Transformation of tobacco with a lupin chitinase gene under control of a stress inducible promoter

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Summary:

Chitinases are a diverse family of proteins occurring in plants. Their function varies considerably, with certain chitinases having been associated with development. The majority however, are pathogenesis related (PR) proteins that have been shown to play a role during plant pathogen interactions. This has lead to many investigations on the use of chitinases in providing transgenic disease resistance. These studies are usually done using a constitutive expression system. This however stands in contrast with the natural defense system were PR gene expression is usually only upregulated when the plant is exposed to abiotic and/or biotic stress factors. The constitutive expression is therefore not ideal as it increases 'cost' penalties due to the energy being spent expressing the gene. In this study however, an inducible expression system was applied using a stress inducible promoter *AtGSTF6* derived from *Arabidopsis thaliana*, to drive *Lupinus albus* IF3 chitinase expression when the plants are under pathogen attack. The construct *AtGSTF6-IF3* was inserted into the binary vector pCAMBIA 2300 and transformed into *Nicotiana tabacum* cv JR6 by *Agrobacterium*-mediated transformation. To demonstrate the functionality of such a construct, an expression study was done on transgenic *N. tabacum* to determine transcription and *in vitro* chitinase enzyme activity. The data revealed that IF3 chitinase gene transcription from lupin plants was achieved in *N. tabacum.* Nine of the twelve lines that tested positive for chitinase gene transcription after hydrogen peroxide treatment, showed increased chitinase activity. With the success of showing increased chitinase activity, these lines were subjected to a detached leaf assay with *Rhizoctonia solani* AG2, which causes leaf target spot disease. The assay showed that six of the nine lines identified as having increased chitinase activity showed reductions in lesion areas. More specifically, three of the four lines showing more than a five-fold increase in chitinase activity compared to the untransformed *N. tabacum*, showed significant lesion reduction. The *AtGSTF6*-IF3 construct can therefore be recommended to increase disease resistance in *N. tabacum* towards *Rhizoctonia solani* AG2 after showing both expression and increased disease resistance in certain transgenic lines.

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List of Abbreviations:

AG:	Anastomosis group
AtGSTF6:	*Arabidopsis thaliana* Glutathion *S*-transferase F (phi)6 gene
bp:	basepair
CaMV:	Cauliflower Mosaic Virus
CSIR:	Council of Scientific and Industrial Research
cv:	cultivar
dH_2O :	distilled water
DNA:	Deoxyribonucleic acid
EDTA:	Ethylenediamine tetraacetic acid
IF:	Intercellular fluid
kbp:	kilobasepair
kDa:	kilodalton
MS:	Murashige and Skoog
MWM:	Molecular weight Marker
*Npt*II:	Neomycin phosphotransferase II
PCR:	Polymerase Chain Reaction
PR:	Pathogenesis response
R gene:	Resistance gene
RNA:	Ribonucleic acid
RNase:	Ribonuclease
RT PCR:	Reverse Transcription Polymerase Chain Reaction
Rpm:	Revolutions per minute
SAR:	Systemic Acquired Resistance
T DNA:	Transfer deoxyribonucleic acid
Tm:	Melting temperature
Tris:	Tris hydroxy methyl aminoethane
vir:	virulence

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Chapter 1

Introduction and aim of study

In nature, survival is determined by how well an organism can approach various challenges presented in its natural environment. One of the major challenges facing plants, are pathogen interactions. Plants being relatively sessile organisms are particularly pressured on an evolutionary basis to withstand these challenges.

This arms race is generally equal from both plants and pathogens however with the advent of globalisation, specifically plant material exchange, many pathogens have been relocated to new parts of the world. There, in the absence of resistant cultivars, they can cause tremendous crop losses. One such case includes Panama disease in bananas caused by Fusarium Wilt, which spread from South-East Asia to tropical America causing enormous crop losses of the susceptible cultivar ‘Gros Michel’ (Stover, 1962).

The problem is however not only limited to the introduction of novel pathogens but also the exchange of spatially separated pathogen populations. O'Donnell *et al*. (1998) showed that Panama disease does not have a monophyletic origin but that there are independent evolutionary origins for the lines. This means that the introduction of more successful pathovars of a specific pathogen can expose previously resistant cultivars to disease (Jones, 2000).

Plant breeding has provided an important avenue in crop protection and pathogen resistance to combat these diseases. The advantages include higher crop yield as well as the reduction in agrochemical application. Conventional plant breeding can however be problematic. It is very time consuming, especially in long lifecycle crops such as trees (Merkle and Dean, 2000) and must be continuously revised as pathogens evolve to evade the new resistance (Datta *et al.* 2002). In certain cases crosses using different species can create undesirable crop characteristics due to linkage drag, and genetic recombination can be very low in hybrid crosses (Grover and Gowthman, 2000).

The advent of genetic modification (GM) has provided a way to alleviate these issues. Probably the most important advantage is that it allows the introduction of resistance genes that are otherwise not available in taxonomically related species. This gene (or genes) can be introduced to provide disease resistance by selectively targeting pathogens.

Currently the application of GM crops is restricted largely due to public concern regarding the various consequences that such relatively new technology can or could have. Nonetheless there are well-known agricultural crops currently available where this technology has proved successful. One of the most successful applications is Bt crops where the insecticidal protein from *Bacillus thuringiensis*, expressed in crop plants, can provide broad spectrum insect resistance (Federici, 2005).

The introduction of disease resistance genes into plants to provide increased resistance in plant-pathogen interactions has been investigated intensively. With the advent of gene-mining techniques such as microarrays, new genes are continuously being discovered that can be potentially utilized.

The aim of this study was to access whether transgenic tobacco expressing a classIII chitinase under a stress inducible promoter was more resistant to the necrotrophic pathogen *Rhizoctonia solani* than untransformed tobacco. This will be tested by producing a gene construct containing the class III IF3 chitinase from *Lupinus albus* under the control of the stress inducible promoter GST1. This construct will be transformed into tobacco and tested for expression. The disease resistance will then be tested using a detached leaf assay for transgenic and nontransgenic lines to determine a possible difference in disease levels. The hypothesis of the study was that the introduction of the stress inducible expression system driving the IF3 chitinase expression would confer increased disease resistance to target leaf spot caused by *Rhizoctonia solani* in transgenic plants expressing and producing the functional transgenic chitinase gene compared to untransformed *Nicotiana tabacum* lines.

This review therefore provides a brief overview of plant-pathogen interactions focusing on Active Oxygen Species signalling found with the stress inducible promoter, PR proteins and more specifically chitinases. The review will also cover the plant pathogen *Rhizoctonia solani* and evaluate the techniques used to genetically engineer transgenic plants with pathogen resistance genes towards this pathogen.

Chapter 2

Literature Review

2.1 Plant –Pathogen interactions

Plants do not have an adaptive circulatory defense system and have therefore evolved other defense mechanisms. These include preformed and induced responses. Preformed responses, also known as nonspecific responses, are based on either presynthesized structural elements such as cell walls and presynthesized secondary metabolites aimed at reducing or inhibiting pathogen attack (Osbourn, 1996).

Induced responses on the other hand have coevolved with pathogens that are able to overcome the preformed responses. These mechanisms rely on the recognition of the invading pathogen. This recognition is converted into a signal cascade pathway that induces changes in gene expression. These genes appear to be relatively important as a large proportion of a plants genome is dedicated to this challenge. This can be illustrated from genome sequence data derived from *Arabidopsis* which has shown that approximately 14% of the genome (in this case coding for about 21000 genes) is dedicated to this activity (Bevan *et al.* 1998).

2.1.1 Types of fungal pathogens on plants:

Fungal plant pathogens can be classified necrotrophic, biotrophic or hemitrophic (Glazebrook, 2005). Necrotrophic pathogens act by killing and destroying plant tissue through toxins and cell wall degrading enzymes. An example of this is *Rhizoctonia solani* (http://www.apsnet.org/online/common/names/tobacco.asp). The dead tissue produced is then utilized as an energy source. Biotrophic pathogens on the other hand can only complete their lifecycle in a living host. These pathogens seldom cause the

death of their hosts but they can impact severly on the growth. An example of a biotrophic fungal pathogen affecting tobacco is *Erysiphe cichoracearum* causing powdery mildew (http://www.apsnet.org/online/common/names/tobacco.asp) .

Hemitrophic pathogens usually have a biotrophic phase in the early infection cycle, which ends up becoming necrotrophic.

2.1.2 Plant defense response activation:

The pathogen invasion needs to be recognised by the plant in order for a defense response to be induced. This can occur either through specific recognition of elicitors produced by the pathogen, such as peptides(Scheel, 1998). This is commonly called a gene for gene resistance were single dominant genes (avirulence genes) are recognised by a single dominant resistance (R) gene (Flor, 1972). The pathogens that are recognised this way fail to initiate disease and are known as avirulent pathogens making the interaction incompatible. It should however be noted that the effectivity of the response is based on how fast the plant is able to mount a response once the pathogen has been detected (Yang *et al.* 1997). On the other hand those avirulence genes not recognised allow the pathogen to interact with the plant (compatible interaction) and will cause disease.

In the case of avr-R gene interaction, a defense reponse is triggered. This response leads to calcium and ion fluxes, leading to an oxidative response with active oxygen species (AOS) being produced. Active oxygen species can be defined as reactive oxygen molecules, usually O^-, H_2O_2 and OH^-, that have a biochemical effect in the cell (Wu *et al.* 1997).

Active oxygen species ultimately lead to transcriptional reprogramming and also often to programmed cell death (Belkhadir *et al.* 2004). The hypersensitive response (HR) is thought to confine the growth of biotrophic pathogens by preventing the pathogen from obtaining living tissue that it requires (Thatcher *et al.* 2005). In necrothrophs this mechanism can actually be detrimental as it can aid the pathogen in obtaining its food source (Glazebrook 2005).

If a specific avr-R recognition interaction does not occur the plant also has the ability to activate a basal defense response. This can occur through pathogen associated molecular patterns (PAMPS), which can include compounds such as flagellin or liposaccharides(Gomez-Gomez and Boller 2002). This type of defense response is

however slower than the avr-R interaction and is also weaker than the avr-R defense interaction. In this case colonisation is not prevented but pathogen spread is limited (Glazebrook *et al.* 1997).

2.1.3) Signalling pathways available after pathogen recognition

Pathogen recognition normally leads to the activation of a defense-signalling pathway. These pathways can be interlinked and form part of a large signalling network. There are several pathways including salicyclic acid (SA), nitric oxide (NO), ethylene (ET) and jasmonic acid (JA) dependent pathways. These pathways can interact extensively and can be either synergistic or antagonistic.

In biotrophic pathogen interactions the signalling pathway appears to be linked to SA (Glazebrook 2005). The stimuli leading to this pathway are based on gene for gene interaction and the occurance of HR. The SA level increase has been linked to *PAD4* and *EDS1* genes in *Arabidopsis thaliana* (Christine *et al.* 2001; Falk *et al.* 1999), which in turn are activated by SA pathway inducing stimuli. The increase in SA in turn activates a series of defense response genes including PR1, PR2 and PR5 families. In necrothrophs the plant defense response appears to be linked to JA and ET signalling (Glazebrook, 2005). The signalling pathway is also commonly observed in wounding responses and results in SA independent defense responses being activated.

This review will focus mostly on signalling that is achieved through active oxygen species (AOS).

2.1.4 Gene activation through Active Oxygen Species signalling:

The production of AOS can be elicited through pathogen attack. Pathogen recognition specifically leads to the production of hydrogen peroxide (H_2O_2), which forms part of the oxidative burst as shown in Figure 2.1. This oxidative burst occurs within 2-3 minutes after infection (Sutherland 1991; Tenhaken *et al.* 1995).

The production of H_2O_2 can have dual functions. It can act directly as a pathogen toxicant (Baker and Orlandi, 1995; Lin *et al.* 2005) as well as reinforcing physical barriers and aiding in lignification of cell walls against invading pathogens (Tenhaken *et al.* 1995). However a more important function is its role in signaling later defense reactions in plant pathogen interaction. It has been know to act in a signal cascade

pathway during various pathogen attacks including bacterial attack (Adam *et al.* 1989), fungal attack (Vera-Estrella *et al.* 1992) and elicitor recognition (Apostol *et al.* 1989; Baker *et al.* 1993).

This signal transduction pathway causes a change in the genetic regulation and results in the activation of a variety of genes. In tobacco it was shown that H_2O_2 leads to PR-1 protein induction and systemic resistance (Chen *et al.* 1995). It does however seem that salicylic acid is required for *PR 1* gene expression (Neuenschwander *et al.* 1995). In potato elevated levels of H_2O_2 caused the increased expression of an acidic chitinase (Wu *et al.* 1997). In *Arabidopsis thaliana* similar results were observed leading to the production of phenylalanine lyase (PAL) defense proteins, *PR* and *glutathione-S-transferase* genes(Bi *et al.* 1995; Desikan *et al.* 1998). These *PR* include chitinases and glucanases genes (Lin *et al.* 2005; Thatcher *et al.* 2005). It should also be noted that H_2O_2 is an important contributor towards the hypersensitive cell death response. The contribution is made in association with the nitric oxide pathway and it has been shown that an appropriate concentration balance of these two elicitor molecules is the key factor to cell death initiation (Delledonne *et al.* 2001).

2.1.5 The activation of Glutathione-*S*-transferases through AOS

Glutathione-*S*-transferases (GSTs) play an important role in primary metabolism, secondary metabolism, signaling and stress metabolism in plants(Dixon *et al.* 2002). During stress metabolism, which includes pathogen attack, the induction of GSTs usually aids in protecting the plants macromolecules against harmful xenobiotics and reactive oxygen species (Yang *et al.* 1997).

Generally GSTs are induced through various signals originating from a stress response such as ethylene, pathogen attack, auxin, salicylic acid, dehydration, high salt and wounding (Itzhaki *et al.* 1994; Yang *et al.* 1998) but primarily by the signal from H_2O_2 (Tenhaken *et al.* 1995). This induction is enabled through the interaction of specific signal molecules that bind to response element motifs, which are found on the glutathione-*S*-transferase (GST) promoter and include ethylene responsive elements, TCA motifs and G-box motifs. Thus induction is often achieved by more than one signal molecule (Droog, 1997).

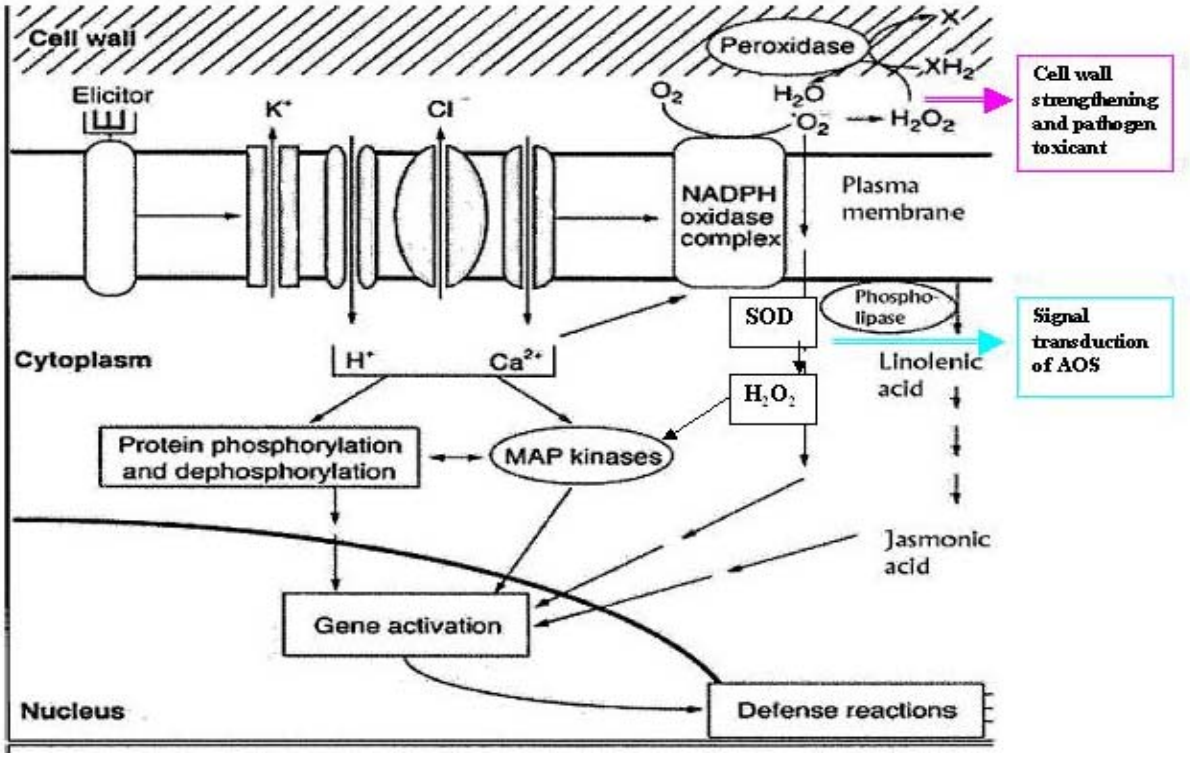


Figure 2.1: A simplified representation showing elicitor perception leading to the signal transduction pathways that enable gene activation. The recognition of the elicitor results in an ion flux, which activates mitogen activated protein (MAP) kinases and also results in the reactive oxygen burst. The oxygen radicle formed by the NADPH oxidase complex is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) allowing further signalling. Note that other AOS signalling exist but were excluded from the figure to simplify the representation. Adapted from (Somssich and Hahlbrock, 1998).

The GST promoter used in this study was originally derived from *Arabidopsis thaliana* ecotype Ws driving the gene *AtGSTF6*. Initially this gene was known as gst I (Grant and Loake 2000) but it has since been renamed *AtGSTF6* (Wagner *et al.* 2002). It belongs to the class I *GST*. This specific class is thought to function as a cellular protectant and is induced by pathogen attack wounding and lipid peroxidation (Grant and Loake 2000). All three response elements mentioned above, were found to occur in the *Arabidopsis thaliana* ecotype Landsberg promoter (Yang *et al.* 1998). Two ethylene response elements (ATTTCAAA) were inversely repeated at position –183bp and –737bp (Yang *et al*. 1998) while two TCA motifs were found to position

–845 and + 32 of the *AtGSTF*6 gene promoter. A G-box motif was also found at position –369 (Yang *et al.* 1998).

A further study was done by Grant and Loake (2000) which showed that this *AtGSTF6* promoter is highly inducible in the presence of AOS. More specifically the study indicated that hydrogen peroxide enables the activation of the *AtGSTF6* promoter. An NADPH oxidase and peroxidase type enzyme provides the redox cues. Nitric oxide, methyl jasmonate and ethylene on the other hand did not seem to influence *AtGSTF6* promoter induction however the activity of a 48-kDa MAP kinase caused an induction signal correlating with the promoter induction.

2.2 Biochemistry of activated plant defense response mechanisms

The signalling transduction pathways induced by pathogen detection lead to the transcriptional activation of several genes involved in some way with defense response. The resultant proteins can have a primary and secondary defense mechanism. A primary role would include cell reinforcement, hypersensitive response leading to programmed cell death, the release of toxic metabolites and defense related proteins (Jones, 2001). The secondary immunity role is in the form is in the form of proliferating systemic aquired resistance (SAR). SAR spreads a broad-spectrum immunity from the site of infection to other parts of the plant (Ryals *et al.* 1996)

2.2.1 Primary defense mechanisms observed against fungal pathogens in plants:

Several molecules and proteins have been identified (either directly or indirectly), as being involved in pathogen defense response. These include pathogenesis related proteins, ribosome inactivating proteins, small cysteine rich proteins, lipid transfer proteins, polygalacturonase inhibiting proteins and antiviral proteins.

2.2.2 Pathogenesis related proteins:

Pathogenesis related (PR) proteins were first identified and defined as proteins that are absent in healthy plants but are upregulated in pathogen infected plants (Van Loon and Van Kammen 1970). Since then PR proteins have been found in more than 40 species (Van Loon and Van Strien 1999) being expressed as part of local and systemic response (Heil and Bostock 2002).

Pathogenesis related proteins were first classed into five main classes based on biological and molecular biology techniques that elucidated the expression pattern in infected tobacco (Van Loon and Van Strien 1999). Since then the different classes have been moved to seventeen PR families based on amino acid sequence similarity and also biological and enzymatic similarity.

Currently there are 17 recognised families of pathogen response proteins (Van Loon and Van Strien 1999). This classification requires two criteria to be met:

1) the protein must be induced by a pathogen in tissues that do not normally express the protein and
2) the induced expression must be shown in at least 2 plant-pathogen interactions or expression must be confirmed in at least two independent research labs.

PR proteins tend to fall into two groups, either being acidic or basic proteins. Generally acidic PR proteins are found in intercellular spaces while basic PR proteins, although possessing similar biological function are located in intracellular spaces such as vacuoles (Van Loon and Van Strien 1999).

The induction of PR proteins expression is specifically based on the signalling pathway that activates it. This can allow the plant to produce specific PR proteins that either target biotrophic or necrotrophic pathogens (Glazebrook, 2005). Research in Arabidopsis has shown that the biotrophic organism *Peronospora parasitica* causes the activation of PR-1, PR2 and PR5 due to SA mediated pathway. The necrotrophic organisms such as *Alternaria brassicola* (Penninckx *et al.* 1996) and *Botrytis cinerea* (Thomma *et al.* 1998) require the jasmonic acid signal pathway to activate PR3 and PR4 for induced resistance.

Table 2.1: The family of pathogenesis-related proteins (Adapted from Van Loon and Van Strien 1999)

PR type	Type member	Properties
PR 1	Tobacco PR-1a	unknown
PR 2	Tobacco PR 2	β 1,3 glucanase
PR 3	Tobacco P Q	Chitinase type I II IV V VI VII
PR 4	Tobacco R	Chitinase type I and II
PR 5	Tobacco S	Thaumatin like
PR 6	Tomato Inhibitor I	Proteinase inhibitor
PR 7	Tomato	Endoproteinase
PR 8	Cucumber chitinase	Chitinase type III
PR9	Tobacco 'lignin forming peroxidase'	Peroxidase
PR 10	Parsley PR1	Ribonuclease like
PR 11	Tobacco Class V chitinase	Chitinase type I
PR 12	Radish Rs-AFP3	Defensin
PR 13	Arabidopsis THI2.1	Thionin
PR 14	Barley LTP14	Lipid transfer protein
PR 15	Barley	Oxalate oxidase
PR 16	Barley	Oxalate oxidase-like protein
PR 17	Barley	unknown

2.2.3 Chitinase:

Chitinases (EC 3.2.1.14) have been found to have a wide range of activities. Many have been shown to have direct antifungal activity and are able to destroy the chitin structural molecule, an N-acetyl-*D*-glucosamine, which is a major cell wall component of many fungi and basis of insect exoskeletons (Figure 2). Chitinase, classed as a glycosyl hydrolase, has been placed in family 18 and 19 based in the glycosyl hydrolase classification system (Henrissat, 1991; Henrissat and Bairoch 1993). From a PR protein perspective, they can be classed into PR3, PR4, PR8 and PR 11 (see Table 1).

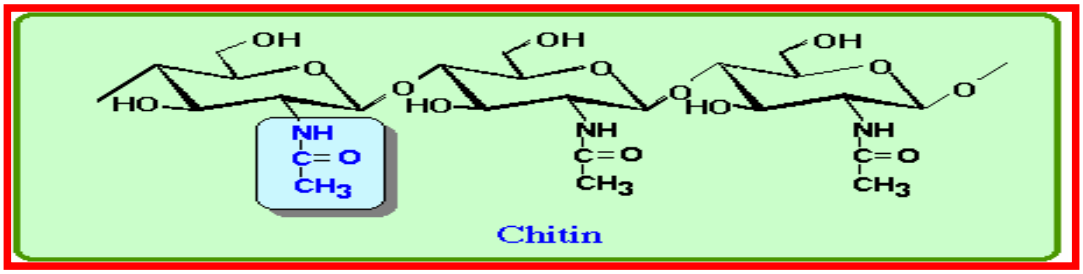


Figure 2.2: Chemical stucture of chitin showing N-acetyl-*D*-glucosamine linked by α 1-4-glycosyl bonds.

Family 18 chitinases occur in bacteria, fungi, yeast, viruses, plants and animals while family 19 is almost exclusively found to occur in plants, with the exception of an isolate obtained from *Streptomyces griseus* (Watanabe *et al.* 1999). These families do not share sequence or structure similarties indicating possible convergent evolution (Hamel *et al.* 1997). Other differences include their biochemical properties. Family 18 for example, keeps the catalysis product in the same configuration as the substrate while Family 19 utilizes an inversion mechanism. (Brameld and Goddard, 1998). The reaction in this case is based on the hydrolysis of the β 1-4-linkages found in the chitin which releases N,N'-chitobiose and other higher oligomers(Shih *et al.* 2001a).

In plants these families are divided into classes, with classes I, II and IV belonging to family 19 while classes III and V belong to family 18 (Neuhaus and Fritig, 1996). Chitinases occur either as constitutively expressed proteins in tissues vulnerable to attack by pathogens (Samac *et al.* 1990), or are inducibly expressed as pathogenesis

related proteins in response to fungal infection (Collinge *et al.* 1993; Roby *et al.* 1990; Wubben *et al.* 1992). This induction can be based either upon the recognition of a pathogen or is caused by other factors such as the exposure to UV light, salicyclic acid, salts, plant hormones and ozone(Ernst *et al.* 1992), all of which form part of stress factors in plants.

Efficacy of chitinases *in vivo* is however often affected by the presence of other unrelated enzymes such as β-1,3-glucanase that amplify its effectivity (Sela-Buurlage *et al.* 1993). Jach *et al.* (1995) showed that the combination of a constitutively expressed class II chitinase coexpressed with class II β-1,3 glucanase provides improved resistance to *Rhizoctonia solani* in transgenic tobacco, compared to the genes being expressed on their own.

However not all chitinases are involved in plant defense responses. Several articles have shown that chitinases appear to form part of developmentally regulated expression present in certain organs. Yeboah *et al*. (1998) showed that a class III chitinase is expressed in developing seeds, Clendennen and May (1997) found that an endochitinase is produced during banana ripening, while Robinson *et al*. (1997) identified a class IV chitinase being expressed in ripening grape berries. In rice, a class I chitinase was found to be constitutively expressed in floral organs but not significantly in vegetative organs (Takakura *et al.* 2000). Furthermore Baldan *et al.* (1997) showed that the addition of an acidic endochitinase can allow the carrot cell mutant *ts11*, which is unabe to form somatic embryos at temperatures above 32 C°, to form somatic embryos upon addition to the media. Therefore it remains questionable whether all chitinases have a primary role in plant defense.

2.2.4 Class III chitinases:

Class III chitinases are characterized by having bifunctional capabilities, which include lysozyme and chitinase activities (Heitz *et al*. 1994; Majeau *et al*. 1990). Class III chitinases also have very little sequence similarities with other classes of chitinases in plants, except class V (Shih *et al.* 2001). Class III chitinases also exhibit more similarity to fungal and bacterial chitinases, which fall into family 18 of the glycosyl hydrolases.

The comparison of class III chitinases from different plant sources has lead to the distinction of two subcategories of chitinases, namely the ChibI (Neuhaus and Fritig,

1996) and the ChibII (Yeboah *et al.* 1998). The ChibI category is distinguished by having short C-terminal regions while the ChibII category shows only 51-53% amino acid similarity to ChibI chitinases and is represented by the soybean chitinase.

2.2.5 Class III IF3 chitinase from *Lupinus albus*:

The classIII IF3 chitinase from *Lupinus albus* is classed into the ChibI group and the gene was first isolated from *Lupinus albus* as described by (Regalado *et al.* 2000). The enzyme is constitutively expressed in various parts of the plant were it is found to occur in the extracellular spaces (called apoplast) of these organs which include the leaves, roots and hypocotyls (Regalado *et al.* 2000). The study also showed that there is an accumulation of the chitinase during *Colletotrichum gloeosporioides* infection. The chitinase appears to be functional as a general stress response protein as it is inducible by both abiotic and biotic stress factors and may thus not be exclusively linked to a pathogen response (Goormachtig *et al*. 1998). A similar pattern can be seen in the only class III acidic chitinase found in Arabidopsis, ATHCHIA (Samac *et al.* 1990), which appears to be developmentally regulated as well as induced by pathogens (Samac and Shah, 1991).

2.2.6 Chitinase applications for transgenic resistance

The use of chitinases to produce disease resistant crops has been intensively investigated. Various plants have been transformed and tested with chitinases and illustrated examples given in Table 2.

Table 2.2: Examples of the application of chitinases in transgenic plant types.

Plant type	Chitinase	Other genes	Type of trials	Results
Cocoa (Maximova *et al*. 2005)	Chitinase class I from cocoa. Expression under constitutive CaMV35S promoter.	No.	1) In vivo.	Leaf disk assay showed a reduced *Colletotrichum gloeosporioides* growth and reduction in leaf necrosis.
Grapevine (Bornhoff *et al.* 2005)	Chitinase (class not given) from barley. Expression under constitutive CaMV35S promoter.	1)Ribosome inactivation protein.	1) Field Trial	No significant improvement or reduction in disease reistance to powdery mildew.
Oilseed rape(Grison *et al.* 1996)	Chitinase (class not given) hybrid tomato chitinase gene. Expression under constitutive CaMV35S promoter.	No.	1) Field trial	Increased resistance in transgenic lines against *Cylindrosporium concentricum*, *Phoma lingam* and *Sclerotinia sclerotiorum*
Rice (Itoh *et al.* 2003)	Chitinase from *Streptomyces griseus.* Expression under constitutive CaMV35S promoter.	No	1) Greenhouse	Leaves showed increased resistance to *Magnaporthe grisea* compared to untransformed.
Rice (Xu *et al.* 2003)	Chitinase gene from *Phaseolus limensis.*	1) β 1-3 glucanase.	1) Field trial	Some transgenic rice lines showed increased resistance to *Magnaporthe grisea*, especially in association with β 1-3 glucanase.

Plant type	Chitinase	Other genes	Type of trials	Results
Silver birch (Pasonen *et al.* 2004)	Chitinase class IV from sugar beet. Expression under constitutive CaMV35S promoter.	No.	1) Field trial	Some transgenic lines proved more susceptible to leaf spot disease *Pyrenopeziza betulicola*, all lines showing no improved resistance. Resistance was increased in some lines for the pathogen *Melampsoridium betulinum*
Tobacco (Carstens *et al.* 2003)	Chitinase from *Saccharomyces cerevisiea*. Expression under constitutive CaMV35S promoter.	No.	1) In vitro detached leaf assay	Transgenic lines showing increase chitinase expression showed a decrease in susceptibility to *Botrytis cinerea*
Tobacco (Fung *et al.* 2002)	Chitinase from *Brassica juncea.* Expression under constitutive CaMV35S promoter.	No	1) In vitro	Transgenic lines expressing chitinase showed a reduction in growth in *Trichoderma viride*
Wheat (Anand *et al.* 2003)	Chitinase class VII and IV genes from wheat. Expression under constitutive Ubiquitin promoter.	1)Thaumatin like protein. 2)β 1-3 glucanase.	1)Greenhouse trial. 2) Field trial.	1)Combination of β 1-3 glucanase and chitin reduced *Fusarium graminearum* infection. 2) No resistance.

2.3) Fungal disesases affecting *Nicotiana tabacum*

The following list of fungal pathogens known to affect *Nicotiana tabacum*, are listed below:
(http://www.apsnet.org/online/common/names/tobacco.asp):

1) Anthracnose
   Colletotrichum destructivum O'Gara
   (teleomorph: *Glomerella glycines* F. Lehm. and F.A. Wolf)
2) Barn spot
   Cercospora nicotianae Ellis & Everh.
3) Barn rot
   Several fungi and bacteria
4) Black root rot
   Thielaviopsis basicola (Berk. & Broome) Ferraris
5) Black shank
   Phytophthora parasitica Dastur var. *nicotianae* (Breda de Haan) Tucker
   P. nicotianae Breda de Haan var. *nicotianae* G.M. Waterhouse
6) Blue mold (downy mildew)
   Peronospora tabacina D.B. Adam
   = *P. hyoscyami* de Bary f. sp. *tabacina*
7) Brown spot
   Alternaria alternata (Fr.:Fr.) Keissl.
8) Charcoal rot
   Macrophomina phaseolina (Tassi) Goidanich
9) Collar rot
   Sclerotinia sclerotiorum (Lib.) de Bary
10) Damping-off, Pythium
   Pythium spp.
   P. aphanidermatum (Edson) Fitzp.
   P. ultimum Trow

11) Frogeye leaf spot

Cercospora nicotianae Ellis & Everh.

12) Fusarium wilt

Fusarium oxysporum Schlechtend.:Fr. (several f. sp.)

13) Gray mold

Botrytis cinerea Pers.:Fr.

(teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel)

14) Olpidium seedling blight

Olpidium brassicae (Woronin) P.A. Dang.

15) Phyllosticta leaf spot

Phyllosticta nicotiana Ellis & Everh.

16) Powdery mildew

Erysiphe cichoracearum DC.

17) Ragged leaf spot

Phoma exigua Desmaz. var. *exigua*

= *Ascochyta phaseolorum* Sacc.

18) Scab

Hymenula affinis (Fautrey & Lambotte) Wollenweb.

= *Fusarium affine* Fautrey & Lambotte

19) Sore shin and damping-off

Rhizoctonia solani Kühn

(teleomorph: *Thanatephorus cucumeris* (A.B. Frank) Donk)

20) Southern stem rot (southern blight)

Sclerotium rolfsii Sacc.

(teleomorph: *Athelia rolfsii* (Cursi) Tu & Kimbrough)

21) Stem rot of transplants

Pythium spp.

22) Target spot

Rhizoctonia solani Kühn

23) Verticillium wilt

Verticllium albo-atrum Reinke & Berthier

V. dahliae Kleb.

2.4 *Rhizoctonia solani*

2.4.1 The biology of *Rhizoctonia solani*

The pathogen *Rhizoctonia* is a soil inhabiting fungus and is classed into the Basidiomycetes because they produce basidiospores as their sexual spores. These spores are however not commonly produced and *Rhizoctonia* is often referred to as sterile mycelia with mostly asexual sclerotia being produced (Agrios, 1997).

Rhizoctonia diseases represent a major portion of stem and root rot disease found on crops worldwide. Losses can occur in almost any crop plant with symptoms varying between crops based on environmental conditions and stage of growth (Agrios, 1997).

In the field *R. solani* usually overwinters as mycelium or sclerotia in the soil or crevises in plant containers. The spread of the fungus can be through physical forces such as rain but also poor agricultural practices such as tools containing contaminated soil. It can also be transmitted through infected tubers as well as in infected seeds. Once the fungus is established in soil it can remain there indefinitely. For most races the optimum infection temperature is between 15 and 18 C° but other races can be active up to 35 C°. The soils usually need to be moderately wet but the disease symptoms are most severe if plant growth is faced with challenging environmental conditions. The symptoms commonly seen include damping-off in seedlings and root rot, stem rot as well as stem canker in growing or grown plants. Certain environmental conditions can also lead to storage organ rots as well as foliar blights and spots of leaves situated close to the ground (Agrios, 1997).

Rhizoctonia solani and other species appear to be ‘collective’ species representing unrelated strains. This observation was made based on the whether or not fusion of touching hyphae, called anastomosis, occurs. If anastomosis occurs between isolates they can be classified into the same anastomosis group (AG). This feature shows that genetic isolation occurs within the *Rhizoctonia solani* population (Agrios, 1997).

The presence of anastomosis groups in *Rhizoctonia solani* is not linked to host specific interaction, however anastomosis groups do show certain defined tendencies, based on host range and disease symptoms(Agrios, 1997).

Anastomosis group 1 (AG1) causes mostly seed and hypocotyl rot as well as aerial sheath blight. AG2 is linked to canker in root crops, stem narrowing and brown patch on turf grass. AG3 affects mostly potato resulting in stem canker and stolon lesions with black sclerotia lesions. AG4 infects various plant species resulting in seed and hypocotyl rot of almost all angiosperms and stem lesions in sugar beet, cotton and legumes. Several other anastomosis groups exist in *Rhizoctonia* (Agrios, 1997) with 12 AG's officially being recognised (Nicoletti *et al.* 1999).

The infection of leaves by *Rhizoctonia* is usually characterised by reddish-brown, slightly sunk in lesions, which develop along petioles and midribs. These lesions tend to spread causing the entire leaf to become dark brown and slimy (Agrios, 1997).

In tobacco the symptoms on the foliage caused by *Rhizoctonia solani* are known as target spot. Target spot symptoms begin as small 2 – 3 mm round watersoaked spots (www.ces.ncsu.edu/depts/pp/notes/**Tobacco**/tdin007/tb07.html).

Under favourable conditions, constituting high temperatures and high humidity, these lesions can enlarge rapidly forming irregular, browning, watersoaked zones usually surrounded by chlorotic halos. The entire leaf can be destroyed (Agrios, G. N. 1997). In nature these lesions usually arise from the basidiospores of *Thanatephorus cucumeris* (sexual stage of *Rhizoctonia solani*).

2.4.2 Cultural and Chemical control

The control of *Rhizoctonia* is fairly difficult in part due to its soil borne origins. The most effective control measure are preventative practises, which include the use of clean soil free equipment in different growth areas, the use of disease free seeds and culture practices which ensure vigorous and fast growth of seedlings as well as providing good drainage and aeration at all times. Soil should also be sterilised if possible through steam or chemicals such as methyl bromide. The exclusion of herbicide applications on certain crops has also lead to decreased *Rhizoctonia* infections (Agrios, 1997).

In the event of an infection no effective fungicides exists that can cure infected plant material. Fungicide applications utilising contact fungicides such as iprodione and chlorothalonil or systemics such as carboxin and triadimefon appear to provide effective control in turf grasses (Agrios, 1997).

2.4.3 Biological control:

There are several mechanisms, which can reduce *Rhizoctonia* disease incidence through biological control. These include the introduction of mycophagous nematodes such as *Aphelenchus avenae* and mycoparasitic fungi such as *Trichoderma harzianum* (Lorito *et al.* 1998) to soil that can reduce *Rhizoctonia* by mycoparasitic interactions.

Rhizoctonia infections can also be reduced through *Rhizoctonia* decline. *Rhizoctonia* decline is caused by two or three infectious doublestranded RNA molecules that can be transmitted from hypovirulent *Rhizoctonia* to virulent *Rhizoctonia* during anastomosis. These doublestranded RNA molecules lead to a reduced infection capability of previously virulent *Rhizoctonia* as well as a reduced survival capability. Many of the abovementioned biological controls have however not been commercialised yet (Agrios, 1997).

2.4.4 Transgenic crops in *Rhizoctonia* control:

The application of transgenic plants to control *Rhizoctonia* infections has been explored.(Punja 2005) showed that the introduction of a thaumatin-like gene into carrot provided increased resistance to *Rhizoctonia solani* while in a similar study (Krishnan *et al.* 2006) showed that the thaumatin-like gene provided increased resistance to rice. (Patkar and Chattoo, 2006) demonstated that a nonspecific lipid transfer protein can improve *Rhizoctonia solani* resistance in rice and (Maddaloni *et al*. 1997) illustrated that tobacco expressing ribosome inactivating protein b32 provided tolerance to *Rhizoctonia solani.* The introduction of pokeweed antiviral protein II showed resistance in transgenic tobacco (Wang *et al*. 1998).

The use of chitinase transgenic plants confering resistance to *Rhizoctonia solani* has been explored. (Lorito *et al.* 1998) showed that the introduction of an endochitinase gene isolated from *Trichoderma harzianum*, a fungus used in biocontrol experiments, resulted in significant disease resistance increase of potato and tobacco to *Rhizoctonia solani*. A similar study done on transgenic cotton, utilising an endochitinase isloated from *Trichoderma viride*, showed a significant increase in resistance to *Rhizoctonia solani*(Emani *et al.* 2003). The combination of a constitutively expressed class II chitinase from barley with a class-II β 1,3 glucanase and a type I ribosome inactivating protein showed increased resistance to *Rhizoctonia solani* (Jach *et al.* 1995). Further transgenic chitinase trial applications to combat *Rhizoctonia solani*

include soybean (Salehi *et al.* 2005), potato (Chye *et al.* 2005), rice (Anand *et al*. 2003; Datta *et al.* 2002; Mao *et al.* 2003; Patkar and Chattoo, 2006), bentgrass (Hao *et al.* 2005), maize (Kim *et al*. 2003) and tobacco (Maddaloni *et al.* 1997; O'Brien, *et al*. 2001)

2.5 Methods for producing transgenic plants

2.5.1 Genetic transformation of plants

The successful introduction and expression of genes forms an essential component in molecular research on plants. The most important criteria include stable integration and consistent transformation. Several techniques will reviewed that can be used to transform plants

2.5.2 Agrobacterium transformation

The first evidence of *Agrobacterium tumefaciens* transformation of the T-DNA fragment into the genome of the host plant was found in 1977 (Chilton *et al.* 1977). This lead scientists to contemplate on the usefulness of Agrobacterium as a vector for recombinant gene transformation and in 1983 the first plant was successfully transformed (Zambryski 1983). Since then research has shown that Agrobacterium is not only limited to plant transformation but can also transform yeast, fungi and human cells (Lacroix *et al*. 2006).

The broad host range and well-studied transformation applications of *Agrobacterium* have made it the most favoured tool for genetic tranformation of plants. The wild type plasmids cannot be used directly for transformation purposes. This is because they are very large, making them difficult to manipulate. They also do not contain unique and multiple restriction enzyme sites and also contain tumor-inducing genes, which are not desirable. Instead certain important genes and sequences need to be incorporated into a specifically designed vector, called a disarmed vector, which eliminates the formation of tumors. These include the *vir* genes as well as the left and right Border sequences, which normally flank the tumorigenic genes and which define the inserted T DNA fragment. An overdrive sequence, flanking the right border repeat, is also necessary as it ensures high efficiency transfer (Peralta and Ream, 1985)

There are currently two types of vectors that can be used. The first is known as a cointegrate vector. The principle is based on subcloning the T-DNA into a conventional *E.coli* plasmid, thereby producing a so-called intermediate vector which is easier to manipulate (Matzke and Chilton, 1981). The recombinant plasmid is then produced, by allowing homologous recombination to occur between the disarmed *Ti* plasmid, containing the *vir* genes, an origin of replication and the left border, and the intermediate vector, which contains the gene of interest, the right border, a plant selectable marker, an origin of replication for *E.coli* as well as a bacterial selectable marker. The end result is a large cointegrate disarmed *Ti* plasmid.

The use of cointegrate vectors has however decreased in recent years, due to their large size and lack of unique restriction enzyme sites. They are also not able to replicate extensively in *Agrobacterium.* These features make the plasmids difficult to manipulate (Primrose *et al*. 2001).

The binary vector system is the alternative vector system, which is a lot smaller than the co-integrate vector and thus much easier to manipulate. The principle is based on the presence of a disarmed Ti plasmid in an *Agrobacterium* strain. This disarmed plasmid lacks the T-DNA region but the *vir* genes located on this plasmid can act in trans on any T-DNA fragment present in the *Agrobacterium* cell. The T-DNA is subcloned on a small *E. coli* plasmid that can easily be manipulated and it is called a mini or micro Ti plasmid. If the mini Ti plasmid, derived from *E.coli*, is transformed into *Agrobacterium* containing the disarmed plasmid, the T-DNA fragment on the mini plasmid can intern be transformed to the appropriate host plant (Bevan, 1984). The size of the T-DNA in this case can be up to 50 kb.

2.5.3 ‘Non Agrobacterium’ mediated genetic transformation:

Agrobacterium is not the only bacteria that can perform genetic transformation. (Broothaerts *et al*. 2005) showed that three other bacteria species *Rhizobium sp.* NGR234, *Sinorhizobium meliloti* and *Mesorhizobium loti* are also capable of gene transfer to plants. This was achieved by transforming these species with the virulence genes encoded by the Agrobacterium T*i* plasmid and a small binary vector containing a T DNA segment.

The non-agrobacterium technology is still new and is being further developed at the present time. It promises to alleviate some problems associated with Agrobacterium

mediated transformation. These include difficulties in Agrobacterium mediated monocot transformation, bypassing many patents as well as overcoming Agrobacterium-tissue incompatibility, which can occur in some plants.

2.5.4 Biolistic transformation

The use of biolistics transformation is second to Agrobacterium transformation in application. The technology plays an important role in transforming recalcitrant crops, which are difficult or impossible to transform with Agrobacterium mediated transformation. Recalcitrant crops include cereals (f.e. rice and wheat) and legumes (f.e. soybean). The importance of biolistics can also be seen as it provided the first transgenic maize, wheat and soybean plants (Taylor and Fauquet, 2002).

Biolistic transformation is based on the forceful introduction of a highly inert; target DNA covered microprojectiles such as gold or tungsten beads into plant cells. Not all plant cells are receptive for the technology and need to be carefully selected based on the following characteristics (Taylor and Fauquet, 2002):

1) the tissue must be capable of receiving and integrating the delivered DNA.
2) the tissue must be receptive to undergo transgenic selection for transformants.
3) the tissue must be totipotent.

Tissues such as meristems and embyogenic tissues meet the above-mentioned criteria and allows the transgenes to integrate in the genome (Finer *et al*. 1999a).

The technique is however limited in that large DNA fragments are only rarely successfully integrated (Hansen and Wright, 1999) and that multicopy and superfluous DNA insertions are commonly found (Finer *et al.* 1999b). Due to the force of the bombardment, biolistics can also result in broken transgene fragments being formed and integrated in the genome (Taylor and Fauquet, 2002). With the availability of more virulent *Agrobacterium* strains that can traget previously recalcitrant crops and the above-mentioned problems with biolistics many transformation experiments are nowadays being done with *Agrobacterium*. Biolistic technology does however still remain viable for certain recalcitrant crops.

2.5.5 Protoplast transformation

Protoplast transformation is a highly technical technique that is based on the generation of individual plant cells, which lack cell wall structures. The removal of

the cell walls is achieved either through the use of mechanical or enzymatic processes. The protoplasts cells can then be easily transformed either through *Agrobacterium* mediated transformation or with the use of direct DNA uptake enabled by either liposomes, electroporation or poly ethylene glycol treatment (Saul *et al*. 1987)

2.6 Constitutive vs Inducible genetic regulation in transgenic plants

The design of an expression vector is dependent on a variety of genetic elements that will allow its proper functioning in an organism. One such element is a promoter, which regulates gene transcription. These promoter elements can be either constitutive or inducibly expressed.

2.6.1. Constitutive promoters

The use of constitutive promoters is well established in the engineering of disease resistance in plants and can allow constitutively active transcription of the gene. Commonly used promoters in dicotyledonous plants include the CaMV35S promoter (Smirnov *et al.* 1990) derived from the Cauliflower Mosaic Virus, and in monocotyledons the ubiquitin promoter derived from maize (Toki *et al.* 1992). In practice however the promoters can show expression differences in different tissues and also under different stimuli. This is due to the transformation processes were foreign DNA is randomly integrated into the genome with the integration site, copy number and configuration of the gene as well as environmental and metabolic factors all being able to influence the transgene expression (Cervera *et al.* 2000; Fladung, 1999; Levine *et al*. 1999). The use of constitutive promoters to express disease resistance gene can also result in plant abnormalities such as reduced size (Chen and Chen 2002), altered morphology (Li *et al*. 2004) and symptom development in the abscense of pathogens (Heather *et al.* 2004). The strong constitutive expression of a gene can also increase fitness costs as resources and energy reserves are diverted in the continous synthesis during gene expression (Pasonen *et al.* 2004). The high level of proteins present can also interfere in cell functioning (Purrington and Bergelson, 1997).

2.6.2 Inducible promoters

Alternatives to constitutive promoters are inducible promoters. The use of inducible promoters is still being investigated but there are several options that can be explored to activate disease resistance. One of the most relevant options are pathogen inducible promoters.

The ideal pathogen inducible promoter would be able to be activated by a broad range of pathogens, thereby allowing a broadspectrum response. However the different signalling pathways activated by the different biologies of pathogens(Glazebrook, 2005) can prevent broadspectrum activation. Many of these promoters are active under disease free conditions (McDowell and Woffenden, 2003), causing a leaky expression of the transgene. Very few promoters have thus proved suitable for biotechnological applications. Currently a lot of research is being done in transcriptomics, which identifies up and down regulated genes in plant pathogen interactions. Genome walking could thus be used to identify the promoters driving the gene expression and with this technique approximately fifty candidate promoters have been identified in Arabidopsis (Eulgem, 2005).

The use of inducible promoters in disease resistant transgenic plants has been investigated. (Keller *et al*. 1999) showed that the use of the pathogen inducible promoter *hsr 203J* promoter driving a *Phytophthora cryptogea* gene encoding the highly active elicitor cryptogein, was able to provide increased resistance towards *Thielaviopsis basicola, Erysiphe cichoracearum*, and *Botrytis cinerea* in tobacco. The promoter was native to tobacco and, as expected, silent during uninduced conditions.

A study by (Yevtushenko *et al*. 2005) showed that the pathogen inducible promoter *win3.12*T from poplar driving the expression of a cecropin A-melittin hybrid gene produced strong antimicrobial resistance in tobacco. The promoter was shown to be inducible and functional in the tobacco after wounding, with the expression being systemic. The transgenic plants showed increased *in vitro* resistance to *Fusarium solani* challenges.

2.7 Summary

This chapter provided background information covering the basics of plant defense response in the presence of a fungal pathogen. It reviewed the roles that Glutathion-*S*-transferases play during active oxygen species signalling highlighting the role of the *At*GSTF6 promoter. It also reviewed pathogenesis related proteins with emphasis placed on chitinases and their role in plants. The review also addressed the fungal pathogens known to attack tobacco and included an in-depth analysis of *Rhizoctonia solani*, used for pathogen assays in this study. A brief overview of issues relating to the production of transgenic crops as well as expression systems used, was presented.

Chapter 3

Materials and Methods

The reagents and chemicals used were either of analytical or molecular biology grade. All aqueous buffers and solutions were prepared with double distilled water. All buffers, solutions and media components are outlined in Appendix A.

All chemicals used were supplied by Sigma (Sigma, Minneapolis, USA) unless otherwise indicated.

Restriction enzymes, RNase A and antibiotics were obtained from Roche Diagnostics (Roche, Indianapolis, USA) unless otherwise indicated.

All plasmid maps are shown in Appendix B.

All primer sequences are shown in Appendix C.

3.1 Plasmid DNA isolation

Minipreparations of plasmid DNA were done with *E.coli* (JM 109) using Qiagen Mini plasmid purification kits and manual instructions (Qiagen, Hilden, Germany), adapted from Birnboim and Doly (1979). Bacterial colonies were grown overnight at 37 °C with shaking in 50 ml Luria Bertani (LB) broth (see Apppendix A.1) with appropriate antibiotics (100µg/ml ampicillin for pBluKSII (-) IF3 and pUC19 constructs, and 50µg/ml ampicillin for the pRTL2-GUS and pCAMBIA 2300 constructs. Plasmid DNA concentrations were determined using the Nanodrop Spectrophotometer (Nanodrop, Delaware, USA) according to manual specifications.

3.2 Nucleotide sequence analysis

Sequence analysis was performed to determine the correct orientation of the insert and sequence integrity. The constructs pUC19-*At*gstF6-IF3-CaMV35S terminator as well as pCAMBIA2300-*At*gstF6-IF3 were sequenced with the Big Dye Terminator ver. 3.1 (Applera Corporation, Foster City, California, USA) kit at the sequencing facility at the University of Pretoria. The sequencing reaction consisted of quarter reactions containing 2 µl of BigDye ready reaction mix (Applera Corporation, Foster City, California, USA), 10 picomol primer, 2 µl 5 x dilution buffer and 100 ng DNA template per 1000bp DNA fragment. The reaction volume was made up to 10 µl using double distilled water. The cycle sequencing for all reactions was as follows; 25 cycles with each cycle consisting of 10 seconds at 96 °C, 5 seconds at 50 °C and 4 minutes at 60 °C. The sequencing reaction was cleaned by adding 2 µl of 3 M sodium acetate (pH 4.6) and 50 µl 95 % Ethanol to the completed sequencing reaction mix. The solution was centrifuged at 13000 x*g* for 20 minutes at 4 °C, followed by the removal of the supernatent. The pellet was washed with 250µl of 70 % ice cold Ethanol, centrifuged for 5 minutes at 13000*g* and the supernatent aspirated from the tube. The dried pellet was handed in at the sequencing facility at the University of Pretoria. was done the primers M13 Forward, M13 Reverse, ClaI-IF3 and SalI-IF3 primers. The sequences were analysed and aligned with the Geneious version 1.2.1 free software (Biomatters and Vector NTI 6.0.)

3.3 Preparation of competent *Escherichia coli* JM109 cells

Overnight cultures of *E.coli* JM109 were resuspended 1:100 in LB medium and incubated at 37 C° until an OD_{600} of 0.3 to 0.4 was reached. A 1 x vol of TSS solution (see Appendix A.2) was added and the cell suspension was mixed on ice. The competent cells were stored at -70 °C.

3.4 Transformation of ligation reaction into *Escherichia coli* JM109

Competent *E.coli* JM109 cells were thawed on ice. One hundred microliters of competent cells were transformed with 30 ng of ligated product/plasmid by addition of the DNA to the cells. The cells were incubated at 4 °C for 5 minutes. Ninehundred microliters of LB (see Appendix A.1) containing 20 mM glucose was added to the cell suspension, which was grown for 60 minutes at 37 °C. The cell suspension was plated out on LB agar plates (see Appendix A.1) containing the appropriate selective agents and grown overnight at 37 °C.

3.5 PCR and restriction enzyme screening of transformed *Escherichia coli* JM109

Colony PCR was performed by picking colonies with a sterile toothpick and suspended in 1ml of double distilled water by vortexing for 1 minute. One microliter of the mixture was used for PCR amplification with a 1 x PCR buffer (Bioline, London, England) using 1 Unit Taq polymerase (Bioline, London, England). PCR reactions were performed with an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing for 30 seconds (temperature depends on primers) and elongation at 72 °C for 45 seconds. A final extension step at 72 °C for 5 minutes was included. Primers used were M 13 Forward and M 13 Reverse, and products were run on a 1.5 % (w/v) agarose gel.

Plasmid DNA was isolated from PCR positive bacterial clones and digested with enzymes that specifically excised the inserts from the plasmids. The digestion mix was resolved on a 1.5 % (w/v) agarose gel.

3.6 Construction of the pUC19-*At*gstF6-IF3 construct

The *Lupinus albus* IF3 chitinase gene (accession number CAA 76203; donated by Regalado, Lissabon, Portugal) was excised from the plasmid pBluKSII (-) IF3 (see Appendix B.2) with the restriction enzymes *Eco* RI and *Kpn* I. The *Arabidopsis thaliana* *At*GST*F6* promoter (accession number Y11727; donated by Loake, Edinburgh, United Kingdom) was also cleaved from AtgstF6-prom-applePGIP cassette-pCAMBIA2300 (Maritz, ARC-Roodeplaat, South Africa)(see Appendix B.3) constructed from pCAMBIA2300 (www.cambia.org) (see Appendix B.1) using *Sac* I and *Eco* RI. The pUC19 plasmid (see Appendix B.4) was digested with *Sac* I and *Kpn* I. Each restriction enzyme digestion contained 2 Units of restriction enzyme and 2.5 µg of DNA made up to a reaction volume of 20 µl with 1x restriction buffer. The reaction was incubated for 1 hour at the specified temperature for the restriction enzymes. The digested products were separated through agarose gel electrophoresis (0.8 % w/v) and desired fragments were isolated from the gel with a Qiagen Gel extraction purification kit (Qiagen, Hilden, Germany). A three-way ligation with the 1075 bp chitinase gene fragment, the 916 bp *AtGSTF6* promoter fragment and the digested pUC19 plasmid was set up and incubated overnight at 15 °C with 1U of T4 DNA ligase (Fermentas Life Science, Ontario, Canada) in 20µl reaction volume. Different ratios of insert : vector were used namely 3:1, 1:1, 1:3, 8:1. Ligated products were transformed into competent *E.coli* JM109 as described in 2.4. Successful constructs yielded the plasmid pUC19-*At*gstF6-IF3 (see Appendix B.5).

3.7 Construction of the pCAMBIA2300-*At*gstF6 -IF3 binary vector

The 222 bp CaMV35S terminator fragment excised from pRTL2-GUS INT plasmid (Berger unpublished) constructed from pRTL2 (donated by Carrington, A & M University, Texas, USA; see Appendix B.6) with *Xba* I and *Pst* I restriction enzymes. The digested plasmid was run on a 0.8 % (w/v) agarose gel. The pUC19-*At*gstF6-IF3 plasmid was digested with *Xba* I and *Pst* I restriction enzymes and ligated to

CaMV35S overnight at 15 °C with 1U of T4 DNA ligase (Fermentas Life Science, Ontario, Canada). The ligated product was transformed into competent *E.coli* JM109

cells, and presence of CaMV35S terminator verified with plasmid restriction enzyme digestion with *Xba* I and *Pst* I.

The pUC19-*At*gstF6-IF3-CaMV35S terminator (Appendix B.7) was digested with *Sac* I and *Pst* I, which yielded a 2233bp fragment containing the *At*GSTF6 promoter, IF3 chitinase gene and CaMV35S terminator. pCAMBIA2300 was linearised by digestion with *Sac* I and *Pst* I into which the 2233bp fragment was inserted. The fragments were ligated overnight at 15 °C with 1U of T4 DNA ligase (Fermentas Life Science, Ontario, Canada) to produce the pCAMBIA2300-*At*GSTF6-IF3 plasmid (see Appendix B.8). The ligated product was cloned into JM109 competent *E.coli* cells, and presence of the fragment verified with plasmid restriction enzyme digestion with *Eco* RI and *Hind* III.

3.8 Preparation of competent *Agrobacterium tumefaciens* LBA 4404

A. tumefaciens LBA4404 (Ooms *et al.* 1982) cultures were streaked out on LB agar plates containing 50µg/ml rifampicin. The cultures were grown for 48 hours at 27 °C in dark conditions. One colony was picked and inoculated in 5ml LB liquid media containing 50µg/ml rifampicin for 48 hours on a shaker. LB liquid media containing 50µg/ml rifampicin was reinoculated with the starter culture and was grown at 27 °C. The optical density OD_{680} of the culture was analysed using the Nanodrop spectrophotometer and was checked until readings fell between 0.5 and 1. The cell cultures were briefly chilled before being centrifuged for 10 min at 4 °C at 1600 *g*. The supernatant was discarded and the cell pellet resuspended in 1.5 x vol of ice cold $CaCl_2$. Cells were aliquoted in 0.3ml batches in frozen 1.5ml Eppendorf tubes and frozen at –80 °C until required.

3.9 Transformation of *Agrobacterium tumefaciens* LBA 4404

The binary vector pCAMBIA2300-*At*gstF6-IF3 was mobilized into competent *Agrobacterium tumefaciens*-disarmed helper strain LBA4404 using the plasmid layering method (Ooms et al. 1982). Five micrograms of binary construct were layered on 300 µl of frozen competent *A. tumefaciens* cells. The mix was allowed to thaw by incubation at 37 °C for 5 minutes, followed by the addition of 1 ml of LB liquid media. Onehundred microliter aliquots were plated out on LB agar and transformants were selected using 50 µg/ml rifampicin and 25 µg/ml kanamycin.

3.10 PCR screening of transformed *Agrobacterium tumefaciens* LBA 4404

Positive colonies were verified for plasmid presence using colony PCR with nptII forward and reverse primers (see Appendix C) and ClaI IF3 and SalI IF3 primers (see Appendix C). The following thermal cycling conditions were employed: denaturation for 2 minutes at 94 °C, 30 cycles of 20 seconds denaturation at 94 °C, followed by 30 seconds annealing at 63 °C for the ClaI-IF3 and SalI-IF3 primers and 72 °C for the *nptII* forward and reverse primers and 45 seconds elongation at 72 °C. A final extension time of 7 minutes at 72 °C was added. PCR products were visualised using gel electrophoresis.

3.11 Tobacco transformation

Tobacco (*Nicotiana tabacum* cv JR6) seeds were kindly provided by Dr Bridget Crampton (CSIR, Pretoria). These plants were previously transformed with an apple polygalacturonase inhibiting protein (*pgip1*) and seeds obtained were in the T1 generation for apple *pgip1*.

The seeds were sown on ½ strength MS media (see Appendix A.3). The tissue culture seedlings were used for *Agrobacterium* mediated leaf disk transformation (Horsch *et*

al. 1985). The leaf disks were cut using a scalpel in 1 x 1 cm leaf disks. The leaf explants were immersed in the prepared *Agrobacterium tumefaciens* cell suspension with an optical density of OD_{620} of 0.68 for 20 minutes. Nontransformed *Agrobacterium* was used as a negative control. Thereafter the leafdisks were blotted on sterile filterpaper to remove excess bacteria before being replated onto regeneration media. Transgenic tobacco was regenerated from transformed leaf disks following selection on regeneration media (see Appendix A.4) supplemented and selected with 100 µg/ml Kanamycin. The rooted shoots were hardened of in a growth room in 1 part coconut peat and 1 part silica sand with the temperature maintained between 24 °C and 26 °C and 12 hour dark/light cycle.

3.12) PCR screening for *Agrobacterium tumefaciens* contamination

Agrobacterium tumefaciens contamination was determined using Mia Forward and Reverse primers (see Appendix C), which are specific for Agrobacterium detection. The protocol followed was for direct leaf PCR. The composition for this type of PCR per reaction was as follows; 1 x PCR buffer (Bioline, London, England), 0.10mM dNTP, 1.5mM $MgCl_2$, 1.25 mM forward and reverse primers, 2 units Bioline Taq (Bioline, London, England) made up to a reaction volume of 50µl with ddH_2O. A leaf disk of 1x1 mm was punched out and added to the mix before proceeding with the reaction. The following thermal cycling conditions were employed: denaturation for 3 minutes at 94 °C , 35 cycles of 20 seconds denaturation at 94 °C, followed by 30 seconds annealing at 72 °C and 45 seconds elongation at 72 °C. A final extension time of 7 minutes at 72 °C was added. PCR products were visualised using gel electrophoresis.

3.13) RNA extraction

Plant leaf tissue was sprayed with 2mM hydrogen peroxide and left for 1 hour before being harvested. Total RNA was extracted from the leaf tissue using the PLANT RNA Purification Reagent (Invitrogen, Carlsbad, California, USA). RNA quality was assessed by agarose gel electrophoresis or Nanodrop spectrophotometry (supplied by Inqaba Biotechnical Industries, Pretoria, South Africa). Total RNA was treated with 1 Unit RNase free DNaseI (Fermentas Life Science, Ontario, Canada) for 30 minutes at 37 °C. The RNA was cleaned with 1/10 3M sodium acetate pH 5.2 and 2.5 times volume of 100% ethanol and washed with 75% Ethanol. Cleaned total RNA samples were stored at –80 °C in RNase-free water until used.

3.14) cDNA synthesis

RNA was treated with RNase free DNaseI. The RNA was first quantified using the Nanodrop spectrophotometer and 3 µg of total RNA loaded. The sample was made up to 17 µl with distilled water followed by the addition of 2 µl of DNaseI reaction buffer (see Appendix A.5), 1 Unit of DNaseI (Fermentas Life Science, Ontario, Canada) and 0.2 µl of RNase inhibitor. The reaction was incubated at 37 °C for 30 minutes. The single stranded cDNA was synthesised from 1.5 µg total DNaseI treated RNA using the ImProm-II^{TM} Reverse Transcriptase System kit (Promega, Madison, USA) using Oligo $(dT)_{15}$ primers to selectively amplify mRNA. The synthesis cycles were 25 °C for 10 minutes, 42 °C for 60 minutes and 70 °C for 15 minutes.

The cDNA was assayed for DNA contamination using an intron-exon spanning primer pair: Actin F (Forward) and Actin R (Reverse) originally designed for *Musa x paradisiaca* (donated by Dr N. van den Berg, University of Pretoria, South Africa) (see Appendix C). The expression of the IF3 chitinase was determined using a PCR containing 20 nanograms of tobacco cDNA under the following thermal conditions; denaturation for 2 minutes at 94 °C, 30 cycles of 20 seconds denaturation at 94 °C, 30 seconds annealing at 60 °C and 45 seconds elongation at 72 °C. A final extension time of 7 minutes at 72 °C was added. The primers used were ChiF RT and ChiR RT (see Appendix C). Products were analysed on 1.5 % agarose gel electrophoresis.

3.15) Crude protein extractions from *Nicotiana tabacum* cv JR6 for chitinase assays

Plants that were treated with hydrogen peroxide were sprayed with 2mM hydrogen peroxide until the leaf was completely wetted. Total protein was extracted from T_0 plants by grinding the young leaves in liquid nitrogen and placing 1 gram of grindate into 6.25 ml of Buffer, consisting of 0.5 M sodium acetate pH5.2, 0.1 % β mercapto-ethanol and 1% polyvinylpolypyrrolidone. The mixture was centrifuged for 20 minutes at 4 °C in the Eppendorf Centrifuge 5810 R (Eppendorf, Hamburg, Germany) at 18000 *g*, and the supernatant was precipitated with 75% ammonium sulfate. The solution was left overnight at 4 °C to precipitate. The precipitate was centrifuged for 30 minutes at 18000 x*g* at 4 °C. The pellet was resuspended in 2 ml 20mM sodium acetate pH 5.2, and was dialysed overnight using a 10000 MWCO Snakeskin pleated dialysis tubing (Pierce, Illanois, USA) in double distilled water.

3.16) Protein concentration determination

After dialysis the protein concentration was determined using the Bio-Rad Bradford Reagent kit (Biorad, Hercules, California, USA) for microtitre plate applications as outlined by the manufacturer. The protein concentration was determined with 595nm Optical Density spectrophotometry using the Multiskan Ascent spectrophotometer (Thermo Labs, New York, USA). Bovine serum albumin (BSA) was used to generate the standard curve using the known BSA concentrations of 0.05, 0.1, 0.35 and 0.5 mg/ml.

3.17) Chitinase assay

The chitinase assays were performed on total protein extractions. Twenty microgram of total protein was serially diluted in 96 well V bottom plates (Greiner Bio-one, Kremsmuenster, Austria) in the order of $(1/2)^n$ with a dilution series of n = 11 and a final volume of 100µl per well. The serial protein dilution was suspended in a final concentration of 0.05 M sodium acetate pH 5.2 and 50 µl CM-Chitin-RBV solution

(LOEWE blue substrates, Sauerlach, Germany) to yield a final volume of 200 µl/well. The plates were incubated for 3 hours at 37 °C. The reaction was terminated with the addition of 50 µl of 1M HCl. The plates were chilled on ice for 20 min and then placed in a -20 °C freezer for 20 minutes followed by centrifugation of 1520 *g* for 15 minutes to sediment undigested substrate. Thereafter 175 µl of supernatant was transferred to 96 well flat bottom polypropylene plates (Greiner Bio-one, Kremsmuenster, Austria) and the absorbance measured at 550nm using the Multiskan Ascent (Thermo Labs, New York, USA).

3.18) Chitinase data analysis

The chitinase enzyme activity was defined as the amount of enzyme needed to yield an absorbance of 0.1 at 550nm absorbance spectrum (Appel *et al.* 1995). The amount of chitinase was expressed on a linear scale as percentage of the undiluted fraction of the total protein extract. The graph generated was used to plot a linear trend line in Microsoft Excel (Microsoft Office 2000). A trend line formula was generated and used to determine enzyme unit activity according to the definition. The unit activity obtained for a transgenic line was reported as a fraction of the untransformed control unit activity. The fraction difference between the transformed line and the untransformed line was plotted using a histogram chart in Microsoft Excel.

3.19) Detached leaf assays

The *Rhizoctonia solani* AG2 isolate was kindly donated by the Centraal Bureau vir Schimmel Kultuuren, Wageningen (see Appendix D) and was initially isolated from *Nicotiana alata* in 1999 (CBSnr 101772). The fungal cultures were maintained on Oatmeal Agar (OA) (see Appendix A.5).

For the pathogen assays, the *R.solani* isolate was inoculated into Czapex Dox containing 100 µg/ml ampicillin to inhibit bacterial growth. The culture was incubated in dark room conditions and shaken at 125 rpm at 24 °C for 5 days after which equal sized pieces of mycelium were removed and freshly inoculated in pectin medium (see Appendix A.9). The flasks were kept at 24 °C in dark room conditions

and shaken at 125 rpm for 7 days. The cultures were then filtered through Whatman #1 filter paper (Whatman, Middlesex, United Kingdom) and weighed. After weighing, the fungal mass was transferred to distilled water and immediately homogenised in a Waring blender until the fungal mass appeared homogenised.

Young and fresh leaves were harvested from rooted tobacco plants. The leaves were placed onto 0.8% water agar in Magenta tissue culture vessel (Sigma, Minneapolis, USA). Each leaf was inoculated with 0.1 mg of blended *R.solani* culture, which was continuously stirred during the application of inoculum to prevent settling of the blended culture. The inoculation was done on the adaxial side of the leaf and four equidistant inoculum drops were placed onto the leaf. The leaves were incubated in constant cool white fluorescent light and kept at 24 °C. Seven days post inoculation, the leaves were photographed. The infected leaves were then transferred into a plastic tub and covered with diluted trypan blue solution (see Appendix A.6) and were placed in a water bath and boiled for 1 minute. The tissue was left in the solution overnight. The next day the staining solution was replaced with saturated chloral hydrate solution to destain the tissue and tissue was stored in the chloral hydrate solution (see Appendix A.7) at room temperature.

3.20) Analysis of the lesion size

The destained tissue was placed onto a water soaked Whatman #1 filter paper (Whatman International) in a Petri dish and analysed using the Versadoc (Biorad, Hercules, California, USA) with Quantity One Software (Biorad, Hercules, California, USA). The settings for all photos were as follows; Exposure time of 12 seconds, area of photograph 900 x 600 mm, Coomassie blue setting and Image colour = spectrum. Lesion areas were identified by eye and marked with the volume contour tool which coalesed all pixels with same colour intensity and measuring the number of pixels inside the generated area.

Chapter 4

Results

4.1) Development of a binary vector with a T-DNA cassette containing a *Arabidopsis thaliana AtGSTF6* promoter, *Lupinus albus* IF3 chitinase gene and Cauliflower Mosaic Virus 35S terminator.

The gene construct, containing *AtGSTF6* promoter and IF3 chitinase, was first cloned into the multiple cloning site of pUC19 to obtain a larger range of restriction enzyme sites. Figure 4.1 shows the digested plasmid products obtained for pKSII (-) IF3 (Genbank accession number CAA 76203; donated by Regalado, Lissabon, Portugal), pUC19 and *At*gstF6-prom-applePGIPcassette-pCAMBIA2300plasmid (*AtGSTF6* Genbank accession number Y11727; donated by Loake, Edinburgh, United Kingdom) (pCAMBIA2300 plasmid donated by CAMBIA®, Canberra, Australia, www.cambia.org). More specifically, lane 3 shows the release of the IF3 chitinase gene (1075 bp product) excised using *Kpn* I and *Eco* RI while lane 7 shows linearised pUC19 plasmid (2680 bp product) digested with *Sac* I and *Kpn* I. Lane 11 shows the *AtGSTF6* promoter (916 bp product) released from *At*gstF6-prom-applePGIPcassette-pCAMBIA2300 plasmid after *Sac* I and *Eco* RI digestion. A product of slightly larger size is observed in Lane 13 (926 bp) that also releases the *AtGSTF6* promoter with only *Eco* RI digestion. The *AtGSTF6* promoter fragment (916 bp fragment) and IF3 chitinase fragment (1075 bp fragment) were ligated into linearised pUC19 and transformed into competent JM109 *Escherischia coli* cells.

White colonies, indicative of clones harbouring recombinant plasmids containing an insert, were selected and PCR screened with M13 Forward and Reverse primers. PCR product was run on an agarose gel and visualised under UV light (Figure 4.2). The

colony PCR was not very successful due to the large size of the fragment amplified, with only 1 of 16 colonies yielding a positive result (lane 10, fragment 2003 bp).

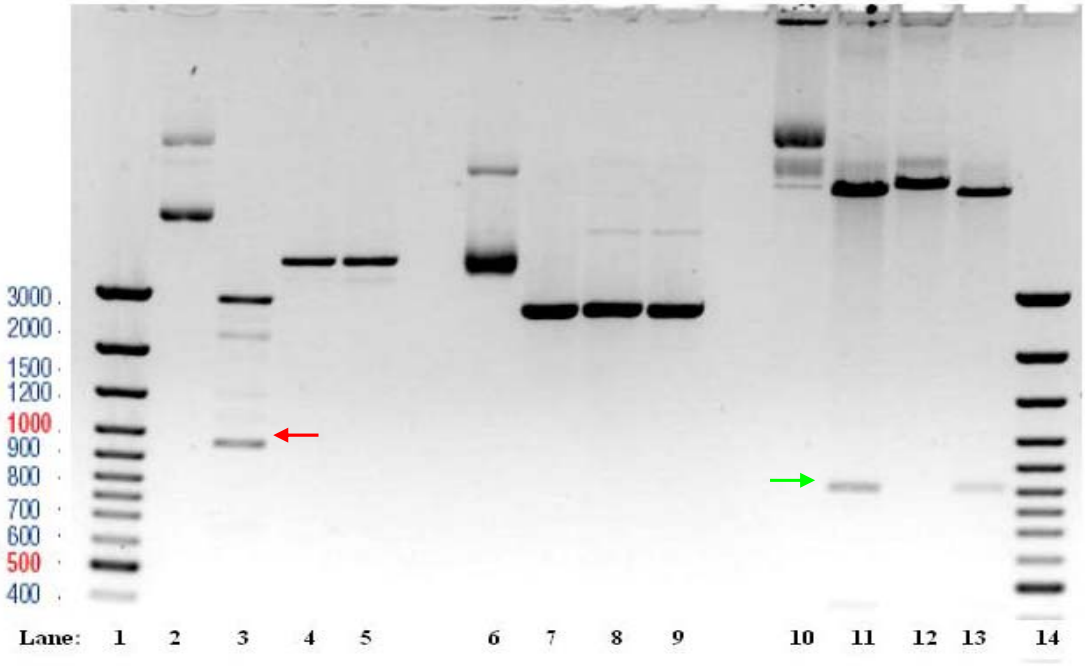


Figure 4.1: Gel electrophoresis of digested cloning plasmids. Lane 1 and 14, 100 bp DNA ladder plus (Fermentas); lane 2, undigested plasmid pKSII (-) IF3; lane 3, *Kpn* I and *Eco* RI digestion of pKSII (-) IF3 with IF3 gene fragment clearly visible (red arrow indicating 1075bp fragment); lane 4 and 5, showing *Kpn* I and *Eco* RI digestion of pKSII (-) IF3; lane 6, undigested pUC19, lane 7 showing *Sac* I and *Kpn* I digestion of pUC19; lane 8 and 9, *Sac* I and *Kpn* I digestion of pUC19 respectively; lane 10, undigested *At*gstF6 prom-applePGIP cassette-pCAMBIA2300plasmid; lane 11, (green arrow indicating a 916bp promoter fragment) *At*gstF6 prom-applePGIP cassette-pCAMBIA2300 plasmid digested with *Sac* I and *Eco* RI showing 916 bp *AtGSTF6* promoter fragment; lane 12 and 13, *Sac* I and *Eco* RI digestion of GST 1 prom-applePGIP cassette-pCAMBIA2300 respectively. Lane 13 yields a 926 bp fragment, which contains the *AtGSTF6* promoter excised with *Eco* RI.

Some colonies were therefore selected, regrown and plasmid isolated and digested with *Eco*RI to confirm insertion. Figure 4.3 shows 2 regrown colonies yielding plasmids with the correct insert being released when digsted with *Eco* RI, shown in lane 2 and 3 (926 bp fragment).

The CaMV35S terminator was then cloned downstream of the IF3 gene into the pUC19-*At*gstF6-IF3 plasmid to complete the cloning cassette, yielding the pUC19-*At*gstF6-IF3-CaMV35S plasmid (Appendix B.7). The construct was not checked using colony PCR with M13 Forward and Reverse primers as the amplified product of 2238 bp is too large for PCR amplification (as shown in Figure 4.2), therefore the plasmid was checked by restriction enzyme mapping. Figure 4.4 shows the excision of the CaMV35S terminator using *Xba* I and *Pst* I from plasmids amplified and extracted from 2 selected colonies. The sizes correspond to the expected size of 222 bp (Lane 2 and 5). In the last cloning step the cloning cassette, composed of the *AtGSTF6* promoter, IF3 chitinase and CaMV35S promoter, was cleaved out of the pUC19-*At*gstF6-IF3-CaMV35S plasmid and CaMV35S terminator plasmid and ligated into pCAMBIA 2300 to yield the pCAMBIA2300-*At*gstF6 -IF3 plasmid. Bacterial clones were selected using blue/white selection and the ligation confirmed with restriction enzyme mapping. One bacterial clone contained the insert. Figure 4.5 shows that the nonlinearised plasmid of pCAMBIA 2300 (lane 3) and pCAMBIA2300-*At*gstF6-IF3 (lane 4) exhibit a noticeable size difference. The restriction enzyme analysis of the plasmid isolated from the bacterial clone, shown in Figure 4.6, indicated that it was pCAMBIA2300-*At*gstF6-IF3 as the *Eco* RI digestion product yielded a 926 bp product containing the *AtGSTF6* promoter (lane 1) and 2 restriction enzyme products of 656bp and 1491 bp size (lane 3).

pCAMBIA2300-*At*gstF6-IF3 plasmid, was isolated from *E.coli* and transformed into *A. tumefaciens* LBA 4404. Colony PCR of transformed Agrobacterium clones showed high transformation efficiency with only 2 colonies out of 17 not being positive for the chitinase PCR product (Figure 4.7). Colonies 1, 4, 5 , 12 and 17 were checked for the presence the *npt*II (Kanamycin resistance) gene (Figure 4.8)

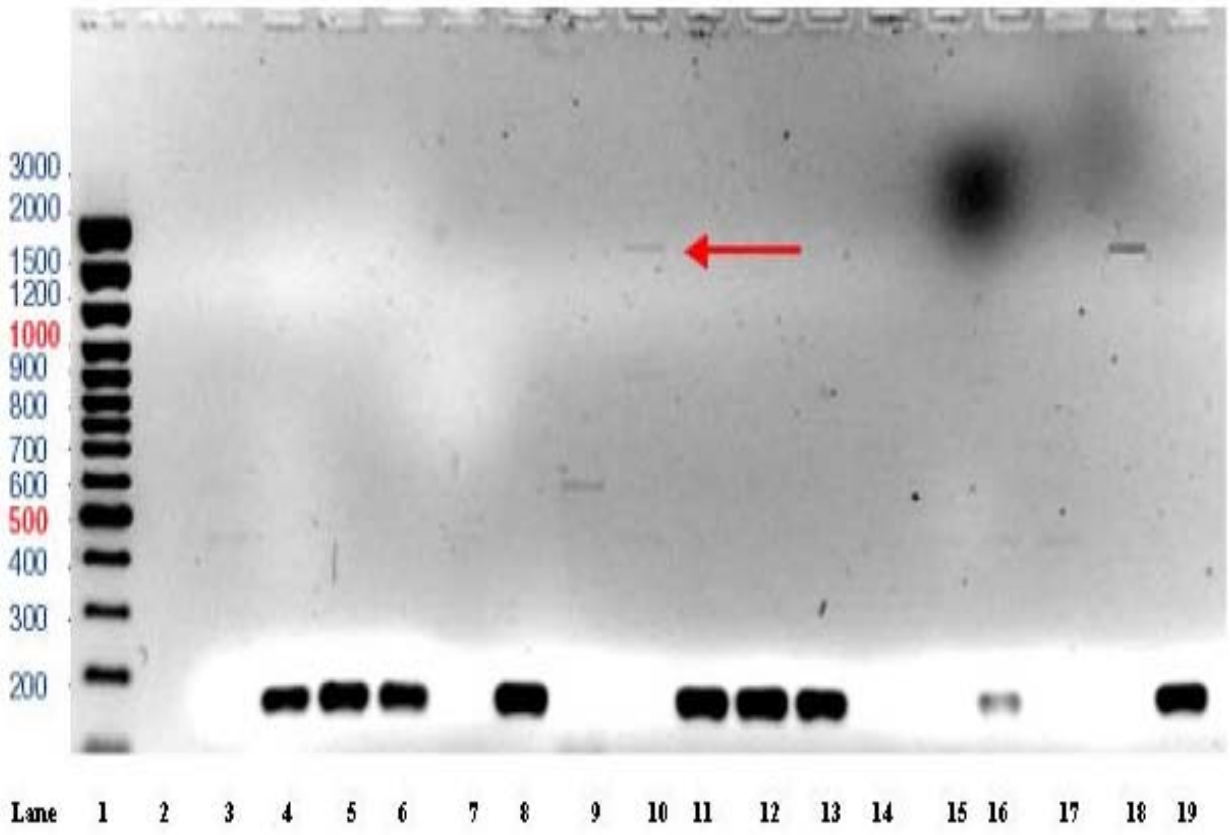


Figure 4.2: Gel electrophoresis of PCR amplification from colonies using M13 Forward and Reverse primers showing amplified gene product (red arrow showing 2003 bp fragment). Lane 1, 100 bp DNA ladder plus (Fermentas); Lane 2 to 17, colony PCR of selected colonies; Lane 10, indicates a positive colony with the other lanes not showing no amplicication product, Lane 18 is a positive colony PCR control and lane 19 is a negative PCR control with untransformed *E.coli* JM 109.

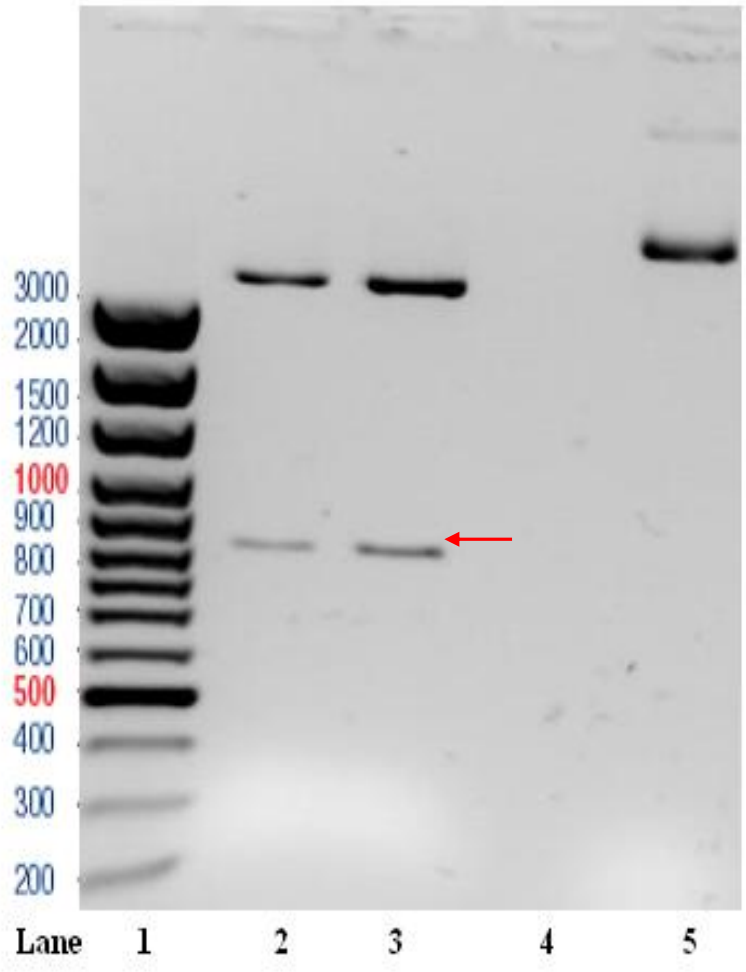


Figure 4.3 Restriction enzyme digestion of plasmids isolated from blue/white selection of positive clones. Lane 1, 100 bp DNA ladder plus (Fermentas); lane 2 and 3, pUC19-*At*gstF6-IF3 plasmids from different clones cut with *Eco*RI to release the *At*gstF6 promoter (red arrow showing 926 bp fragment); lane 4, empty and lane 5 shows uncut pUC19 -*At*gstF6-IF3 plasmid.

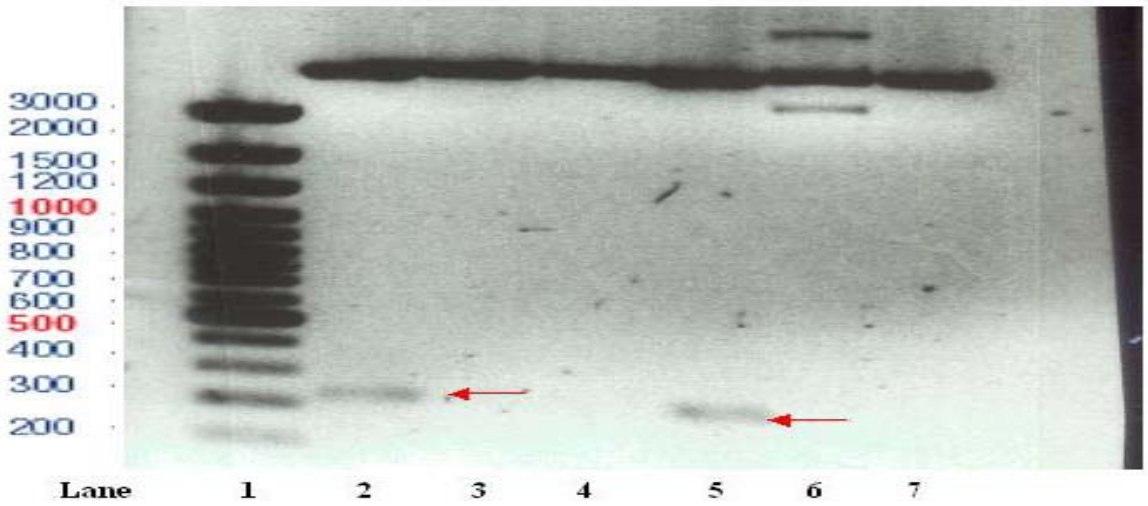


Figure 4.4: Restriction enzyme digest of pUC19-*At*gstF6-IF3 and CaMV35S terminator with *Xba* I and *Pst* I. Lane 1, 100 bp DNA ladder plus (Fermentas); Lane 2 and 5, pUC19-*At*gstF6-IF3-CaMV35S terminator plasmid, isolated from two white colonies selected with blue/white selection, cut with *Xba* I and *Pst* I, which releases the CaMV35S, terminator (red arrow showing 222 bp fragment); Lane 3 and 6, the plasmid linearised with *Xba* I and lane 4 and 7 represent the plasmid cut with *Pst* I.

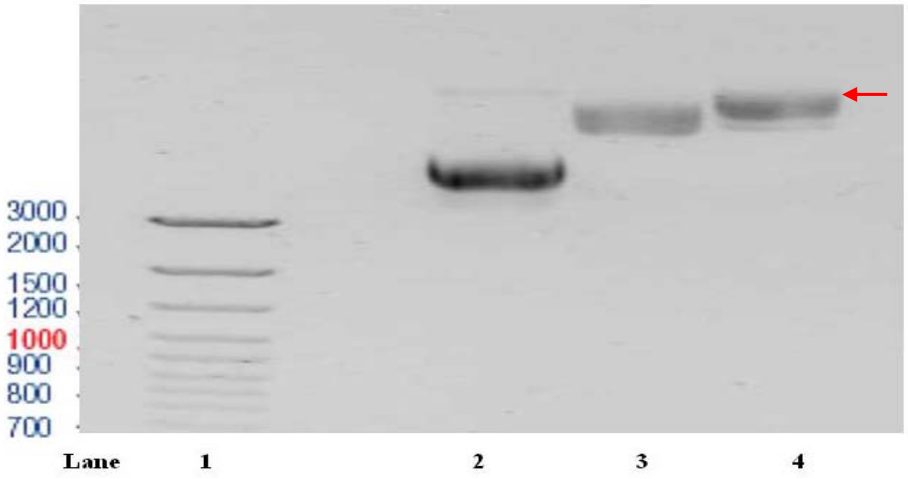


Figure 4.5: Plasmid extraction of various non-linearised plasmids. Lane 1, 100bp DNA ladder plus (Fermentas); lane 2, pUC19-*At*gstF6-IF3-CaMV35S terminator plasmid (4881bp); Lane 3, pCAMBIA 2300 (8742bp); Lane 4, pCAMBIA 2300 with cloning cassette (*At*gstF6-IF3-CaMV35S terminator) (10937bp). Note the difference in size of pCAMBIA 2300 and pCAMBIA2300-*At*gstF6-IF3 plasmid (see red arrow).

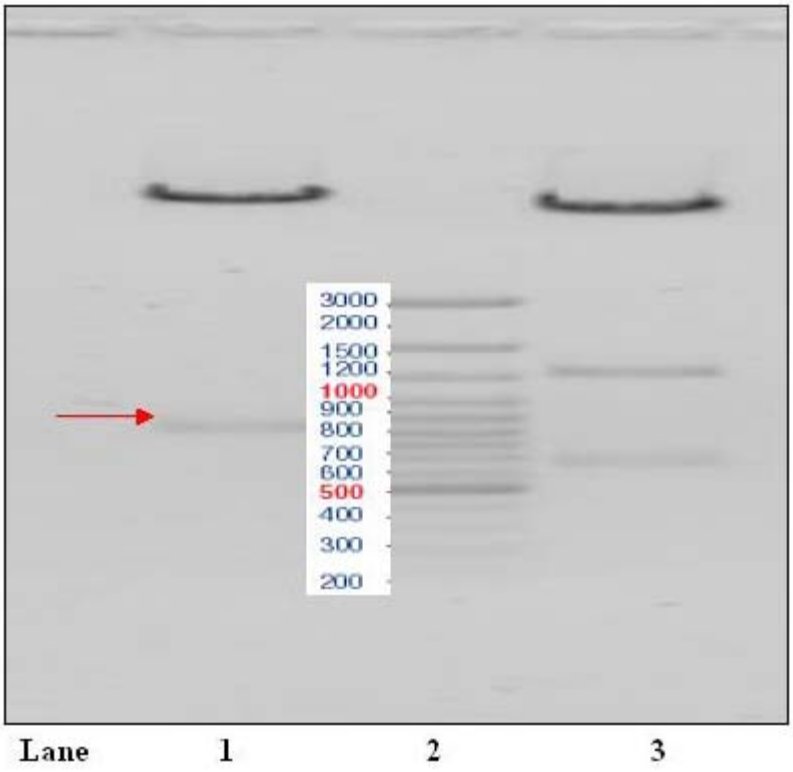


Figure 4.6: Plasmid restriction enzyme mapping of pCAMBIA2300-*At*gstF6 -IF3 plasmid. Lane 1, pCAMBIA2300-*At*gstF6 -IF3 plasmid digested with *Eco*RI, which releases the *AtGSTF6* promoter (red arrow showing 926 bp fragment); Lane 2, 100bp DNA ladder plus (Fermentas); Lane 3, pCAMBIA2300-*At*gstF6-IF3 plasmid digested with *Hind* III that releases two fragments (656bp and 1491bp).

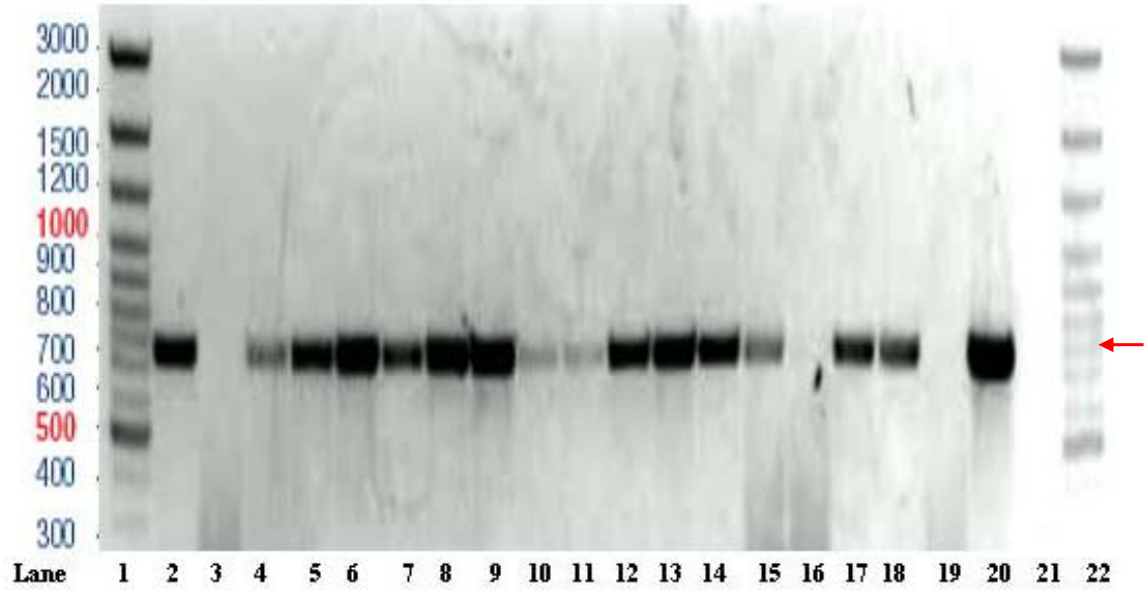


Figure 4.7: Gel electrophoresis showing colony PCR from transformed *A.tumefaciens* colonies using primers PCGIF and PCG2R (see Appendix). Lane 1 and 21 represent 100bp DNA ladder plus (Fermentas). Lane 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17 and 18 represent transformed *Agrobacterium* colonies (red arrow shows a 727bp product) while Lane 3 and 16 represent non-transformed Agrobacterium. Lane 20 represents a positive control PCR, while lane 21 is a water control.

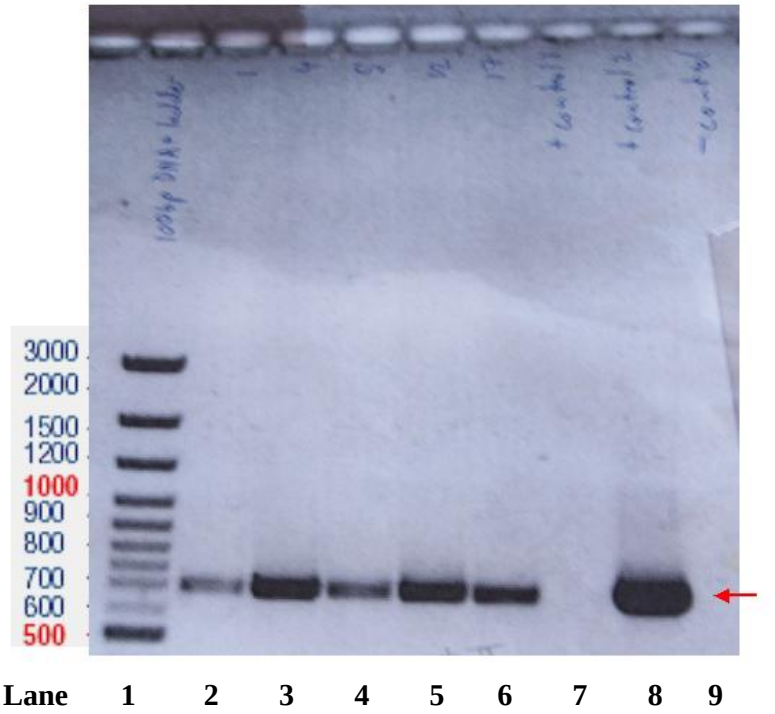


Figure 4.8: Colony PCR of Kanamycin resistance gene (*nptII*) (red arrow shows 729bp product) in transformed *Agrobacterium*. Lane 1 represents a 100bp DNA ladder. Lane 2, 3, 4, 5 and 6 represent colonies 1, 4, 5, 12 and 17 respectively, which are positive for the presence of the *nptII* gene. Lane 7 shows a colony PCR on untransformed Agrobacterium yielding no product, Lane 8 shows Agrobacterium spiked with plasmid DNA and lane 9 shows a negative water control yielding no product.

Sequencing of the IF3 gene using the internal primers ClaI-IF3 and SalI-IF3 showed that there is an inversion mutation at position 551 of the IF3 CDS sequence as shown in Figure 4.9. The sequencing was done on the cloning vectors pUC19-*At*gstF6-IF3, pUC19-*At*gstF6-IF3-CaMV35S and pCAMBIA2300-*At*gstF6-IF3 plasmids and compared to the sequence data provided by (Regalado, Lissabon, Portugal; Genbank accession number CAA 76203). It is not known if the mutation already occurred in pBLUKSII (-) IF3. The inversion mutation was analysed for amino acid changes and showed that the protein sequence was changed at position 212 from a cysteine residue to a serine residue shown in Figure 4.10. The changed amino acid sequence was used

to search for homologues using BlastP (Gen Bank) Neighbor-joining tree was created to identify the relationship of the IF3 chitinases and identify the closest class III relatives in *Nicotiana tabacum* (Figure 4.11)

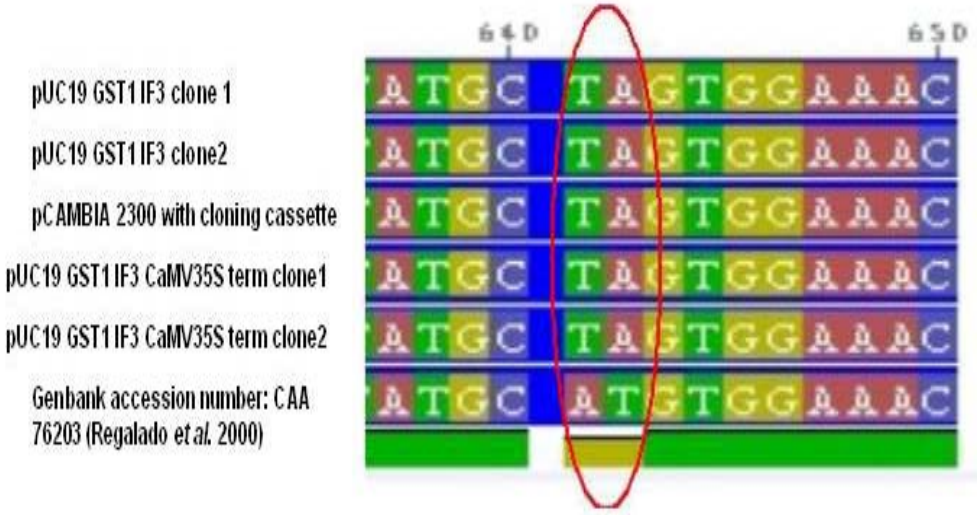


Figure 4.9: A section (637 –650 bp) of the nucleotide sequence alignment of IF3 chitinase in various vectors, showing the inversion of AT to TA using the ClaI-IF3 primer for sequencing. The CDS sequence was aligned to the IF3 from 5 repeated sequencing experiments, which used the ClaI-IF3 primer.

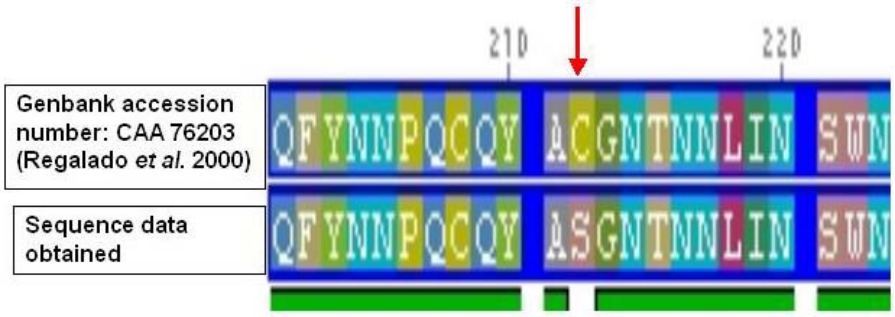
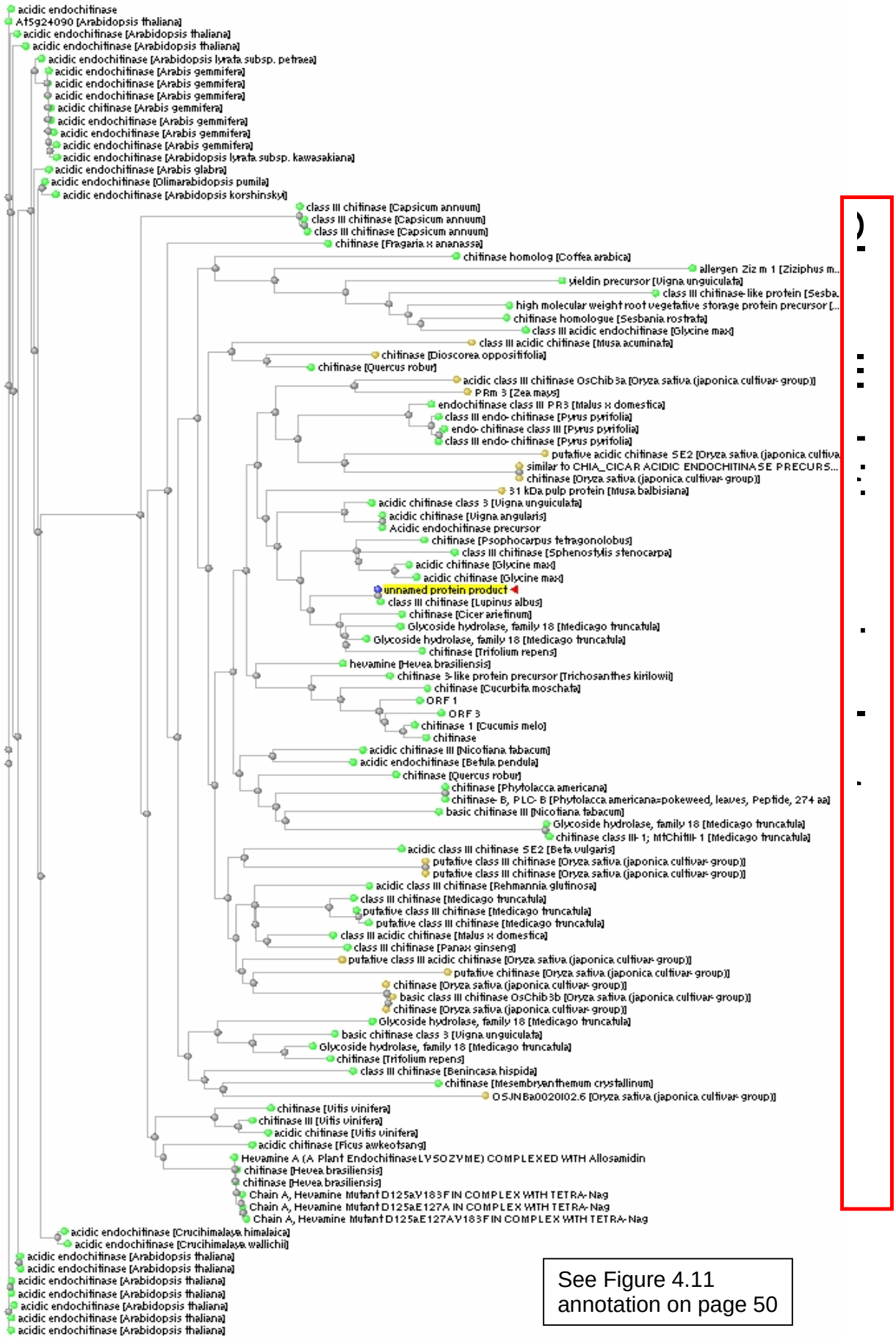


Figure 4.10: Amino Acid sequence alignment of the original sequence (Regalado *et al.* 2000) with the mutation sequence showing the amino acid change from cysteine to serine at amino acid position 212.



See Figure 4.11 annotation on page 50

Figure 4.11: Distance tree of the IF3 chitinase amino acid sequence (highlighted in yellow) and BlastP amino acid sequences obtained using BlastP (www.ncbi.nlm.nih.gov). The BLAST computes a pairwise alignment between a query and the database sequences searched. It does not explicitly compute an alignment between the different database sequences (i.e., does not perform a multiple sequence alignment). For purposes of this amino acid sequence distance tree presentation an implicit alignment between the database sequences is constructed, based upon the alignment of those (database) sequences to the query. It may often occur that two database sequences align to different parts of the query, so that they barely overlap each other or do not overlap at all. In this case it is not possible to calculate a distance between these two sequences and only the higher scoring sequence is included in the tree. The maximum sequence difference allowed is 65% and distances are real and calculated using the Kimura method.

4.2) Transformation and Molecular Analysis of Transformed Tobacco cv. JR6

Plants were transformed with transgenic *A. tumefaciens* containing the binary vector pCAMBIA2300-*At*gstF6-IF3 (Figure 4.7 and 4.8). Shoots regenerated from leaf disks on regeneration media (Figure 4.12) and developed shoots were allowed to develop roots in regeneration media (Figure 4.13). The explants were established and showed no visible phenotypic difference to the untransformed control plants.

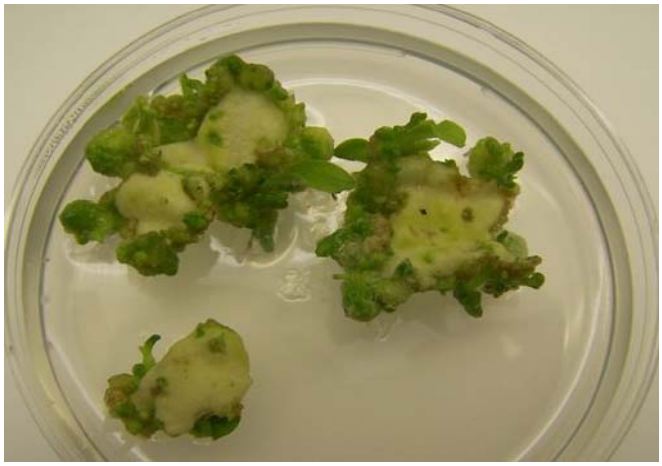


Figure 4.12: Regeneration of leaf disks on shooting media after Agrobacterium transformation with pCAMBIA2300-*At*gstf6-IF3. The development of the shoots can be seen on the edges of the leaf disks.

Figure 4.13: Individual shoots on regeneration media showing active growth after Agrobacterium mediated transformation and root initiation (red arrow)

In order to determine whether the T_0 lines could be tested for the presence of the introduced constructs using conventional PCR, the explants were first tested for Agrobacterium contamination through PCR using Mia Forward and Reverse primers (Grayburn and Vick, 1995) . The sample material that was tested for Agrobacterium contamination showed that plants were still positive for the presence of Agrobacterium (Figure 4.14). For this reason PCR screening for transformed shoots for transgene presence had to be altered to qualitative reverse transcription (RT) PCR approach. To test the qualitative RT PCR approach, 10 different uncharacterised, regenerated, rooted shoots from independent transgenic events were selected. The selected rooted shoots were put through different time points of harvesting, which included untreated, 4 hours post treatment with 2mM hydrogen peroxide and 24 hours post treatment with 2mM hydrogen peroxide. The hydrogen peroxide treatment was included as Grant *et al.* (2000) showed that the *AtGSTF6* gene transcripts (described as *gst1*) naturally accumulate in the presence of hydrogen peroxide in *Arabidopsis thaliana*. It was assumed that the same induction pattern would be found in the transgenic *Nicotiana tabacum* and that at least one of the ten uncharacterised transgenic events selected, would contain a functional construct. The leaf material of all 10 shoots was bulked for each time point and total RNA was extracted. To assess

the quality of the RNA, it was run on an agarose gel elctrophoresis (Figure 4.15). cDNA was synthesized from total DNase treated total RNA from each of the bulked samples.

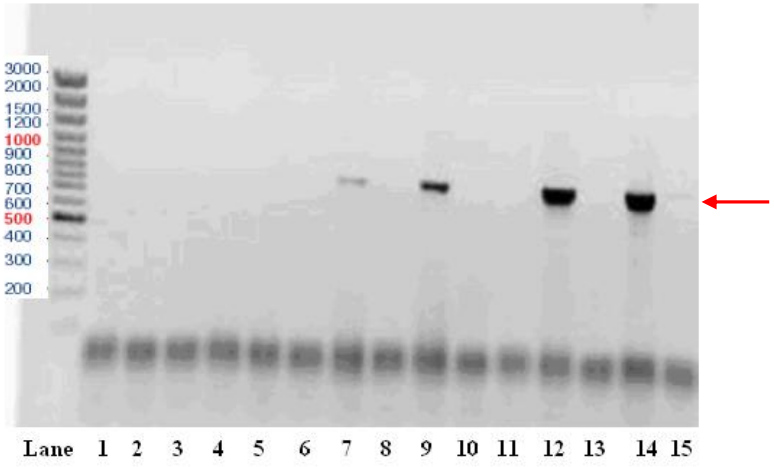


Figure 4.14: Gel electrophoresis showing the PCR detection of Agrobacterium contamination on different explant samples. Lane 1, 100bp DNA plus ladder. Lane 7, 9 and 12, Agrobacterium contamination as a PCR product for Mia primers is obtained. Lanes 1,2,3,4,5,6,8 and 11 are negative. Lane 13 represents a negative control while lane 14 represents an Agrobacterium control line. Lane 15 is a leaf disk with spiked with Agrobacterium (faint line).

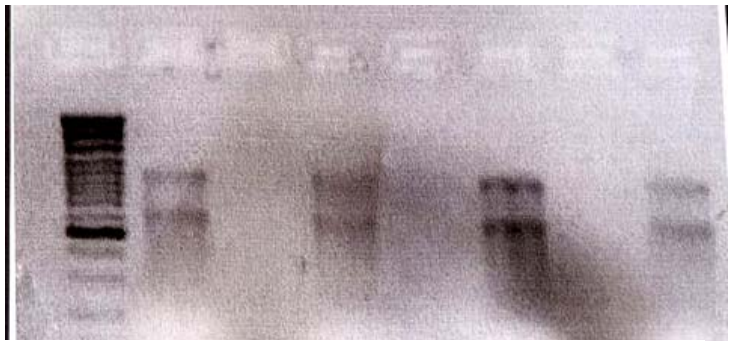


Figure 4.15: Gel Electrophoresis of RNA extraction showing lane1 100 bp DNA ladder plus, Lane 2 untransformed tobacco, Lane 3 untreated, Lane 4, 4hours post-treatment, Lane 5 24 hours post-treatment

The single stranded cDNA synthesized from DNase treated total RNA, was used to test the presence of IF3 chitinase mRNA transcript using PCR. The primers target a section of the IF3 chitinase transcript using ChiF (Forward) RT and ChiR (Reverse) RT primers to yield a 402bp fragment following PCR amplification. This was done for all the bulked samples. The negative control was untransformed *N.tabacum* JR6.

Figure 4.16 (Panel A) shows that the chitinase gene is transcribed to mRNA, due to the presence of PCR generated bands of size 402bp in all the assayed bulked cDNA samples. To check for genomic DNA contamination, PCR was also performed on DNase treated RNA (lane 3, 5, 7, 9). The absence of bands confirmed that genomic DNA was absent. The transcript presence could therefore be confirmed but the level of transcription could not be determined. The absence of bands in the untransformed tobacco line cDNA shows that no endogenous chitinase genes are detected with the chitinase PCR. The results also show that the expression is triggered in the absense of hydrogen peroxide treatment as the untreated bulked samples show transcript presence. This is indicative of leaky chitinase gene expression from the *At*GSTF6 promoter in *N. tabacum.*

The actin PCR is used to identify genomic (g) DNA contamination (Figure 4.16, Panel B). In the presence of genomic DNA multiple bands are formed while in the presence of cDNA one band is generated. This is due to the actin primers spanning introns which are removed during mRNA processing and are thus not present in cDNA generated from mRNA. In Figure 4.16 all cDNA show single bands while the gDNA control shows multiple bands, all larger then the cDNA actin bands.

The results prompted the use of this technique to individually identify transgenic lines. Twenty lines were treated with a foliar spray of 2mM hydrogen peroxide and left for 1 hour before harvesting to allow expression of the chitinase gene. Figure 4.17 and 4.18 show 12 individual lines that were positive for transcript presence. Thus 12 out of 20 lines were shown to be tansformed with the construct showing a 60% transformation efficancy.

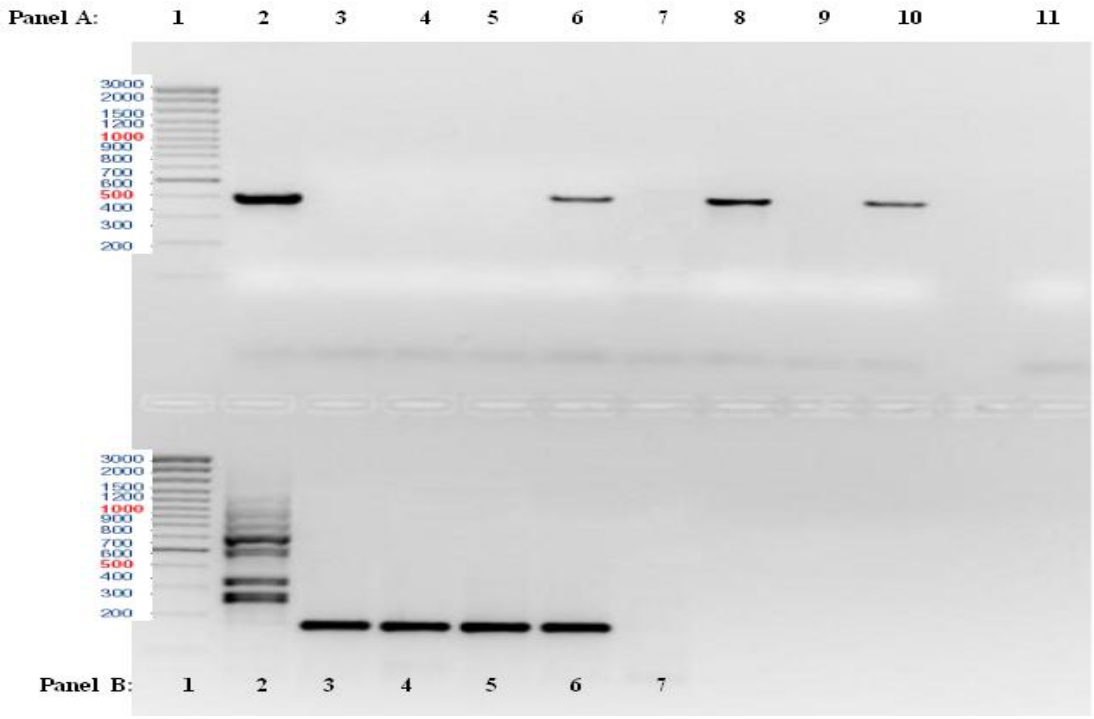


Figure 4.16: Gel electrophoresis showing qualitative RT PCR of the IF3 chitinase gene and actin gene from samples bulked from 10 plants of transgenic tobacco events. Panel A shows the PCR for the IF3 chitinase gene and Panel B shows the actin endogenous control.

Panel A: Lane 1, 100 bp DNA ladder plus (Fermentas); Lane 2 represents a positive pCAMBIA2300-*At*gstF6-IF3 plasmid DNA control; Lane 3, PCR done on untransformed JR6 RNA; Lane 4, PCR on untransformed JR6 cDNA; Lane 5, PCR performed on untreated bulked sample RNA; Lane 6, PCR untreated bulked samples on cDNA; Lane 7, 4 hour post treatment samples with PCR done on RNA; Lane 8, 4 hour post treatment samples with PCR done on cDNA; Lane 9, 24 hour post treatment samples with PCR done on RNA; Lane 10, represents 24 hours post treatment samples with PCR done on cDNA; Lane 11, water control.

Panel B: Lane 1, 100 bp DNA ladder plus (Fermentas); Lane 2, positive gDNA control from untransformed tobacco JR6 (254 bp); Lane 3, JR6 cDNA (200 bp) ; Lane 4, untreated bulked sample cDNA (200 bp); Lane 5, 4 hour post-treatment bulked sample cDNA (200 bp); Lane 6, 24 hour post-treatment bulked sample cDNA (200 bp); Lane 7, water control.

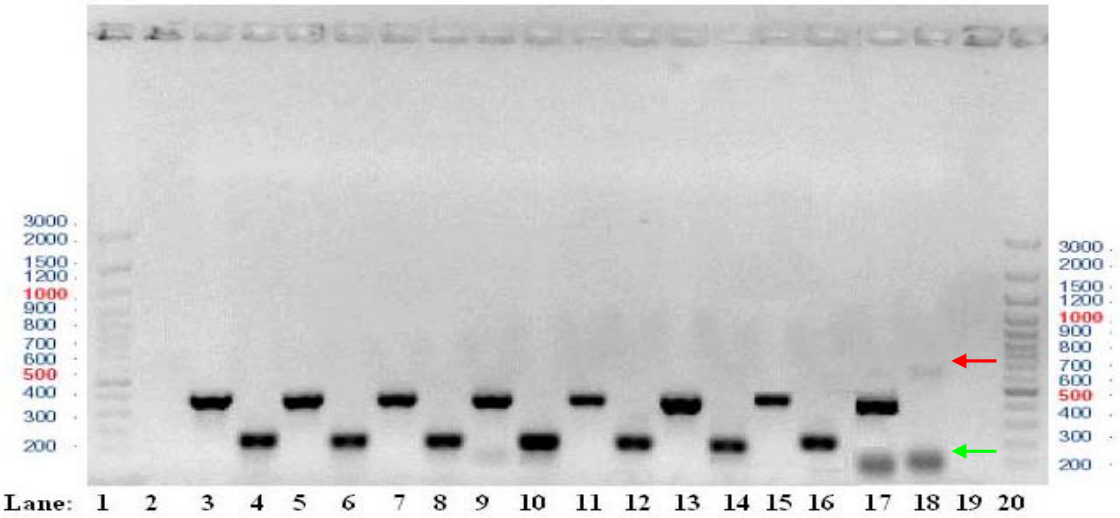


Figure 4.17: Gel electrophoresis showing qualitative RT PCR to test individual transgenic lines treated with 2mM hydrogen peroxide and left for 1 hour before harvesting. Lane 1 and 20, 100bp DNA ladder plus (Fermentas); Lane 2, empty; Lane 3,5,7,9,11,13,15 represent lines J6 #1, J6 #2, J6 #4, 5.1 #2, 5.3 #1, 5.3 #2, 5.4 #2 respectively, all showing chitinase PCR product of 402 bp; Lane 4,6,8,10,12,14,16 show corresponding actin endogenous controls for PCR with 200bp PCR fragment for lines J6 #1, J6 #2, J6 #4, 5.1 #2, 5.3 #1, 5.3 #2, 5.4 #2 respectively; Lane 17, positive PCR control for chitinase from pCAMBIA2300-*At*gstF6-IF3 DNA showing primer dimers at the base of the gel; Lane 18, Positive actin PCR control from gDNA from untransformed tobacco(faint line at red arrow) showing primer dimers at the base of the gel. Lane 19 shows a water control for the chitinase and actin PCR. The lowest bands shown in Lane 17 and 18 (green arrow) represent primer dimers.

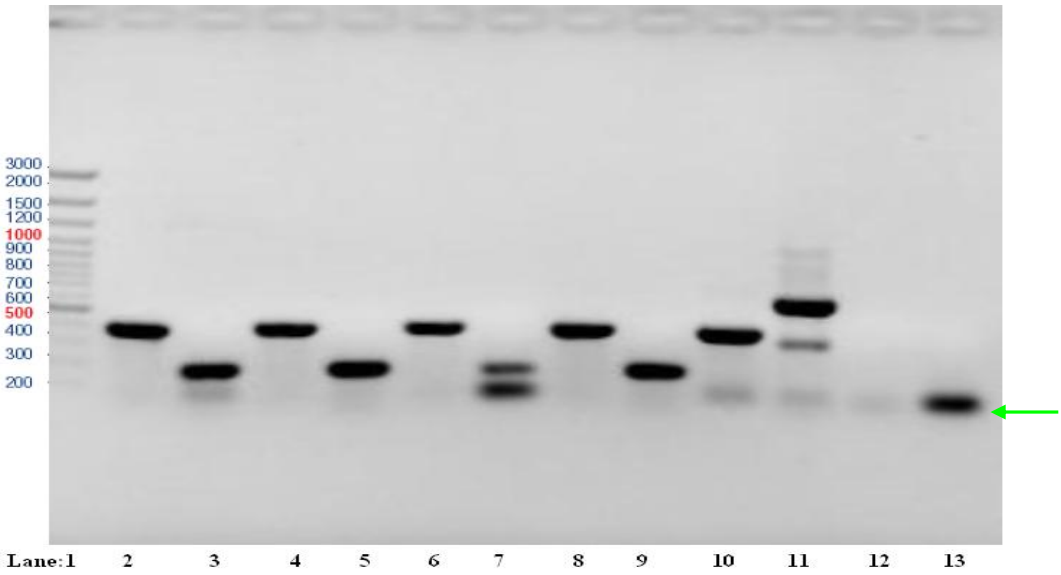


Figure 4.18: Gel electrophoresis showing qualitative RT PCR to test individual transgenic lines treated with 2mM hydrogen peroxide and left for 1 hour before harvesting. Lane 1,100bp DNA ladder plus (Fermentas). Lane 2, 4, 6 and 8 represent chitinase transcript tested lines for 5.4 #3, 5.5a #1, 5.5a #2 and 5.5a #5 respectively, which are PCR positive for the IF3 chitinase. Lane 3, 5 ,7 and 9, actin endogenous controls for the lines 5.4 #3, 5.5a #1, 5.5a #2 and 5.5a #5 respectively. Lane 10 represents positive PCR control for chitinase from pCAMBIA2300-*At*gstF6-IF3 DNA and lane 11 represents a positive actin PCR control from gDNA from untransformed tobacco. Lane 12 is water control for chitinase and lane 13 is water control for actin. The lowest bands shown in Lane 3, 7, 10, 11, 12 and 13 (green arrow) represent primer dimers.

Table 4.1: Following RT PCR screening of all the putative transgenics, 12 lines were found to exhibit IF3 chitinase expression following treatment with 2mM hydrogen peroxide, left for 1 hour before harvesting.

Line	IF3 chitinase expression (+/-)
J6 #1	**+**
J6 #2	**+**
J6 #4	**+**
5.1 #2	**+**
5.3 #1	**+**
5.3 #2	**+**
5.4 #2	**+**
5.4 #3	**+**
5.5a #1	**+**
5.5a #2	**+**
5.5a #5	**+**
5.5a #7	**+ (results not shown)**

4.3) Testing chitinase enzyme activity

The twelve lines that were found to transcribe the gene through the RT PCR approach, needed to be characterised for their chitinase activity. To test whether the chitinase enzyme was functional, 10 different uncharacterised, regenerated, rooted shoots from independent transgenic events were selected. The leaf material used for the chitinase assay, was of the same origin as that used initially for the RT PCR approach. The selected rooted shoots were put through different time points of harvesting, which included untreated, 4 hours post treatment with 2mM hydrogen peroxide and 24 hours post treatment with 2mM hydrogen peroxide. The hydrogen peroxide treatment was included as Grant *et al.*(2000) showed that the *AtGSTF6* gene transcripts (described as *gst1*) naturally accumulates in the presence of hydrogen peroxide in *Arabidopsis thaliana*. It was assumed that the same induction pattern would be found in the transgenic *Nicotiana tabacum* and that at least one of the ten uncharacterised transgenic events selected, would show increased chitinase activity.

The absorbance at 550nm was plotted against the percentage-undiluted protein (Figure 4.18) i.e. the amount of protein present in the dilution sample from the original protein concentration.

Appel *et al.* (1995) showed that the linearity between absorbance values and enzyme concentration was limited to intermediate enzyme concentrations. The paper also showed that an absorbance of 0.1 always occured within the concentration range were absorbance-concentration linearity was valid. This necessitated the application of serial dilution of the total protein. Higher protein concentrations were assayed, but only the linear section of the graph is represented. This section was used to calculate a linear regression line.

One unit activity is defined as the amount (µg) of protein required to yield an absorbance of 0.1 at 550nm with a substrate concentration of (0.5mg/ml) in a volume of 200 µl incubated at 37 C° for 3 hours using the untransformed line *Nicotiana tabacum* cv. JR6 as reference.

The unit activity was calculated as follows:

Example:

Untransformed cv JR6 line:

Formula obtained $y = 0.02x + 0.0116$ / y = 0.1 $Absorbance_{550nm}$ based Unit enzyme definition

$0.1 = 0.02x + 0.0116$ / -0.0116

$0.0884 = 0.02x$ / %0.02

4.42% (undiluted protein) = x

(20µg of total protein represents 100% percentage undiluted value)

Solve for z

4.42% = z µg

100% = 20 µg

z = 0.884µg

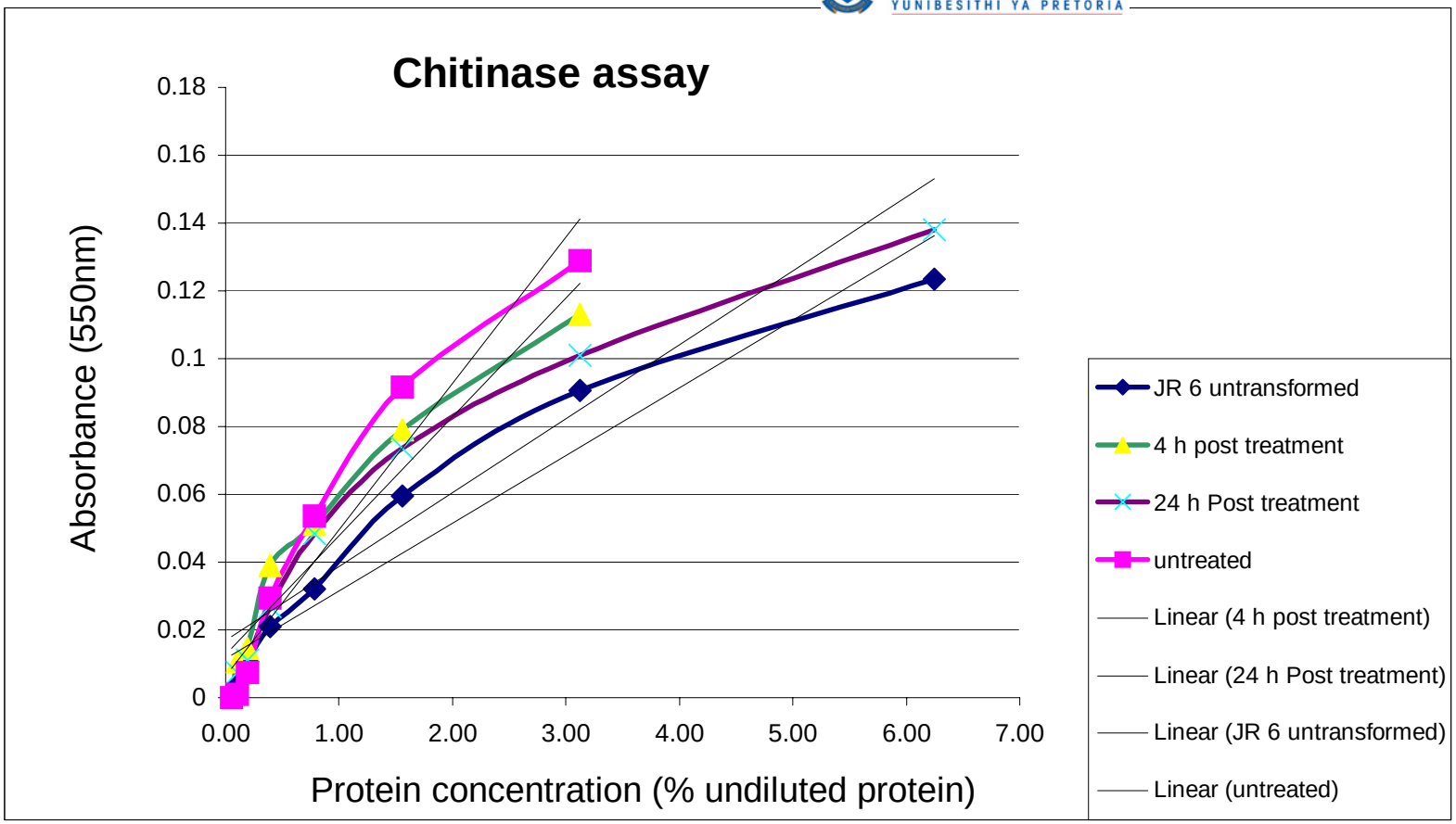


Figure 4.19: Graph showing chitinase assays from one repeat done on untransformed, untreated, 4 hour post-treatment and 24 hour post-treatment leaf material bulked from ten different plants. The absorbance at 550nm is plotted against the percentage undiluted protein extract. Trendlines were plotted on the graphs and the formulas obtained. From this data one can see that the untransformed line JR6 requires a higher total protein concentration than the untreated bulked leaf material to reach an absorbance of 0.1.

Untreated transformed bulk sample:

Formula obtained y = 0.0432x + 0.0064 / y = 0.1 $Absorbance_{550nm}$ based Unit enzyme definition (Appel et al. 1995)

0.1 = 0.0432x + 0.0064 / -0.0064

0.0936 = 0.0432x / 0.0432

2.16% (undiluted protein) = x

(20μg of total protein represents 100% percentage undiluted value)

Solve for z

2.16% = z μg

100% = 20 μg

z = 0.43 μg

Chitinase activity of the untreated transformed line relative to the untransformed cv JR6 control line

-0.884 μg of protein is required to yield an Absorbance of 0.1 in the untransformed control line and is defined as one Unit.

-0.43 μg of protein is required to yield the equivalent Absorbance of 0.1 in the untreated transformed bulk sample, therefore relative to the untransformed cv JR6 line the untreated transformed bulk sample can be expressed as:

$\frac{0.884\mu g}{0.43\mu g}$ = 2.1 Units greater chitinase activity is found in the untreated transformed bulk sample.

The assay was repeated in triplicate on the same bulked tissue samples as part of a technical repeat. The CM-Chitin-RBV substrate can precipitate under certain conditions such as time and exposure to freezing temperatures, causing the substrate concentration to be variable between different experiments. Also, the exposure to HCl during the termination step of the reaction results in CM-Chitin-substrate hydrolysis.

This means that the absorbance readings for different experiments can differ due substrate concentration differences, which in turn affect the unit readings obtained. In order to obtain comparable results between assays, the readings were always compared to the untransformed lines by diving the Unit activity for the transgenic line with that of the Unit activity of the nontransgenic line. The fraction obtained was then compared between the different lines (Figure 4.19). The results show that an increased chitinase activity is observed between the untreated bulked sample and the 4hour post treatment bulked sample compared to the untransformed line. The 24-hour post treatment bulked sample does not show significant difference in chitinase activity when compared with the other samples using the error bars.

The assay was repeated for individual lines as above except that all lines (transgenic and nontransgenic) were sprayed with 2mM hydrogen peroxide and left for 1 hour before harvesting to allow gene expression. The trendlines obtained and calculation performed for the triplicate biological repeat experiment can be seen in Appendix E.

The results show that 9 lines exhibit increased chitinase expression (Figure 4.20 and Table 4.1) while 3 lines do not indicate increased gene expression.

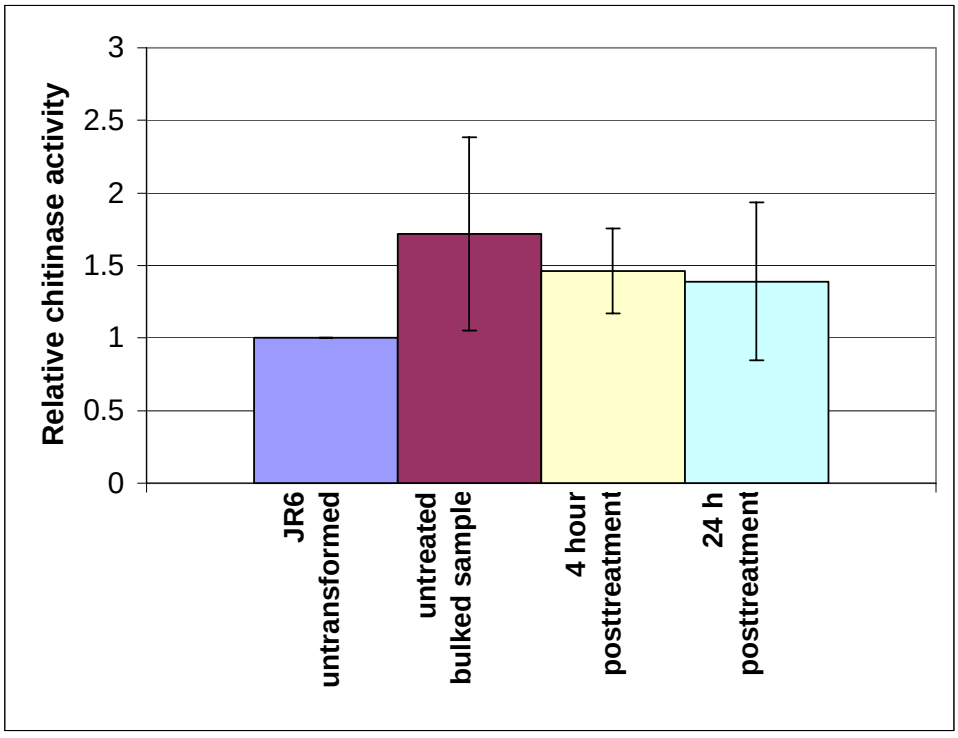


Figure 4.20: Bar chart showing the relative chitinase enzye activity between the untransformed line JR6 and untreated bulk, 4 hours post treatment bulk and 24 hours post treatment bulk samples respectively of the triplicate technical repeat done on the bulked leaf material.

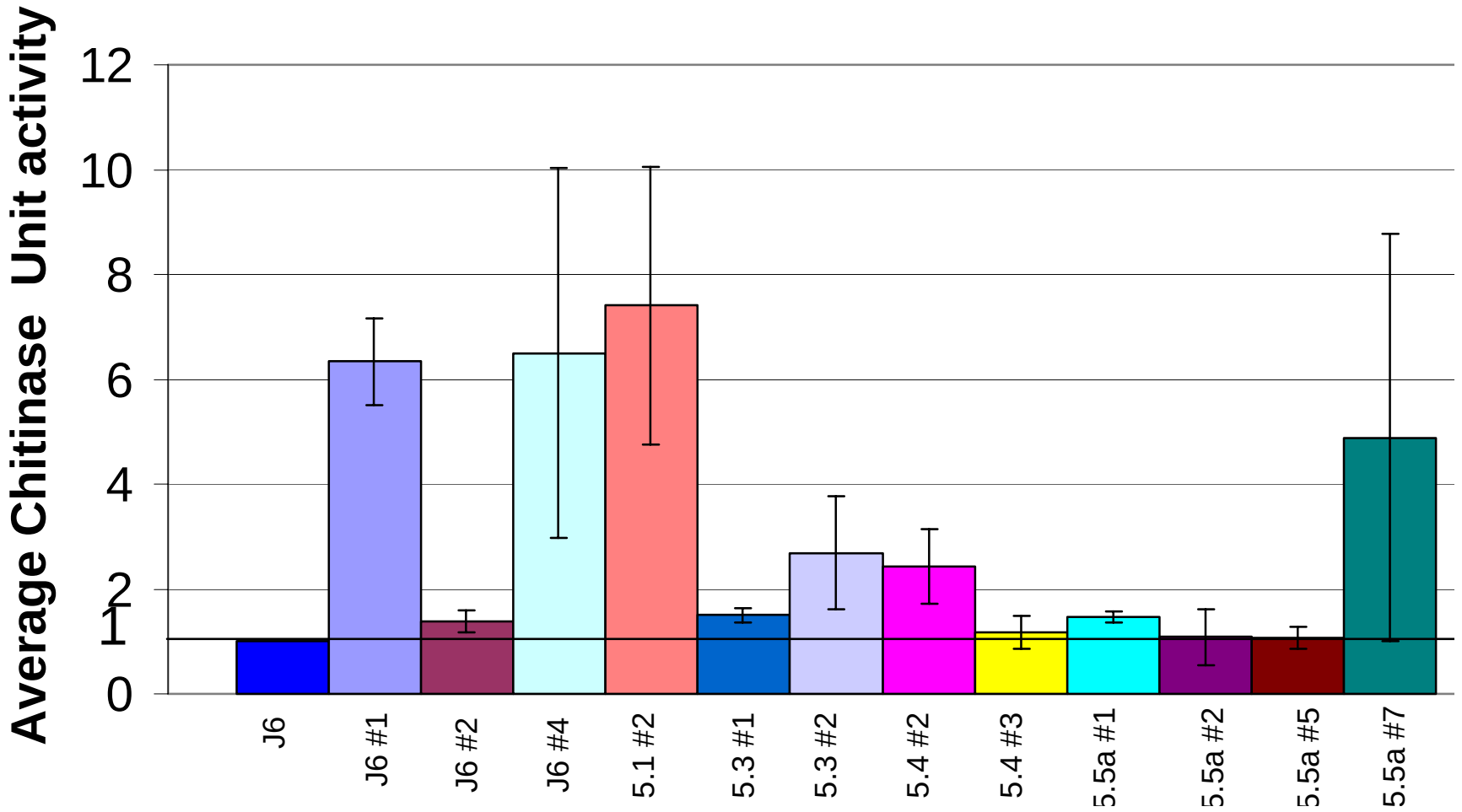


Figure 4.21: Bar chart showing the difference in Unit activity of the transgenic plants in relation to the untransformed JR6 control. The representation is composed of a triplicate repeat for each of the transgenic lines and is a comparison to the JR6 untransformed.

Table 4.2: Transgenic lines showing increased chitinase expression compared to untransformed control plants.

Transgenic line	Increased chitinase expression Yes (Y)/ No (N)
J6 #1	Y
J6 #2	Y
J6 #4	Y
5.1 #2	Y
5.3 #1	Y
5.3 #2	Y
5.4 #2	Y
5.4 #3	N
5.5a #1	Y
5.5a #2	N
5.5a #5	N
5.5a #7	Y

4.4) *Rhizoctonia solani* pathogen assay

The necrotrophic pathogen *Rhizoctonia solani* (AG 2) was chosen for the detached leaf assays. The culture was obtained from the Centraal Bureau vir Schimmel Kultuuren, Wageningen and was isolated from *Nicotiana alata*. The first experiment was to determine whether the isolate was able to cause disease in *Nicotiana tabacum.* This was done by placing a 5 x 5 mm piece of hyphae on a detached untransformed tobacco leaf maintained on water agar. Symptoms developed as shown, after 5 days Figure 4.1. The symptoms for target spot (the name for the disease symptom caused by *Rhizoctonia solani* on leaves) under very humid conditions show watersoaked, spreading opaque necrotic lesions, which leave disintegrated tissue behind www.ces.ncsu.edu/depts/pp/notes/**Tobacco**/tdin007/tb07.html.

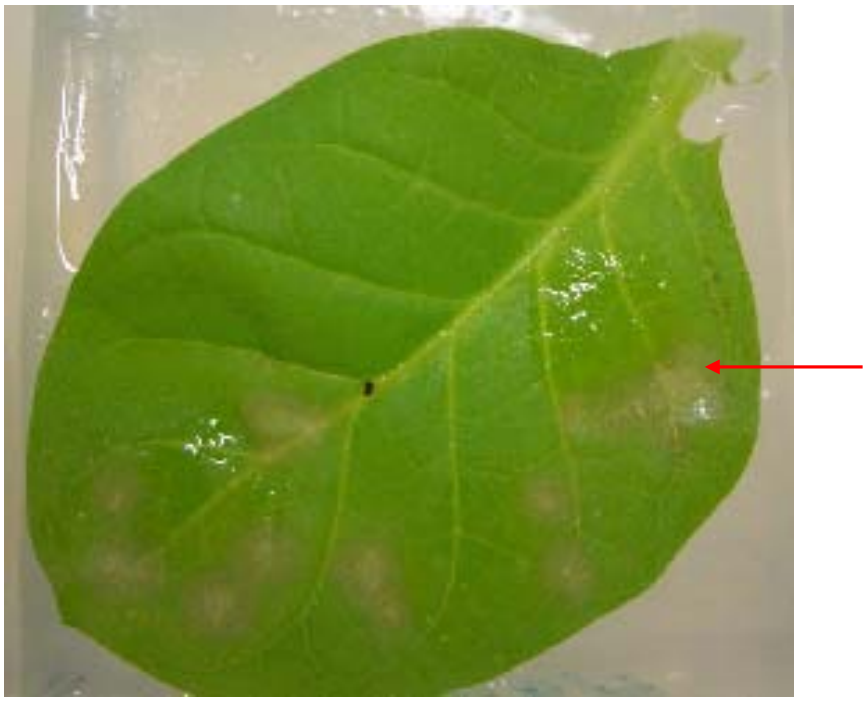
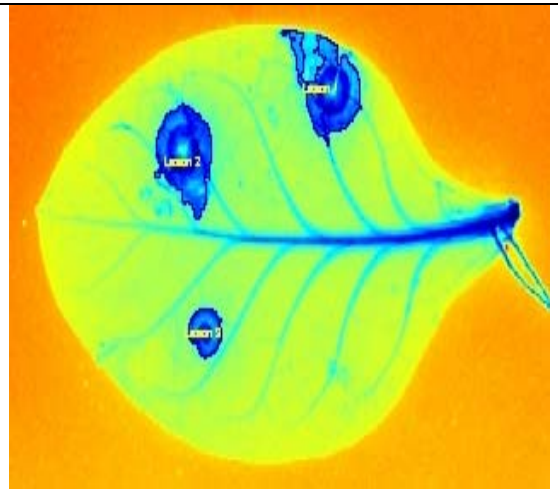
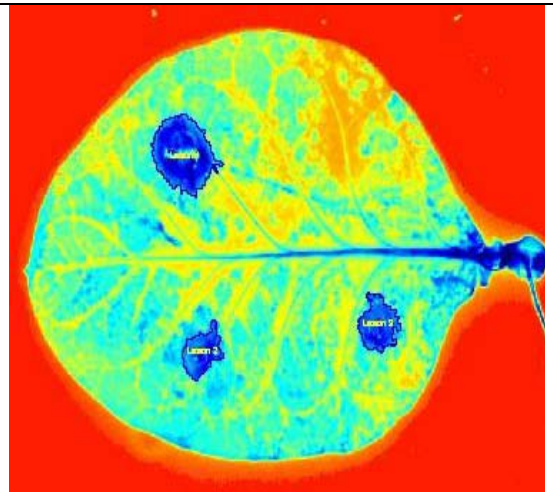
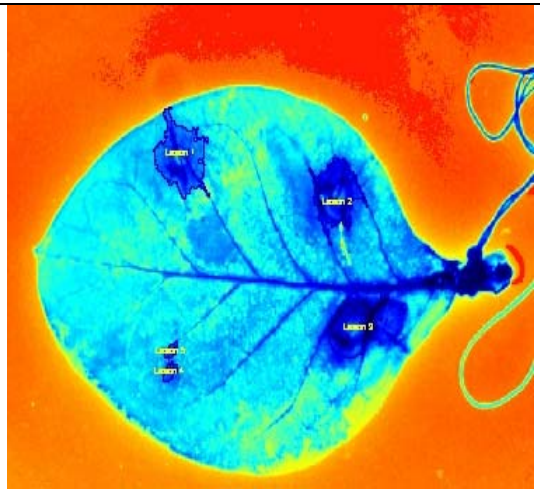
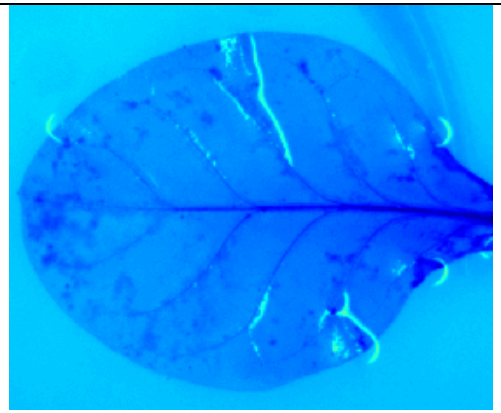


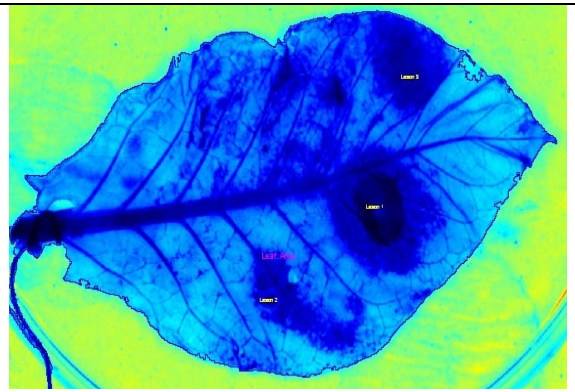
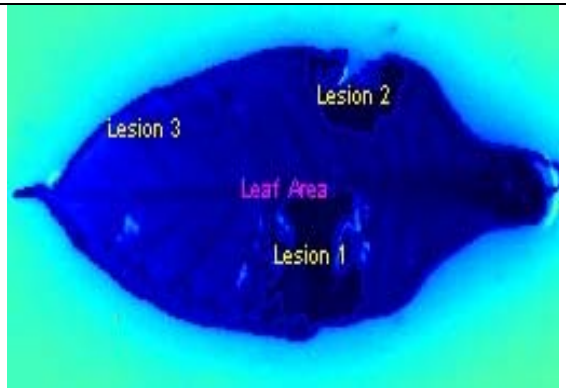
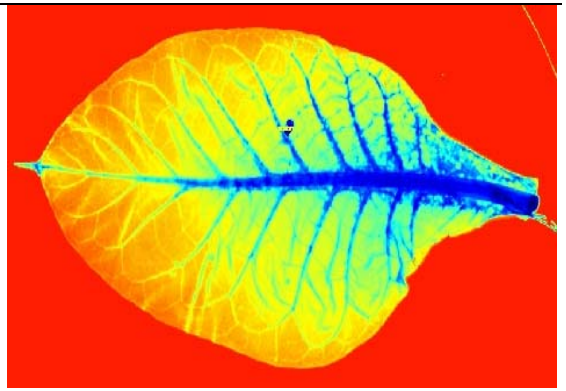
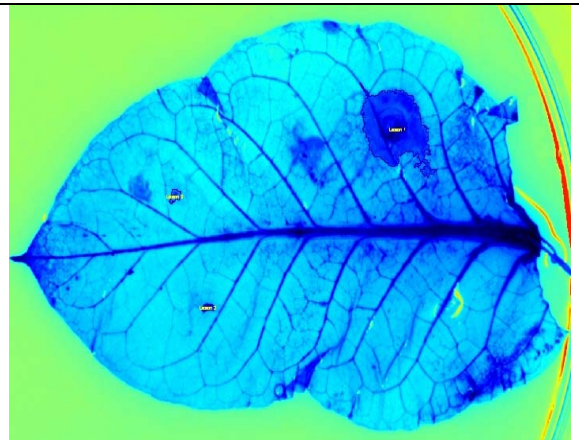
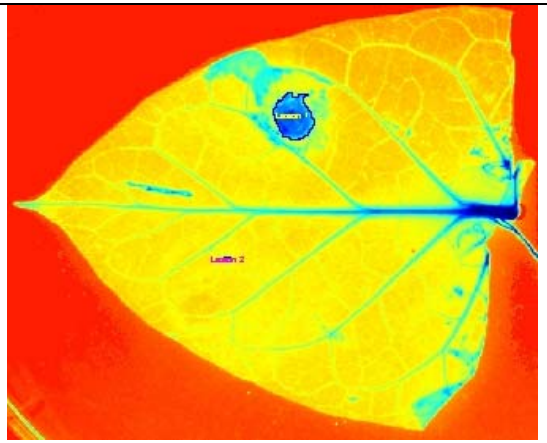
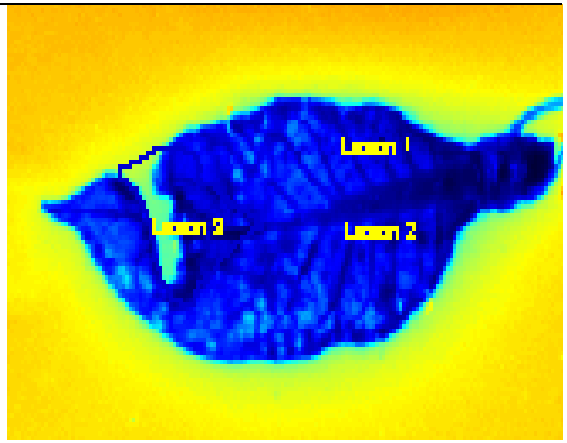
Figure 4.22: Detached untransformed tobacco leaf showing lesion development (red arrow) after infection with *Rhizoctonia solani*. The photo was taken five days after inoculation of the leaf with hyphae and lesion shows a typical watersoaked, browning lesion, which is characteristic for *Rhizoctonia solani* infection.

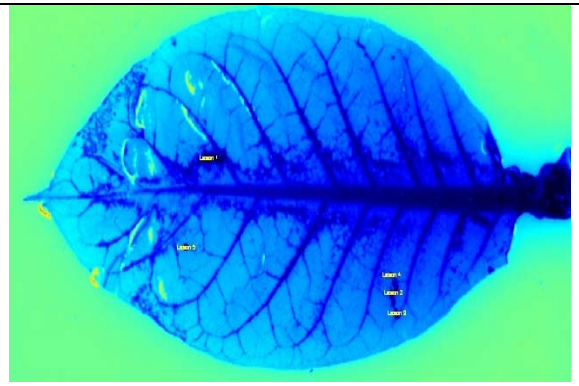
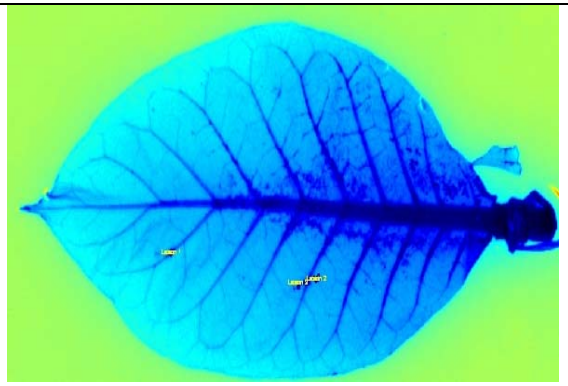
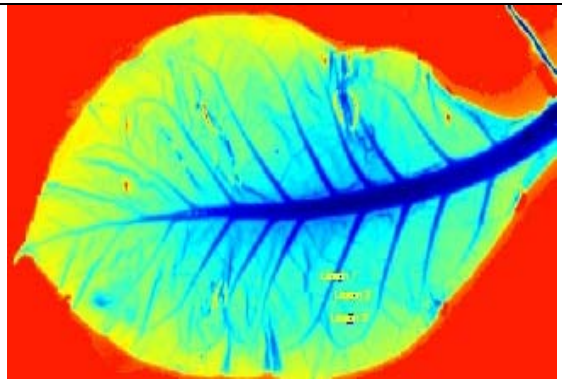
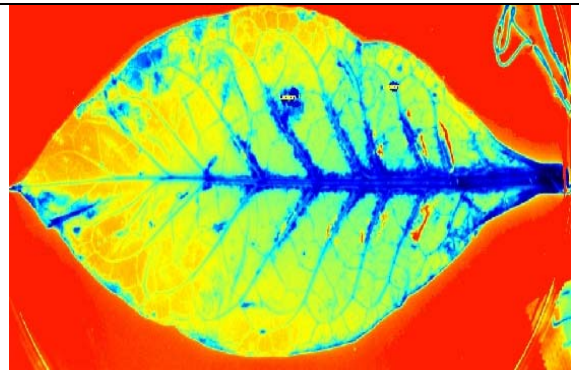
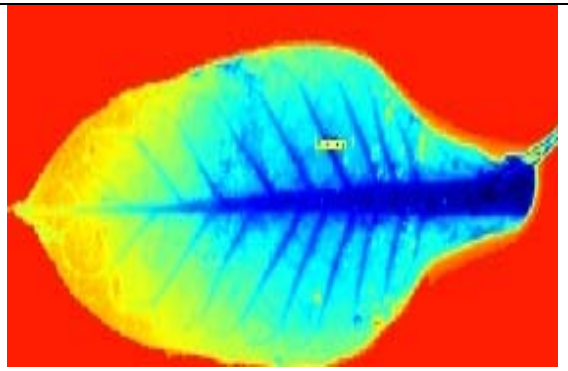
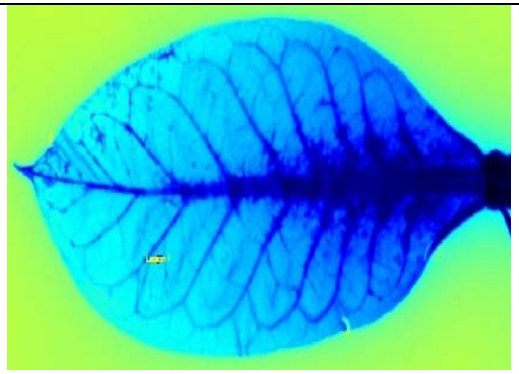
Rhizoctonia solani does not readily make sexual spores as it is classed into the sterile fungi. The inoculations were therefore done using homogenised mycelia suspended in autoclaved double distilled water. The next step was to determine the correct amount of homogenised hyphae mass to elicit a disease response on the detached leaf. Amounts corresponding to 0.05 and 0.1 mg total mycelia mass yielded desired results while the amount of 0.01 mg yielded few disease symptoms and 0.2 and 0.3 mg yielded undesirably strong symptom development (Data not shown). It was decided that an amount of 0.1 mg *Rhizoctonia* was used for the detached leaf assay.

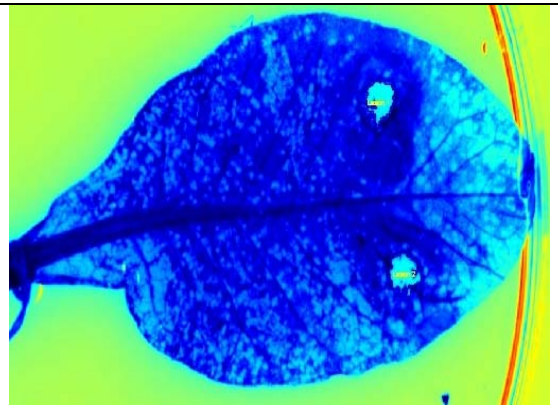
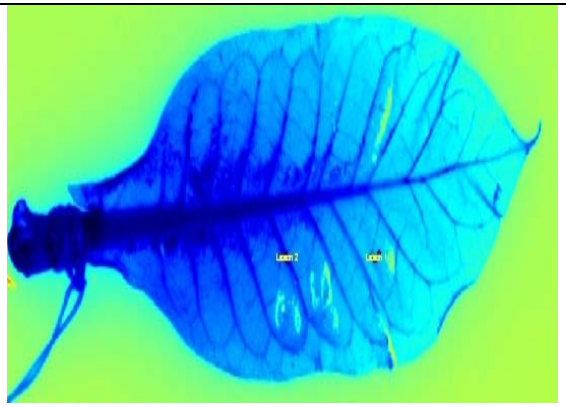
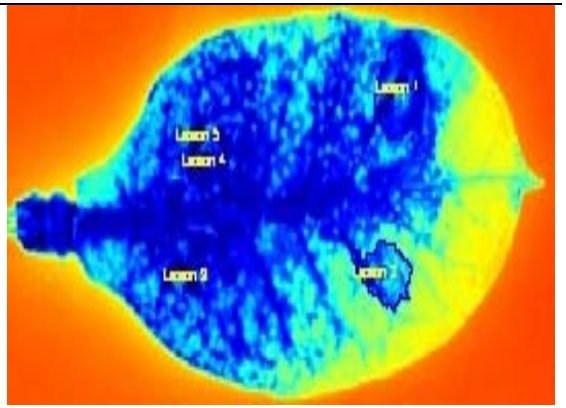
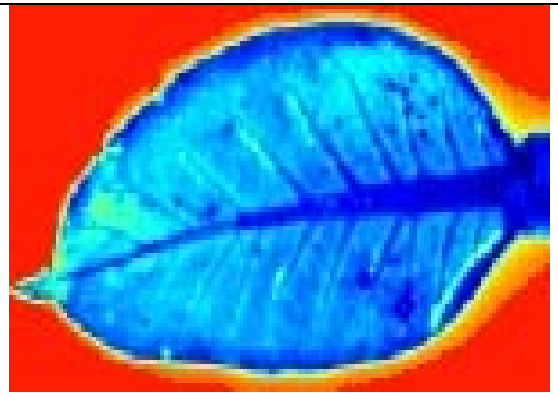
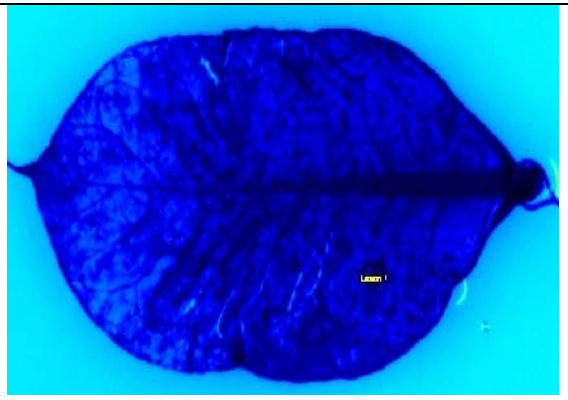
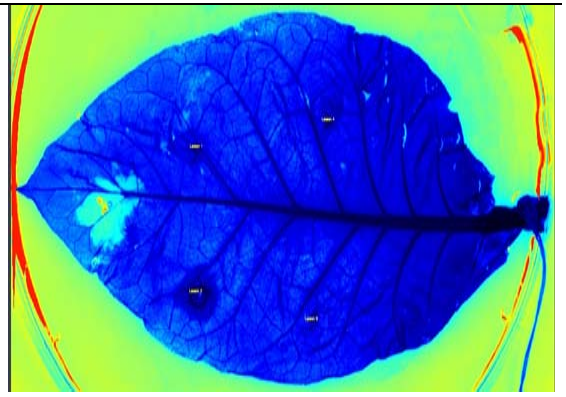
The lines that were characterised for gene expression and IF3 chitinase activity were used in a detached leaf assay. *Rhizoctonia solani* mycelia were weighed, homogenised and applied on four equidistant spots on each leaf. Not all spots developed lesions on the leaf. The lesions were stained using trypan blue, which stains hyphal structures and dead plant cells blue. The results of the staining are shown in Table 4.3.

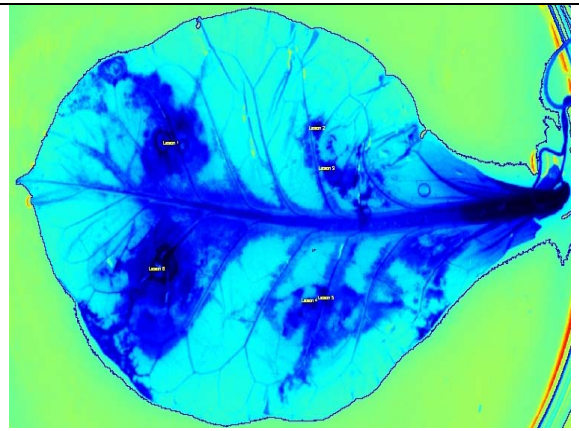
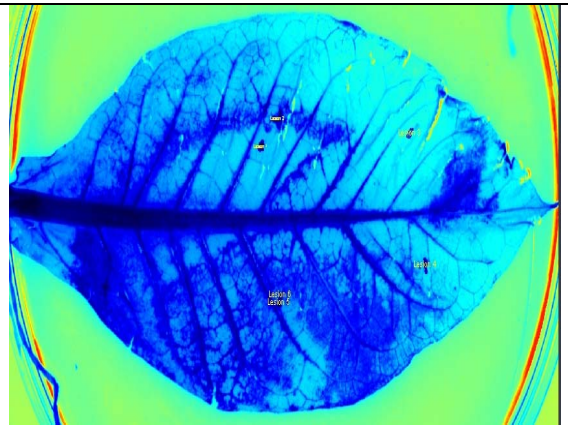
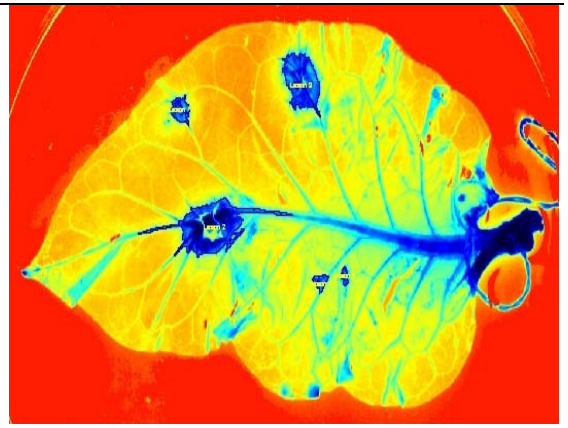
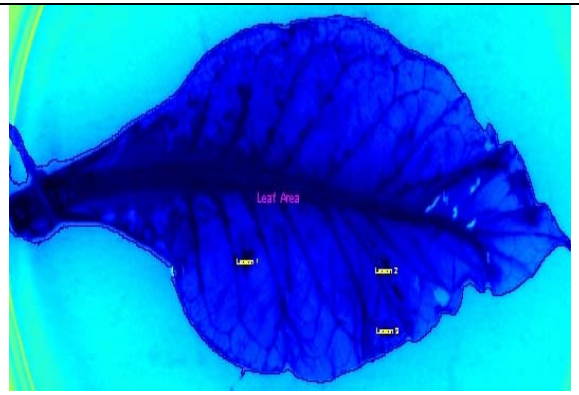
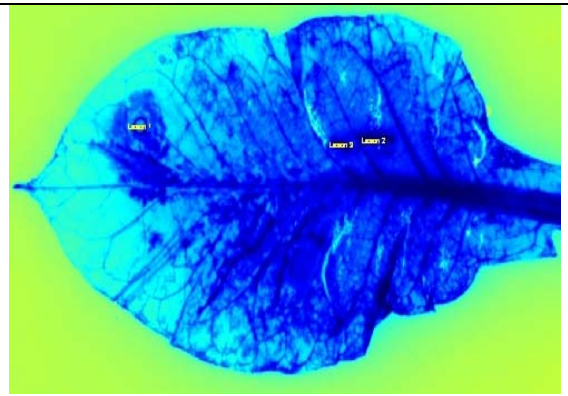
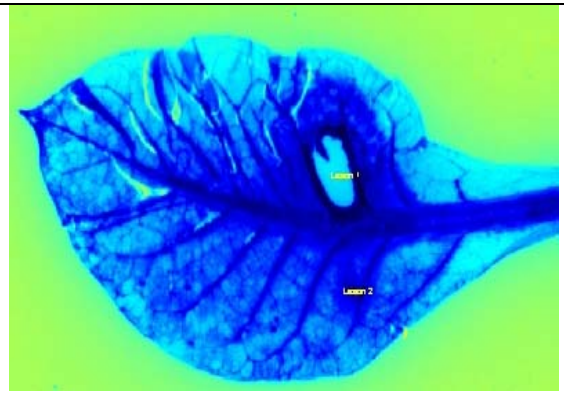
Table 4.3: Photographs showing detached leaves stained with trypan blue from tobacco following target spot leaf infection with *Rhizoctonia solani* AG2

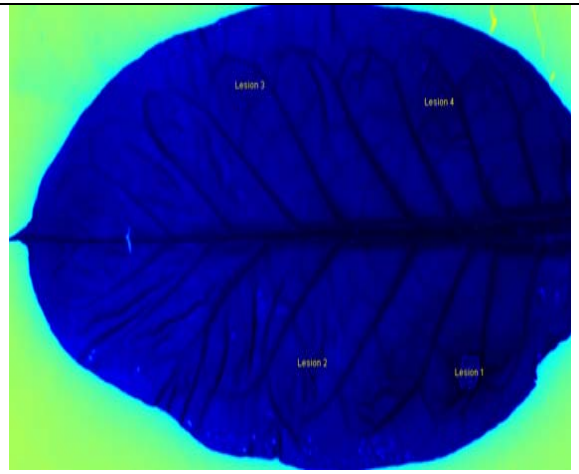
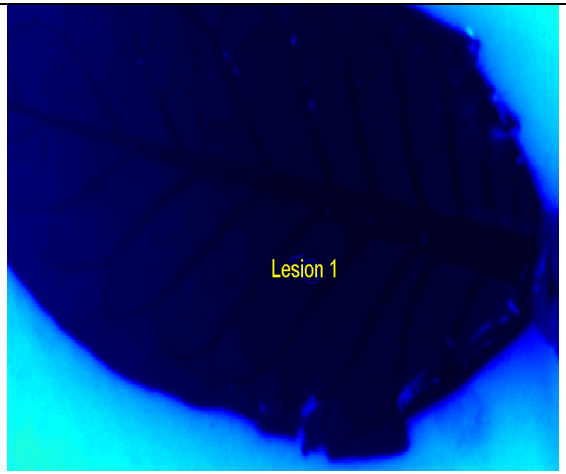
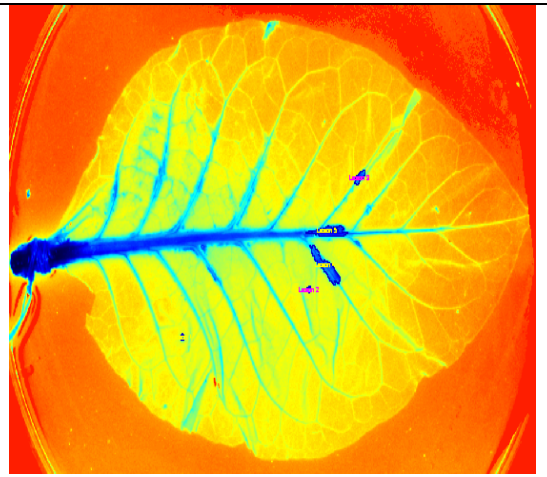
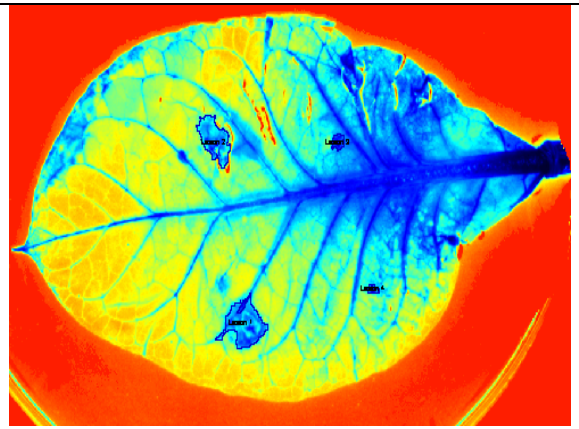
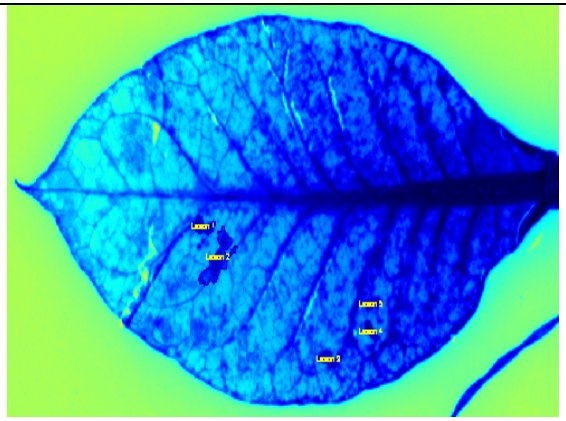
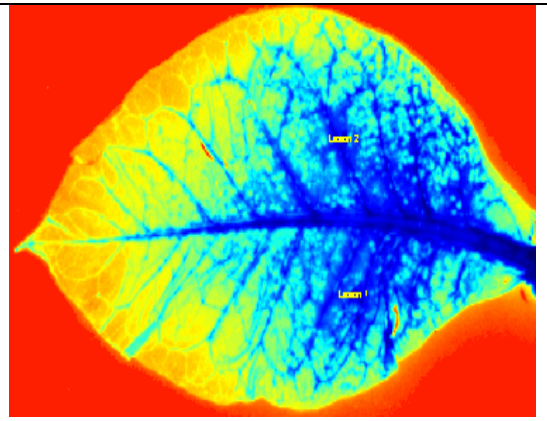
Line	Repeat 1	Repeat 2	Repeat 3
JR6			
Negative control (boiled fungus mix)			

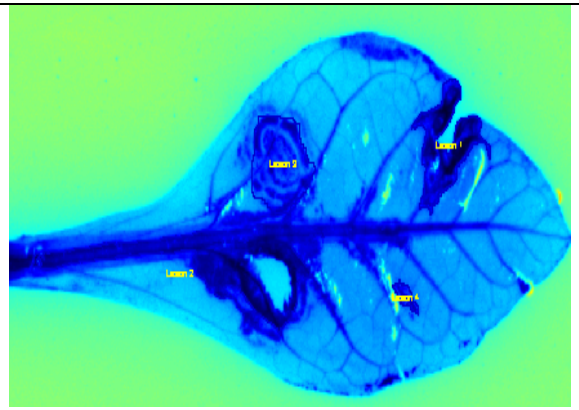
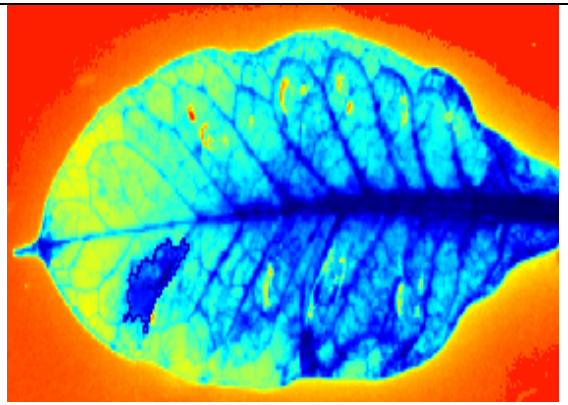
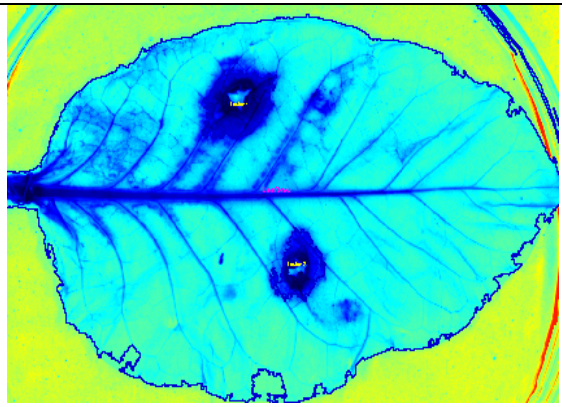
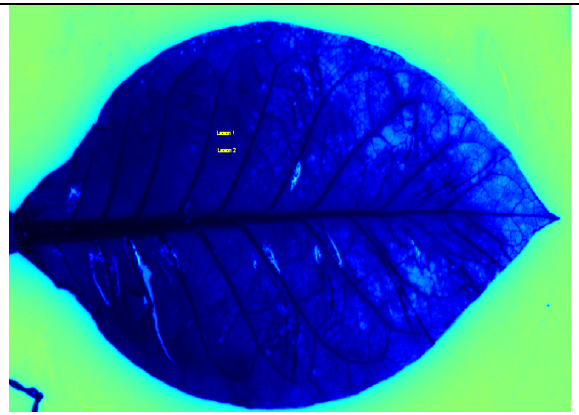
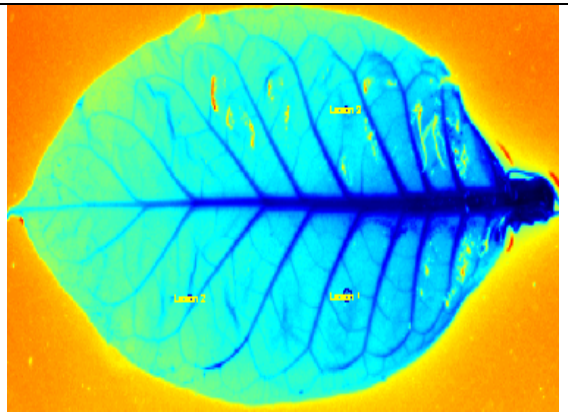
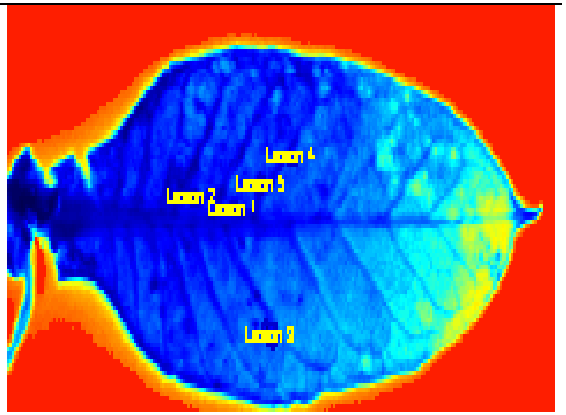
Line	Repeat 1	Repeat 2	Repeat 3
J6 #1			
J6 #2			

Line	Repeat 1	Repeat 2	Repeat 3
J6 #4			
5.1 #2			

Line	Repeat 1	Repeat 2	Repeat 3
5.3 #1			
5.3 #2			

Line	Repeat 1	Repeat 2	Repeat 3
5.4 #2			
5.4 #3			

Line	Repeat 1	Repeat 2	Repeat 3
5.5a #1			
5.5a #2			

Line	Repeat 1	Repeat 2	Repeat 3
5.5a #5			
5.5a #7			

The lesions that were generated on the leaves were quantified using the Bio-Rad Versadoc and Quantity One Software (Bio-Rad). This was done to determine whether the size in lesion generated would correlate with the chitinase gene expression and enzyme assay data generated and vice versa. The average area for the triplicate repeat was obtained and is represented in figure 4.3.

Figure 4.23 indicates that the following lines show a significant decrease in lesion area compared to the untransformed line JR6. These lines include 5.1 #2, 5.3 #1, 5.5a #1, 5.5a #2, 5.5a #7 and J6 #4.

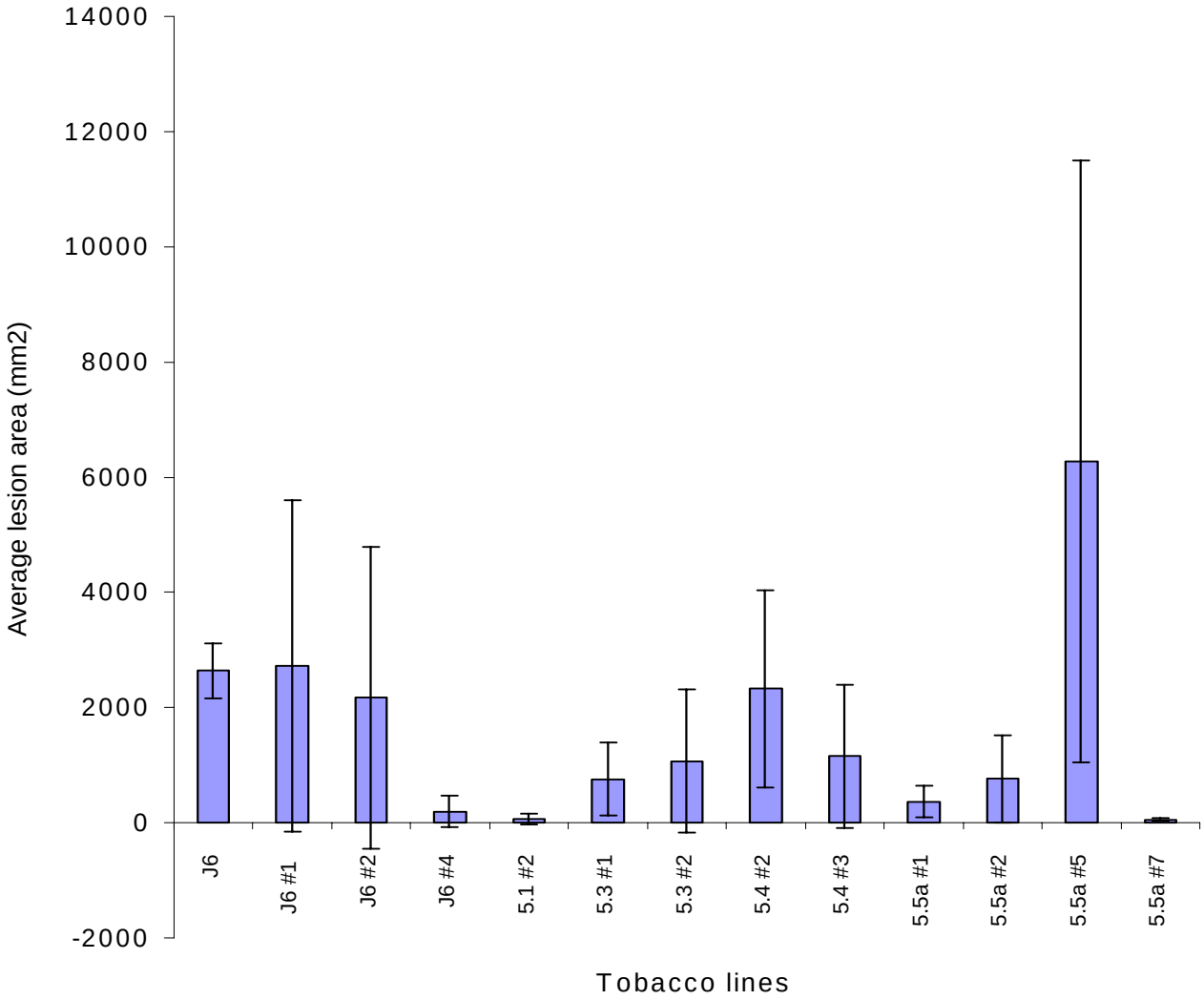


Figure 4.23: Bar chart showing the average lesion areas that were measured for the triplicate repeats for various tobacco lines. The lesion areas were obtained using the Quantity One Software (Bio-Rad) and Volume contour tool.

Chapter 5

Discussion

The research aim was to determine whether the introduction of a classIII chitinase from *Lupinus albus,* driven by a stress inducible promoter *AtGSTF6* element, would yield increased resistance to the fungal pathogen *Rhizoctonia solani.*

This was achieved by constructing an expression cassette containing the *AtGSTF6*-IF3 chitinase gene contained in the binary vector pCAMBIA2300. The binary vector was successfully transformed into *Agrobacterium tumefaciens* LBA 4404 and thereafter the expression cassette was transformed into *Nicotiana tabacum* cv JR6. The transformation yielded 12 different transgenic lines. Verification of the gene integration and expression was achieved through qualitative RT PCR performed on T_0 plants. The T_0 transgenic lines were tested for chitinase expression using a colorimetric chitinase assay. This was followed by a detached leaf assay on transgenic lines that were tested for disease resistance to *Rhizoctonia solani.*

5.1) Subcloning of the *AtGSTF6* promoter and IF3 chitinase gene into pCAMBIA 2300

The cloning strategy was designed so that gene element orientation problems were eliminated.

The first step was to clone the *AtGSTF6* promoter in front of the IF3 chitinase gene. The *AtGSTF6* promoter was already present in a pCAMBIA2300 construct called *At*gstF6-prom-apple PGIP cassette-pCAMBIA2300 (See Appendix B.3). The apple PGIP could however not be excised using available restriction enzymes, combined

with the insertion of the IF3 chitinase as a one step ligation. The plasmid size is 10999bp, which would also have made genetic modification more difficult.

A cloning strategy was thus designed that would allow the construction of the expression cassette in a small carrier plasmid pUC19. A three-way ligation was designed between the *AtGSTF6* promoter (excised by SacI and EcoRI restriction enzymes), IF3 chitinase gene (excised by EcoRI and KpnI restriction enzymes) and linearised pUC19 plasmid (cleaved using SacI and KpnI). The cloning procedure was successful as verified with PCR verification of the cloned insert (Figure 4.2) and restriction enzyme digestion (Figure 4.3).

This was followed by the successful integration of the terminator sequence (Figure 4.4). The completed cloning cassette was successfully introduced in pCAMBIA 2300 (Figure 4.6). The binary vector was then transformed successfully into *Agrobacterium tumefaciens* LBA 4404 and confirmed with colony PCR (Figure 4.7).

5.2) Genetic integrity of the chitinase gene

The IF3 chitinase was obtained from the plasmid pBluKSII(-) IF3 (see Appendix B.2) (Genbank accession number CAA 76203; donated by Regalado, Lissabon, Portugal) with the plasmid sequence available on Vector NTI and was assumed to be correct.

After the gene was cloned, the complete gene was sequenced using primers such as M13 Forward and Reverse primers (data not shown) and ClaI IF3 (Figure 4.9) and SalI IF3 (data not shown) primers. The sequencing data conclusively showed the presence of an inversion from AT to TA at position 641-641 (Figure 4.9). This inversion is nonsynonymous resulting in an amino acid change of cysteine to serine (Figure 4.10). The change was worrying initially although subsequent chitinase activity assays did show that functionality was maintained. The error could either have been generated as part of a typing error in the sequence database or less probable, a mutation arising during the first cloning step into *E.coli* for plasmid multiplication.

The amino acid sequence derived from the chitinase gene was compared to other plant chitinase amino acid sequences using Blast P (Pubmed), and doing a pairwise alignment with the sequences (Figure 4.11). Therefore the distances generated only

relate the similarity of the lupin chitinase sequence with other chitinase sequences and cannot be seen as a multiple alignment.

The figure shows that IF3 chitinase groups closely to Leguminosae characterised chitinases such as *Mediocago truncatula* and *Cicer arietinum*, which was expected as *Lupinus albus* also belongs to the Leguminosae.

One native *Nicotiana tabacum* acidic classIII chitinase clustered further away. This chitinase was isolated from the intercellular fluid in tobacco leaves that were uninfected and infected with Tobacco Mosaic Virus (Lawton *et al.* 1992), thereby showing constitutive expression. A similar pattern was also observed in the IF3 lupin chitinase used in this study, which is constitutively expressed and present in vegetative organs (more specifically the intercellular spaces) of *Lupinus albus* (Regalado *et al.* 2000).

5.3) Tobacco transformation and qualitative RT PCR verification of IF3 chitinase expression

Tobacco leaf disks were co cultivated with *Agrobacterium tumefaciens* LBA 4404 containing the binary vector. The leaf disks were then plated on regeneration media containing kanamycin. This allowed for the selection of transgenic shoots expressing the *npt* II kanamycin resistance gene (Figure 4.12 and 4.13).

The selection method used to obtain transgenic plants requires the addition of the bacteriocidal antibiotic cefotaxime. This antibiotic prevents the *Agrobacterium* from overgrowing the leaf disks during root and shoot formation. However cefotaxime is light sensitive and degrades if exposed to light, often causing the *Agrobacterium* to remain present on leaf surfaces, especially during the regeneration process when leaf disks are exposed to light (http://products.sanofi-aventis.us/claforan/claforan.html). Selection of regenerated transgenic T_0 lines transformed with *Agrobacterium* using PCR of transgenes can therefore result in false positives obtained from genetic material present in the *Agrobacterium*. The presence on putative transgenic plants was confirmed with PCR using *Agrobacterium* specific Mia primers (Grayburn and Vick, 1995), which showed 3 out 12 line samples contained *Agrobacterium* contamination

on the leaf surface (Figure 4.14). This gene forms part of the house-keeping genes in Agrobacterium and encodes a isopentenyl transferase and is highly conserved (Gray *et al.* 1996). Direct PCR to verify transgene presence was therefore not a viable option due to the possibility of *Agrobacterium* contamination.

The outcome of this result meant that an alternative strategy had to be developed. Qualitative reverse transcription (RT) PCR was chosen, as it would yield two results. Firstly it would identify the presence of the chitinase gene in transgenic lines and more importantly that the chitinase gene is transcribed.

The transgenic lines used and tested were T_0 generation as the plants took approximately one year to reach flowering stage to produce a T_1 generation. The initial approach was designed to determine whether the chitinase constructs were being expressed under the control of the inducible *AtGSTF6* promoter element. RNA extracted from leaves of ten different regenerated lines was bulked. Prior to RNA extraction, the plants used in the trial were subjected to 2mM hydrogen peroxide foliar spray treatment and leaves were harvested 4 hours and 24 hours post treatment. The application of hydrogen peroxide was used, as GST promoter elements are known to be induced by hydrogen peroxide (Tenhaken *et al.* 1995). Foliar hydrogen peroxide spray treatment is commonly used to induce *gst* promoters. Wagner *et al.* (2002) used a 5mM foliar spray treatment to probe *gst* induction in *Arabidopsis thaliana* while Chen *et al.* (1996) used 1 mM and 5mM treatments. The treatments included untreated, four hours post treatment and 24 hours post treatment leaves. Figure 4.16 shows that all three treatments express the gene, including the untreated sample. The negative control was untreated untransformed *Nicotiana tabacum* cv JR6, which did not show chitinase PCR amplification, thereby ruling out endogenous chitinase gene amplification. The untreated sample also exhibits chitinase expression, which inturn shows that the promoter possibly has some residual constitutive expression. The leaky expression of inducible expression systems in transgenics is not uncommon (Primrose *et al.* 2001). This can arise due to low level of transcription factor binding on the *AtGSTF6* promoter and/or enhancer or can be due to inadvertent induction resulting from sensitivity to low levels of signal molecules within the cell (Salter *et al.* 1998). Considering the inducible characteristics of the *AtGSTF6* promoter, the latter may be possible. The *AtGSTF6* promoter has a wide-ranging induction pattern, which includes dehydration, low temperature, high salt, wounding

and pathogen attack (Yang *et al.* 1998) driven by mitogen activated protein kinase (MAPK) activity (Grant *et al*. 2000).

The technique was then applied for screening individual lines. All individual lines were treated with 2mM hydrogen peroxide and left for one hour to allow expression activation. The untransformed tobacco line JR6 was treated in a similar manner. This screening technique identified 12 transgenic events showing chitinase presence and gene expression (Figure 4.17, 4.18 and Table 4.1).

5.4) Chitinase assays

The chitinase assays were performed to determine the *in vitro* chitinase activity of the lines identified in Table 4.1. The first experiment was conducted on the same bulked leaf material discussed in Section 5.3.

The chitinase data obtained for the bulked samples can be seen in Figure 4.20. This figure shows that the untreated and 4 hours posttreatment samples exhibit a slight increase in chitinase activity compared to the untransformed line, which was untreated. The increased chitinase activity compared to the untransformed control was a good indicator that the chitinase construct was properly expressed and active. The increased chitinase activity in the untreated sample can also indicate possible transient expression due to a leaky inducible promoter.

The chitinase activity data generated from the bulked samples cannot be seen as quantitative but rather as qualitative due to variability in the sampling process. The variability includes the quantity of leaf material harvested per line due to size differences in the leaves.

Individual T_0 lines were then tested for their chitinase activity. All lines were leaf spray treated with 2mM hydrogen peroxide, including the untransformed line. The induction of endogenous chitinases found in tobacco by the hydrogen peroxide treatment cannot be ruled out, however as all lines assayed were treated with hydrogen peroxide, a direct comparison of increased chitinase activity could be made between the untransformed and transformed tobacco lines. The assay was repeated in

duplicate with 3 biological repeats per line. The biological repeats were conducted over a period of months. This meant that the substrate CM-Chitin-RBV solution (LOEWE blue substrates, Germany), which is influenced by precipitation over time and hydrolysis of dye molecule from chitin substrate based on time exposure to HCL, could give variable results between biological repeat experiments (LOEWE blue substrates, Germany). This variability meant that chitinase Unit activities between different biological repeats could not be compared directly.

The alternative was to analyse the Unit activity between the transgenic line and the untransformed line within a biological repeat. Figure 4.21 shows that the lines J6 #1, J6 #4, 5.1 #2 and 5.5a #7 have several fold increase in chitinase activity compared to the untransformed control. Other lines that show a slight increase in chitinase activity are lines J6 #2, 5.3 #1, 5.3 #2, 5.4 #2 and 5.5a #1.

There are several factors, which can cause differential gene expression in independent transformants. Position effect can affect both constitutive and inducible expression systems and is based on a variation of the site of integration of the transgene in the genome (Blundy *et al.* 1991, Peach and Velten, 1991). Another possibility is gene dosage effect were the number of transgene inserts as well as their zygosity (in this case being hemizygous genotype in the T_0 generation) can influence gene expression in transgenic lines with multiple copies of the transgene being able to increase the expression (Beaujean *et al*. 1998). *Agrobacterium* mediated transformation with T-DNA often results in the integration of more than one copy of T-DNA. Multiple T-DNA inserts have been linked to the down regulation of gene expression. Hobbs *et al.* (1990) found that multiple T-DNA inserts in *Nicotiana tabacum* showed increased methylation, which leads to genetic down-regulation, while single copy T-DNA insertions did not. Other possible explanations for transcriptional gene silencing and down regulation include ectopic DNA-DNA interactions, promoter methylation and aberrant mRNA leading to transcript degradation (Kooter *et al.* 1999; Matzke and Matzke, 1998; Meza, *et al.* 2002). This form of transgene silencing in undesireable as it reduces the efficiency and reliability of transgene expression in transgenics.

5.5) Detached leaf assays with *Rhizoctonia solani*

The T_0 lines characterised on a transcription and chitinase activity level were tested for their resistance towards *Rhizoctonia solani.* The use of *Rhizoctonia solani* to test chitinase activity is well documented, as the cell wall of the pathogen is readily accessible to chitinase enzymes (Benhamou *et al.* 1993) making it a useful model organism in pathogen resistance assays testing chitinase effectivity.

The *Rhizoctonia solani* was grown in pectin media for 7 days. This media was selected to enable the harvesting of polygalacturonase produced by the fungus for another study. After harvesting, the mycelia were used for the pathogen assay.

Known weights of homogenised mycelia were placed on four equidistant spots per leaf. Mycelia was used instead of spores, as *R.solani* makes spores only under certain conditions (and is thus classed as a sterile fungus) (Agrios, 1997). Many disease resistance experiments with *R.solani* are made using soil soak techniques (Datta *et al.* 2002; Emani *et al.* 2003; Jach *et al.* 1995; Lorito *et al.* 1998). The soil soak technique is based on dispensing a known weight of fungal mycelium into the growth medium in which the plants are grown. This allows for a more natural simulation of an infection study. In this case the soil soak technique was not viable as the lines were in T_0 stage and could not be sacrificed, nor did the individual transgenic lines allow significant statistical inference. *R.solani* is however also known to cause leaf target spot on tobacco leaves when placed under high relative humidity (Nicoletti *et al.* 1999). Leaf target spot is known to only develop in conditions of very high humidity and rainfall. The symptoms show watersoaked, spreading opaque necrotic lesions, which leave disintegrated tissue behind.

The first step of the experiment was to determine if the *R.solani* AG2 isolate, isolated from *Nicotiana alata*, was able to infect the leaves of *Nicotiana tabacum* cv JR6. Figure 4.22 shows that lesions develop 5 days after infection with the *R. solani* isolate, showing the characteristic water-soaked browning lesions associated with leaf target spot. In order to quantify the infection, the infected area was measured using the Versadoc in combination with Quantity One Software (Bio-Rad). The Versadoc is an imaging device that allows detailed photography of a variety of objects under different lighting conditions. This software however had problems distinguishing the browning lesion area from the uninfected area. Therefore, trypan blue staining was used, to create better definition between the infected and uninfected area. This staining technique is used to reveal hyphal structures and dead plant cells in plant

tissues and is commonly applied in *Arabidopsis thaliana* (commonweb.unifr.ch/biol/pub/mauchgroup/staining.html).

Fungal structures and dead plant cells are stained blue while other uninfected parts are bleached. This staining allowed the software to identify the infected areas successfully (Table 4.3) and quantify the infected lesion area (Figure 4.23).

From this data one can see that line 5.1 #2, 5.3 #1, 5.5a #1, 5.5a #2, 5.5a #7 and J6 #4 show a decreased lesion area compared to the untransformed *Nicotiana tabacum* cv JR6. Not all the spots developed lesions. This could be due to the fungus not being able to find a site of entry in the leaf. Another possibility is possible disease resistance provided by the chitinase expression. The lines 5.1 #2, 5.5a #7 and J6 #4, having shown several fold increase in chitinase activity also show a strong reduction in the lesion area. This correlation is indicative that the combination of the *AtGSTF6* promoter with the IF3 chitinase can provide increased disease resistance. The increased chitinolytic activity can also result in secondary, indirect protection mechanisms. The degraded glycosidic compounds resulting from chitin hydrolysis are know to act as pathogen elicitors (Benhamou *et al.* 1996; Nishizawa *et al.* 1999; Takai *et al.* 2001; Tanabe *et al.* 2006).

Line J6 #1, which also shows a several fold increase in chitinase activity does not indicate a reduced lesion size. One phenomenon that was found in transgenic *Arabidopsis thaliana* can explain the result. van Leeuwen *et al.* (2001) showed that constitutive transgenic expression can vary within and between a leaf (known as genetic variegation). This genetic variegation does not allow a single pattern of expression to be assigned to a leaf. Different leaves could therefore exhibit different levels of expression of the chitinase gene, which in turn could cause different lesion size developments. Line 5.5a#2 shows no significant increased chitinase activity but shows a reduced lesion size in response to *R.solani* infection. This could be due to a variety of factors including the fungi not finding a site of entry for infection or conditions' not being optimal for fungal infection for the specific repeats.

5.6) Conclusion

The hypothesis of the study was that the introduction of the stress inducible expression system utilising the promoter *AtGSTF6*, driving the IF3 chitinase expression, would confer increased disease resistance to target leaf spot caused by *Rhizoctonia solani* in transgenic plants expressing and producing the functional transgenic chitinase.

The summarised data obtained to test this hypothesis is presented in Table 5.1. From this data one can see that seven of the twelve transgenic events show correlation with the hypothesis while five events do not. The lines J6 #4, 5.1 #2 and 5.5a #7 which showed more than 5 fold increase in chitinase activity also showed a strong resistance towards *Rhizoctonia solani* while J6 #1, which showed a 6.3 fold chitinase activity increase, did not yield a significant decrease in lesion size as discussed in section 5.5.

The variability has previously been documented by (Grison *et al* 1996) who showed that resistance in chitinase transgenic plants is influenced by tissue and cellular localisation of the chitinase, chitinase expression kinetics and period of infection. Other important aspects that can play a role include genetic variegation within leaves (van Leeuwen *et al.* 2001).

In the investigation presented, resistance to *Rhizoctonia solani* AG2 appeared to be increased in transgenic lines showing a fivefold increase in chitinase activity while lower chitinase activity did not result in significant increased disease resistance. The hypothesis appears to be valid in lines were high enzyme activity is registered.

5.7) Future Studies

Future studies would include the investigation of selfed T_1 generation plants from the transgenic lines characterised in this study. PCR can be done to confirm the transgene presence in the T_1 generation. Southern Blots done on these plants could yield information on the copy number of the transgenes in the different lines. This data could in turn explain possible expression deviation, such RNA silencing. The inducible *AtGSTF6* expression system used, also needs to be characterised further in the T_1 generation, to determine if the chitinase has low transient expression or is truly inducible. This data could clarify whether the *AtGSTF6* inducible expression system is viable in *Nicotiana tabacum* to provide an inducible disease resistance response when driving other pathogen response protein expression. Furthermore, experiments

could include disease resistance assays such as soil soak experiments (Emani *et al.* 2003; Jach *et al.* 1995) with *Rhizoctonia solani* AG2 to access resistance of the transgenic lines to the usual symptoms such as dampening off and sore shin (Nicoletti *et al.* 1999). Disease resistance assays could be expanded to other chitin containing pathogenic fungi such as *Sclerotinia sclerotiorum*, *Cercospora nicotiana* and *Fusarium oxysporum*, all of which are pathogens known to infect *N. tabacum*. This data could allow the evaluation of the construct in providing disease resistance in transgenic plants towards pathogenic fungi containing chitin. The study could be stacking principle and whether they can act synergistically to reduce *R.solani* infection.

Table 5.1: Summary of chitinase enzyme activity and *Rhizoctonia solani* resistance assay for *Nicotiana tabacum* cv. JR6 T_0 events.

Transgenic line	IF3 chitinase gene expression*	Chitinase activity**	Increased chitinase activity***		Resistance to *Rhizoctonia solani* ****		Correlation to hypothesis
JR6 untransformed	-	1		Rank		Rank	
J6 #1	+	6.3	Y	3	N	-	X
J6 #2	+	1.4	Y	9	N	-	X
J6 #4	+	6.5	Y	2	Y	3	√
5.1 #2	+	7.4	Y	1	Y	2	√
5.3 #1	+	1.5	Y	7	Y	5	√
5.3 #2	+	2.7	Y	5	N	-	X
5.4 #2	+	2.4	Y	6	N	-	X
5.4 #3	+	1.2	N	-	N	-	√
5.5a #1	+	1.5	Y	8	Y	4	√
5.5a #2	+	1.1	N	-	Y	6	X
5.5a #5	+	1.1	N	-	N	-	√
5.5a #7	+	5	Y	4	Y	1	√

* Presence of IF3 chitinase transcript, based on RT-PCR (data from Figure 4.17 and Figure 4.18)
** Average fold difference in chitinase activity 1 hour after hydrogen peroxide treatment compared to the untransformed tobacco cv. JR6 subjected to the same treatment.

*** Significant increase or non-significant increase in chitinase activity compared to untransformed control (Data from Figure 4.20).

**** Significant increase (Y) or non-significant increase (N) in lesion area compared to untransformed control (Data from Figure 4.22).

Chapter 6

Appendices

Appendix A

Buffers, Solutions, Reagents and Culture Media

A.1) Luria-Bertani (LB) Broth:

Composition per 1l:

10g Tryptone
5g Yeast extract
5g NaCl
for LB agar, add 15 g Bacteriologial Agar

A.2) TSS solution

Composition:

Sterile 40% (w/v) polyethylene glycol (PEG) 3350 diluted to 20% PEG in sterileLB.
100 mM $MgCl_2$
10% (v/v) dimethyl sulfoxide (DMSO)
pH 6.5

A.3) Murashige and Skoog (MS) media:

Composition per 1l:

4.42 g MS Basal Salt mixture with vitamins (Highveld Biological Ltd)
30g Sucrose
8 g Agar
pH 5.8

A.4) Regeneration media

Composition per 1l:

4.42 g MS Basal Salt mixture with vitamins (Highveld Biological Ltd)
30g Sucrose
0.5 mg 3-Indolylacetic acid (IAA)
1 mg 6-Benzylaminopurine (BAP)
8g Agar

A.5) DNase I 10 x reaction buffer:

100 mM Tris pH7.5
25 mM $MgCl_2$
1 mM $CaCl_2$

A.5) Oatmeal Agar:

Composition per 1 l:

60 g Oatmeal
12.5 g Agar

A.6) Trypan blue solution:

Stock solution:

10 g Phenol
10 ml glycerol
10 ml lactic acid
10 ml water
0.02g trypan blue

Working solution:
Mix 96% ethanol with stock solution (2:1 v/v respectively)

A.7) Chloral hydrate solution:

Composition per 400 ml:

1kg chloral hydrate dissolved overnight at 37 C °.

A.8) Citrate/Phosphate buffer

Composition per 100ml:

26.7 ml 0.1 M Citric Acid
23.3ml 0.2 M Na_2HPO_4
50 ml dH_20
pH 6

A.9) Pectin media:

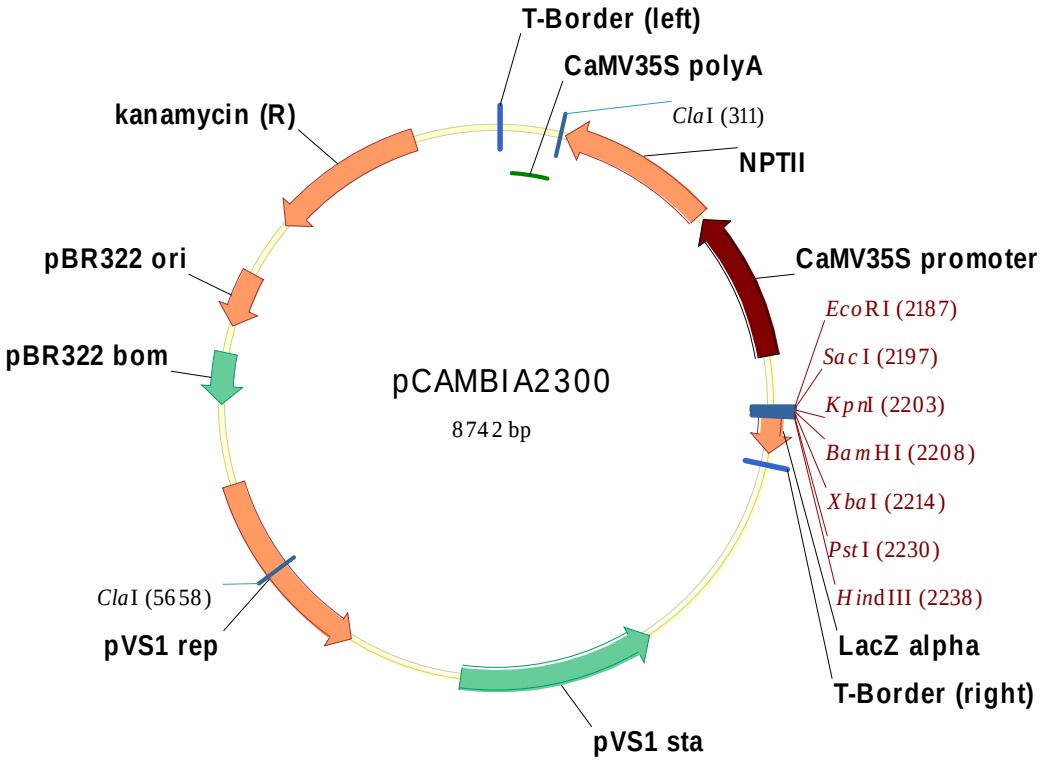
Composition per 25ml:

24 ml Citrate/Phosphate buffer
0.25 g washed pectin (Sigma)
2mM $MgSO_4$ x $7H_2O$
0.1ppm $MnSO_4$ x $7H_2O$
25mM KNO_3
1ppm $ZnSO_4$
0.15ppm $CuSO_4$
1ppm $FeSO_4$
1mg/ml ampicillin

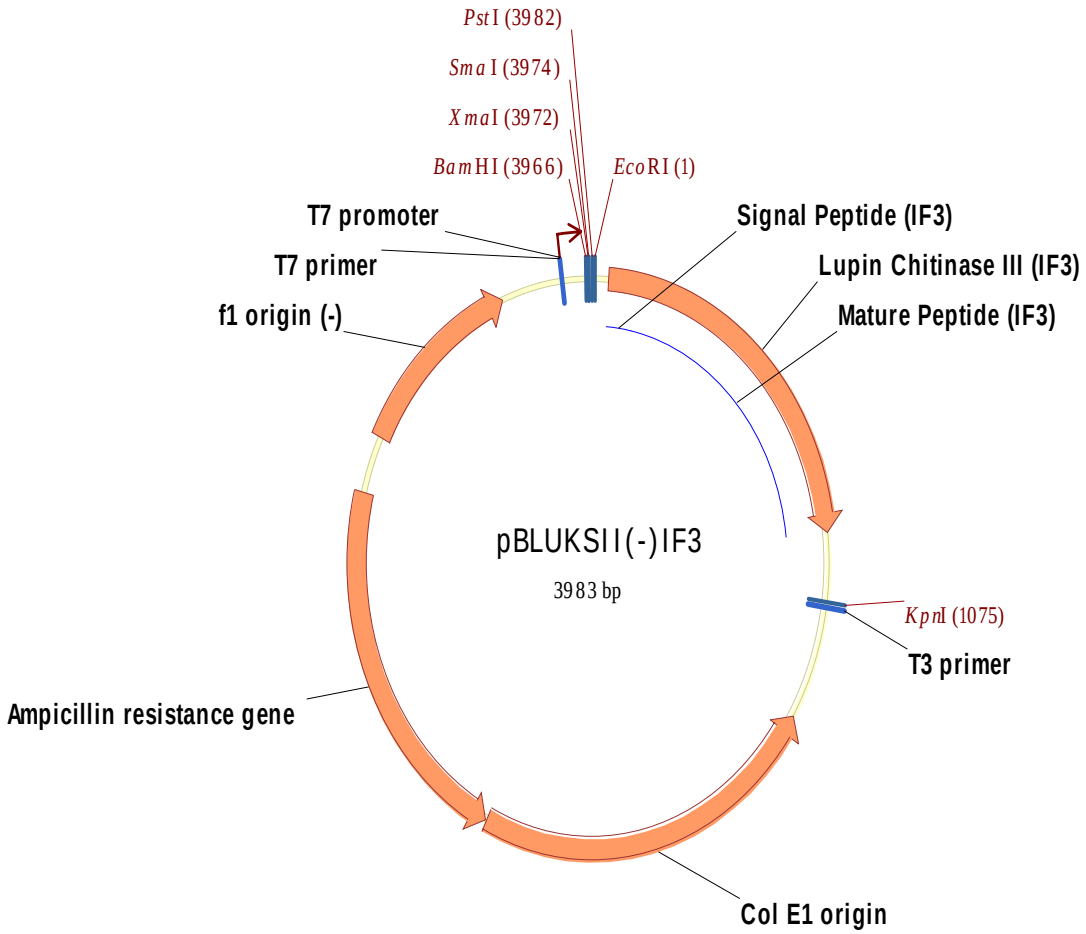
Appendix B

Plasmid Maps

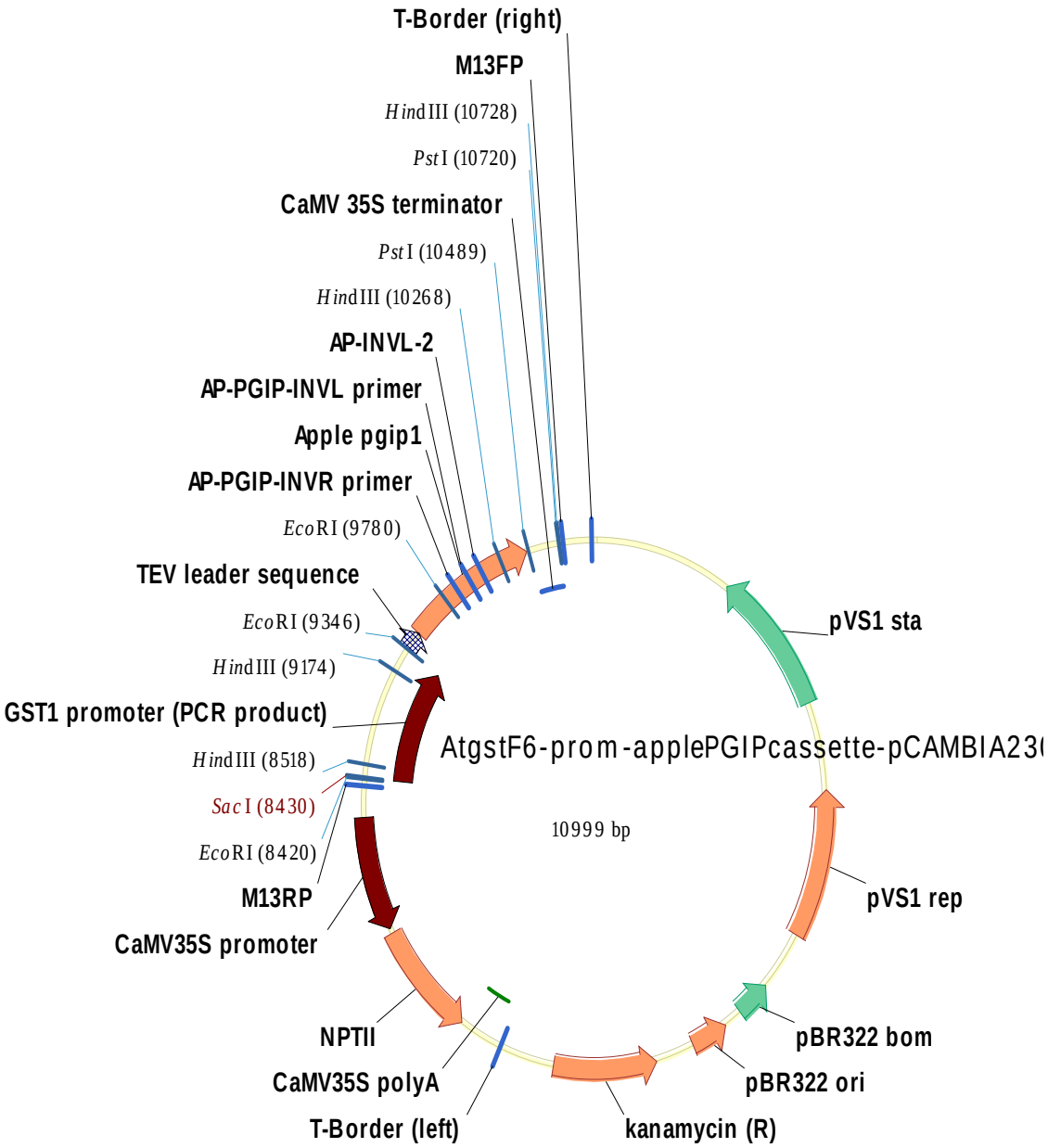
B.1) pCAMBIA 2300(pCAMBIA2300 plasmid donated by CAMBIA®, Canberra, Australia; www.cambia.org):



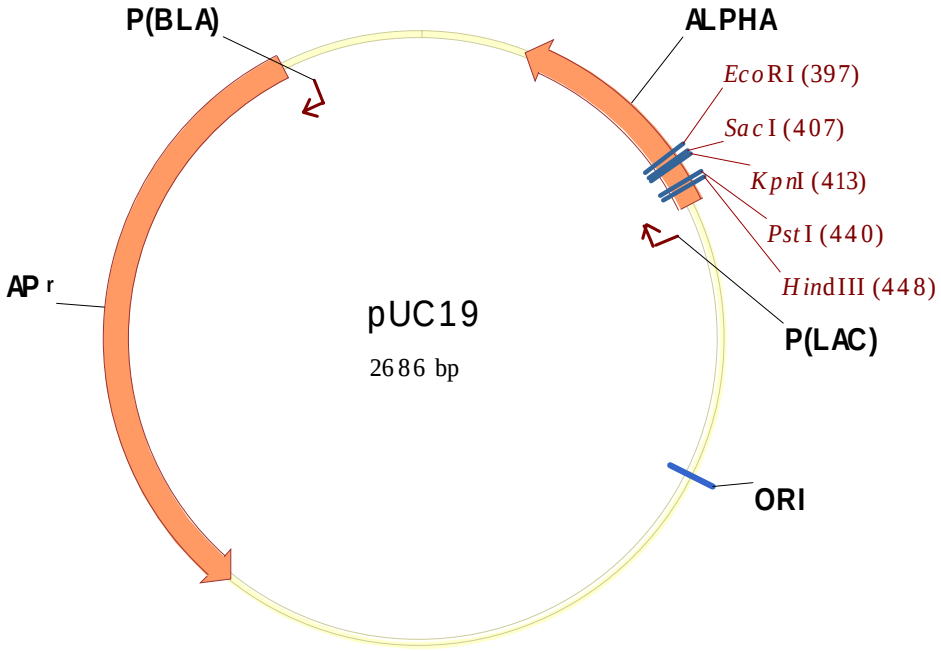
B.2) pBLUKSII (-) IF3(Genbank accession number CAA 76203; donated by Regalado, Lissabon, Portugal):



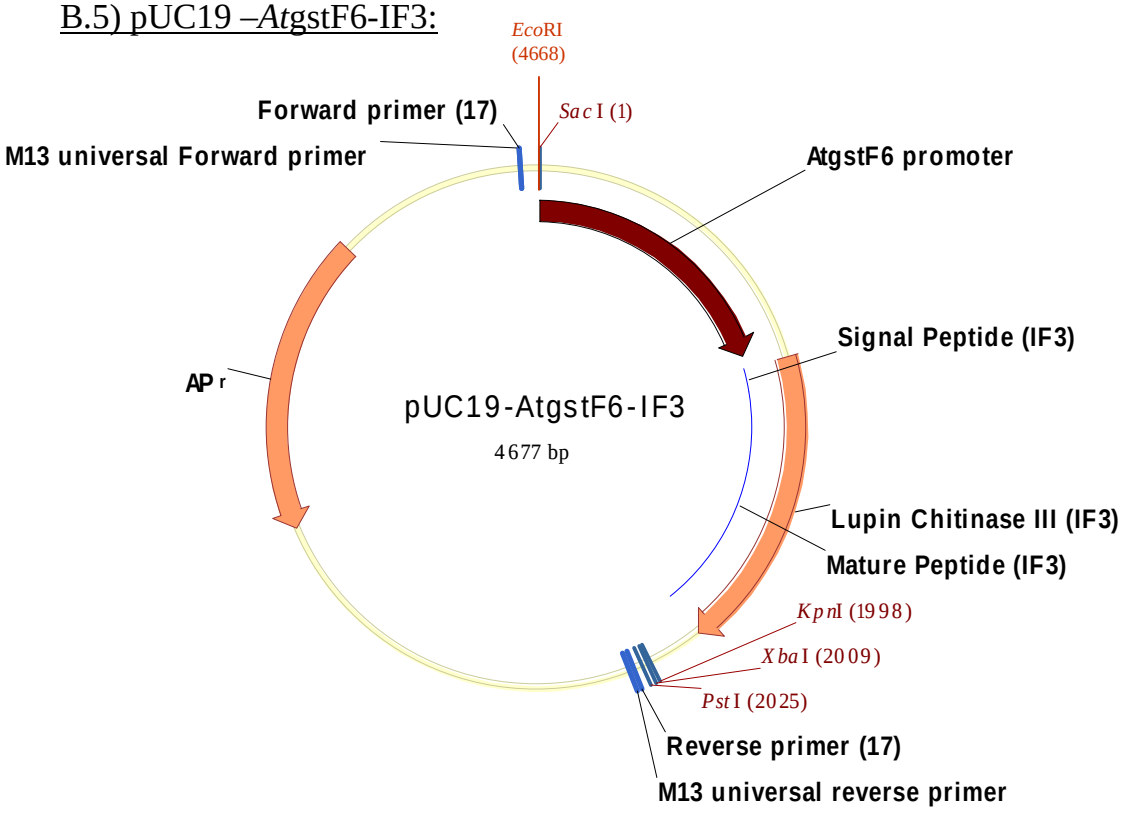
B.3) *At*gstF6-prom-applePGIP cassette-pCAMBIA2300(Maritz, ARC-Roodeplaat, South Africa)(*At*gstf6 Genbank accession number Y11727; donated by Loake, Edinburgh, United Kingdom):



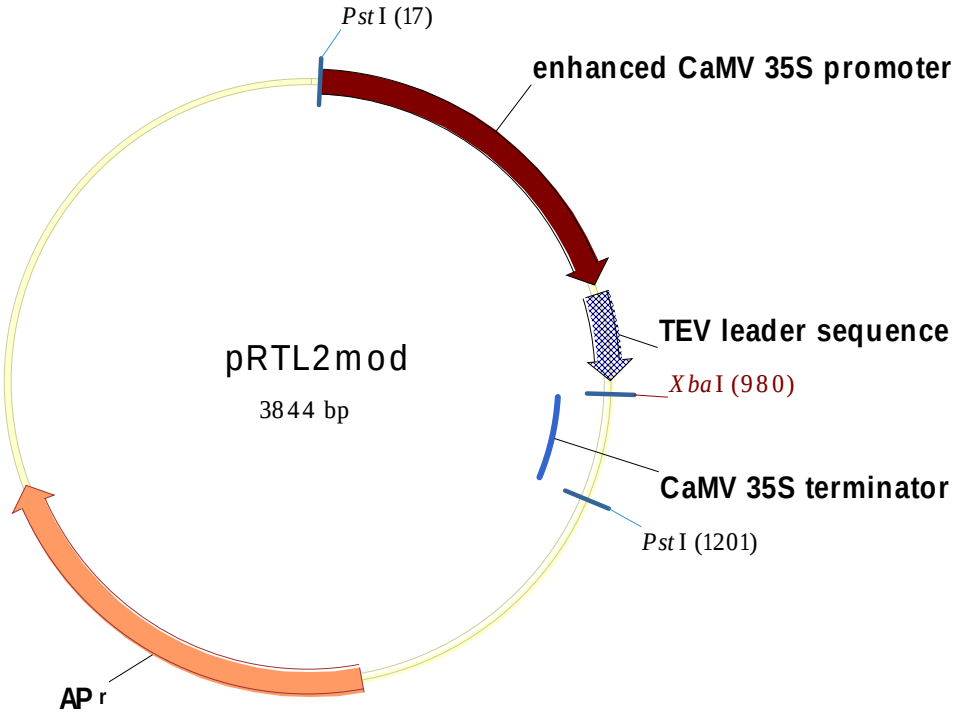
B.4) pUC19(Promega, Madison, USA):



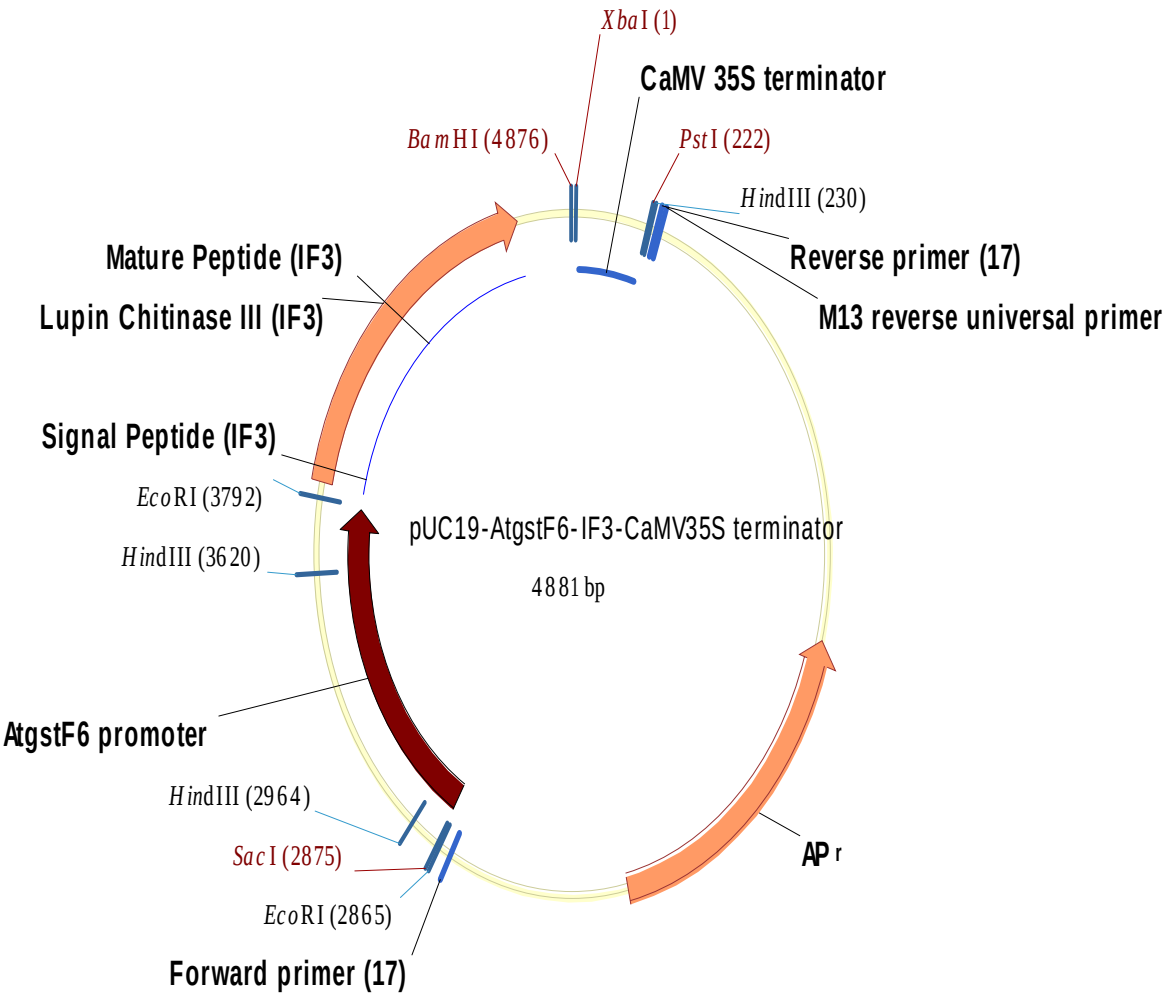
B.5) pUC19 –*At*gstF6-IF3:



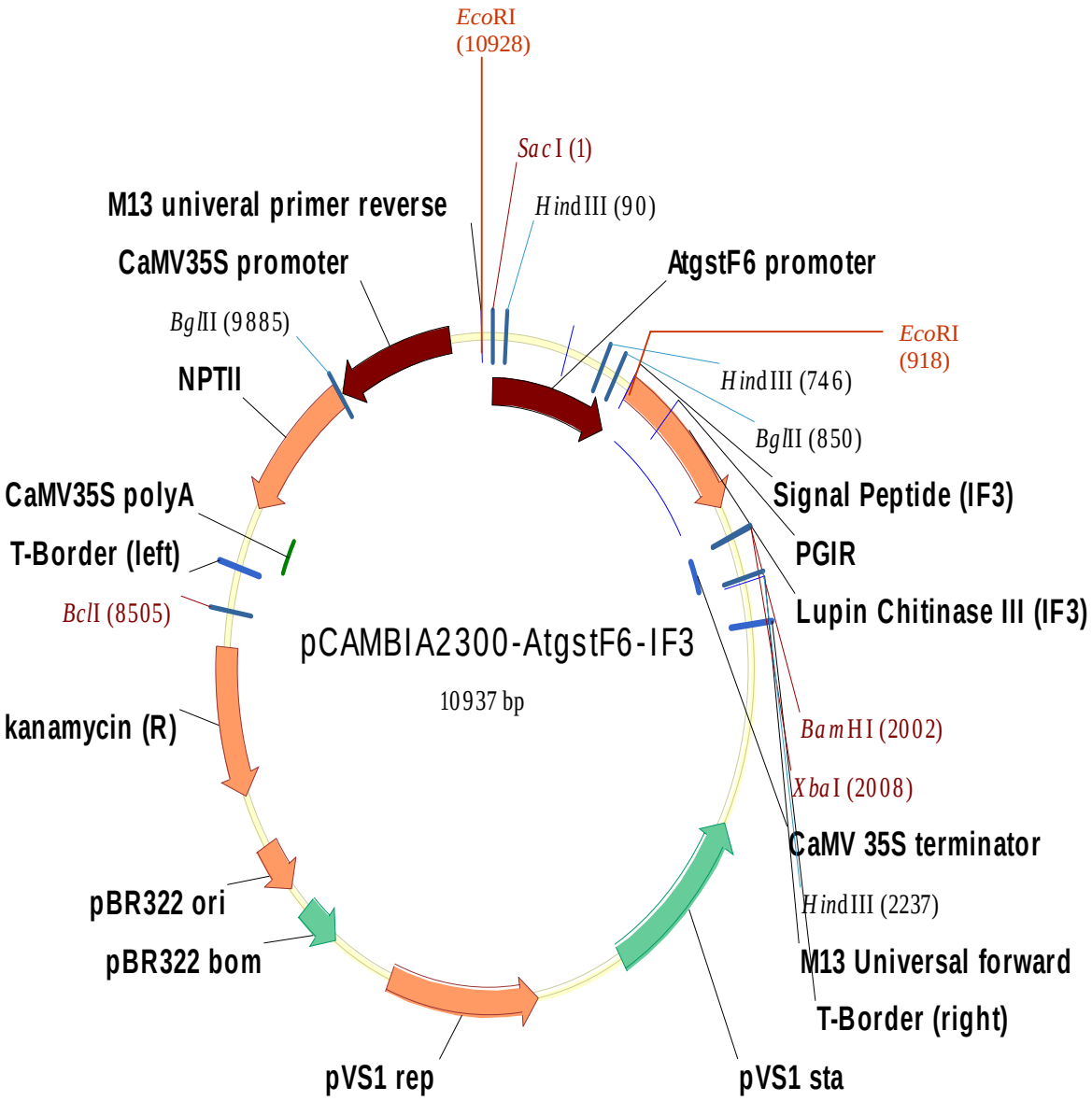
B.6) pRTL2 (pRTL2 plasmid donated by Dr. J. Carrington of Texas A. & M University):



B.7) pUC19-*At*gstF6-IF3-CaMV35S terminator:


XbaI (1)
CaMV 35S terminator
BamHI (4876)
PstI (222)
HindIII (230)
Mature Peptide (IF3)
Reverse primer (17)
Lupin Chitinase III (IF3)
M13 reverse universal primer
Signal Peptide (IF3)
EcoRI (3792)
pUC19-AtgstF6-IF3-CaMV35S terminator
HindIII (3620)
4881 bp
AtgstF6 promoter
HindIII (2964)
SacI (2875)
AP r
EcoRI (2865)
Forward primer (17)

B.8) pCAMBIA2300-*At*gstF6-IF3 plasmid:



Appendix C

Primers:

Table 6.1: Primer names and sequences and their application.

Primer name	Primer sequence (5'- 3')	Application
M13 Forward	GTTTCCCAGTCACGACGTTG	Universal plasmid primer
M13 Reverse	TGAGCGGATAACAATTTCACACAG	Universal plasmid primer
Chi F RT	GTCACTCTCGTGATGGGTTA	Chitinase fragment (402bp)
Chi R RT	GGCATTGAGGGTTGTTGTAG	Chitinase gene fragment (402bp)
MiaA Forward	CCGGCCCGACGGCAAGCGGC	Agrobacterium specific
MiaA Reverse	CGGCTGGATGCGCGTCCAG	Agrobacterium specific
PCGIF	GCAGCCAGTCTCAAACTGAT	Chitinase gene fragment (727 bp)
PCG2R	CAAGTTGTCTCGTGCCGAAT	Chitinase gene fragment (727 bp)
Actin F	GCTATTCAGGGCGTCCTTTC	Endogenous Actin PCR control
Actin R	GCTGACACCATCACCAGAATC	Endogenous Actin PCR control
ClaI IF3	CAAACAAGTTTCACTCAT	Sequencing primers IF3 gene
SalI IF3	ATGCCTGCAGGTCGA	Sequencing primers IF3 gene
NPTII-L	GAGGCTATTCGGCTATGACTG	Amplification of Kanamycin resistance gene
NPTII-R	ATCGGGAGCGGCGATACCGTA	Amplification of Kanamycin resistance gene

Appendix D

Rhizoctonia solani accession number (Centraal Bureau vir Schimmel Kultuuren, Wageningen):

CBSnr 101772 Name *Rhizoctonia solani* J.G. Kühn Collection nrs IPO 2tabakR01 Collected by Nicoletti, No. RT 13 Isolated from Nicotiana alata Location Italy Deposited by IPO-DLO, Wageningen Date of accession Aug 1999 VegCompat AG 2tabak Conds for growth MEA, OA Preservation LN,LY,MO Supply Active.

Appendix E

Table E.1: Chitinase assay results for the first biological repeat showing the line, trendline formula obtained, Units obtained and the factor difference of the Unit of the transgenic line compared to the non transgenic line.

December	Formula obtained	% undiluted Protein	Micrograms Protein	Enzyme Units
J6 #1	y= 0.0346x + 0.0235	2.2	0.44	7.3
J6 #2	y = 0.0093x + 0.0036	10.4	2.08	1.54
J6 #4	y = 0.0359x + 0.0151	2.4	0.47	6.80
J6 negative control	y = 0.0059 +0.0052	16.1	3.21	1
5.1 #2	y = 0.0352x + 0.0252	2.1	0.43	7.54
5.3 #1	y = 0.0083x +0.003	11.7	2.34	1.38
5.3 #2	y = 0.0184x + 0.0252	4.1	0.82	3.92
5.4 #2	y = 0.0095x + 0.0047	10.0	2.01	1.60
5.4 #3	y = 0.0051x - 0.0007	19.7	3.95	0.81
5.5A #1	y = 0.0092x + 0.0064	10.2	2.03	1.58
5.5A#2	y = 0.0025x + 0.0141	34.4	6.87	0.47
5.5A #5	y = 0.0047x + 0.0089	19.4	3.88	0.83
5.5A#7	y = 0.0056x + 0.0118	15.8	3.15	1.02

Table E.2: Chitinase assay results for the second biological repeat showing the line, trendline formula obtained, Units obtained and the factor difference of the Unit of the transgenic line compared to the non transgenic line.

April	Formula obtained	% undiluted Protein	Micrograms protein	Enzyme Units
J6 #1	y = 0.0052x + 0.0191	15.6	3.12	5.81
J6 #2	y = 0.0012x + 0.0046	79.5	15.9	1.14
J6 #4	y = 0.0023x + 0.0261	32.1	6.42	2.82
J6 negative control	y = 0.0011x +0.0003	90.6	18.12	1
5.1 #2	y = 0.0044x +0.0151	19.3	3.86	4.69
5.3 #1	y = 0.0015x + 0.0078	61.5	12.3	1.47
5.3 #2	y = 0.0022x +0.0114	40.3	8.06	2.25
5.4 #2	y = 0.0027x + 0.0143	31.7	6.34	2.86
5.4 #3	y = 0.0014x + 0.0035	68.9	13.78	1.31
5.5A #1	y=0.0014x + 0.0082	65.6	13.12	1.38
5.5A#2	y = 0.0015x + 0.0028	64.8	12.96	1.40
5.5A #5	y= 0.0012x + 0.0047	79.4	15.88	1.14
5.5A#7	y = 0.0046x + 0.0143	18.6	3.72	4.87

Table E.3: Chitinase assay results for the third biological repeat showing the line, trendline formula obtained, Units obtained and the factor difference of the Unit of the transgenic line compared to the non transgenic line.

May	Formula obtained	% undiluted Protein	Micrograms protein	Enzyme Units
J6 #1	y = 0.0059x + 0.0102	15.2	3.04	5.90
J6 #2	y = 0.0015x + 0.0078	61.5	12.3	1.46
J6 #4	y = 0.0088x + 0.0198	9.1	1.82	9.86
J6 negative control	y = 0.0011x + 0.0012	89.8	17.96	1
5.1 #2	y = 0.0098x + 0.0119	9	1.8	9.98
5.3 #1	y= 0.0017x +0.0073	54.5	10.9	1.65
5.3 #2	y= 0.0018x + 0.0149	47.3	9.46	1.9
5.4 #2	y = 0.0028x + 0.0112	31.7	6.34	2.83
5.4 #3	y = 0.0015x + 0.003	64.7	12.94	1.39
5.5A #1	y = 0.0015x + 0.0062	62.5	12.5	1.44
5.5A#2	y = 0.0014x + 0.0088	65.1	13.03	1.38
5.5A #5	y = 0.0013x + 0.0043	73.6	14.72	1.22
5.5A#7	y = 0.0091x + 0.0111	10.2	2.05	8.78

Table E.4: Combined data for the triplicate biological repeat showing the average Factor difference in Unit activity as well as the standard deviation.

Combined data	Average Unit Activity	Standard Deviation Average Unit Activity
J6 #1	6.34	0.84
J6 #2	1.39	0.21
J6 #4	6.5	3.53
J6 negative control	1	0
5.1 #2	7.40	2.64
5.3 #1	1.5	0.14
5.3 #2	2.69	1.08
5.4 #2	2.43	0.72
5.4 #3	1.17	0.31
5.5A #1	1.47	0.10
5.5A#2	1.08	0.53
5.5A #5	1.06	0.20
5.5A#7	4.89	3.88

Chapter 7

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