Construction and testing of an endochitinase/β-1,3-glucanase fusion gene for ability to confer Fusarium wilt resistance in tomato

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October 25, 2010

A thesis submitted for the degree of Master of Philosophy of the Australian National University
For my family and Rosamunde — my laptop computer and my lady.
Statement of sources

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of higher education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given. Materials obtained for use in this study that were generated by others have been acknowledged accordingly in the text.

Cahya Prihatna

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Abstract

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) is one of the most devastating diseases of tomato. The most effective, practical, and environmentally friendly strategy to control the disease would be to generate resistant tomato cultivars. Such a strategy can be achieved through the introduction of resistance genes or genes that function in defence, such as chitinase and $\beta$-1,3-glucanase, into susceptible tomato cultivars. This study reports an attempt to generate transgenic tomato carrying endochitinase (*ech42*) and $\beta$-1,3-glucanase (*gluc78*) genes from the biocontrol fungus *Trichoderma atroviride* strain P1 and test their combined ability to confer resistance to Fusarium wilt. Several steps involving various technically challenging approaches were performed to try to achieve this objective. First, an endochitinase/$\beta$-1,3-glucanase fusion gene was constructed using PCR-driven overlap extension to obtain a transcriptional and translational fusion. A modified PCR-driven overlap extension method was developed and it proved efficient in creating the chimeric endochitinase/$\beta$-1,3-glucanase fusion gene. A red fluorescent protein (*rfp*) reporter gene construct was also generated using this method. Second, *Fol* race 3 was transformed with a green fluorescent protein (*gfp*) reporter gene to enable the growth of *Fol* race 3 to be monitored in tomato roots. Third, transformation of tomato hairy roots and whole tomato transformation were attempted with the endochitinase/$\beta$-1,3-glucanase fusion gene using the *rfp* gene as a reporter.

*Fol* race 3 was transformed with *gfp* under the control of the constitutive *gpd* promoter (*gpd promoter-gfp*) and the inducible *SIX1* (*Avr3*) promoter (*SIX1 promoter-gfp*) using *Agrobacterium tumefaciens*-mediated transformation. The *SIX1* promoter-*gfp* construct would allow the expression of *gfp* only when *Fol* is growing within its tomato host [164]. *Fol* race 3 transformation with *gpd*
promoter-\textit{gfp} was efficient and transformants were obtained at high frequency. On the other hand, transformation of \textit{Fol} race 3 with \textit{SIX1} promoter-\textit{gfp} occurred at low frequency and there was little evidence for GFP fluorescence when putative transformants were used to infect tomato roots. The pathogenicity of both transformed lines was not altered towards resistant and susceptible tomato cultivars but the \textit{SIX1} promoter-\textit{gfp} \textit{Fol} transformants showed reduced avirulence towards the resistant tomato line. The general lack of \textit{SIX1} promoter-\textit{gfp} transformants able to express \textit{gfp} may be attributed to the lack of 300 bp of the \textit{SIX1} promoter region that could contain necessary elements for efficient expression of the \textit{SIX1} promoter-\textit{gfp} construct. To examine this hypothesis, the additional 300 bp of the \textit{SIX1} region should be added to the \textit{SIX1} promoter-\textit{gfp} construct and used to transform \textit{Fol}.

The \textit{rfp} reporter gene was incorporated along with the chimeric gene into the T-DNA of binary vectors to enable screening of transformed hairy roots with the chimeric gene. However, only weak expression of \textit{rfp} transcript and no expression of chimeric gene transcript was detected following transient expression analysis in \textit{Nicotiana benthamiana}.

Hairy root transformation employing \textit{Agrobacterium rhizogenes} was performed both \textit{in vitro} and \textit{ex vitro}. However, the lack of RFP fluorescence prevented detection of hairy roots transformed with the chimeric gene. Hairy roots may have been transformed but the \textit{ocs} promoter was probably not strong enough to drive sufficient \textit{rfp} expression for detection of RFP fluorescence. Replacing the \textit{ocs} promoter with a stronger promoter like the 35S promoter could have helped to enhance \textit{rfp} expression. Transformation was also attempted using \textit{A. tumefaciens} carrying the chimeric gene but it failed to generate any transformed tomato plants.

Although the ability of the chimeric endochitinase/\(\beta\)-1,3-glucanase gene to confer resistance against Fusarium wilt in tomato remained untested, it remains a valuable objective for future research.
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<th>Definition</th>
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<tbody>
<tr>
<td>ABA</td>
<td>absisic acid</td>
</tr>
<tr>
<td>AFM</td>
<td>Armstrong <em>Fusarium</em> medium</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CC-NB-LRR</td>
<td>coiled-coil nucleotide binding leucine-rich repeat</td>
</tr>
<tr>
<td>CDA</td>
<td>Czapek Dox agar</td>
</tr>
<tr>
<td>CM</td>
<td>co-cultivation medium</td>
</tr>
<tr>
<td>CWDE(s)</td>
<td>cell wall degrading enzyme(s)</td>
</tr>
<tr>
<td>Fol</td>
<td><em>Fusarium oxysporum</em> f. sp. <em>lycopersici</em></td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GIP(s)</td>
<td>glucanase inhibitor protein(s)</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
</tr>
<tr>
<td>IFB</td>
<td>infiltration buffer</td>
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<tr>
<td>IM</td>
<td>induction medium</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<tr>
<td>MM</td>
<td>minimal medium</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
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<tr>
<td>Term</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>PR protein</td>
<td>pathogenesis-related protein</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>R gene</td>
<td>resistance gene</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>rol</td>
<td>root loci</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse-transcriptase PCR</td>
</tr>
<tr>
<td>SOE</td>
<td>spliced by overlap extension</td>
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<tr>
<td>TB</td>
<td>transformation buffer</td>
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<tr>
<td>TF</td>
<td>transcription factor</td>
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Chapter 1

General introduction

Fusarium wilt disease in tomato, caused by *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*), is one of the most devastating diseases of tomato and it has caused major losses in tomato production worldwide. The disease is characterised by wilting and browning of the leaves, yellowing, stunted growth, and eventual death of the plant. Crop yield could be minimal or nearly absent in highly infected tomato plantings. Control against epidemic spread of Fusarium wilt involves three main strategies: first, control through plant husbandry practices such as avoiding contamination of healthy plants and rotating tomato plantings with different crops; second, the development of resistant cultivars by conventional breeding; and lastly, the use of agrochemicals such as methyl bromide and thiophanate-methyl. The plant husbandry technique is not effective because of the widespread and persistent nature of the fungus. Conventional breeding has successfully generated resistant cultivars, but the technique is time-consuming and genetic resources are limited. Chemical use may be practical and effective in the short term, but limited in the long term, and too late if the disease has already occurred. It is also potentially damaging to the environment. Another strategy employs competing non-pathogenic strains of *F. oxysporum* as biological control agents [53, 85, 118]. These strains colonise the rhizosphere and roots of potential host plants but do not induce any local or systemic symptoms. Many attempts have been made to engineer durable resistance in important crop plants employing genes involved in disease resistance. However, in some cases, this approach has failed to generate durable and broad-spectrum disease-resistant transgenic
plants. Nevertheless, future development of transgenic approaches remains possible as our understanding of pathogenesis and plant defence improves.

Establishment of disease is actually a failure of the plant to recognise pathogen challenge and to induce a rapid defence response. Plants already possess several layers of defence — from physical barriers and pre-formed antimicrobials, to surveillance systems encoded by innate immunity genes and disease resistance genes, which are capable of responding to pathogen attack by activating an array of defence responses, mainly characterised by the hypersensitive response and the production of pathogenesis-related (PR) proteins. Thus, a plant constantly monitors for pathogen attack. According to the gene-for-gene hypothesis, a plant is resistant to a potential pathogen if it carries a resistance (R) gene cognate to an Avr determinant from the pathogen [52]. Approximately 40 disease resistance genes involved in recognition of specific pathogens by a variety of host plants have been cloned and characterised (reviewed in [24]). In tomato, four resistance genes have been shown to confer resistance to known Fol races. The only resistance gene effective against Fol that has been cloned and characterised so far is the I-2 gene, which confers resistance to Fol race 2 [148, 119]. The I-2 gene encodes a nucleotide-binding leucine-rich repeat protein with a coiled-coil domain (CC-NB-LRR) and is expressed mainly in the vascular tissue of the roots, stems, and leaves of tomato [110].

Defence responses induced upon perception of pathogen avirulence proteins by plant resistance proteins are complex and overlapping regardless of the resistance protein involved and the nature of the attacking pathogen. One of the major characteristics of plant defence responses is the expression of a set of plant defence-related genes including those encoding PR proteins. Chitinases and glucanases are examples of PR proteins that are often found in plants undergoing hypersensitive response and other types of defence responses against pathogens. These enzymes degrade chitin and glucan, major constituents of fungal cell walls [107, 86, 165, 103, 5]. Evidence from several studies showed that, compared with the introduction of either chitinase or β-1,3-glucanase alone, constitutive expression of both chitinase and β-1,3-glucanase genes as a double
gene construct resulted in higher levels of resistance to several pathogenic fungi [72, 75, 109, 5]. However, despite such improvements, the transgenic constructs tested so far have not yet been able to confer effective resistance against a wide range of fungal pathogens. Moreover, in some cases, introduction of exogenous genes caused deleterious effects to the plants [95]. Although effective transgenic resistance against insects and viruses has been achieved and used commercially, no commercially useful levels of fungus-resistant crops have been achieved to date. Therefore, novel alternative strategies that would circumvent the problems found to date are vital to generate durable fungus-resistant crops.

1.1 The tomato-*Fusarium oxysporum* f. sp. *lycopersici* interaction

1.1.1 The gene-for-gene model for *Fol*-tomato interaction

*Fusarium oxysporum* is a soilborne fungus that exists as host-specialised pathogenic strains and non-pathogenic root-colonising strains. The pathogenic strains are clustered into formae speciales based on their host range. For instance, *F. oxysporum* f. sp. *lycopersici* (*Fol*) causes vascular wilt of tomato plants; *F. oxysporum* f. sp. *cubense* causes vascular wilt in banana; and *F. oxysporum* f. sp. *vasinfectum* causes vascular wilt in cotton. *Fol* isolates are further subdivided into races based on their interaction with different tomato cultivars carrying various resistance genes.

The interaction between *Fol* and tomato is race-cultivar specific. A classical gene-for-gene relationship has been proposed as a basic concept underlying the interaction between *Fol* races and their host cultivars based on dominant monogenic resistance triggered by challenge with known *Fol* races. Races in *Fol* are assigned according to their order of discovery, namely races 1, 2, and 3. On the other hand, four resistance genes that confer resistance to known *Fol* races have been described in tomato: *I*; *I-1*; *I-2*; and *I-3* [70]. *Fol* race 1 is countered by the *I* gene or the unlinked *I-1* gene. *Fol* race 2 is virulent on *I* or *I-1* tomato
lines, and resistance to this race is conferred by the I-2 gene. Likewise, race 3 is virulent on the I/I-1 and I-2 lines, and is countered by the I-3 gene. However, the I-2 gene could also provide resistance against a subset of Fol race 1 isolates, thus further subdividing race 1 into separate virulence groups [111].

The I gene was introgressed from the wild tomato Lycopersicon pimpinellifolium accession 160 (PI79532) [13] and behaves as a dominant gene that resides in chromosome 11 [120]. The I-1 gene, which also confers resistance to Fol race 1, was found later in another wild tomato L. pennellii [17, 137]. The I-2 gene, which confers resistance to Fol race 2, was discovered in a hybrid of L. esculentum × L. pimpinellifolium PI126915 and is located on chromosome 11 [138]. The I-2 gene has been identified and cloned, and it encodes a CC-NB-LRR protein [148]. The I-3 gene that confers resistance to Fol race 3 was introgressed from L. pennellii accession LA716 and is located on chromosome 7 tightly linked (about 2.5 cM) to the Got-2 isozyme marker [17]. Recent genetic and physical mapping places the I-3 gene within a 0.38 cM interval between RGA332 and bP23/gPT molecular markers with 50-60 kb in length [93]. A cluster of five S-receptor like kinase (SRLK) genes is present within the I-3 region, and a cluster of SRLK genes is also found in the microsyntenous region of grape chromosome 12 [93, 92].

In Fol, the first Avr gene discovered was SIX1 [131]. The gene encodes a small, cysteine-rich protein with an approximate mass of 32 kDa. The central part of the SIX1 protein is probably processed to form a 12 kDa protein found in the xylem sap of tomato. Gene knock-out and complementation revealed that the SIX1 gene is required for I-3-mediated resistance of tomato to Fol. However, deletion of SIX1 led to reduced virulence of Fol on susceptible tomato lines [130]. This suggests that the SIX1 gene is also required for Fol pathogenicity. Three other secreted proteins were also found in the xylem sap of tomato infected with Fol race 1, namely SIX2, SIX3, and SIX4. The nomenclature of these proteins was revised based on gene-for-gene specificity with the corresponding R genes in tomato plants. SIX1 is now named Avr3, SIX4 is Avr1, and SIX3 is Avr2. Avr1, Avr2, and Avr3 genes are present in Fol race 1; Avr1 is absent in Fol race 2 and
race 3 strains; *Fol* race 2 harbours *Avr*2 and *Avr*3; and race 3 harbours the *Avr*3 gene. *Fol* race 3 also carries *Avr*2 gene but with point mutations. Nevertheless, most *Fol* race 1 strains can cause disease in tomato plants containing the *I*-3 resistance gene. Interestingly, *SIX1* is still found intact and functional in some *Fol* race 1 isolates that are virulent on an *I*-3 tomato line [131, 130]. Deletion of *Avr1* renders *Fol* race 1 avirulent towards tomato plants containing the *I*-3 gene, while introduction of *Avr1* in race 2 or race 3 leads to gain of virulence towards *I*-3 plants. This suggests that *Avr1* suppresses *I*-2 and *I*-3-mediated resistance in tomato [68]. However, other factors in the genetic background of the fungus may contribute to the suppression of *R* gene-mediated immunity by *Avr1* since not all *Fol* race 1 strains are virulent on *I*-2 and/or *I*-3 tomato plants.

### 1.1.2 Infection and disease progression

In addition to studying the molecular interaction between *Fol* and tomato, studies on physical interaction at the microscopic level would provide invaluable information about the onset and development of Fusarium wilt disease in tomato as well as the response of the host plants to *Fol* infection. The most common visual markers used in plant-pathogen interaction studies are β-D-glucuronidase (GUS) and green fluorescent protein (GFP) [69]. GFP, in particular, has been widely used to visualise the interaction between pathogens and host plants [96]. Before the advent of fluorescent proteins, histochemical staining was used to study the *in planta* interaction between *F. oxysporum* and its hosts [31]. Unlike foliar and fruit diseases, plant root and vascular diseases pose a unique challenge in the observation of disease onset and progression because special techniques are required to avoid invasive examination of the roots.

*Fusarium oxysporum* infects plants via the roots primarily through penetration into the meristematic region of primary and lateral roots [117]. Before contact with the host cells, pathogen hyphae may release phytotoxin(s) or other proteins that compromise host cell defence [34]. The first step of *Fol* colonisation and development in tomato is the adhesion of *Fol* conidia onto the roots. In
Regardles of the strain, Fol conidia attach firmly to the tomato roots after 15 minutes [128]. Within 24 hours the surface of the root tip region is colonised and hyphae grow along anticlinal walls. Hyphae penetrate into the epidermis as early as 24 hours and subsequently colonise the root cortex. At this stage, physical barriers such as thickening and coiling of the plant cell walls are usually formed in the cortex and extend to regions proximal to xylem vessels, which are never colonised by nonpathogenic F. oxysporum. These barriers generally fail to prevent the centripetal growth of pathogenic strain towards the stele [117]. Colonisation of the cortex by a pathogenic strain is more extensive than by a nonpathogenic one. A pathogenic strain is able to penetrate barriers in the hypodermis before it finally reaches and colonises the xylem. Once the fungus colonises the xylem vessels, it grows preferentially within the xylem vessels of the vascular bundle and spreads throughout the plant via these vessels. Heavy Fol colonisation in these vessels causes blocking of water and nutrient transport throughout the plant. Heavy Fol infection results in an excessive proliferation of parenchyma and tyloses as a response to the pathogen, and these contribute to further clogging of the xylem vessels. As a result, loss of water causes the plant to lose turgor and collapse leading to death of the plant. Fol also produces cell wall degrading enzymes (CWDEs) and toxins that contribute to the death of the plant [7]. Physiological responses in tomato towards Fol infection include the production of callose, tyloses, and phytoalexins [7]. However, these responses occur in both compatible and incompatible interactions. Thus, it is not known what the exact mechanisms are that restrict Fol colonisation in resistant plants.

1.2 The role for pathogenesis-related proteins in defence responses

Pathogenesis-related (PR) proteins were first identified in tobacco leaves undergoing hypersensitive response to Tobacco Mosaic Virus (TMV) infection. They were found to share common biochemical properties: being low molecular weight, acidic, and relatively protease resistant [167, 166]. Subsequently, many more PR
proteins have been identified and they display diverse properties. PR proteins were originally classified by their characteristic induction in pathological and related situations. Related proteins that are expressed in the absence of pathogen infection are referred to as PR-like proteins. However, the levels of certain PR-like proteins increase after pathogen infection and some PR proteins are occasionally found in healthy plants. The term pathogenesis-related proteins has now become a collective term for all microbe-induced proteins and their homologues.

To avoid confusion, the term inducible defence-related proteins has been proposed to distinguish those PR proteins that are only induced after pathogen infection and related to the defence response [165]. Until recently, there were 17 recognised families of PR proteins classified according to their serological relatedness or biological activity. Of these, PR-1, -2, -3, -4, -5, -8, -11, -12, 13, and -14 are all thought to have antifungal activity. PR-2, -3, -4, -8, and -11 have been shown to have a role in limiting pathogen growth, activity, and spread. These specific proteins are also designated antifungal proteins because of their fungitoxic or fungistatic capacity. The PR-2 family have been characterised as β-1,3-glucanases, whereas PR-3, -4, -8, -11 are recognised as endochitinases. Several chitinases of the PR-8 family also possess lysozyme activity and thus may have antibacterial activity. Defensins (PR-12) and thionins (PR-13), both have broad antifungal and antibacterial activities [87, 45]. Some PR proteins were also identified as being localised intra- and extracellularly in plant tissue or cultured cells upon pathogen attack.

In both compatible and incompatible interactions, several PR genes are upregulated, but some are differentially induced only during an incompatible interaction [54]. Expression of several PR genes is induced more rapidly and strongly in incompatible interactions than in compatible interactions [157]. The incompatibility-associated induction of some PR genes suggests that they might play a role in defence against pathogens. Mutants deficient in regulatory pathways controlling PR protein production show enhanced pathogen susceptibility [56]. Moreover, constitutive expression of PR genes in transgenic plants has provided evidence for a direct antimicrobial role for some PR proteins.
Many PR genes are also co-ordinately induced at the onset of systemic acquired resistance (SAR) (reviewed in [134, 153]). However, many PR genes are also expressed in response to abiotic stress or developmental cues [48, 30, 101, 25]. This overlapping expression in response to defence and other signals has made the elucidation of their specific roles complicated.

1.2.1 The role for chitinases and β-1,3-glucanases in defence responses

1.2.1.1 Antifungal effects of chitinases and β-1,3-glucanases

Chitinases and β-1,3-glucanases have long been thought to have a major role in the disruption of chitin and β-1,3-glucan, the backbone of the fungal cell wall. This is true in many cases. Large numbers of publications have reported the effects of these enzymes in limiting the growth of fungi in vitro [171, 23, 98, 100, 107, 142]. However, the mechanisms by which these enzymes arrest pathogen growth in plants and contribute to resistance or tolerance to disease are not well understood. The mechanisms and components involve in disease resistance are complex and multifaceted, making it difficult to dissect the particular functions of these proteins in defence responses.

A large number of chitinases and β-1,3-glucanases have been purified and the genes have been cloned. β-1,3-glucanases are monomeric proteins ranging from 25-35 kDa in size and have acidic or basic isoelectric points (pI) (reviewed in [154]). The basic isozymes contain a C-terminal extension that is cleaved off during their targeting to the vacuoles, whereas the acidic isozymes do not contain the extension and are located extracellularly. Based on deduced amino acid sequences, tobacco β-1,3-glucanases are divided into three classes [122, 94, 175].

Several tobacco β-1,3-glucanases display significant differences in their activities towards laminarin as a substrate [143]. Enzymes that show the highest activity towards laminarin are not effective towards cell walls from the soy-
bean pathogen *Phytophthora megasperma*, whereas a soybean β-1,3-glucanase that also has high activity towards laminarin, is also effective in degrading the *P. megasperma* cell walls. This suggests that β-1,3-glucanases within a given plant species or β-1,3-glucanases from different plant species have different substrate specificities. The soybean β-1,3-glucanase shares 63% amino acid sequence identity with tobacco class III β-1,3-glucanase, and only 55% and 51% with tobacco β-1,3-glucanases class I and class II, respectively [156]. The subtle differences in substrate specificity may distinguish glucanase isoforms that produce elicitors from pathogen cell walls [185] versus those that degrade elicitors that have been produced [135].

Plant chitinases are divided into seven classes (reviewed in [79]). Plant chitinases are generally endochitinases that have molecular masses of around 30 kDa. The majority of plant chitinases are family 19 chitinases that possess chitosanase and lysozyme activities [63, 113]. The antifungal effects of chitinases have long been studied, and the best studied chitinases are of tobacco origin. The antifungal effect of chitinase was first reported by Schlumbaum and colleagues [140]. They found that chitinase was the main proteinaceous inhibitor of fungal growth found in bean leaves infected with *F. solani f. sp. solani*. Chitinases, like β-1,3-glucanases have different substrate specificities [19]. Tobacco undergoing hypersensitive response to Tobacco Mosaic Virus produces at least 10 chitinases that belong to five distinct classes. *In vitro* analysis of purified chitinase isoforms showed that class I isoforms are the most active towards chitin, whereas basic class III isoforms are the most effective in hydrolising bacterial cell walls. Chitinases of class V and VI appear to be effective in degrading chitin oligomers. Tested against *F. solani in vitro*, only class I vacuolar chitinase and β-1,3-glucanase result in hyphal tip lysis and growth inhibition. Transgenic tobacco carrying modified class I chitinase and β-1,3-glucanase that enable these enzymes to be targeted extracellulary, showed inhibition towards *F. solani* comparable to that of the *in vitro* test [142]. Although it has been shown that chitinases are able to degrade chitin as a substrate, the effect towards pathogenic fungi is rather transient [18], and a more significant effect is achieved when chitinase is combined with β-1,3-glucanase [107, 103].
Participation of chitinases and \( \beta-1,3 \)-glucanases in the defence responses of plants to pathogens was proposed even before these proteins were characterised as PR proteins [1, 16]. Antimicrobial activity of chitinases and \( \beta-1,3 \)-glucanases \textit{in vivo} is inferred from localised accumulation of these proteins in the vicinity of infection sites [9, 10], and there is generally a spatio-temporal regulation leading to simultaneous expression of chitinases and \( \beta-1,3 \)-glucanases [76, 182], although in potato infected by \textit{P. infestans}, the temporal and spatial patterns of chitinases and \( \beta-1,3 \)-glucanases are similar between compatible and incompatible interactions [141].

1.2.1.2 Chitin and glucan degradation and pathogen perception

Besides the direct antifungal effect of chitinases and \( \beta-1,3 \)-glucanase towards pathogens, enzymatic degradation of fungal chitin and \( \beta \)-glucan releases oligosaccharides that appear to function as elicitors of numerous downstream defence response genes. Plants perceive these elicitors as pathogen-associated molecular patterns (PAMPs) that enable discrimination between self and nonself structures leading to the activation of defence responses (reviewed in [73, 11]).

Chitin, a major component of fungal cell walls, is a linear polymer of N-acetyl-D-glucosamine. Chitin oligomers are general elicitors of plant defence responses [15]. Plants lack chitin, therefore the functions of chitinases are probably to degrade chitin from non-plant organisms such as fungi, and the degradation products could act as non-self signals. Chitooligosaccharides, the products of chitin degradation, are perceived by specific systems leading to induction of genes involved in defence responses [174]. In rice, CEBiP (Chitin Elicitor Binding Protein), a plasma membrane glycoprotein with LysM motifs has been shown to be involved in binding and perception of chitin [78]. CEBiP lacks any intracellular domains, suggesting that an additional component(s) is required for signalling through the plasma membrane into the cytoplasm. A signalling partner for CEBiP is not known, but in \textit{Arabidopsis}, LysM receptor like kinase 1 (LysM RLK1) and CERK1, have been shown to be essential in chitin signalling [173, 112].
LysM domain-containing receptor-like kinases (Nod Factor Receptor1 [NFR1] and NFR5) were found in legumes to be critical for modified chitooligosaccharide (Nod factor) perception leading to nodule formation during legume-rhizobium symbiotic association. However, in legume and non-legume plants, these proteins seemed to activate different signalling pathways resulting in strikingly different types of plant-microbe interactions. Recent data suggest that the defence response resulting from chitin perception is mediated by mitogen-activated protein kinases (MAPKs) [147, 172, 187]. Transcription factors (TF) are also involved in cellular reprogramming of plant cells into defence mode. In response to chitin elicitation, plant cells can reprogram gene expression [35, 127]. Recent data show that at least 118 TF genes are responsive to chitin [91], and many of them are WRKY TFs, which have been implicated in plant defence [46]. These types of response are also found in defence response upon recognition of the bacterial PAMPs flagellin and EF-Tu. Therefore, it is suggested that the chitin signalling pathway and bacterial PAMP signalling pathways converge into a conserved pathway. However, unlike bacterial flagellin and EF-Tu signalling pathways, the chitin signalling pathway seems to be independent of jasmonic acid- (JA), salicylic acid- (SA), and ethylene-mediated signalling pathways [173, 187].

Receptors for β-1,3-glucan are not well studied. The putative β-glucan binding proteins (GBPs) in soybean are thought to be part of a β-glucan elicitor receptor complex that perceives 1.6-β-linked and 1.3-β-branched heptaglucoside (HG) that is present in cell walls of the pathogenic oomycete Phytophthora sojae [51]. One of the main outcomes of β-glucan elicitor perception is induction of phytoalexin production [29, 145, 146, 184].

1.2.2 Chitinase and β-1,3-glucanase in defence response to Fusarium oxysporum

In response to F. oxysporum infection, both chitinase and β-1,3-glucanase genes have been shown to be expressed concomitantly in tomato in both compatible and incompatible interactions. Studies on the xylem sap proteome of
Fol-infected tomato found at least 4 isoforms of PR-2 (β-1,3-glucanase) and 2 isoforms of PR-3 (chitinase) [129]. The glucanase isoforms specific for infection included both basic and acidic forms, while the acidic chitinase isoform was not correlated with Fol infection. In another study, chitinase was detectable in the intercellular fluids of untreated carnation and carnation treated with F. o. f. sp. dianthi [168]. In contrast, β-1,3-glucanase was not detectable in the intercellular fluids of untreated leaves, stems, and roots. It was only induced during compatible and incompatible interactions of carnation with F. o. f. sp. dianthi. These findings may suggest differential regulation of chitinases and β-1,3-glucanases in different plants in response to pathogen infection.

Immunocytochemical studies on chitinase and β-1,3-glucanase localisation in tomato plant tissues upon F. o. f. sp. radicis-lycopersici infection showed that in incompatible interaction, β-1,3-glucanase accumulation was an early event in both colonised and uncolonised tissues, whereas chitinases were mainly found in the host cells adjacent to invading fungal hyphae and around damaged fungal cell walls [10]. Thus, it was suggested that β-1,3-glucanases play an early role in the protection of plants against pathogens, whereas chitinases were induced as a response after elicitors were released by β-1,3-glucanases. The main difference between resistant and susceptible tomato plants towards F. o. f. sp. radicis-lycopersici was that β-1,3-glucanase was induced earlier in the resistant tomato plants compared to the susceptible one [9]. In contrast, although the activation and accumulation of chitinases and β-1,3-glucanase were rapid and correlated with the onset of infection in potato infected with Phytophthora infestans, the mRNA and protein expression were indistinguishable between resistant and susceptible potato plants [141].

### 1.3 Transgenic approaches to improve Fusarium wilt resistance

Recent advances in genetic engineering have made it possible to transform plants with almost any gene. This has enabled significant advances to be made
towards the improvement of disease resistance in plants by creating transgenic plants with genes involved in disease resistance. The profound effect of chitinase and \(\beta\)-1,3-glucanase in suppressing the growth of several pathogenic fungi \textit{in vitro} has long underpinned the heterologous expression of chitinase and \(\beta\)-1,3-glucanase genes from various sources as a mean of engineering fungal resistance. Large numbers of publications have reported efforts to improve disease resistance by employing chitinases and glucanases from plants and microorganisms [151, 41, 108, 100, 38]. However, weak antifungal effects and the narrow antifungal spectrum of the transgenes has hampered the application of transgenic approaches [125, 155].

1.3.1 Chitinase and \(\beta\)-1,3-glucanase genes from mycoparasitic fungi

The fact that plant chitinases and \(\beta\)-1,3-glucanase are not always effective in limiting pathogen growth and spread might also be caused by PR inhibitors produced by the pathogen. This was first discovered in \textit{Colletotrichum lindemuthianum}, a causal agent of anthracnose in French bean [2]. The fungus released an effective inhibitor to an endo-\(\beta\)-1,3-glucanase produced by its host to protect its cell wall, which is constituted largely of glucan. A later report provided detailed information about glucanase inhibitors that are secreted by the oomycete \textit{Phytophthora sojae}. The proteins are termed glucanase inhibitor proteins (GIPs) and although homologous with the trypsin class of serine proteases they are proteolytically nonfunctional because one or more residues of the essential catalytic triad is absent [132].

One possible way to overcome this problem is to employ non-plant chitinases and \(\beta\)-1,3-glucanase. The inhibitors of plant chitinases and \(\beta\)-1,3-glucanase may not have adapted to counteract the activity of non-plant chitinases and \(\beta\)-1,3-glucanase. \textit{Trichoderma} species have long been used as biocontrol agents against numerous pathogenic fungi, not only because some of them are beneficial plant symbionts, but also because they are parasites of other fungi [62]. The latter
characteristic strongly suggests that *Trichoderma* species possess highly efficient antifungal molecules including chitinases and β-1,3-glucanase. *Trichoderma harzianum* possesses at least seven distinct chitinases: three endochitinases, two exochitinases, and two N-acetylglucosaminidases [33, 162, 61, 60, 81, 161, 99]. Chitinases of this fungus have been shown to be strongly antifungal against several pathogenic fungi. Transgenic rice expressing *ech42* (encodes an endochitinase, CHIT42), *nag70* (encodes an exochitinase, N-acetylglucosaminidase), and *gluc78* (encodes a β-1,3-glucosidase, GLUC78), from *T. atroviride* strain P1 (previously designated *T. harzianum*) showed significant levels of resistance to sheath blight and blast diseases [108]. Resistance to several pathogenic fungi and bacteria was attained in transgenic tobacco expressing two chitinases, CHIT42 and CHIT33 from *T. harzianum* [38].

1.3.2 A chimeric gene strategy

Rapid recognition of an invading pathogen and robust deployment of defence responses is the key determinant of resistance in plants (reviewed in [179]). Although signal transduction mechanisms are largely shared between incompatible and compatible interactions, the more rapid signalling after pathogen infection during incompatible interaction is the key event in the resistant plant [157, 8]. Specific *R* gene-dependent and basal defence responses are genetically overlapping. Specific *R* gene-mediated immunity superimposes one or more basal defence pathways and is thought to have a function in accelerating the deployment of effective defence responses [157]. Therefore, to become resistant, a plant must be able to arrest pathogens growth by deploying defence responses in the right time and place.

The failure of resistance can be attributed to the late recognition and induction of the defence response. Similarly, failures in the strategy of expressing plant defence proteins as transgenes have occurred because of the inefficiency in which the transgenes were expressed rather than the lack of activity of proteins. Therefore, construction of multiple transgenes allowing rapid defence response is
pivotal to conferring resistance against diseases [75]. Additionally, introduction of a single gene is often inadequate to achieve effective resistance [75]:

Transgenes are constructed mostly by linking genes within a T-DNA in such a way that each individual gene is regulated by its own promoter and terminator. However, linked transgenes are not necessarily expressed co-ordinately resulting in substantial variation in the expression levels of the individual genes using the above strategy [4, 39]. Transgene construction is mostly performed by combining genes of interest through a cut and paste strategy employing restriction endonucleases, which is time-consuming and limited in the availability of convenient restriction sites. It can also introduce artefacts because of the restriction sites. For instance, non-silent mutations were introduced to create desired specific restriction sites required for fusion of isoform 2b and 4b of a plasma membrane Ca\textsuperscript{2+} pump protein [6]. Consequently, the mutations have to be taken into account in data interpretation.

Polymerase chain reaction (PCR) has enabled a wide range of applications in molecular biology. PCR allows the fusion of two or more sequences of DNA without the use of restriction endonucleases and ligase [67]. This new approach is called gene splicing by overlap extension, and is often abbreviated as SOE. In addition to the omission of restriction enzymes and ligase, the advantages of this approach over the cut and paste strategy is that the DNA sequences can be fused at precise junctions irrespective of nucleotide sequences at the recombination site. Briefly, the SOE method uses overlapping primers, which are complementary to each other to form a junction between two or more DNA fragments to be joined. The overlapping regions between the two or more fragments will hybridise and will be extended by PCR to form fused DNA sequence. Several modifications to the original method have been implemented [183, 74, 65]. This method will allow the creation of transcriptional and translational fusion genes. Thus, this approach may enable the construction of transcriptional and translational chitinase/glucanase genes fusion driven under one promoter, which, in turn, may allow simultaneous expression of the chimeric gene.
1.4 Hairy root plant transformation

*Agrobacterium*-mediated plant transformation has been widely used to generate transgenic plants. Genetic transformation of plants employs two closely-related species of *Agrobacterium*: *A. tumefaciens* and *A. rhizogenes*. The two differ in the phenotypic outcomes of their virulence towards plants. *A. tumefaciens* causes crown gall tumours, whereas *A. rhizogenes* causes hairy roots. Crown gall and hairy roots are traits associated with Ti (tumour inducing) and Ri (root inducing) plasmids, respectively.

1.4.1 The biology of *Agrobacterium rhizogenes*

*Agrobacterium* is a rod-shaped Gram negative bacterium. *Agrobacterium* species are divided according to disease symptomology, metabolic and physiological traits, ribosomal DNA sequence, and other classification criteria. Based on disease symptomology, *Agrobacterium* species are classified into: *A. tumefaciens* that causes crown gall disease, *A. rhizogenes* that causes hairy roots, *A. vitis* that causes cane galls on grape, and non-pathogenic *A. radiobacter*. Based on metabolic and physiological properties, *Agrobacterium* species are divided into biovars and biotypes. From phytopathological point of view, the classification of *Agrobacterium* strains based on chromosomally determined biotypes is of little use. The diseases provoked by *A. tumefaciens* and *A. rhizogenes* are plasmid transmissible traits and do not reflect the real species divisions that correspond to biovars. The diseases are exchangeable as the Ti and Ri plasmids can be exchanged between strains belonging to different biotypes. Genome sequencing of several *Agrobacterium* species including *A. tumefaciens* strain C58, *A. rhizogenes* strain A4, *A. radiobacter* strain K84, and *A. vitis* strain S4 has shed light on further classification of these bacteria [57, 181].

*A. rhizogenes*, like *A. tumefaciens*, incites the formation of neoplastic tissue at the infected area and allows growth of transformed tissue in the absence of exogenous plant growth regulators. However, unlike undifferentiated tumours
incited by *A. tumefaciens*, *A. rhizogenes* induces highly branched, ageotropic roots, which emerge from sites of infection. The host range of *A. rhizogenes* seems to be limited, but under *in vitro* condition, it can incite hairy roots in a wide range of plants, from dicotyledonous to monocotyledonous plant families [36, 160]. The ability of *A. rhizogenes* to incite hairy root disease is determined by a large Ri plasmid similar to that found in *Agrobacterium tumefaciens* which causes crown gall tumours of plants. For example, *A. rhizogenes* strain A4 possesses a large Ri plasmid called pRiA4.

*A. rhizogenes* can also be classified based by the type of opines produced in the hairy roots they incite. Most *A. rhizogenes* characterised direct the production of the opine agropine. These include strains carrying pRiA4, pRi1855, pRiHRI, pRi15834, and pRiLBA9402 plasmids. The second type directs the production of mannopine and includes the strain harbouring pRi8196. The other groups are cucumopine- and mikimopine-types, which can be represented by *A. rhizogenes* strains possessing pRi2659 and pRi1724 plasmids, respectively. Agropine-type Ri plasmids possess two T-DNA regions, the left T-DNA (T<sub>L</sub>-DNA) and the right T-DNA (T<sub>R</sub>-DNA), whereas mannopine-, cucumopine-, or mikimopine-type Ri plasmids contain only one T-DNA corresponding to the T<sub>L</sub>-DNA. The T<sub>L</sub>-DNA contains *rol* (root loci) genes that are involved in root initiation and development, whereas the T<sub>R</sub>-DNA carries *aux* genes homologous to genes encoding auxin synthesis in the T-DNA of the Ti plasmid [178, 71, 77]. Expression of T<sub>R</sub>-DNA alone induces hairy roots in some plants but the phenotype is not as strong as if both T<sub>L</sub>-DNA and T<sub>R</sub>-DNA are expressed together [169]. Testing of hairy root induction of several *A. rhizogenes* strains on carrot discs revealed that *A. rhizogenes* can be further divided into polar and non-polar types. All agropine-type strains are non-polar as they are able to induce root proliferation on both apical and basal surfaces of carrot discs, whereas all other strains are polar as they can only incite root formation on the apical surface not on the auxin-depleted basal surface of carrot discs [22].
1.4.2 Molecular and physiological basis of hairy root formation

The mechanism of how A. rhizogenes incites the formation of hairy roots is not completely understood. Most of the early processes of hairy root formation are similar to those of A. tumefaciens pathogenesis, but the later stages that lead to genetic transformation and hairy root formation still remain poorly understood. The Ri plasmid is a large plasmid that contains root inducing and opine biosynthesis genes in the T-DNA and opine catabolism and virulence (vir) genes outside the T-DNA. Ri plasmid root-inducing genes share extensive functional homology with Ti plasmid genes and the underlying mechanisms for hairy roots and crown gall tumorigenesis are likely to be similar. In both cases, the T-DNA of Ri or Ti plasmid is transferred and integrated into the plant genome and T-DNA genes are expressed as polyadenylated mRNA [40]. The T-DNA enables the transformed plant cells to grow in the absence of exogenous phytohormones. It also directs the plant to produce specific amino acid conjugates that can be utilised only by the infecting A. rhizogenes as carbon, nitrogen, and energy sources.

The ability of A. rhizogenes to transfer T-DNA and to incite hairy roots has made it an alternative vehicle to A. tumefaciens for genetic transformation of plants. Transformation is achieved by co-transferring (co-transformation) a binary vector along with the Ri plasmid and transgenic plants can be regenerated from the transformed hairy roots. However, the transgenic plants usually have abnormal morphologies including stunted growth, shorten internodes, wrinkled leaves, reduced apical dominance, atypical flower morphology, and reduced fertility [159, 158, 21, 150, 64]. Hairy root cultures have been exploited for research purposes including plant-pathogen interactions. However, hairy root cultures require obligate in vitro confinement that made this approach limited on its use especially for the study of plant-pathogen interactions, in which some of them are not easily amenable to life in the Petri dish. Another tool has been developed that can overcome some problems concerning hairy root cultures. In this technique, hairy roots are generated ex vitro from the stem from which they originated. This ‘composite plant’ consists of hairy roots attached to the wild
type shoot [26]. Ex vitro composite plants have been used for the study of the interactions between plants and root-invading pathogens such plant-nematode interactions. To our knowledge, the use of hairy roots for the study of Fol-tomato interactions has not been reported.

1.5 Research objectives

The aim of this study is to introduce a chimeric endochitinase/β-1,3-glucanase fusion gene to tomato and to test its ability to confer resistance in tomato to Fusarium wilt disease. The chitinase and glucanase genes used were ech42 and gluc78 from a biocontrol fungus Trichoderma atroviride strain P1.

The genes were fused using PCR-driven overlap extension method that allowed a transcriptional and translational fusion of the genes. Hairy root and whole tomato transformations were performed to transfer the chimeric gene to tomato employing A. rhizogenes and A. tumefaciens, respectively. To enable selection of transformed hairy roots, a red fluorescent protein (rfp) gene was incorporated into the T-DNA along with the chimeric gene. The levels of resistance of tomato to Fusarium wilt can be assessed by monitoring the growth of Fol race 3 in the roots of tomato. To monitor the growth of Fol race 3 in the roots, the fungus was transformed with gfp under the control of constitutive and inducible promoter using A. tumefaciens-mediated transformation.

The ability of the chimeric gene in conferring resistance to Fusarium wilt was not examined because of several problems. The chimeric endochitinase/β-1,3-glucanase gene was constructed but transient expression analysis in N. benthamiana failed to detect the expression of the chimeric gene. Hairy roots were generated in tomato both in vitro and ex vitro but RFP fluorescence in the hairy roots was not observed. The attempt to generate stable transgenic tomato using A. tumefaciens-mediated transformation failed to produce any transformants. On the other hand, Fol race 3 was transformed with gfp under constitutive gpd promoter and inducible SIX1 promoter. Although our hypoth-
esis that introducing combined chitinase and \( \beta \)-1,3-glucanase from *Trichoderma* would confer resistance in tomato to Fusarium wilt is untested, further research would be valuable to test this hypothesis.

Chapter 2

General materials and methods

2.1 Bacterial and fungal strains and culture conditions

2.1.1 *Escherichia coli* strains

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Chapter 2

General materials and methods

2.1 Bacterial and fungal strains and culture conditions

2.1.1 *Escherichia coli* strains

*Escherichia coli* strains used for molecular cloning were DH5α and TOP10. All strains were grown in Luria-Bertani (LB) broth or on LB agar medium at 37 °C. For liquid culture, cells were grown at 37 °C in 15 mL tubes or 250 mL flasks with constant shaking at 200 rpm in a rotary shaker. Antibiotic selection was applied as appropriate for the vector used.

2.1.2 *Agrobacterium tumefaciens* strains

*Agrobacterium tumefaciens* strains used in this study were strains AGL1 (AGL0 *recA::bla* pTiBo542ΔT Mop+ CbR) [88] and GV3101 (*rpoH*+ *hrcA*+) [82]. *A. tumefaciens* AGL1 was used for *Fol* race 3 transformation, whereas GV3101 was employed for tomato transformation. Cultivation media used were LB agar or broth, or yeast extract-peptone (YEP) medium supplemented with carbenicillin (50 μg/mL) for Ti plasmid selection or rifampicin (50 μg/mL) for chromosomal selection of AGL1 and gentamycin (50 μg/mL) for Ti plasmid selection of GV3101 and antibiotics corresponding to the binary vectors used.
2.1.3 *Agrobacterium rhizogenes* strains

For tomato hairy root transformation, *Agrobacterium rhizogenes* strain A4 harbouring the agropine-type Ri plasmid (pRiA4) was used. The bacterium was propagated in MGML agar or broth medium containing rifampicin (50 µg/ml) at 27 °C. For liquid culture, cells were grown in 15 mL tubes or 250 mL flasks with constant shaking at 200 rpm in a rotary shaker. *A. rhizogenes* ARqual was also used for hairy root transformation. This strain is a Ti-plasmid cured *A. tumefaciens* C58 that carries pRiA4 [126]. ARqual was grown on LB agar or in LB broth supplemented with 100 µg/mL spectinomycin or streptomycin at 27 °C.

2.1.4 *Trichoderma atroviride*

*Trichoderma atroviride* strain P1 (ATCC 74058 formerly known as *T. harzianum* P1) was used as the source of the endochitinase (ech42) and β-1,3-glucanase (gluc78) genes used in this study. For the production of mycelia, the fungus was grown in potato dextrose broth (PDB) or on potato dextrose agar (PDA). For chitinase and β-1,3-glucanase production, the fungus was grown in Minimal medium (MM) supplemented with 0.2% (w/v) dried *Fol* race 3 mycelia as sole carbon source. Liquid cultures were incubated in 250 mL flasks at 25 °C with constant shaking at 200 rpm in a rotary shaker.

2.1.5 *Fusarium oxysporum* f. sp. *lycopersici* race 3

The *Fol* race 3 isolate used in this study was Australian isolate no. 1943, provided by the Queensland Department of Primary Industry, Plant Pathology Section. *Fol* race 3 is maintained as a spore suspension in 15% (v/v) glycerol solution and frozen at -80 °C, or as dried-spores blotted on sterile filter papers stored at -20 °C. For routine culturing, *Fol* race 3 was grown on PDA at 25 °C and stored at 4 °C. For biomass or conidial production, the fungus was grown in PDB at 25 °C on a rotary shaker (200 rpm) for 3-5 days. Different media were
used depending on the purpose, and details are provided in the following chapters.

2.2 Bacterial plasmid DNA protocols

Plasmid DNA was isolated using spin column kits provided by QIAGEN Corporation according to the manufacturer’s instructions. A QIAprep® Spin Miniprep Kit was used for small-scale isolation of high-copy plasmids, whereas for low-copy plasmids, medium-scale isolation was performed using a QIAGEN Plasmid Midi Kit. For all plasmid preparations, water was used as eluent instead of buffer. Plasmids were stored at -20 °C until further use.

2.3 Fungal genomic DNA protocols

Fungal biomass was produced in PDB. Mycelia were recovered by filtration through four layers of miracloth and air-dried. Dried mycelia were sliced into small pieces and weighed. 200 mg of dried mycelia was frozen using liquid nitrogen and immediately ground with a pestle and mortar. This step was repeated twice until the mycelia were ground into a fine powder. 5 mL extraction buffer (200 mM Tris-Cl pH 8.0; 50 mM EDTA pH 8.0; 200 mM NaCl; 2% (w/v) sodium N-laurylsarcosinate; 0.1% (v/v) 2-(\(\beta\))-mercaptoethanol) was added to the powder and mixed gently. The mycelial mush was transferred to 1.5 mL tubes and incubated at 65 °C for 30 min. The tubes were cooled down to room temperature and an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) solution was added. The suspension was mixed gently until an emulsion formed and was then centrifuged at 6,000 g for 10 min. Supernatants were transferred to new tubes and an equal volume of chloroform:isoamylalcohol (24:1) solution was added. The solution was mixed gently until an emulsion formed and centrifuged again at 6,000 g for 10 min. Supernatants were transferred to new tubes and an equal volume of isopropanol was added and mixed. To enhance precipitation, DNA solutions were stored at -20 °C for 1 hour. High molecular weight DNA
appeared as an aggregate, and was spooled out with a glass rod or hook and washed several times with 70% (v/v) ethanol. DNA was air-dried and redisolved in Tris-Cl buffer pH 8.0 supplemented with 20 μg/ml RNase A.

2.4 Polymerase chain reactions (PCR)

PCR was generally performed in a reaction volume of 10 μL for colony screening and 20 μL for amplification of DNA from plasmid or genomic DNA for cloning or sequencing in 200 μL thin-walled polypropylene PCR tubes using a PTC-200 Peltier thermal cycler (MJ Research, Inc.). PCR reagents contained 1× PCR buffer (SIGMA Corp.) (10 mM Tris-HCl pH 8.3; 50 mM KCl; 1.1 mM MgCl$_2$; and 0.01% (w/v) gelatin), 250 μM of each dNTP, 500 nM of each oligonucleotide primer, 0.05 U/μL REDTaq DNA polymerase (SIGMA Corp.), and 0.01-0.05 pmol/μL of DNA template. PCR was carried out with an initial denaturing step at 94 °C for 1 minute followed by 25-30 cycles of denaturing at 94 °C for 20-30 sec, annealing of primers at 50-60 °C for 30 sec, and primer extension at 72 °C for 1 minute per kb of product expected. The PCR reaction was completed by a final extension at 72 °C for 10 min. For high complexity DNA such as genomic DNA, the reaction was carried out for 30 cycles and a 30 sec denaturation time was employed. Colony PCR was carried out for 30 cycles with the same conditions as DNA amplification from plasmids, except the initial denaturation time was 2 minutes. The quality of the primers and PCR conditions were analysed using an online tool OligoAnalyzer 3.2 hosted by Integrated DNA Technologies (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Primer design was performed manually or using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).
2.5 Molecular cloning procedures

2.5.1 DNA gel electrophoresis

DNA samples, along with DNA size markers, were size-separated through 0.8-2.5% (w/v) agarose gels (depending on the size of the DNA fragments to be analysed) containing 0.5 μg/mL of ethidium bromide using electrophoresis apparatus supplied by BIO-RAD. Routinely, 1× TAE buffer pH 8.0 (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) was used as a running buffer. Normally, the gel was run at 65 V for 45-50 min. Nucleic acids were visualised using a UV transilluminator and documented using a Gel Doc 1000 system and Molecular Analyst software (BIO-RAD).

2.5.2 Preparation of chemically competent *E. coli*

A single colony of *E. coli* DH5α grown on an LB plate was used as inoculant for an overnight culture of *E. coli* grown in 1.5 mL SOB medium at 37 °C. This initial culture was then used to inoculate 200 mL SOB media in conical flasks. The culture was grown at 25 °C in a rotary shaker with constant shaking at 200 rpm until the optical density at 600 nm (OD<sub>600</sub>) reached 0.4-0.8. The culture was then chilled on ice for 10 min, and centrifuged at 3,000 g at 4 °C for 10 min. The pellet was resuspended in 67 mL ice-cold, filter-sterilised, transformation buffer (TB) (9.9 mM Piperazine-N,N-bis[2-ethanesulfonic acid]; 15 mM CaCl<sub>2</sub>-2H<sub>2</sub>O; 0.25 M KCl; and 55 mM MnCl<sub>2</sub>-4H<sub>2</sub>O, pH 6.7). The suspension was incubated on ice for 10 min before centrifuging for a second time at 3,000 g at 4 °C for 10 min. The pellet was resuspended again with 16 mL of ice-cold TB with an addition of 1.2 mL dimethyl sulfoxide (DMSO). Competent cells were prepared as 50 μL aliquots and frozen in liquid nitrogen before being stored at -80 °C. Competency of the cells was checked by transforming the cells with a known amount of plasmid containing a selectable marker. Competent cells were also checked for contamination using a control without plasmid. Chemically competent TOP10 cells were obtained from Invitrogen.
2.5.3 Preparation of *Agrobacterium* electro-competent cells

Media and antibiotics used to prepare *Agrobacterium* competent cells are described in section 2.1.2 and 2.1.3. All *Agrobacterium* strains were grown on agar plates and incubated at 27 °C for 2-3 days. Single colonies were inoculated into 3 mL liquid media and grown at 27 °C for 36 hours with constant shaking at 200 rpm in a rotary shaker. After 36 hours, 1 mL of this culture was used to inoculate 100 mL liquid media. The culture was grown overnight at 27 °C with constant shaking at 200 rpm until the OD₆₀₀ reached a value between 0.6-0.8. The culture was chilled on ice for 10 min and harvested by centrifugation at 5,000 g for 10 min at 4 °C. The pellet was resuspended in 100 mL MilliQ water and harvested again by centrifugation at 5,000 g for 10 min at 4 °C. This washing step was repeated three times. After the final wash with water, the cells were resuspended in 50 mL sterile ice-cold 10% (v/v) glycerol, and pelleted again. The pellets were resuspended in 1 mL ice-cold 10% (v/v) glycerol. After this final wash with glycerol, 50 µL aliquots of the suspended cells were transferred to 1.5 mL pre-chilled tubes and frozen in liquid nitrogen before storing at -80 °C.

2.5.4 DNA restriction digestion and ligation

Restriction digestion and ligation of DNA fragments and vectors was carried out using standard protocols [136] and the enzyme manufacturers' instructions. Generally, restriction digestion was performed in a 20 µL reaction containing 0.5-2 µg of DNA, 20-30 units of the required enzyme, and 1× restriction enzyme-specific buffer. When recommended, the restriction digestion mix was supplemented with (100 µg/mL) bovine serum albumin (BSA). The reaction mix was incubated for 1-4 hours at the specified temperature depending on the enzyme used. In most cases, directional cloning was performed. For double digestion, the buffer that facilitated optimal activity for both enzymes was used. In the absence of such a buffer, or if the enzymes required very different optimum temperatures, sequential digestion was performed. In the case of different temperatures with the same
buffer, the enzyme used for the first restriction digestion was heat-inactivated and followed by digestion with the second enzyme. In the case of different buffers, the DNA from the first digestion was ethanol-precipitated or spin column-purified and resuspended in water, followed by the second digestion reaction.

The restriction digestion products or PCR products were separated by size in 0.8-2.5% (w/v) agarose gels as described in section 2.5.1. Upon visualisation of DNA using a UV transilluminator, the section of the gel containing the desired size of DNA was sliced and transferred to a 1.5 mL tube. The DNA from the excised gel was extracted using a QIAquick Gel Extraction Kit (QIAGEN Corp.) according to the manufacturer's instructions before ligating into vectors. The purity and amount of the recovered DNA were estimated by gel electrophoresis as described above. For most ligation reactions, an insert:vector molar ratio of 3:1 was used based on 20-30 fmol of vector per reaction. The ligation was performed in a 10 μL reaction containing 0.4 Weiss unit of T4 DNA ligase and 1× ligation buffer (60 mM Tris-HCl pH 7.5; 60 mM MgCl₂; 50 mM NaCl; 1 mg/mL BSA; 70 mM β-mercaptoethanol; 1 mM ATP; 20 mM dithiothreitol; and 10 mM spermidine). The reaction was carried out at 14 °C for a minimum of 4 hours. Cloning of PCR-amplified DNA fragments generated using a polymerase that lacks 3' exonuclease activity, such as Taq polymerase, was performed using a TA Cloning® Kit from Invitrogen with pCR® 2.1 as the vector. DNA fragments amplified by proofreading DNA polymerase required the addition of 3' adenine overhangs to enable cloning into the TA-vector. For this purpose, 0.05 U/μL REDTaq DNA polymerase (SIGMA Corp.) and 500 μM of dNTPs mix were added directly to the previous PCR mixture and incubated at 72 °C for 10 min. The volume of this reaction mixture that would give a three fold molar ratio relative to the vector was used for ligation.

2.5.5 Transformation of chemically competent *E. coli*

Chemically competent *E. coli* strains used were DH5α and One Shot® TOP10 Chemically Competent *E. coli* from Invitrogen. The transformation
protocol was the same for both strains. Vials containing the ligation reactions were centrifuged briefly and placed on ice. Competent cells vials were thawed on ice before transformation. Depending on the concentration of DNA in the ligation mixture, a volume that gave approximately 25 ng of vector was transferred to the competent cells and mixed by stirring gently with a pipette tip. The vials were left on ice for 30 min. Cells were heat-shocked in a water bath for 1 minute at 42 °C without shaking, and were immediately transferred to ice. 250 μL of room temperature SOC medium was added to each vial, and the vials were incubated at 37 °C for 1 hour with constant shaking at 200 rpm in a rotary shaker. 100 μL aliquots of the cell suspension from each transformation vial were spread on LB agar plates containing 40 μL of 40 mg/mL X-Gal and antibiotics appropriate to the vector used. A pCR® 2.1 TA Cloning® Kit from Invitrogen was used routinely for TA cloning and kanamycin (50 μg/mL) was used for plasmid selection. Plates were incubated overnight at 37 °C. The incubation temperature was shifted to 4 °C to allow proper blue colour development before screening of transformants.

2.6 Screening for recombinant plasmids

2.6.1 Initial screening of colonies by PCR

If the transformation efficiency was sufficiently high, a minimum of six colonies from each transformation were screened. Cells were added directly to the PCR mixture by touching colonies at the top using a pipette tip and swirling the cells gently in the PCR mixture. PCR was performed as described in section 2.4. PCR products were run on agarose gels to check for DNA corresponding to the expected sizes of amplified DNA fragments. Pipette tips that were used to transfer cells to the PCR mixture were also used to inoculate 5 mL LB broth containing appropriate antibiotics for plasmid selection. Recombinant plasmids were isolated from cultures that corresponded to positive colonies in the colony PCR.
2.6.2 Restriction digestion analysis of the recombinant plasmids

Plasmids were isolated from PCR-positives cultures using a QIAprep® Spin Miniprep Kit according to the manufacturer’s instructions. The isolated plasmids were then digested to check for cloned DNA inserts as described in section 2.5.4. The products of restriction digestion were analysed by gel electrophoresis on 0.8-1.0% (w/v) agarose gels.

2.6.3 DNA sequencing and analysis

The recombinant plasmids were sequenced at the Australian Genome Research Facility (AGRF), Brisbane, Australia to authenticate insert integration and sequence correctness. Sample preparation was performed as recommended by AGRF. Samples were prepared as a 10 μL mixture of 6.4-10 pmol of each primer, 400-1000 ng plasmid DNA, and MilliQ water. Tubes were sealed with parafilm to avoid evaporation before being dispatched to AGRF. Sequence data were analysed using the ContigExpress module of Vector NTI Advance 10 software package (Invitrogen) or the Staden software package.

2.7 Transfer of binary vectors to Agrobacterium

The binary vectors used in this study were transferred to disarmed A. tumefaciens strain AGL1 for Fol race 3 transformation and to wild type A. rhizogenes strain A4 as well as A. rhizogenes strain ARqua1 for tomato hairy root transformation. Plasmids were transferred to electro-competent cells by electroporation. The electro-competent cells were thawed on ice before electroporation. Approximately 100 ng of plasmid DNA was mixed gently with the cells. For binary vectors that are derivatives of pGreen, an equal amount of pSoup was co-electroporated with the binary vectors into Agrobacterium to enable replication of the binary vectors in Agrobacterium [66]. The mixture
was transferred to a pre-chilled 2 mm-gap electroporation cuvette (BIO-RAD). Electroporation was performed using a GenePulser electroporator (BIO-RAD) with the following conditions: 2.5 kV electromotive force; 25 μF capacitance; 400 Ω resistance; and 8-10 msec pulse length. After electroporation the cells were revived immediately with 1 mL ice-cold LB broth and grown at 27 °C with constant shaking at 200 rpm for 4 hours. Cells were spread on LB agar plates containing appropriate antibiotics for chromosomal selection and binary vector selection. The cultures were incubated at 27 °C for 2-3 days. The colonies were screened for the presence of DNA inserts in the binary vectors by colony PCR and restriction digestion analysis.

2.8 Fungal inoculation onto tomato seedlings

Fol race 3 was inoculated onto tomato seedlings using the root-dip method. Tomato seedlings grown in UC soil mix (per cubic yard of mix: 50% sand; 50% peat moss; 125 g potassium nitrate; 125 g potassium sulphate; 1.25 kg single superphosphate; 3.75 kg dolomite limestone; 1.25 kg calcium carbonate lime) were removed at the three-week stage, dipped into $10^6$/mL conidial suspension for 1 min, and replanted to new UC soil mix. After replanting, the seedlings were watered immediately to recover turgor. The inoculated tomato seedlings were incubated at 25 °C with a 16 hours light/8 hours dark photoperiod for three weeks in a growth chamber. The seedlings were watered daily. A scoring system of 0 - 5 was used to evaluate the symptoms at 21 dpi: 0 = no reaction or healthy plant; 1 = localised vascular staining in tip of primary root; 2 = staining to cotyledonary node; 3 = staining beyond cotyledonary node but with no external symptoms; 4 = external symptoms and severe stunting; 5 = complete collapse and death. Scores of 0 - 2 were classified as resistant, whereas, scores of 3 - 5 were classified as susceptible. For disease scoring, seedlings were checked for obvious external symptoms of wilting and stunting before examining the roots. Seedlings that showed external symptoms but did not die were scored 4 and those that collapsed and died were scored 5. If there were no obvious external
symptoms, the seedlings were carefully uprooted and the roots were washed with water prior to examining the tip of the primary root and cotyledonary node for brown discolouration (brownish lesions). Stems in the cotyledonary nodes were also sectioned transversely and longitudinally and examined for brown discolouration.
Chapter 3

Transformation of *Fusarium oxysporum* f. sp. *lycopersici* with green fluorescent protein reporter genes

3.1 Introduction

One of the key strategies to understanding the mechanism of Fusarium wilt disease development in tomato is to monitor the development of *Fol* within its host. Unlike foliar and fruit pathogens that cause conspicuous symptoms readily visible on exposed aboveground surfaces, their root infecting counterparts by virtue of being concealed underground, pose a unique challenge in monitoring disease onset and progression.

Some *in vivo* methods have been developed that use transmitted light or fluorochromes to accentuate fungal structures inside the host. Such techniques generally employ histochemical/cytochemical markers or genetically encoded markers. In most cases, the latter is superior to the former. The $\beta$-D-glucuronidase (GUS) gene from *E. coli* is a versatile and widely used visual marker in phytopathological studies. Although GUS has been a significant tool, there are limitations to its use: penetration of the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) into plant tissue can be limited or of variable efficiency and diffusion of the reaction product can reduce the spatial resolution. However, the success
of genetic transformation of *Fol* with genes expressing green fluorescent protein (GFP) has increased the potential of *in vivo* imaging with an improved spatio-temporal determination of the pathogen within the host tissue [84]. GFP-tagging is also practically much simpler, non-invasive and non-destructive, and only requires oxygen and UV or blue light to induce fluorescence.

Formation of the fluorescent chromophore of GFP is species-independent, allowing it to be produced heterologously in many systems. Several pathogenic filamentous fungi have been transformed with *gfp* to monitor their development within their plant hosts. Fungal transformation is generally achieved through electroporation or polyethylene glycol treatment of fungal protoplasts (reviewed in [133]). Since the first report that *A. tumefaciens* was able to transform yeast [20], filamentous fungal transformation has also been achieved by employing disarmed *A. tumefaciens* [37]. *A. tumefaciens*-mediated fungal transformation was found to be more efficient than either of the other two methods. *A. tumefaciens*-mediated transformation was up to 600 fold more efficient than the standard transformation protocols.

Many fungal *gfp* expression systems utilise the *gfp* gene fused to the *E. coli* hygromycin B phosphotransferase (*hph*) gene constitutively regulated by the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter and anthranilate synthase (*trpC*) terminator. Although constitutive expression of *gfp* is often desirable, in certain cases, inducible expression is more informative than constitutive expression e.g. in studies of gene expression.

A number of genes including genes encoding effector proteins are expressed during *Fol* pathogenesis of tomato. For example, SIX1 is an effector protein that is secreted into the xylem sap of tomato during colonisation of xylem vessels and is required for *Fol* virulence. It also triggers recognition that leads to resistance in tomato plants carrying the *I-3* resistance gene. Fusion of *gfp* with the *SIX1* promoter revealed that the *SIX1* gene is expressed immediately upon penetration of the root cortex and it is expressed only when the fungus is inside the plant [164]. This property of the *SIX1* promoter provides a technical advantage because it enables discrimination of hyphae that have penetrated plant tissue from those
that have not.

One aim of this study was to generate *Fol* race 3 *gfp* transformants and employ them for infection of transgenic tomato hairy roots carrying the chimeric endochitinase/β-1,3-glucanase gene derived from *T. atroviride*. Two lines of *Fol* race 3 *gfp* transformants were generated in this study: transformants with constitutive expression of *gfp* driven by the *gpd* promoter and transformants with inducible expression of *gfp* driven by the *SIX1* promoter.

### 3.2 Materials and methods

#### 3.2.1 Transformation of *Agrobacterium tumefaciens* strain AGL1 with binary vectors harbouring *gfp*

The binary vector pPK2HPHGFP was obtained from Martijn Rep (Swammerdam Institute for Life Science, University of Amsterdam, The Netherlands). This plasmid was derived from pPK2 [32] with an insertion of *gfp* as an ApaI cassette downstream of the *hph* gene. Expression of the *gfp* gene was controlled by the constitutive *gpd* promoter. The binary vector contains the *nptII* gene for kanamycin-resistance selection in bacteria, and the *hph* gene for hygromycin-resistance selection in fungi. Another binary vector, designated pSIX1GFP, was also obtained from Martijn Rep. The binary vector is a derivative of pPZP200 [59] that contains the *SIX1* promoter-*gfp*-SIX1 terminator construct. The 3' of this construct contains a 1.7 kb *Fol* genomic DNA fragment derived from a region proximal to the *SIX1* locus to facilitate homologous recombination. The whole *gfp* construct is present in the vector as a HindIII-EcoRI cassette [164]. The binary vector contains the *aadA* gene, which confers bacterial resistance to spectinomycin/streptomycin, and the *ble* gene, which confers fungal resistance to Zeocin™.

The binary vectors were transferred to electro-competent *Agrobacterium tumefaciens* strain AGL1 by electroporation using the protocol described in section 2.7. Electroporated cells were plated out on LB agar containing 50
μg/mL of carbenicillin and 50 μg/mL kanamycin for selection of cells carrying pPK2HPHGFP, and 50 μg/mL of carbenicillin and 100 μg/mL of spectinomycin for selection of cells carrying pSIX1GFP. Colonies that grew on the antibiotic selection media were screened for the presence of each binary vector (pPK2HPHGFP and pSIX1GFP) by colony PCR and restriction digestion analysis as described in section 2.6.1 and 2.6.2, respectively. An 811 bp fragment of the \textit{hph} open reading frame (ORF) from pPK2HPHGFP was amplified by colony PCR using the oligonucleotide primers HPH1F (gtccggggcaaggaag) and HPH1R (tccagatgtcaagctgttt). PCR was carried out with initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 20 sec, primer annealing at 56 °C for 30 sec, and primer extension at 72 °C for 1 min. PCR amplification was completed by a final extension at 72 °C for 10 min. The presence of pSIX1GFP in spectinomycin-resistant colonies was confirmed by amplifying a 318 bp fragment that spans the junction between the \textit{SIX1} promoter and \textit{gfp}. PCR amplification was performed as above using oligonucleotide primers SIX1PF (gcctcaactctgctgtctt) and GFPR (gggcggccgtttacttgta) and a primer annealing temperature 55 °C instead of 56 °C. The success of transformation was further verified by restriction digestion of the binary vectors. Binary vectors were isolated and digested from cultures derived from colonies that showed positive results in colony PCR. pPK2HPHGFP was digested with ApaI to release the \textit{gfp} fragment, whereas pSIX1GFP was digested with HindIII and EcoRI.

### 3.2.2 \textit{Agrobacterium tumefaciens}-mediated transformation of Fol race 3

The method for \textit{Fol} race 3 transformation with \textit{gfp} described herein is adapted from a method described by Martijn Rep (personal communication) with some modifications.
3.2.2.1 Preparation of *Agrobacterium tumefaciens* strain AGL1 culture for use in *Fol* race 3 transformation

The transformed *A. tumefaciens* strain AGL1 was grown on LB agar containing appropriate antibiotics for 3 days at 27 °C. A single colony from this culture was used to inoculate 5 mL of 2×YT medium supplemented with appropriate antibiotics. The culture was grown for 24 hours at 27 °C with a constant shaking at 200 rpm. The cultures were pelleted and resuspended to an OD$_{600}$ of 0.45 in 10 mL induction medium (IM, Appendix E) supplemented with 200 μM acetosyringone and incubated at 27 °C for 6 hours without shaking.

3.2.2.2 *Fol* race 3 culture and conidia preparation

*Fol* race 3 was grown on PDA at 25 °C for a week until sporulation. The cultures were then flooded with 5 mL sterile water and scraped to obtain conidial suspensions. 100 μL of the conidial suspension was used to inoculate 100 mL PDB. The culture was grown on a rotary shaker (200 rpm) at 27 °C for 5 days. Conidia were harvested by filtration over four layers of miracloth. Conidia were pelleted by centrifugation at 6,000 g for 10 min and washed with water. The conidial suspension was then centrifuged again at 6,000 g for 10 min and washed again with water. This washing step was repeated three times. The concentration of conidia was then estimated using a hemacytometer. The concentration of the conidia was adjusted by diluting the conidial suspension with IM containing AGL1 (see above) to give a final concentration of $1 \times 10^5$ conidia/mL in a 10 mL AGL1-*Fol* race 3 mixture.

3.2.2.3 Co-cultivation of *Fol* race 3 and *A. tumefaciens*

Three pieces of sterile ME25 filter papers (Metrical® Membrane Filter, Gelman Sciences Inc.; 25 mm; 0.45 μm) were placed on each co-cultivation medium (CM, Appendix E) plate supplemented with 200 μM acetosyringone. 25 μL of the AGL1-*Fol* race 3 mixture was spotted on each membrane. The plates were
After 2 days, membranes that contained AGL1 transformed with pPK2PHHGFP were transferred to Czapek Dox agar (CDA) supplemented with 100 µg/mL of hygromycin and 200 µM cefotaxime, whereas membranes that contained AGL1 transformed with pSIX1GFP were transferred to CDA supplemented with 100 µg/mL Zeocin™ and 200 µM cefotaxime. For Zeocin™, the CDA was buffered with 0.1 M Tris pH 8.0. Cefotaxime was used to kill the A. tumefaciens. Membranes were incubated for 5-6 days at 25 °C until putative transformants grew out of the filter onto the media. Putative transformants (hyphae that were able to grow on the media) were transferred again to the same media and incubated for 2 days at 25 °C to allow further growth. Sections of mycelia from putative transformants were transferred to the same media supplemented with appropriate antibiotics and grown until the fungus covered the surface of the media.

3.2.3 Screening of Fol race 3 gfp transformants

For putative Fol race 3 transformants expressing gfp under the control of the gpd promoter, cultures grown on plates were observed using a dissecting fluorescence microscope (Leica MZ FLIII) for GFP fluorescence with magnification of 8 to 20 times. A GFP3 filter with an excitation filter of 470±40 nm and a barrier filter 525±50 nm was used to screen the transformants. All images were captured using a Leica DC200 camera and processed with a Leica IM50 software.

For putative Fol race 3 transformants carrying gfp under the SIX1 promoter, gfp expression was observed in Fol-infected root tissue. Fol conidia were prepared as a 1×10^6 conidia/mL suspension, and 50 mL of this conidial suspension was poured into tubs. 10-day-old tomato seedlings grown on germination media in tubs were removed and placed into tubs containing conidial suspension. The seedlings were positioned in such a way that the whole root system was immersed in the conidial suspension with the aerial parts of the seedling supported in an upright position by the vertical wall of the tub (Figure 3.1). The seedlings
were incubated for 2-5 days in a tissue culture chamber at 25 °C with a 16 h photoperiod. The roots of the seedlings were observed for \textit{gfp} expression everyday using a dissecting fluorescence microscope (Leica MZ FLIII) from the second to the fifth day post inoculation (dpi) using GFP3 filter with magnification of 8 to 20 times.

### 3.2.4 Pathogenicity test of \textit{Fol} race 3 transformants in tomato

Stable \textit{Fol} race 3 transformants were tested for their virulence and avirulence in susceptible and resistant tomato cultivars. Transformants and wild type \textit{Fol} race 3 were grown in PDB for 5 days at 25 °C with constant shaking at 200 rpm. 100 \( \mu \text{L} \) of this culture was used to inoculate 100 mL Armstrong Fusarium Media (AFM, Appendix F), and the culture was incubated for 3 days at 25 °C with a constant shaking at 200 rpm.

Conidia were harvested by filtering the culture through four layers of miracloth, followed by centrifugation at 6,000 \( g \) for 10 min. The pellets were washed with water, and the suspension was centrifuged again at 6,000 \( g \) for 10 min. This washing step was repeated three times. The concentration of the conidia was estimated by counting the spores using an Improved Neubauer hemacytometer at 160\( \times \) magnification. The concentration of the conidia was adjusted to \( 1 \times 10^6 \) conidia/mL in sterile water.

For pathogenicity tests, we used the IL7-3 tomato line that is known to be resistant to \textit{Fol} race 3 and the M82 tomato line that is susceptible to \textit{Fol} race 3. Four independent \textit{Fol} race 3 transformants from each transformation line (\textit{SIX1} promoter-\textit{gfp} and \textit{gpd} promoter-\textit{gfp}) were tested for their pathogenicity in M82 and IL7-3 tomato cultivars. Fifteen seedlings of each cultivar was used for each individual transformant. Wild type \textit{Fol} race 3 was used as a control. Water was also used to mock-inoculate seedlings. Inoculation of tomato seedlings was performed under the supervision of Des McGrath (Queensland Department of Primary Industries and Fisheries). Fungal inoculation was performed using the
Figure 3.1: Screening of *Fusarium oxysporum* f. sp. *lycopersici* race 3 transformed with the SIX1 promoter-*gfp* reporter gene. 10-days-old tomato seedlings in tubs were infected with *Fol* race 3 conidia. The tomato roots were immersed in conidial suspension (1×10⁶ conidia/mL) and incubated for 5 days at 25 °C with 16 h photoperiod. Observation of *gfp* expression was performed using a dissecting fluorescence microscope everyday starting at the second day post inoculation.

root-dip method described in Section 2.8.

3.3 Results

3.3.1 Screening of *Fol* race 3 *gfp* transformants

*A. tumefaciens*-mediated transformation of *Fol* race 3 was found to be efficient for both pPK2HPHGF and pSIX1GFP binary vectors. In general, transformed colonies were distinguishable from non-transformed colonies by five days after transfer from co-cultivation medium (CM) to Czapek Dox agar containing antibiotics. The transformed colonies usually grew out of the membrane onto the media, while the non-transformed colonies grew very slowly, and generally never reached the edge of the membrane. On subsequent transfer to antibiotic selection media, transformed colonies grew faster than non-transformed colonies (Figure 3.2). However, there was a difference in the selection efficiency between
Figure 3.2: *Agrobacterium tumefaciens*-mediated transformation of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) race 3. A: Co-cultivation of *A. tumefaciens* AGL1 and *Fol* race 3 on cellulose membrane filter. B: Growth of eight putative transformants on Czapek Dox agar supplemented with Zeocin™. Putative transformants number 4 and 6 did not grow after being transferred to selection media, therefore they were presumed not to be transformants. WT = wild type *Fol* race 3.

Hygromycin B and Zeocin™. Hygromycin B was more efficient in suppressing the growth of non-transformed *Fol* race 3 than Zeocin™. The growth of non-transformed *Fol* race 3 on hygromycin B was more restricted, and in some cases the growth was completely blocked. Whereas on Zeocin™, the non-transformed *Fol* colonies were still able to grow to some extent, but the transformants were distinguishable from the non-transformants by their rapid growth. The hyphae of transformed *Fol* race 3 grown on CDA containing Zeocin™ were slightly aerial and coarse.

In total, there were 35 independent *Fol* gpd:gfp colonies that were resistant to hygromycin B, and all expressed gfp. In all isolates, GFP fluorescence was readily visible by fluorescence microscopy and appeared uniform throughout the hyphae. GFP fluorescence was also observed in conidia with the same intensity as that observed in the hyphae (Figure 3.3). The stability of the four transformants that were tested for their pathogenicity was tested by culturing them several times on PDA without antibiotics and examining their GFP fluorescence. All the tested isolates were still expressing gfp fluorescence with the same intensity and
Figure 3.3: Expression of gfp in transformed *Fusarium oxysporum* f. sp. *lycopersici* (Fol) race 3. A and B: Hyphae of Fol race 3 grown on potato dextrose agar expressing gfp regulated by the *gpd* promoter. *gfp* was expressed in both hyphae (A and B) and conidia (A). C - F: Hyphae of transformed Fol race 3 expressing gfp regulated by the *SIX1* promoter in tomato roots. In panel C, hyphae were observed at the junction between the stem and the root at the third day post inoculation. However, the localisation of hyphae in the root could not be determined because the resolution of the image was not sufficient to differentiate root tissues. In panel D, hyphae were seen growing along the xylem vessels at the fifth day post inoculation. In panels E and F, the hyphae seemed to grow along the vascular tissue, but appeared discontinuous and uneven.
uniformity as they were before subculturing.

Fourteen independent Fol colonies that showed resistance to Zeocin™ were obtained from the Fol transformation with the SIX1 promoter-gfp construct. Of these, eight isolates were used to infect tomato seedlings in vitro as described in section 3.2.3. At 2 dpi, there was no GFP fluorescence observed. This was probably because there was no infecting hyphae in the tomato roots at that time point, or, infection may have occurred but was missed because of the amount of hyphal growth in the roots was very low. GFP fluorescence was observed at 3 dpi and from that time onwards (Figure 3.3). Infection occurred infrequently, as there were only four infection points in four seedlings (one infection point per seedling) out of 16 seedlings used. These four infection sites were from four different transformants, and these transformants were then used for pathogenicity testing in tomato. One out of the four infection patches was observed at 3 dpi (Figure 3.3:C), and the other three were observed at 5 dpi (Figure 3.3:D-F).

Autofluorescence was observed in the roots, and sometimes the autofluorescence was quite intense and resembled GFP fluorescence (Figure 3.4:A-C). However it was usually possible to differentiate autofluorescence from GFP fluorescence. The rare occurrence of GFP-tagged Fol infection in the roots could be due to poor colonisation of the roots by Fol or poor GFP fluorescence. To test these hypotheses, the roots were observed using a stereo fluorescence microscope with long exposure times (up to 900 msec). Root colonisation was frequently found especially at the fifth day of incubation, and in some areas, the roots were colonised by a dense network of hyphae (Figure 3.4:D). In areas where the root was heavily colonised, the fluorescence was usually very strong, making it difficult to distinguish GFP fluorescence from autofluorescence. Thus, it could not be determined whether there were any infecting hyphae that expressed gfp in areas where the root was colonised heavily.
Figure 3.4: Green autofluorescence in tomato roots and root colonisation by *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) race 3 isolate transformed with the SIX1 promoter-\(gfp\) fusion. A - C: green autofluorescence observed in tomato roots. Panel A shows an example of strong autofluorescence; Panels B and C show normal autofluorescence commonly found in the roots. Panel D: long exposure times revealed heavy surface colonisation of the roots by the *Fol* race 3 transformant. Surface colonisation of the roots by the *Fol* transformant was obvious only when the exposure time was set high. However, because of strong autofluorescence in this region, even without long exposure, no infecting hyphae observed. Intense fluorescence was always observed wherever there was a high amount of surface colonisation.
3.3.2 The spectrum of pathogenicity of *Fol* race 3 *gfp* transformants was not altered in resistant and susceptible tomato

Pathogenicity testing of four selected *Fol* race 3 *gpd* promoter-*gfp* transformants and four *SIX1* promoter-*gfp* transformants revealed that they did not have an altered spectrum of pathogenicity on susceptible or resistant host plants compared with wild type *Fol* race 3 (Figure 3.5; Table 3.1). They were all still virulent on the susceptible tomato cultivar and avirulent on the resistant tomato cultivar. Early symptoms of Fusarium wilt in seedlings inoculated with *Fol* race 3 *gpd* promoter-*gfp* transformants were evident 2 weeks after inoculation. The leaves yellowed and the whole plants appeared to lose turgor. At 3 weeks after inoculation, most of the susceptible plants were dead and the survivors showed severe wilting, whereas all the resistant plants showed only mild symptoms of Fusarium wilt. The only conspicuous symptom was yellowing at the edges of the leaves.

In contrast, all four of the *SIX1* promoter-*gfp* transformants caused some wilting on resistant tomato, but the symptoms were milder than those observed on susceptible tomato inoculated with either the *SIX1* promoter-*gfp* transformants or the wild type progenitor, as reflected by an intermediate average disease index (Table 3.1). Based on the disease index, these transformants were only weakly avirulent on the resistant tomato line IL7-3.

3.4 Discussion

3.4.1 Transformation of *Fusarium oxysporum* f. sp. *lycopersici* with *gfp*

The recent discovery that *gfp* under the control of the *SIX1* promoter was expressed in *Fol* only when *Fol* was growing within the tissues of its host prompted
Figure 3.5: Pathogenicity test of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) race 3 *gfp* transformants on tomato seedlings. Figures on the left show resistant tomato lines, whereas figures on the right show susceptible tomato lines. Mock-inoculated tomato seedlings are shown in figures A and B. Figures C, E, and G correspond to resistant tomato seedlings infected with: C = wild type Fol race 3; E = Fol race 3 transformed with *gpd* promoter-*gfp*; G = Fol race 3 transformed with *SIX1* promoter-*gfp*. Figure D, F, and H correspond to susceptible tomato seedlings infected with: D = wild type Fol race 3; F = Fol race 3 transformed with *gpd* promoter-*gfp*; H = Fol race 3 transformed with *SIX1* promoter-*gfp*. Pictures were taken at three weeks post inoculation.
Table 3.1: Disease severity in tomato seedlings infected with *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) race 3 *gfp* transformants. Four isolates of each transformation line (*gpd*-*gfp* and *SIX1*-*gfp*) were tested for their virulence on three-week-old resistant and susceptible tomato lines. The data were recorded at three weeks post inoculation. Each datum is a mean of the disease score assessment ± the standard error. Fifteen seedlings of each cultivar were used for each individual transformant. The experiment was not replicated. Disease was evaluated using the following criteria: 0 = no reaction, healthy plant; 1 = localised vascular staining in tip of primary roots; 2 = staining to cotyledonary node; 3 = staining beyond cotyledonary node but with no external symptoms; 4 = external symptoms and severe stunting; 5 = complete collapse and death. Scores of 0 - 2 are classified as resistant, scores of 3 - 5 are classified as susceptible. *Fol3WT* = wild type *Fol* race 3; *Fol3gpdgfp1 - Fol3gpdgfp4 = Fol* race 3 isolates 1 - 4 transformed with *gpd* promoter-*gfp*. *Fol3SIX1gfp1 - Fol3SIX1gfp4 = Fol* race 3 isolates 1 - 4 transformed with *SIX1* promoter-*gfp*.

<table>
<thead>
<tr>
<th>Transformants</th>
<th>Resistant tomato</th>
<th>Susceptible tomato</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock-inoculated</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Fol3WT</td>
<td>0.00±0.00</td>
<td>4.69±0.31</td>
</tr>
<tr>
<td>Fol3gpdgfp1</td>
<td>0.13±0.00</td>
<td>5.00±0.00</td>
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<td>4.23±0.77</td>
</tr>
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<td>4.40±0.40</td>
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<td>Fol3gpdgfp4</td>
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<td>4.71±0.29</td>
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<tr>
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<td>2.64±0.50</td>
<td>4.71±0.29</td>
</tr>
</tbody>
</table>
the use of this system to assess the ability of the endochitinase/β-1,3-glucanase fusion gene to confer resistance to *Fol* race 3 in tomato. Potentially, this would serve as a better system for assessing the levels of resistance to *Fol* race 3 in transgenic hairy roots of tomato compared to the use of *Fol* expressing *gfp* constitutively, because the former would only allow observation of infecting hyphae and exclude any non-infecting hyphae.

In this study, *Fol* race 3 was transformed with high efficiency using the *gpd* promoter-*gfp* fusion. Antibiotic resistant colonies occurred at a high frequency, and, in general, expressed *gfp*. Although Zeocin™-resistant colonies also occurred at high frequency in transformations using the *SIX1* promoter-*gfp* fusion, transformants expressing *gfp* were only observed at low frequency.

There are several possible reasons for the low frequency of infections expressing *gfp* observed when screening the *SIX1* promoter-*gfp* transformants. One possible reason for the low frequency of infections expressing *gfp* was that *gfp* was not expressed in most of the transformants tested, or that it was expressed at such low levels that GFP fluorescence was indistinguishable from root autofluorescence. Poor *gfp* expression could perhaps be explained by positional effects, in which the construct was inserted at positions in the *Fol* genome incompatible with strong expression of the transgene. Poor *gfp* expression could perhaps also be explained by the nature of the *SIX1* promoter-*gfp* construct, which contains sequences flanking *gfp* that enable homologous recombination at the original *SIX1* locus at a frequency less than 3% [130] (Figure 3.6).

The *SIX1* promoter-*gfp* construct contains approximately 900 bp of promoter sequence between the NcoI site used for cloning and the start codon of the *SIX1* gene. Sequence of the *SIX1* region reveals another 300 bp upstream of the NcoI site and downstream of a repeat region (miniature impala repetitive elements) that could contain additional promoter elements necessary for efficient *SIX1* expression. For example, this region contains a qa-1F-like element (CGCAAAACATTCATCC on the minus strand), which in *Neurospora crassa* binds qA-1F, an activator that positively regulates expression of genes in the quinic acid (qa) gene cluster. Ectopic insertion of the *SIX1* promoter-*gfp* fusion
might lead to poor gfp expression because the promoter is truncated but homologous recombination between the SIX1 promoter-gfp fusion and the SIX1 gene might lead to good gfp expression, as found by van der Does et al. (2008) [164], because the full-length SIX1 promoter is restored (Figure 3.6). Pathogenicity tests (see below) confirm the absence of homologous recombination at the SIX1 locus. To examine this hypothesis, the additional 300 bp of the SIX1 region can be added to the SIX1 promoter-gfp construct and used to transform Fol. The transformed Fol can then be used to infect tomato seedlings.

However, the possibility of poor gfp expression was not tested by expression analysis e.g. by RT-PCR, therefore it is not possible to conclude whether the low frequency of GFP fluorescence in infected roots was due to low Fol infection in the roots or to low expression levels of gfp. However, pathogenicity tests (discussed below) indicate that the pathogenicity of these transformants was unaffected suggesting that the ability to infect tomato roots had not been reduced significantly, thereby perhaps favouring poor gfp expression as an explanation. In this study, infected roots were observed using a stereo dissecting fluorescence microscope, which is more limited in its ability to capture details in the root system compared to a confocal laser scanning microscope, which van der Does et al. (2008) [164] used in their study. It is possible that this technical difference may have also contributed to the poor detection of GFP fluorescence.

van der Does et al. (2008) [164] reported that gfp under the control of the SIX1 promoter was expressed and GFP fluorescence detected in infecting Fol as early as 2 days after inoculation, shortly after Fol actually enters the root, and infections were found along the root with roughly 20 infection patches per root system. In this study, Fol expressing gfp was observed later at three days post inoculation, and only four infection patches were found in 16 tomato seedlings inoculated with Fol. In this study, a Fol race 3 isolate from Australia was used for transformation whereas in the study by van der Does et al. (2008) [164] a Fol race 2 isolate from France was used. It is possible that genetic differences between the two races may have contributed to both the rate and extent of infection as well as the level of SIX1 promoter activity driving gfp expression.
Figure 3.6: Homologous recombination of the SIX promoter-gfp construct at the SIX1 locus. SIX1 open reading frame (ORF) of Fusarium oxysporum f. sp. lycopersici race 2 was replaced by the hygromycin resistance gene [131]. Analysis of the SIX1 sequence reveals another 300 bp that could contain necessary elements for good SIX1 expression. This additional 300 bp sequence is absent in the SIX1 promoter of the SIX1 promoter-gfp construct (see text). Homologous recombination of the SIX1 promoter-gfp construct at the SIX1 locus restores the full length SIX1 promoter. If homologous recombination occurs at the SIX1 locus, the full length SIX1 promoter will be restored, and this would lead to good gfp expression as observed by van der Does et al. (2008) [164]. On the other hand, if homologous recombination does not occur at the SIX1 locus, gfp will be expressed under the control of the truncated SIX1 promoter that could lead to poor gfp expression.

3.4.2 Pathogenicity of Fol race 3 gfp transformants was not altered

Pathogenicity tests showed that all tested Fol race 3 gpd promoter-gfp transformants were still virulent towards susceptible tomato plants and avirulent towards resistant tomato plants. This result confirmed that gpd promoter-gfp transformation did not alter pathogenicity on the tomato cultivars that we tested. Most of the tested transformants caused wilt symptoms on susceptible tomato comparable to that of wild type Fol. One exception was the gpd promoter-gfp transformant, designated Fol3gpdgfp1, which caused more severe disease in susceptible tomato. This isolate also caused mild symptoms of Fusarium wilt in resistant tomato plants compared to the other transformants. Some other gpd promoter-gfp transformants, on the other hand, caused less severe disease on susceptible plants compared to the wild type. However, this experiment was not repeated, so it could not be concluded whether these differences were due to genetic alteration or experimental variation.
Pathogenicity tests of *Fol* race 3 *SIX1* promoter-*gfp* transformants showed that the transformants were still fully virulent on susceptible tomato plants comparable to that of wild type *Fol*. On the other hand, the transformants showed reduced avirulence towards resistant tomato plants compared to the wild type ones. Even though the transformants caused obvious wilt symptoms on the resistant tomato line, the transformants were still considered avirulent (plants resistant) based on the disease score (Table 3.1). These results imply that homologous recombination at the *SIX1* locus had not occurred, but instead that the *SIX1* promoter-*gfp* construct had most likely inserted ectopically as a T-DNA insertion. However, this hypothesis remained untested because disruption of the *SIX1* gene was not examined. If homologous recombination had occurred at the *SIX1* locus, the *Fol* transformants would be expected to show both reduced avirulence on resistant tomato plants and reduced virulence on susceptible tomato plants because *SIX1* is required for *I-3*-mediated resistance and contributes to *Fol* pathogenicity [131, 130]. Given that the major purpose of this study was to employ the transformants that retained full pathogenicity to infect susceptible tomato plants, these transformants would be still useful except for the uncertainty about their ability to express sufficient *gfp* to enable sensitive detection of *Fol* in tomato roots.

To test the hypothesis that poor *gfp* expression might be due to the lack of the additional 300 bp sequences of the *SIX1* region in the *SIX1* promoter-*gfp* construct, it would be useful to add back the missing sequences to the construct and test the *Fol* race 3 transformants for *gfp* expression and pathogenicity in tomato. Moreover, it would have been interesting to carry out a qPCR analysis of *SIX1* gene expression in the transformants.
Chapter 4

Construction of an endochitinase/β-1,3-glucanase fusion gene and a red fluorescent protein reporter gene

4.1 Introduction

4.1.1 Engineering the defence machinery in plants with chitinase and glucanase genes from *Trichoderma*

Following the discovery of genes and molecules involved in disease resistance, various attempts have been made to improve resistance to diseases especially in economically important crops. The ability to transfer genes of interest between plants has been accelerated by the advent of genetic engineering and plant transformation techniques. Genetic engineering technology also allows the transfer of genes from non-plant species, something that was not available using conventional breeding techniques. However, in contrast to the successful production of insect-resistant crops such as Bt corn or cotton, no transgenic crops are available that show good levels of resistance to microbial pathogens. Major problems with these approaches are the complexities of disease-resistance signalling and the complex lifestyles of pathogen. The elucidation of many disease resistance processes in plants has allowed different approaches or targets for manipulation to improve disease resistance. One of the oldest strategies in the engineering of
resistance to pathogens is the overexpression of antifungal proteins, mainly chitinases and glucanases. This approach has proven successful in some cases, but broad spectrum durability has not been achieved. In addition, deleterious effects on plant physiology owing to overexpression of transgenes have been reported [116, 50, 95, 108].

In many applications, the chitinases and glucanases used as antifungal transgenes are of plant origin. One of the major limitations of this approach is that these antifungal proteins are often highly specific for only a few pathogens, and generally they do not provide broad-spectrum control [3, 125]. One possible reason is that pathogens might have adapted to plant chitinases and glucanases. Pathogens have evolved to counteract plant chitinases and glucanases by producing proteinaceous inhibitors such as Avr4 from *C. fulvum*, which protects the fungal cell wall from plant chitinases, and glucanase inhibitor proteins (GIPs) produced by *Colletotrichum lindemuthianum* and oomycetes including *Phytophthora sojae* [163, 132]. Also, it is possible that transgenes derived from another plant encode chitinases and glucanases that are adapted to attack only pathogens with which they have co-evolved. From this evolutionary arms race point of view, the use of chitinases and glucanases of non-plant origin could potentially circumvent the problem because pathogens may not be adapted to counteract non-plant chitinases and glucanases. Moreover, chitinases from *Trichoderma* have been shown to have a much higher activity (up to 100 times) to a wider range of plant pathogenic fungi than plant chitinases [98].

*Trichoderma* species have been used as biocontrol agents to protect plants from diseases. There are several characteristics of *Trichoderma* species that qualify them as biocontrol agents. They are rhizosphere inhabitants and have been shown to be beneficial plant symbionts, engaging in intimate relationships with plants, whereby they increase plant growth and can also induce resistance to pathogens [102, 106, 180, 62]. Secondly, they are antagonists of some fungal pathogens. The latter property is generally referred to as mycoparasitism because *Trichoderma* penetrates host fungi, grows inside the hyphae and eventually kills the fungal host. The mechanism of parasitism involves expression of genes
encoding cell wall-degrading enzymes, most notably chitinases and glucanases [186, 139, 23, 83, 97]. Although the role of these proteins in mycoparasitism is not completely understood, some chitinases and glucanases from *T. atroviride* have been identified as being upregulated during contact with fungal hosts and are suggested to function in host cell wall degradation [23, 42]. Therefore, chitinases and glucanases from *Trichoderma* may be a good source of transgenes to confer resistance against pathogenic fungi in plants.

Chitinases from *T. atroviride* have been introduced into several model and crop plants, and these plants exhibited increased levels of resistance to some pathogenic fungi [41, 108, 100, 38]. However, unlike the chitinases, the β-1,3-glucanase (*gluc78*) gene from this strain caused deleterious effects on transgenic plants. A transgenic rice line carrying the *gluc78* gene, or in combination with the *ech42* endochitinase gene and/or an exochitinase gene (*nag70*) from this fungus showed abnormal phenotypes such as stunted growth, brown specks that mimic blast lesions, and lack of spike differentiation [95, 108]. Moreover, *gluc78* expression caused a reduction in transformation efficiency and survival rate of transgenic rice. Constitutive expression of this gene in transgenic rice is thought to destroy β-1,3-glucan, which is a minor component of mature vegetative cell walls and a major component of mature endosperm walls unique to the Poaceae family. Since the cell walls of dicotyledonous plants, such as tomato, are not known to contain β-1,3-glucan, the *gluc78* gene might not cause deleterious effects in these plants.

### 4.1.2 Transgene strategies for coordinate expression of fusion genes

A common strategy to express transgenes *in planta* is to use strong promoters that enable constitutive overexpression of the transgenes. If multiple transgenes are co-transformed they are usually regulated by separate promoters. These strategies pose several drawbacks. Firstly, overexpression of defence-related genes often results in deleterious effects on transgenic plants, such as abnormal mor-
phology and reduced yield. Uncontrolled defence reactions in uninfected cells are costly to plants. Therefore, for many strategies, pathogen-inducible promoters might be more useful because they limit the cost of resistance by restricting expression to infection sites. Secondly, stacking transgenes with separate promoters often results in uncoordinated expression of the individual transgenes [4, 39]. Although coordinated expression is not always desirable, enabling simultaneous expression of the transgenes would overcome the variation of expression levels between individual transgenes that normally occurs when they are controlled by separate promoters [75].

One possible solution to the above problems is to engineer a transgene construct that enables transcriptional and translational fusion of two or more genes under the control of an inducible, tissue-specific promoter. PCR-driven overlap extension [67] facilitates the construction of transcriptional and translational gene fusions. This method allows for amplification and fusion of DNA fragments at virtually any point in the DNA sequences. Therefore, two or more genes can be fused as one open reading frame (ORF) by removing the stop codons in the upstream genes. However, translational fusions could result in the production of aberrant proteins that fail to fold into their native conformation due to interference between the proteins fused to one another. One way to avoid this problem is to use a glycine-alanine linker between the genes.

Glycine and alanine are the simplest amino acids and make relatively little contribution to structural changes because they fit readily into all structures and do not favour helix formation in particular [12]. Therefore, insertion of a glycine-alanine repeat in between individual proteins of the chimeric protein should not alter the proper folding of each protein because it would not form secondary structures such as an alpha helix or a beta sheet. Instead, it would simply act as a structural spacer between the two proteins. Moreover, glycine-alanine repeats have been shown to prevent protein unfolding by proteasomes [89, 90, 188]. In this case, the ech42 and gluc78 coding sequences were fused as one open reading frame by removal of the ech42 stop codon and insertion of five glycine-alanine repeats between ech42 and gluc78. In this way, the transgene construct encodes
a chimeric endochitinase/β-1,3-glucanase protein.

The $I-2$ resistance gene of tomato is expressed in low levels particularly in the vascular tissue of roots and stems of tomato. Thus, its expression is localised to the site of $Fol$ containment [110]. Therefore, the promoter of the $I-2$ gene would be an ideal regulator for the chimeric $ech42/gluc78$ fusion gene as it would provide spatial expression of the fusion gene related to the site of $Fol$ development.

4.1.3 Red fluorescent protein as a transgene reporter

Red fluorescent protein (RFP) has been widely used as a visual reporter; either as an alternative or as a counterstain to GFP. RFP has excitation/emission maxima of 584/607 nm, thus it is readily distinguished from GFP in coexpression studies. Incorporating an $rfp$ reporter gene into the T-DNA carrying a chimeric chitinase/β-1,3-glucanase construct would facilitate the identification of transgenic tomato hairy roots carrying the chimeric gene.

The $ocs$ promoter was chosen to drive the expression of the $rfp$ gene in planta. The $ocs$ elements comprise a family of related 20-bp DNA sequences that are important components of the promoters of a number of Agrobacterium genes expressed in plants, such as the octopine synthase ($ocs$) gene. They have been used to drive inducible expression of genes, especially in plant roots in the presence of auxin [43]. Therefore, the $ocs$ promoter would seem to be an ideal promoter to drive the expression of the $rfp$ gene in hairy roots of tomato.

This chapter describes the construction of a chimeric endochitinase/β-1,3-glucanase fusion gene and an $rfp$ reporter gene construct by PCR-driven overlap extension and the testing of these constructs by transient expression in planta.

4.2 Materials and methods

The construction of the endochitinase/β-1,3-glucanase chimeric gene and
the _rfp_ reporter gene construct was accomplished using the PCR-driven overlap extension method. This method required the design of oligonucleotide primers that allow the creation of overlapping DNA sequences between genes that are to be spliced. In a PCR reaction, the overlapping DNA sequences hybridise, and the hybridised DNA is then extended to a full length fused DNA by the thermostable DNA polymerase. The oligonucleotide primers used in this study are listed in Table 4.1. All PCRs were carried out employing iProof™ High-Fidelity DNA Polymerase (BIO-RAD) in a 20 μL reaction volume. Unless otherwise stated, the PCR reaction consisted of 0.02 U/μL iProof™ DNA polymerase, 1× buffer HF (BIO-RAD), 250 μM dNTP mix, 500 nM of each primer, and 0.05 pmol of DNA templates. The annealing temperature of primers and the polymerisation time varied depending on the melting temperature of the primers and the length of the DNA templates. All PCR reactions were terminated by a final extension at 72 °C for 10 min.

### 4.2.1 Amplification of endochitinase (_ech42_) coding sequence from _Trichoderma atroviride_ strain P1 cDNA

#### 4.2.1.1 Culture conditions to induce chitinase gene expression

_Trichoderma atroviride_ strain P1 was obtained from the American Type Culture Collection (ATCC number 74058). To induce the expression of the endochitinase (_ech42_) gene in _T. atroviride_ strain P1, the fungus was grown in minimal medium (MM, Appendix D) with Fol race 3 biomass as the sole carbon source. One mL of 10⁶/mL conidia of Fol race 3 was inoculated into 100 mL of PDB (Difco) and incubated for 5 days at 25 °C with constant shaking at 200 rpm. Mycelia were harvested by filtration over four layers of miracloth and dried on paper towel overnight. The dried mycelia were sliced into small pieces, ground in liquid nitrogen into a fine powder using a mortar and pestle and autoclaved. One mL of 10⁶/mL conidia of _T. atroviride_ was inoculated into PDB and incubated for 48 hours at 25 °C with constant shaking at 200
rpm. Mycelia were harvested by filtration through four layers of miracloth, and washed with sterile water. Mycelia were transferred to MM supplemented with 0.2% (w/v) dried *Fol* race 3 mycelia as the sole carbon source, and incubated for 24 hours at 25 °C with constant shaking at 200 rpm. Mycelia were recovered, washed with sterile MilliQ water, and immediately frozen with liquid nitrogen before being used for RNA extraction.

4.2.1.2 Total RNA isolation and cDNA synthesis

Approximately 100 mg of *T. atroviride* strain P1 mycelia was used as the starting material for total RNA extraction. Mycelia were ground into a fine powder in liquid nitrogen using a pestle and mortar, and the powder was then decanted into 1.5 mL tubes. Total RNA isolation was performed using the RNeasy® Mini Kit (QIAGEN Corp.) according to the manufacturer’s instructions.

Total RNA was DNase-treated to remove contaminating DNA before proceeding to cDNA synthesis. 12 U/µL of RQ1 DNase (Promega), 1.2 µL of 10× RQ1 reaction buffer (Promega), and 0.75 µL of RNase inhibitor (Amersham) were added to approximately 1.2 µg of total RNA to give a final volume of 12 µL of reaction mixture. The mixture was incubated at 37 °C for 2 hours. The reaction was terminated by the addition of 1× RQ1 DNase stop solution (Promega), and the enzymes were then heat-inactivated by incubating the reaction mixture at 65 °C for 20 min.

The first strand cDNA was generated using SuperScript™ II Reverse Transcriptase supplied by Invitrogen. Approximately 1 µg of DNase-treated total RNA was used as a template for the reverse transcriptase reaction. The reaction mixture consisted of 1× 1st strand buffer (Invitrogen) (50 mM Tris-HCl pH 8.3; 75 mM KCl; and 3 mM MgCl₂), 0.01 M dithiothreitol (DTT), 500 µM dNTP mix, and 10 ng/µL oligo dT in a 40 µL reaction volume. The reaction mixture was incubated at 65 °C for 5 min and followed by incubation at 37 °C for 10 min. After incubation, 2 µL of SuperScript™ II Reverse Transcriptase was added to the mixture and incubated at 37 °C for 60 min, followed by incubation
### Table 4.1: Oligonucleotide primers used to create the endochitinase/β-1,3-glucanase fusion gene and the RFP construct using PCR-driven overlap extension method as well as for detection of their transient expression in *Nicotiana benthamiana*. Italicised sequences are restriction endonuclease sites, and underlined sequences are overlapping sequences with the sequences of DNA fragments to be joined. The underlined sequences will introduce the 3' sequences of the adjoining DNA fragments to the DNA templates. ech42FRT, gluc78RRT, UNIVRFPR (along with ocs-RFP F) were used to detect the transient expression of the chimeric endochitinase/β-1,3-glucanase fusion gene and RFP.

<table>
<thead>
<tr>
<th>Oligonucleotide primers</th>
<th>Oligonucleotide primer sequences (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>I2ProEcoF</td>
<td>GAATTCATATGTTGCTCTTCTATTTCT</td>
</tr>
<tr>
<td>I2ProClaR</td>
<td>ATCGATAAAATCTGCAAAAAAGCAAAAAACAG</td>
</tr>
<tr>
<td>CP02F</td>
<td>GGCAGTGATAGTACGCAAACG</td>
</tr>
<tr>
<td>CP02R</td>
<td>GTTGAGACCGCTCTCGAGTGT</td>
</tr>
<tr>
<td>CP03F</td>
<td>GCGACAAGCTGGTGGCTT</td>
</tr>
<tr>
<td>CP03R</td>
<td>CTATGAGCTCTCATATGAATGGCA</td>
</tr>
<tr>
<td>CP04F</td>
<td>ATCGATGGGATTTTCCTTCTTTTTCAC</td>
</tr>
<tr>
<td>CP04R</td>
<td>GTTTGCGTATCCACTGCGGCGCACGAAAGATGGGATAATT</td>
</tr>
<tr>
<td>CP05F</td>
<td>TCCCACTCTGCGGCGCCGCGACTGGATACGAAACGGCG</td>
</tr>
<tr>
<td>CP05R</td>
<td>AGCACCAGCACAGCACAGGCCAGCGAGCAGGGTTAGAGCCTGAGG</td>
</tr>
<tr>
<td>CP06F</td>
<td>GGTGCTGGTGCTGGTGCTGGTGCTGGCAGAAAGCTGGTGCTGGCTTCCCA</td>
</tr>
<tr>
<td>CP06R</td>
<td>TCTAGAATATGAGCTCTCATATGAAATGG</td>
</tr>
<tr>
<td>OCS_pro_ApaIF</td>
<td>GGCCCACGGTCTTACGCTACATGAATATTC</td>
</tr>
<tr>
<td>ocs-RFP F</td>
<td>CCATTATATTGGGATACCTAAACCAACATGCTCTTCGAGGAGCTTCATC</td>
</tr>
<tr>
<td>ocs-RFP R</td>
<td>ATGACGTGGAGCTCAGCTGTGGAGTGAGTGGTCTAGAATATACG</td>
</tr>
<tr>
<td>RFP-ocs F</td>
<td>GCCACTCAGGGGCGCTAAGCTTTATCAATCTGCTGTTAATTCG</td>
</tr>
<tr>
<td>RFP-ocs R</td>
<td>CATTAAAGGCTAGCTGAGATTTGATAAGCTTTAGAGGAGCCTGAGGAGCCTGAGGAG</td>
</tr>
<tr>
<td>OCS_ter_HindIII F</td>
<td>AAAGCTAAATCCAACTAATGCTTCCAGCTGGTTAATTCG</td>
</tr>
<tr>
<td>OCS_ter_EcoRI R</td>
<td>GGTAACATATGAAATGTGAAAGAG</td>
</tr>
<tr>
<td>ech42FRT</td>
<td>GCTTAGACTTACATGAAATGTGAAATGTG</td>
</tr>
<tr>
<td>gluc78RRT</td>
<td>CAGCCTGGATGGGCTGAGCTGGCATT</td>
</tr>
<tr>
<td>UNIVRFPR</td>
<td>CTGGGCAATAGTTAAGTGGCTT</td>
</tr>
<tr>
<td>ApaIF35S-RFP</td>
<td>GGCCCACATCCACAAACAAATCTGAGCTTAACAGC</td>
</tr>
<tr>
<td>EcoRIF35S-RFP</td>
<td>GAATTCATACACCACAAAAATCTGAGCTTAACAGC</td>
</tr>
<tr>
<td>35S-RFPR</td>
<td>gatgaCGTCTCGGAGGAGGCCATGgttaattgttaattgttaattgtg</td>
</tr>
</tbody>
</table>
| RFP-35SF                | caacattacacattacattacattacattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattat
at 95 °C for 5 min before the mixture was cooled down to 4 °C.

4.2.1.3 Amplification of the endochitinase ($ech42$) coding sequence

$ech42$ cDNA was amplified using gene-specific primers CP02F and CP02R. The primers were designed to amplify the $ech42$ cDNA excluding the signal peptide sequence and the stop codon. PCR was performed using 1 μL of cDNA synthesis reaction mixture as a template in a 20 μL reaction volume. PCR was performed with an initial denaturing step at 98 °C for 30 sec followed by 25 cycles of denaturation at 98 °C for 8 sec, annealing of primers at 55 °C for 30 sec, and primer extension at 72 °C for 30 sec. Since the $ech42$ gene has three introns, the cDNA product was confirmed by comparing its size with $ech42$ amplified from genomic DNA. The $ech42$ cDNA was then cloned into the pCR® 2.1 vector (Invitrogen).

4.2.2 Amplification of the $\beta$-1,3-glucanase ($gluc78$) gene from $T. atroviride$ strain P1 genomic DNA

The $gluc78$ gene was amplified directly from genomic DNA because the gene is intronless. The primers CP03F and CP03R for $gluc78$ amplification were designed to amplify $gluc78$ excluding the signal peptide sequence. PCR was performed with an initial denaturing step at 98 °C for 30 sec followed by 25 cycles of denaturation at 98 °C for 8 sec, annealing of primer at 55 °C for 30 sec, and primer extension at 72 °C for 90 sec. The PCR product was subsequently cloned into the pCR® 2.1 vector (Invitrogen).

4.2.3 Amplification and cloning of the $I-2$ gene promoter into a binary vector

The $I-2$ promoter was amplified from a binary vector pCBJ257 using a pair of primers I2ProEcoF and I2ProClaR that introduce an EcoRI site at the
5' end and a ClaI site at the 3' end of the promoter. The length of the I-2 promoter including its native ATG is 1290 bp. PCR was performed with an initial denaturing step at 98 °C for 30 sec followed by 25 cycles of denaturation at 98 °C for 8 sec, annealing of primer at 57 °C for 30 sec, and primer extension at 72 °C for 30 sec. The PCR product was subsequently cloned into the pCR® 2.1 vector (Invitrogen). The I-2 promoter was then cloned into binary vector pCBJ352 as an EcoRI-ClaI cassette replacing the 35S promoter. The new binary vector containing the I-2 promoter was designated pCBJ362. The binary vector pCBJ352 contains the CaMV 35S promoter, TMV omega leader and 35S terminator as an EcoRI/BamHI cassette. pCBJ352 was constructed by Peter Vredenbregt (an intern student from Wageningen University, The Netherlands).

4.2.4 Amplification of PR-1a signal peptide

The tobacco PR-1a signal peptide was amplified from pCBJ242 for use as the leader peptide of the chimeric ech42/gluc78 gene. The fragment is 90 bp long, and the amplified product was used directly for gene fusion without subcloning. PCR amplification was carried out using primers CP04F and CP04R. The reverse primer (CP04R) introduces overlapping sequences with the 5' end of the echj42 gene. PCR was performed with an initial denaturing step at 98 °C for 30 sec followed by 25 cycles of denaturation at 98 °C for 8 sec, annealing of primer at 55 °C for 30 sec, and primer extension at 72 °C for 15 sec. The PCR product was run on a 2% agarose gel, recovered, and used directly for fusion with the gluc78 gene using PCR-driven overlap extension.

4.2.5 Gene splicing by PCR-driven overlap extension of PR-1a, ech42, and gluc78

Fusion of the PR-1a signal peptide, ech42, and gluc78 was performed using PCR-driven overlap extension with some modifications. In this method, the initial PCR generates DNA fragments partially overlapping with each other. These
are used as templates for subsequent PCR to make a full-length product. Primers were designed in such a way that the 3' of the sense strand of the first (upstream) gene and the 3' of the antisense strand of the second (downstream) gene contained overlapping complementary sequences after amplification. Gene splicing was accomplished by extending the hybridised segments of the two genes using the hybridised 3' ends of each gene as primers. The fused genes were then amplified using primers flanking the two annealed DNA strands. The basic steps of PCR-driven overlap extension method are illustrated in Figure 4.1. The PR-1a signal peptide, ech42, and gluc78 were fused in two steps. PR-1a and ech42 were first fused to create a PR-1a/ech42 fusion (herein named spliced by overlap extension 1, abbreviated as SOE1), and SOE1 was then fused with gluc78 to create SOE1/gluc78 (herein named SOE2). A five-repeat glycine-alanine linker was inserted between SOE1 and gluc78 as a spacer.

The first round PCR was performed using primers that introduced overlapping segments to create the junction between the adjoining genes. The second round PCR fused the hybridised genes at the junction to create a full-length fusion product. The third round of PCR was performed to amplify the fused gene using primers that flanked the joined genes. These primers introduced a ClaI site at the 5' end of PR-1a and an XbaI site at the 3' end of gluc78 to construct the chimeric gene as a ClaI-XbaI cassette. PR-1a, ech42, and gluc78 were amplified from their corresponding vectors as described in sections 4.2.4, 4.2.1, and 4.2.2.

4.2.5.1 The first round PCR

The first round PCR to amplify the PR-1a signal peptide, ech42, and gluc78 from their corresponding vectors was performed using primer pairs CP04F and CP04R, CP05F and CP05R, and CP06F and CP06R, respectively. The amplification reactions were performed with an initial denaturation step at 98 °C for 30 sec followed by 25 cycles of denaturation at 98 °C for 8 sec, annealing of primer at 57 °C for 30 sec, and primer extension at 72 °C for 15 sec per kb of
product expected. The PCR products were gel purified and used directly as templates for the second round PCR without subcloning.

4.2.5.2 The second round PCR

For the second round PCR, approximately 0.05 pmol/μL of each template derived from the first round PCR was used in the PCR without the addition of primers. PCR was performed with an initial denaturation step at 98 °C for 30 sec followed by 10 cycles of denaturation at 98 °C for 10 sec, annealing of primer at 57 °C for 10 min, and primer extension at 72 °C for 15 sec per kb of product expected. The final extension step was not performed in this PCR. The whole reaction mixture of the second round PCR was used directly as a template for the third round PCR without being gel purified.

4.2.5.3 The third round PCR

The third round PCR was carried out to amplify the fused genes from the second round PCR. Fresh DNA polymerase, dNTP mix, and a pair of primers that flanked the fused genes were added into the reaction mixture of the second round PCR. To amplify SOE1, primers CP04F and CP05R were used, whereas primers CP04F and CP06R were used to amplify SOE2. PCR amplification was carried out with the same conditions as the first round PCR (section 4.2.5.1). The PCR products were analysed by gel electrophoresis. A band of DNA with the expected size of the fused genes was excised from the gel and purified prior to cloning in vector pCR® 2.1. The chimeric gene was digested from the vector and cloned into the pCBJ352 and pCBJ362 plant expression vectors as a Clal-Xbal cassette to generate pCBJ372 and pCBJ382, respectively.
Figure 4.1: Schematic diagram of PCR-driven overlap extension method. In this illustration, two DNA fragments, A and B, will be fused using primers a, b, c, and d. This method consists of three steps. First, amplification of individual fragments (A and B) that will be fused using the corresponding primers for each fragment (fragment A with primers a and b, fragment B with primers c and d). Primer b will introduce sequence overlapping with fragment B on fragment A, and vice versa, primer c will introduce sequence overlapping with fragment A on fragment B. The two fragments are then mixed together in the second round PCR without the addition of any primers because the overlapping regions will hybridise and the 3' ends of the hybridised region will act as primers. Therefore, the product of the second PCR is a full length combination of the fused genes. The product of the second round PCR (the fused genes A and B) is then amplified using primers a and d.
4.2.6 Construction and cloning of an rfp reporter gene

The rfp gene was amplified from pICHRFP and fused to the ocs promoter and terminator using PCR-driven overlap extension as described in section 4.2.5. Firstly, the ocs terminator was amplified with primers RFP-ocs F and OCS_ter_EcoRI_R that introduce sequence overlapping with rfp as well as HindIII and EcoRI sites. The ORF of rfp was amplified using primers ocs-RFP F and RFP-ocs R that introduce sequences overlapping with the ocs promoter and terminator, respectively. The ocs terminator and rfp were fused in the second round PCR and the fused fragments were then amplified in the third round PCR using primers ocs-RFP F and OCS_ter_EcoRI_R.

The ocs promoter was amplified using primers OCS_pro_ApaLF and ocs-RFP R that introduce ApaI and NcoI sites and sequence overlapping with rfp. The rfp/ocs terminator fusion and the ocs promoter were fused in the second round PCR and then amplified in the third round using primers OCS_pro_ApaLF and OCS_ter_EcoRI_R. The PCR products were analysed by gel electrophoresis. Bands of DNA with the expected size of the fusion product were excised from the gel and recovered prior to cloning in vector pCR® 2.1. The rfp construct was digested with ApaI and EcoRI and ligated into pCBJ352, pCBJ362, pCBJ372 and pCBJ382. The resulting binary vectors were then named pCBJ400, pCBJ401, pCBJ402, and pCBJ403, respectively. The rfp construct was located upstream of the chimeric endochitinase/β-1,3-glucanase gene in the binary vectors pCBJ372 and pCBJ382. The final T-DNA construct containing rfp and chimeric ech42/gluc78 is illustrated in Figure 4.2. The binary vectors pCBJ400, pCBJ401, pCBJ402, and pCBJ403 were then used for transient expression of the chimeric ech42/gluc78 gene in N. benthamiana (described in section 4.2.7) as well as for tomato hairy root and stable tomato transformations (Chapter 5). The binary vectors pCBJ400 and pCBJ401 were used as empty binary vector controls.

As will be described in the following sections, transient expression in N. benthamiana failed to detect good rfp expression and microscopic analysis also
failed to detect RFP fluorescence. Therefore, another *rfp* construct under the control of 35S promoter replacing the *ocs* promoter was made using PCR-driven overlap extension method. The 35S promoter was amplified from pCBJ352 using two sets of primer pairs ApaIF35S-RFP and 35S-RFPR, and EcoRIF35S-RFP and 35S-RFPR. The first primer set introduced Apal site to the 5' of the promoter replacing the original EcoRI site in pCBJ352. The first primer set also introduced overlapping sequences with the *rfp* gene and an Ncol site replacing the original Clal site at the 3' of the promoter. The second primer set did not change the original EcoRI site but replaced the Clal site with the Ncol site at the 3' of the promoter and introduced overlapping sequences with the *rfp* gene. The *rfp/ocs* terminator fusion was amplified using primers RFP-35SF and RFP-35SR and fused to the 35S promoter. The *ocs* promoter/*rfp/ocs terminator fusions were cut out from pCBJ402 and pCBJ403 and replaced with the newly constructed 35S promoter/*rfp/ocs terminator fusions. The fusion constructs were cloned into pCBJ402 as an Apal-EcoRI cassette and into pCBJ403 as an EcoRI cassette. The resulting binary vectors were then named pCBJ404 and pCBJ405, respectively. Unfortunately, time constraints did not allow testing of these constructs.

### 4.2.7 Transient expression of the chimeric chitinase/β-1,3-glucanase fusion gene and the *rfp* reporter gene in *Nicotiana benthamiana*

To test the expression of the chimeric chitinase/β-1,3-glucanase fusion gene and the *rfp* reporter gene *in planta*, the constructs were expressed transiently in *N. benthamiana* leaves following infiltration with *A. tumefaciens* strain GV3101 (agroinfiltration). GV3101 isolates carrying the constructs were grown on LB plates containing rifampicin (50 μg/mL), gentamycin (50 μg/mL), and kanamycin (50 μg/mL) at 27 °C for 3 days. A single colony of GV3101 was inoculated into 3 mL of YEP medium supplemented with appropriate antibiotics and incubated for 2-3 days at 27 °C. 150 μL of this culture was used to inoculate 15 mL of YEP containing appropriate antibiotics in 250 mL conical flasks and incubated at 27
°C with constant shaking for 16-24 hrs. Cells from the cultures were collected by centrifugation at 5,000 g for 10 min at room temperature and the pellets were washed once with infiltration buffer (IFB) [1 ℓ IFB: 4.33 g MS basal salts (Sigma), 20 g sucrose, 1.95 g MES, pH adjusted to 5.6 with KOH supplemented with 200 μM acetosyringone]. Optical density of the cells was adjusted to OD$_{600}$ = 1. The cells were incubated in IFB for 2 hours at room temperature before infiltration.

The *N. benthamiana* plants were watered approximately 15 minutes before infiltration. The induced GV3101 cells were infiltrated into the leaves through the abaxial surface using a 1 mL syringe without a needle. The cells were infiltrated into three circular regions per leaf with the diameter of each circle being approximately 2.5 cm. IFB without bacteria was used as a control. At the third day after infiltration, the infiltrated leaves and control were harvested and frozen with liquid nitrogen before storing at -80 °C prior to performing reverse transcriptase PCR to detect transgene expression.

Total RNA extraction and cDNA synthesis were performed as described in section 4.2.1.2. Total RNA was extracted from the frozen, infiltrated leaves. For each sample, the total RNA was isolated from three infiltrated leaves. Approximately 1 μg of DNase-treated total RNA was used as a template for the reverse transcriptase reaction. The primers ech42FRT and gluc78RRT were used to detect the chimeric chitinase/β-1,3-glucanase transcript. These primers covered a 245 bp internal fragment of *ech42*, the glycine-alanine linker and *gluc78*. For detection of *rfp*, a pair of primers ocs-RFP F and UNIVRFPR were used to amplify a 577 bp internal fragment of the *rfp* gene. As a reaction control, each PCR was repeated without the addition of the SuperScript™ II Reverse Transcriptase.

### 4.3 Results

#### 4.3.1 Gene splicing by PCR-driven overlap extension of PR-1a, *ech42*, and *gluc78*

The PR-1a, *ech42*, and *gluc78* genes were PCR-amplified and fused using PCR-
driven overlap extension (Figure 4.3). The ech42 coding sequence was obtained from cDNA and subcloned before being fused with PR-la and gluc78. The primers used to amplify ech42 were designed in such a way that it lacked the signal peptide and stop codon. The native signal peptide of ech42 was replaced by the tobacco PR-la signal peptide to facilitate the secretion of the chimeric gene into the plant apoplast. The gluc78 coding sequence, on the other hand, was amplified directly from the genomic DNA of T. atroviride because it lacks introns.

The construction of the chimeric gene was accomplished in two steps, although theoretically all the three DNA fragments could have been fused together at once. Firstly, the PR-la signal peptide was fused with ech42, and secondly, the PR-la/ech42 (SOE1) fusion was fused with gluc78 resulting in the final PR-la/ech42/gluc78 (SOE2) fusion gene construct. The successful construction of SOE1 was confirmed by comparing the size of SOE1 with ech42 and by DNA sequencing (Figure 4.4). Agarose gel analysis showed that there was only one discrete band produced, indicating that the method and the primers had been used successfully to create the fusion gene. However, gel analysis also revealed non-specific products. A smear of DNA was always observed even when the annealing temperature was increased to an extent that the intensity of the PCR
product was greatly reduced (data not shown).

Similarly, successful generation of the SOE2 construct was also shown by agarose gel analysis, which showed a band that corresponded to the expected size (3,490 bp) (Figure 4.5). The DNA authenticity of SOE2 was confirmed by DNA sequencing. A smearing of DNA and two non-specific bands appeared, which included one that showed the same size as SOE1. It is likely that this band was indeed SOE1, but it is likely a product of multiplication of the SOE1 sense strand because the forward primer that was used to generate SOE1 and SOE1 itself were both included in the PCR mixture to create SOE2. Likewise, the other band, which was faint and showed the same size as the gluc78, was probably gluc78. A range of annealing temperatures (55, 57, and 60 °C) were used to amplify SOE2, but the DNA smear and non-specific DNA bands (SOE1 and gluc78) were always observed except at the annealing temperature of 60 °C, in which there was no amplification.

The rfp construct was also successfully constructed using PCR-driven overlap extension (Figure 4.6). The first fusion of the rfp coding sequence with the ocs terminator produced a single DNA band, but as was the case with SOE2, the final fusion of the rfp/ocs terminator and the ocs promoter produced an intense band of the expected size together with other non-specific bands including a band corresponding to the ocs promoter and the rfp/ocs terminator fusion. The annealing temperature for the primers was not optimised since the final fusion product was intense and sufficient for gel recovery.

All plasmids that have been constructed and used in this study are listed in Table 4.2. The overall size of the T-DNA containing the rfp, and chimeric ech42/gluc78 genes, and nos::nptII cassette is about 8 kb. The binary vectors were not sequenced in entirety but the authenticity of all constructs inserted into the binary vectors was confirmed by DNA sequencing and restriction digestion analysis.
<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Constructs</th>
<th>Remarks</th>
</tr>
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<tbody>
<tr>
<td>pCBJ352</td>
<td>35S promoter::35S 3'</td>
<td>35S promoter and terminator in pGreenII</td>
</tr>
<tr>
<td>pCBJ362</td>
<td>1-2 promoter::35S 3'</td>
<td>pCBJ352 with 1-2 promoter replacing 35S promoter</td>
</tr>
<tr>
<td>pCBJ372</td>
<td>35S promoter::PR-1a::ech42::gluc78::35S 3'</td>
<td>pCBJ352 + chimeric gene without rfp</td>
</tr>
<tr>
<td>pCBJ382</td>
<td>1-2 promoter::PR-1a::ech42::gluc78::3'</td>
<td>pCBJ362 + chimeric gene without rfp</td>
</tr>
<tr>
<td>pCBJ400</td>
<td>ocs::rfp::ocs 3'</td>
<td>pCBJ352 + rfp without chimeric gene</td>
</tr>
<tr>
<td>pCBJ401</td>
<td>ocs::rfp::ocs 3'</td>
<td>pCBJ362 + rfp without chimeric gene</td>
</tr>
<tr>
<td>pCBJ402</td>
<td>ocs::rfp::ocs 3':35S promoter::PR-1a::ech42::gluc78::35S 3'</td>
<td>pCBJ352 + rfp + chimeric gene</td>
</tr>
<tr>
<td>pCBJ403</td>
<td>ocs::rfp::ocs 3':1-2 promoter::PR-1a::ech42::gluc78::35S 3'</td>
<td>pCBJ362 + rfp + chimeric gene</td>
</tr>
<tr>
<td>pCBJ404</td>
<td>35S promoter::rfp::ocs 3':EcoRI</td>
<td>pCBJ352 + rfp under 35S promoter</td>
</tr>
</tbody>
</table>

**Table 4.2:** List of plasmids used in this study. All plasmids were generated from a pGreenII backbone. All constructs were cloned into the multiple cloning site of the lacZ reporter gene. No restriction sites were introduced during construction of the chimeric ech42::gluc78 fusion gene.
Figure 4.3: Amplification of the PR-la signal peptide, ech42 and gluc78. The panel in part A shows the DNA gel analysis of the synthesis of ech42 cDNA that lacks native signal peptide and stop codon. The panels in part B shows the DNA gel analyses of products of the first round PCR of the overlapping PR-la signal peptide, ech42, and gluc78 coding sequences.

4.3.2 Transient expression of the chimeric chitinase/β-1,3-glucanase fusion gene and rfp reporter gene in Nicotiana benthamiana

After the chimeric endochitinase/β-1,3-glucanase and rfp reporter gene constructs were made, expression of the transgenes in N. benthamiana leaves following their delivery by A. tumefaciens was examined using RT-PCR. The chimeric endochitinase/β-1,3-glucanase gene under the control of either the 35S or the I-2 promoter did not appear to be expressed since no DNA that corresponded to the expected size was observed on the agarose gel (Figure 4.7). A smaller fragment, bigger than primer dimer, was evident from leaves infiltrated with A. tumefaciens carrying pCBJ403 although the band was faint. This band was likely a result of non-specific amplification of DNA because amplification from plasmid DNA did not show any non-specific product, but the control without reverse transcriptase did produce the smaller fragment. It should also be noted that the amplification of the expected fragment from plasmid DNA did not give an intense band. This raises the possibility that the primers did not enable efficient amplification of the expected fragment.

Expression of rfp was observed at very low levels in leaves infiltrated with A. tumefaciens carrying pCBJ403 but not in other samples (Figure 4.7). In all
Figure 4.4: Construction of a PR-la signal peptide and ech42 fusion gene (SOE1). Part A shows the DNA gel analysis of SOE1 and part B shows a comparison of DNA fragment sizes between SOE1 and ech42. Whole PCR mixtures were loaded onto the gels for visualisation.

Figure 4.5: Construction of the chimeric endochitinase/β-1,3-glucanase fusion gene (SOE2). Part A shows the gel analysis of SOE2 PCR products generated using annealing temperatures of 55, 57, and 60 °C. The whole PCR mixture was loaded onto the gel for visualisation. The SOE2 product is 3490 bp long. A smear of DNA was always present except at an annealing temperature of 60 °C, where no product was produced. Smaller fragments with sizes similar to SOE1 and gluc78 were also observed, and these fragments probably correspond to SOE1 and gluc78. Part B shows the gel-purified SOE2 used for cloning.
Figure 4.6: Construction of an rfp reporter gene. Lane 1 shows the rfp/ocs terminator fusion, whereas lane 2 shows the final ocs promoter/rfp/ocs terminator fusion.

samples, apart from plasmid DNA, smaller fragments with very strong signals were evident on the gel. From their size it is likely that these fragments were primer dimers. However, none of the observed fragments were checked by DNA sequencing, therefore, the identity of the fragments was not known.

These experiments lacked the appropriate controls for cDNA synthesis so it is not possible to conclude unequivocally that the transgenes were not transcribed in planta. These experiments could not be repeated because of a lack of time.

4.4 Discussion

4.4.1 Gene splicing by PCR-driven overlap extension of PR-1a, ech42, and gluc78

The chimeric endochitinase/β-1,3-glucanase and rfp reporter gene constructs were created using PCR-driven overlap extension. In theory, the method is fast, efficient and only requires standard PCR reagents. The use of proof-reading DNA polymerase is essential to minimise mutations caused by polymerisation errors that normally occur when using DNA polymerases that lack 3′-5′ exonu-
Buffer 402 403 p
+ - + - + - 

**Figure 4.7:** Transient expression of the chimeric endochitinase/β-1,3-glucanase fusion and the rfp reporter genes in *Nicotiana benthamiana*. The leaves of *N. benthamiana* were infiltrated with *Agrobacterium tumefaciens* GV3101 carrying the binary vectors pCBJ402 or pCBJ403. The leaves were also infiltrated with infiltration buffer (IFB) without bacteria as a control. The infiltrated leaves were harvested and examined for the expression of the chimeric endochitinase/β-1,3-glucanase fusion and rfp reporter genes on the third day post-infiltration. The expression of the transgenes was examined using reverse transcriptase PCR (RT-PCR). Buffer: RT-PCR of leaves infiltrated with IFB; 402: RT-PCR of leaves infiltrated with GV3101 carrying pCBJ402; 403: RT-PCR of leaves infiltrated with GV3101 carrying pCBJ403; p: PCR amplification of fragment targeted for detection directly from pCBJ402 plasmid DNA; ±: with or without the addition of reverse transcriptase.

Since the original method proposed by Horton *et al.* (1989) [67], several modifications to the method have been proposed for the creation of gene fusions. However, these modifications have not been adopted in many applications, probably because of inefficient annealing of short overlapping segments [170]. Moreover, several published methods were tried in the attempt to create the chimeric endochitinase/β-1,3-glucanase gene, but none of the methods worked. Here, a modified PCR-driven overlap extension method is presented that enabled efficient generation of the chimeric endochitinase/β-1,3-glucanase and rfp reporter gene constructs.

There were three critical steps for the successful construction of the two fusion genes. Firstly, the design of oligonucleotide primers was critical for the creation...
of the chimeric gene. Because the primers need to create an overlap between adjoining fragments, the design was performed manually and the primer sequences were then analysed in silico for their quality. The primers need to contain sufficient overlap to enable annealing of adjacent fragments but must not contain regions of self complementarity that would cause the formation of complex secondary structures interfering with annealing of the overlaps between adjacent fragments. This is particularly important when creating the fragments that contain the overlapping sequences in the first round PCR, because the primer that contains overlapping sequence must have a significantly higher melting temperature than the flanking primer.

Secondly, the second round PCR (the fusion PCR) was found to be critical. This reaction is not actually an amplification reaction but an extension of the hybridised overlapping sequences in both directions. It is omitted in some of the existing methods because, theoretically, it is not necessary to run this reaction separately as the overlap extension can occur in the third round PCR along with the final amplification of the fusion product. However, in practice this additional reaction was found to be necessary because, without this step, the final fusion product was never produced. In the second round PCR, a ten minute annealing time was sufficient, but an annealing time less than five minutes failed to generate the final fusion product. However, the annealing time was not optimised to the shortest time that could generate the final fusion product.

Thirdly, the use of the whole reaction mixture from the second round PCR as components for the third round PCR (standard PCR) was also found to be critical. The use of the whole PCR mixture from the second round PCR for the third round PCR will add unnecessary components that would interfere the following reaction, but on the other hand, it will give sufficient templates for the next PCR. In theory, there will be three different combinations of hybridisation in the reaction mixture; hybridisation of overlapping sequences at the 3' ends of the first and second fragments; hybridisation of overlapping sequences at the 5' ends of the first and the second fragments; third, hybridisation of complementary strands from the same fragment. The first hybridisation is the required arrangement
for the fusion PCR, but the third arrangement would likely dominate. Even assuming that all of these combinations were equally represented, the fusion-generating arrangement (the first combination) would be only a quarter of all the arrangements. In reality it would be much less. Consequently, a large volume of reaction mixture was required to ensure that there was sufficient gene-fusion template for the third round PCR. Therefore, the whole reaction mixture from the second round PCR was used in the third round PCR, with the addition of fresh DNA polymerase, dNTP mix, and oligonucleotide primers. The necessity of this requirement was shown empirically. The addition of a small amount of the mixture from the second round PCR to the third round PCR failed to produce the final fusion product. However, the minimum amount sufficient to produce the final product was not determined since the third round PCR always produced the final product when the whole mixture from the previous PCR was added.

Although the agarose gel analysis showed that the gene fusion products were always accompanied by either a DNA smear or nonspecific bands, the expected products were always recoverable in isolation from other non-specific bands of DNA. The non-specific bands observed in the final fusion of endochitinase/β-1,3-glucanase were probably due to nonspecific priming and multiplication of the non-fused DNA fragments as described in Section 4.3.1.

The rfp reporter gene was constructed under the control of the ocs promoter and ocs terminator. The fusion of the rfp coding sequence to the ocs promoter and terminator was also accomplished in two steps. First, the ocs terminator, was fused with the rfp, and then the rfp/ocs terminator fusion was fused with the ocs promoter. Agarose gel analysis of the final rfp fusion product also revealed nonspecific bands. These were also probably due to nonspecific priming and multiplication of the non-fused DNA fragments as described in Section 4.3.1. Overall, the modified PCR-driven overlap extension method was efficient in creating the endochitinase/β-1,3-glucanase and rfp reporter fusion gene constructs.
4.4.2 Transient expression of the chimeric chitinase/β-1,3-glucanase fusion and rfp reporter genes in *Nicotiana benthamiana*

Agroinfiltration is a common method used to examine the transient expression of transgenes *in planta* using *A. tumefaciens*. The expression of the chimeric chitinase/β-1,3-glucanase fusion and rfp reporter genes was examined in *N. benthamiana* after agroinfiltration using *A. tumefaciens* GV3101. RT-PCR analysis showed no evidence that the chimeric chitinase/β-1,3-glucanase fusion gene under the control of the 35S promoter or the I-2 promoter was expressed in *N. benthamiana*. There are a number of possible explanations for this observation. The transgene may not be expressed or it was expressed at such low levels that it could not be detected by RT-PCR. Sampling time after agroinfiltration may have been critical for the detection of mRNA because the transgenes might not be transcribed at levels sufficient for detection if sampled too early, or post-transcriptional gene silencing may already have occurred if sampled too late. A time-course expression analysis was not performed to determine if the transgenes were expressed at a later timepoint. The third day post infiltration might not have been the time when the transgene mRNAs were most abundant.

Transient expression of the chimeric chitinase/β-1,3-glucanase fusion gene under the control of the I-2 promoter could have been low in leaves of *N. benthamiana* but no transgene expression was detected even under the control of 35S promoter. It should be noted that the RT-PCR primers did not amplify the internal fragment of the chimeric chitinase/β-1,3-glucanase fusion gene from the binary vector very well. Therefore, the absence of an RT-PCR product might also be due to inefficient amplification because of the poor quality of the primers. Since the primers were not re-designed to improve their quality, this explanation can not be ruled out. Another reason for the absence of an RT-PCR product could be failure of the cDNA synthesis reaction. A positive control, for instance the amplification of a cDNA derived from housekeeping gene such as the glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene, was not included. A positive control should have been included to ensure that the cDNA synthesis
reaction was working properly.

Transient expression of the \textit{rfp} gene under the control of the \textit{ocs} promoter was detected in leaves infiltrated with \textit{A. tumefaciens} carrying pCBJ403 but not with \textit{A. tumefaciens} carrying pCBJ402, although the expression level was very low. The differing results from the two plasmids could be due to experimental variations, such as variation in the performance of \textit{Agrobacterium} infiltrations by the investigator. This difference could perhaps have been resolved by repeating the experiment several times. Unlike detection of the chimeric chitinase/\(\beta\)-1,3-glucanase fusion gene, the internal fragment of \textit{rfp} amplified very well in the positive plasmid control PCR. Therefore, the absence of \textit{rfp} expression might not be due to inefficient amplification but rather a lack of expression. But again, an endogenous housekeeping gene control was not included, therefore the reason for the absence of \textit{rfp} expression in leaves infiltrated with \textit{A. tumefaciens} carrying pCBJ402 could not be determined unequivocally. Despite the lack of an appropriate control, these results point to a conclusion contrary to the hypothesis that the \textit{ocs} promoter would be an ideal promoter to drive the expression of the \textit{rfp} gene.

Transient expression of the chimeric chitinase/\(\beta\)-1,3-glucanase fusion and the \textit{rfp} reporter gene constructs in \textit{N. benthamiana} revealed that they were not expressed apart from low level expression of \textit{rfp} from one of the constructs. However, the failure to detect most of the transgenes in this experiment does not necessarily imply that the transgenes were not expressed. Further experiments are required to confirm these results. Unfortunately, time constraints did not allow further experimentation.
Chapter 5

Tomato hairy root transformation

5.1 Introduction

5.1.1 The biology and applications of hairy roots

*Agrobacterium rhizogenes* is the causal agent of hairy-root disease. It incites the formation of neoplastic, plagiotropic roots called hairy roots. Morphologically, hairy roots are different from wild type roots in the following ways: root hairs are longer and more numerous, and root systems are more branched and display an agravitropic phenotype [159]. Similar to *A. tumefaciens*, *A. rhizogenes* transfers T-DNA from a root-inducing (Ri) plasmids or from an engineered binary vector into the plant genomic DNA. Thus, transgenic roots can be formed that usually contain T-DNA from either the Ri plasmid or from both the Ri plasmid and the binary vector. Creation of such transgenic roots is achieved through cotransformation, in which *A. rhizogenes* that already contains the Ri plasmid is transformed with a binary vector, and the transformed *A. rhizogenes* is then used to transform plants. The T-DNA confers on the plant cell the ability to grow in the absence of exogenous plant hormones and the ability to produce modified amino acids (opines), which, in turn are utilised only by the inciting bacteria as carbon, nitrogen, and energy sources.

Hairy roots are widely used as a transgenic tool to produce metabolites and
to study gene function in plants. One of the applications of transgenic hairy roots is to study root-biotic interactions such as the interaction between roots and root-pathogens. Hairy roots can be cultured *in vitro*, or generated directly from plants to form composite plants that consist of wild type shoot from which transgenic roots are induced to develop. Hairy root cultures *in vitro* have been exploited mainly for studies on secondary metabolite production and metabolic engineering (reviewed in [55]). In contrast, interest in *ex vitro* composite plants has mainly been directed at generating whole-plant information [26]. Hairy root cultures *in vitro* have several properties that make them amenable to plant biotechnological applications. For instance, hairy roots can be generated in a relatively short time, and they can grow fast. However, *in vitro* conditions can preclude hairy roots from being a versatile tool for many areas of research such as plant-microbe interactions. *Ex vitro* composite plants offer advantages over hairy root cultures grown *in vitro*, not only because they can provide whole-plant information, but also the non-sterile conditions allow the rapid and efficient production of composite plants with little requirement for specialised facilities or materials. This system also brings benefits over conventional stable transgenic approaches as it reduces the time required for generating transgenic roots from several months for stable transgenic plant production via *A. tumefaciens* to several weeks for hairy root production. Secondly, composite plants allow the testing of transgenes in roots in the context of a whole plant.

5.1.2 The use of hairy roots in the study of plant-pathogen interactions

*A. rhizogenes*-induced hairy-roots have been used to study the interactions between roots and nitrogen fixing bacteria [126], mycorrhizal fungi [114], and nematodes [80, 115]. Transgenic hairy roots have been an important tool in understanding the function of genes, especially those that are predicted to function or be involved in interactions between plants and microbes. Hairy roots generated *in vitro* and *ex vitro* can allow testing genes of interest for their efficacy in conferring resistance in roots against pathogens. Hairy roots have been success-
fully used to study nematode resistance genes [26]. Silencing the Mi1.2 resistance gene using dsRNA in transgenic hairy roots of a nematode-resistant cultivar of tomato produced a susceptible phenotype. Hairy roots transgenic for selected defence peptides have been employed to study the efficacy of the peptides in the induction of premature zoospore encystment of Phytophthora capsici [47]. Nevertheless, despite its established usefulness in the studies of nitrogen-fixing bacteria, mycorrhizal fungi, and nematodes, the use of hairy roots in the studies of root-pathogenic fungi is not well established.

Experiments dealing with the interaction between Fol and tomato mostly use wild type roots or roots of stably transformed tomato plants. Transgenic tomato hairy roots would be an ideal system to study the Fol-tomato interactions because Fol interacts with the roots of tomato at the early stages of Fusarium wilt disease development. Nonetheless, reports regarding the use of hairy roots for the study of tomato-Fol interactions are still lacking. Tomato hairy root cultures have been used to study chemical modifications of the plant cell wall as a mechanism of defence against fungal pathogens or as a response to fungus-derived elicitors. In tomato hairy root cultures, F. oxysporum extracts induce the release of phenolic compounds such as ferulic acid, 4-hydrobenzoic acid and 4-coumaric acid, and induce cell wall strengthening by lignin deposition [105, 104].

We have developed an RFP-tagged chimeric endochitinase/β-1,3-glucanase construct to be tested for its efficacy in conferring resistance to Fusarium wilt in tomato. We have also obtained transformed Fol race 3 expressing gfp under the control of either a constitutive or inducible promoter. Use of these constructs would allow the generation and detection of transgenic hairy roots carrying the chimeric gene and the testing of the transgenes for their ability to confer resistance against Fusarium wilt by monitoring the growth of GFP-tagged Fol race 3 in the transgenic hairy roots.
5.2 Materials and methods

For hairy root and tomato transformations, the binary vectors pCBJ400, pCBJ401, pCBJ402, and pCBJ403 were used. The binary vectors pCBJ400 and pCBJ401 were used as empty binary vector controls for the chimeric endochitinase/β-1,3-glucanase gene.

5.2.1 Generation of tomato hairy roots in vitro

5.2.1.1 Tomato explant preparation

Tomato cultivar M82 was used as the plant material. This tomato cultivar lacks resistance to Fol race 3. To surface-sterilise, tomato seeds were put in 15 mL screw-top Falcon tubes containing 5 mL of 70% (v/v) ethanol and soaked in ethanol for 2 min to remove gelatinous materials on the surface of the seeds, before washing with sterile water. The seeds were then surface-sterilised with 7 mL of 10% (v/v) bleach (Domestos®, Unilever, Australia) containing 49.9 g/ℓ sodium hypochlorite and put on an orbital shaker with gentle shaking for 3 hours. The bleach was decanted and the seeds were washed with sterile water 4 to 8 times. The seeds were left in the final wash of water overnight and gently shaken on an orbital shaker. The water was decanted, and the seeds were blotted on sterile filter papers to remove the remaining water. After they were blotted dry, seeds were transferred using sterile forceps to Sigma Phytacon tubs containing 100 mL of solid seed germination medium (20 seeds per tub). Seeds were grown at 25 °C with a 16 hour light/8 hour dark photoperiod for 9 to 14 days until seedlings were large enough for explant excision. Seedlings were removed from the germination media and placed on Petri dishes containing sterile water. Cotyledons were cut transversely to give approximately 1 cm² explants. The base and tip of each cotyledon was cut off and the remainder of the cotyledon was then cut into two pieces. Cotyledons were cut under water with a rolling action using sterile scalpel blades.
5.2.1.2 *In vitro* inoculation of tomato explants with *Agrobacterium rhizogenes*

*Agrobacterium rhizogenes* strain A4 and ARqual were employed to generate hairy roots from the tomato explants. *A. rhizogenes* A4 isolates carrying the binary vectors were grown in MGML medium containing rifampicin (50 μg/mL) and kanamycin (50 μg/mL), whereas *A. rhizogenes* ARqual isolates carrying the binary vectors were grown in LB broth supplemented with spectinomycin or streptomycin (100 μg/mL) and kanamycin (50 μg/mL). The cultures were incubated at 27 °C with constant shaking at 200 rpm until the optical density of the cells at 600 nm reached between 0.6-0.8. Cells were pelleted by centrifugation for 10 min at 5,000 g. The pellets were then resuspended in Min A medium (Appendix G). The excised cotyledons were submerged in hormone-free Murashige and Skoog (MS) medium containing 100 μM acetosyringone for 5 min. After immersion, the excised cotyledons were submerged in *A. rhizogenes* cell suspension for 10 min to allow attachment of cells onto the explants. Explants were then blotted off on sterile filter papers to remove excess bacterial suspension before being placed adaxial side downward on antibiotic-free feeder plates. Explants were incubated on feeder plates for 3 days at 25 °C with a 16 hours light/8 hours dark photoperiod to allow bacterial infection and explant transformation to occur. After three days, the explants were transferred, abaxial side downward, to regeneration media containing 500 μg/ml augmentin and 50 μg/mL kanamycin. Explants were periodically transferred onto new regeneration media every 10 days, or whenever there were explants overgrown by *A. rhizogenes* to prevent spreading of the bacteria to other explants.

5.2.2 Generation of tomato hairy roots *ex vitro*

5.2.2.1 Tomato seedlings preparation and *ex vitro* experimental setup

In these experiments, the bacteria were injected into the stems of whole seedlings. Hairy roots were generated at the infection sites, thus producing
composite plants consisting of transgenic roots and wild-type shoots and roots. An initial experiment was done using two-weeks-old tomato seedlings grown in UC mix soil. The bacteria were inoculated onto the hypocotyl of the young seedlings. The inoculated seedlings were put in a ‘humid chamber’ with a 15 cm high transparent lid and manual vent in a glasshouse (Figure 5.1). However, hairy roots were not generated from these seedlings because the wounding on the stems caused them to collapse and most of the seedlings to die. The seedlings were too young, and the stems were too small for injection. In the second experiment, older seedlings were used. Seedlings of tomato cultivar M82 were grown in UC mix soil fertilised with Osmocote® for two weeks in a glasshouse. After two weeks, the seedlings were transferred to bigger pots and grown for a further three weeks until the stems were big enough for injection with needles. Prior to inoculation with \textit{A. rhizogenes}, the seedlings were placed inside a wooden-framed plastic growth chamber to maintain humidity (day temperature: 25 °C; night temperature: 20 °C; relative humidity at day: 90%; relative humidity at night: 70%; CO₂ level: ambient; light intensity: 210 \mu E\cdot m^{-2}\cdot s^{-1}; 16 hour light/8 hour dark photoperiod). However, hairy roots formed poorly under these conditions. Therefore, a different set of experiments was performed. For the third experiment, the seedlings were placed inside a metal-framed plastic hood in a glasshouse (Figure 5.2) and the pots containing the seedlings were put on trays permanently filled with water. Air circulation in the glasshouse was not as strong as in the growth chamber. This enabled higher humidity to be maintained in the chamber because evaporated water could be trapped and the resulting water condensation was an indicator that the humidity was indeed 100%.

5.2.2.2 \textit{Ex vitro} inoculation of tomato explants with \textit{Agrobacterium rhizogenes}

Cultures of \textit{Agrobacterium rhizogenes} strains A4 and ARqua1 were prepared as described in Chapter 2, section 2.1.3. For inoculation, the bacteria were prepared as liquid cultures in 100 mL of MGML (A4) or LB broth (ARqua1) and as bacterial lawns on MGML or LB plates. For liquid cultures, a single colony
Figure 5.1: Inoculation of two-week old tomato seedlings with *Agrobacterium rhizogenes* strain A4 and *A. rhizogenes* strain ARqua1. (A) Tomato seedlings were grown on UC mix soil for two weeks in a glasshouse prior inoculation with *A. rhizogenes*. (B) The bacteria were injected onto the hypocotyls of the seedlings using 1 mL syringes with needles. (C) The inoculated seedlings were then put in a manually ventilated, humidity chamber in a glasshouse. Hairy roots were not produced in this experiment.
Figure 5.2: Experimental set up for *ex vitro* composite plants in glasshouse and growth chamber. *A. rhizogenes*-inoculated seedlings were put inside plastic hoods to maintain high humidity. (A-B) humidity chamber in glasshouse; (C) humidity chamber in growth chamber.
was inoculated into 3 mL liquid medium containing appropriate antibiotics and
grown at 27 °C for 3 days. One mL of each culture was used to inoculate 100 mL
of MGML or LB supplemented with antibiotics, and the cultures were grown at
27 °C for 3 days with constant shaking at 200 rpm on a shaking incubator. For
cultures on plates, one loop of bacterial cells from liquid cultures was streaked
onto solid media containing the appropriate antibiotics and the inoculated plates
were incubated for 3 days at 27 °C. The presence of the binary vectors pCBJ400,
pCBJ401, pCBJ402, and pCBJ403 in A. rhizogenes ARqua1 was checked am-
plifying the ocs promoter/rfp/ocs terminator construct from colonies that will
be used for inoculation onto tomato seedlings. Whereas, the presence of these
binary vectors in A. rhizogenes A4 was checked by restriction digestion analy-
sis to release the ocs promoter/rfp/ocs terminator construct and the chimeric
endochitinase/β-1,3-glucanase construct (SOE2) from pCBJ403.

For inoculation of whole seedlings, the stems above the cotyledons were
inoculated with bacteria from liquid cultures by injecting the bacteria using a 1
mL syringes with a needle (Figure 5.3A). The bacteria were injected carefully
several times at different spots on the stem. Bacterial lawns were used to
inoculate seedlings with roots removed (apical stem sections) by swiping the
cut surface of the stems across the bacterial lawn so that the cut surface was
completely coated with bacteria (Figure 5.3B). The roots were removed by
cutting the stems diagonally to increase the surface area available for coating
with bacteria. After coating with bacteria, the seedlings were replanted into
soil and watered immediately. As controls for all experiments, A. rhizogenes A4
and ARQua1 that did not carry binary vectors were used to inoculate seedlings.
Water was also used to mock-inoculate seedlings. Four seedlings were used for
each treatment. After inoculation, the seedlings were covered with the plastic
hoods and incubated until hairy roots formed.

5.2.3 Screening of transgenic tomato hairy roots

The expression of rfp in the hairy roots was observed using both a UV
Figure 5.3: Generation of *ex vitro* composite tomato plants. Stems of tomato plants were inoculated with *Agrobacterium rhizogenes* A4 and *A. rhizogenes* ARqua1. (A) The bacteria were injected onto the stems above the cotyledons using 1 mL syringes with needles. (B) In other experiments, apical stem sections were inoculated by swiping the cut basal ends of seedling stems over the bacterial lawn so that the cut area was coated with the bacteria.

Illuminator for preliminary observation and a stereo dissecting microscope for more detailed observation. Microscopic observation was performed using a Leica MZ FLIII fluorescence stereomicroscope (Leica Microsystems Ltd., Switzerland) using a Cy3\textsuperscript{TM} filter (555/50 nm excitation and 610/75 nm barrier). A Leica DC200 camera was used to capture fluorescence images and Leica IM50 software (version 1.20) was used to record the images.

5.2.4 Tomato stable transformation

Although transgenic hairy roots would be an ideal system to examine the efficacy of the chimeric endochitinase/β-1,3-glucanase fusion gene to confer resistance to Fusarium wilt in tomato, generation of stable transgenic tomato plants could serve as a more suitable tool. Therefore, an attempt was made to produce whole transformed tomato plants expressing the chimeric gene.

5.2.4.1 Tomato explant preparation

Tomato cultivar Moneymaker Cf0 Tm-2\textsuperscript{2} was used for stable tomato trans-
This tomato cultivar lacks any resistance towards known Fol races, but is known to be resistant to tobacco mosaic virus. Tomato explants used for stable transformation were prepared as described in section 5.2.1.1. The cotyledon pieces were blotted on sterile filter paper and put on feeder plates adaxial side down. The plates were incubated at 25 °C under low light intensity for at least eight hours.

5.2.4.2 Inoculation and co-cultivation of tomato explants with *Agrobacterium tumefaciens* GV3101

*Agrobacterium tumefaciens* GV3101 carrying the binary vectors was grown overnight in 5 mL YEP containing rifampicin (50 μg/mL), gentamycin (50 μg/mL), and kanamycin (50 μg/mL). Two mL of the overnight culture was inoculated into 50 mL YEP containing appropriate antibiotics and the culture was grown at 27 °C for 4-6 hours with constant shaking at 200 rpm until an OD₆₀₀ of 0.5-1.0 was reached. The cells were then centrifuged at 8,000 g for 10 min at 4 °C. The pellets were resuspended with MS medium containing 3% (w/v) sucrose to an OD₆₀₀ of 0.4-0.5.

For co-cultivation, tomato explants from feeder plates were removed and immersed in *A. tumefaciens* GV3101 cell suspension in a Petri dish. The explants were then blotted off on sterile filter papers and returned to the feeder plates abaxial side downwards. A fresh bacterial suspension was used for each plate. The plates were incubated at 25 °C under low light intensity for 40 hours.

5.2.4.3 Regeneration of tomato stable transformants

After co-cultivation on feeder plates, the explants were transferred to regeneration plates to generate shoots. The explants were positioned abaxial side downwards on the media. The regeneration plates were supplemented with 500 μg/mL augmentin to kill the bacteria and 100 μg/mL kanamycin to select transformed shoots. The plates were incubated at 25 °C with a 16 hour light/8
hour dark photoperiod. The explants were transferred to fresh media every 2-3 weeks or whenever any explants were overgrown by bacteria.

5.3 Results

5.3.1 Generation of tomato hairy roots \textit{in vitro}

\textit{Agrobacterium rhizogenes} strains A4 and ARqua1 were employed to generate hairy roots in tomato explants \textit{in vitro}. Both strains were able to generate hairy roots but they showed different levels of rhizogenicity and incited hairy roots with distinct morphology (Figure 5.4). \textit{A. rhizogenes} A4 incited more numerous hairy roots than ARqua1. Hairy roots incited by ARqua1 were scarce and did not differentiate completely into roots as the colour of most hairy roots produced was greenish and less hairy compared to the hairy roots incited by A4. Moreover, brownish pigmentation was observed in media containing explants infected by ARqua1. Hairy roots emerged on explants inoculated with A4 at the third day post-inoculation, whereas hairy roots in explants inoculated with ARqua1 emerged at the fourth day post-inoculation.

Of 120 explants for each strain, 95% of the explants inoculated with A4 generated hairy roots, whereas only 15% of the seedlings inoculated with ARqua1 generated hairy roots. On average, each explant infected by A4 produced four hairy roots, whereas most explants infected by ARqua1 failed to generate hairy roots, with an average of two hairy roots per explant on those that did produce hairy roots. Therefore, \textit{in vitro}, A4 incited hairy roots more efficiently in tomato than ARqua1 under the conditions tested.
Figure 5.4: Production of tomato hairy roots *in vitro*. Cotyledons of two-week old tomato seedlings were inoculated with *Agrobacterium rhizogenes* strains A4 and ARqua1 carrying binary vectors pCBJ400, pCBJ401, pCBJ402, pCBJ403 as well as strains that did not carry binary vector. A4 incited more pronounced hairy root formation, whereas ARqua1 incited fewer and less differentiated hairy roots. Hairy roots appeared at the third day post-inoculation in explants that were inoculated with A4, whereas in explants that were inoculated with ARqua1 hairy roots appeared at the fourth day post-inoculation.
Before proceeding with the *ex vitro* hairy root transformation, the presence of the binary vectors in *A. rhizogenes* A4 and *A. rhizogenes* ARqual was checked by PCR and restriction digestion analysis. The presence of the binary vectors in A4 could not be confirmed by colony PCR because it failed to amplify the *rfp* construct directly from cells of A4. This is probably because of high amount of polysaccharides produced by A4 when it is grown on MGML medium that contains high amount of mannitol. This could cause cell lysis difficult during the PCR or the polysaccharides interfered with the PCR. Nonetheless, the fragment was detected by restriction digestion analysis from binary vectors isolated from PCR-negative colonies (Figure 5.5). The *rfp* construct fragment was detected from colonies of ARqual (Figure 5.6).

The first attempt to produce hairy roots *ex vitro* in a growth chamber was unsuccessful. Hairy roots were produced but poorly and only in seedlings infected by A4, not ARqual. The hairy roots were stunted and did not develop well (Figure 5.7A). The number of hairy roots generated was low with only around 4 hairy roots per plant on average. Overall, the system was not suitable to generate hairy roots *ex vitro*.

The inefficiency of hairy root generation in the growth chamber was likely due to insufficient levels of humidity despite keeping the inoculated seedlings under a plastic hood to try to maintain high humidity. Therefore, the second experiment was conducted in a glasshouse as described in section 5.2.2.1. *Agrobacterium rhizogenes* strains A4 and ARqual were both able to incite hairy roots in stems of tomato seedlings grown in a glasshouse (Figure 5.7). These hairy roots were incited by Ri plasmids, and not as a general response to wounding since mock-inoculation with water did not produce any roots. As observed in the *in vitro* experiment, A4 and ARqual incited hairy roots *ex vitro* with different efficiency and the hairy roots produced exhibited distinct morphology. All seedlings inoculated with A4 generated hairy roots, whereas only 50% of seedlings inoculated with ARqual generated hairy roots. However, about 80% of these produced poor hairy roots with imperfect differentiation. The hairy roots were scarce, slender,
Figure 5.5: Restriction digestion analysis of *rfp* construct and the chimeric endochitinase/β-1,3-glucanase gene (SOE2) in binary vectors used for hairy root transformation isolated from *Agrobacterium rhizogenes* A4 prior inoculation onto tomato seedlings. The *ocs* promoter/*rfp*/*ocs* terminator fusion was digested with Apal and EcoRI from binary vectors pCBJ400, pCBJ401, pCBJ402, and pCBJ403. SOE2 was digested only from pCBJ403. 400: pCBJ400, 401: pCBJ401, 402: pCBJ402, 403: pCBJ403.

Figure 5.6: PCR amplification of *ocs* promoter/*rfp*/*ocs* terminator fusion construct from colonies of *Agrobacterium rhizogenes* ARqua1 carrying binary vectors pCBJ400, pCBJ401, pCBJ402, and pCBJ403 prior inoculation onto tomato seedlings. 400: pCBJ400, 401: pCBJ401, 402: pCBJ402, 403: pCBJ403. p400: PCR amplification of fragment targeted for detection directly from pCBJ400 plasmid DNA.
greenish, and less hairy. Hairy roots appeared 10 days post-inoculation in stems inoculated either with A4 or ARqual. Roots were also induced in uninfected regions of the stems usually further up towards the apical zone. These were found only in seedlings inoculated with A4.

Roots were also generated from the cut stems (Figure 5.8) but the roots were likely to be a mixture of wild-type roots and hairy roots incited by the bacteria because the mock-inoculated stems also produced roots, although to a lesser extent. The morphology of the roots in the cut stems inoculated with either A4 or ARqual and mock inoculated stems was similar. Because the origin of the roots was not determined, it was not possible to distinguish roots induced by Ri plasmids from those arising by normal tissue differentiation.

5.3.3 Screening of transgenic tomato hairy roots

Hairy roots generated from tomato explants in vitro and from stems of seedlings ex vitro incited by A. rhizogenes A4 and A. rhizogenes ARqual carrying binary vectors pCBJ400, pCBJ401, pCBJ402, and pCBJ403 as well as A. rhizogenes that did not carry binary vector were observed using a stereo dissecting microscope to detect RFP fluorescence. Before being observed using a microscope, preliminary observation was performed by direct observation of the hairy roots by eye or by UV illumination. Direct observation by eye (RFP is visible under ambient light conditions) or by UV illumination failed to reveal any red fluorescence from the hairy roots. Microscopic observation was then performed to enable more sensitive screening for rfp expression.

The use of a Cyan filter to observe RFP fluorescence resulted in an intense red coloured background after long exposure (Figure 5.9). The red coloured background was observed in all hairy root samples. The figure only shows hairy roots incited by A. rhizogenes A4 and A. rhizogenes ARqual carrying binary vector pCBJ403 as well as A. rhizogenes A4 that did not carry binary vector. The Cy3™ filter was not the proper filter for RFP, but it can be used to detect RFP fluorescence since it filters a range of wavelength (555/50 nm excitation and
Figure 5.7: *Ex vitro* composite tomato plant production. Hairy roots were generated by inoculating the stems of tomato seedlings with *Agrobacterium rhizogenes* A4 and *A. rhizogenes* ARqual. Yellow arrows indicate the inoculation sites and hairy roots produced at the sites. (A) Stunted, poorly developed hairy roots produced in seedlings inoculated with *A. rhizogenes* A4 incubated in a growth chamber. (B-C) Hairy root production in seedlings infected by *A. rhizogenes* A4 incubated in a glasshouse. (D-E) Hairy root production in seedlings infected by *A. rhizogenes* ARqual incubated in a glasshouse. (F) Hairy roots produced in uninfected regions of stems, usually towards the apical zone. The stems were inoculated with *A. rhizogenes* A4.
Figure 5.8: Hairy roots produced in rootless seedlings. *Agrobacterium rhizogenes* strains A4 and ARqual were inoculated by swiping excised apical stem sections of seedlings over a bacterial lawn so that the cut stems were coated with bacteria. All treatments including mock inoculation produced roots with similar morphology, except fewer roots were produced in mock inoculated seedlings than those inoculated with bacteria. It is likely that the hairy roots produced on apical stem sections inoculated with *A. rhizogenes* were a mixture of wild type roots and hairy roots.

610/75 nm barrier) overlapping with the wavelength required for RFP detection (546/12 nm excitation and 560 long-pass). The red colour signals observed in hairy roots incited by A4 and ARqua1 carrying the *rfp* gene were also observed in hairy roots incited by A4 that did not carry the *rfp* gene (did not carry any binary vectors). Therefore, the red colouration was not RFP fluorescence. The roots were observed under long exposure because shorter exposure times did not give clear images.

5.3.4 Tomato stable transformation

In order to produce transgenic tomato plants, *Agrobacterium*-mediated stable tomato transformation was performed. However, the experiment failed to generate any transformants. A number of shoots was generated, but either the shoots died or were later overgrown by *Agrobacterium* (Figure 5.10). Most of the explants were overgrown by *Agrobacterium*, indicating that the concentration of the antibiotic, augmentin, was not sufficient to kill or suppress the growth of the bacteria. All bacteria-free explants showed chlorophyll loss accompanied by expanding necrotic areas. Some of the dying explants were still able to produce shoots but the shoots did not grow, turned chlorotic, and eventually died. The frequency of callus-forming explants was very low; approximately 3% of the total number of explants formed calli, and about half of these formed shoots. Shoots
Figure 5.9: Observation of RFP fluorescence in tomato hairy roots. Hairy roots incited by *Agrobacterium rhizogenes* A4 and ARqua1 carrying binary vector pCBJ403, and *A. rhizogenes* that did not carry binary vector, were screened for RFP fluorescence using a stereo dissecting microscope with a Cy3\textsuperscript{TM} filter. Long exposures using the Cy3\textsuperscript{TM} filter resulted in intense red background, which was indistinguishable from the control without RFP. RFP fluorescence was not observed in any of the hairy root samples including hairy roots incited by *A. rhizogenes* carrying binary vectors pCBJ400, pCBJ401, and pCBJ402 (data not shown).
were generated three months after inoculation.

5.4 Discussion

5.4.1 Generation of tomato hairy roots *in vitro*

To complete this study, a bioassay is needed to test whether the chimeric endochitinase/β-1,3-glucanase gene can confer resistance against *Fol* in tomato. The bioassay should be consistent with the lifestyle and pathogenicity of *Fol*, which colonises and infects the roots of tomato plants to gain access to the vascular bundles where it causes severe wilting by obstructing the flow of water through xylem vessels. Therefore, the roots are the first place where *Fol* starts to compromise host defences. Resistance to *Fol* could be achieved by engineering an inducible and localised defence response in the roots. Therefore we chose to express the fusion construct in tomato root tissue using *A. rhizogenes*-mediated hairy root transformation.

In order to obtain transgenic hairy roots carrying the chimeric endochitinase/β-1,3-glucanase gene, the first experiment was to infect tomato
explants (sliced cotyledons) with *A. rhizogenes* strains A4 and ARqua1 *in vitro*. Hairy roots were successfully generated from the tomato explants. *A. rhizogenes* A4 was more efficient in generating hairy roots than ARqua1. Moreover, ARqua1 incited hairy roots with aberrant morphology. The differences were probably caused by the genetic background of ARqua1. Although ARqua1 has acquired its Ri plasmid from A4, the C58 chromosomal background may affect its rhizogenicity.

About 95% of the explants infected by *A. rhizogenes* A4 produced hairy roots with each explant having around 4 hairy roots on average. In contrast, less than 15% of explants infected by *A. rhizogenes* ARqua1 produced hairy roots, and the number of hairy roots on each explant was lower than the number of hairy roots incited by A4. *A. rhizogenes* ARqua1 is known to be less virulent than most other wild-type or modified *A. rhizogenes* strains [126]. This strain is often used to transform legumes for the study of root nodulation. *A. rhizogenes* ARqua1 is a Sm<sup>r</sup>-derivative of strain R1000 that carries pRiA4b, a derivative of the large Ri plasmid pRiA4c (also called pArA4c) from *A. rhizogenes* A4. The reduced virulence of ARqua1 might be due to the different genetic background of R1000 compared to A4 but could also be due to the absence of components that are present in pRiA4c but not pRiA4b. pRiA4c can dissociate into two smaller plasmids pRiA4a and pRiA4b [177]. pRiA4a confers sensitivity to agrocin and the ability to catabolise opines [123], whereas pRiA4b functions in virulence, opine production, and the ability to catabolise agropine [176].

In a study of root nodulation in *Vicia hirsuta*, although ARqua1 and other *Agrobacterium* strains carrying pRiA4b-type plasmids incited hairy roots in over 90% of the plants, the number of hairy roots on each plant was much lower than those incited by *Agrobacterium* carrying the pRi15834-type plasmid, which is homologous to pRiA4c, although the plasmids were all transferred to the C58 background [126]. The phenotype of the hairy roots induced by the pRi15834-type plasmid was more pronounced, whereas the hairy roots from pRiA4b-type formed in numbers proportional to the wild type roots and showed normal morphology. Therefore, ARqua1 was suitable for the study of
root nodulation in legumes since it was able to generate hairy roots that were phenotypically more similar to wild type roots. However, this strain was found unsuitable for our study because its performance in tomato was poor and it induced hairy roots with aberrant morphology.

5.4.2 Generation of tomato hairy roots *ex vitro*

The generation of composite tomato plants was conducted in a glasshouse and growth chamber under high humidity. Hairy root generation in the growth chamber was not successful even though the ambient humidity was set high. The level of humidity in the growth chamber was probably not sufficiently high to allow efficient induction hairy roots. Although the humidity was set to a high level, air circulation was strong, thus preventing humidity rising sufficiently to allow water condensation indicative of 100% humidity. On the other hand, water condensation was evident on the plastic hood used to cover the plants in the glasshouse. Thus, it seems likely that high humidity is a prerequisite for the induction of hairy roots on parts of the plant exposed to the air. A study by Collinge (2002) [27] also shows that high humidity is required for *ex vitro* hairy root formation in tomato. High humidity might be required by the bacteria for their growth to a certain level where they reach a quorum that enables them to transfer the T-DNA to the genome of the tomato plants.

Similar to the hairy roots generated *in vitro*, *A. rhizogenes* A4 incited hairy roots that were more pronounced than those incited by ARqual in the *ex vitro* transformation. The morphology of the hairy roots between the strains was also different. The inefficiency of *A. rhizogenes* ARqual to incite hairy roots and the defective morphology of the hairy roots may be attributed to the lack of a complete set of genes required for normal hairy root formation compared to *A. rhizogenes* A4. pRiA4a, the missing element in ARqual that is present in A4 is probably necessary for the complete development of hairy roots.

Inoculation sites also influence the ability of *A. rhizogenes* to incite hairy roots. Boisson-Dernier *et al.* (2001) [14] showed that in *Medicago truncatula*, the
majority of hairy roots produced from stab inoculation of the plantlet hypocotyl or epicotyl with *A. rhizogenes* grew poorly. Improved growth of hairy roots was obtained when *A. rhizogenes* was inoculated onto *M. truncatula* seedling radicles that had been freshly sectioned to remove the tip. The influence of the site of inoculation on hairy root formation could be attributed to the spatial gradient of hormonal composition at different locations in the plants.

Root formation was occasionally found in uninfected regions of the stem, and most of these areas of root formation were located above infection sites. This was observed only in plants inoculated with A4. Some bacterial cells might be carried upward by water and nutrient flux through the vascular bundles and may have incited hairy roots at the locations where they were carried upward.

### 5.4.3 Screening of transgenic tomato hairy roots

Hairy roots generated *in vitro* and *ex vitro* were screened for the presence of T-DNA carrying the endochitinase/β-1,3-glucanase chimeric transgene by looking for the expression of the *rfp* reporter gene carried on the same T-DNA using a stereo dissecting fluorescence microscope. The absence of RFP fluorescence in the hairy roots does not necessarily imply that the *rfp* was not expressed because the use of a Cy3™ filter may not have been optimal for detection of RFP fluorescence. The *rfp* gene may have been expressed, but the level of expression may have been insufficient to allow detection of a fluorescence signal. To examine *rfp* expression, RT-PCR could have been performed but time did not permit.

### 5.4.4 Tomato stable transformation

The attempt to generate stable transgenic tomato plants using *A. tumefaciens*-mediated transformation failed to produce any transformants. One factor that likely contributed to the failure was the inefficiency of the antibiotic
augmentin to suppress the growth of *Agrobacterium* after co-cultivation. Most of the explants were overgrown by bacteria, and thus could not be rescued. A concentration of augmentin of 500 μg/mL should have been sufficient to kill the bacteria given that this concentration of antibiotic has been used in a number of published tomato transformation methods. It should be noted however, that the antibiotic augmentin used was out of date, therefore it could have deteriorated. Increasing the concentration of augmentin or using another antibiotic such as cefotaxime could help to improve the suppression of *Agrobacterium* overgrowth.

Shoots that were generated from some explants were presumably not transformed because they could not survive kanamycin selection and eventually died. Factors such as plant variety [44], explant material [49], growth regulators [124], bacterial concentration [144], and *Agrobacterium* virulence gene inducers [152] influence the transformation efficiency. A previous attempt to transform tomato cultivar M82, the cultivar that was used for hairy root transformation in this study, was not successful (Lim, 2009, unpublished data). This tomato cultivar might be recalcitrant to stable tomato transformation, so in this study the tomato cultivar Moneymaker CfO Tm-2^2^ was used as an alternative to M82. Despite the ineffectiveness of augmentin to control *Agrobacterium* growth, the lack of kanamycin resistant shoots might imply that this cultivar was also recalcitrant although others have reported the production of transgenic plants using this line.

Growth regulators used for plant tissue culture are diverse, but generally the auxin IAA and the cytokinin zeatin riboside are the growth regulators of choice for tomato transformation because they have been proven to improve transformation frequency. Park *et al.* (2003) [121] reported that increasing the concentration of zeatin riboside also increased the frequency of transformation. Irrespective of the genotype tested, tomato transformation frequencies greater than 20% were achieved when 2 mg/ℓ of zeatin was used. The concentration of zeatin riboside used in this study was 0.5 mg/ℓ, much lower than that used by Park *et al.* (2003) [121]. The use of phenolic compounds, such as acetosyringone, also improves the transformation efficiency. Acetosyringone replaces the use of feeder cells to providing phenolic molecules because it improves transformation efficiency greater
than feeder cells [28]. However, in this study, the use of acetosyringone did not appear to help to transform tomato with the chimeric endochitinase/β-1,3-glucanase gene. Presumably, other factors were involved, and those factors contributed to the difficulty of transforming tomato with the chimeric endochitinase/β-1,3-glucanase gene in this study.

In summary, hairy roots of tomato were generated in vitro and ex vitro using A. rhizogenes A4. The generation of ex vitro composite plants required high levels of humidity. The integration of the chimeric endochitinase/β-1,3-glucanase gene into the genome of cells of root tissue could not be determined because the expression of the reporter gene (rfp) was not observed in the hairy roots. However, this result was not confirmed by expression analysis e.g. by RT-PCR, therefore it is not possible to conclude whether the roots generated were transgenic or not. An attempt to generate stable tomato transformants carrying the chimeric gene failed because of the ineffectiveness of bacterial growth suppression and of the transformation itself.
Chapter 6

General discussion

6.1 Transformation of *Fusarium oxysporum* f. sp. *lycopersici* with green fluorescent protein reporter genes

Research on *Fol*-tomato interactions has been aided by the use of GFP to monitor the development of *Fol* inside infected plants. The aim of transforming *Fol* race 3 with *gfp* in this study was to examine the resistance of tomato carrying the chimeric endochitinase/β-1,3-glucanase gene against Fusarium wilt by monitoring the growth of GFP-tagged *Fol* in planta. Transformation of *Fol* race 3 with *gfp* was achieved by employing *A. tumefaciens* in a procedure that was found efficient and relatively simple. In this study, *Fol* race 3 was successfully transformed with *gfp* driven by the constitutive *gpd* and inducible *SIX1* promoters.

Transformation of *Fol* race 3 with *gfp* driven by the *gpd* promoter was achieved with high frequency and transformants showed good *gfp* expression whilst maintaining their pattern of avirulence and virulence on resistant and susceptible tomato plants. On the other hand, transformation of *Fol* with *gfp* under the control of the *SIX1* promoter resulted in fewer confirmed transformants as indicated by a general lack of *Fol* transformants able to express *gfp* inside the roots. This may be attributed to a number of possible causes, including the rareness of *Fol* infection of roots under the experimental conditions used, inefficient expression of *gfp*, or that most of the *Fol* isolates tested were not true transformants although they were apparently resistant to the antibiotic Zeocin™. One way to confirm
whether they were true transformants or not would have been to perform PCR or a Southern blot.

Pathogenicity tests revealed that the avirulence/virulence phenotypes of *Fol* race 3 *gpd* promoter-*gfp* transformants were not altered on resistant and susceptible tomato lines. In contrast, *Fol* race 3 *SIX1* promoter-*gfp* transformants caused some wilting symptoms on resistant tomato plants, but the symptoms were milder than those observed on susceptible tomato inoculated with either the *SIX1* promoter-*gfp* transformants or the wild type progenitor. It is unlikely that the *SIX1* promoter-*gfp* construct was integrated at the *SIX1* locus because this would be expected to reduce not increase virulence. It is more likely that the *SIX1* promoter-*gfp* construct integrated randomly into the *Fol* genome and this could create positional effects, in which the construct was integrated at positions incompatible with efficient expression of *gfp* driven by the *SIX1* promoter. The promoter sequence in the construct lacks 300 bp of the *SIX1* promoter region that could contain necessary elements for efficient *SIX1* expression. Homologous recombination of the construct at the *SIX1* locus restores the complete sequence of the promoter and therefore gives good *gfp* expression as observed by van der Does et al. (2008) [164]. This could explain the poor *gfp* expression in this study. To test this hypothesis, the *SIX1* promoter-*gfp* construct could be re-engineered to include the missing 300 bp of *SIX1* promoter sequence and then test the new construct for *gfp* expression.

6.2 Construction of a chimeric endochitinase and \(\beta-1,3\)-glucanase fusion gene and red fluorescent protein reporter gene

In this report, construction of an endochitinase/\(\beta-1,3\)-glucanase fusion gene is described. To the best of my knowledge, this is the first report that describes the construction of a transcriptional and translational fusion of endochitinase and \(\beta-1,3\)-glucanase genes. The PCR-driven overlap extension method was used to construct the chimeric endochitinase/\(\beta-1,3\)-glucanase gene and the *rfp* construct.
Several steps were found to be critical for successful construction of chimeric genes using this method. Primers with a balance between sufficient length of overlapping sequences and minimal probability of secondary structure formation are essential for the creation of fusion genes. Theoretically a separate overlap extension step can be omitted, but in this study it proved to be necessary for creation of the fusion genes. Finally, a sufficiently large amount of sample from the overlap extension step was required as a template for the third round PCR because of the low probability of overlap hybridisation compared to self hybridisation. The method presented herein is a modification of several existing methods, and this study showed it to be capable of creating a fusion product of 3.5 kb. The chimeric endochitinase/β-1,3-glucanase gene and the rfp construct were each fused in two steps. To improve efficiency, these genes could have been fused together in one step because, in theory, PCR-driven overlap extension allows fusion of two or more genes at once. This could reduce the time required to create fusion genes using this method.

Unfortunately, transient expression of the chimeric gene was not detected in *N. benthamiana* and expression of the rfp construct was barely detected. This does not necessarily imply a lack of expression because a suitable positive control for the reverse-transcriptase PCR was not included. These experiments need to be repeated with a complete set of controls and performed at different time points after infiltration with *Agrobacterium*. Western blot analysis would also be useful to detect protein levels, and to check for the stability of the fusion protein. Stability of the fusion protein is a prerequisite for the successful use of this approach. The chimeric gene could also be expressed in a heterologous protein expression system such as *E. coli* and the protein could be purified and checked for its chitinase and/or glucanase activities as well as its antifungal activity *in vitro*.

### 6.3 Tomato hairy root transformation

Hairy root transformation can be a useful alternative to whole plant trans-
formation especially for the study of root biology. Transgenic plants can be generated from hairy roots but the resulting transgenic plants often show abnormal morphology and/or physiology. For many applications, hairy root cultures or composite plants are used because these can be generated within weeks. Hairy roots can be used to study plant resistance to root pathogens. For example, composite tomato plants have been used to study plant resistance to nematodes [26]. Potentially, hairy roots can also be used to study resistance in tomato to Fusarium wilt.

Resistance to Fusarium wilt could be evaluated by examining the growth of Fol in hairy roots expressing a specific transgene of interest compared to hairy roots lacking the transgene. We have generated Fol transformed with gfp to monitor the infection of hairy roots transformed with the chimeric endochitinase/β-1,3-glucanase gene. Hairy roots were generated from tomato explants and from inoculated stems of whole tomato plants. However, the lack of RFP fluorescence in the hairy roots suggests that they were not transformed with the chimeric gene or that they were transformed but the rfp gene did not function properly as a reporter. Despite the use of a non optimal filter set to detect RFP fluorescence, the system used should still have been able to detect RFP fluorescence. The lack of RFP fluorescence was probably due to weak expression of the rfp gene. Although expression of the transgenes was not examined by PCR, the rfp reporter gene construct probably needs to be improved to achieve a reliable reporter system for transformation. For example, a strong promoter like the 35S promoter could be used to replace the ocs promoter to enhance rfp expression.

In some plant species, including tomato, the frequency of hairy roots co-transformed by a binary vector T-DNA is high [149]. One study of co-transformation with a binary vector containing gfp showed that more than 60% of hairy roots generated from tomato cotyledon explants showed GFP fluorescence [27]. In this study, the lack of RFP fluorescence in the roots was probably not due to low transformation frequency by the T-DNA carrying the rfp reporter but rather a problem with the rfp construct itself.

In this study, Agrobacterium rhizogenes strains A4 and ARqual were used to
produce hairy roots both in vitro and ex vitro. A. rhizogenes A4 was more efficient in inciting hairy roots than ARqual. Despite its low virulence, A. rhizogenes ARqual is often used to generate hairy roots in legumes, but our results show that A. rhizogenes ARqual was not suitable for hairy root transformation in tomato because it was a poor inducer of hairy roots and it generated hairy roots with imperfect differentiation. Ex vitro experiments showed that high humidity is required for hairy roots to form efficiently. A previous study has also shown a high humidity requirement for efficient hairy root formation in tomato [27].

The attempt to transform tomato with the chimeric gene failed to generate any transformed tomato plants. The use of A. tumefaciens strain GV3101, which is used routinely for agroinfiltration experiments, might not have been efficient to transform the tomato line that we used with the chimeric gene. The use of another A. tumefaciens strain such as LBA4404, which has been used routinely for stable transformation of tomato, may have helped to improve transformation efficiency. Moreover, the composition of plant culture media, especially the types and concentration of growth regulators, and technical aspects of the transformation procedure could perhaps have been varied to improve transformation efficiency.

6.4 Conclusion

In this study, an attempt was made to engineer tomato plants with transgenic endochitinase and β-1,3-glucanase genes from T. atroviride to confer resistance against Fusarium wilt caused by Fol. To accomplish this objective, the endochitinase and β-1,3-glucanase genes were constructed in such a way that they were fused transcriptionally and translationally. A combination of chitinase and glucanase has been reported to increase the levels of resistance against several pathogenic fungi compared to the use of either chitinase or glucanase alone [72, 75, 109, 5]. A transcriptional and translational fusion would ensure a coordinated and simultaneous expression of the transgenes in planta. Two constructs were created, one driven by the strong constitutive 35S promoter and the other
driven by the \textit{I-2} promoter expected to target expression to the region of \textit{Fol} attack, but their expression could not be detected in \textit{N. benthamiana} transient assays. However, given that the expression analysis did not include a positive control, it could not be concluded whether the chimeric gene was expressed or not.

Following the construction of the chimeric endochitinase/\(\beta\)-1,3-glucanase fusion gene, a bioassay system to test the efficacy of the transgene is required. To this end, \textit{Fol} was transformed with \textit{gfp} to enable the efficient monitoring of its growth and development \textit{in planta}. Two lines of \textit{Fol gfp} transformants were obtained: one that carries \textit{gfp} under the control of the constitutive \textit{gpd} promoter and the other \textit{gfp} under the control of \textit{SIX1} promoter, which drives the expression of \textit{gfp} only when \textit{Fol} is growing inside infected tomato plants. As \textit{Fol} infects through the roots to gain access to the vascular tissue, hairy root transformation was performed to obtain transgenic roots carrying the chimeric gene. Hairy roots were generated using the wild-type \textit{A. rhizogenes} A4, but hairy roots carrying the chimeric gene could not be identified because their detection via RFP was problematic. These experiments should be repeated to include a positive control for RFP fluorescence. In addition, the \textit{rfp} construct also needs to be improved. For example, replacing the \textit{ocs} promoter with the 35S promoter would provide stronger expression of \textit{rfp}. Stable transformation was also attempted, but it failed to produce any transformed shoots.

Testing of the ability of the chimeric endochitinase/\(\beta\)-1,3-glucanase gene to confer resistance in tomato to Fusarium wilt could not be carried to completion because of the problems described above. Nonetheless, the experiments presented herein may be continued and improved to enable the testing of the chimeric gene to be completed, and the results obtained so far may be useful for other related applications and purposes.
Appendix A

Pathogenicity test of transformed *Fusarium oxysporum* f. sp. *lycopersici* with *gfp* reporter genes on tomato

| Sample | Disease severity | Plant height | Disease severity | Plant height | Disease severity | Plant height | Disease severity | Plant height | Disease severity | Plant height | Disease severity | Plant height | Mean
|--------|-----------------|--------------|-----------------|--------------|-----------------|--------------|-----------------|--------------|-----------------|--------------|-----------------|--------------|------
| 1      | 0               | 32           | 0               | 32           | 0               | 32           | 0               | 32           | 0               | 32           | 0               | 32           | 32  |
| 2      | 0               | 27           | 0               | 27           | 0               | 27           | 0               | 27           | 0               | 27           | 0               | 27           | 27  |
| 3      | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 28  |
| 4      | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 28  |
| 5      | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 28  |
| 6      | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 28  |
| 7      | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 28  |
| 8      | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 28  |
| 9      | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 28  |
| 10     | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 28  |
| 11     | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 28  |
| 12     | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 28  |
| 13     | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 28  |
| 14     | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 28  |
| 15     | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 0               | 28           | 28  |

Figure A.1: Pathogenicity test of transformed *Fusarium oxysporum* f. sp. *lycopersici* with *gfp* under the control of constitutive *gpd* promoter on susceptible tomato cultivar M82 and resistant tomato cultivar IL7-3. Plant height is in cm.
### Figure A.2:

Pathogenicity test of transformed *Fusarium oxysporum* f. sp. *lycopersici* with *gfp* under the control of inducible *SIX1* promoter on susceptible tomato cultivar M82 and resistant tomato cultivar IL7-3. Plant height is in cm.
Appendix B

Map of binary vectors

Figure B.1: pCBJ352

pCBJ352
6316 bp
Figure B.2: pCBJ362

pCBJ362
6187 bp
Figure B.3: pCBJ372

pCBJ372
9783 bp
Figure B.4: pCBJ382

pCBJ382
9654 bp
Figure B.5: pCBJ400

pCBJ400
7523 bp
pCBJ401
7449 bp

Figure B.6: pCBJ401
Figure B.7: pCBJ402
Figure B.8: pCBJ403
Appendix C

Media used for tomato transformation

1. Seed germination media (per liter)
   - 1× Murashige and Skoog (MS) media
   - 10 g glucose
   - 4 g gelrite
   - pH to 5.8 in 1 l with 1 M KOH

2. Feeder plates (per liter)
   - 1× MS media
   - 4 g gelrite
   - 0.5 mg (500 μL of 1 mg/mL stock) 2,4 dichlorophenoxyacetic acid (2,4-D)
   - pH to 6.0 in 1 l with 1 M KOH

3. Co-cultivation medium (per liter)
   - 1× MS media
   - 20 g sucrose
   - 4.25 g agargel
   - pH to 6 with 1 M KOH in 1 l
   - 1 mg 1-naphthalene acetic acid (NAA)
• 1 mg 6-benzylaminopurine (BAP)
• 200 mM acetosyringone

4. Regeneration plates (per liter)

• 1× MS media
• 100 mg myo-inositol
• 1× Nitsch vitamins
• 20 g sucrose
• 4.25 g agargel
• pH to 6 with 1 M KOH in 1 ℓ
• 0.5 mg 3-indolylacetic acid (IAA)
• 100 µL of 5 mg/mL zeatin riboside
• 500 µg/mL augmentin
• 100 µg/mL kanamycin

1. 1 mg/mL 2,4-dichlorophenoxyacetic acid (2,4 D)

• dissolve 100 mg in 1 mL 100% ethanol
• add 3 mL 1 N KOH
• adjust volume to 80 mL with water
• pH to 6.0 with 1 N HCl
• adjust volume to 100 mL
• (2,4 D can be autoclaved and stored at 4 °C)

2. 20 mg/mL zeatin riboside

• dissolve 2 mg zeatin riboside in 1 N NaOH
• filter sterilise, use 100 µL for 1 ℓ

3. 1000× Nitsch vitamins

• 50 mg thiamine
- 200 mg glycine
- 500 mg nicotinic acid
- 50 mg pyridoxine HCl
- 50 mg folic acid
- 5 mg biotin

not all vitamins will go into solution, keep at 4 °C and shake well before use
Appendix D

Minimum media (MM) used for induction of endochitinase production in *Trichoderma atroviride* strain P1

Composition of Minimum media (MM) for *Trichoderma atroviride* strain P1

- $5.17 \times 10^{-3} \text{ M K}_2\text{HPO}_4$
- $2.68 \times 10^{-3} \text{ M KCl}$
- $1.25 \times 10^{-2} \text{ M NH}_4\text{NO}_3$

autoclave and add into final concentration:

- $8.11 \times 10^{-4} \text{ M MgSO}_4\cdot7\text{H}_2\text{O}$ (filter sterilised)
- 2 ppm FeSO$_4\cdot7\text{H}_2\text{O}$ (filter sterilised)
- 2 ppm ZnSO$_4\cdot7\text{H}_2\text{O}$ (filter sterilised)
- 2 ppm MnCl$_2\cdot7\text{H}_2\text{O}$ (filter sterilised)
- 0.05% (w/v) asparagine (filter sterilised)

add 2% of ground and autoclaved *Fol* biomass to the media.
Appendix E

Media used for transformation of *Fol* race 3 with *gfp*

1. **Induction medium (IM)**
   - 10 mM K$_2$HPO$_4$
   - 10 mM KH$_2$PO$_4$
   - 2.5 mM NaCl
   - 4 mM (NH$_4$)$_2$SO$_4$
   - 0.5% glycerol

   autoclave 120 °C 20 mins

   - 9 μM FeSO$_4$·7H$_2$O
   - 40 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.3

   filter sterilise

   - 0.7 mM CaCl$_2$
   - 2 mM MgSO$_4$·7H$_2$O
   - 10 mM glucose

   autoclave and add into final concentration 200 μM acetosyringone

2. **Co-cultivation Medium (CM)**

   Same as IM, except:
• 5 mM glucose
• 1.5% (w/v) Bacto agar

add into final concentration 200 \( \mu \text{M} \) acetosyringone

3. Czapek Dox Agar

• 36 \( \mu \text{M} \) Fe\( \text{SO}_4 \cdot 7\text{H}_2\text{O} \)
• 2 mM Mg\( \text{SO}_4 \cdot 7\text{H}_2\text{O} \)
• 7 mM KCl
• 6 mM K\(_2\)HPO\(_4\)
• 40 mM NaNO\(_3\)
• 3% (w/v) sucrose
• 1.5% agar

autoclave, add into final concentration:

• 100 \( \mu \text{g/mL} \) hygromycin or Zeocin\(^\text{TM}\) (see text)
• 200 \( \mu \text{M} \) cefotaxime
Appendix F

Armstrong *Fusarium* medium (AFM)

Composition of Armstrong *Fusarium* medium (AFM) for cultivation of *Fol* for pathogenicity test (per liter)

- 1.1 g KH$_2$PO$_4$
- 1.6 g KCl
- 7.27 g KNO$_3$
- 0.05 mL Tween 80
- 2% (w/v) sucrose

Autoclave and add into final concentration:

- 0.4 g MgSO$_4$•7H$_2$O (filter sterilised)
- 2 ppm FeSO$_4$•7H$_2$O (filter sterilised)
- 2 ppm ZnSO$_4$•7H$_2$O (filter sterilised)
- 2 ppm MnSO$_4$•2H$_2$O (filter sterilised)
- 1.0 mg thiamine–HCl (filter sterilised)

Adjust the pH of the medium to 4.5
Appendix G

Media for cultivation of bacteria

1. MGML medium
   Per liter:
   • 5 g yeast extract
   • 0.5 g casamino acid
   • 8 g mannitol
   • 2 g ammonium sulphate
   • 5 g NaCl
   Adjust pH to 6.6

2. Min A medium
   Per liter:
   • 10.5 g KH$_2$PO$_4$
   • 4.5 g K$_2$HPO$_4$
   • 1 g (NH$_4$)$_2$SO$_4$
   • 0.5 g sodium citrate
   • 0.1 g MgSO$_4$·7H$_2$O
   • 0.1 g L-arginine
   • 10 g glucose

3. Yeast extract-peptone (YEP) medium
   Per liter:
4. **Luria-Bertani (LB) medium**

Per liter:

To 950 mL of deionised H\textsubscript{2}O, add:

- 10 g tryptone
- 5 g yeast extract
- 10 g NaCl

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH. Adjust the volume of the solution to 1 liter with deionised H\textsubscript{2}O.

5. **2× yeast extract-tryptone (YT) medium**

Per liter:

To 900 mL of deionised H\textsubscript{2}O, add:

- 16 g tryptone
- 10 g yeast extract
- 5 g NaCl

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH. Adjust the volume of the solution to 1 liter with deionised H\textsubscript{2}O.

6. **SOB medium**

Per liter:

To 950 mL of deionised H\textsubscript{2}O, add:

- 20 g tryptone
- 5 g yeast extract
- 0.5 g NaCl

Shake until the solutes have dissolved. Add:
• 10 mL of a 250 mM KCl

Adjust pH to 7.0 with 5 N NaOH, adjust volume to 1 liter, autoclave, and add:

• 5 mL of 2 M MgCl₂ (autoclaved)

7. SOC medium

SOC medium is identical to SOB medium except that it contains 20 mM glucose. After the SOB medium has been autoclaved, allow it to cool down to 60 °C or less and add 20 mL of filter-sterilised 1 M of glucose.
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