Functional analysis of a lignin biosynthetic gene in transgenic tobacco

by

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DECLARATION

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SUMMARY

Necrotrophic fungi infect many economically important crop plants. This results in great losses in the agricultural sector world-wide. Understanding the nature by which plants respond to pathogens is imperative for genetically enhancing disease resistance in plants. Research tools have significantly contributed to our understanding of how the plant responds to pathogen attack, identifying an array of defence mechanisms used by plants upon attack.

Many fungal pathogens secrete endopolygalacturonases (endoPGs) when infecting plants. These hydrolytic enzymes are inhibited by polygalacturonase-inhibiting proteins (PGIPs) associated with plant cell walls. PGIPs are well characterised and their current known functions are all linked to endoPG inhibition and the subsequent upregulation of plant defence pathways. Work on grapevine PGIPs have shown that apart from being efficient antifungal proteins, leading to protection of the plant against *Botrytis cinerea* when overexpressed, PGIPs might also have additional functions linked to cell wall strengthening. This working hypothesis formed the motivation of this study where a *cinnamyl alcohol dehydrogenase* (CAD) (1.1.1.195) gene was targeted for functional analysis in tobacco (*Nicotiana tabacum*). Some previous work and genetic resources obtained is relevant to this study, specifically previously characterized transgenic tobacco lines overexpressing the *Vitis vinifera pgip1* (*Vvpgip1)* gene. These lines have confirmed PGIP-specific resistance phenotypes against *B. cinerea*, as well as increased levels of CAD transcripts in healthy plants. Moreover, preliminary evaluations indicated increased lignin levels as well as differential expression of several other cell wall genes in these overexpressing lines (in the absence of infections).

In this study we generated a transgenic tobacco population, overexpressing the native *CAD14* gene, via *Agrobacterium* transformations. The transgene was overexpressed with the Cauliflower Mosaic Virus promoter (CaMV 35Sp). The CAD transgenic population was analyzed for transgene integration and expression and showed active transcription, even from leaves that normally don't express CAD to high levels. These lines, together with the untransformed control, and a representative transgenic VvPGIP1 tobacco line previously characterized with elevated expression of CAD were used for all further analyses, specifically CAD activity assays of stems and leaves, as well as whole plant infections with *B. cinerea*. CAD enzyme activity assays were performed on healthy uninfected plant lines, without inducing native CAD expression or resistance phenotypes (i.e. without *Botrytis* infection). CAD activity was detected in leaves and stems, but a statistically sound separation between the CAD population and the untransformed control was only observed in the stems. The CAD assays also confirmed previous results that indicated that CAD transcription was upregulated in the PGIP line in the absence of infection. Overall, in all plant lines the stems exhibited 10-fold higher levels of CAD activity than the leaves, but the transgenic VvPGIP1 line showed a further 2-3-fold increase in CAD activity in

the stems, when compared to the untransformed control and the majority of the CAD overexpressing lines.

Disease assessment by whole plant infections with *B. cinerea* of the CAD transgenic plants revealed reduced disease susceptibility towards this pathogen. A reduction in disease susceptibility of 20 – 40% (based on lesion sizes) was observed for a homologous group of transgenic lines that was statistically clearly separated from the untransformed control plants following infection with *Botrytis* over an 11-day-period. The VvPGIP1 transgenic line displayed the strongest resistance phenotype, with reduction in susceptibility of 47%. The reduction in plant tissue maceration and lesion expansion was most pronounced in the VvPGIP1 line compared to the CAD transgenic plants, while the CAD transgenic plants showed more reduction than the untransformed control. In combination, the data confirms that CAD upregulation could lead to resistance phenotypes. Relating this data back to the previously observed upregulation of CAD in the VvPGIP1-overexpressing lines, the findings from this study corroborates that increased CAD activity contributes to the observed resistance phenotypes, possibility by strengthening the cell wall.

In conclusion, this study yielded a characterized transgenic population overexpressing the *CAD14* gene; this overexpression contributed to increased RNA transcription compared to the untransformed control plant, increased CAD activity (most notably in the stems) and a disease resistance phenotype against *Botrytis*. These findings corroborates the current working hypothesis in our group that PGIPs might have a role in preparing the plant cell for attack by contributing to specific cell wall changes. The exact mechanisms are still currently unknown and under investigation. The transgenic lines generated in this study will be invaluable in the subsequent analyses where these various phenotypes will be subjected to profiling and accurate cell wall analyses.

OPSOMMING

Nekrotrofiese swamme infekteer en beskadig verskeie ekonomies belangrike gewasse. Dit lei wêreldwyd tot groot verliese vir die landbousektor. Dit is noodsaaklik om te verstaan hoe plante reageer teenoor patogene, sodat die siekteweerstand van plante verbeter kan word. Navorsingshulpbronne het beduidend bygedra tot die kennis van plantreaksies tydens patogeniese aanvalle, en het sodoende ‘n reeks van weerstandmeganismes, wat die plant inspan tydens ‘n aanval, geïdentifiseer.

Verskeie patogeniese swamme skei endopoligalakturonases (endoPGs) af tydens plant-infeksie. Hierdie hidrolitiese ensieme word geïnhibeer deur poligalakturonase-inhiberende proteïene (PGIPs) wat met die plantselwand geassosieerd is. PGIPs is goed gekarakteriseerd en al hulle huidiglik bekende funksies is gekoppel aan endoPG inhibisie en die daaropvolgende opregulering van plant weerstandspaaie. Navorsing op wingerd PGIPs het gewys dat, afgesien van die feit dat PGIPs goeie antifungiese proteïene is wat lei tot beskerming van die plant teen *Botrytis cinerea* wanneer dit ooruitgedruk word, PGIPs ook moontlik addisionele funksies verrig wat verwant is aan selwandversterking. Hierdie werkshipotese vorm die motivering vir hierdie studie waarin ‘n *sinnamiel alkohol dehidrogenase* (SAD) (1.1.1.195) geen geteiken is vir funksionele analise in tabak (*Nicotiana tabacum*). Vorige navorsing en genetiese hulpbronne daardeur verkry is belangrik vir hierdie studie, spesifiek die gekarakteriseerde transgeniese tabaklyne wat die V*itis vinifera pgip1* (*Vvpgip1*) geen ooruitdruk. Hierdie lyne besit bevestigde PGIP-spesifieke weerstandsfenotipes teen *B. cinerea*, sowel as hoër vlakke van SAD transkripte in gesonde plante. Voorlopige analises het ook aangedui dat hierdie ooruitdrukkende lyne hoër lignien vlakke het, asook differensiële uitdrukking van verskeie ander selwandgene (in die afwesigheid van infeksie).

In hierdie studie is ‘n transgeniese tabakpopulasie gegenereer wat die natuurlike tabak *SAD14* geen ooruitdruk, deur middel van *Agrobacterium* transformasie. Die transgeen is ooruitgedruk deur die Blomkool Mosaïek Virus promoter (CaMV 35Sp). Die SAD transgeniese populasie is geanaliseer vir transgeen integrasie en uitdrukking en het aktiewe transkriptering getoon, selfs in blare waar daar normaalweg nie hoë vlakke van SAD uitgedruk word nie. Hierdie lyne, die ongetransformeerde wilde-tipe kontrole sowel as ’n verteenwoordigende transgeniese VvPGIP1 tabaklyn wat vooraf gekarakteriseerd was met hoë SAD uitdrukking, is gebruik vir alle verdere analises, spesifiek SAD aktiwiteitstoetse in stingels en blare, asook heelplantinfeksies met *B. cinerea*. Aktiwiteitsanalises van die SAD ensiem is gedoen op gesonde ongeinfekteerde plantlyne, sonder om natuurlike tabak SAD uitdrukking of weerstandsfenotipes te induseer (dus sonder *Botrytis* infeksie). SAD aktiwiteit is waargeneem in beide die blare en stingels, maar ‘n statisties betekenisvolle skeiding is slegs gevind tussen die SAD populasie en die ongetransformeerde kontrole in die stingels. Die SAD toetse het ook

vorige resultate bevestig wat aangedui het dat SAD transkripsie opgereguleer word in die PGIP lyn in die afwesigheid van infeksie. Die stingels het oor die algemeen ‘n 10-voudige vermeerdering in SAD aktiwiteitsvlakke getoon in vergelyking met die blare, maar die transgeniese VvPGIP1 lyn het ‘n verdere 2-3-voudige verhoging in SAD aktiwiteit gehad in die stingels ,in vergelyking met die ongetransformeerde kontrole en die meerderheid van die SAD-ooruitdrukkende lyne.

Siekteweerstand ondersoeke deur middel van heelplantinfeksies met *B. cinerea* van die SAD transgeniese plante, het verminderde vatbaarheid aangedui ten opsigte van hierdie patogeen. ‘n Afname in siekte-vatbaarheid van 20 – 40% (gebaseer op wondgroottes) is waargeneem vir ‘n homoloë groep transgeniese lyne wat statisties betekenisvol geskei kon word van die ongetransformeerde kontrole plante na infeksie met *Botrytis* in ‘n infeksietoets wat 11 dae geduur het. Die VvPGIP1 transgeniese lyn het die mees weerstandbiedende fenotipe gehad, met ‘n afname in siekte-vatbaarheid van 47%. Die afname in plantweefselafbreking en wondgrootte was die duidelikste in die VvPGIP1 lyn in vergelyking met die SAD transgeniese plante, terwyl die SAD transgeniese plante ‘n groter afname aangedui het as die ongetransformeerde kontrole. In kombinasie het die data bevestig dat SAD opregulasie kan lei tot weerstandbiedende fenotipes. Hierdie data, in vergelyking met die vorige bevinding van opregulasie van SAD in die VvPGIP1-ooruitdrukkende lyne, bevestig dat hoër SAD aktiwiteit bydra tot die waargenome weerstandbiedende fenotipes, moontlik deur versterking van die plantselwand.

Ter afsluiting, hierdie studie het ‘n gekarakteriseerde transgeniese populasie wat die *SAD14* geen ooruitdruk gelewer; hierdie ooruitdrukking het bygedra tot hoër RNA transkripsie in vergelyking met die kontrole, verhoogde SAD aktiwiteit (veral in die stingels) en siekte-weerstandbiedende fenotipes teenoor *Botrytis*. Hierdie bevindinge ondersteun die huidige werkshipotese in ons groep dat PGIPs moontlik ‘n rol speel in die voorbereiding van die plantsel teen infeksie deur spesifieke selwandveranderinge te veroorsaak. Die spesifieke meganismes is steeds onbekend en word verder ondersoek. Die transgeniese lyne wat tydens hierdie studie gegenereer is, sal baie belangrik wees in opvolgende analises waar hierdie verskillende fenotipes gebruik kan word om die profiel van selwandkomponente, maar ook die akkurate selwandsamestelling te bestudeer.

This thesis is in honor of the Mbewana family

BIOGRAPHICAL SKETCH

Sandiswa Mbewana was born in Transkei, South Africa on the 24th of March in 1982. She matriculated at Greenpoint Secondary School, East London in 2000. Sandiswa enrolled for a BSc Biotechnology degree in 2001 at the University of the Western Cape and completed her studies majoring in Biochemistry, Microbiology and Botany in 2005. A degree in HonsBSc (Wine Biotechnology) was subsequently awarded to her in 2005 at the University of Stellenbosch. Thereafter she enrolled for an MSc-degree in Wine Biotechnology.

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PREFACE

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately and is written according to the style of the Plant Physiology. Chapter 3 is part of a study that will be submitted for publication.

Chapter 1 **GENERAL INTRODUCTION AND PROJECT AIMS**

Chapter 2 **LITERATURE REVIEW**

A general overview of plant defense with a specific focus on polygalacturonase-inhibiting protein (PGIPs) and lignin

Chapter 3 **RESEARCH RESULTS**

Analysis of a possible fungal resistance phenotype in transgenic tobacco plants overexpressing a native *cinnamyl alcohol dehydrogenase* gene

Chapter 4 **GENERAL DISCUSSION AND CONCLUSIONS**

In Chapter three, I performed all experiments, captured and compiled the data and drafted the manuscript. My supervisors Prof MA Vivier and Dr DA Joubert were involved in the conceptual development of study, the continued evaluation of the data and the conclusions of the research, as well as providing guidance to improve the compiled manuscript.

CONTENTS

Chapter 1

GENERAL INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 Introduction

Plants are exposed to environmental stress factors which negatively affect their growth and development. These factors are known as biotic and abiotic stress factors. Over the years, plants have developed defense mechanisms in order to adapt to their continuously changing environment. Advances in molecular biology and biotechnology have provided very valuable tools and approaches to study and understand the nature by which plants defend themselves against the multitude of stress factors that impact their growth and development.

Plants defend themselves against pathogens mainly via two mechanisms: by using the structural characteristics of the plant body which act as primary barriers, and by using their cellular biochemistry to produce defense compounds before, during and after attack. The specific combination of structural and biochemical defenses will be influenced by the specific plant-pathogen interaction.

The plant cell surface forms the first line of defense against invading pathogens (Collinge, 2009; Lagaert *et al*., 2009). Pectin, one of the major components of the primary cell wall is found in dicotyledonous and monocotyledonous plants (Tomassini *et al*., 2009). In order to successfully penetrate and colonize the host tissue to obtain required nutrients, many fungal pathogens secrete a wide range of hydrolytic enzymes including pectate lyases, pectin methyl esterases, beta-galacturonase and polygalacturonases (PGs) (Cervone *et al*., 1989; Garcia-Brugger *et al*., 2006). In pathogenesis strategies relying on PGs, the enzymes are exported from the pathogens to the host tissue. PGs cleave the α-(1→4) linkages between D-galacturonic acid residues in non-methylated homogalacturonan, which is a major component of pectin, causing the separation of plant cells from each other and tissue maceration of the host cells thereby facilitating penetration and colonization (Esquerré-Tugaye *et al*., 2000; De Lorenzo and Ferrari, 2002; Ferrari *et al*., 2003, Gomathi and Gnanamanickam, 2004). PGs are considered important pathogenicity factors of several fungi on their respective hosts (Shieh *et al*., 1997; ten Have *et al*., 1998; Isshiki *et al*., 2001; Oeser *et al*., 2002; Li *et al*., 2004; Kars *et al*., 2005).

Following pathogen recognition, signal transduction pathways are activated involving “amongst other” ion fluxes, protein kinases and generation of active oxygen species, all resulting in the expression of defense related genes encoding enzymes responsible for phytoalexin biosynthesis and pathogenesis related proteins (Heath 2000). These responses are associated with the hypersensitive response (HR) (Heath 2000), which is considered to be an important event for limiting the speed of infection by pathogens (ten Have *et al*., 1998; Poinssot *et al*., 2003). The HR responses include the synthesis of a range of antimicrobial proteins.

Polygalacturonase-inhibiting protein (PGIPs) are considered antifungal proteins that interact with fungal PGs in inhibition reactions that not only reduce the invasive action of the fungi, but also activate defense responses in the surrounding healthy tissues not yet colonized by the pathogen (De Lorenzo *et al*., 2001). The role of PGIP in plant defense has been demonstrated by overexpression of their encoding genes in different plant genetic backgrounds (Powell *et al*., 2000; Ferrari *et al*., 2003; Agüero *et al*., 2005; Manfredini *et al*., 2005; Joubert *et al*., 2006). These studies have proven that PGIP reduces disease susceptibility when overexpressed and that PGIP levels could be correlated to the level of resistance (Abu-Goukh *et al*., 1983; Johnston *et al*., 1993).

PGIPs have been studied extensively, yet the interactions of these proteins with activated defense responses have not been determined. To determine *how* PGIPs contribute to increased disease resistance, our laboratory has been studying the grapevine PGIP (Joubert, *et al*, 2006; Becker, 2007; Joubert *et al*., 2007). Transgenic tobacco plants overexpressing the *Vitis vinifera pgip1* gene (*Vvpgip1*) (Joubert *et al*., 2006) have been shown to exhibit PGIP-specific resistance phenotypes when challenged with *B. cinerea* in whole-plant infection assays. These plant lines revealed changes in expression patterns of cell wall related genes, as well as indications of increased lignin content when compared to untransformed control plants (Becker, 2007). Remarkably, these changes were observed in the absence of pathogen infection, indicating a possible new role of PGIPs in defense "priming" (Becker, 2007). A microarray analysis of the PGIP overexpressing lines (in comparison with untransformed controls) indicated that several genes involved in lignin formation were differentially regulated. One of the genes affected was the *cinnamyl alcohol dehydrogenase* (CAD) gene which was upregulated in some of the overexpressing lines (Becker 2007). CAD is widely accepted as a marker for lignin-formation (Walter *et al*., 1988; Mitchell *et al*., 1994). In tobacco and other species, several studies have evaluated phenotypes linked to downregulation of *CAD* gene expression, specifically in relation to altered lignin levels for biotechnological targets in the pulp and paper industry (Baucher *et al*., 1998; Selman-Housein *et al*., 1999; Farrokhi *et al*., 2006; Vanholme *et al*., 2008).

The current project was designed to study the effect of CAD upregulation in transgenic tobacco, specifically to evaluate whether or not increased expression of the encoding gene might contribute to resistance phenotypes. The previous findings are important to the rationale of this study is linked to the observed upregulation of CAD expression in PGIP overexpressing lines and the fact that these lines exhibit a PGIP-specific resistance phenotype that is correlated with increased PGIP activity. The proposed outcome of the study would be to further our understanding of the mechanism(s) underlying the observed cell wall strengthening phenotype in the PGIP overexpressing lines by studying the possible functional role of CAD in defense.

1.2 Specific Project Aims

The aim of this study was to perform a functional analysis of tobacco plants overexpressing the native *CAD* gene and to characterize the possible resistance phenotypes. The objective of this study is to understand one of the mechanisms by which PGIP reduced disease susceptibility and to establish a phenotype linked to lignin deposition in stable *CAD* transgenic lines. Elevated lignin levels in the plant cell wall could make the plant less susceptible to *Botrytis* infection because lignified cell walls have been proven to be more resistant to enzymatic hydrolysis by fungal polygalaturonases (Bruce and West, 1989). It is hoped that the analysis of the contribution of CAD towards reduced disease susceptibility will also begin to shed some light into the very complex role of PGIPs in plant defense.

Specific aims of this study included:

1) To isolate and clone the *cinnamyl alcohol dehydrogense* (*CAD*) gene from *Nicotiana tabacum*;

2) To sub-clone the isolated CAD encoding gene into a plant expression vector and transform tobacco with the overexpression construct via *Agrobacterium tumefaciens*;

3) To generate a transgenic tobacco population overexpresssing the *CAD* gene and to genetically analyze the population (alongside untransformed controls) by evaluation of the transgene integration and expression;

4) To determine the CAD enzyme activity in the transgenic plant lines in comparison with the untransformed control, as well as plant lines overexpressing the grapevine PGIP gene;

5) To evaluate possible resistance phenotypes in the CAD lines in comparison with untransformed controls, as well as the existing PGIP-overexpressing (VvPGIP) tobacco lines (as positive controls of resistance phenotypes). The evaluations will involve whole plant infections with *Botrytis* spore suspensions in a time course evaluation over 11 days.

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Chapter 2

LITERATURE REVIEW

A general overview of plant defense, with a specific focus on Polygalacturonase-inhibiting proteins (PGIPs) and lignin

A GENERAL OVERVIEW OF PLANT DEFENSE, WITH A SPECIFIC FOCUS ON POLYGALACTURONASE-INHIBITING PROTEINS (PGIPS) AND LIGNIN

This chapter is written according to the style of *Plant Physiology*

2.1 Introduction

Plants are sessile organisms that cannot hide or escape when attacked by pathogens. They become infected by different types of pathogens and attacked by a variety of pests. Plants have become well adapted to defend themselves against pathogens and pests. The defense pathways utilized depend on the type of pathogen and its mode of action to colonize and cause disease. This review will briefly summarize plant-pathogen interactions in general and then focus on two well known antifungal strategies, namely the role of polygalacturonase-inhibiting proteins (PGIPs) and the formation of lignin. The discussion will further focus on the plant cell wall and the changes that occur in cell wall metabolism during infection.

2.2 General summary of plant-pathogen interactions

An interaction of pathogens with their host plant could be categorized as compatible if the pathogen overcomes the plant's defense barriers and establishes symptoms or incompatible when plants release an array of defenses that efficiently limit pathogen growth (Glazebrook, 2005). An infection by a pathogen involves breaching the primary defense of the plant by either mechanical or enzymatic means. Plant infections are caused by micro-organisms such as bacteria, viruses, fungi, nematodes and protozoa. Some of the best studied organisms infecting plants are classified as bio- and necrotrophic pathogens (Nürnberger and Brunner, 2002). Biotrophic pathogens are parasitic organisms that specialize in feeding on living plant's tissues. These pathogens have a narrow host range and they adapt to a specific line of a given plant species. They do not directly penetrate the plant epidermis. They typically germinate on the plant surface, enter via the stomata or other natural openings and subsequently penetrate mesophyl cell walls to gain access to the nutrients. Biotrophs live in the intercellular space between the leaf mesophyll cells and some produce haustoria as feeding structures that invaginate the plasma membrane of the host cells. By their feeding activities, they create a nutrient sink to the infection site, which will wound the host, but not kill it. The most common and important group of biotrophic plant pathogens are the rust fungi (*Basidiomycota*) and the powdery mildew fungi (*Ascomycota*).

Necrotrophic pathogens on the other hand are parasitic organisms that obtain their nutrients from dead cells and tissues of the host organisms. They grow on plant tissues that are

wounded or weakened and frequently produce toxins to kill host tissues prior to colonization (de Wit, 2007; Hématy *et al*., 2009).

To defend themselves, plants rely on recognition of pathogens to ultimately culminate in induced defense responses. One of the levels of recognition relates to the presence of conserved microbial molecules, referred to as pathogen- (or microbe-) associated molecular patterns (PAMPs/MAMPs). Pattern recognition receptors (PRRs) are involved in this process. (Nürnberger and Brunner, 2002; Jones and Dangl, 2006). The PRRs are proteins expressed by the cells innate immune system to identify molecules associated with pathogens and cellular stress (Day and Graham, 2007; de Wit 2007). This first level of recognition is referred to as PAMP-triggered immunity (PTI). Intracellular responses associated with PTI include rapid ion fluxes across plasma membrane, MAP kinase activation, production of reactive-oxygen species, rapid changes in gene expression and cell wall reinforcement (Zipfel, 2008; Hématy *et al*., 2009). To counteract these plant defense responses, pathogens suppress PTI by secreting effectors in the apoplast or directly into the cytoplasm of the host cells (Gohre and Robtzek, 2008). When these effectors are recognised, hypersensitive responses (HR) are induced (Heath 2000; Jones and Dangl, 2006) and expression of disease resistance (R) genes are activated to recognise the effectors directly or indirectly, leading to effector-triggered immunity (ETI) (Jones and Dangl, 2006; Zipfel, 2008, 2009). Often ETI is quantitatively stronger that PTI (Jones and Dangl 2006).

2.3 The importance of the cell wall in infection and defense

2.3.1 Structure of plant cell walls

The plant cell wall is an exoskeleton surrounding the protoplast. It is composed of a highly integrated and structurally complex network of polysaccharides, including cellulose, hemicelluloses and pectin (Cosgrove, 2005) (see Fig 1). The cell wall connects cells to tissues, signals the plant to grow and divide and controls the shape of the plant organs. It also facilitates water transport and defense (Cosgrove, 2005). The polysaccharide content forms a major primary barrier against pathogenic fungi due to its polysaccharide-rich cell wall. The polysaccharides are cross-linked with both ionic and covalent bonds into a network that resists physical penetration (Carpita and McCann, 2000).

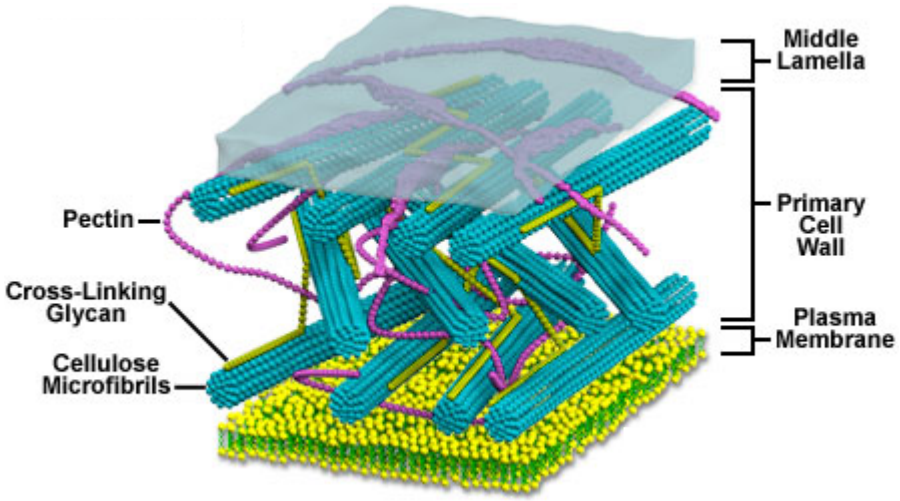

Figure 1: A diagrammatical representation of the plant cell wall structure (image adapted from: micro.magnet.fsu.edu/cells/plants/cellwall.html).

Other substances found in the cell wall are lignin and a waxy cuticle. Lignin is found in all vascular plants, mostly between the plant cells and in the cell walls. It is a complex, insoluble polymer. Lignin is a strengthening material in all cell walls and is associated with cellulose (Lodish *et al*., 2000).

2.3.2 Penetration of plant cell walls by pathogens

The majority of plant-pathogenic bacteria and fungi must penetrate the cell walls in order to initiate and expand necrotic infections or to establish colonization within the plant (Powell *et al*., 2000). Most fungal pathogens secrete hydrolytic enzymes capable of degrading the cell wall polymers. Pectin degrading enzymes weaken the cell wall and expose other polymers to degradation by hemicellulases and cellulases (D'Ovidio *et al*., 2004). They are the first cell wall degrading enzymes secreted by the pathogens (English *et al*., 1971; Jones *et al*., 1971; Tomassini *et al*., 2009). Among these enzymes are the pectate lyases, pectin lyases, pectin methylesterase, glucanases and polygalacturonases (PGs) which are produced for cell wall hydrolysis (Cervone *et al*., 1989; Stotz *et al*., 1993; Lagaert *et al*., 2009). PGs are secreted by almost all phytopathogens and a wide variety of isoenzymes exist. They are important virulence factors in *Botrytis cinerea* (ten Have *et al*., 1998; Kars *et al*., 2005), *Claviceps purpurea* (Oeser *et al*., 2002), *Alternaria citri* (Isshiki *et al*., 2001) and *Aspergillus flavus* (Shieh *et al*., 1997). PGs from *B. cinerea* will be discussed to illustrate the importance and mode of action of these pathogen-derived enzymes.

B. cinerea is a plant pathogenic ascomycete that causes pre- and post harvest diseases on many economically important crops (ten Have *et al*., 2001). It is a necrotrophic fungus that affects many plant species. For successful penetration and colonization, *B cinerea* secretes hydrolytic PG enzyme. PGs randomly cleave the α-(1→4) linkages between D-galacturonic

acid residues in pectin and other galacturonases in non-methylated homogalacturonan, a major component of pectin. This causes tissue maceration of the host cells, thereby facilitating penetration and colonization by the pathogen (De Lorenzo and Ferrari, 2002; Ferrari *et al*., 2003, Cheng *et al*., 2008; Lagaert *et al*., 2009). Degradation of the plant cell wall by the fungus facilitates fungal growth and provides the fungus with nutrients (ten Have *et al*., 2001).

PGs of *B. cinerea* are encoded by a family of six members (ten Have *et al*., 1998; Wubben *et al*., 1999; 2000). These genes are differentially expressed, depending on the stage of infection and host (ten Have *et al*., 2001). *Bcpg1* and *Bcpg2* are expressed constitutively; *Bcpg3* is induced by a change in pH; *Bcpg4* and *Bcpg6* are induced by D-galacturonic acid, whereas *Bcpg5* is induced by xylogalacturonic acid (Wubben *et al*., 1999, 2000). *Bcpg1* and *Bcpg2* are required for full virulence (ten Have *et al*., 1998, Joubert *et al*., 2007) in *B. cinerea.* Gene inactivation studies indicated that *Bcpg2* activity is involved in the penetration step by breaching the cell wall (Kars *et al*., 2005); whereas *Bcpg1* activity is required during colonization to breach the pectin-rich cell wall of the middle lamella (ten Have *et al*., 1998). *Bcpg3* is the only PG enzyme that has displayed a broader pH optimum and is active between pH 3.2 and 4.5 (ten Have *et al*., 2001; Wubben *et al*., 2000).

PG activity is inhibited by polygalacturonase-inhibiting proteins (PGIPs), present in the plant cell wall. PGIPs play an important role in resistance against phytopathogenic fungi by interacting with PGs to limit fungal penetration by reducing the hydrolytic activity of PGs (De Lorenzo *et al*., 2001; Powell *et al*., 2000; Ferrari *et al*., 2003; Agüero *et al*., 2005; Manfredini *et al*., 2005; Joubert *et al*., 2006; Janni *et al*., 2008).

2.3.3 Inhibition of fungal penetration and colonization by polygalacturonase-inhibiting protein (PGIP)

To counteract the action of PGs, plants have evolved many PGIPs with different recognition capabilities against the various PGs secreted by pathogenic fungi (Di Matteo *et al*., 2003). PGIPs are soluble glycoproteins found in the extracellular matrix of dicotyledonous and monocotyledonous plants and are bound to the cell wall by ionic interactions (Jones and Jones, 1997; De Lorenzo and Ferrari, 2002). They have a molecular mass of around 44 kDa, with N-linked glycosylation accounting for 10 kDa of the mass (when de-gylcosylated the mass is around 34 kDa). They contain a signal peptide that is processed through the endo-membrane system, targeting the protein to the apoplast (Chimwamurombe *et al*., 2001; Gomathi and Gnamanikam, 2004). These genes are usually clustered in the plant genome and their expression is spatially and temporally regulated during development and in response to several stimuli (De Lorenzo *et al*., 2001). PGIPs belong to a superfamily of leucine-rich repeat (LRR) proteins (Jones and Jones, 1997; Leckie *et al*., 1999; Kobe and Kajava, 2001). The LRR is a versatile structural motif responsible for many protein-protein interactions and is involved in

many cellular functions such as receptor dimerization, domain repulsion, regulation of adhesion and binding events (Buchanan and Gay, 1996; Gomathi and Gnanamanickam, 2004). They play a role in the recognition of pathogens (Dangl and Jones, 2001) and non-host general resistance (Nürnberger *et al*., 2004). LRRs are characterized by the tandem repetition of leucines in a consensus motif (xxLxLxx) (Mattei *et al*., 2001) in which the x residues are solvent exposed and involved in the interaction of ligands (Kobe and Deisenhofer, 1995; Leckie *et al*., 1999; Mattei *et al*., 2001).

PGIPs expression is induced in response to biotic and abiotic stress stimuli as well as by treatment with SA, elicitors such as fungal glucan or oligogalacturonase (OGAs) in response to wounding (Bergmann *et al*., 1994). The PG-PGIP interaction retards the hydrolytic activity of PGs, thus limiting the aggressive potential of the PG and is hypothesized to favor the accumulation of elicitor-active OGAs in the apoplast (Cervone, 1989; Ridley *et al*., 2001; Federici *et al*., 2006). OGAs are elicitors of plant defense responses that lead to the accumulation of phytoalexins, the synthesis of lignin, the expression of β-1, 3-glucanases, proteinase inhibitors and the production of ROS (De Lorenzo *et al*., 2001; Ridley *et al*.,2001; D'Ovidio *et al*., 2004). They contribute to the basal resistance against pathogens through a signaling pathway activated by PAMPs (Cervone *et al*., 1989). The PG-PGIP interaction is highly specific and varies with each PG-PGIPs individual inhibition interaction (Stotz *et al*., 1993; Yao *et al*., 1995).

PGIPs have been shown to limit *B. cinerea* invasion in a variety of plants, including tomato and grapevine (Powell *et al*., 2000; Agüero *et al*., 2005), tobacco (Manfredini *et al*., 2005; Joubert *et al*., 2006), *Arabidopsis* (Ferrari *et al*., 2003), and wheat (Janni *et al*., 2008). PGIPs interfere with the hydrolytic action of the PGs by blocking sites of the substrates subject to enzyme action. A change in pH in the apoplast as a result of blocking of carboxyl groups by PGIP can change the activity of the enzymes localized in the call wall, thus limiting pathogenic function (Protsenko *et al*., 2008). In a recent study the role of PGIP in transgenic tobacco plants overexpressing the grapevine PGIP encoding gene was examined (Joubert *et al*., 2006). The plants have been shown to exhibit PGIP-specific resistance phenotypes when challenged with *B. cinerea* in whole-plant infection assays. These plant lines revealed changes in expression patterns of cell wall related genes, as well as indications of increased lignin content when compared to untransformed control plants (Becker, 2007). Remarkably, these changes were observed in the absence of pathogen infection, indicating a possible new role for PGIPs in defense priming (Becker, 2007). The overexpression of PGIP seems to trigger changes leading to a strengthened cell wall, thus reinforcing the cell wall prior to infection.

2.4 A focus on lignin

2.4.1 The importance of lignin

Lignin is the second most abundant plant compound after cellulose (Bruce and West, 1989; Knight *et al*., 1992; Ferrer *et al*., 2008). It represents a quarter of the terrestrial biomass and accounts for up to 35% (dry weight) of secondary xylem in woody species. It is therefore one of the most abundant natural polymers, along with cellulose and chitin (Bruce and West, 1989; Knight *et al*., 1992; Hawkins and Boudet, 1994; Whetten and Sederoff, 1995). Lignin imparts rigidity and structural support to the plant cell wall (Higuchi *et al*., 1981). The importance of lignin ranges from its fundamental roles in evolution of land plants, global carbon cycling, plant growth and development, its role in biotic and abiotic stress resistance of plants to the potential importance of lignin in agriculture and the use of plant material.

Several industries are affected by lignin. Because of the sizable economic benefits that might be achieved, these industries have resorted to plant biotechnology and genetic engineering as well as conventional breeding techniques to fundamentally modify lignin quality and quantity (Baucher *et al*., 1998; Jung and Ni, 1998; Selman-Housein *et al*., 1999; Farrokhi *et al*., 2006). Lignin engineering can improve the processing efficiency of plant biomass for pulping, forage digestibility and biofuels (Vanholme *et al*., 2008). Efforts have been mainly focused on the modification of expression levels of specific genes in the biosynthetic pathway leading to lignin (Piquemal *et al*., 1998; Baucher *et al*., 1996; Atenassova *et al*., 1995). Reducing the lignin content of fiber and forage is aimed at reducing the cost for preparing fiber and improved digestibility of fodders and forage.

Plants synthesize high levels of lignin which are required for general structural support of the plant body and strengthening of the tissues involved in water transport (Boudet and Chabannes, 2001; Lauvergeat *et al*., 2001; Ferrer *et al*., 2008). Lignin forms an integral part of the cell walls especially in the tracheas, phloem fibers, periderm and vessel elements of the xylem and sclerenchyma (Goujon *et al*., 2003; Rogers and Campbell, 2004; Dean, 2005). It is deposited within the cell wall carbohydrate matrixes as well as the cellulose and hemicellulose microfibrils. These components have a mechanical influence on lignin development (Donaldson, 2001).

Lignification takes place when cell growth is completed and the cells undergo secondary growth (Fukuda and Komamine, 1982). The lignin polymer is produced by the dehydrogenative polymerization of three different cinnamyl alcohols that differ in the degree of methoxylation at the C3 and C5 positions of the aromatic ring. When incorporated into lignin, these alcohols are called the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of the polymer, respectively (Sederoff *et al*., 1999; Boerjan *et al*., 2003). The monolignol building blocks are synthesized via the phenylpropanioid pathway (Walter *et al*., 1988; Bruce and West, 1989), which also provides precursors to a wide range of products playing a key role in plant development and defense

such as flavanoids, coumarins, phytoalexins and tannins (Whetten and Sederoff, 1995; Lauvergeat *et al*., 2001; Boerjan *et al*., 2003; Ferrer *et al*., 2008).

To determine the structure or monomeric composition of lignin in plants is extremely difficult because of the heterogeneity of the polymer and high proportion of the covalent bonds that link different monomers. During isolation lignin also could undergo secondary modifications such as condensation, oxidation or substitution (Baucher *et al*., 2003).

2.4.2 Monolignol Biosynthesis

The lignin monolignols are produced intracellularly, and then exported to the cell wall and subsequently polymerized (Baucher *et al*., 2003) (see Fig 2 for a representation of the lignin biosyntheitic pathway). Monolignols are C6 – C3 phenylpropanoid compounds that differ from each other by the degree of methoxylation of the phenyl ring (Campbell and Sederoff, 1996, Baucher *et al*., 1998; Rogers and Campbell., 2004). The proportion of these monolignols in the plant cell walls vary depending on the plant species, monomeric composition, cytological origin, conditions of growth and stage of development (Mitchell *et al*., 1994; Baucher *et al*., 1996; Sibout *et al*., 2005; Farrokhi *et al*., 2006). The monolignols are the by-product of the phenylpropanoid pathway, starting from phenylalanine and tyrosine (Sato *et al*., 1997; Whetten *et al*., 1998; Baucher *et al*., 2003). Biosynthesis starts with the deamination of phenylalanine and involves successive hydroxylation reactions in the aromatic ring, followed by phenolic *O*-methlyation and conversion of the side-chain carboxyl to an alcohol group (Kawaoka *et al*., 2000; Boudet *et al*., 2004). The enzymes involved in monolignol synthesis are hydroxycinnamyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) (Kawaoka *et al*., 2000; Boudet *et al*., 2004). The first step of monolignol synthesis is catalyzed by CCR and the second by CAD (Sibout *et al*., 2003). These enzymes are abundant in areas where the secondary cell wall is formed (Takabe *et al*., 2001). CCR catalyzes the reduction the of hydroxycinnamoyl-CoA thioester to its corresponding aldehyde (Whettern and Sederoff, 1995; Lauvergeat *et al*., 2001). These aldehydes in turn are substrates for the CAD enzyme.

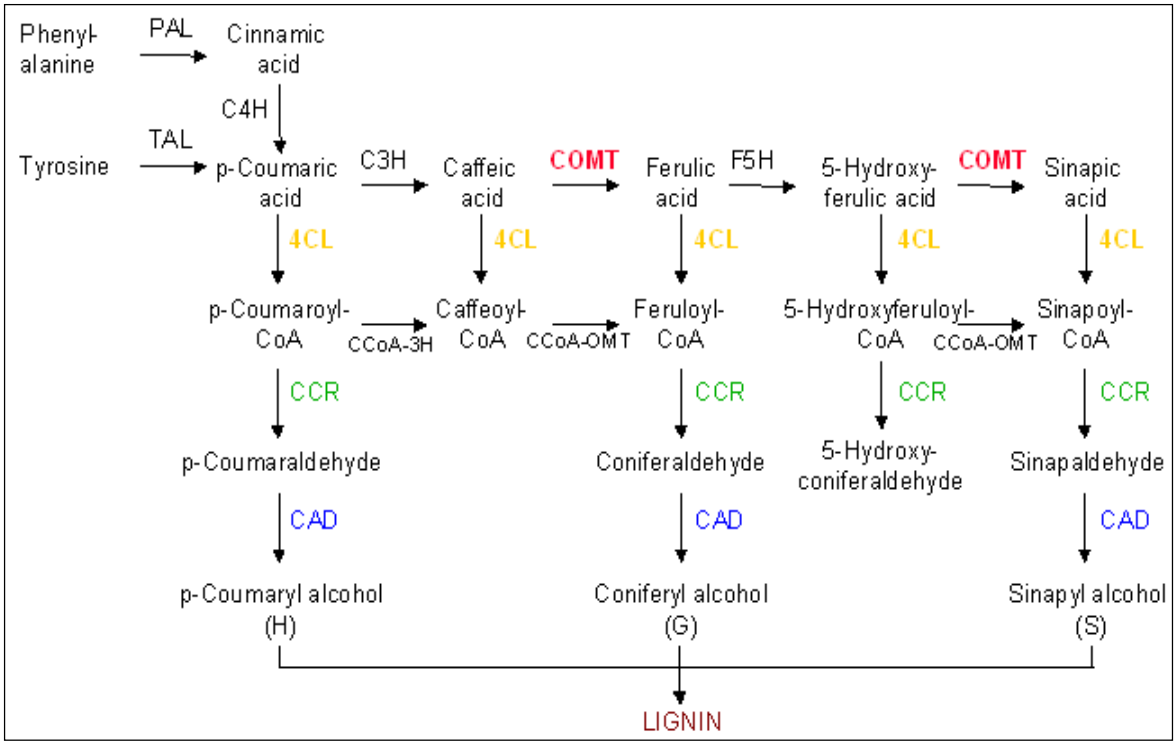

Figure 2: Biosynthetic pathway of monolignols and lignin. The general flow of the pathway from deamination of the phenylalanine, followed by hydroxylation of the aromatic ring, methylation and the reduction of the terminal acidic group to an alcohol is shown. Abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3H, *p*-coumarate 3-hydroxylase; COMT, Caffeic *O*-methyltransferase; CCoAOMT, caffeoyl-CoA *O*-mathytrasferase; F5H, ferulate 5-hydroxylase; 4CL, 4-coumarate-CoA ligase; CCR, cinnamyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase (from Spangenberg *et al*., 2001).

CADs form a part of the alcohol dehydrogenase multi-gene family (De Melis *et al*., 1999). They catalyze the conversion of *p*-hydroxycinnamyaldehydes to their corresponding hydroxycinnamyl alcohols in the presence of NADP as cofactor (Ralph *et al*., 1997; Smith and Dubery, 1997; Jung and Ni, 1998; De Melis *et al*., 1999; Ferrer *et al*., 2008). This enzyme is low in abundance, comprising approximately 0.05% (w/v) of total soluble protein in plant stems (Halpin *et al*., 1992). A CAD homolog from Aspen (*Populus tremuloides*), sinapyl alcohol dehydrogenase (SAD), which preferably reduces sinapyl aldehyde to sinapyl alcohol, was identified (Li *et al*., 2001). Aspen CAD preferably reduces coniferaldehydes, therefore SAD may be the enzyme responsible for the final step in the biosynthesis of sinapyl alcohol (Li *et al*., 2001).

CAD genes have been isolated and characterized from tobacco (Halpin *et al*., 1992; Knight *et al*., 1992), *Eucalyptus* (Goffner *et al*., 1992; Grima-Pettanati *et al*., 1993), maize (Halpin *et al*., 1998) and *Aspen* (Li *et al*., 2001). In tobacco, two CAD isoforms (CAD14 and CAD19) were identified. Although the two genes display high sequence similarity (Halpin *et al*., 1992; Knight *et al*., 1992), the proteins have peptide sizes of 42.5 and 44 kD respectively. Both require NADP as cofactor and have high affinity for coniferaldehyde. They are present in equal amounts in tobacco. Their observed differences in amino acid composition suggest that they derive from separate gene products. The genetic lineage of *Nicotiana tabacum,* which is an

allotetraploid hybrid containing chromosomes from two parental species supports this observation (Halpin *et al*., 1992; Knight *et al*., 1992).

CAD is present during different stages of plant development (Raes *et al*., 2003) and is also expressed in response to stress (Galliano *et al*., 1993), pathogen elicitors (Campbell and Ellis, 1992) and wounding (Lauvergeat *et al*., 2001), thus being regulated by both developmental and environmental stress factors. The monolignols are relatively toxic and unstable compounds that do not accumulate to high levels within living plants. Glycosylation of the phenolic hydroxyl groups to produce monolignol glucosides stabilizes the compounds and renders them nontoxic to the plant (Whetten and Sederoff, 1995).

In addition to the three monolignols, lignin contains traces of acetates, *p*-coumarates, *p*-hydroxybezoates and tyramine ferulate (Sederoff *et al*., 1999; Boerjan *et al*., 2003; Rastogi and Dwivedi, 2008). A variety of chemical links include ether and carbon-carbon bonds that connect the units of lignin (Ralph *et al*., 1997; Boerjan *et al*., 2003). The current concept of the monolignol biosynthetic pathway envisages a metabolic grid that leads to the formation of monolignols in the cytoplasm. This would occur through successive side chain reductions and ring hydroxylations, or methylation reactions and conversion of the carboxyl side chains to alcohol groups (Rastogi and Dwivedi, 2008).

2.4.3 Enzymatic oxidative polymerization of monolignols to form lignin

Polymerization of lignin occurs in the plant cell, so monolignols need to be transported from the cytosol where they are synthesized, to the cell wall. Lignin is formed by dehydrogenative polymerization of the monolignols (Mäder and Füssl, 1982; Baucher *et al*., 1998; Boerjan *et al*., 2003). Polymerization of lignin occurs through an oxidative coupling mechanism, whereby monolignol radicals react with radical sites on the lignin polymer. Polymerization is attributed to different enzymes such as polyphenol oxidases, coniferyl alcohol oxidase, laccase and peroxidases (Evans and Himmelsbach, 1991; Sato *et al*., 1997). The different classes of enzymes display broad substrate specificities and this has complicated the identification of isoforms that are specifically involved in lignification during normal growth and development of plants (Vanholme *et al*., 2008).

2.4.4 The role of lignin in plant defense

Lignification is a well known mechanism of disease resistance in plants (Vance *et al*., 1980). Plants with high lignin content have been shown to be less susceptible to microbes and insect herbivores (Kawasaki *et al*., 2006). During defense responses, lignin and lignin-like phenolic compound accumulation was shown to occur in a variety of plant-pathogen interactions (Vance *et al*., 1980; Nicholson and Hammerschmidt, 1992). The phenylpropanoid pathway that

provides the lignin-building monolignol unit is strongly activated after infection by the pathogen or treatment with elicitors (Pakusch and Matern, 1991; Jaeck *et al*., 1992). In the infected plant, deposition of phenylpropanoid compounds is part of the cell wall reinforcement that restricts pathogen invasion (Nicholson and Hammerschmidt, 1992).

Lignification of plant cells around the site of infection or lesion has been reported to be a defense response of plants that can potentially slow down the spread of a pathogen (Nicholson and Hammerschmidt, 1992; Knight *et al*., 1992). Lignin is synthesized locally in the plant epidermal cell walls in response to attempted penetration (Knight *et al*., 1992). Polymerization of monomer forms cross-links with carbohydrates and proteins. This renders the cell wall highly resistant to mechanical and enzymatic degradation (Bruce and West, 1989). Lignification chemically modifies the cell wall to be more resistant to cell degrading enzymes. It also restricts the diffusion of toxins from the pathogen to the host and of nutrients from the host to the pathogen through the cell wall (Nicholson and Hammerschmidt, 1992). Other elicitors of lignin include chitosan, extracts from fungal cell walls, and suspensions of chitin or fungal cell walls and fungal lipids (Bruce and West, 1989). Hyper-lignification is often seen in cellulose-deficient plants (Hématy *et al*., 2007) or in response to pathogen attack (Hückelhoven, 2007) to reinforce the cell wall.

Callose a plant polysaccharide composed of glucose residues linked together through β-1,3-linkages**,** is also deposited also deposited along the edges of pathogen-derived wounds and on certain occasions, it completely encases the attacked cells. This polysaccharide is also thought to act as a physical reinforcement at the site of damage. It plays a role in sealing breaks in the cell wall. This suggests that callose is potentially an important factor in cell wall integrity signaling in plants (Hématy *et al*., 2009). In combination, these results indicate an important role of cell wall strengthening by deposition of lignin as an inducible defense response (He *et al*., 2002; Cavalcanti *et al*., 2006).

Despite the described importance of lignin in plant defense, direct genetic evidence of this is rare. This might be attributed to the high level of redundancy observed for enzymes involved in the monolignol pathway. Some evidence is available from transgenic (overexpressing/silencing) studies, i.e. gene silencing of monolignol genes lead to a susceptible phenotype against powdery mildew in wheat lines normally resistant to the pathogen (Bhuiyan *et al*., 2009). Also, in potato, a decrease in phenolic compounds (including lignin) rendered the tissue more susceptible to *Phytophthora infestans* (Yao *et al*., 1995).

2.5 Conclusions and Perspectives

Studies into plant defense mechanisms and plant-pathogen interactions have lead to a wealth of knowledge on how plants defend themselves and how pathogens infect their hosts. Linking all this knowledge to molecular pathways and studies on the genetic level has gained

momentum in plant biology and plant pathology, specifically since genomes have been sequenced of both crop plants and their pathogens. It is now possible to study these complex aspects on a systems level to gain understanding of the underlying pathways, their control points and the various interactions with other factors and pathways. These studies typically generate new hypotheses that could then be followed with forward and/or reverse genetic approaches.

This review focused on two well-known role-players in plant defense, PGIPs and lignification as a mechanism of plant defense. Through work performed in our laboratory, some evidence exists that show that overexpression of PGIPs might lead to cell wall strengthening in anticipation of plant infection. One of the pathways affected is the lignin biosynthetic pathway. Current and ongoing work (also described in Chapter 3 of this thesis) aims to elucidate this interaction further to facilitate our understanding of the *in vivo* roles of PGIPs and plant defense responses in general.

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Chapter 3

Research Results

Analysis of a possible fungal resistance phenotype in transgenic tobacco plants overexpressing a native *cinnamyl alcohol dehydrogenase* gene

RESEARCH RESULTS

Analysis of a possible fungal resistance phenotype in transgenic tobacco plants overexpressing a native *cinnamyl alcohol dehydrogenase* gene

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3.1 Abstract

Polygalacturonase-inhibiting proteins (PGIPs) are cell-wall associated plant inhibitors of fungal endopolygalacturonases (PGs). The PG-PGIP inhibition interaction contributes to plant defense by limiting colonization by the pathogen, as well as by up-regulating the inducible plant defense responses. In a recent study in our laboratory, the role of PGIPs was studied in transgenic tobacco plants overexpressing the grapevine (*Vitis vinifera*) PGIP encoding (*Vvpgip1*) gene. It was previously confirmed that these lines had a PGIP-specific resistance phenotype when infected with *Botrytis cinerea*, but when these lines were compared with the untransformed control on the transcriptomic level, it was found that in the absence of infection, genes involved in cell wall strengthening were differentially regulated. From the lignin biosynthetic pathway the *cinnamyl alcohol dehydrogenase* (*CAD*) (1.1.1.195) and *lignin peroxidase* (*POD*) genes were differentially regulated.

In this study the aim was to investigate the biological relevance of the up-regulation of the *CAD* gene and its possible contribution to resistance phenotypes in tobacco. The *CAD14* gene was isolated from *Nicotiana tabacum*, sequenced, cloned and transformed back into tobacco under the control of the constitutive CaMV 35S promoter and NOS terminator. A T_1 population was generated and maintained under green house growth conditions. No obvious morphological phenotypes were observed in the population. The transgenic plants were analyzed to confirm gene integration and expression as well CAD activity. A previously characterized VvPGIP1 transgenic line with increased levels of CAD was included in the assays for comparison. The highest activity levels were recorded from stems. The assays confirmed that CAD overexpression lead "to a small", but statistically significant increased CAD activity in the stems of the transgenic population. A similar trend was observed in the younger leaves, although the activity levels were very low and the differences were not statistically significant. The VvPGIP1-line also exhibited elevated CAD activities, specifically in the stem. In this line, overexpression of PGIP was observed previously to induce CAD expression; the assays confirmed that this PGIP-driven up-regulation lead to increases in CAD activity.

Disease assessment by whole plant infections with *B. cinerea* of the *CAD* transgenic plants revealed reduced disease susceptibility towards this pathogen. A reduction in disease susceptibility of 20 – 40% was observed for a homologous group of transgenic lines that was statistically clearly separated from the untransformed control plants following infection with *Botrytis* over an 11-day-period. The VvPGIP1 transgenic line displayed the strongest resistance phenotype, with reduction in susceptibility of 47%. In combination, the data confirms that CAD up-regulation could lead to resistance phenotypes. Relating this

data back to the previously observed up-regulation of CAD in the PGIP-overexpressing lines, the findings from this study corroborates that increased CAD activity could contribute to the observed resistance phenotypes, possibility by strengthening the cell wall.

3.2 Introduction

Botrytis cinerea is a phytopathogenic fungus that causes gray mould in plant species. During infection, it secretes several endopolygalacturonases (PGs) to degrade cell wall pectin, and among them, BcPG1 and BcPG2 are important virulence factors (ten Have *et al*., 1998, Kars *et al*., 2005; Joubert *et al*., 2007). To counteract the activity of PGs, plants have evolved polygalacturonase-inhibiting proteins (PGIPs). PGIPs are cell-wall associated plant inhibitors that belong to the superfamily of leucine-rich repeat (LLR) proteins (D'Ovidio *et al*., 2004). The structure of LLR repeats is conserved in many disease resistance genes (Bergelson *et al*., 2001) and is characteristic of protein-protein interactions (Shanmugam, 2005). PGIP retards polygalacturonase (PG) action by preventing cell wall degradation and limiting fungal growth and colonization (Juge, 2006). PGIP is typically induced during infection and apart from its inhibition interactions on PGs, it has been linked to the active defense responses of plants under attack. *In vitro*, the inhibition of PGs by PGIPs prolongs the existence of oligogalacturonides (OGs), which is thought to induce plant defense responses (Cervone *et al*., 1989).

Overexpression of PGIP genes in tobacco (*Nicotiana tabacum*) (Menfradini *et al*., 2005; Joubert *et al*., 2006), tomato (*Lycepersicon esculentum*) (Powell *et al*., 2000), grapevine (*Vitis vinifera*) (Agüero *et al*., 2005) and *Arabidopsis* (Ferrari *et al*., 2003) lead to reduced disease symptoms against *Botrytis cinerea*. The PGIP encoding gene from *Vitis vinifera* (*Vvpgip1*), when overexpressed in tobacco contributed to a reduction in disease susceptibility of 47-69% towards *Botrytis* infection (Joubert at al., 2006). Moreover, when these lines were further analyzed, differential regulation of genes involved in cell wall strengthening was observed (Becker, 2007). In most lines there was a significant increase in expression of the *cinnamyl alcohol dehydrogenase* (*CAD*) and *lignin peroxidase* (*POD*) genes, both members of the lignin biosynthetic pathway. Significantly, this differential expression was observed in healthy, uninfected plants (thus in the absence PGs and activated defense responses). This is a novel finding since all functions previously linked to PGIPs formed part of active defense in plants.

The CAD enzyme catalyzes the last step of the lignin biosynthesis pathway (Ralph *et al*., 1997; Jung and Ni, 1998), and is thus considered a highly specific marker for lignification (Walter *et al*., 1988; Mitchell *et al*., 1994). Lignification is a common plant response to infection and wounding. It provides an effective physical barrier to growth and entry of plant pathogens (Vance and Sherwood, 1976; Knight *et al*., 1992). Lignin deposition has been shown to interfere with enzymatic hydrolysis and mechanical penetration by fungal pathogens and may also impair the movement of water and diffusible molecules between the plant and the fungus

(Bruce and West, 1989; Valentines *et al*., 2005). This polymer is also synthesized locally in the plant epidermal cell walls in response to attempted penetration (Knight *et al*., 1992).

In the study of Becker (2007), preliminary biochemical evidence corroborated the gene expression data, showing that the VvPGIP1 transgenic lines had increased lignin deposition in the stems. This lead to the hypothesis that in PGIP-overexpressing lines, cell wall strengthening might be initiated by the increased levels of PGIP, even before infection occurs, thus contributing to the observed resistance phenotypes against pathogens.

To improve our understanding of the biological relevance of the observed *CAD* gene up-regulation in PGIP-overexpressing lines, and its contribution to the PGIP-specific resistance phenotypes, we performed a functional analysis of transgenic tobacco plants overexpressing the native *CAD* gene. A T_1 transgenic population was established and analyzed for transgene integration and expression. CAD activity assays in leaves and stems included a representative VvPGIP1 line previously shown to induce increased transcription of *CAD*. The activity assays confirmed increased activity of CAD in the overexpressing lines and was followed with infection studies, clearly showing that the increased CAD activities contributed to a decrease in disease susceptibility. The data obtained is valuable in evaluating the hypothesis that PGIP overexpression lead to possible priming through cell wall strengthening in anticipation of infection.

3.3 Materials and Methods

3.3.1 Bacterial strains and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli D*H5α was grown at 37˚C in Luria Bertani (LB) medium (Sambrook *et al*., 1989) supplemented with 100 µg/ml ampicillin as selection agent. *Agrobacterium tumefaciens* EHA105 were grown at 28˚C in LB medium supplemented with antibiotics; 50 µg/ml kanamycin and 10 µg/ml rifampicin for selection of transformants or to maintain selection pressure.

Table 1: Strains and plasmids used in this study

Strain/Plasmid	Description	Reference
Strains		
Escherichia coli DH5α	supE44lacU169[ø80lacZM15hsdR17recA1gyrA96thi-1relA1]	Life technologies (GIBCO/BL)
Agrobacterium tumefaciens EHA 105	Disarmed, succinomopine-type strain	Hood *et al*., 1993
Plasmids		
pGEM –T-Easy	pGEM5Zf(+)-based PCR cloning vector	Promega, Madision, USA
pART7	Cloning vector, CaMV 35S promoter, OCS 3' terminator	Gleave, 1992
pART27	Binary vector	Gleave, 1992
pGEM-*CAD14*	*CAD14* gene cloned into pGEM-T-Easy vector	This study
pART7-*CAD14*	*CAD14* gene cloned into pART7 vector	This study
pART27-*CAD14*	pART7-*CAD14* gene and LB-RB from pART7 cloned into pART27 vector	This study

3.3.2 Maintenance of plants

In vitro tobacco (*Nicotiana tabacum* cv Petite Havana SR1) (Lehle seeds, USA) plants were cultivated from sterile seeds on MS media (Murashige and Skoog, 1962) at 26˚C with 16 h-light/8 h dark photoperiod. A light intensity of 120 $\mu mol.m^2.s^{-1}$ was maintained for the photoperiod. In the green house, wild-type and transgenic tobacco seeds were sown into peat moss soil (Jiffy Products Inc AS, Kritiansand, Norway) under natural light at 26°C and 65% relative humidity. Plants were supplemented with a commercially available liquid organic fertilizer every two weeks (Nitrosol®, Fleuron (Pty) Ltd, South Africa).

3.3.3 RNA extraction from tobacco, cDNA synthesis and cloning of the tobacco *CAD* gene into a plant expression vector

All DNA manipulations were performed according to Sambrook *et al*. (1989), unless otherwise stated. RNA was extracted from six-week old tobacco plantlets (leaf material) using a sodium perchlorate-based method. An extraction buffer consisting of 5 M sodium perchlorate, 0.3 M Tris-HCl (pH8.3), 8.5% (w/v) polyvinylpolyprollidone (PVPP), 2% (w/v) PEG 4000, 1% (v/v) β-mercapto-ethanol and 1% (w/v) SDS was added to the finely ground plant material . The tissue was allowed to thaw in this buffer and agitated for 30 min at room temperature. Following centrifugation, the supernatant was passed through a syringe plugged with cotton wool to remove insoluble reagents and plant debris. Several phenol/chloroform extractions were performed before precipitating the RNA overnight with 2.5 M LiCl at -20ºC. The pellet was

washed with 70% ethanol and re-suspended. RNA was purified using the RNeasy Mini Kit (Qiagen GnbH, Hilden, Germany) and quantified using the Nanodrop (Wilmington, DE, USA). Two cDNA synthesis reactions of 25 µg each were set up for each test or reference RNA sample in a total volume of 30 µl. Before denaturation at 70ºC for 10 min, 2 µl of oligo d(T) primers (500 µg/ml) were added. Following denaturation, first strand buffer and DTT were added according to the manufacturer's recommendation (InVitrogen, Carlsbad, CA, USA). A 2:3 (aa-dTTP:dTTP) aminoally-dNTP (Ambion, Austin, TX, USA) mix was added to a 1X concentration (0.5 mM each of dATP, dCTP and dGTP, 0.2 mM aa-dUTP and 0.3 mM dTTP) before incubation at 46ºC for 2 min after which 200 U of SuperScript III Reverse Transcriptase (InVitrogen, Carlsbad, CA, USA) was added. The same amount of enzyme was added following incubation for 4 h at 46ºC, after which cDNA synthesis proceeded overnight.

A sequence encoding the *CAD* gene, as described by Knight *et al* (1992) was used to design gene specific primers (details below). The cDNA *CAD14* gene (1.3 kb) was amplified by polymerase chain reaction (PCR) from the synthesized cDNA. All PCR reactions were conducted in a Whatman Biometra Tri-thermoblock automated temperature cycler (Göttingen, Germany). The reactions were performed in a final volume of 50 µl with 2.5 U ExTaq DNA polymerase (Takara, Japan), 10X buffer, 200 µM dNTPs, 2 mM $MgCl_2$, 200 µM of primer 5´-GAA TTC ATG GGT GGC TTG GAA GTT-3´ and 5´-GGA TCC TTA CTG GTC AAG CTT GCT-3´ which included the *Eco*RI and *Bam*HI sites (underlined) respectively, and 5-10 ng of the template DNA. Thermal cycling was performed with the following parameters: an initial denaturation step at 95˚C for 5 min followed by 30 cycles of, 95˚C for 45 s, 56˚C for 30 s and 72˚C for 1 min, the amplification cycle was completed by a single incubation step at 72˚C for 5 min. The PCR products were fractionated on a 0.8% (w/v) TAE agarose gel.

The gel-purified PCR product was ligated into the cloning vector pGEM-T-Easy (Promega, Madison, USA) vector. The resulting plasmid, pGEM-*CAD*, was sequenced in both directions at Inqaba Biotech (Pretoria, South Africa) with the T7 and SP3 primers. Comparative sequence analysis was conducted by using the advanced basic local alignment search tool (BLAST) of the National Center for Biotechnological Information (NCBI) (www.ncbi.nlm.nih.gov).

The gene was excised from the pGEM-*CAD14* vector with *Bam*HI and *Eco*RI (Fermentas, Hanover, MD) and was sub-cloned into the cloning vector pART7 (Gleave, 1992) between the cauliflower mosaic virus (CaMV 35S) promoter and nopaline synthase (NOS) terminator using the *Bam*HI and *Eco*RI sites, resulting in the pART7-*CAD14* expression cassette. Small scale plasmid preparations and restriction mapping with *Bam*HI, *Eco*RI and *Pvu*I confirmed the presence of the desired gene expression cassettes in the correct orientation. The gene expression cassette was removed from pART7-*CAD14* with *Not*I and sub-cloned into the binary vector pART27 (Gleave, 1992) with the same site, yielding the pART27-*CAD14* expression vector. The integrity of the expression vector was verified by restriction mapping using *Sal*I, *Pst*I and *Bam*HI and sequencing.

3.3.4 Stable transformation of *Nicotiana tabacum* with the CAD expression cassette

The pART27-*CAD14* expression vector was transformed into *A. tumefaciens* EHA105 by electroporation (Hood *et al*., 1993). PCR was performed using *CAD* gene specific primers to confirm the presence of the binary vector in the transformed *Agrobacterium* strain. *N. tabacum* leaf discs were transformed with the pART27-*CAD14* construct via *A. tumefaciens* mediated leaf disc transformation according to Gallios and Morinho (1995) with various modifications. Ten milliliters (ml) *A. tumefaciens* cells containing pART27-*CAD14* were grown to an OD_{600} of 1.0, harvested by centrifugation and were resuspended in 10 mM Mg_2SO_4. Twenty ml of the *Agrobacterium* suspension was pipetted into a sterile petri dish. The leaf discs were placed in the *Agrobacterium* suspension and incubated at room temperature (RT) for 20 min. The discs were then transferred to a co-cultivation medium and left in the light for three days. After three days, the leaf discs were washed in MS liquid media containing 400 µg/ml cefotaxime to suppress *Agrobacterium* growth. Shoot formation was initiated on MS medium supplemented with 2 mg/ml BAP (6-benzyl-aminopurine) (Sigma, Aston Manor, South Africa), 100 µg/ml kanamycin and 400 µg/ml cefotaxime. The leaf disks and budding shoots were transferred to fresh medium every week. Differentiated shoots were excised and rooting was induced on MS media supplemented with 2 mg/ml NAA and 100 µg/ml kanamycin. Rooted primary (T_0) transformants were transferred into peat moss soil and maintained in a green house. Plants flowered naturally and T_0 population seeds were collected. The segregation of the T_1 progeny for kanamycin resistance was established by kanamycin selection on MS medium supplemented with kanamycin. All subsequent analysis were performed on T_1 progeny plant lines.

3.3.5 Southern Blot analysis

Genomic DNA was extracted from 0.1 g of tobacco leaves according to the method described by McGarvey and Kaper (1991). The transformed and WT plant material were ground in liquid nitrogen. Genomic DNA was extracted with 800 µl of extraction buffer (3% (w/v) CTAB (Cetyltrimethylammonium bromide), 1.4 M NaCl, 0.02 M EDTA (Ethylenediaminetetracetic acid), 1 M Tris/HCl (pH 8.0)). For Southern blot analysis, total genomic DNA was digested with *Xba*I (Fermentas, Hanover, MD) and the fragments were fractionated on a 0.8% (w/v) TBE (Tris-borate-EDTA) agarose gel by electrophoresis. The fractionated DNA was transferred from the agarose gel onto a Hybond N+ nylon membrane (Amersham, Bioscience, UK) by capillary transfer in TBE buffer and then fixed by irradiation with ultraviolet light. Pre-hybridization (2 h) was performed with a DIG Easy Hyb solution at 37°C and hybridization was with the same solution with the addition of a randomly DIG labeled CAD probe. Detection was performed according to the DIG manufacturer's instructions (Roche Diagnostics GmbH, Mannheim,

Germany). *Xba*I an enzyme that does not have a recognition site within the *CAD* gene was used, thus each band corresponds to a single integration event.

3.3.6 Northern Blot analysis

Total RNA was extracted from tobacco leaves according to the method of Chang *et al*. (1993). Five micrograms of total RNA per lane were fractionated on a 1.2% (w/v) agarose gel in 1 X FA buffer, with 0.7% (v/v) formaldehyde. The fractionated RNA was transferred from the agarose gel onto a Hybond N+ nylon membrane (Amersham, Bioscience, UK) by capillary transfer in 20 X SSC and then fixed to the membrane by irradiation with ultraviolet light. Pre-hybridization (2 h) was performed at 50°C in high SDS hybridization buffer (7% (w/v) SDS (Sodium dodecyl sulfate); 50% (w/v) formamide; 2% (w/v) casein blocking solution; 0.1% (w/v) N-lauroyl sarcosine). Hybridizations (16 – 20 h) were in the same solution with the addition of a PCR DIG-labeled CAD probe. Detection was performed according to the DIG manufacturer's instructions.

3.3.7 Measuring CAD enzyme activity in transgenic tobacco

Enzyme assays were performed in triplicate as described by Raes *et al*. (2001), with few modifications. Plant leaf positions 4 and 5, and whole stems (200 mg) from six-week-old plants were harvested and frozen in liquid nitrogen. Crude protein was extracted with 1 ml Tris/HCl (100 mM, pH 8.8) and centrifuged at 3000 g for 15 min, 4°C. The assay was performed in a microtiter plate with 125 µl Tris/HCl (100 mM, pH 8.8), 25 µl coniferyl alcohol (200 mM) as substrate and 75 µl crude protein. The plate was incubated in the dark at 30 °C for 10 min. The assay was initiated by addition of 100 µl NADP (50 mM) and readings were taken at two minute intervals for ten minutes. The 10 min readings were used for analysis. CAD enzyme activity was assayed spectrophotometrically by measuring the increase in absorbance at 400 nm when coniferyl alcohol is oxidized to coniferaldehyde (Wyrambrik and Grisebach, 1975). For each plant line, three biological repeats were assayed and three technical repeats were performed. Protein concentration was determined using a Bradford dye-binding assay in triplicate using bovine serum albumin (BSA) as a standard (Bradford, 1976). The enzyme activity was expressed as $mM.mg^{-1}$ protein.min^{-1} ($A = \varepsilon.c.L$). The transmittance and absorbance of a sample depends on the molar concentration (c), the light path length in centimetres (L) and molar absorptivity (ε) for the dissolved sample. The values shown are averages of all biological and technical repeats.

3.3.8 Whole plant infections of transgenic plants

Infection assays were performed on untransformed controls, *CAD* transgenic (T_1 population) plants, as well as a *Vvpgip1* transgenic line with a previously characterized PGIP-specific phenotype (Joubert *et al*, 2006). *Botrytis cinerea* pathogenic cultures were grown as described in Joubert *et al*. (2006). Six to eight week old plants were placed in transparent Perspex humidity chambers at 100% relative humidity and a light intensity of 120 $\mu mol.m^2.s^{-1}$ for a 16 h period, after which 5 µL of a *B. cinerea* spore suspension (1×10^3 spores in a 50% grape juice medium) was spotted on the adaxial surface of the leaves, without wounding or detachment. Three leaves of each plant (leaf position 3-5) were inoculated with four spots per leaf. Four plants per plant line were inoculated. Measurements of lesions were performed according to Joubert *et al*. (2006). Significant differences and homogeneous groups were calculated by performing a one way Analysis of Variance (ANOVA) using the STATISTICA 7 (StatSoft Inc, Tulsa, OK, USA) software package at 95% confidence. Mean values were used to determine significant differences ($p \leq 0.05$) between transgenic lines.

3.4 Results

3.4.1 Isolation of the tobacco *CAD14* gene and overexpression of the gene in tobacco plants

The *CAD* gene was amplified from tobacco cDNA; sequence analysis showed a 100% sequence homology to *CAD14* (GenBank Accession no. X62343.1) and 94% similarity to *CAD19* (GenBank Accession no.X6344.1) of *Nicotiana tabacum*. The gene is 1398 nucleotides long with an open reading frame of 357 amino acids. The *CAD14* gene was further sub-cloned into a plant expression vector pART27 resulting in the pART27-*CAD14* expression cassette (Figure 1). The pART27-*CAD14* plasmid map was drawn using Vector NTI ®.

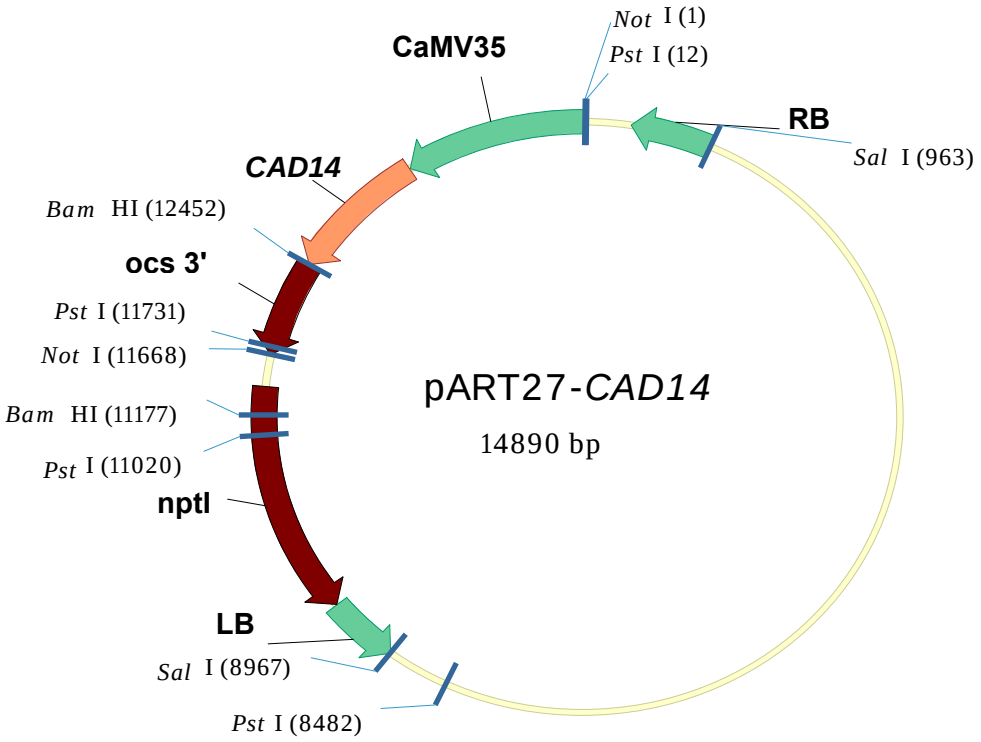


Figure 1: Plasmid map of pART27-*CAD14* expression vector. The vector contains the tobacco *cinnamyl alcohol dehydrogenase* gene (*CAD14*). RB and LB correspond to the right and left T- DNA borders respectively; CaMV 35Sp denotes the constitutive Cauliflower Mosaic Virus 35S promoter; ocs_T the octopine synthase terminator; nptII the neomycin phosphotransferase II gene encoding antibiotic resistance and regulated by the nopaline synthase promoter and terminator.

The pART27-*CAD14* expression vector was transformed back into tobacco and putative transgenic plants were regenerated. From the initial extensive putative transgenic population (more than a hundred lines), a subset of approximately 20 primary transgenic plants (T_0) were randomly selected. Their self pollinated seeds were collected for further analysis. From the self-pollinated seeds, ten T_1 kanamycin resistant transgenic plants were randomly selected and grown in the green house for further analysis. All the plants showed normal germination and growth rates as well as normal vegetative and reproductive morphology.

3.4.2 Evaluation of CAD integration and expression in transgenic plants overexpressing the *CAD14* gene

The plant lines were subjected to Southern blot analysis to confirm gene integration (Figure 2). The probe detected two bands corresponding to the two tobacco *CAD* native genes, *CAD14* and *CAD19* in the SR1 and empty vector controls. All other signals correspond to the integrated transgenes. From lines CAD 30, 33 and 44 the insert could not be detected, while CAD 4, 31, 32, 42, and 43 showed unique integration patterns compared to the SR1 control plants. CAD 38 had the same integration pattern as CAD 31 and was thus considered clonally derived.

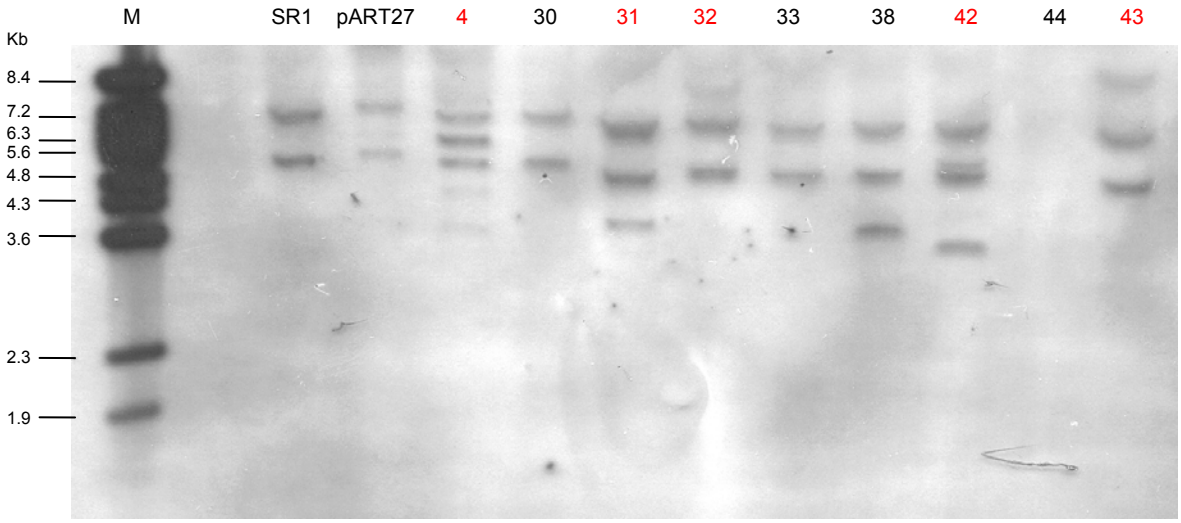


Figure 2: Gene integration analysis by Southern Blot of T_1 generation plants overexpressing the tobacco *cinnamyl alcohol dehydrogense* (*CAD14*) gene. Genomic DNA was isolated as described above. Total gDNA was digested with *Xba*I and fractionated in a 0.8% TBE gel then blotted onto a positively charged nylon membrane. The membrane was hybridized with a DIG-labeled *CAD* gene specific probe. The probe detected bands that correspond to the *CAD* tobacco native gene copies and the pART27-*CAD* integration gene event. Each band corresponds to a single gene integration event. The numbers indicate *CAD* transgenic lines tested. The marker lane (M) contains lambda DNA digested with *Bst*EII, was used as a marker. Sizes for the standard DNA fragments are indicated in kb. The numbers highlighted in red indicate the plants used for further analysis. pART27 indicates plants transformed with an empty vector. SR1 indicate untransformed control.

Expression of the CAD14 transcripts in the transgenic tobacco plant lines was determined by northern blot analysis. The blots were hybridized with a DIG-labeled *CAD* gene specific probe. The probe detected a single band of a size corresponding to the *CAD14* transcripts of approximately 1398 nt in the transformed plants (Figure 3A) (Damiani *et al*., 2005). CAD 4, 32 and 42 were the highest expressing lines followed by CAD 42, 31 and 43. All these lines were used for further analysis. The native CAD expression could only be seen in the control plants when a higher concentration of RNA was used for analysis (Figure 3B). The two native *CAD* genes express RNA transcripts of similar size.

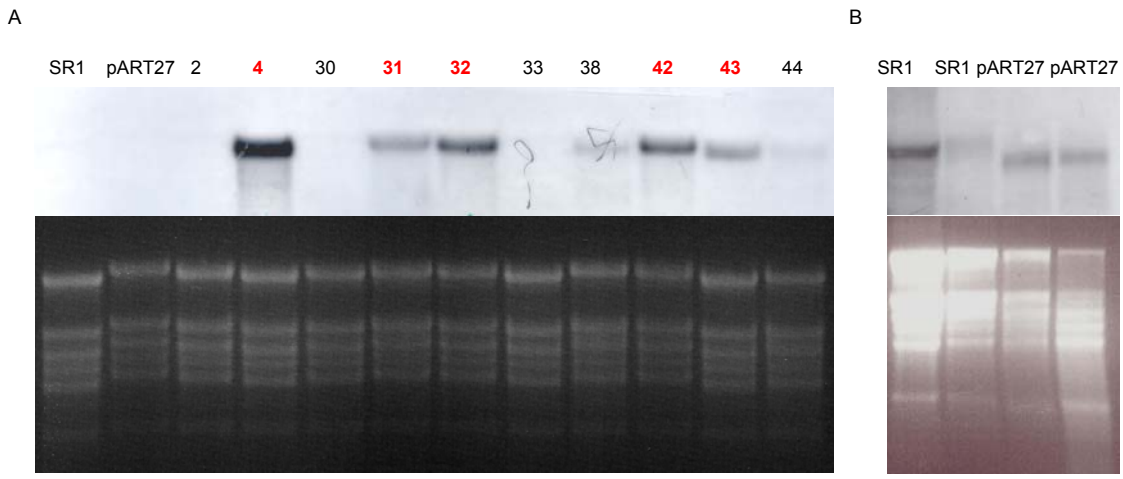
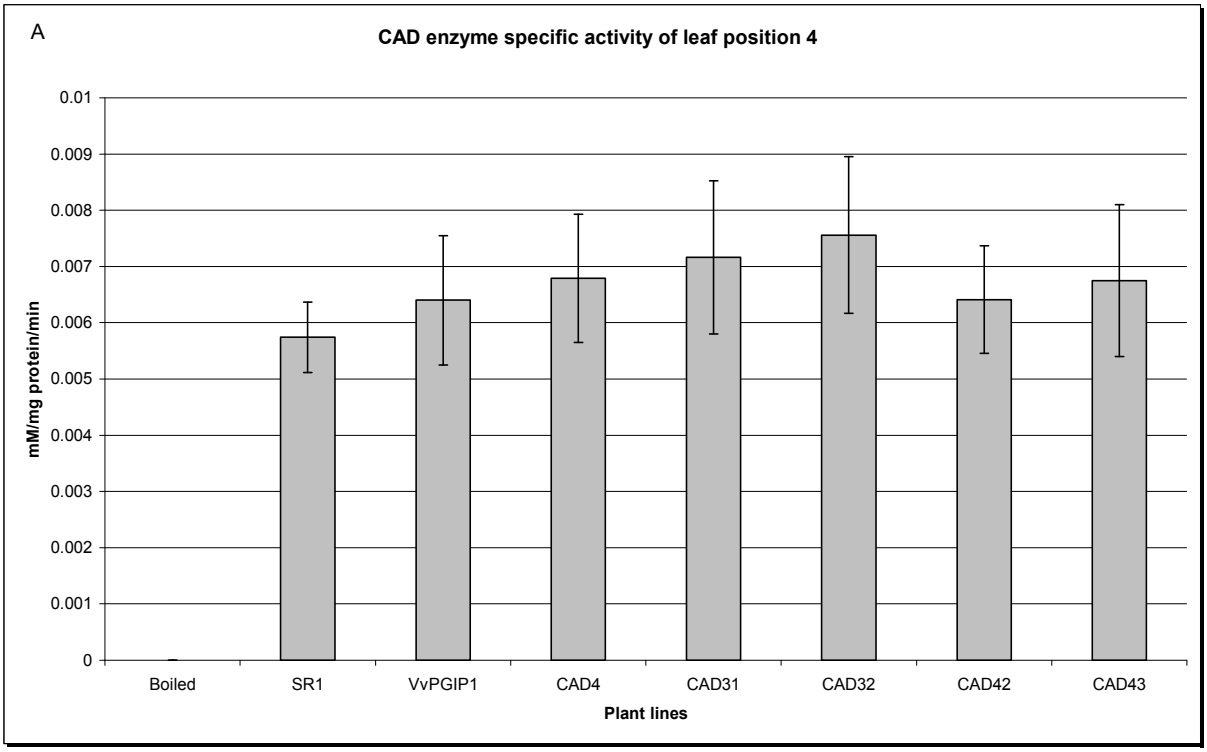
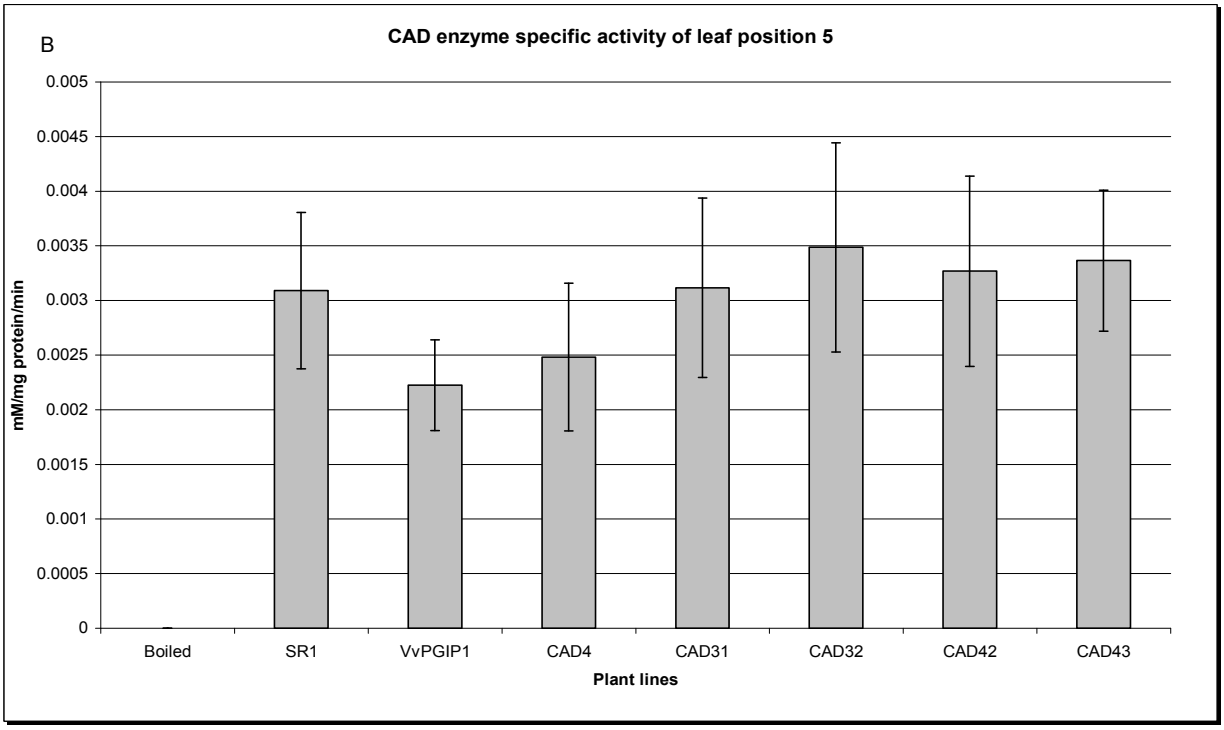


Figure 3: Gene expression of tobacco *cinnamyl alcohol dehygrogenase* (*CAD*) genes in the T_1 generation of CAD transgenic tobacco and controls. Five micrograms of total RNA from leaves was run on an agarose-formaldehyde gel and blotted onto a positively charged nylon membrane. The membrane was hybridized with a DIG-labelled *CAD* gene specific probe. The probe detected a single band of a size corresponding to *CAD* gene transcripts. The RNA gel shows comparative loading in each lane. Ethidium bromide staining of the formaldehyde agarose gel is shown. The numbers highlighted in red indicate the plants used for further analysis. pART27 indicates the plants transformed with an empty vector. (A) Quantitative analysis of *CAD* gene expression in T_1 generation. (B) The expression of the *CAD* gene in SR1 and pART27 empty vector control plants when a higher concentration of RNA (approximately 15 µg) was used. SR1 indicates an untransformed control.

3.4.3 Evaluation of the CAD enzyme activity

To assess enzyme activity of CAD in transgenic plants overexpressing the *CAD14* gene, crude protein extracts were prepared from six week old transgenic (T_1 population) and untransformed SR1 control plants. The enzyme activity of the transgenic plants was compared relative to the untransformed control plants. A representative transgenic VvPGIP1 tobacco line previously characterized with elevated expression of *CAD* was also included in the study. Coniferyl alcohol was used as a substrate throughout. The assays revealed that the overexpression of the *CAD* gene lead to small, but statistically significant increases in CAD activity when the untransformed control and the transgenic lines were compared (Figure 4), specifically in whole stems (Figure 4C). PGIP1 and CAD4 had the highest CAD activity in the stems compared to other transgenic plant lines. The tobacco stems had 10 fold higher CAD activity compared to leaves. Comparing the stems of the transgenic and control populations, it was clear that the individual transgenic lines were not statistically significantly different from each other, but as a population they could be separated from the untransformed control. The younger leaves displayed a similar trend (Figure 4A) although the overall values were very low and did not statistically separate due to fairly large error margins. In the slightly older leaves CAD activity was similar in the control and transformed lines (Figure 4B). CAD enzyme activity was also lower in leaf position 5 compared to leaf position 4. The assays also confirmed that the VvPGIP1 (line 45) plants had elevated CAD activity, specifically in the stems and young leaves (Figure 4A, C). In the older leaves the activity was lower than that of the wild type (Figure 4B).


A
CAD enzyme specific activity of leaf position 4
mM/mg protein/min
0.01
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
Boiled
SR1
VvPGIP1
CAD4
CAD31
CAD32
CAD42
CAD43
Plant lines


B
CAD enzyme specific activity of leaf position 5
mM/mg protein/min
0.005
0.0045
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
Boiled
SR1
VvPGIP1
CAD4
CAD31
CAD32
CAD42
CAD43
Plant lines

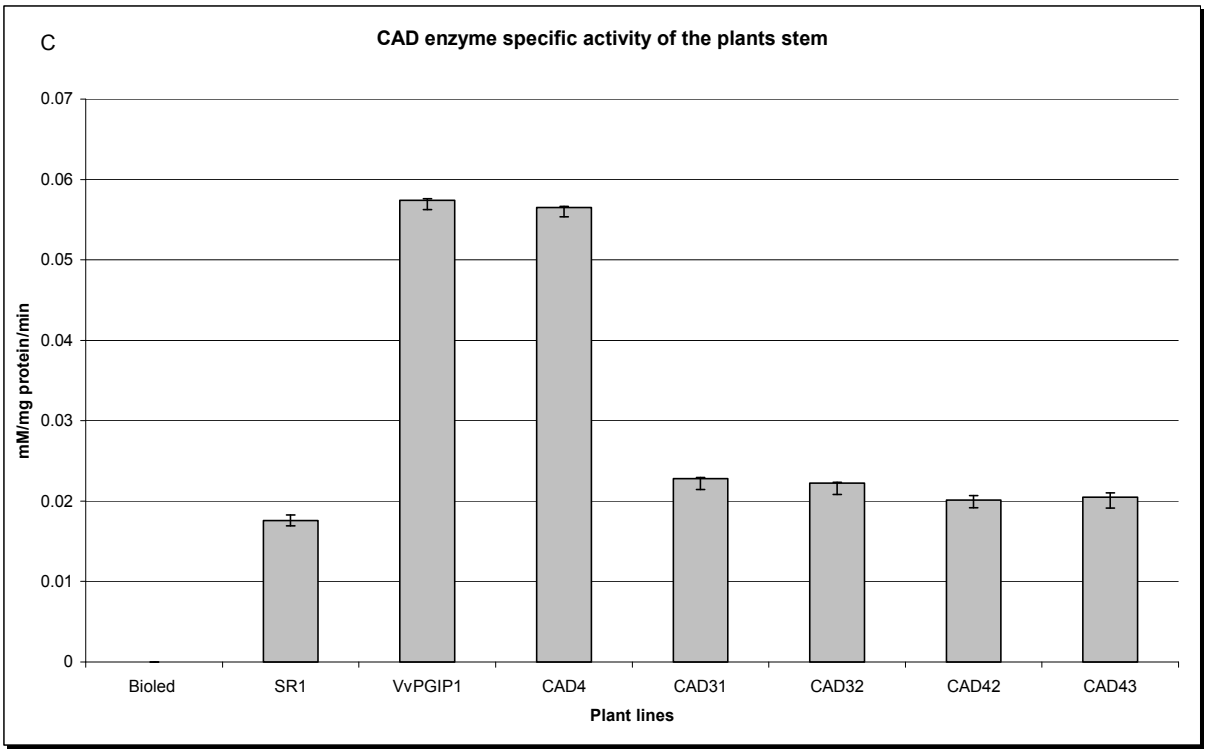


Figure 4: Cinnamyl alcohol dehydrogenase (CAD) activity of CAD-transgenic tobacco and control populations. PGIP refers to a transgenic plant overexpressing the grapevine *PGIP1* gene (VvPGIP1 transgenic tobacco line 45, as described in Joubert *et al*., 2006). The assays were performed on six-week old green house acclimatized transgenic plants. The CAD activities (in mM/mg protein/min) recorded from leaf position 4 (A); leaf position 5 (B) and whole stems (C). Three biological repeats and three technical repeats were performed per plant line. The boiled sample is the enzyme assay negative control. SR1 indicates an untransformed control.

3.4.4 Whole plant infections with *Botrytis cinerea*

To assess whether transgenic tobacco plants overexpressing the *CAD14* gene would show reduced disease susceptibility to *B. cinerea*, a whole plant infection assay was conducted on the transgenic and control lines. Five plant lines overexpressing the tobacco *CAD14* gene, the VvPGIP1 (line 45), and an untransformed SR1 control plant were infected with *Botrytis* spores. The infections were followed for 11 days and 93 - 96% of the inoculums sites developed into primary lesions within 3 days post inoculation (dpi) (Table. 2).

Table 2: Analysis of lesion development on transgenic tobacco lines overexpressing the *CAD* gene following whole plant infections with *Botrytis cinerea*

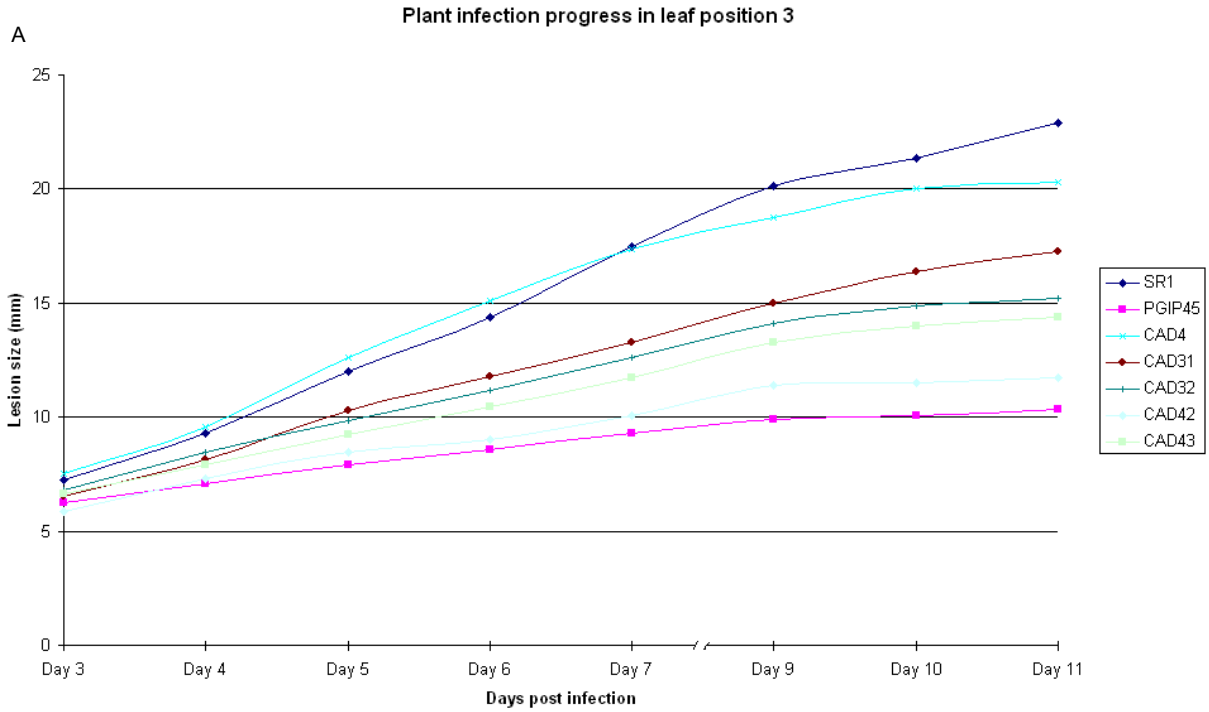
Plant lines	Infection rate (%)	Average lesion size (mm)				Percentage decrease in disease susceptibility (compared to SR1)[a]	Homogenous groups[a]
		Pos 3	Pos 4	Pos 5	combined		
VvPGIP1	97	10.4 (±1.5)	14.5 (±2.8)	12.8 (±2.3)	12.6 (± 2.1)	48%	C
CAD32	93	15.2 (± 3.1)	14.4 (±4)	14 (± 3.2)	14.5 (±3.4)	40%	A, C
CAD43	97	14.4 (± 2.8)	18.3 (± 3.7)	14 (± 3.2)	15.6 (± 3.2)	35%	A,B,C
CAD42	97	11.8 (± 0.8)	17.4 (± 4.4)	19.2 (± 5.8)	16.4 (± 3.6)	32%	A,B,C
CAD31	100	17.2 (± 3.7)	18.5 (± 4)	15 (± 3.1)	16.8 (± 2.6)	30%	A,B,C
CAD4	93	20.3 (± 4.7)	17.1 (± 5.1)	15.7 (± 5.8)	17.7 (± 5.2)	27%	A,B,C
SR1	97	22.9 (± 10.4)	24.6 (± 6.3)	24 (± 5.4)	24.0 (± 7.3)	0%	D

The decrease in disease susceptibility was calculated by comparing the average lesion size at 11 dpi of all plant lines to the untransformed control plant. Leaf positions 3, 4 and 5 are indicated.

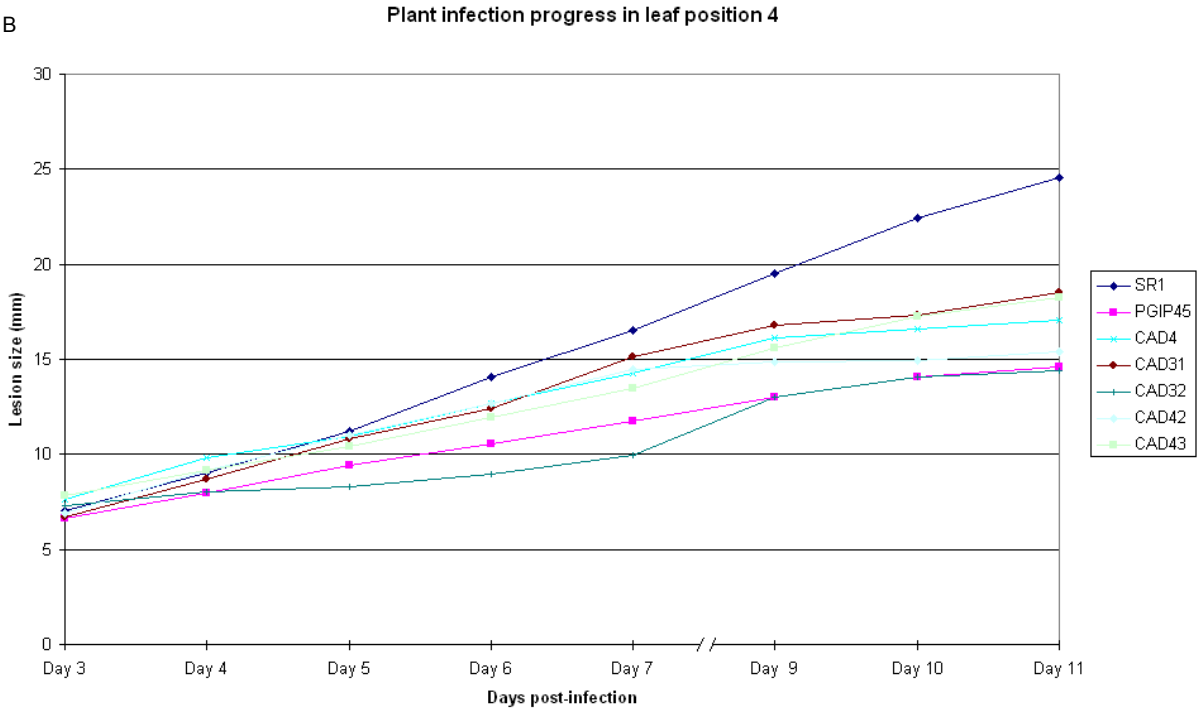
[a]Three independent whole plant infections were carried out on the different plant lines. Significant differences and homogeneous groups were calculated by performing a one way Analysis of Variance (ANOVA) on the combined data using the STATISTICA 7 (StatSoft Inc, Tulsa, OK, USA) software package at 95% confidence.

Lesion sizes were measured daily from day three to eleven dpi and compared per leaf (Table. 2 and Figure 5 A-C), as well as combining the results of all leaves per line to evaluate the level of susceptibility per line (Figure 5D). Comparing disease progression over the 11 days, the untransformed controls developed severe symptoms and actively spreading infections, also leading to *Botrytis* spore formation on the infected surfaces (Figure 6). The different leaf positions all yielded the same trend: the untransformed control was heavily infected, while the PGIP overexpressing line consistently lead to a strong resistance phenotype. The CAD overexpressing lines also showed reduced susceptibility with smaller and more confined lesions developing than the wild type (Figure 6). Moreover, the appearance of the lesions were quite different in the CAD overexpressing lines; these lines developed dry and confined lesions which were almost always surrounded by a spreading yellow halo. The PGIP overexpressing line had the smallest lesions sizes, and with a dry and restricted appearance without any chlorotic or yellowing zones on the leaves. The VvPGIP1 lines lead to a decrease in disease susceptibility of 48%, whereas the CAD population decreased susceptibility by 20-40%, with an average reduction of 33% compared to the SR1 control plant. Data from all the individual leaves were combined at 11 dpi and subjected to statistical analysis. Statistical analysis grouped the transgenic lines into four homogenous groups based on lesion size at 11 dpi (Table 2). The VvPGIP1 line and the SR1 control grouped separate groups to any of the transgenic lines with the VvPGIP1 line in a separate grouping that overlapped with some lines of the CAD population.

A


Plant infection progress in leaf position 3
Lesion size (mm)
25
20
15
10
5
0
Day 3
Day 4
Day 5
Day 6
Day 7
Day 9
Day 10
Day 11
Days post infection
SR1
PGIP45
CAD4
CAD31
CAD32
CAD42
CAD43

B


Plant infection progress in leaf position 4
Lesion size (mm)
30
25
20
15
10
5
0
Day 3
Day 4
Day 5
Day 6
Day 7
Day 9
Day 10
Day 11
Days post-infection
SR1
PGIP45
CAD4
CAD31
CAD32
CAD42
CAD43

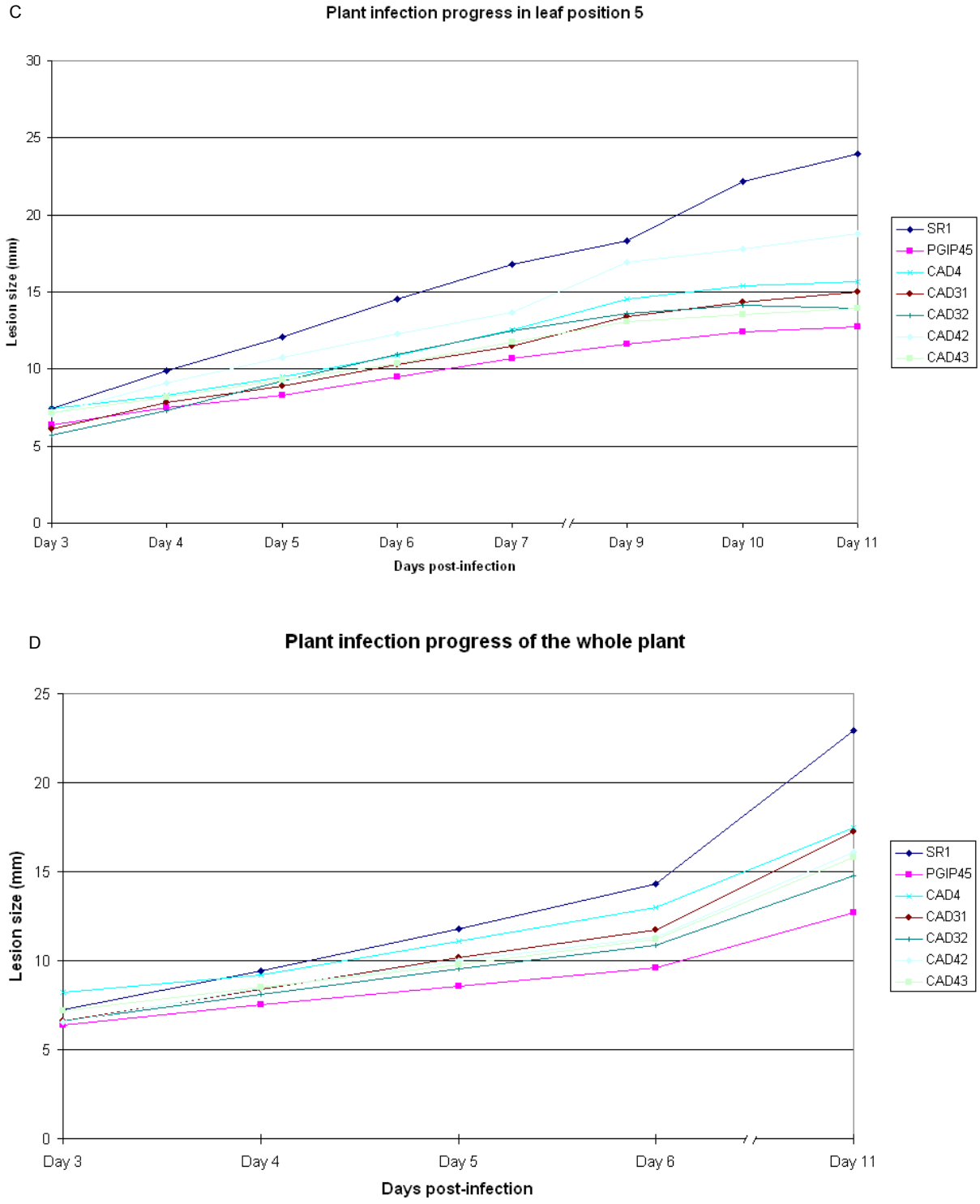
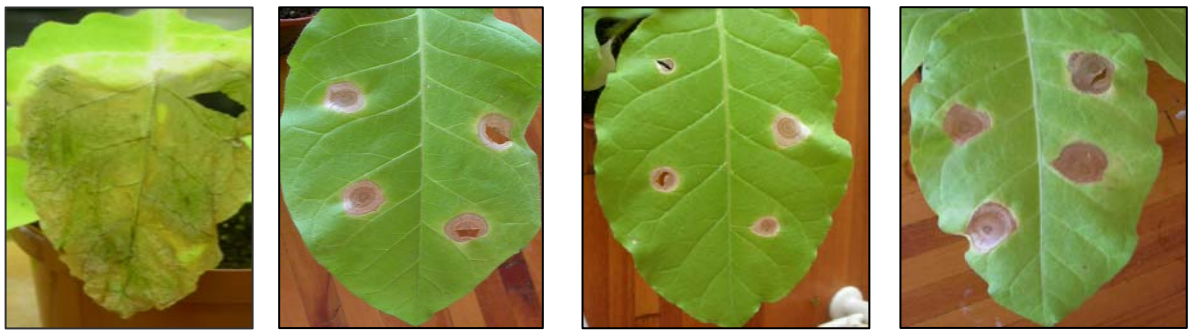


Figure 5: *Botrytis cinerea* lesion development during a whole plant infection assay of CAD transgenic plants and non-transgenic SR1. VvPGIP1 is a transgenic line with a confirmed PGIP-specific resistance phenotype against *B. cinerea* (as described in Joubert *et al*., 2006). The lesion sizes were measured at the indicated days post-inoculation. The mean lesion diameter of the developing lesions at the indicated days post inoculation of transgenic plants and untransformed control plant are shown. Lesions were recorded at leaf position 3 (A), leaf position 4 (B), and leaf position 5 (C) .The combined results of all the leaf positions are shown in (D). One-way Anova indicated significant differences between the mean lesion diameter of the plant lines at 11 dpi (t-test, $P<0.05$).



SR1 VvPGIP1 CAD32 CAD43

Figure 6: Healthy transgenic tobacco plants were inoculated with *Botrytis cinerea* spores. Five lines of CAD progeny were inoculated with 1 x 10^3 *Botrytis* spores in a 50% grape juice medium. In each plant three leaves were inoculated with four spots per leaf. Lesion development was assessed three days post-inoculation until day 11 when the lesions were statistically significant between the different plant lines. The necrotic lesions of the CAD transgenic plants were brown, dry and with distinct yellowing around the plant lesion, whereas the SR1 lesions were rapidly spreading water-soaked with visible fungal survival structures and sporulation. VvPGIP1 is a transgenic line overexpressing a grapevine *PGIP* gene and with a confirmed PGIP-specific resistance phenotype against *Botrytis* (Joubert *et al*., 2006). CAD32 and CAD43 are transgenic lines overexpressing the tobacco *CAD14* gene. Pictures were taken 11 days post-inoculation, when it became impossible to measure distinct lesions on the WT.

3.5 Discussion

The defense mechanisms of plants comprise many different components, including preformed, passive defenses, as well as inducible active defense responses. Lignification is well established as an effective means to structurally reinforce plant tissues to protect against disease and damage. In the current study, the effect of CAD overexpression in tobacco was evaluated, specifically to establish whether or not increased CAD activity would lead to disease resistant phenotypes. The motivation to study CAD overexpression stems from a previous observation that under uninducing conditions (i.e. in the absence of infection or induction of resistance responses); CAD transcripts were upregulated in transgenic tobacco lines that were engineered to overexpressed a grapevine PGIP (Becker, 2007). These lines were highly resistant against *B. cinerea* infection (Joubert *et al*., 2006).

A transgenic tobacco population overexpressing the native *CAD14* gene was established and genetically characterized. In this study, a native *CAD* gene known as a marker for lignification, was isolated and overexpressed (see Figure 1 for construct) in tobacco. The isolated cDNA sequence showed 100% homology with *CAD14* from tobacco. In Southern blot analysis, both native *CAD* genes (*CAD14* and *CAD19*) (Halpin *et al*., 1992) was observed in the SR1 control line, the empty vector control line, as well as all the transgenic lines. These genes are derived from the two parental lines of *N. tabacum, N. sylvestris* and *N. tomentositormis*. The close similarity between the two *CAD* genes suggests that the two genes in *N. tabacum* are the same gene originating from the two parental lines, rather than two distinct isoforms of CAD (Knight *et al*., 1992). The enzymes have a specific cofactor requirement for NADP and have

high affinity for coniferylaldehyde which is converted to coniferyl alcohol (Halpin *et al*., 1992; Damiani *et al*., 2005).

The *CAD14* gene was transformed into tobacco via *A. tumefaciens* and a large putative transgenic population was generated. A randomly selected subset of plant lines were subjected to further analyses. The transgenic population was characterized by confirming transgene presence and integration (Figure 2) and expression (Figure 3). Northern blot analysis of the transgenic population confirmed *CAD* expression in the leaves, but did not pick up *CAD* transcripts in leaves from the untransformed and vector controls (Figure 3A), unless very high concentrations of RNA was used (Figure 3B). It is known that *CAD* expression and activity is highest in stem tissue (Tavares *et al*., 2000, Reas *et al*., 2003, Sibout *et al*., 2003; Damiani *et al*., 2005) with very low basal expression levels in other organs such as leaves. High transcript levels were also found in the petiole and leaf veins by Damiani *et al*. (2005). *CAD* expression is typically regulated by developmental and environmental stress factors. The results obtained with the expression analysis confirmed that the transgenic lines accumulated *CAD* transcripts in organs (such as leaves) that do not normally express *CAD* under uninducing conditions.

Did CAD overexpression contribute to increased CAD activity? CAD catalyzes the reduction of cinnamyl aldehydes to the corresponding cinnamyl alcohols which are the direct monomeric precursors to the lignin polymer (Ralph *et al*., 1997, Jung and Ni, 1998, Mitchell *et al*., 1999). In this study, overexpression of a *CAD* gene lead to small, but significant increases in CAD activity compared to the wild type in the stems. These enzyme activity assays were performed on healthy uninfected plants, without inducing native *CAD* expression or resistance phenotypes (i.e. without *Botrytis* infection). CAD activity is known to be highest in stems and actively growing organs, which was clearly confirmed by the CAD activity assays (Figure 4). The CAD transgenic population showed a statistically significant increase in CAD activity in the stems (as a population) when compared to the untransformed control. A similar, but less pronounced trend was observed for young leaves, and in older leaves the CAD activities of the control and CAD population could not be separated. Although increased levels of transcripts were present in the leaves in the transgenic population, the overall CAD enzyme activity under these conditions was very low, suggesting that the regulatory mechanisms impacting on CAD activity is not only transcriptionally derived. It has been shown that lignin biosynthesis and the control of the process is complex (Vermerris and Nicholson, 2008). Moreover, the very low levels of CAD activity in the leaf samples probably requires further optimization of the CAD activity assay to accurately determine the absolute levels – the assay was probably not linear in these ranges, contributing to the large variance in the samples. The data from the leaf samples should therefore be treated as a trend analysis rather than statistically confirmed data. The activity levels in the stems fell within the linear range of the assay and displayed small error bars and could be statistically analyzed. The high CAD activity observed in theCAD4 line could

be due to the specific integration site of the transgene in this line, possibly leading to a very favorable position in the genome.

A transgenic tobacco line overexpressing the grapevine PGIP that has been shown to exhibit strong PGIP-specific resistance phenotypes against *B. cinerea* was also included in the CAD activity study. The CAD assays confirmed previous results that indicated that CAD transcription was upregulated in the PGIP line in the absence of infection. The PGIP lines displayed very high levels of CAD activity in the stems. Overall, in all plant lines the stems exhibited 10-fold higher levels of CAD activity than the leaves, but the transgenic PGIP line showed a further 2-3-fold increase in CAD activity in the stems, when compared to the untransformed control and the majority of the CAD overexpressing lines. PGIPs have been shown to move apoplastically in the plant body and accumulate in the vascular tissues of stems (Agüero *et al*., 2005); the high levels of CAD activity in the stems of the PGIP overexpressing line might be due to an accumulation of PGIPs in the stems and a subsequent upregulation of *CAD* expression. How an abundance of PGIPs trigger CAD expression in these lines is currently not known.

Another aspect that still needs to be investigated relates to the lignin content in the various transgenic lines. Preliminary lignin assays were inconclusive (results not shown) and although the trend was that the transgenic lines displayed higher levels of lignin, these assays will have to be optimized and repeated before a correlation can be drawn between CAD activity and lignin content. It is known that the overall lignin content could appear unaffected even if gene expression in the biosynthetic pathway has been manipulated (Rastogi and Dwivedi, 2008). Lignin composition could however change (Rastogi and Dwivedi, 2008) and also needs to be evaluated before a conclusion can be drawn regarding the changes that might be linked to the overexpression of the *CAD* gene.

The CAD overexpressing lines were more resistant against *Botrytis* than the WT control. Having established that *CAD* gene expression and enzyme activity were higher in VvPGIP1 and CAD plant lines compared to untransformed control plants specifically in the stems, the decrease in disease susceptibility was assessed with whole plant infections. Plants were inoculated with *B. cinerea* spore suspension on the adaxial surface of the leaves. A very high spore load and high humidity conditions ensured effective infection rates. The CAD overexpressing lines showed a decrease in disease susceptibility compared to the control. An average decrease in disease susceptibility of 33% was recorded in the transgenic population. The increase in resistance compared to the control was more pronounced in younger leaves. The transgenic population displayed confined and dry lesions that formed distinctive yellow zones surrounding the infection spots. The plant lines overexpressing VvPGIP1 and *CAD* not only showed a reduction in lesion size and spread, but also a reduced development of fungal biomass. The reduction in plant tissue maceration and lesion expansion was most pronounced

in the VvPGIP1 line compared to the CAD transgenic plants, while the CAD transgenic plants showed more reduction than the SR1 control plant. This whole plant infection assay revealed very encouraging results and should be repeated in an independent infection experiment to confirm the data.

The results obtained confirmed that increased *CAD* expression could contribute to resistance phenotypes in tobacco. The fact that the PGIP overexpressing line clearly performed better in the infection assay is probably due to the fact that in this line, the CAD overexpression contributes to the defense in addition to the normal defense functions of PGIPs.

Increased CAD activity in response to PGIP overexpression: indications of "primed" cell wall re-inforcements? Polygalacturonase-inhibiting proteins (PGIP) play a crucial role in the plant defense process by interacting with hydrolytic enzymes secreted by the invading fungal pathogens (De Lorenzo *et al*., 2001), thereby limiting the invasion of the fungi as well as triggering subsequent defense responses in the plant. Overexpression of PGIP in transgenic plants inhibited the destructive potential of the fungi (Powell *et al*., 2000; Ferrari *et al*., 2003; Agüero *et al*., 2005; Manfredini *et al*., 2005; Joubert *et al*., 2006; Joubert *et al*., 2007; Janni *et al*., 2008). PGIP transcription levels are crucial at the time of pathogen infection (Yao *et al*., 1999) as demonstrated by Johnston *et al*. (1993) and Salvi *et al*. (1990).

In a previous study, the grapevine (*Vitis vinifera*) PGIP encoding gene was isolated, cloned and stably transformed into *N. tabacum* (De Ascensao, 2001). PGIP1 was also shown to be highly active against PGs of *B. cineara* (Joubert *et al*, 2006; Joubert *et al*., 2007). The overexpression of the grapevine PGIP rendered the transgenic plants less susceptible to infection by *Botrytis*. A reduction in disease susceptibility of 47-69% was observed in a comprehensive whole plant infection (Joubert *et al*., 2006). Transcriptomic analysis revealed that under uninduced or uninfecting conditions, the overexpression of grapevine *pgip* gene in tobacco resulted in induced expression of seemingly non-associated genes, such as the *CAD* gene involved in lignin formation (Becker, 2007).

CAD is known as a marker for lignin-formation (Walter *et al*., 1988; Mitchell *et al*., 1994) and lignification is known to play a role in plants as a pre-formed defense response (Vermerris and Nicholson, 2008). Up-regulation of the CAD enzyme could allow reinforcement of the cell wall by lignification prior to fungal ingress, therefore enhancing the plants primary defense. The VvPGIP1 transgenic plants with reinforced cell walls might thus be "primed" before pathogen ingress, contributing to the decrease in disease susceptibility observed in lines accumulating high levels of PGIP. Priming is a mechanism by which plants show induced resistance to pathogens after a conditional treatment (Beckers and Conrath, 2007). In this mechanism, defense responses are not activated directly, but are accelerated upon pathogen or insect attack, resulting in enhanced resistance (Wees *et al*., 2008) and increasing the plants ability to perceive stress stimuli more effectively (Conrath *et al*., 2006). Although priming has been

known for many years as a component of defense response in plants (Kuć, 1987), it has never been associated with PGIP overexpression. The results of this study contributes to the current working hypothesis that the decrease in *Botrytis* symptoms in grapevine PGIP overexpressing lines is not only due to the ability of VvPGIP1 to strongly inhibit fungal PGs (Joubert *et al*. 2006; 2007), but also the putative changes in cell wall metabolism that are triggered before infection occurs. Future work will benefit from accurate cell wall profiling analyses and associated enzyme activities to evaluate this hypothesis. The various PGIP and CAD transgenic lines are valuable genetic resources to clarify the role of PGIPs and cell wall components in plant defense.

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Chapter 4

General discussion and conclusions

GENERAL DISCUSSION AND CONCLUSIONS

4.1 General discussion and conclusions

Genetic engineering is applied to a wide range of plants, including the most economically important crops worldwide. The development and use of molecular biology as a scientific discipline and tool have also opened possibilities for scientific study that was previously unimaginable. The fields of molecular genetics and molecular physiology are currently undergoing a change in paradigm from a 'vertical' analysis of the role(s) of one or few genes to horizontal holistic approaches studying simultaneously the function of many or all the genes of an organism. Especially within the agricultural fields, new technologies that compliment traditional crop improvement strategies have been implemented with great effect. One of the most important targets in genetic engineering strategies is increased resistance of plants towards fungal pathogens. This is partly driven by the discovery, isolation and characterization of plant antifungal proteins and their encoding genes.

The adoption and implementation of molecular biological tools to further elucidate the interaction between plants and pathogens are also used to extend our current knowledge of two classical role-players in plant-pathogen interactions. These role-players are the endo-polygalacturonases (PGs) secreted by fungal pathogens during infection and their inhibitors present in the plant cell walls, the endo-polygalacturonse-inhibiting proteins (PGIPs). At the Institute for Wine Biotechnology, one of the research initiatives entails the study of plant-pathogen interaction, utilizing the PG-PGIP inhibition interaction as a model. The first grapevine PGIP (*Vitis vinifera* PGIP1, *VvPGIP1*) was isolated, cloned and transformed into *Nicotiana tabacum* (De Ascensao, 2001). Transgenic tobacco plants overexpressing the *VvPGIP1* gene (Joubert *et al*., 2006) exhibit PGIP-specific resistance phenotypes when challenged with *B. cinerea* in whole-plant infection assays. These plant lines revealed changes in expression patterns of cell wall related genes, as well as indications of increased lignin content when compared to untransformed control plants (Becker, 2007). Remarkably, these changes were observed in the absence of pathogen infection, indicating a possible new role of PGIPs in defense "priming" (Becker, 2007). A microarray analysis of the PGIP overexpressing lines (in comparison with untransformed controls) indicated that several genes involved in lignin formation was differentially regulated. One of the genes affected was the *cinnamyl alcohol dehydrogenase* (*CAD*) gene which was upregulated in some of the overexpressing lines (Becker 2007). CAD is widely accepted as a marker for lignin-formation (Walter *et al*., 1988; Mitchell *et al*., 1994). In tobacco and other species, several studies have evaluated phenotypes linked to downregulation of *CAD* gene expression, specifically in relation to altered lignin levels for biotechnological targets in the pulp and paper industry (Baucher *et al*., 1998; Selman-Housein *et al*., 1999; Farrokhi *et al*., 2006; Vanholme *et al*., 2008).

In this thesis the functional role of CAD in plant defense was further investigated, specifically to evaluate the biological relevance of increased transcription of the *CAD* gene. The motivation for this study stems from the pertinent observation that PGIP overexpressing tobacco lines display differential expression of specific genes that might indicate a cell wall strengthening phenotype (Becker, 2007 and as described in Chapters 1 and 2 of this thesis). The goal of this study was to investigate the biological relevance of the upregulation of the *CAD* gene in PGIP-overexpressing lines and its possible contribution to PGIP resistance phenotypes in tobacco and to establish a phenotype linked to lignin biosynthsis in this a population.

Development and characterization of a CAD14 overexpressing tobacco population: The *CAD14* gene was isolated from *Nicotiana tabacum* and was transformed back into tobacco via *Agrobacterium* mediated transfer (as described and discussed in chapter 3 of this thesis). Gene integration and expression were confirmed by Southern and northern blot, respectively (Figs.2 and 3 in Chapter 3). Working with a genetically characterized CAD overexpressing population, as well as a VvPGIP1 tobacco line with known elevated transcription of CAD (Becker, 2007), the functional relevance of the transcriptional upregulation of CAD was investigated by performing CAD activity assays on leaves and whole stems. From literature it is known that CAD activity is highest in stems and actively growing organs (Halpin *et al*., 1992; Knight *et al*., 1992), which was clearly confirmed by the CAD activity assays. The assays on the stems yielded data that was statistically significant, whereas the activities of the young and older leaves were not statistically separable from the control. The overall CAD levels in leaves were very low, despite strong CAD transcript signals in these organs. The overexpression lead to strong transcription in these organs that normally do not exhibit CAD expression, but this did not translate into significant levels of CAD activity. These results confirmed again that the lignin biosynthetic pathway is under strict control mechanisms that are not only linked to transcription. The activity assay will have to be optimized further to accommodate the low levels of activity in the leaves before a statistically significant dataset can be obtained for leaves.

Importantly, The CAD assays confirmed that the observed increase in CAD transcription in PGIP overexpressing lines lead to increased levels of CAD activity. The *VvPGIP1* lines displayed very high levels of CAD activity in the stems. Overall, in all plant lines the stems exhibited 10-fold higher levels of CAD activity than the leaves, but the transgenic *VvPGIP1* plant line showed a further 2-3-fold increase in CAD activity in the stems, when compared to the untransformed control and the majority of the CAD overexpressing lines. It is currently not understood how an abundance of PGIPs trigger CAD expression in these lines, but the confirmation of increased CAD activity provides further proof that PGIP overexpression might lead to altered activities of cell wall proteins. Most importantly, these increased activities were observed under uninducing conditions which, specifically in the PGIP lines might indicate a novel *in vivo* role for PGIPs.

CAD overexpression reduced diseases susceptibility to *Botrytis* infection: The characterized population of overexpressing CAD lines, as well as the WT tobacco and the VvPGIP1 line were used to evaluate the potential resistance phenotypes linked to CAD overexpression. The VvPGIP1 line has a confirmed PGIP-specific resistance phenotype against *B. cinerea* (as described in Joubert *et al*., 2006). The CAD overexpressing lines showed a marked decrease in disease susceptibility compared to the WT control (Figs 3.5 and 3.6 of Chapter 3). An average decrease in disease susceptibility of 33% was recorded in the transgenic population. The reduction in plant tissue maceration and lesion expansion was most pronounced in the *VvPGIP1* line compared to the *CAD* transgenic plants, while the *CAD* transgenic plants showed more reduction than the SR1 control plant. Since CAD is considered a marker for lignifications and increased CAD activity was confirmed in these lines, increased lignifications could contribute to the stronger resistance phenotype in the CAD overexpressing population. Analysis of lignin levels and lignin content in this population is currently ongoing.

Conclusions and perspectives

It is well known that pathogen attack leads to increases in lignin deposition (Oelofse and Dubery, 1996; De Ascensao *et al.,* 2000; Cohen, 2002; Kawasaki *et al*., 2006; Mender *et al*., 2007). In this study a PGIP overexpresising line with a known resistance phenotype and previously observed increase in CAD transcription has been evaluated alongside a CAD overexpressing tobacco population to clarify the functional relevance of CAD overexpression. The results also obtained confirmed that overexpression of PGIP induced CAD gene expression and activity in the absence of pathogen infection or other inducing signals. These results confirm that CAD gene upregulation in PGIP overexpressing plant lines contributes to plant defense by possibly re-enforcing the plant's primary defense prior to pathogen invasion. Importantly, the results obtained supports the current working hypothesis that PGIPs might also play a role in preparing the plant for pathogen attack by increasing cell wall strength prior to infection (hypothesis based on results obtained by Becker, 2007).

Future work will involve detailed cell wall analyses, including biochemical, chemical and microscopical analyses to confirm the changes in lignin content and deposition, as well as overall cell wall architecture. *In situ* lignifications and specifically lignin composition will be studied in the various populations that have been generated in this and other related studies in our environment to understand PGIP-related disease resistance phenotypes better. These evaluations should provide interesting information as to what is changed in the cell walls and how these changes contribute to disease resistance in plants. The results obtained in this thesis and the characterized transgenic populations generated in this study will be invaluable in these and subsequent analyses.

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