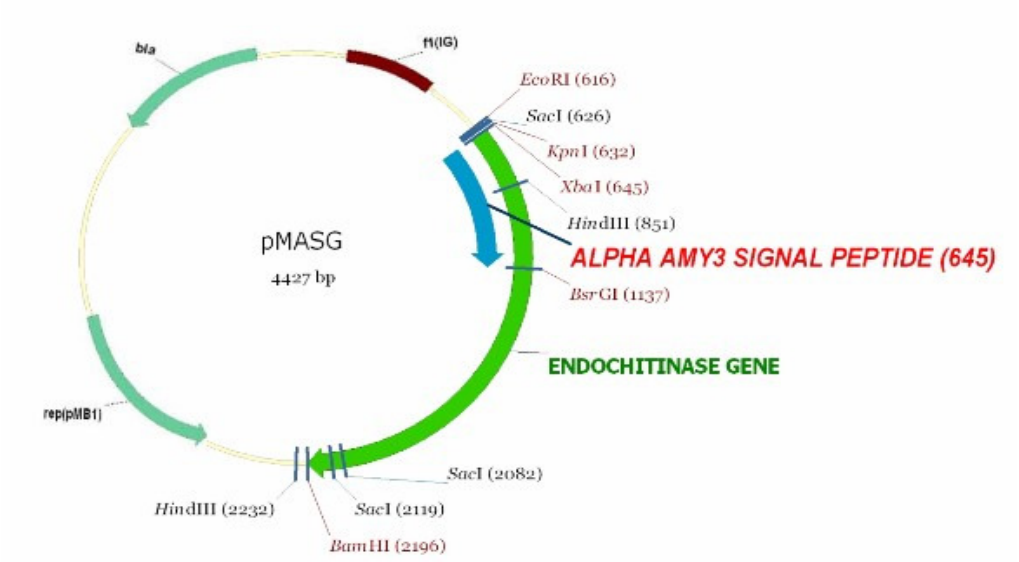


Fig.3. Vector map of pMASG

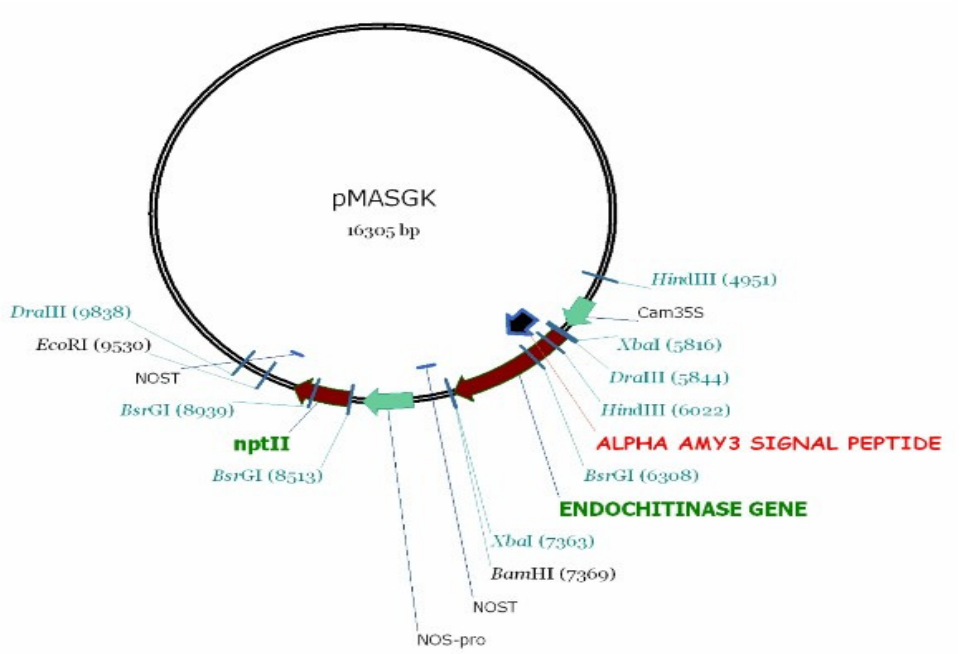


Fig.4. Vector map of pMASGK

chloride. The leaves were cut into 1 to 2 cm² disks and incubated in acetosyringone (200 µM) treated *Agrobacterium* culture and left for 20 min for agroinfection.

These explants were dried by blotting onto the sterile filter paper and placed on solid Murashige and Skoog (MS) medium (Appendix VIII) without any hormones and kept in dark for 2 days for co-cultivation. Leaf disks were later transferred to test tubes containing MS medium with Napthalene acetic acid, NAA (0.5 mg/l), benzyladenine, BAP (1 mg/l) for callus induction and cephotaxime (200 mg/l) to avoid *Agrobacterium* growth and kanamycin (200 mg/l) as selection pressure. The cultures were incubated at 25℃ and 16 hrs photoperiod for 3-4 weeks. The shoots and excised calli were subcultured and incubated for another 4 weeks at same culture conditions. Young shoots were then transferred to hormone free MS medium with 200µg/ml cefotaxime and 200µg/ml kanamycin as selection for rooting. Cultures were incubated for 3-4 weeks and survived plants were transferred to pots containing peat and acclimatized in green house. DNA was extracted from those plants by rapid method (Appendix IX) and checked through PCR with signal peptide specific forward and gene specific reverse primers for the presence of PSP*α-amyech*42 gene and with gene specific (TVR) primers for the presence of *ech*42*SP* gene.

TVR primers:

Forward	5'-ACCATGTTGAGCTTCCTCGGCA -3'
Reverse	5'-TCCCCTGAAAAGAAGCCACCT-3'

3.7 Establishment of cell suspension culture

3.7.1 Establishment of callus cultures

Calli were initiated from leaves of five young greenhouse-grown primary transformants with endochitinase having plant signal peptide (PSP*α-amyech*42), two transformants with endochitinase having fungal signal peptide (*ech*42*SP*) and from untransformed plants. To initiate callus formation, leaf pieces were placed on MS medium containing 3 mg/l NAA, 0.25 mg/l kinetin, and 8.8 g/l Bacto agar. Subcultured callus was maintained on KCMS medium (MS medium with 0.1 mg/l kinetin, 0.2 mg/l 2, 4-D and 0.2 g/l KH_2PO_4) at a 16 h light/8 h dark photoperiod.

3.7.2 Initiation of cell suspensions from callus cultures

Cell suspensions were initiated from established callus cultures 2 weeks after transfer. Approximately 1.5 g callus was placed in 30 ml modified liquid KCMS medium (1.5 mg/l of 2, 4-D instead of 0.2 mg/l). Flasks were maintained at 24 ºC and a 16 h light/8 h dark photoperiod on a rotary shaker at 100 rpm. Every 3 days, large clumps were removed by filtering the suspension through a single layer of sterile cheesecloth. Cells were allowed to settle, 10 ml culture medium was removed, and 10 ml fresh medium was added. After the level of cell clumping decreased, 5 ml cell suspension was transferred to 45 ml fresh KCMS medium once a week. Chitinase activity was checked from cell suspension extract and callus.

3.8 Assay for chitinase activity

3.8.1 Protein extraction from callus and liquid medium

Total protein was extracted from tobacco transformants with PSP*α-amyech*42 *or ech*42*SP* using the procedure described by Velasquez and Hammerschmidt (2004) with slight modification. Non transformed plant was taken as control. Callus tissues were ground using a mortar and pestle using liquid nitrogen and homogenized in 0.01M sodium acetate (pH 5.0) in a ratio of 3 ml of buffer for 1 g of fresh tissue. The homogenate was centrifuged at 12000 rpm for 15 min at 4℃ and the supernatant was filtered using 0.22 µ pore size membrane filter. The total protein was estimated using Lowry's method and equal quantity of protein was used for further assays. For studies of cell suspension medium, 7-day-old suspension cultures were centrifuged for 5 min at 1,400 *g*. The supernatant was collected from PSP*α-amyech*42 and *ech*42*SP* culture and assayed for endochitinase activity. A non-transformed tobacco cell suspension was used as a negative control. For each treatment, three replications were used. ANOVA was applied to compare the results. Data are presented as mean ± S.E., $P< 0.05$ was considered as statistically significant.

3.8.2 Estimation of protein by Lowry's method

3.8.2.1 Alkaline copper reagent

Alkaline copper reagent was prepared by mixing 2% sodium carbonate in 0.1 N NaOH, 1% sodium potassium tartarate and 0.5% copper sulfate in 100: 1: 1 proportion respectively.

3.8.2.2 Protein standard solution

A stock of protein standard was prepared by dissolving 50 mg of bovine serum albumin (BSA) in distilled water and making up the volume to 50 ml with distilled water in a volumetric flask. This solution contains 1 mg of protein per ml. From this stock working standard was prepared by taking 10 ml of stock solution and diluted to 100 ml distilled water in a volumetric flask. This solution contains 100 µg of proteins per ml.

3.8.2.3 Protein estimation

Working standard solution (20-100 microgram) was pipetted out and transferred to labeled test tubes. Volume was made up to 1 ml with distilled water in each tube. One blank tube was maintained with 1 ml distilled water. To these test tubes, 5 ml of alkaline copper reagent was added, mixed thoroughly and kept at room temperature for 10 min. To all the tubes 0.5 ml of 1 N FCR (Folin-Ciocalteau Reagent) was added, mixed and kept in dark for 30 min. Culture filtrates having induced glucanase as samples were treated as explained above. Percent transmission (T) values of the standard and sample against reagent blank which is set to 100 at 660 nm were read using spectrophotometer. Using standard graph mg of protein per gram of sample was calculated.

3.8.3 Estimation of reducing sugars by dinitro-salicylic acid method

3.8.3.1 DNSA reagent

DNSA reagent was prepared by dissolving 1 g of 3, 5-dinitrosalicylic acid in a little amount of 2 N NaOH. 30 g of sodium potassium tartarate was added and made up to 100 ml with 2 N NaOH.

3.8.3.2 Preparation of standard

100 mg of D-glucose was dissolved in water and made up to 100 ml with water in a volumetric flask. This solution contains 1 mg of glucose per ml, and was used as stock. In a series of labeled test tubes, suitable aliquots from stock (100-1000 microgram) were pipetted out and made up to 1 ml in all the tubes. A blank with 1 ml distilled water was maintained. 0.5 ml of DNSA reagent was added to all the tubes, mixed well and kept in boiling water bath for 5 min. Tubes were cooled and made upto 10 ml. The per cent transmission of the standard and the samples was recorded against reagent blank which was adjusted to 100%T at 540 nm. Reducing sugars present per gram of the sample were calculated based on the glucose standard graph.

In order to identify plants having higher chitinase activity, reducing sugars released by 300 µl of culture filtrate in 30 min have been converted into microgram of reducing sugars released per microgram of crude protein (from each culture filtrate) per min.

3.9 Bioassay against pathogen

3.9.1 Bioassay against *Sclerotium rolfsii*

Sclerotium rolfsii was used for the pathogen inhibition assay. Two different concentration of liquid media extract (PSP*α-amyech*42*, ech*42*SP and* control) *viz.* 100 µg and 200 µg total protein were used. First, extract was spread over the PDA (Appendix X) plate uniformly in two replications for each concentration of proteins and a single sclerotial body of *S. rolfsii* was placed on the center of the plate (Khanzada *et al.,* 2006). This was incubated 28ºC for three days and observed for the inhibition of germination of spore, diameter of fungal growth and lysis of mycelium under microscope at magnification of 100X. Per cent inhibition of fungal growth was calculated by formula (Rini and Sulochana, 2007).

$$\% \text{ inhibition of growth} = \frac{\text{Colony diameter of control} - \text{Colony diameter of treatment}}{\text{Colony diameter of control}} \times 100$$

3.9.2 Bioassay against *Rhizoctonia*

Another pathogen used for bioassay was *Rhizoctonia bataticola.* Liquid media extract of concentration 200 µg of total protein was spread over the PDA plate uniformly in three replications. This was incubated for 3 days at 28ºC and observed for the inhibition of diameter of fungal growth and lysis of mycelium under microscope at magnification of 45X. Percent inhibition was also calculated by the formula described in section 3.9.1.

4. EXPERIMENTAL RESULTS

The present study was conducted to fuse plant signal peptide (PSPαamy3) sequence with *Trichoderma* endochitinase gene (*ech*42) for efficient secretion in intercellular spaces. Chimeric endochitinase gene (PSP*α-amyech*42) was cloned into plant transformation vector pBI121 and tobacco plants were transformed with this construct and further analysed for secretion into intercellular spaces and pathogen inhibition. Results of various experiments conducted are presented below.

4.1 Confirmation of plant signal peptide (PSPαamy3) in pCR2.1 vector

For confirmation of synthesized α-amylase signal peptide nucleotide sequence, lyophilized product was diluted and transformed into *E. coli* DH5α. Two colonies were picked and streaked on Luria agar containing kanamycin (50µg/ml) and ampicillin (100 µg/ml). On restriction of plasmid DNA isolated from these clone with *Xba*I and *Bsr*GI enzymes, a linearized insert of 501bp was released (Plate 1).

4.2 Fusion of α-amylase signal peptide with *Ech*42

In order to fuse α-amylase signal peptide with *ech*42, signal peptide sequence was released from pCR2.1 with *Bam*HI and *Bsr*GI enzymes and was ligated with linearized pSUM1 carrying *ech*42 gene with its own signal peptide (*ech*42*SP*), and transferred into *E.coli* DH5α cells. Ten transformants were picked and streaked on Luria agar containing ampicillin (100 µg/ml). PCR analysis with signal peptide specific forward and gene specific reverse primers and restriction analysis of the plasmids isolated from these clones confirmed the presence of insert of 1.6 kb (Plate 2 and 4). Further orientation of insert was confirmed by restricting with *Pst*I enzyme which gave sharp bands of 900 bp and 3.3 kb (Plate 5). Chimeric endochitinase was named as PSP*α-amyech*42 and clones were named as pMASG (Fig 3).

4.3 Construction of plant transformation vector carrying endochitinase gene (PSP*α-amyech*42)

For cloning of endochitinase gene fused with plant signal peptide (PSP*α-amyech*42) into pBI121, pMASG was restricted with *Xba*I enzyme and was ligated with pBI121 vector linearized with same enzyme (treated with alkaline phosphate), and transferred to *E.coli* DH5α cells. Single transformant colony was picked and streaked on Luria agar containing kanamycin (50 µg/ml). PCR with signal peptide specific forward and gene specific reverse primers and restriction analysis of plasmid DNA isolated from the transformants with *Xba*I enzyme confirmed the presence of insert size of 1.6 kb (Plate 4). Further orientation of PSP*α-amyech*42 was confirmed by using *Sac*I enzyme which released two bands which were of 2.1kb and 14.4kb (Plate 4) and the clone was named as pMASGK.

4.4 Transformation of *Agrobacterium tumefaciens* strain LBA4404 with plant transformation vector (pMASGK)

The confirmed plant transformation vector pMASGK with endochitinase gene (PSP*α-amyech*42) was transferred into *Agrobacterium tumefaciens* LBA4404 via tri-parental mating using *E.coli* (pRK2013) as helper strain. Patch mating in the ratios of 1:1:1 donor: helper: recipient was done. Three transconjugants were picked on YEMA (Yeast Extract Mannitol Agar) containing kanamycin (50 µg/ml), streptomycin (1200 µg/ml) and rifampicin (25 µg/ml). Presence of 1.6 kb amplicon size (Plate 6) in the PCR analysis of plasmids obtained from recombinant *A. tumefaciens* (with pMASGK) vector) using signal peptide specific forward and gene specific reverse primers confirmed the presence of PSP*α-amyech*42.

4.5 *Agrobacterium* mediated transformation of tobacco with endochitinase gene (PSP*α-amyech*42)

Forty to sixty leaf discs were co-cultivated with *Agrobacterium tumefaciens* LBA4404 containing pMASGK construct in three batches and forty leaf discs were co-cultivated with pSUM1C (carrying endochitinase gene, *ech*42*SP*) in two batches for 48 hours on MS

Legends

Plate.1: Restriciton of alpha-amylase plant signal peptide

M :1kb DNA ladder (NEB)

1-2 :pCR 2.1 cut with Xba1 and BsrG1

Plate.2: PCR Confirmation pMASG

M : 1kb DNA ladder

N :Negative control (without vector)

1-5 :Amplification of PSPα-amy ech42

Plate.3: PCR Confirmation of pMASGK

M :1kb DNA ladder

P :Positive control (pMASG clone)

N :Negative control (without vector)

I :Amplification of PSPα-amy ech42

Plate.4: Restriction confirmation of pMASG and pMASGK

M1 :λHindIII/ EcoRI double digest DNA ladder

1 :Positive control (pTZ57R/T vector backbone)

2 :pMasg cut with BamHI and BsrG1

3 :Positive control (pBI121 vector backbone

4 :pMASGK cut with XbaI

5 :pMasgk cut with SacI

M2 :1kb ladder

Plate.5: Orientation confirmation of pMASG

M :1kb DNA ladder

1-7 :pMASG cut with PstI

Plate.6: PCR confirmation of pMASGK in Agrobacterium

M :1kb DNA ladder

P :Positive control (pMASG)

N :Negative control (Agrobacterium without gene)

1-3 :Amplification of PSPalpha-amy ech42

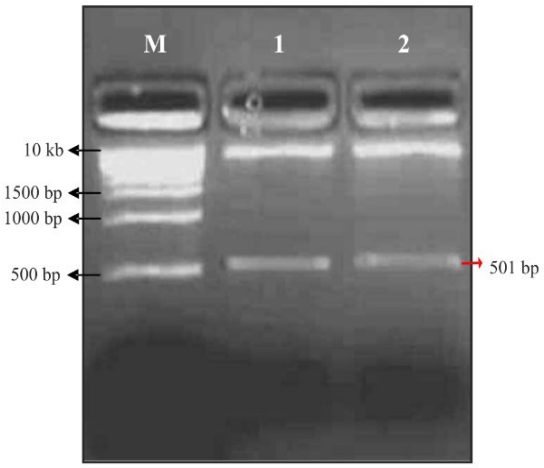


Plate.1:Restriction confirmation of alpha-amylase plant signal peptide

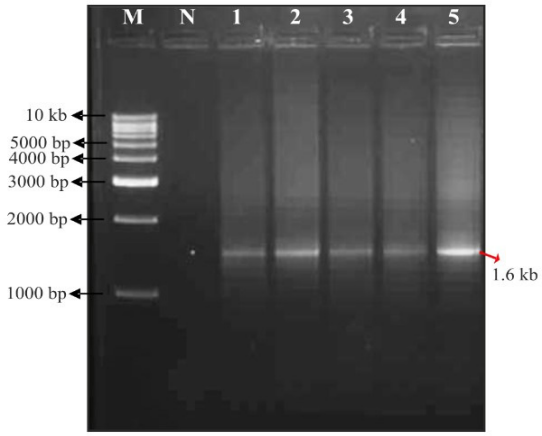


Plate.2:PCR Confrmation pMASG

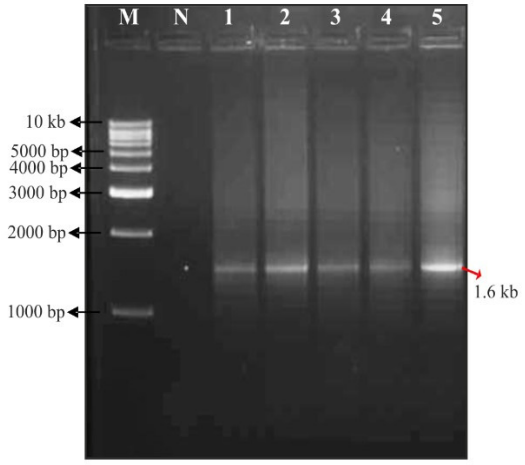


Plate.3: PCR confirmation of pMASGK

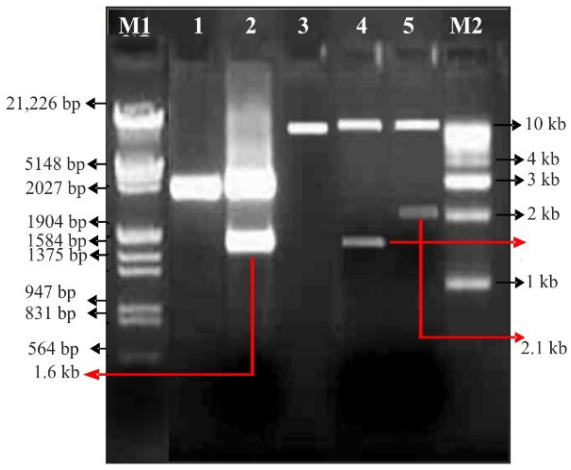


Plate.4: Restriction confirmation of pMASG and pMASGK

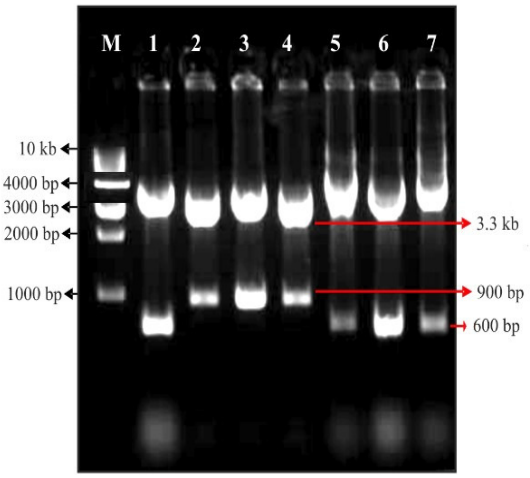


Plate.5: Orientation confirmation of pMASG

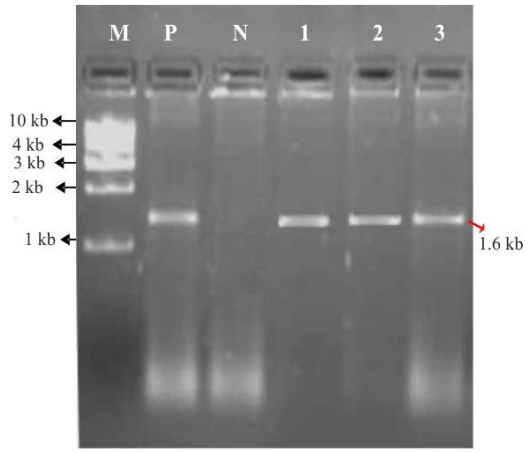


Plate.6: PCR confirmation of pMASGK in Agrobacterium

medium. Ninety one (pooled from three batches) explants (PSP*α-amyech*42*)* and twenty six explants (*ech*42*SP*) were further transferred to the MS medium with NAA (0.5 mg/L), BAP (1 mg/L) and cefotaxime (200 mg/L) which produced calli and direct shoots within three weeks. The shoots and calli were excised and transferred to hormone free MS medium with 200µg/ml cefotaxime and 200 µg/ml kanamycin. Majority of the cultures turned albino on both callus induction and shoot regeneration medium. Only 75 (PSP*α-amyech*42*)* and 15 (*ech*42*SP*) surviving green shoots were transferred to rooting medium with 200 µg/ml kanamycin after about 4 weeks. Plants having well developed roots were transferred to sterilized peat and shifted to green house (Table 1). Out of 8 survived plants (*ech*42*SP*), 5 showed the presence of amplicon size of 1.6 kb (*ech*42) in PCR analysis using gene specific (TVR) primers (Plate 8). Also, PCR analysis of DNA isolated from 50 (PSP*α-amyech*42) plants using signal peptide (PSPαamy3) specific forward and gene specific reverse primers; indicated the presence of transgene only in 29 plants (Plate 9).

4.6 Assay for intercellular secretion of endochitinase

4.6.1 Establishment of cell suspension culture

From the confirmed transgenic plants, calli were obtained from 5 plants positive for PSP*α-amyech*42, 2 plants positive for *ech*42*SP*, and 1 non transformed plant by culturing leaf explants on MS medium containing 3 mg/l NAA, 0.25 mg/l kinetin, and 8.8 g/l Agar) for one week followed by subcuturing on KCMS medium (MS medium with 0.1 mg/l kinetin, 0.2 mg/l 2, 4-D and 0.2 g/l KH_2PO_4 (Plate 10) for two weeks. Calli could be obtained from explants of all the plants used for culturing. Further these calli were transferred to liquid KCMS medium and well dispersed cell suspensions were obtained in 7 days (Plate 11).

4.6.2 Estimation of chitinase activity

Dinitro-salicylic acid method was used to quantitatively estimate endochitinase activity with glycol chitin as a substrate. Total protein in media extracts and cell aggregates of 7 days old cell suspension culture was estimated by Lowry's method. The amount of reducing sugar released after 30 minutes incubation at 50^0C using 300 µg protein from transgenic plants was read at 540 nm (Table 2). All the PCR positive transgenic plants (PSP*α-amyech*42) tested shown higher level of enzyme activity compared to transgenic plants with *ech*42*SP* and non transgenic plants (Plate 12).

Endochitinase activity was low in the medium extracts (from cell suspension culture) of control plants (0.98 µg/ µg of total protein). Enzyme activity varied in cell suspension cultures of different transgenic plants with PSP*α-amyech*42. The amount of reducing sugars released in these plants varied between 9.49 and 11.78 µg per µg of total protein. The amount of reducing sugars released was 7.29 and 8.22 µg per µg of total protein in two transgenic plants with *ech*42*SP* and did not differ significantly with each other. Among PSP*α-amyech*42 plants, cell suspensions from two plants, A_3 and A_9 showed significantly higher activity and the amount of reducing sugar released was 11.78 and 11.33 µg per µg of total protein respectively. Endochitinase activity in the media extracts of transgenic PSP*α-amyech*42 suspension was 10-12 times higher than the control cell suspension while media extracts from transgenic *ech*42*SP* suspension showed 7-8 times higher activity compare to control cell suspension (Table 2). Coefficient of variations was calculated as 5.24%.

Endochitinase activity was also studied in cell extracts of suspension cultures and endochitinase activity observed was low (0.57 µg/µg of total protein) compared to media extracts. Similar to media extracts, chitinase activity varied in cell extracts of different transgenic plants with PSP*α-amyech*42 and which ranged from 1.53-2.00µg/µg of total protein. Two plants with *ech*42*SP* showed 1.13 and 1.07 µg of reducing sugar/µg of total protein and did not differ significantly from each other. Among PSP*α-amyech*42 plants, A_3 and A_9 showed significantly more endochitinase activity *i.e.* 1.9 µg/µg of total protein. Endochitinase activity in the cell extract of transgenic PSP*α-amyech*42 callus was 2.68-3.51 times higher than in the cell extract of control callus. Whereas, cell extract of transgenic *ech*42*SP* showed 1.88-1.98 times higher endochitinase activity than in the cell extract of control callus (Table 2). Coefficient of variation was calculated as 6.63%.

Legends

Plate.7: Various stages of tobacco transformation

A :Shoot initiation

B :Root initiation

C :Putative transgenic plant of ech42 SP

D :Putative transgenic plant of PSPα-amy ech42

Plate.8: PCR confirmation of transgenic (ech42) tobacco plant

M :λHindIII/EcoRI double digest DNA ladder

P :Positive control (pSUM1)

N :Negative control (genomic DNA of control plant)

1-3 :Amplification of ech42 SP

Plate.9: PCR confirmation of transgenic (PSP alpha-amylase ech42) tobacco plant

M :1kb DNA ladder

P :Positive control (pMASGK)

N :Negative control (genomic DNA of control plant)

1-5 :Amplification of PSPα-amy ech42

(A) (B)

(C) (D)

Plate.7: Various stages of tobacco transformation

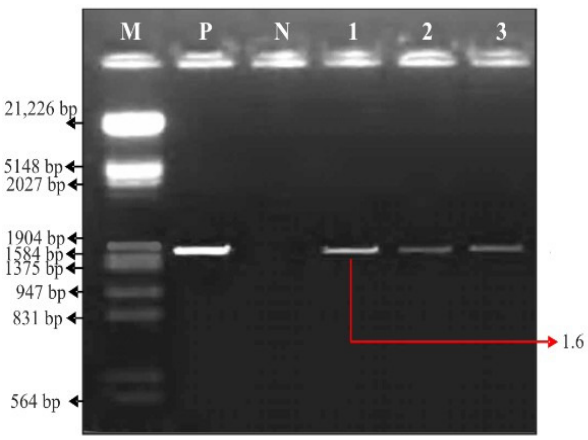


Plate.8: PCR confirmation of transgenic (ech42) tobacco plant

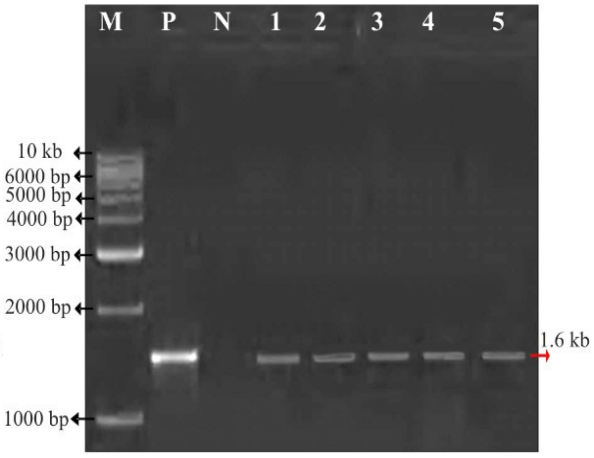


Plate.9: PCR confirmation of transgenic (PSP alpha-amylase ech42) tobacco plant

Legends

Plate.12: Assay for chitinase activity in media and cell extracts of cell suspension culture

A : Blank

B :Cell extract of control plant (callus)

C :Media extract of control plant

D :Cell extract of ech42 SP plant (callus)

E :Media extract of ech42 SP plant

F :Cell extract of PSP α-amy ech42 plant (callus)

G :Media extract of PSP α-amy ech42 plant

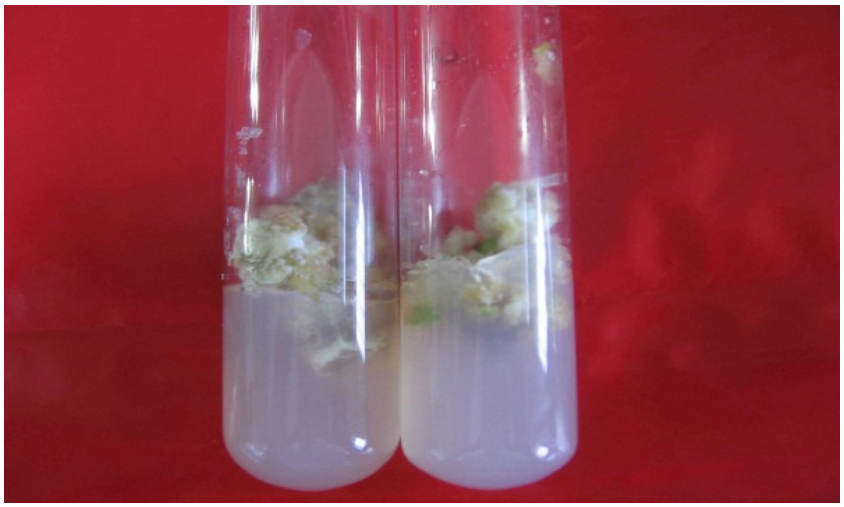


Plate.10: Initiation of callus for cell suspension

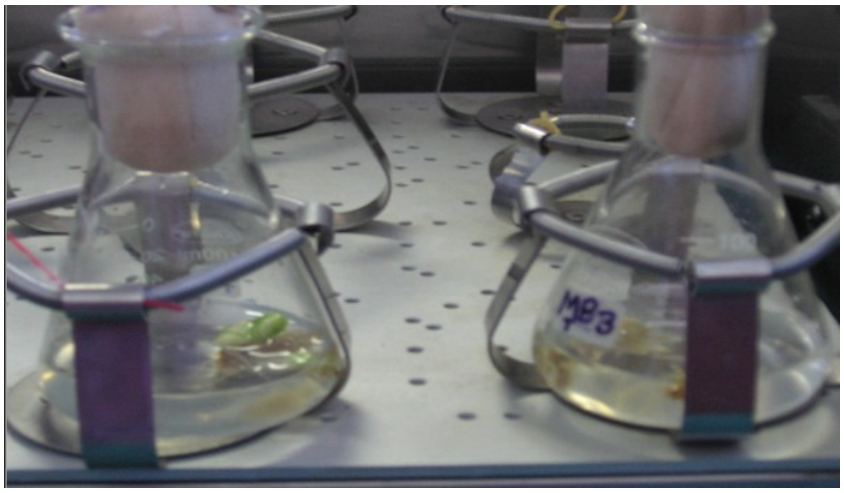


Plate.11. Cell suspension culture

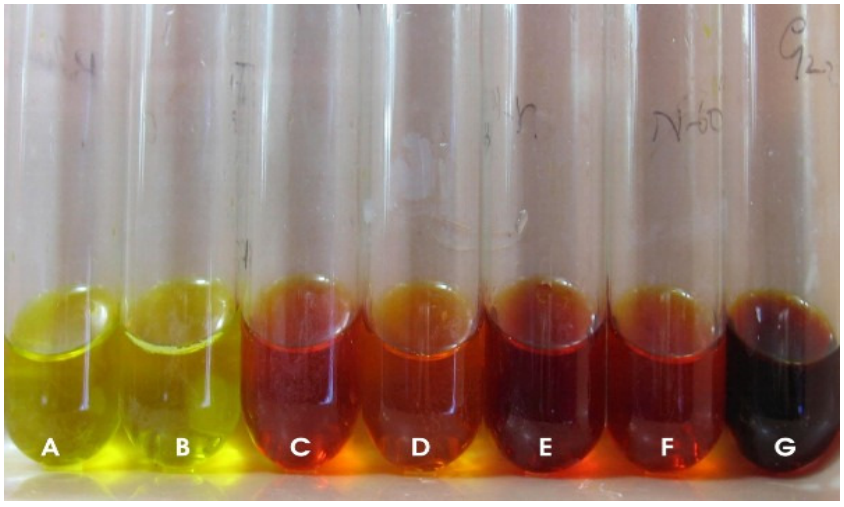


Plate.12. Assay for chitinase activity in media and cell extracts of cell suspension culture

Table 1: Co-Cultivation of leaf explants and recovery of endo*chitinase* PSP*α-amyech*42 and *ech*42*SP* positive tobacco plants

Sl. NO.	Batch	Number					
		Explants Co-cultivated	Explants showing shoot initiation	Shoots elongated	Rooting	Plants survived	PCR (+ve)
PSP*α-amyech*42*)*							
1	B1	40	35 (87.5)	30 (86.0)	25 (83.0)	18 (72.0)	7 (39.0)
2	B2	60	31 (52.0)	28 (90.0)	26 (93.0)	22 (85.0)	16 (73.0)
3	B3	40	25 (63.0)	17 (68.0)	15 (88.0)	10 (67.0)	6 (60.0)
*ech*42*SP*							
1	B1	40	26 (65.0)	15 (58.0)	11 (73.0)	8 (73.0)	5 (63.0)

Figures in parenthesis represent percent values

Table 2: Endochitinase activity (µg of reducing sugar / µg of total protein) in transgenic tobacco cell suspension cultures

Sl. No.	Plant I.D.	Cell extract		Media extract	
		Enzyme activity ±S.E.	Times that of control	Enzyme activity ±S.E.	Times that of control
1.	Control	0.57±0.08	1	0.98±0.01	1
2.	*ech*42(1)	1.13±0.08	1.98	8.22±0.14	8.39
3.	*ech*42(2)	1.07±0.08	1.88	7.29±0.19	7.44
PSP*α-amyech*42					
4.	A_3	1.9±0.05	3.33	11.78±0.09	12.02
5.	A_6	2.0±0.06	3.51	10.41±0.53	10.62
6.	A_7	1.53±0.08	2.68	9.49±0.14	9.68
7.	A_9	1.9±0.1	3.33	11.33±0.18	11.56
8.	A_{11}	1.87±0.06	3.28	10.69±0.33	10.91

4.6.3 Bioassay against pathogen

4.6.3.1 Bioassay against *Sclerotium rolfsii*

Addition of media extract from transgenic plants to the PDA plate inhibited the radial growth of *S. rolfsii* at two different concentrations tested compared to extracts from non transgenic plants. At low concentration *i.e.* 100 µg of total protein, no considerable difference was observed in the mycelial growth of fungus. In contrast, addition of 200 µg of total protein from media extracts of transgenic plants inhibited fungal mycelial growth on PDA plate compared to control (Plate 13). On PDA plate with medium extract from plant carrying *ech*42*SP (ech*42 its own signal peptide) percent inhibition was 9-11 per cent at 100 µg of total protein and 12-14 per cent at 200 µg of total protein. Media extract from plant carrying PSP*α-amyech*42 showed percent inhibition of 26-41 per cent at a100 µg of total protein and it was 58-73 per cent at 200 µg of total protein. Among plants carrying PSP*α-amyech*42 gene, A_3 and A_9 was significantly inhibiting the mycelial growth of fungus (69% and 73% respectively). Observation of mycelia under light microscope at magnification of 100X revealed the bulging and lysis of mycelium in plates with medium extracts from transgenic plants (PSP*α-amyech*42) compared to control plant (Plate14).

4.6.3.2 Bioassay against *Rhizoctonia bataticola*

Media extracts of transgenic and control suspensions were also used to study the inhibition of mycelial growth of *Rhizoctonia bataticola* on PDA plate. Per cent inhibition of mycelia growth of fungus on PDA plate with *ech*42*SP* suspension was 20-24 per cent whereas percent inhibition recorded with PSP*α-amyech*42 suspension was 36-53 per cent (Table 4). Delayed growth of mycelia can be observed with PSP*α-amyech*42 suspension compared to control suspension. Mycelial growth on PDA plate with *ech*42*SP* suspension was more compared to PSP*α-amyech*42 suspension (Plate 15). Plants carrying PSP*α-amyech*42 gene, A_3 and A_9 showed significant inhibition with 49% and 53% respectively. Observation of mycelia under light microscope at 45X showed shrinkage and shriveling in media extract of PSP*α-amyech*42 suspension compared to media extract of control suspension in which normal shape of mycelia was observed. In case of PDA plate with media extract of *ech*42*SP* suspension, the effect is less (Plate 16).

Legends

Plate.13: growth inhibition of Sclerotium rolfsii

A(I) :Media extract of control suspension (100µg)

A(ii) :Media extract of control suspension (200 µg)

B(i) :Media extract of ech42 SP suspension (100 µg)

B(ii) :Media extract of ech 42 SP suspension (200 µg)

C(i) :Media extract of PSP α-amyech42 suspension (100 µg)

C(ii) :Media extract of PSP α-amyech42 suspension (200 µg)

D(i) :Plain PDA

D(ii) :Media extract of control suspension (200 µg)

D(iii) : Media extract of control suspension (200 µg)

D(iv) : Media extract of PSP α-amyech42 suspension (200 µg)

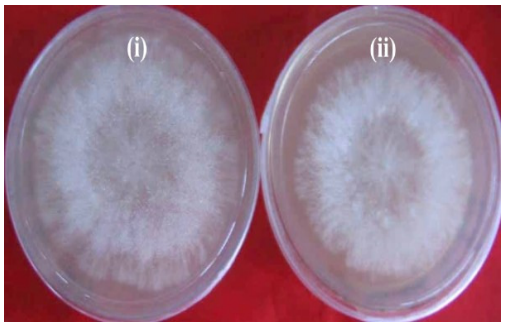
Plate.14: Microscopic view of mycelium of S. rolfsii (100X)

A :On Plain PDA

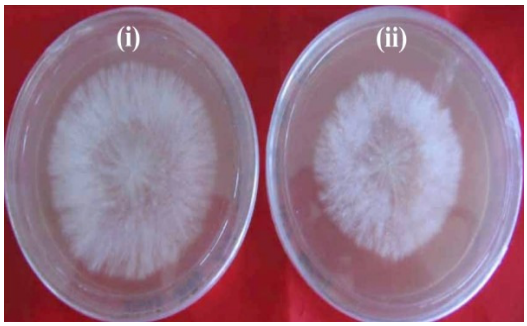
B :On PDA with media extracts of control cell suspension

C :On PDA with media extracts of ech42 SP cell suspension

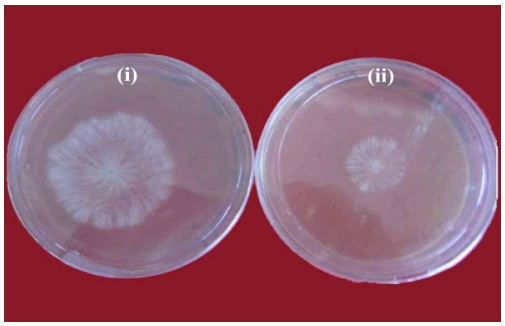
D :On PDA with media extracts of PSP α-amy ech42 cell suspension



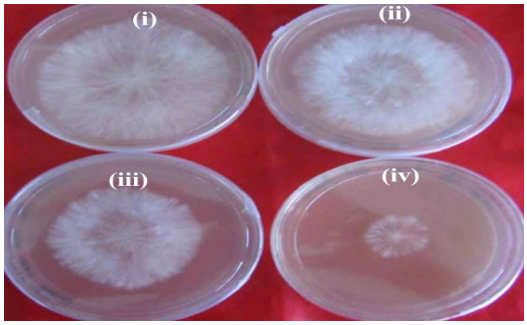
(A)



(B)

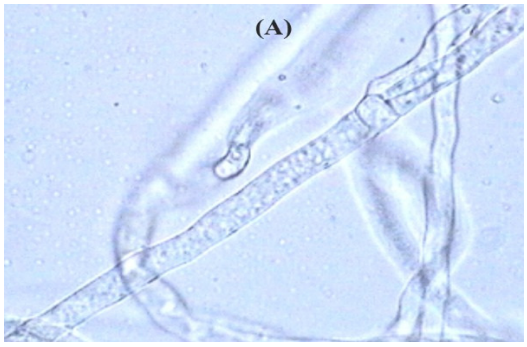


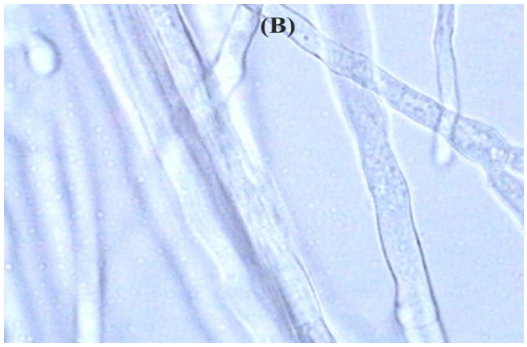
(C)

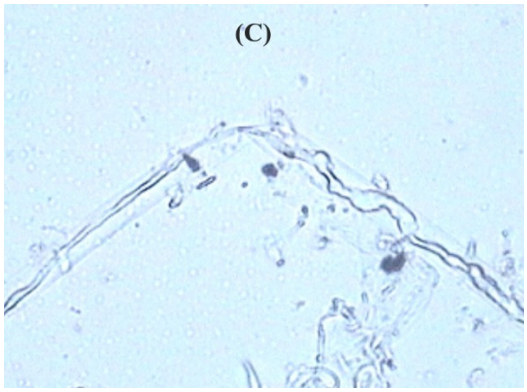


(D)

Plate.13. Growth inhibition of Sclerotium rolfsii







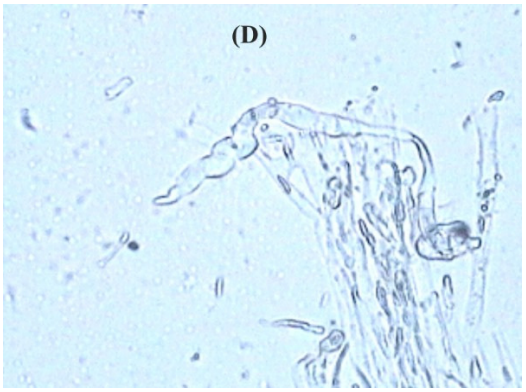


Plate.14: Microscopic view of mycelium of Sclerotium rolfsii

Legends

.

Plate.15: Growth inhibition Rhizoctonia bataticola

A :Plain PDA

B :Media extract of control suspension (200µg)

C :Media extract of ech42 SP suspension (200µg)

D :Media extract of PSP α-amy ech42 suspension (200µg)

Plate.16: Microscopic view of mycelium of Rhizoctonia bataticola(45x)

A :On plain PDA

B :On PDA with media extracts of control cell suspension

C :On PDA with media extracts of ech42 SP cell suspension

D :On PDA with media extracts of PSP α-amy ech42 cell suspension

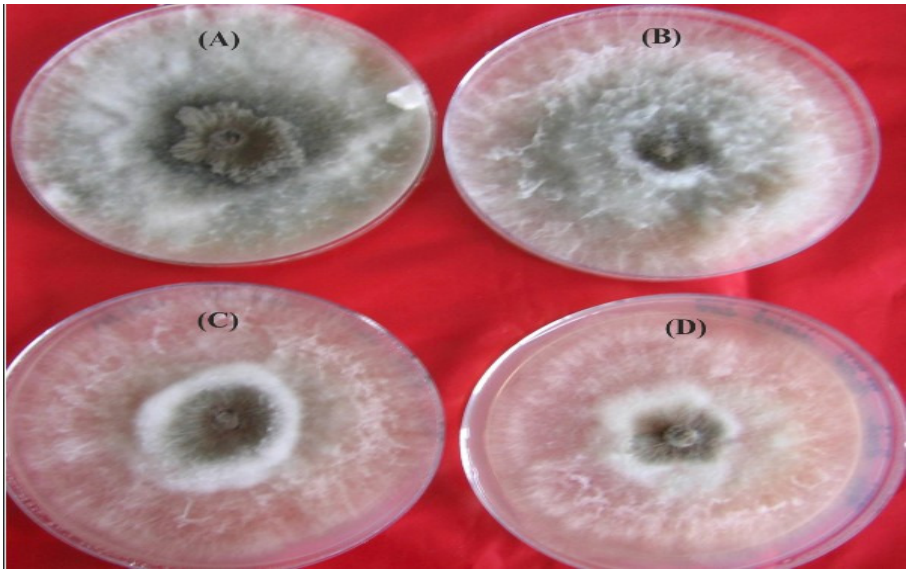
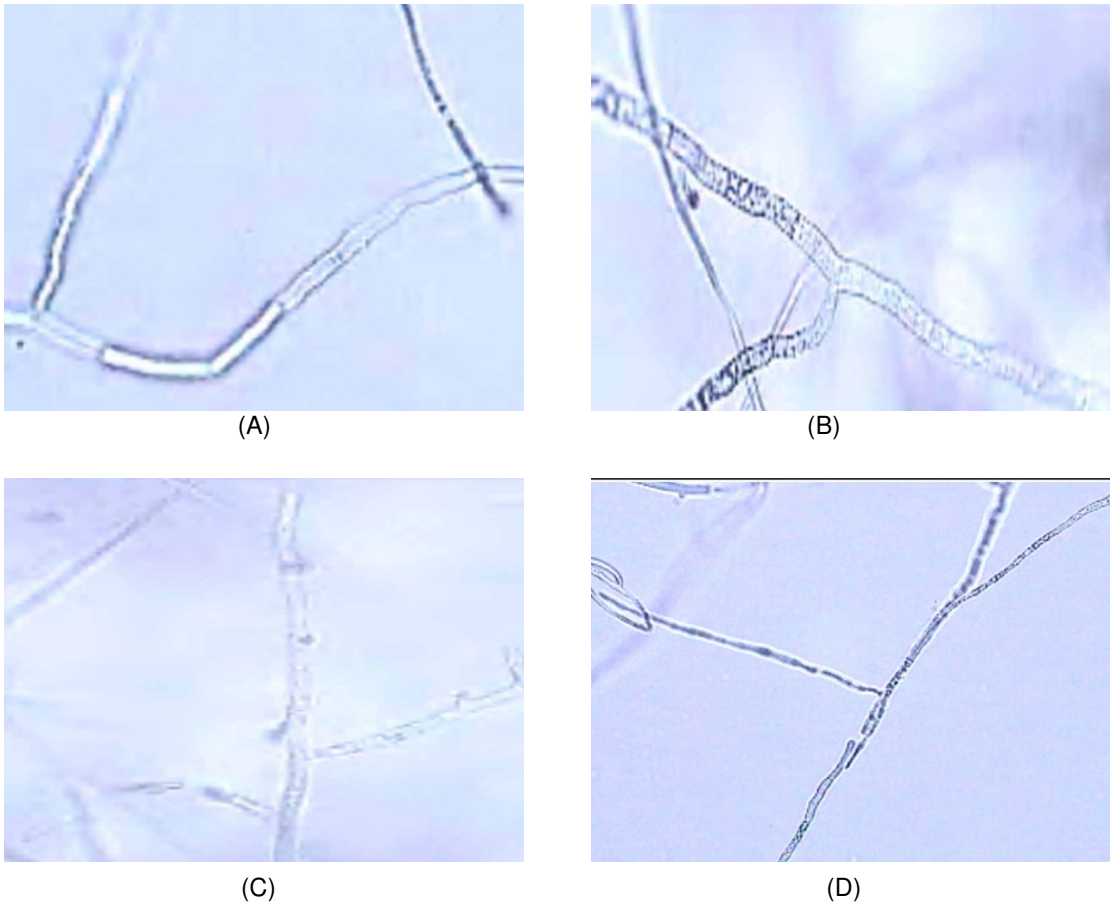


Plate.15. Growth inhibition of Rhizoctonia bataticola



(A) (B)

(C) (D)

Plate.16: Microscopic view of mycelium of Rhizoctonia bataticola

Table 3: Percent inhibition of *Sclerotium rolfsii* on PDA plate with medium extract

Sl. No.	Plant I.D.	Concentration (µg of total protein)	
		100	200
1.	Control	0	0
Per cent Inhibition (%)			
2.	*ech*42 (1)	11	14
3.	*ech*42 (2)	9	12
PSP*α-amyech*42			
4.	A_3	35	69
5.	A_6	26	58
6.	A_7	41	67
7.	A_9	36	73
8.	A_{11}	38	59

Table 4: Percent inhibition of *Rhizoctonia bataticola* on PDA plate with medium extract

Sl. No.	Plant I.D.	Concentration (200 µg of total protein)
1.	Control	0
Per cent Inhibition (%)		
2.	*ech*42 (1)	24
3.	*ech*42 (2)	20
PSP*α-amyech*42		
4.	A_3	49
5.	A_6	36
6.	A_7	42
7.	A_9	53
8.	A_{11}	44

5. DISCUSSION

Thousands of insects, fungi, viruses, bacteria, nematodes and other living forms are potential hazards to agricultural crops. Several crop protection methods were developed to control these pests of which chemical control measures are most widely practiced by farmers. However, control through chemical pesticides suffers from several drawbacks such as cost of production, environment and health hazards *etc.* Therefore in recent years alternative control methods are being developed which are safer and relatively less costly. One such strategy is the breeding for resistant crop varieties. Genetic engineering techniques are being used recently to make specific improvement in the crop varieties retaining all the other desirable features. Therefore, this technique can be used to improve disease or pest resistance in an otherwise popular genotype by transferring one or few genes from distant sources. In order to develop fungal disease resistance in crop plants, genes encoding hydrolytic enzymes such as chitinases and glucanases from diverse sources are being transferred to plants (Jach *et al*., 1995; Lorito *et al*., 1998; Hong and Hwang, 2006). Acidic chitinases are located extracellularly, and they may be involved in the recognition process during pathogen infection upon making contact with the pathogen, recognize the cell wall component using chitinolytic or lysozyme activity, and release oligosaccharides as elicitors, and thus trigger the plant defense system (Graham and Sticklen, 1994). In addition, acidic chitinase may also act as the first line of defense by interfering with the pathogen invading structure and therefore limiting its spread, which means enzymes should be secreted extracellularly and signal peptides play an important role in this process (Dore *et al*., 1989; Iturriaga *et al*., 1989). The present investigation was carried out to compare α-amylase plant signal peptide and signal peptide of *Trichoderma ech*42 for efficient secretion of endochitinase outside. The results are discussed in this chapter.

5.1 Tobacco transformation and establishment of cell suspension

In this study, efforts were made to fuse the alpha amylase (αamy3) signal peptide sequence with the endochitinase (*ech*42) gene sequences cloned from potential biocontrol agent *Trichderma virens.* This was further cloned into plant transformation vector pBI121 and was transferred to *Agrobacterium*. *Agrobacterium* mediated transformation was used to transform a model plant tobacco with this construct. In order to compare the secretion, tobacco was also transformed with *ech*42 carrying its own signal peptide. Tobacco is used as model plants to study various aspects in 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 (Helgeson, 1979). 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 (Sommer *et al.,* 1998). In the present study aim was to use tobacco as a expression system for comparison of signal peptides (αamy3 and *Trichoderma ech*42*SP*) for extracellular secretion of endochitinase enzyme.

Alpha amylase (αamy3) signal peptide from rice was used since it was known to secrete the protein of interest in intercellular spaces in various studies (During *et al*., 1989, 1990; Chen *et al*., 1994; Chan *et al*., 1994; Chiang *et al*., 2005; Park *et al*., 1997; Yu *et al*., 2005; Liu *et al*., 2004). Using αamy3 SP secretion of a chimeric T_4 lysozyme in transgenic plants was reported (During *et al*., 1990; Hippe *et al*., 1989). Comparable experiments in *E. coli* and yeast have shown that assembly of an active antibody is possible in both organisms only in the presence of signal peptides in the foreign precursors (Better *et al*., 1988; Horwitz *et al*., 1988; Skerra and Pluckthun, 1988; Wood *et al*., 1985). Similarly, Chen *et al*. (1994) analyzed the subcellular localization of α-amylases in cultured rice (*Oryza sativa*) suspension cells and revealed that α-amylases are localized in cell walls as well as in starch granules within amyloplasts. The dual localization of α-amylases disagrees with the general belief that the translocation of proteins to chloroplasts or amyloplasts and the extracellular compartments is carried out by different targeting signals and via different pathways (Verner and Schatz, 1988). Liu (2006) compared different signal peptides for secretion of GFP in rice suspension culture; found that αAmylase3 is the better signal peptide for secreting the GFP out. As efforts to fuse α-amylase signal peptide with *ech*42 has not been made earlier, this investigation has been carried out in this study.

Since, the biocontrol fungus *Trichoderma harzianum* produces many chitinolytic enzymes (Chet, 1987), including endochitinase which randomly cleaves chitin and *Trichoderma* endochitinase tends to be more effective in controlling fungi than chitinases found in plants or other fungi (Harman *et al*. 1993; Lorito *et al*. 1993), the present study was conducted with *Trichoderma* endochitinase gene. It was reported by Schickler and Chet, (1997) that heterologous chitinase gene expression is used in various plants to enhance their defense mechanisms against fungal pathogens. A number of studies have successfully demonstrated that chimeric genes can protect plants against infection by fungal pathogens (Broglie *et al.* 1991; Jach *et al.* 1995). Similarly, Lorito *et al.* (1993; 1994) demonstrated that in *in vitro* experiments there are no chitin-containing phytopathogens that would be resistant to chitinases of *Trichoderma.* Introduction of a single fungal gene into a cultured plant is expected to produce a transgene with increased resistance to a broad range of pathogenic fungi (multiple introductions are required if plant genes are involved). The gene *ech*42 from *T. harzianum*, (Garcia *et al*., 1994; Hayes *et al*., 1994), codes for a chitinase with significantly higher activity against a broad range of phytopathogenic fungi than other chitinolytic enzymes (Lorito *et al*., 1993; 1994). Lorito *et al*. (1998) reported that the endochitinase gene (*chit* 42) of *T. harzianum* P1 and CECT 2413 has been transferred into tobacco and potato, resulting in a high level of constitutive expression in various tissues, which has no detectable effect on the plant growth and development. The transgenic strains thus obtained are highly or completely resistant to the leaf pathogens *Alternaria alternaria, A. solani*, and *B. cinerea* and the soil phytopathogen *R. solani*. The high level and broad spectrum of the resistance acquired with the endochitinase gene from *Trichoderma sp*. surpasses the efficiency of transgenic expression of plant or bacterial chitinases in plants.

In the present study, co-cultivated explants were transferred to shoot induction medium having kanamycin as selection pressure. About 80 per cent of explants showed shoot initiations which were transferred to shoot elongation medium (with selection pressure) where only 70 per cent of shoot initials elongated normally. Further, shoots were transferred to rooting media (with selection pressure) and only 80 per cent of them rooted. Out of 50 plants, only 29 plants were positive for PSP*α-amyech*42 and out of 8 plants, only 5 plants were positive for *ech*42 with its own signal peptide. Occurrence of escapes on selection medium is a most common phenomenon during selection of transformants on medium with antibiotics. Cell suspension cultures were established from green house grown positive plants. Callus cultures were initiated from five plants carrying PSP*α-amyech*42, two plants with *ech*42*SP* and from non transformed pants. Cell and cell suspensions were obtained upon transferring callus to liquid KCMS medium in two weeks. Similar approach has been followed by Brants and Earle, (2001). They established calli and cell suspensions from tobacco plants transformed with an endochitinase encoding cDNA from the biocontrol fungus *Trichoderma harzianum.* Calli from four primary transformants had high levels of endochitinase activity, like the plants from which they were derived compared to control callus. Cell suspensions were initiated from 3 weeks old callus cultures after transferring to liquid KCMS medium in 2 weeks. Similarly, Lund and Dunsmuir, (1992) conducted experiment to improve secretion of ChiA by plant cells using ChiA (bacterial) or PR1b (tobacco) signal peptide. They assayed the level of secretion of ChiA from different transgenic tobacco cells by analyzing the media from plant cell suspension cultures and by comparing the profiles of protein extracts from leaf protoplasts and corresponding whole leaves. The data from the analysis of suspension culture media suggest that in the absence of any signal sequence (pChiA-M) the ChiA which is expressed is not secreted. In the presence of a signal sequence, either the ChiA signal (pChiA), or the PRIb signal (pPRSSChiA), ChiA protein is glycosylated and secreted. The observation that higher levels of ChiA protein appear in the medium from pPRSSChiA transformants than from pChiA transformants suggests that secretion is more efficient when the PRIb signal is fused to ChiA.

5.2 Assay for endochitinase activity

In this study, media extract was collected after 7 days and checked for chitinase activity. Media extract obtained from PSP*α-amyech*42 containing plants showed higher chitinase activity compared to plants with *ech*42*SP*. Endochitinase activity detected in media extracts of PSP*α-amyech*42 cell suspensions was 10-12 times more compared to extracts of control suspension. On the other hand, media extracts from *ech*42*SP* suspensions showed 8-9 times chitinase activity that of control. The elevated protein concentration and endochitinase

activity in media extracts of transgenic samples are further indications that endochitinase is secreted from the transgenic cells. Endochitinase activity in the cell extract of transgenic PSP*α-amyech*42 callus was 2.68-3.51 times higher than in the cell extract of control callus. Whereas, cell extract of transgenic *ech*42*SP* showed 1.88-1.98 times higher endochitinase activity than in the cell extract of control callus. This further confirms that the endochitinase is secreted in the liquid medium. The correlation coefficient between activity in the calli and in the medium was 0.9, indicating that calli with higher endochitinase activity tend to have higher endochitinase activity in the surrounding medium. Similarly, Brants and Earle, (2001) reported higher chitinase activity in the medium from the transgenic suspensions (42 and 37 n*M* MU/min per mg of protein). 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. Likewise, During *et al.* (1990) constructed chimeric genes containing the coding sequence of the barley α-amylase signal peptide which has been fused to cDNAs coding for either the mature light or the mature heavy chain of a monoclonal antibody. A plasmid was constructed linking both chimeric genes under the control of plant active promoters in an expression cassette. This DNA fragment was stably integrated into the genome of *Nicotiana tabacum* by *Agrobacterium tumefaciens* mediated gene transfer. Synthesis of light and heavy chains and assembly to antibodies was detected in transgenic tobacco tissue using specific secondary antibodies. By electron microscopic immunogold labeling, the presence of assembled antibody could be detected within the endoplasmic reticulum.

5.3 Bioassay against pathogen

In order to further confirm the extracellular secretion of endochitinase from transgenic and control cell suspensions, media extracts were used in the PDA plate to see its effect on growth of two selected soil borne plant pathogen *S. rolfsii* and *R. solani*. On PDA plates with media extracts of PSP*α-amyech*42*,* percent inhibition of *S. rolfsii* was 26-41 per cent and 9-11 per cent with media extracts of *ech*42*SP* when 100µg of total protein was added, it was 58-73 per cent with media extracts of PSP*α-amyech*42 and 12-14 per cent with media extracts of *ech*42*SP* when 200µg of total protein was added. With media extracts of PSP*α-amyech*42 *on* PDA plate, percent inhibition of *R. solani* was 36-53 per cent and media extracts of *ech*42*SP* showed percent inhibition of 20-24 per cent when 200µg of total protein was used. Microscopic observation of the mycelia of pathogenic fungi grown on PDA plates with media extracts revealed the degradation and shrinkage of mycelium. Katatny *et al.* (2000) tested antifungal activity of *T. harzianum* chitinase and β-1, 3-glucanase in culture filtrates against *S. rolfsii.* Growth of *S. rolfsii* was significantly inhibited (upto 61.8 %) by enzyme preparations from *T. harzianum*. Likewise, Kucuk and Kivanc, (2003) tested antifungal activity of *T. harzianum* isolates against *S. rolfsii.* They reported that *T. harzianum* isolates T_3 and T_{19} showed 70-88% inhibition rate.

Krishnamurthy *et al.* (1999) screened thirty-five strains of *Trichoderma viride* and *T. harzianum* for their antagonistic ability against the rice sheath blight pathogen, *Rhizoctonia solani* using culture filtrates. Observation of interacting mycelia under Leitz inverted light microscope revealed the antagonism of the hyphae of effective strains of *Trichoderma* towards those of *R. solani.* In later stages, *R. solani* hyphae showed extreme shrinkage and shriveling, probably an irrecoverable state. The role of glucanases and chitinolytic enzymes in fungal cell wall degradation during mycoparasitism by *Trichoderma* spp. is well documented (Lorito *et al* 1993; Ridout *et al* 1986; Sivan and Chet, 1989). Kumar *et al.* (2009) analyzed resistance in transgenic cotton plants against *R. solani* expressing an endochitinase gene from *Trichoderma virens.* Transgenic seedlings were protected from infection by *R. solani*, while the wild-type (WT) seedlings developed severe collar lesions and many were stunted. Although *R. solani* is not a foliar pathogen of cotton, they tested whether the constitutively-expressed endochitinase would confer resistance in mature leaves. Five days after inoculating the leaves with the pathogen, non-transgenic control leaves showed large, dark-colored necrotic lesions. On the other hand, the size of the lesion was either very small, or it was completely absent in the leaves from transgenic plant. Even the mycelial growth from the agar plugs was less extensive on the transgenic leaves.

The localization of chitinases may have functional significance and be attributed to a balanced plant defense system. The sudden release of enzymes can have a dramatic effect on the pathogen, and overwhelm the natural balance of cell wall construction in the growing tip, and can cause a fungicidal effect, digest cell walls, lyse hyphal tip, inhibit spore germination, and kill the pathogen.

The chimeric gene can be transferred to commercial crops like tomato for validation of results and enhanced expression of gene for better fungal resistance. The fused gene *(*PSP*α-amyech*42*)* can be further fused to GFP reporter gene which will further facilitate the detection of localization of endochitinase (cloned gene product) for further confirmation of role of signal peptides considered for this study. The transgenic plants obtained can be further evaluated in next (T_1) generation.

6. SUMMARY AND CONCLUSIONS

The present investigation was carried out to compare endochitinase signal peptide with plant signal peptide for better secretion into intercellular spaces in plants. In this research plant signal peptide α-amylase was synthesized and fused with *Trichderma virens* endochitinase gene *ech*42. A plant transformation vector was constructed carrying this construct and transferred to tobacco. Further cell suspension was established from plants carrying *ech*42*SP* and PSP*α-amyech*42. Chitinase activity was checked in media extracts and cell extracts of suspension culture and later these extracts were used for bioassay against *Sclerotium rolfsii* and *Rhizoctonia solani*. The results of various experiments conducted are summarized below:

- α-amylase plant signal peptide was fused with *ech*42 present in pSUM1 (previously cloned) (Upendra, 2006). The chimeric gene was named as PSP*αamyech*42 and the recombinant vector was named as pMASG.
- A plant transformation vector was constructed carrying PSP*αamyech*42 and was named as pMASGK. It was transferred to *Agrobacerium tumefaciens* LBA4404 via triparental mating.
- The *Agrobacterium* clones carrying PSP*α-amyech*42 and *ech*42*SP* were separately used for tobacco transformation. Putative transformants were selected on medium with kanamycin (200 mg/ml) and further screened for the presence of PSP*αamyech*42 using signal peptide specific forward and gene specific reverse primers and also for the presence of *ech*42*SP* using gene specific primers. Out of 50 survived plants, only 29 were positive for PSP*α-amyech*42 and out of 8 survived plants, only 5 were positive for *ech*42*SP.*
- Well established Callus was initiated from five transformants (PCR positive) carrying PSP*α-amyech*42, two transformants (PCR positive) carrying *ech*42*SP* and non transformed plants in three weeks. Cell suspension cultures were established from initiated callus in one week.
- Chitinase activity was checked from media and cell extracts of suspension cultures. Plants carrying PSP*α-amyech*42 showed higher endochitinase activity (9-12 times) than plants carrying *ech*42*SP* (7-8 times) in both media and cell extracts.
- Media extracts (200 µg of total protein) of PSP*α-amyech*42 suspension on PDA plate showed per cent inhibition of 73% and 53% against *Sclerotium rolfsii* and *Rhizoctonia solani* respectively. Media extracts (200 µg of total protein) of *ech42SP* suspension showed per cent inhibition of 14% and 24% against *Sclerotium rolfsii* and *Rhizoctonia solani* respectively.
- Use of α-amylase signal peptide helped better secretion of endochitinase which was confirmed by chitinase activity in media and cell extracts and bioassay against *Sclerotium rolfsii* and *Rhizoctonia solani.*

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Appendix I

a. Luria agar

Ingredients	Concentration (g/l)
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	5.0
Agar	18.0
pH	7.2

Luria agar Amp_{50} : To 100 ml Luria agar 50 µl of Amp_{100} (antibiotic) was added at 50℃.

Luria agar Amp_{100} : To 100 ml Luria agar 100 µl of Amp_{100} (antibiotic) was added at 50℃.

b. Recipe for 0.7 per cent Agarose gel (40 ml)

Agarose :280 mg

1x TAE : 40 ml

EtBr (10 mg/ml) : 2 µl

Appendix II: Reagents for plasmid isolation

STET buffer

Tris-Cl (pH 8.0)	: 10 mm
NaCl	: 0.1 M
EDTA (pH 8.0)	: 1.0 mM

Autoclaved and stored at 4℃

Alkaline lysis solution I

Glucose	: 50 mM
Tris-Cl (pH 8.0)	: 25 mM
EDTA (pH 8.0)	: 10 mm

Autoclaved and stored at 4℃

Alkaline lysis solution II

NaOH	: 0.2 N
SDS	: 1% (w/v)

(Prepared fresh and used at room temperature)

Alkaline lysis solution III

5 M potassium acetate	: 60 ml
Glacial acetic acid	: 11.5 ml
Double distilled water	: 28.5 ml

Autoclaved and stored at 4℃

Appendix III: Restriction Recipes

A) Restriction of pCR2.1 and pSUM1 vector

plasmid DNA	:	1.5 µl
Enzyme *Bsr*GI (5U)	:	0.5 µl
Enzyme *Bam*H1 (5U)	:	0.5 µl
10x buffer (C)	:	2 µl
Sterile water	:	15.5µl
Total	:	20 µl

B). Restriction of pSUM1 and pBI121 vector

plasmid DNA	:	1.5 µl
Enzyme *Xba*I (5U)	:	1.0 µl
10x buffer (C)	:	2 µl
Sterile water	:	15.5µl
Total	:	20 µl

C). Restriction of pMASG vector

plasmid DNA	:	1.5 µl
Enzyme *Bsr*GI (5U)	:	0.5 µl
Enzyme *Bam*H1 (5U)	:	0.5 µl
10x buffer (C)	:	2 µl
Sterile water	:	15.5µl
Total	:	20 µl

plasmid DNA	:	1.5 µl
Enzyme *Pst*I (5U)	:	0.5 µl
10x buffer(C)	:	2 µl
Sterile water	:	15.5µl
Total	:	20 µl

D). Restriction of pMASGK vector

plasmid DNA	:	1.5 µl
Enzyme *Xba*I (5U)	:	0.5 µl
10x buffer(C)	:	2 µl
Sterile water	:	15.5µl
Total	:	20 µl

plasmid DNA	:	1.5 µl
Enzyme *Sac*I (5U)	:	0.5 µl
10x buffer(C)	:	2 µl
Sterile water	:	15.5µl
Total	:	20 µl

Appendix IV

Ligation of pCR2.1 insert and pSUM1

pCR2.1 insert	3 µl
Plasmid DNA (pSUM1)	1.5 µl
T_4 DNA ligase enzyme (10 U)	1 µl
Buffer (10 x)	1 µl
Sterile H_2O	3.5 µl
Total	10 µl

Ligation of pMASG insert and pBI121

pMASG insert	3 µl
Plasmid DNA (pBI121)	1.5 µl
T_4 DNA ligase enzyme (10 U)	1 µl
Buffer (10 x)	1 µl
Sterile H_2O	3.5 µl
Total	10 µl

Appendix V: PCR reaction mixture

Template DNA	1.0 µl
Primer forward (5pM)	1.0 µl
Primer Reverse (5pM)	1.0 µl
dNTP's (2.5 mM)	2.0 µl
Taq buffer A (10X)	2.0 µl
Taq DNA polymerase	0.3 µl
Sterile water	12.7 µl
Total	20.0 µl

Appendix VI

a. Loading dye composition

Loading dye (6x) : 0.25% bromophenol blue
40% (w/v) sucrose in water

b. Ethidium bromide
10 mg/ml in distilled water. Stored at 4℃ in dark bottle.

c. Recipe for 1 per cent Agarose gel (40 ml)

Agarose :400 mg

1x TAE : 40 ml

EtBr (10 mg/ml) : 2 µl

d. 50x TAE composition

Tris base : 242 g

Glacial acetic acid : 57.1 ml

0.5 M EDTA (pH 8.0) : 100 ml

Total volume 1000 ml with double distilled water.

Appendix VII: Composition of yeast extract mannitol agar (YEMA) for 100 ml

D-Mannitol	-	1 g
KH_2PO_4	-	20 mg
K_2HPO_4	-	20 mg
Yeast Extract	-	100 mg
$MgS0_4$. $7H_2O$ (1 M)	-	80 µl
$CaCl_2$ (1 M)	-	40 µl
Agar	-	1.8 g

Appendix VIII: Components of Murashige and Skoog (1962) medium (modified)

	Component	mg/l concentration
Macronutrients	NH_4NO_3	1650.00
	KNO_3	1900.00
	$MgSO_4 . 7H_2O$	370.00
	KH_2PO_4	170.00
	$CaCl_2 . 2H_2O$	440.00
Micronutrients	$FeSO_4 . 7H_2O$	27.80
	Na_2 EDTA	60.00
	$MnSO_4 . 4H_2O$	22.30
	$ZnSO_4 . 7H_2O$	8.60
	H_3BO_3	6.30
	KI	0.83
	$Na_2MoO_4 . 2H_2O$	0.25
	$CuSO_4 . 5H_2O$	0.025
	$CoCl_2 . 6H_2O$	0.025
Organics	Thiamine HCl	10.00
	Pyridoxine HCl	1.00
	Nicotinic acid	1.00
	Glycine	10.00
	Myo*inos*itol	100.00
	Biotin	0.50

Appendix IX: Extraction buffer for DNA isolation (RAPID method)

Tris-HCl (pH 7.5)	200 mM
NaCl	250 ml
EDTA (pH 8.00)	25 mM
SDS	0.5%

Appendix X

Potato Dextrose Agar (PDA)

Peeled potato	200 g/l
Dextrose	20 g/l
Yeast Extract	0.1 g/l
Agar (for solid medium)	20 g/l
Distilled water	1000 ml

ENHANCEMENT OF *Trichoderma* ENDOCHITINASE SECRETION IN TOBACCO CELL CULTURE USING α-*amylase* SIGNAL PEPTIDE

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ABSTRACT

The present investigation was carried out to compare *Trichoderma* endochitinase signal peptide (SP) with α-amylase signal peptide (plant) for better secretion into intercellular spaces in plants. α-amylase signal peptide was synthesized and fused with endochitinase gene (*ech*42) cloned from *Trichoderma virens* previously (pSUM1C). The chimeric gene was named as PSP*α-amyech*42 and recombinant vector was named as pMASG. A plant transformation vector (pMASGK) was constructed carrying PSP*α-amyech*42 and transferred to *Agrobacterium tumefaciens* strain LBA4404. Tobacco leaf discs (cv. White Burley) were co-cultivated with separately with *Agrobacterium tumefaciens* strain LBA4404 carrying pMASGK and *Agrobacterium tumefaciens* strain LBA4404 carrying pSUM1C to compare the two signal peptides for efficient secretion of endochitinase. Putative transformants were selected on MS medium with kanamycin (200 mg/l). Only 29 out of 50 survived plants were positive for PSP*α-amyech*42 and 5 out of 8 survived plants were positive for *ech*42.

For confirmation of secretion of endochitinase, well established callus was initiated from 5 transformants with PSP*α-amyech*42, 2 transformants with ech42 having its own SP and non transformed plant in three weeks. Cell suspension cultures were established from initiated callus in one week. Chitinase activity was checked from media and cell extracts of suspension cultures. Plants carrying PSP*α-amyech*42 showed higher endochitinase activity (9-12 times) than plants carrying *ech*42SP (7-8 times) in both media and cell extracts. For further confirmation of secretion of endochitinase, bioassay against pathogen was done. Media extracts (200 µg of total protein) of PSP*α-amyech*42 suspension on PDA plate showed 73% and 53% growth inhibition of *Sclerotium rolfsii* and *Rhizoctonia solani* respectively whereas media extracts (200 µg of total protein) of *ech*42SP suspension showed 14% and 24% growth inhibition of *Sclerotium rolfsii* and *Rhizoctonia solani* respectively. The chimeric gene can be further used in important crop plants to have better resistance to fungal pathogens.