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Expression of Multi-Domain Lytic Peptide Genes in Transgenic Plants for Disease Resistance

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EXPRESSION OF MULTI-DOMAIN LYTIC PEPTIDE GENES IN TRANSGENIC PLANTS FOR DISEASE RESISTANCE

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EXPRESSION OF MULTI-DOMAIN LYTIC PEPTIDE GENES IN TRANSGENIC PLANTS FOR DISEASE RESISTANCE

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Many organisms produce small proteins which exhibit antimicrobial activities. In recent decades, the biological role of antimicrobial peptides (AMP) has been recognized as the main factor in the defense mechanisms against a broad range of pathogenic microbes. The increased worldwide incidence of microbial resistance to antibiotics and pesticides, makes AMPs promising alternative for the control of microbial disease.

In the last decades, synthetically designed novel non-natural APMs have become an option for use in the improvement of agriculture for crops. The synthetic AMPs have enhanced activities against a wide spectrum of pathogens with low non-specific toxicity, good stability, and bioavailability. Exploring the potential of the AMPs in transgenic crops could lead to the development of new and improved cultivars which are resistant to various pathogenic diseases.

In the present study, four different non-plant multi-domain lytic peptide genes, which code for antimicrobial peptides and are expressed in *Nicotiana benthamiana* tobacco plants were tested against three fungal pathogens: S*clerotinia sclerotiorum, Rhizoctonia solani,* and *Pythium* sp. Detached-leaf bioassay was performed for the transgenic plants carrying multi-domain lytic peptide constructs (ORF13, RSA1, CN77, ORF12), transgenic vector control

plants (1234), and wild control plants (WT) against the three fungal pathogens. Symptom area of each leaf was measured with high accuracy. Data was recorded and processed by statistical analyses. The results showed that transgenic plant lines ORF13 and RSL1 have substantial resistance to S*clerotinia sclerotiorum* infection, producing significant smaller symptom area compared to controls: vector plant line 1234 and wild type WT. However, these lines were not effective against two other fungal pathogens *Rhizoctonia solani,* and *Pythium* sp

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INTRODUCTION

Antimicrobial peptides (AMPs) are natural components of many living organisms' defense system. The common characteristic of these peptides is their ability to suppress a wide range of pathogenic microbes. AMPs are peptides which consist of up to 100 amino acids (AAs). In nature, AMPs are molecules with a high degree of biochemical and structural variability, which strongly correlates with the environmental diversity and the richness of living organisms. Despite their variability, AMPs share common characteristics such as positively charged AAs and the existence of hydrophobic or hydrophilic secondary structures. The cationic nature of AMPs determines their ability to interact selectively with negatively charged microbial surfaces, which result in disruption or inhibition of microbial cells. Based on their secondary structure, AMPs can be grouped into the following types: α–helical, β–sheets, β–loop and random coil. α–Helical structure is a form of a right hand-spiral protein conformation. To form this shape, each N-H backbone group in the protein donates a hydrogen bond to the C=O backbone group of the protein. The distance between the bonds is three to four residues along the protein sequence. As in the α–helical, β–sheets assembly consists of two or three hydrogen bonds which connect two parallel β–strands molecules. In the random coil structure, a group of protein strands is bonded randomly with an indefinite sequence.

Mode of Action of Antimicrobial Peptides

Bacteria Cell Wall Interaction

Unlike the higher living organisms, bacterial membrane is often surrounded by a tight cell wall composed of lipopolysaccharides (LPS), phospholipids and peptidoglycans (PGN). The presence of an outer membrane in gram-negative bacteria is a typical characteristic that does not exist in gram-positive bacteria. Both bacterial types have periplasmic space or periplasm. In gram-positive bacteria periplasm is located between the cell wall and the cytoplasmic membrane. Periplasmic space in Gram-negative bacteria is situated between the plasma membrane and the outer membrane.

The cell wall of both bacterial types contains PGN layers. The gram-positive bacteria cell wall is made of several PGN layers with a thickness of about 40-80 nm. In Gram-negative bacteria, the PGN layer is 7-8 nm thick and is locked in the periplasmic space (1). The outer membrane of Gram-negative bacteria is composed of a lipid bilayer with phospholipid inner leaflet and lipopolysaccharide outer leaflet (2–4).

AMPs can penetrate the gram-negative bacterial cell wall by a charge-exchange mechanism. During this process, the cationic peptides compete with Ca^{2+} and Mg^{2+} and bind to the lipopolysaccharide (LPS) layer of the outer membrane. Later, these peptides penetrate the bacterial cells by an interaction with the cytoplasmic membrane phospholipids. Due to their hydrophobic domains, peptides translocated across the bacterial membrane, entering into the cell and binding to DNA and other cell membranes (5).

AMPs interaction with gram-positive bacterial cell wall is not well understood. AMPs can disrupt the synthesis of PGN by interfering the enzymatic processes of the synthesis. AMPs

also target the negatively charged cell wall components such as teichoic acid and lipoteichoic acid. The semisynthetic lipoglycopeptide oritavancin can be used as a model interaction of AMPs against the gram-positive bacterial cell wall. It displays the effect of inhibition of PGN synthesis (1).

Fungal Cell Wall Interaction

The fungal cell wall is a layered structure, with the inner layers performing a predominantly mechanical function, while the outer layers are associated with the physiological features of particular fungal species. In most species, the inner cell wall is composed of covalently attached branched β-(1, 3) glucan with chitin. This branched glucan is tied to proteins and polysaccharides which compose outer cell wall, and their conformation varies with the fungal species (6).

AMPs interaction with the fungal cell wall varies depending on the AMPs type. Some AMPs disrupt the function and structure of the cell wall. Defensin NP-1 binds tightly to the chitin layer, which can lead to selective plasma membrane lysis. Nikkomycin can block chitin biosynthesis. Echinocandin and its synthetic analogs can inhibit synthesis of branched β-(1,3) glucan (7).

Phospholipid Membrane Interaction

Antimicrobial peptides undergo conformational changes when they interact with a target phospholipid membrane. In water the AMPs structure is hydrophilic. However, their conformation has to be changed because they must expose a hydrophobic area to the lipidic constituent of the membrane. The conformation change happens when a monomeric

peptide adopts a random structure in solution but gains an amphipathic structure when it is reaching the membrane. The other scenario is when peptides form an oligomer, they exposed their hydrophilic regions to the solution and bury their hydrophobic regions in a formed lumen. Upon reaching the membrane surface, the oligomer reverses its organization such that the hydrophobic part is exposed to a lipid constituent of the cell membrane (8). Antimicrobial peptides act in different ways when they interact with the membranes, but the main action mode of AMPs is the permeabilization of phospholipid membranes. This process is described through several models: toroidal-pore model, carpet model, and barrel-stave model.

Toroidal-Pore Model

In this model, the lipid monolayer is bent continuously by attached alpha helix AMPs. To forming a pore, the hydrophobic surface of the peptide interacts with lipid head groups of the membrane (9). In the formed toroidal pore, the polar faces of the peptide are associated with the polar head groups of the lipid constituents (10). Lipids constituting the pore tilt from the normal lamellar structure, and connect the two leaflets of the membrane, creating an uninterrupted bend from top to bottom in a mode of the toroidal hole (11).

Carpet Model

In this model, peptides are accumulated on the phospholipid membrane by strong electrostatic forces. The anionic phospholipid head groups of the plasma membrane attract the cationic antimicrobial peptides, which are oriented parallel to the membrane surface. Peptides are attached to the anionic phospholipid head groups at the multiple sites covering the membrane surface in carpet-like mode. At high peptide concentration, surface-attached peptides are capable of disrupting the membrane bilayer (11).

Barrel-Stave Model

In this model alpha-helix peptides are linked together in an oligomer molecule which forms transmembrane pores. The hydrophilic surfaces of the peptides are oriented to the lumen of the pore; the hydrophobic surface is oriented to the lipid layer of the cell membrane. The transmembrane pore formation process starts with binding of peptide monomers to the phospholipid membrane surface. Subsequently, the monomers are inserted into the lipid core of the membrane. The peptide monomer concentration is critical for the pore size formation. The high accumulation of the monomers increases the pore size and consequently leads to cytoplasmic cell content release (12).

Intracellular Targets Interaction

In addition to the ability to interact with cell walls and the cell membranes, there are more indications for specific interactions of the AMPs with intracellular targets. The most common effect of AMPs is the inhibition of both cell wall and protein synthesis due to DNA and RNA binding. By translocating to the cytoplasm, AMPs can alter the cytoplasmic membrane formation by inhibition of main metabolic processes such as cell wall formation, enzyme activity, nucleic acid and protein synthesis (13).

History of Antimicrobial Peptides

In 1939 an antimicrobial peptide named gramicidins was isolated from the prokaryotic cells of *Bacillus brevis* (14). Gramicidin, a heterogeneous mixture of six antibiotic peptides, was successfully used to treat infected wounds on the guinea-pig skin which proved their potential for clinical use (15). The gramicidin was the first commercially produced AMPs (16).

Eukaryotic organisms also produce AMPs. Previous research in plants showed the existence of four different groups of AMPs which play a significant role in the plant defense system (17). These groups include plant defensins, lipid transfer peptides, thionins, and chitin-binding peptides (17).

The increase of multidrug-resistance in microbial pathogens in the early 1960s raised the interest in host defense molecules (18, 19). Studies with human neutrophils during that period proved that the oxygen-independent mechanisms which are not part of the adaptive immune system are responsible for the elimination of pathogenic bacteria. The mechanisms are based on cationic proteins contained in the neutrophil cells (20, 21).

The isolation of bombinin AMP in 1962 from the orange speckled frog *Bombina variegata*, is the first reported animal antimicrobial peptide (22). At the same time during the 1960s the antimicrobial peptide, lactoferrin was isolated from milk (23). Several research papers from the late 1970s and 1980s reported isolation of AMPs from rabbits' and humans' leukocytes; these groups of antimicrobial peptides are well known nowadays as α–defensins (24–27).

In an experiment carried out in 1981 by Boman *et al.* pupae of the moth *Hyalophora cecropia* were injected with bacteria. Later, inducible cationic antimicrobial peptides

named P9A and P9B were isolated from the hemolymph of the pupae. The proteins were sequenced, characterized, and renamed as cecropins. The cecropins were the first reported α–helical AMPs (28).

In the late 1980s, β–defensins and θ-defensins were isolated from bovine granulocytes (29). The first anionic AMPs was isolated and identified from the frog species *Xenopus laevis* in the mid-1990s (30). During the same period, more evidence had proved the view that lysozyme activities are non-enzymatic with a similar mode of action to that of antimicrobial peptides (31). These findings convinced some authors to believe that AMPs are the main defense factor of organisms lacking an adaptive immune system (32). These theories were confirmed in an experiment with the fruit fly, *Drosophila melanogaster*, during the mid-1990s. After the deletion of the genes responsible for encoding AMPs, the fly became susceptible to fungal infections (33). The extensive study of the AMPs encompasses not only plants and insects but also the invertebrate organisms which do not possess an adaptive immune system (34–37). Most of the current findings and understanding for the AMPs function and structure are based on experiments conducted with amphibian skin secretions, which are a rich source of AMPs (38–41). Research work proved that AMPs are typical for all multicellular organisms (42), and these peptides have an essential role in the mammalian immune system (43–46).

Antimicrobial Peptides as Inhibitors of Plant Pathogenic Microorganisms in Transgenic Plants

Diseases caused by microbial pathogen constitute a significant problem in crop production in the United States and worldwide. Pesticide application is a common practice to combat crop diseases. In the United States, over one billion pounds of agrochemicals are used annually for pest control, and the worldwide usage is approximately 5.6 billion pounds per year (47). The systemic use of pesticides harms the environment and increases the risk of pesticide resistance incidents. Therefore, alternative means to combating crop diseases are being tested and employed.

Genetically engineering techniques have shown excellent results in plant-pathogen inhibition in recent decades. Successfully created commercial lines of genetically modified (GM) crops which are resistant to a broad range of pathogens have proven the effectiveness of this alternative approach. For example, experiments with model plants contained gene construct with AMPs coding sequences have been carried out by some authors. Results have shown the great capability of the AMPs genes constructs for protection against diseases caused by plant pathogens (48–53).

In a carried study, human lactoferrin cDNA has been used for tobacco plants *Nicotiana tabacum* transformation (48). The integration of cDNA in the plant genome was analyzed by Southern, Northern, and Western blots. After inoculation with the bacterial pathogen *Ralstonia solanacearum*, most transgenic plants showed substantial delays of bacterial wilt symptoms. The positive relationship between the expressed lactoferrin gene and disease resistance was proved by enzyme-linked immunosorbent assay.

Further examples include investigations of the expression of the lactoferrin (LF) gene in transgenic tomato plants (49). The plants were tested for resistance to the pathogen *Ralstonia solanacearum* Smith that causes bacterial wilt in tomato. Susceptible tomato line F7926-96 was transformed by *Agrobacterium transformation* with modified LF cDNA and gene introgression confirmed by Southern blot. Additionally, lactoferrin expression was detected by northern and western blots. In T1 and T2 generations, kanamycin resistance was observed based on Mendelian segregation verifying a single locus insertion. Furthermore, Southern blot analysis showed a single corresponding band of LF cDNA in T1 and T2 kanamycin resistant plants. The two transgenic tomato lines inoculated with the pathogen ($1x10^7$ and $1x10^8$ colony-forming units (CFU)) exhibited an early resistance and subsequent susceptibility. The survival rate until maturity was observed at 44% to 55% of the plants when they are inoculated with $1x10^5$ CFU. These results demonstrated the potential of the lactoferrin AMP gene for control of bacterial wilt of tomato.

Bovine lactoferrin cDNA construct was used for transformation of wheat plants, and the transformants were inoculated with head blight disease fungus *Fusarium graminearum* (54). Significant reduction of disease incidence was observed in the transgenic plants. Highly susceptible cultivar Bobwhite expressed high resistance compared to control Bobwhite plants and two untransformed commercial wheat cultivars (Wheaton and ND 2710). The positive correlation between lactoferrin gene expression and the levels of disease resistance were proved by an ELISA experiment.

Two tomato proteins Snakin-2 (SN2) and extensin-like protein (ELP) were over-expressed in transgenic tomato plants to test their ability to induce resistance to *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) (51). The tomato plants, cultivar Mountain

Fresh, were transformed via an *Agrobacterium tumefaciens* transformation. Tomato transgenic lines that accumulated a high level of SN2 or ELP mRNA showed significant tolerance to Cmm by a delay of the wilt symptoms and a reduction in the size of canker lesions compared to control. In the transgenic lines over-expressing SN2 or ELP, bacterial populations were significantly lower (100-10,000-fold) than in control plants. The results suggested a potential biotechnological application of these two defense proteins.

Substantial advances have been made to develop efficient methods for synthesis of new antimicrobial peptides and their derivatives. These designed peptide modifications can be assayed for the desired antimicrobial activity. By molecular engineering techniques, their properties can be improved regarding the pathogen specificity, the toxic side effect and the range of action.

The novel AMPs originate from naturally occurring peptides via synthetic modifications. It is well known that the most natural AMPs have nonspecific toxicity against plant or animal cells at certain concentration levels. This adverse effect can be avoided by modulation and redesigned of the natural AMPs. Membrane-active AMPs cecropins, magainins, and melittin have toxic properties, which make their practical use very difficult (55). Cecropins are the first class of AMPs in which activity ratio against microbes versus toxicity to plant cells, was modulated. The primary sequences of cecropin B analogs: SB-37 and Shiva-1 were engineered in a way that reduced their toxicity to plant protoplasts (52, 56).

Transgenic plants with broad-spectrum resistance to bacterial and fungal phytopathogens were created by combining two or more AMPs genes. In one study, a synthetic gene coding cecropin-melittin cationic hybrids chimera (MsrA1) is expressed in two potato cultivars

Desiree and Russet Burbank (53). The potato cultivars demonstrated very high resistance to bacterial and fungal phytopathogens.

Expression of multiple genes in a single open reading frame (ORF) is an alternative approach for combining desirable AMPs in a single polyprotein. This method is exploited from several animal Picornavirus and plant Potyviruses. Their polyproteins are processed co-translationally and post-translationally to mature forms by virus-encoded proteases (57). In an experiment, the Foot-and-mouth disease virus (FMDV) 2A protein, which is responsible for the 'cleavage' at its carboxyl-terminus, is used as cleavage of artificial polyproteins (58). Multiple copies of chloramphenicol acetyltransferase (CAT) and β−glucuronidase (GUS) genes were fused in a single open reading frame (ORF) with a copy of the 2A protein gene between them. The ORF construct was expressed in transgenic tobacco plants. Consistent detection in the transgenic tobacco of CAT and GUS proteins with efficiency ranged from 80% to 100% proved that FMDV 2A protease functioned properly in plant cells. This work proved that creating a single ORF gene construct with merged multiple AMPs genes can be used in transgenic plants for efficient protection against various plant pathogens.

In an effort to achieve stronger resistance than our previous work with lactoferrin and lactoferricin, we fused the lactoferricin domain of lactoferrin with multiple active domains of other AMPs using FMDV 2A protease. Transgenic tobacco and tomato plants were generated from these constructs and grown several generations to obtain homozygous lines. In this work four selected lines were tested for resistance against three economically important fungal pathogens.

MATERIALS AND METHODS

Multi-domain lytic peptide constructs and transgenic lines

Previous work in our laboratory generated several multi-domain constructs by fusing two synthetic or naturally occurring AMPs with lactoferricin. A large number of transgenic tobacco and tomato lines were obtained expressing these constructs. Seeds from four transgenic tobacco lines were used in this work to obtain plants and to test them for fungal disease resistance.

Preparation of transgenic and control *Nicotiana benthamiana* plants

Seeds sterilization

In this study, six *N. benthamiana* lines have been used: four transgenic lines containing lytic peptide genes (ORF13, RSA1, CN77, ORF12) along with one empty vector line containing a viral gene construct (1234) in the same binary construct, and one wild type control non-transgenic (WT) line. Seeds were sterilized by the following protocol: First seeds were placed in 2 ml Eppendorf tubes. One milliliter of 10 % solution of commercial bleach (NaOCl) was added to each tube. The seeds were immersed for five minutes. After removal of bleach from the tubes, the seeds were washed with distilled water three times. Washed seeds were immersed in 70% Ethanol for 30 seconds followed by three washes with distilled water. The distilled water was removed carefully from the Eppendorf tubes with a pipette.

Seeds Germination on Murashige-Skoog Solid Plant Media

The seeds were placed in magenta boxes on 50 ml Murashige-Skoog (MS) solid plant media (Murashige-Skoog Plant Media: MS Salt 4.3 g, B1 — Inositol (1 g Inositol, 10 mg Thiamin HCl, 100 ml ddH_2O) 10 ml, Millers I (6g KH_2PO_4, 100ml ddH_2O) 3 ml, Sucrose 30 g and Phytagar 8 g per Liter having pH 5.8) under aseptic condition in a Laminar Air Flow chamber. After placement on to the MS media, the seeds were streaked gently with a sterile glass loop. Antibiotic Kanamycin was added (100 mg per liter) to the MS media for transgenic plant seeds ORF13, RSA1, CN77, ORF12 and empty vector control 1234. No antibiotics were added in MS media for the wild type control (WT) plant seeds.

Magenta boxes were placed in a plant growth chamber at 26 °C. The first germination was detected ten days after placing the seeds on MS media. The plants were kept in the magenta boxes for forty-five days after germination.

Transfer of the seedlings from magenta boxes to pots

After forty-five days of growth in magenta boxes, the plants were transplanted in 500 cm^3 pots filled with soil. Each pot was filled with approximately 400 cm^3 steam-dissinfested potting soil (The potting mix was prepared in the UNL Plant Pathology Greenhouse staff). A single plant was placed in each pot. Ten plants were prepared for each transgenic line and the wild type control. The pots were watered gently after planting. All plants were placed in a room with a steady temperature of 23 °C, ~30% relative humidity, and artificial lighting with a day/night ratio of 14h/10h.

Neomycin phosphotransferase II Enzyme-linked-Immunosorbent Assay (NPTII ELISA)

Neomycin phosphotransferase II (NPTII) is a selectable marker gene used in the transgenic lines ORF13, RSA1, CN77, ORF12, and empty vector control 1234. Seven days after the seedlings transfer to the pots, the presence of the NPTII protein product was qualitatively evaluated by an ELISA assay using an NPTII (Neomycin phosphotransferase II) ELISA kit from Agdia, Inc (52642 Co Rd 1, Elkhart, IN 46514). Leaf biomass from each plant was planced in an Eppendorf tube. The biomass was ground in the tubes with the presence of 1x PEB1 extraction buffer in a 1:10 ratio (tissue weight in grams: buffer volume in milliliters).

One hundred μl of prepared samples were dispensed into the sample wells. The negative control well was filled with 100 μl of 1x Phosphate Buffered Saline (PBS). The sample wells were incubated overnight (16 hours) in the humid box at temperature 4 °C). After incubation, wells were washed seven times with 1x PBS buffer. 100 μl enzyme conjugate (mixed one-part MRS-2 component to four-part 1x PBS buffer and enzyme conjugate bottles A and B with dilutions 1:100 for each bottle) was dispensed in the cleaned wells. The wells were incubated for two hours in the humid box at room temperature, ~ 23 °C. After the incubation, cells were washed eight times with 1x PBS buffer. 100 μl of TNB substrate solution was dispensed in the washed wells. The wells were incubated for fifteen minutes in the humid box at room temperature. The results were evaluated after the incubation.

DNA extraction from plants

The DNA from the transgenic plant lines ORF13, RSA1, CN77, ORF12, 1234 empty vector control, and WT control was extracted by use of Thermo Fisher Plant DNAzol Reagent. For this procedure, the protocol provided by the manufacturer was used. The samples were ground using a mortar and pestle. After the pulverization, tissue was moved into Eppendorf tubes by using a sterile spatula. The Eppendorf tubes contained tissues were weighed on a scale as the scale was tared by the weight of an empty tube. 300 µl plant DNAzol was added in each Eppendorf tube per 100 mg plant tissue, mixed thoroughly and incubated for five minutes at 25 °C by shaking. After incubation, 300 µl of chloroform was added to each tube and mixed by vortex for approximately 30 seconds, then incubated for five minutes at 25 °C with shaking, followed by centrifugation at 12000 RPM at 4 °C for 10 minutes. The supernatant was transferred into a fresh Eppendorf tube and mixed with 225 µl of 100 % ethanol for DNA precipitation. The samples were inverted eight times and then incubated at room temperature for five minutes. Subsequently, the tubes were centrifuged at 5000 RPM for four minutes, and the resulted supernatant was discarded and the DNA pellet retained. The DNA pellets were washed with 300 µl Plant DNAzol-ethanol wash (mix of 1 volume DNAzol with 0.75 volume 100% ethanol) and the tubes were vortexed and incubated for five minutes, then centrifuged at 5000 RPM for 4 minutes and the supernatant was removed. The same washing step was repeated two more times. Following the washing steps, 300 µl of 75 % ethanol was added to the tubes, vortexed and then centrifuged at 5000 RPM for 4 minutes. The supernatant was removed, and the tubes were stored vertically for five minutes to remove the remaining ethanol. If some ethanol still existed in the tubes, it was removed with a micro-pipette. Finally, 70 µl TE (Tris —

EDTA) buffer was added in the tubes for dissolving DNA. The tubes were stored at -80 °C for further analysis.

DNA quality check

The quality of extracted DNA was checked by 0.8% agarose gel electrophoresis.

Neomycin phosphotransferase II - Polymerase Chain Reaction (*NPTII* - PCR)

Polymerase Chain Reaction (PCR) assay was performed for the transgenic plant lines ORF13, RSA1, CN77, ORF12, 1234 empty vector control, and WT control, targeting Neomycin phosphotransferase II (*NPTII*) selectable marker gene. GoTaq® Green Master Mix (bacterial derived Taq DNA polymerase, dNTPs, $MgCl_2$, and reaction buffers) was used following manufacturer's instructions. 2 µl DNA of each transgenic plant sample was added to18 µl reaction mixture containing 6 µl ddH_2O, 1 µl Forward Primer, 1 µl Reverse Primer along with 10 µl GoTaq® Green Master Mix. The NPTII primers had the following sequence: Forward Primer; 221 5′-GAGGCTATTCGGCTATGAC-3′, Reverse Primer 921 5′-ATCGGGAGCGGCGATACCG-3′ (The primers were designed by Mitra Lab). The thermal cycle was set to: 1) 94 ºC for 3 min., 2) 94 ºC for 30 sec., 3) 55 ºC for 40 sec., 4) 72 ºC for 1 min. 40 sec., 5) 72 ºC for seven min., 6) 4 ºC forever, and steps two, three and four were repeated 35 times. The size of the amplified fragment of the NPTII tn5 gene was 700 bp.

Lytic peptide gene - Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) assay was performed for the transgenic plant lines ORF13, RSA1, CN77, ORF12, targeting lytic peptide gene. GoTaq® Green Master Mix solution which contains bacterial derived Taq DNA polymerase, dNTPs, $MgCl_2$, and reaction buffers has been used for this assay. Sample were prepared by the protocol

provided by the GoTaq® Green Master Mix manufacture. Each sample had a final volume of 20 µl: 2 µl template DNA, 6 µl ddH_2O, 1 µl Forward Primer, 1 µl Reverse Primer, and 10 µl GoTaq® Green Master Mix. The thermal cycle was set to: 1) 94ºC for 3 min., 2) 94ºC for 30 sec., 3) 55ºC for 40 sec., 4) 72ºC for 1min. 40 sec., 5) 72ºC for 7 min., 6) 4ºC forever, and steps 2, 3 and 4 were repeated 35 times altogether during the cycle.

Primer's sequences:

Primer	Sequence
ORF13	F 5′ GGC TGT TCT TTG GGC TCT TA 3′ R 5′ CCC CTA ACG GAA AAC ACA GC 3′
RSA1	F 5′ GGA AGG GGA TTT TCT GCT GC 3′ R 5′ GCA GCC TCT CTT TCT TCA GC 3′
CN77	F 5′ GCT CTT TAC AGG GGA TGC CT 3′ R 5′ CCC TCA CAC CAT CCA CGA TT 3′
ORF12	F 5′ GCT ACT GGA ACT TCT GCT GC 3′ R 5′ TCA AGG CTG ATT ACC TCC CC 3′

Table 1. Primer sequences used to amplify Lytic peptide genes. The primers were designed by Mitra Lab.

Agarose gel electrophoresis

The amplified DNA product was analyzed with 1% agarose gel (100 mg Agarose to 100 ml 1x Tris-Borate-EDTA buffer, and 0.6 µl Ethidium Bromide solution 10 mg/ml) electrophoresis for 100 minutes.

Detached-leaf bioassay

Detached-leaf bioassay was performed to screen the transgenic plants ORF13, RSA1, CN77, ORF12 carrying the lytic peptide genes, empty vector control plants 1234, and wild type control (WT) plants against three fungal pathogens: S*clerotinia sclerotiorum*, *Rhizoctonia solani*, *Pythium* sp.

Rhizoctonia solani is a soil borne plant pathogenic basidiomycete fungus. It has wide range hosts. *Rhizoctonia solani* attacking primarily the roots and lower stems of plants and cause serious plant losses especially during seedling stage.

S*clerotinia sclerotiorum* is a plant pathogenic ascomycete fungus. It causes diseases known as white mold, cottony rot, soft rot or stem rot. S*clerotinia sclerotiorum* has a wide host range in different life-stages. The disease can spread very quickly from plant to plant in the field. It can affect crops in the storage facilities after harvest. Common crops which are affected and suffer deleterious economic impacts are soybeans, cannola and peanuts.

Pythium species are fungus-like eukaryotic microorganisms belonging to the parasitic oomycetes. Formally they were classified as fungi. *Pythium* cause diseases with economic importance such as root rots.

Each transgenic variety contained ten plants. For this experiment, two detached leaves per plant were used. Each bioassay had three replications per plant from each line. Sixty experiments for each plant lines per pathogen have been performed. The pathogens were grown in a petri dish on a solid Potato Dextrose Agar media (PDA) (39 g PDA powder to 1liter ddH_2O autoclaved on 120 ^oC for 20 minutes). The PDA powder was procured from Sigma-Aldrich company. S*clerotinia sclerotiorum* and *Rhizoctonia solani* were grown for

four days on the media before the experiment. *Pythium* sp. was grown for two days on the media before the experiment. By using a sterile glass tube, 8 mm plugs were cut in the media after the growth of the pathogen for use as inoculum, see below. The detached leaves were placed in a plastic container with high humidity. Containers size is: 40 cm L x 31.8 cm W x 6 cm H. The inoculation was performed by use of sterile tweezers. Plugs were transferred to the leaves with the pathogen on the upper surface of the plug. The plugs' position on the leaves were approximately 15 mm down from the tip of each leaf. After the inoculation, containers were sealed with a transparent plastic wrap. Sealed containers were moved in a temperature-controlled room with an artificial light with a day/night ratio 14h/10h. The experiments with *Sclerotinia sclerotiorum* and *Rhizoctonia solani* were incubated for three days after inoculation. The *Pythium* sp. experiment was incubated for two days after inoculation.

Data collection

After incubation, leaves were photographed on a scaled photographic field. Each photo was processed with a graphics editor by marking the infected area of each leaf with a single color and the background color was changed to white. Compu Eye software was used (59) to measure the symptom area of each leaf quantitatively with high accuracy. The result of measuring the infected area as well as the total area was recorded in square millimeters.

RESULTS

Seeds Germination on Murashige-Skoog Solid Plant Media

The wild type control *Nicotiana benthamiana* seeds were germinated in magenta boxes with 50 ml solid Murashige-Skoog (MS) plant media. The transgenic plant seeds from lines ORF13, RSA1, CN77, ORF12, and transgenic vector control were germinated on modified MS media with Kanamycin antibiotic. The first germination was observed ten days after the sowing. The plants were kept in the magenta boxes for forty-five days after germination. During that period the plants were in good health and had an excellent growth rate with no signs of discoloration or fading (Fig. 1). The transgenic lytic peptide lines and the transgenic control line grew on Kanamycin fortified MS solid media confirming that the plants carried the *Neomycin phosphotransferase II* marker gene and linked lytic peptide genes (Fig. 1).

Figure 1. Aseptically grown ORF12 transgenic tobacco lines in magenta boxes. Plants show normal growth and development.

Neomycin phosphotransferase II Enzyme-linked-Immunosorbent Assay (NPTII ELISA)

The enzyme activity of NPTII selectable marker gene was evaluated qualitatively with an Enzyme-Linked-Immunosorbent Assay for the transgenic lines ORF13, RSA1, CN77, ORF12, transgenic vector control and wild type (WT) control plants. This test was performed using Agdia NPTII (Neomycin phosphotransferase II) ELISA kit. All transgenic lines: ORF13, RSA1, CN77, ORF12, vector control (1234), showed positive results for the existence of *Neomycin phosphotransferase II* marker gene in the plant's genome. According to the protocol, the appearance of blue color in the test wells is an indication for a positive result. Blue color is seen in the test wells of the transgenic plants' lines (Fig. 2 and Fig. 3). No color was present in the test wells for the wild type control plants and the negative control (Fig. 2 and Fig. 3).

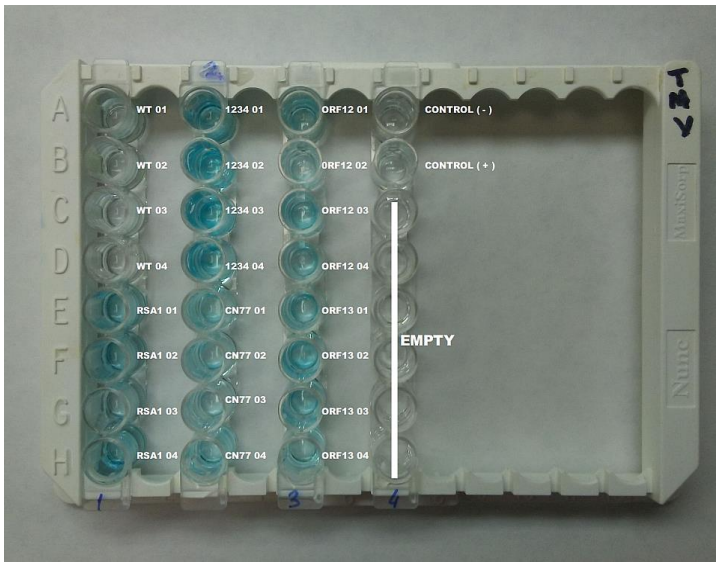

Figure 2. NPTII ELISA assay of transgenic and control plants. The blue colored wells indicate positive result. Marks on the photo correspond with the tested plant varieties.

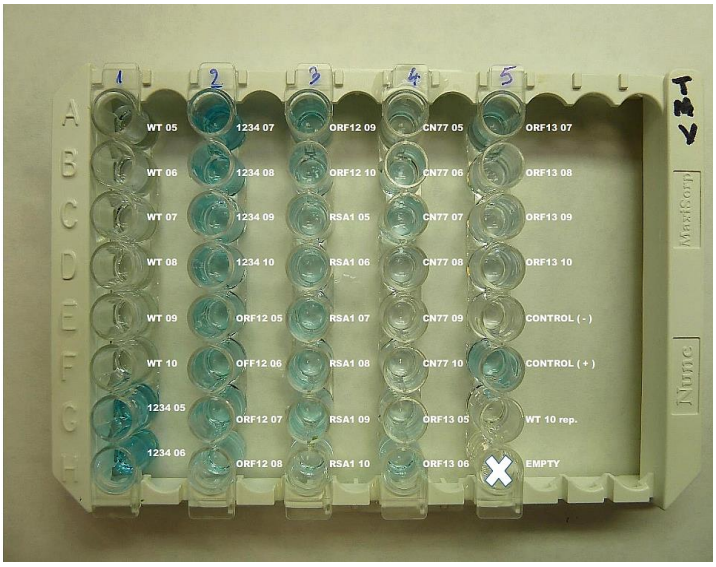

Figure 3. NPTII ELISA assay of transgenic and control plants. The blue colored wells indicate positive result. Marks on the photo, correspond with the tested plant varieties.

Plant DNA extraction

DNA was extracted from the transgenic plant lines ORF13, RSA1, CN77, ORF12, vector control (1234) and WT control. The quality of extracted DNA was tested on 0.75% agarose gel (750 mg Agarose to 100 ml x1 Tris-Borate-EDTA buffer and 0.6µl of Ethidium Bromide solution 10 mg/ml) electrophoresis was for 80 minutes. The gel showed that the quality of the DNA was adequate (Fig. 4 to Fig. 8).

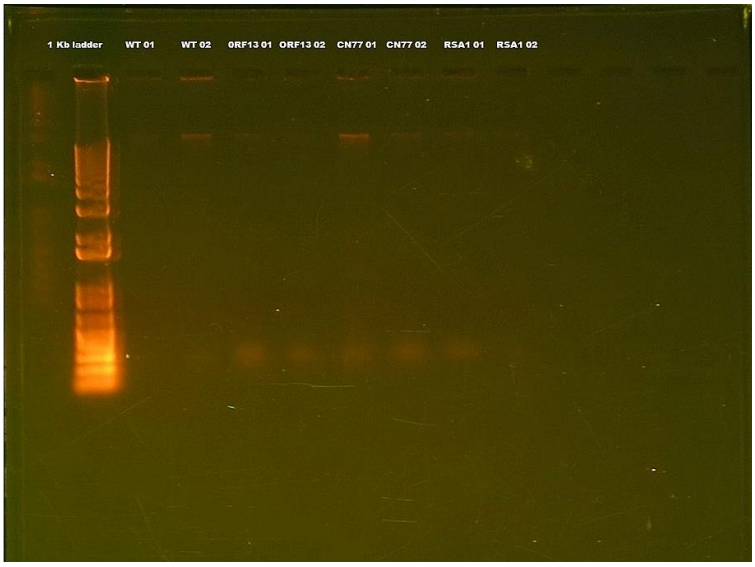

Figure 4. Extracted DNA was evaluated using agarose gel electrophoresis. The photo shows an adequate quality of the DNA. Lanes indicate the molecular weight marker (1 Kb ladder) and genomic DNA samples from respective plants.

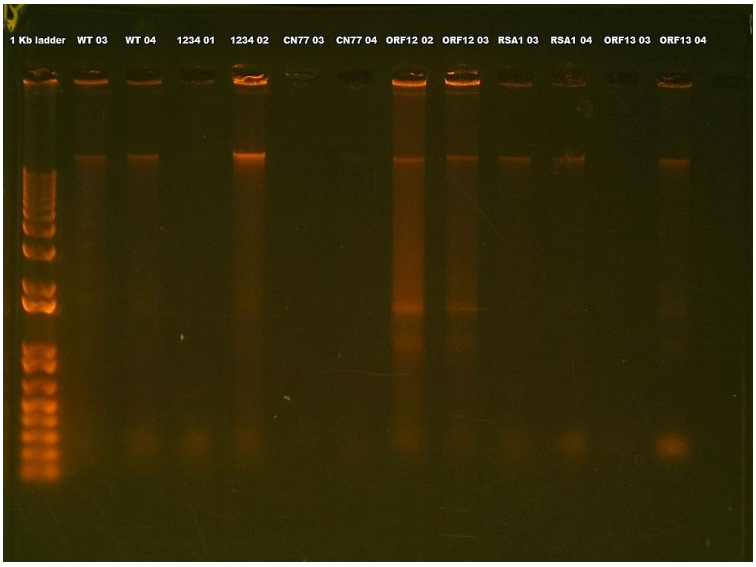

Figure 5. Extracted DNA was evaluated using agarose gel electrophoresis. The photo shows an adequate quality of the DNA. Lanes indicate the molecular weight marker (1 Kb ladder) and genomic DNA samples from respective plants.

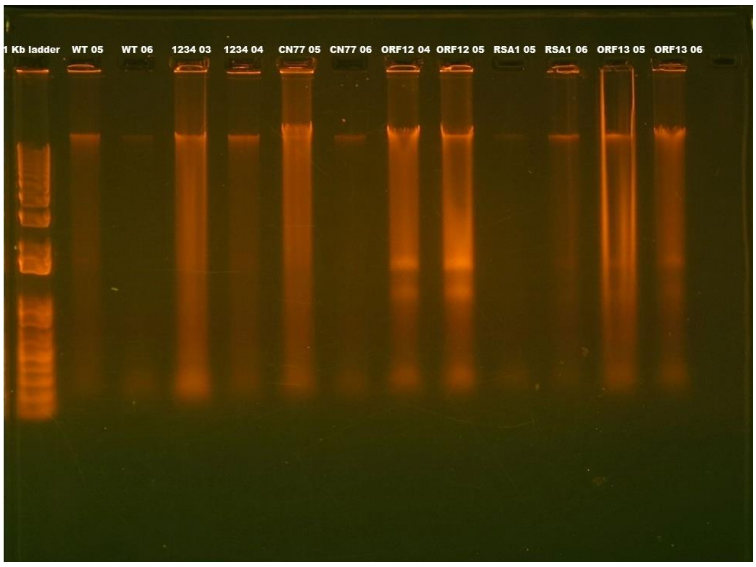

Figure 6. Extracted DNA was evaluated using agarose gel electrophoresis. The photo shows an adequate quality of the DNA. Lanes indicate the molecular weight marker (1 Kb ladder) and genomic DNA samples from respective plants.

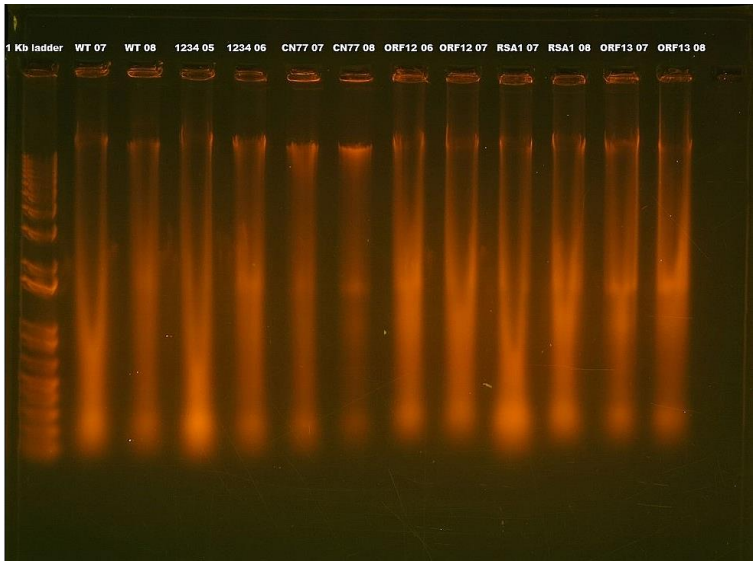

Figure 7. Extracted DNA was evaluated using agarose gel electrophoresis. The photo shows an adequate quality of the DNA. Lanes indicate the molecular weight marker (1 Kb ladder) and genomic DNA samples from respective plants.

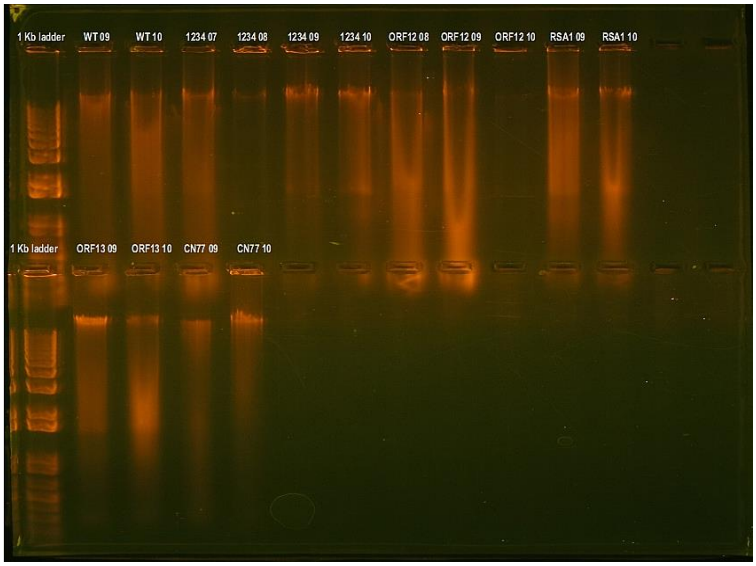

Figure 8. Extracted DNA was evaluated using agarose gel electrophoresis. The photo shows an adequate quality of the DNA. Lanes indicate the molecular weight marker (1 Kb ladder) and genomic DNA samples from respective plants.

Polymerase Chain Reaction to amplify *Neomycin phosphotransferase II* selectable marker gene (NPTII – PCR)

Polymerase Chain Reaction (PCR) assay was performed for the transgenic plant lines ORF13, RSA1, CN77, ORF12 and vector control (1234) along with WT control, targeting the *Neomycin phosphotransferase II* (*NPTII*) selectable marker gene. All samples of the transgenic plants expressed positive results for the *NPTII* marker gene. The transgenic control plants which containing vector control (1234) also showed positive results for the *NPTII* marker gene. All samples were analyzed on 1.00% agarose gel (1000 mg Agarose to 100 ml 1x Tris-Borate-EDTA buffer and 0.6 µl of Ethidium Bromide solution 10 mg/ml) electrophoresis at 100V and 50 amps was for 90 minutes. All *NPTII* positive samples showed a clear band at 700 bp region on the agarose gel (Fig. 9 to Fig.14). All samples from wild type *N. benthamiana* plants displayed negative results for the NPTII marker gene (Fig. 15 and Fig. 16).

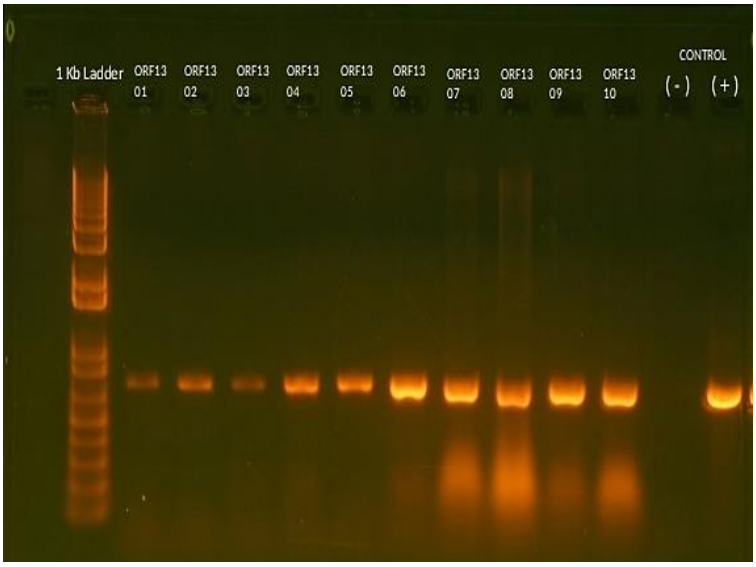

Figure 9. Agarose gel showing PCR amplification for *NPTII* gene in ORF 13 plants. The positive samples showed a clear band at 700 bp region on the agarose gel. Lanes indicate the molecular weight marker (1 Kb ladder) and genomic DNA samples from respective plants.

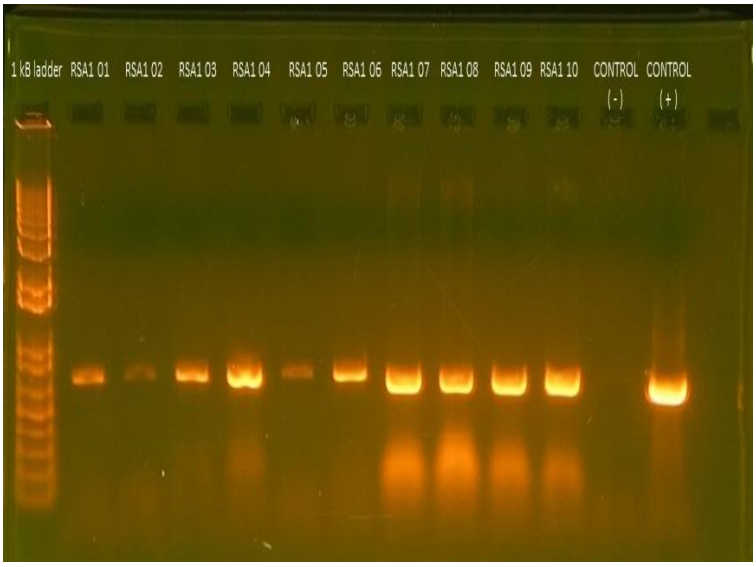

Figure 10. Agarose gel showing PCR amplification for *NPTII* gene in RSA1 plants. The positive samples showed a clear band at 700 bp region on the agarose gel. Lanes indicate the molecular weight marker (1 Kb ladder) and genomic DNA samples from respective plants.

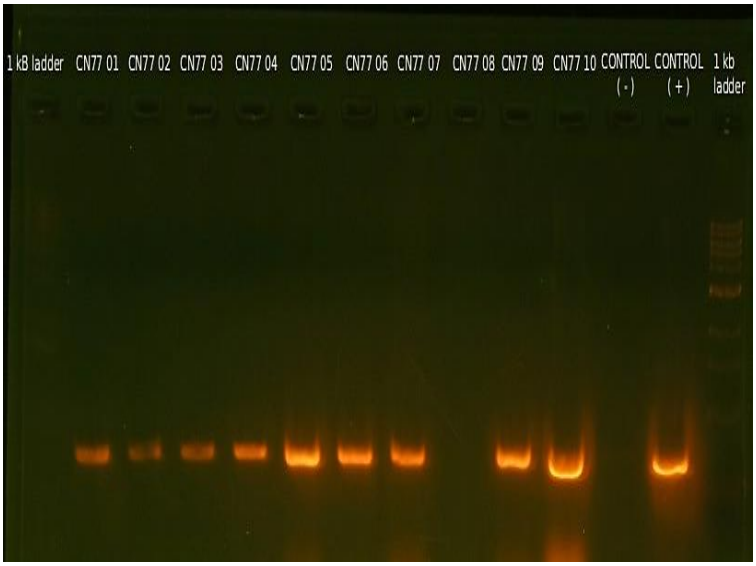

Figure 11. Agarose gel showing PCR amplification for *NPTII* gene in CN77 plants. The positive samples showed a clear band at 700 bp region on the agarose gel. Lanes indicate the molecular weight marker (1 Kb ladder) and genomic DNA samples from respective plants.

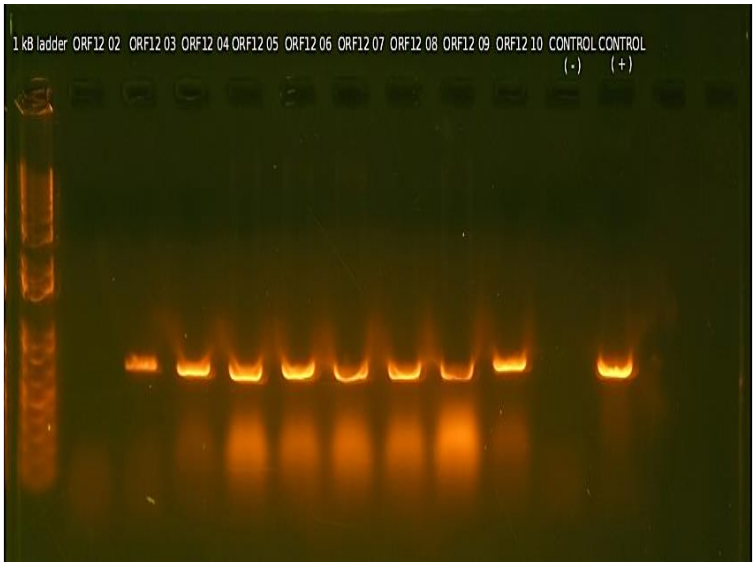

Figure 12. Agarose gel showing PCR amplification for *NPTII* gene in ORF12 plants. The positive samples showed a clear band at 700 bp region on the agarose gel. Lanes indicate the molecular weight marker (1 Kb ladder) and genomic DNA samples from respective plants.

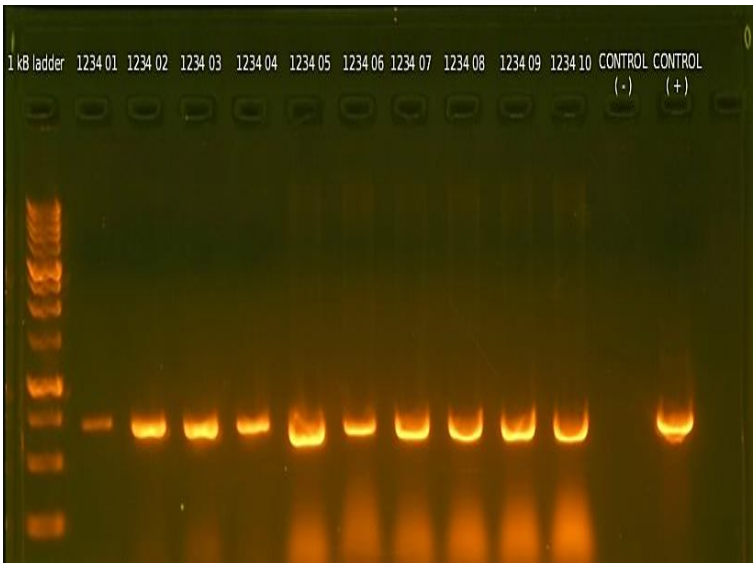

Figure 13. Agarose gel showing PCR amplification for *NPTII* gene in vector control (1234) plants. The positive samples showed a clear band at 700 bp region on the agarose gel. Lanes indicate the molecular weight marker (1 Kb ladder) and genomic DNA samples from respective plants.

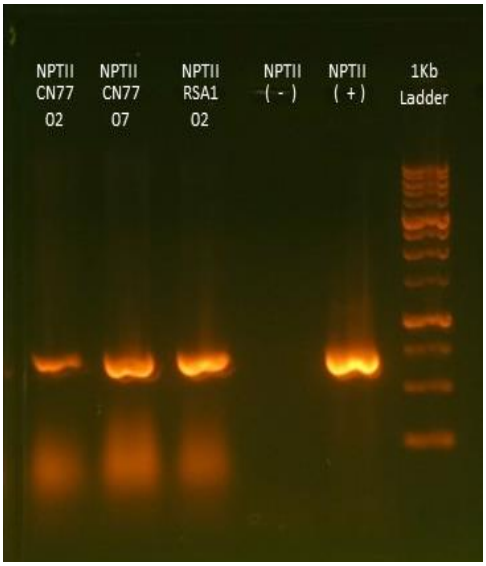

Figure 14. Agarose gel showing PCR amplification for *NPTII* gene in CN77 02 and CN77 07 and RSA1 02. The analysis of these samples was repeated due to missing bands in the previous experiments. The positive samples showed a clear band at 700 bp region on the agarose gel. Lanes indicate the molecular weight marker (1 Kb ladder) and genomic DNA samples from respective plants.

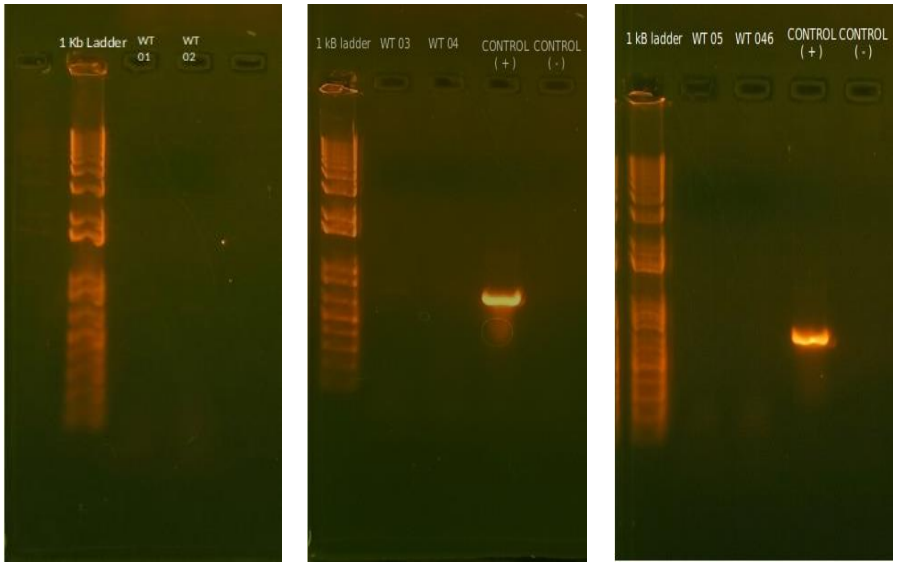

Figure 15. Agarose gel showing PCR amplification for *NPTII* gene in WT control plants. The negative samples showed no band at 700 bp region on the agarose gel. Lanes indicate the molecular weight marker (1 Kb ladder) and genomic DNA samples from respective plants.

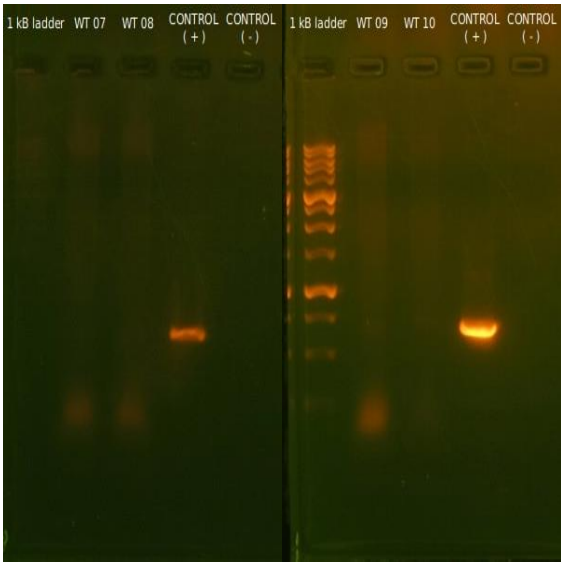

Figure 16. Agarose gel showing PCR amplification for *NPTII* gene in WT control plants. The negative samples showed no band at 700 bp region on the agarose gel. Lanes indicate the molecular weight marker (1 Kb ladder) and genomic DNA samples from respective plants.

Polymerase Chain Reaction to amplify Lytic peptide genes

Polymerase Chain Reaction (PCR) assays were employed for the transgenic plant lines ORF13, RSA1, CN77, ORF12. All four transgenic plant lines (ORF13, RSA1, CN77, ORF12) depicted positive results regarding the targeted lytic peptide genes. Table 1 shows the size of each gene.

Gene	Size
ORF13	213 bp
RSA1	740 bp
CN77	799 bp
ORF12	390 bp

Table 2. Amplified length of Lytic peptide genes.

The PCR product was affirmed with the agarose gel electrophoresis. For all four transgenic plant line (ORF13, RSA1, CN77 and ORF12) samples, clear bands have been observed according to the expected amplicon size (Table 2), (see Fig. 17 to Fig. 21).

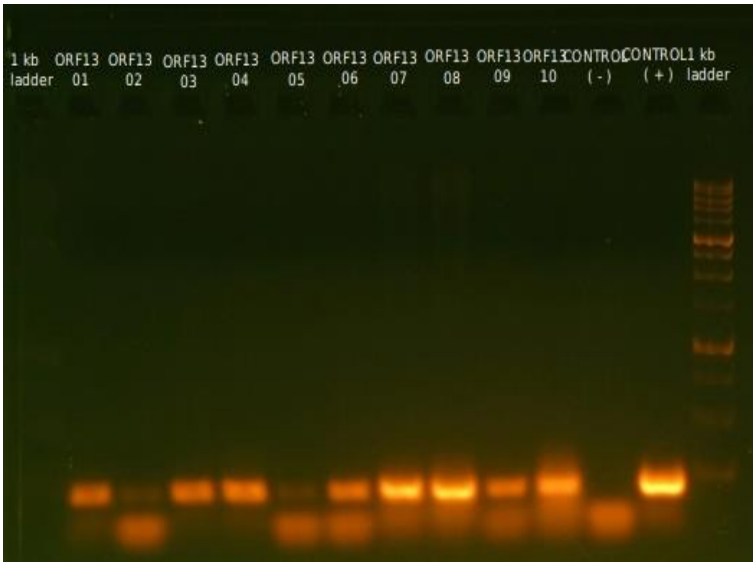

Figure 17. Agarose gel showing PCR amplification for ORF13 lytic peptide gene. The positive samples showed a clear band at 213 bp region on the agarose gel. Lanes indicate the PCR amplification from different ORF13 transgenic lines along with the molecular weight marker (1 kb ladder).

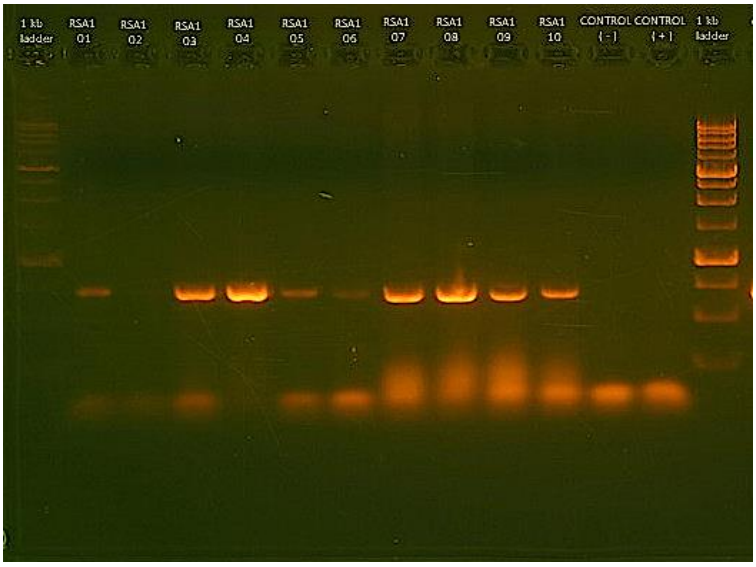

Figure 18. Agarose gel showing PCR amplification for RSA1 lytic peptide gene. The positive samples showed a clear band at 740 bp region on the agarose gel. Lanes indicate the PCR amplification from different RSA1 transgenic lines along with the molecular weight marker (1 kb ladder).

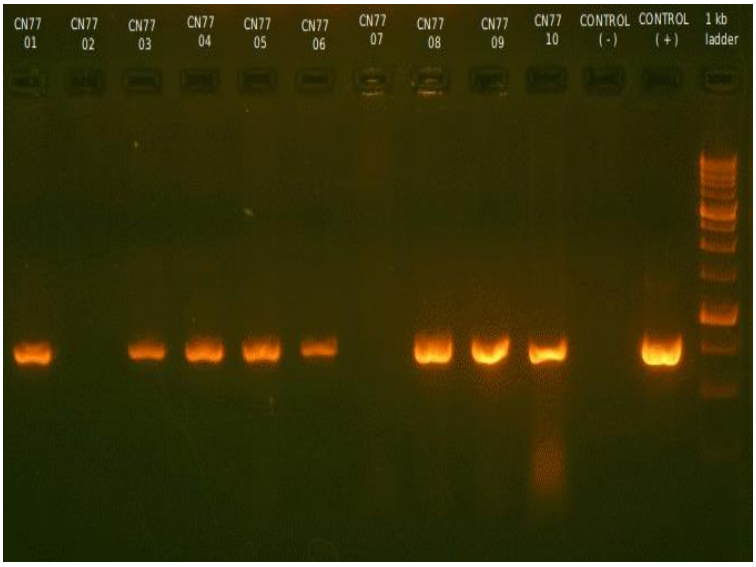

Figure 19. Agarose gel showing PCR amplification for CN77 lytic peptide gene. The positive samples showed a clear band at 799 bp region on the agarose gel. Lanes indicate the PCR amplification from different CN77 transgenic lines along with the molecular weight marker (1 kb ladder).

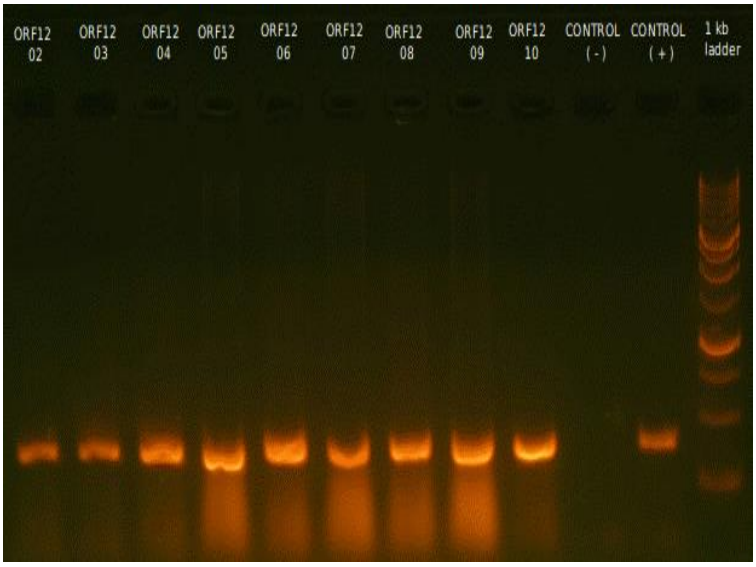

Figure 20. Agarose gel showing PCR amplification for ORF12 lytic peptide gene. The positive samples showed a clear band at 390 bp region on the agarose gel. Lanes indicate the PCR amplification from different ORF12 transgenic lines along with the molecular weight marker (1 kb ladder).

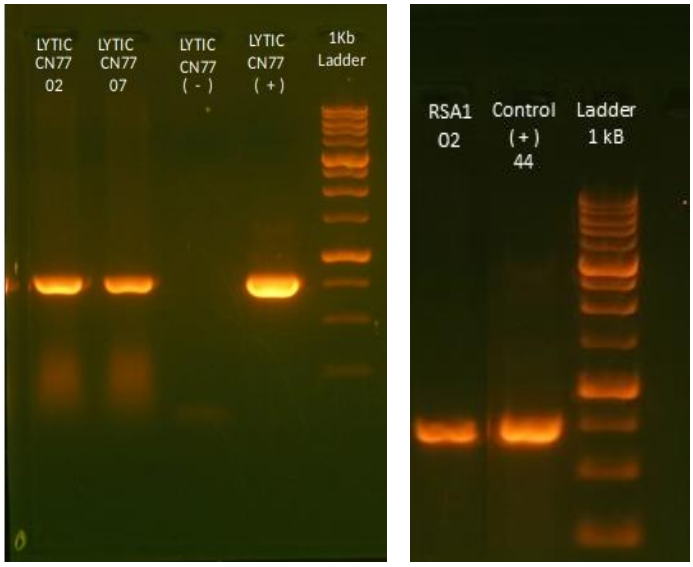

Figure 21. Agarose gel showing PCR amplification for CN77 and RSA1 lytic peptides genes. The analysis was repeated for these samples due to missing bands in previous experiments. The positive samples showed a clear band at 799 bp region for CN77 and 740 bp region for RSA1 on the agarose gel. Lanes indicate the molecular weight marker (1 Kb ladder) and genomic DNA samples from respective plants.

Detached-leaf Bioassay Experiment

Detached-leaf bioassay was performed for the transgenic plants carrying multi-domain lytic peptide constructs ORF13, RSA1, CN77, ORF12, transgenic vector control plants (1234), and wild control plants WT, against three fungal pathogens: S*clerotinia sclerotiorum, Rhizoctonia solani,* and *Pythium* sp. The infected area of each leaf was measured, each individual measurement was recorded in square millimeters (mm^2).

Experimental design

The goal of this experiment is to test four transgenic lines ORF13, RSA1, CN77, ORF12 for resistance to three fungal plant pathogens S*clerotinia sclerotiorum, Rhizoctonia solani,* and *Pythium* sp. compared to vector control (1234) and WT lines.

Three fungal pathogens were tested sequentially for each plant. Three pseudo-replications have been performed for each pathogen. Two leaves from each plant were inoculated per experiment. The leaves from the same plants are not independent, so they are not true replicates, and they were named 'pseudo-replications'. Therefore, the affected area of the six leaves from the same plant were averaged to one response value, then 60 responses per tested pathogen. The experimental design is a completely randomized design (CRD), randomly assigning one plant variety to one pot.

Source of variation	**Degrees of freedom**
Genotype	5
error	54
total	60-1=59

Table 3. Source of variations and degrees of freedom for the all three tested pathogens.

The least square mean model equation: $y_{ij} = \mu + \tau_i + e_{ij}$
y_{ij}: response, $i = 1,2, \dots, 6$, $j = 1, 2, \dots, 10$
μ: overall mean
τ_i: i^{th} variety effect, fixed, $i = 1, 2, \dots, 6$
e_{ij}: error term, random, $e_{ij} \sim N(0, \sigma^2)$

Table 4. The least square mean model equation used in the statistical analyses of the obtained data sets.

Variety name	**Code**
ORF13	ORF1
RSL1	RSL1
ORF12	ORF2
CN77	CN77
1234	CNTR2
WT	CNTR1

Table 5. Variety codes used in the statistical analyses and charts.

***Sclerotinia sclerotiorum* experiment**

A

B

C

D

E

Figure 22. Comparison of detached leaves of *Nicotiana benthamiana* inoculated with *Sclerotinia sclerotiorum.* A) ORF13 leaf with a small infection severity. B) ORF13 leaf with a moderate infection severity. C) ORF13 leaf with a high infection severity. D) Wild Type leaf with high infection severity. E) Vector control (1234) leaf with high infection severity.

A

B

C

D

E

Figure 23. Comparison of detached leaves of *Nicotiana benthamiana* inoculated with *Sclerotinia sclerotiorum.* A) RSL1 leaf with a small infection severity. B) RSL1 leaf with a moderate infection severity. C) RSL1 leaf with a high infection severity. D) Wild Type leaf with high infection severity. E) Vector control (1234) leaf with high infection severity.

A

B

C

D

E

Figure 24. Comparison of detached leaves of *Nicotiana benthamiana* inoculated with *Sclerotinia sclerotiorum.* A) CN77 leaf with a small infection severity. B) CN77 leaf with a moderate infection severity. C) CN77 leaf with a high infection severity. D) Wild Type leaf with high infection severity. E) Vector control (1234) leaf with high infection severity.

A

B

C

D

E

Figure 25. Comparison of detached leaves of *Nicotiana benthamiana* inoculated with *Sclerotinia sclerotiorum.* A) ORF12 leaf with a small infection severity. B) ORF12 leaf with a moderate infection severity. C) ORF12 leaf with a high infection severity. D) Wild Type leaf with high infection severity. E) Vector control (1234) leaf with high infection severity.

The least square mean was calculated for all varieties (Table 6).

Variety Least Squares Means								
Variety	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr >|t|**	**Alpha**	**Lower**	**Upper**
CN77	830.17	111.11	53	7.47	<.0001	0.05	607.32	1053.02
CNTR1	953.21	111.11	53	8.58	<.0001	0.05	730.36	1176.06
CNTR2	838.23	111.11	53	7.54	<.0001	0.05	615.37	1061.08
ORF1	537.98	111.11	53	4.84	<.0001	0.05	315.13	760.83
ORF2	713.14	117.12	53	6.09	<.0001	0.05	478.23	948.04
RSL1	416.18	111.11	53	3.75	0.0004	0.05	193.33	639.03

Table 6. Estimated Least Squares Means and the Standard Error for each variety.

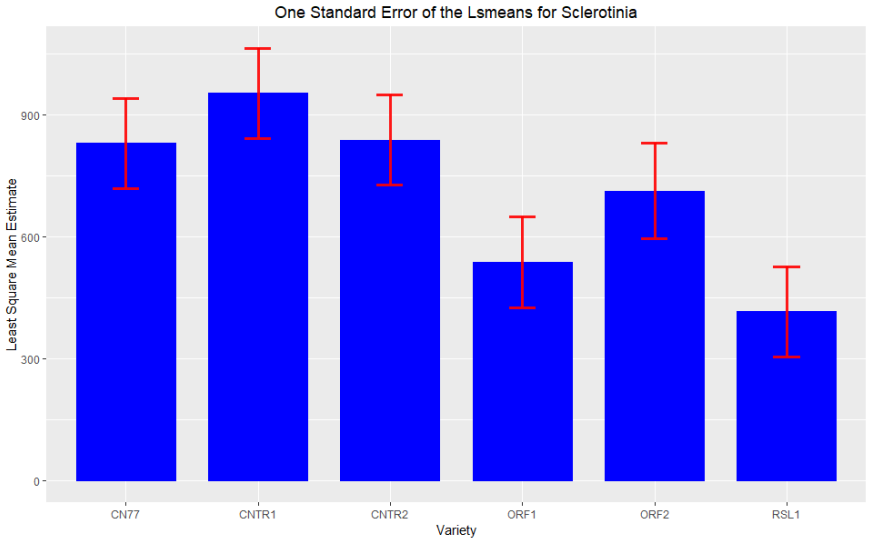

Chart 1. *Sclerotinia sclerotiorum* experiment**.** The estimated Least Squares Means and the Standard Error for each variety. The blue bars indicate estimated Squares Means for the varieties, the red bars indicate standard error.

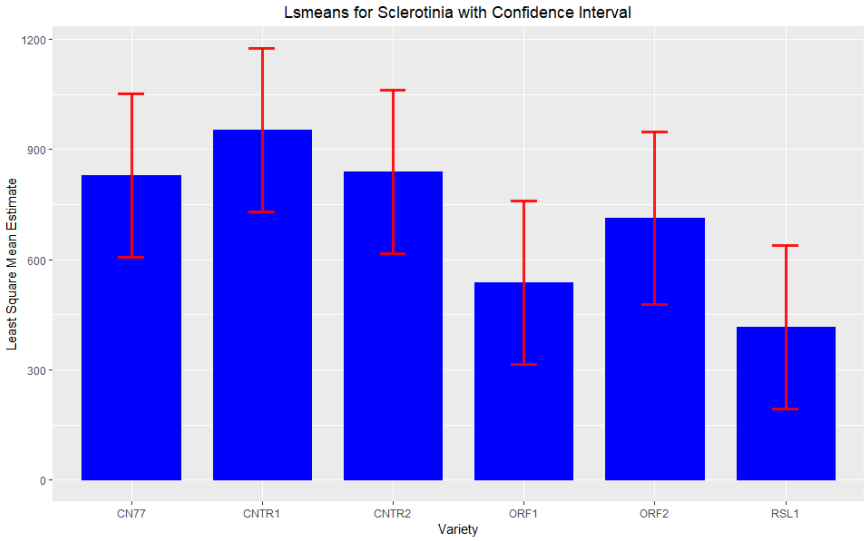

Chart 2. *Sclerotinia sclerotiorum* experiment**.** Least squares mean with confidential interval. The blue bars indicate estimated Squares Means for the varieties, the red bars indicate the confidence interval.

All four transgenic plant varieties ORF13, RSA1, CN77, ORF12 were compared to control wild type variety WT (Table 7).

Differences of Variety Least Squares Means Adjustment for Multiple Comparisons: Dunnett						
Variety	**Variety**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Adj P**
CN77	**CNTR1**	-123.04	157.13	53	-0.78	0.8982
CNTR2	**CNTR1**	-114.98	157.13	53	-0.73	0.9203
ORF1	**CNTR1**	-415.23	157.13	53	-2.64	0.0444
ORF2	**CNTR1**	-240.07	161.43	53	-1.49	0.4397
RSL1	**CNTR1**	-537.03	157.13	53	-3.42	0.0055

Table 7. Estimated mean differences between wild type WT control and the transgenic varieties ORF13, RSA1, CN77, ORF12.

Comparing the differences of the mean, ORF1 and RSL1 variety have significant smaller symptom area than the wild type control variety WT (Table 7). The transgenic plants demonstrated less susceptibility to the pathogen. The estimated p-values RSL1 = 0.0055, and ORF1= 0.0444 with significance level alpha 0.05, indicates substantial difference comparing to the wild control variety.

The four transgenic plant varieties ORF13, RSA1, CN77, ORF12 were compared to the transgenic control line 1234 (Table 8).

Differences of Variety Least Squares Means Adjustment for Multiple Comparisons: Dunnett						
Variety	**Variety**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Adj P**
CN77	**CNTR2**	-8.0586	157.13	53	-0.05	1.0000
CNTR1	**CNTR2**	114.98	157.13	53	0.73	0.9203
ORF1	**CNTR2**	-300.25	157.13	53	-1.91	0.2167
ORF2	**CNTR2**	-125.09	161.43	53	-0.77	0.9020
RSL1	**CNTR2**	-422.04	157.13	53	-2.69	0.0399

Table 8. Estimated mean differences between transgenic vector control 1234 and the lytic peptide transgenic varieties ORF13, RSA1, CN77, ORF12.

Comparing the differences of the mean, RSL1 variety have significant smaller symptom area than the transgenic vector control variety 1234 (Table 8). This transgenic variety demonstrated less susceptibility to the pathogen. The estimated p-values RSL1= 0.0399 with significance level alpha 0.05, indicates substantial difference comparing to the transgenic control variety 1234.

***Rhizoctonia solani* experiment**

A

B

C

D

E

Figure 26. Comparison of detached leaves of *Nicotiana benthamiana* inoculated with *Rhizoctonia solani.* A) ORF13 leaf with a small infection severity. B) ORF13 leaf with a moderate infection severity. C) ORF13 leaf with a high infection severity. D) Wild Type leaf with high infection severity. E) Vector control (1234) leaf with high infection severity.

A

B

C

D

E

Figure 27. Comparison of detached leaves of *Nicotiana benthamiana* inoculated with *Rhizoctonia solani.* A) RSL1 leaf with a small infection severity. B) RSL1 leaf with a moderate infection severity. C) RSL1 leaf with a high infection severity. D) Wild Type leaf with high infection severity. E) Vector control (1234) leaf with high infection severity.

A

B

C

D

E

Figure 28. Comparison of detached leaves of *Nicotiana benthamiana* inoculated with *Rhizoctonia solani.* A) CN77 leaf with a small infection severity. B) CN77 leaf with a moderate infection severity. C) CN77 leaf with a high infection severity. D) Wild Type leaf with high infection severity. E) Vector control (1234) leaf with high infection severity.

A

B

C

D

E

Figure 29. Comparison of detached leaves of *Nicotiana benthamiana* inoculated with *Rhizoctonia solani.* A) ORF12 leaf with a small infection severity. B) ORF12 leaf with a moderate infection severity. C) ORF12 leaf with a high infection severity. D) Wild Type leaf with high infection severity. E) Vector control (1234) leaf with high infection severity.

The least square mean was calculated for all varieties, four lytic peptides transgenic ORF13, RSA1, CN77, ORF12 and two controls 1234, WT (Table 9).

Variety Least Squares Means								
Variety	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
CN77	489.09	61.9526	53	7.89	<.0001	0.05	364.83	613.35
CNTR1	390.30	61.9526	53	6.30	<.0001	0.05	266.04	514.56
CNTR2	498.84	61.9526	53	8.05	<.0001	0.05	374.58	623.10
ORF1	299.63	61.9526	53	4.84	<.0001	0.05	175.37	423.89
ORF2	702.10	65.3038	53	10.75	<.0001	0.05	571.12	833.08
RSL1	345.43	61.9526	53	5.58	<.0001	0.05	221.17	469.69

Table 9. Estimated Least Squares Means and the Standard Error for each variety.

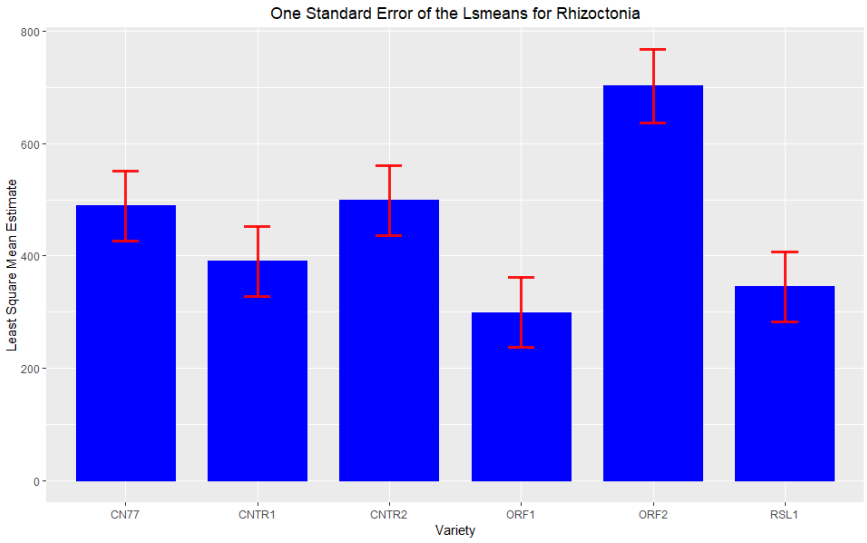

Chart 3. *Rhizoctonia solani* experiment. The estimated Least Squares Means and the Standard Error for each variety. The blue bars indicate estimated Squares Means for the varieties, the red bars indicate standard error.

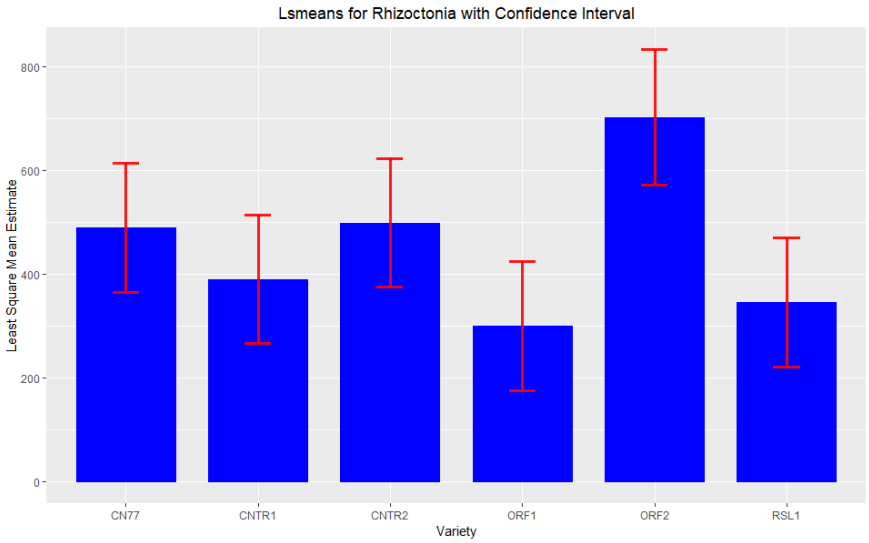

Chart 4. *Rhizoctonia solani* experiment. Least squares mean with confidential interval. The blue bars indicate estimated Squares Means for the varieties, the red bars indicate the confidence interval.

All four transgenic lytic peptide plant varieties ORF13, RSA1, CN77, ORF12 were compared to control wild type variety WT (Table 10).

Differences of Variety Least Squares Means Adjustment for Multiple Comparisons: Dunnett						
Variety	**Variety**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Adj P**
CN77	**CNTR1**	98.7890	87.6142	53	1.13	0.6890
CNTR2	**CNTR1**	108.54	87.6142	53	1.24	0.6099
ORF1	**CNTR1**	-90.6706	87.6142	53	-1.03	0.7528
ORF2	**CNTR1**	311.80	90.0151	53	3.46	0.0048
RSL1	**CNTR1**	-44.8656	87.6142	53	-0.51	0.9810

Table 10. Estimated mean differences between wild type WT control and the transgenic varieties ORF13, RSA1, CN77, ORF12.

The estimate mean values indicates no significant smaller symptom area for the transgenic lines ORF13, RSA1, CN77, ORF12 comparing to control the wild type WT (Table 10).

The four transgenic lytic peptide varieties ORF13, RSA1, CN77, ORF12 were compared to the transgenic control line 1234 (Table 11).

Differences of Variety Least Squares Means Adjustment for Multiple Comparisons: Dunnett						
Variety	**Variety**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Adj P**
CN77	**CNTR2**	-9.7526	87.6142	53	-0.11	1.0000
CNTR1	**CNTR2**	-108.54	87.6142	53	-1.24	0.6099
ORF1	**CNTR2**	-199.21	87.6142	53	-2.27	0.1041
ORF2	**CNTR2**	203.26	90.0151	53	2.26	0.1076
RSL1	**CNTR2**	-153.41	87.6142	53	-1.75	0.2887

Table 11. Estimated mean differences between transgenic control 1234 and the lytic peptide transgenic varieties ORF13, RSA1, CN77, ORF12.

The comparison analysis of the mean indicates no significant smaller symptom area for the transgenic lines ORF13, RSA1, CN77, ORF12 comparing to the transgenic control line 1234 (Table 11).

***Pythium* sp. experiment**

A

B

C

D

E

Figure 30. Comparison of detached leaves of *Nicotiana benthamiana* inoculated with *Pythium* sp. A) ORF13 leaf with a small infection severity. B) ORF13 leaf with a moderate infection severity. C) ORF13 leaf with a high infection severity. D) Wild Type leaf with high infection severity. E) Vector control (1234) leaf with high infection severity.

A

B

C

D

E

Figure 31. Comparison of detached leaves of *Nicotiana benthamiana* inoculated with *Pythium* sp. A) RSL1 leaf with a small infection severity. B) RSL1 leaf with a moderate infection severity. C) RSL1 leaf with a high infection severity. D) Wild Type leaf with high infection severity. E) Vector control (1234) leaf with high infection severity.

A

B

C

D

E

Figure 32. Comparison of detached leaves of *Nicotiana benthamiana* inoculated with *Pythium* sp. A) CN77 leaf with a small infection severity. B) CN77 leaf with a moderate infection severity. C) CN77 leaf with a high infection severity. D) Wild Type leaf with high infection severity. E) Vector control (1234) leaf with high infection severity.

A

B

C

D

E

Figure 33. Comparison of detached leaves of *Nicotiana benthamiana* inoculated with *Pythium* sp. A) ORF12 leaf with a small infection severity. B) ORF12 leaf with a moderate infection severity. C) ORF12 leaf with a high infection severity. D) Wild Type leaf with high infection severity. E) Vector control (1234) leaf with high infection severity.

The least square mean was estimated for all varieties in the *Pythium* sp. experiment (Table 12).

Variety Least Squares Means								
Variety	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
CN77	1257.12	79.4394	52	15.82	<.0001	0.05	1097.71	1416.52
CNTR1	864.45	79.4394	52	10.88	<.0001	0.05	705.05	1023.86
CNTR2	1012.26	79.4394	52	12.74	<.0001	0.05	852.86	1171.67
ORF1	1035.43	79.4394	52	13.03	<.0001	0.05	876.03	1194.84
ORF2	1055.42	88.8159	52	11.88	<.0001	0.05	877.19	1233.64
RSL1	1019.90	79.4394	52	12.84	<.0001	0.05	860.49	1179.31

Table 12. Estimated Least Squares Means and the Standard Error for each variety.

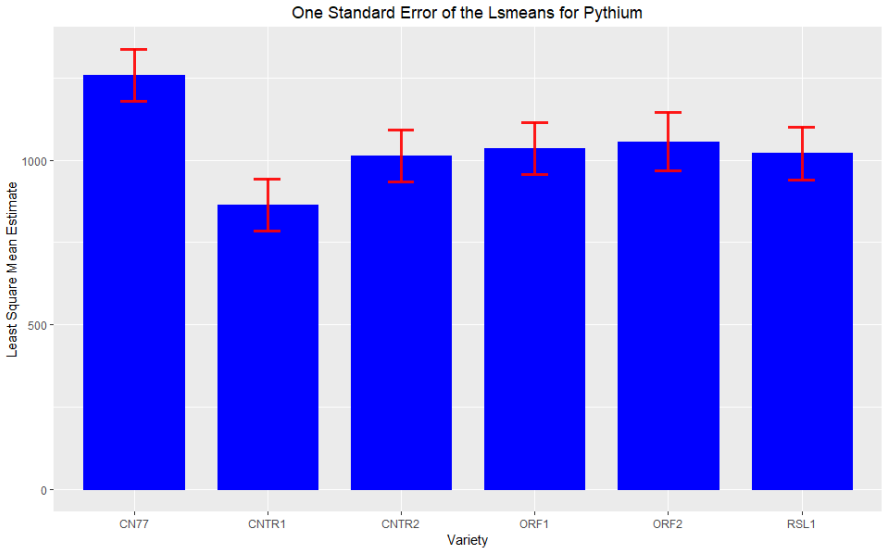

Chart 5. *Pythium* sp. experiment. The estimated Least Squares Means and the Standard Error for each variety. The blue bars indicate estimated Squares Means for the varieties, the red bars indicate standard error.

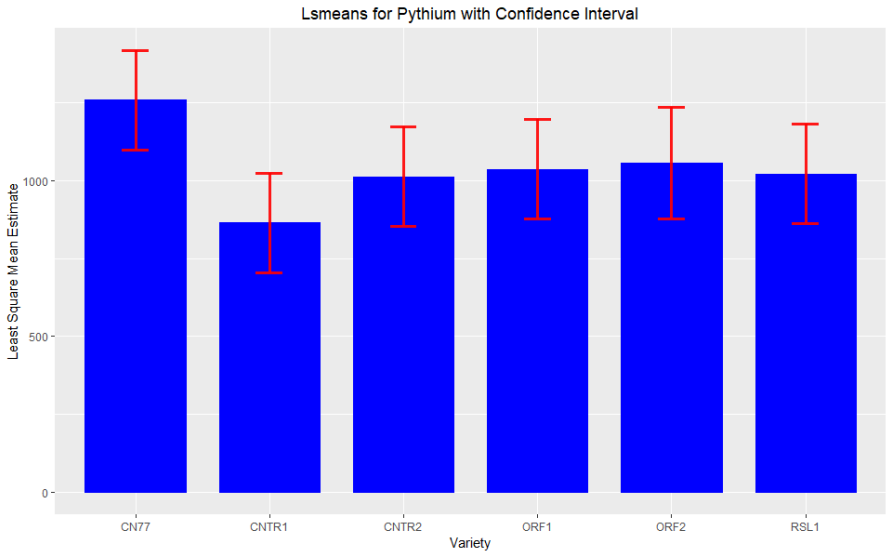

Chart 6. *Pythium* sp. experiment. Least squares mean with confidential interval. The blue bars indicate estimated Squares Means for the varieties, the red bars indicate the confidence interval.

The four transgenic lytic peptide plant varieties ORF13, RSA1, CN77, ORF12 were compared to control wild type variety WT (Table 13).

Differences of Variety Least Squares Means Adjustment for Multiple Comparisons: Dunnett						
Variety	**Variety**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Adj P**
CN77	**CNTR1**	392.66	112.34	52	3.50	0.0044
CNTR2	**CNTR1**	147.81	112.34	52	1.32	0.5577
ORF1	**CNTR1**	170.98	112.34	52	1.52	0.4197
ORF2	**CNTR1**	190.96	119.16	52	1.60	0.3708
RSL1	**CNTR1**	155.44	112.34	52	1.38	0.5106

Table 13. Estimated mean differences between wild type WT control and the transgenic varieties ORF13, RSA1, CN77, ORF12.

The differences of the mean calculation indicate no significant smaller symptom area for the transgenic lytic peptide lines comparing to control the wild type WT (Table 13).

All four transgenic lytic peptide plant lines ORF13, RSA1, CN77, ORF12 were compared to the transgenic control line 1234 (Table 14).

Differences of Variety Least Squares Means Adjustment for Multiple Comparisons: Dunnett						
Variety	**Variety**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Adj P**
CN77	**CNTR2**	244.85	112.34	52	2.18	0.1281
CNTR1	**CNTR2**	-147.81	112.34	52	-1.32	0.5577
ORF1	**CNTR2**	23.1702	112.34	52	0.21	0.9997
ORF2	**CNTR2**	43.1524	119.16	52	0.36	0.9960
RSL1	**CNTR2**	7.6358	112.34	52	0.07	1.0000

Table 14. Estimated mean differences between transgenic control 1234 and the lytic peptide transgenic varieties ORF13, RSA1, CN77, ORF12.

The statistical analysis disclosed no significant smaller symptom area for the transgenic lines ORF13, RSA1, CN77, ORF12 comparing to the transgenic vector control line 1234 (Table 14).

DISCUSSION

Many organisms produced small proteins which exhibit antimicrobial activities. In recent decades, the biological role of antimicrobial peptides (AMP) was recognized as the main factor in the defense mechanisms against a broad range of pathogenic microbes. The increased incidence of antimicrobial resistance worldwide due to overuse of antibiotics and pesticides makes AMPs promising alternative for the treatment of microbial disease.

In the last decades, the design of novel non-natural APMs is an option for use in agriculture for crops' improvement. The designed AMPs have enhanced properties against wide spectrum of pathogens while they have low non-specific toxicity, good stability, and bioavailability. By exploring the potential of the AMPs in the crop production, could lead to the development of new, improved cultivars which are resistant to various pathogenic diseases. In the present study, four different lytic peptide genes, which codes for antimicrobial peptides, transformed and expressing in tobacco plants were tested against three fungal pathogens: Sclerotinia sclerotiorum, Rhizoctonia solani, and Pythium sp. The resistance of tobacco plant transformants was examined in-vivo with a detached leaves bioassay.

For this study, seeds of four transgenic homozygous N. benthamiana tobacco plants varieties containing lytic peptide genes, one vector control transgenic non-lytic peptide gene tobacco plant variety, and one control wild type N. benthamiana tobacco plant variety were germinated on solid Murashige-Skoog (MS) plant media.

All transgenic varieties were tested through germination on modified MS plant media with Kanamycin antibiotic. The transgenic plant lines did not show any toxic effect during the germination and post-germination period. The observation of the plants showed normal growth and development. There had no discoloration or fading. That experiment proved the existence of NPTII selectable marker gene in the plants' genome.

The NPTII selectable marker gene was evaluated qualitatively with an Enzyme-Linked-Immunosorbent Assay. The obtained results proved the NPTII selectable marker presence in plant genome. The results of Enzyme-Linked-Immunosorbent Assay was confirmed by Polymerase Chain Reaction (PCR) Assay. All transgenic plant lines expressed positive results for Neomycin phosphotransferase II marker gene in their genome. The tested samples revealed a clear band at 700 bp region on the agarose gel.

The DNA of the transgenic lines ORF13, RSA1, CN77, ORF12, carrying non-plant multi-lytic peptide genes, were tested with a Polymerase Chain Reaction assay by using a specific primer for the respective genes. The presence of the lytic peptide genes was determined in all transgenic lines' genome. The tested samples indicated clear band with accordance to amplified length of the lytic peptide genes.

In a previous research, transgenic plant lines with expressed cationic peptides with broad-spectrum antimicrobial activity demonstrated powerful resistance to bacterial and fungal phytopathogens (53). In a study with transgenic tobacco plants expressing an antimicrobial bovine lactoferrin gene, a detached leaf assay proved the potential of the AMPs against fungal pathogens (50). In the current work, a detached leaf assay was performed to test the effect of expressed multi-lytic peptides on three fungal pathogens: S*clerotinia sclerotiorum, Rhizoctonia solani,* and *Pythium* sp. The results clearly showed that

transgenic plant lines ORF13 and RSL1 have substantial resistance to S*clerotinia sclerotiorum* infection by producing significantly smaller symptom area compared to control vector plant line 1234 and WT. The statistical analyzes confirmed the results of the bioassay. Estimated least square mean p-values respectively for the varieties RSL1(0.0055) and ORF1 (0.0444) showed significant difference in the infected area comparing to the control WT. Similar result was observed with RSL1 variety which demonstrated less susceptibility to the pathogen. Estimated p-values RSL1 (0.0399) indicated substantial difference comparing to the transgenic control variety 1234.

The experiments with the other two pathogens *Rhizoctonia solani,* and *Pythium* sp. did not develop significant smaller necrotic area comparing to two controls. Statistical analysis also confirmed these results.

In conclusion, non-plant multi-lytic peptide confers resistance against S*clerotinia sclerotiorum,* causing economically important white mold infection on plants. Further studies are necessary to confirm the potential of the non-plant multi-lytic peptides for control of plant diseases.

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