Assessment of promoter function and enhancing disease resistance in transgenic carrot (*Daucus carota* L.)

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ABSTRACT

Genetic engineering is a promising strategy for creating agriculturally significant resistance in carrot. Several different transgenic strategies, in addition to conducting a detailed quantification of commonly used promoters to achieve disease resistance were investigated. Tissue-specific patterns and levels of protein expression were characterized in transgenic carrot plants transformed with the β-glucuronidase gene driven by five promoters: Cauliflower mosaic virus *35S*, double *35S* (*D35S*), *Arabidopsis* ubiquitin (*UBQ3*), mannopine synthase (*mas2*) or rooting loci promoter (*rolD*). *UBQ3* promoter provided the highest levels of expression in roots, while *D35S* and *35S* promoters had high expression in leaves. *rolD* and *Mas2* promoters had enhanced root expression; however levels were much lower compared to the constitutive promoters, which were subsequently used in this research.

Genes encoding a rice peroxidase (*POC1*), wheat chitinase (383) and β1, 3- glucanase (638) were introduced into carrot. Lines expressing 638 alone had no enhanced resistance to *B. cinerea* or *S. sclerotiorum,* while 383 lines reduced disease symptoms by up to 40%. When 638 and 383 were co-expressed resistance levels were similar to 383 alone. High levels of disease resistance were seen in lines expressing *POC1* with 70-90% reduction in symptoms to *B. cinerea* and *S. sclerotiorum*. *POC1* lines had 20-30% increases in lignin levels in petioles and roots, which was enhanced with pathogen challenge. Several

defence genes exhibited strong induction in *POC1* expressing plants when induced with fungal cell wall elicitor. Additionally, *POC1* lines had reduced H_2O_2 accumulation during oxidative burst response. *POC1* derived resistance was effective towards necrotrophic pathogens.

Over-expression of Arabidopsis *Nonexpressor of Pathogenesis Related protein 1* was studied in carrot, with two independent lines successfully generated and analyzed. There was no detectable activation of the systemic acquire resistance (SAR) pathways in the absence of pathogens, however, the lines exhibited more intense and longer lasting activation of SAR when elicited with fungal cell walls or Salicylic acid. Both lines were highly resistant against biotrophic and necrotrophic foliar pathogens and the roots were resistant towards *A. radicina*, indicating broad-spectrum disease resistance. The results from this study demonstrate the feasibility of engineering disease resistance in carrot using several different approaches.

Keywords: Carrot, disease resistance, transgenic, signalling pathways, systemic acquired resistance, peroxidase, oxidative burst, promoter, chitinase, glucanase

DEDICATION

I would like to dedicate this work to my loving wife Liane for the emotional support and patience while I completed this work.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMS USED

2,4-D	2,4-dichlorophenoxyacetic acid
35S	Cauliflower mosaic virus *35S* promoter
383	Acidic exo-chitinase cloned from wheat
638	Acidic exo β-1, 3-glucanase cloned from wheat
AIR	Alcohol insoluble residue
ANOVA	Analysis of variance
AtNPR1	*Arabidopsis thaliana Non-Expressor of PR genes*
Avr-gene	Avirulence gene
Bar	phosphinothricin N-acetyltransferase gene
BTH	benzothiadiazole S-methyl ester
CWDE	Cell wall degrading enzyme
DAB	3',3-diaminobenzidine
Dai	days after inoculation
DPI	diphenylene iodinum
DSI	Disease severity index
DTT	Dithiothreitol
ET	Ethylene
FAA	Formalin acetic acid alcohol
GUS	β- glucuronidase
Hph	hygromycin phosphotransferase
HR	Hypersensitive response
INA	2,6-dichloroisonicotinic acid
ISR	Induced systemic resistance
JA	Jasmonic acid
LTGA	ligno-thioglycolic acid derivative
MAPK	Mitogen activated protein kinase
Mas2	Mannopine synthase
MCS	Multi-cloning site
MeJA	Methyl jasmonic acid
MES	2-(*N*-morpholino)ethanesulfonic acid
MeSA	Methyl salicylic acid
MU	4-methylumbelliferone
MUG	4-methylumbelliferone-glucuronide
NIM1	Noninducible immunity
Nos	nopaline synthase gene terminator
NPR1	*Non-Expressor of Pathogenesis Related genes*
OD	Optical density
PAL	Phenylalanine ammonium lyase
PCR	Polymerase chain reaction

POC1	Rice cationic peroxidase
POX	Peroxidase
PPT	phosphinothricin
PR	Pathogenesis Related
Pst	*Pseudomonas syringae* pv. *tomato*
Psm	*Pseudomonas syringae* pv. *maculicola*
PVPP	polyvinylpoly pyrolidone
R-gene	Resistance gene
RolD	Rooting loci gene promoter
ROS	Reactive oxygen species
RT-PCR	Reverse-transcriptase PCR
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
SSC	Saline sodium citrate buffer
SS-walls	*Sclerotinia sclerotiorum* purified cell wall extract
TBE	Tris Borate EDTA buffer
TGA transcription factor	TGACG motif binding transcription factor
TLP	Thaumatin-like protein
TMV	Tobacco mosaic virus
Tris	tris(hydroxymethyl)aminomethane
UBQ3	Arabidopsis ubiquitin3 promoter
X-gluc	1mM 5-bromo-3-chloro-3-indoyl-β-D-glucuronide cyclohexamide sodium salt

1: INTRODUCTION

Carrot (*Daucus carota* L. subsp. *sativa*), a member of the family Apiaceae is grown world-wide for its edible taproot. Carrot is a biennial plant requiring one season of vegetative growth, with an inflorescence developing generally during the second year of growth following exposure to a period of vernalization (typically over winter). Carrots are marketed as fresh roots or for processing in canned soups, juice or frozen typically in mixed vegetable products. Nutritionally, carrots are the most important source of *β*-carotene (provitamin A) in the human diet. They also contain vitamins B1 and C, and are a source of dietary fiber (Ammirato 1986). Carrots are the second most important vegetable crop grown world-wide as well as in Canada. Canadian producers harvested more than 300 000 metric tonnes of carrot in 2006 (Food & Agriculture Organization, United Nations).

Cultivated carrot varieties descended from wild carrot grown throughout Europe and the Middle East. Popular orange coloured western grown varieties originated from purple anthocyanin containing lines originally from Afghanistan. There have been major improvements in carrot breeding, resulting in cultivars with enhanced yield and market quality (Punja et al. 2007). Root shape, length and colour, smooth skin texture, flavor and early maturity have all been improved significantly. Despite these breeding improvements, there has been relatively little progress in breeding carrots that are resistant to fungal and bacterial

pathogens, either during the field season or during their long post-harvest storage. The use of genetic engineering may be one of the more promising methods to increase disease resistance in carrots, to add another commercially desirable trait

1.1 Diseases of carrot

Carrots are infected by a range of microbial pathogens that can result in a reduction of both yield and crop value. In order for bunching carrots to be harvested as high value carrots, the plants need to have disease-free tops as well as roots. While healthy carrot foliage is not required for processing carrots, diseased tops affect photosynthate accumulation reducing yield in addition to weakening the tops can lead to carrot breakage, greatly lowering efficiency of mechanical carrot harvesting (Farrar et al. 2004). In the following sections, several of the important pathogens of carrot with different infection strategies will be reviewed.

1.1.1 Botrytis cinerea

Botrytis cinerea Pers. is one of the most widely studied necrotrophic fungal plant pathogens. It causes serious damage and economic losses on more than 200 different plant species world-wide (Williamson et al. 2007). *B. cinerea* causes the grey mould disease in carrots, infecting both the leaves and taproots in both the field and postharvest. Typical infections occur through wounds or natural openings on the carrot, and can rapidly cause cellular collapse followed

by proliferation of the grey conidia on the surface, leading to soft rot. Grey mould is not a major economically important disease during the growing season in Canada but it can be extremely problematic during the post harvest storage period that can last up to one year. *B. cinerea* is difficult to control due to its prolific growth and variety of virulence factors, the number of alternative hosts and ability to survive long periods as sclerotia on crop debris (Williamson et al. 2007).

B. cinerea forms multi-nucleated conidia (macroconidia) and uninucleated microconidia (male spermatia). Branched conidiophores proliferate following collapse of the tissue during infection that serve as further inoculum for disease spread, and impart a grey colour to the infected tissue. Conidia spread through splash dispersal or aerial release through diurnal changes in temperature and humidity resulting in air dispersion and long distance infection (Williamson et al. 2007). Conidia can germinate and obtain nutrients under a range of temperatures and humidity conditions, with optimal germination occurring at 15 ºC with high humidity (Rosslenbroich et al. 2000). Small dark grey sclerotia develop in the decaying tissue following infection, which serve as one of the long term survival structures of the fungi (Rosslenbroich et al. 2000). Sclerotia contain very high levels of β-glucans and melanin, which protect them against desiccation and low temperatures. In Canada, dormancy of the sclerotia is typically broken in the early spring once temperatures rise above 5 ºC with enhanced germination occurring with high humidity (Williamson et al. 2007).

B. cinerea serves as one of the main models for studying necrotrophic infection since it exhibits nearly all of the characteristics associated with necrotrophs. The genome contains multiple copies of genes encoding for cutinases and lipases that degrade the plant cuticle, which serves as a primary defence against infection. These enzymes are secreted at high levels from the appressoria, to facilitate the breach of the plant cells (Rosslenbroich et al. 2000). Down regulation of a single lipase or cutinase gene did not reduce virulence of *B. cinerea*, due to the large amount of redundancy in gene copies (reviewed in Williamson et al. 2007). The developing appressorium also secretes defensive compounds such as superoxide dismutase (BcSOD1). High levels of BcSOD1 serve to protect the penetrating appressoria from the reactive oxygen species (ROS) generated from the oxidative burst initiated by the plant cell (Rolke et al. 2004). Removal of BcSOD1 function reduced virulence of *B. cinerea* (Patel et al. 2008; Rolke et al. 2004).

B. cinerea produces a large number of phytotoxic metabolites, proteins and pathogenesis factors both *in vitro* and during plant infection. Of all the metabolites produced by *B. cinerea*, botrydial exhibits the highest phytotoxic activity (Colmenares et al. 2002). Botrydial is a phytotoxic sesquiterpene secreted from the infecting fungus. Exogenous application of purified botrydial induced chlorosis and cell collapse when applied to plant leaves. Deletion of botrydial biosynthesis resulted in severe reduction of virulence in some *B. cinerea* strains (Siewers et al. 2005), with only minor effects in others (Choquer et al. 2007). These findings indicate that certain strains of *B. cinerea* rely on

botrydial as a key infection mechanism whereas others produce a large number of other toxic metabolites (Siewers et al. 2005). *B. cinerea* also produces a number of phytotoxic peptides during infection, which lead to enhanced cellular necrosis (Staats et al. 2005); however these peptides do not seem to be crucial for pathogenesis.

B. cinerea also produces oxalic acid during host infection or when grown on low pH medium (Rosslenbroich et al. 2000). Oxalic acid serves as a cofactor for infection, functioning through sequestering Ca^{++} ions from intact plant cell walls, thereby facilitating easier cellular breakdown and infection. Oxalic acid deficient mutants of *B. cinerea* or transgenic plants expressing an oxalic acid oxidase resulted in reduced grey mould symptoms (Han et al. 2007; Walz et al. 2008a).

One of the main infection mechanisms of *B. cinerea* involves a complex interaction with the host plant, where the pathogen triggers the production of plant-derived ROS and HR. Successful *B. cinerea* infections are followed by an oxidative burst, where the infecting mycelia are able to survive while the plant cells are heavily damaged (reviewed in Choquer et al. 2007). Studies in Arabidopsis indicated that *B. cinerea* induces the expression of the HR gene Hsr203 (Govrin et al. 2000). Arabidopsis HR deficient *dnd1* mutants exhibited increased resistance towards *B. cinerea* infection, while these plants were hypersensitive to many biotrophic pathogens (Govrin et al. 2000).

Primary control of B. cinerea involves modification of cultural practices such as row trimming typically reduce *B. cinerea* infection in the field. However,

under extended severe cool damp periods, fungicides are used to control the disease spread (Davis et al. 2002).

Mediation of host resistance to *B. cinerea* is through the action of genes involved primarily in Jasmonic acid (JA) and ethylene (ET) dependent responses. However, the salicylic acid (SA) dependent pathways are also involved in local resistance (Ferrari et al. 2003). There are numerous examples of transgenic Arabidopsis, tobacco and tomato plants expressing a range of genes that have resistance to *B. cinerea* (reviewed in Punja 2006). Moderate to high levels of *B. cinerea* resistance have been reported in transgenic carrots expressing a variety of PR-genes, including JA/ET responsive chitinases (Punja et al. 1996; Baranski et al. 2008; Jayaraj et al. 2007) as well as SA responsive thaumatin-like protein (TLP) (Chen et al. 2002). There have been other transgenic carrot lines expressing a variety of genes with resistance to *B. cinerea* (Punja et al. 2007).

1.1.2 Sclerotinia sclerotiorum

Sclerotinia sclerotiorum (Lib) de Bary is the pathogen that causes Sclerotinia rot of carrot; also called watery rot, white mould or cottony rot. S*. sclerotiorum* is one of the most economically important diseases in global and Canadian carrot production (reviewed in Kora et al. 2003). *S. sclerotiorum* causes disease in dozens of other agriculturally important crops, with a host range of more than 400 species comprised of more than 250 genera and 75 families (Davis 2004). *S. sclerotiorum* causes diseases in both the foliage and the taproot of carrot, with the foliage being more susceptible during early senescence and the roots being most susceptible immediately after harvest

(Kora et al. 2005). Early field infection results in seedling damping off, with losses reported as high as 90% in the mid 1930s (reviewed in Kora et al. 2003). However, *S. sclerotiorum* is most devastating as a post-harvest disease with losses as high as 50% being reported in Canada (Kora et al. 2003).

On carrot foliage, the symptoms of Sclerotinia rot begin as dark green water-soaked lesions that rapidly develop and collapse the cell walls, resulting in soft rot. Lesions expand rapidly and can consume the entire leaf rosette, with the development of thick cottony white mycelium as the disease progresses. Large black sclerotia form externally on the decaying leaf tissue that serves as the long-term storage body of the fungus (Davis 2004). The sclerotia typically will germinate myceliogenically without a dormancy period requirement, and further infect carrot leaves or roots. Alternatively, sclerotia can germinate carpogenically after a minimum of 2 weeks of chilling to produce apothecia, typically occurring in the spring. Ascospores are released from the apothecia in a diurnal cycle, causing foliar infection of carrots after a minimum of 48 h exposure to free water (Davis 2004).

S. sclerotiorum produces a wide range of cell wall degrading enzymes (CWDE) as pathogenicity factors. These include proteases and over 20 different pectinases (reviewed in Bolton et al. 2006). Deletion of an individual or combination of 2-3 CWDE did not result in decreased virulence, indicating a large degree of functional overlap (Bolton et al. 2006). The main non-host selective mechanism for *S. sclerotiorum* infection involves the secretion of oxalic acid. Oxalic acid appears to function through a variety of mechanisms, including:

lowering the pH of the plant to near optimal for CWDE activity (Bolton et al. 2006), repression of the oxidative burst (Cessna et al. 2000; Walz et al. 2008b), decreasing the activity of plant defence enzymes, weakening of plant cell walls through chelating Ca^{++} ions, as well as being directly toxic to the plant cells (Rollins et al. 2001), and being a potent mediator of plant programmed cell death (Kim et al. 2008a). Oxalic acid deficient *S. sclerotiorum* mutants are unable to infect many plant species (Bolton et al. 2006), indicating its significance in pathogenicity.

Resistance against *S. sclerotiorum* is difficult to achieve through traditional breeding efforts, with the genes controlling resistance associated with the JA, ET and SA signalling pathways (Guo et al. 2007; Zhao et al. 2007). There are no carrot cultivars available with high levels of *S. sclerotiorum* resistance, some lines exhibit moderate resistance (reviewed in Kora et al. 2003). Some transgenic plants have shown high levels of *S. sclerotiorum* resistance, through detoxification of oxalic acid with oxalate oxidase (Cober et al. 2003; Dong et al. 2008; Hu et al. 2003; Walz et al. 2008a). Moderate increases in *S. sclerotiorum* resistance were observed in transgenic carrot expressing TLP (Chen et al. 2002; Punja 2005). Control of Sclerotinia rot is mainly through cultural or chemical practices (Davis 2004).

1.1.3 Alternaria radicina

Diseases caused by *Alternaria* species, primarily *A. dauci* and *A. radicina*, are among the most important carrot diseases worldwide. Black rot disease of carrot is caused by *A. radicina* Meier. Typical symptoms of Black rot are dry

black sunken lesions on the taproot. Infections generally travel from infected petioles progressing down to the petiole base where necrotic rings initiate the infection of the taproot just below the soil level (Davis 2004). Under conditions of high humidity, *A. radicina* progresses up the petioles and will infect the canopy, causing foliar blight. The weakened petioles due to infection often break during mechanical harvesting, resulting in severe yield reductions (Farrar et al. 2004). In Canada, most of the economic losses due to *A. radicina* occur due to post-harvest infection, since infection can occur at temperatures down to freezing levels. In severe cases, post-harvest losses can reach 100%, completely infecting storage bins (Farrar et al. 2004).

Reproduction of *A. radicina* occurs through asexual conidia, which are produced in abundance on readily formed conidiophores emerging from infected tissues. The conidia are highly melanised with a dark brown colour (Davis 2004). The exact mechanisms of infection are unknown, with *A. radicina* producing a wide range of cell wall degrading enzymes; however, no individual enzymes have been implicated with pathogenicity (Thomma 2003). *A. radicina* has a narrow host range, primarily only infecting carrot. However, close relatives of carrot including; celery, parsley, dill, fennel and parsnip are all susceptible to foliar infection (Farrar et al. 2004). Disease occurs through wind or splash transmission of conidia. Although *A. radicina* can infect carrot at any point of development growth, senescent petioles are the most susceptible to infection (Farrar et al. 2004).Carrot umbels are easily infected resulting in contaminated seed, which serves to complete the disease cycle. Taproots replanted for seed production are

highly susceptible to *A. radicina*, either from the taproot being previously infected or from inoculum that can survive in the soil for up to 8 years (Davis 2004). Infection of the seed occurs along the surface of the seed surface, which can then result in pericarp infection.

Since *A. radicina* is a seed-borne pathogen, it can be very difficult to control and can spread very rapidly to new growing areas. There has been some moderate resistance to *A. radicina* foliar infections in transgenic carrots expressing a *Pseudomonas* antimicrobial peptide (Baranski et al. 2007), a fungal mycoparasitic endochitinase (Baranski et al. 2008), and a rice thaumatin-like protein (Punja 2005). However, there have been no reports of transgenic carrots with *A. radicina* resistance in taproots. Control using fungicides during the growing season is difficult since infection typically begins on older petioles near the crown, making fungicide penetration difficult once the carrot canopy is mature. There are several commercial varieties available with genetic resistance to *A. radicina*, but many of these resistant varieties have heightened susceptibility to other important carrot pathogens (reviewed in Farrar et al. 2004). Ensuring pathogen-free seed stock is the most effective mechanism of controlling the spread of black rot although this is very difficult due to long survival periods of the fungi in the field.

1.1.4 Erysiphe heraclei

The pathogen *Erysiphe heraclei* DC is the most common cause of powdery mildew in carrot. *E. heraclei* can form dense spores and highly branched mycelium on infected tissues, leading to a powdery appearance. The

pathogen can infect all above-ground parts of the carrot plant, with particularly dense infections occurring at the base of the petioles and mature umbels (Davis 2004). Severe leaf infection can lead to chlorosis. Most carrot plants are able to recover from even the most severe infection, however, such infection often leads to pre-mature senescence (Davis 2004). In Canada, powdery mildew is generally not a major concern for growers, although severe field infection can result in decreased yields due to reduced carrot photosynthesis. Powdery mildew provides an interesting model for true biotrophic fungal pathogen that infects carrot.

Mycelia of *E. heraclei* grow primarily along the outer surface of the carrot tissue with only the haustoria penetrating the epidermis. Sporulation of *E. heraclei* begins 7 days following infection. The conidia are mainly wind dispersed and can travel very long distances in the air. Interestingly, *E. heraclei* conidia do not require free water or high humidity to germinate and cause infection (Davis 2004). However, optimal infection occurs at moderately high temperatures and high humidity, with a reduction in mycelial development in direct sunlight (Davis et al. 2002). Heavy rain or irrigation reduces disease symptoms by disrupting mycelial development.

Due to low economic importance, active control of powdery mildew is uncommon in most carrot growing regions (Davis 2004).However, in extreme cases powdery mildew can easily be controlled through use of sulphur based fungicides, since *E. heraclei* are highly sensitive to its application (Davis 2004). There are many sources of genetic resistance to powdery mildew in commercial

carrot varieties. Typically this resistance co-segregates with resistance to another very important pathogen *A. dauci*, however, this also leads to enhanced susceptibility to *A. radicina* (Cornell Univesity et al. 2009; Farrar et al. 2004).

1.1.5 Xanthomonas hortorum

Xanthomonas hortorum pv. *carotae* Kendrick (previously *X. campestris* pv. *carotae*) is the pathogen that causes bacterial leaf blight in carrot. The *X. hortorum* bacterium is a fast growing gram negative rod with polar flagella (Howard et al. 1994). In warmer climates, including the southern USA, this disease can be devastating, resulting in crop losses in individual fields of up to 100% (Davis 2004). In Canada, losses are not as severe; however, losses can be significant during hot and humid growing seasons (Howard et al. 1994). Disease symptoms begin as irregular necrotic water-soaked lesions surrounded by a chlorotic halo. As the disease progresses, the chlorosis is replaced by dry and brittle necrotic lesions (Davis et al. 2002). Infected umbels and petioles under high humidity often produce a thick yellowish exudate, containing extremely high levels of bacteria (Howard et al. 1994).

Carrot is the only known host for *X. hortorum*. The bacteria can persist for long periods of time on carrot debris in the field. Plant to plant infection occurs through splash transfer of the bacteria. Infection can proceed rapidly in less than 7 days under warm (>25 ºC) temperatures and high humidity (Howard et al. 1994), with symptoms typically appearing after 10-12 days.

X. hortorum is a seed-borne pathogen that very commonly infects commercial seed stock. Transfer of contaminated seed is the bacteria's main mechanism of long distance travel (Davis 2004). Control of *X. hortorum* is mainly through heat or chemical treatment of the seeds, with some resistant cultivars grown around the world (Howard et al. 1994). There have been no previous reports of transgenic carrots with resistance to bacterial leaf blight.

1.2 Necrotrophs vs. biotrophs

Commonly, plant pathogens are classified either by phylogeny or through infection mechanisms. Placing pathogens into two major categories of infection mechanisms has been the standard method of classification, either as biotrophic or necrotrophic pathogens. The infection and lifestyles of pathogens differ greatly, with biotrophs deriving all of their energy from living cells, whereas necrotrophs derive energy from consuming killed cells or living saprophytically (reviewed in Glazebrook 2005). A third more rarely used and complex classification category of pathogens are the hemibiotrophs, which infect plant tissue with an initial period of biotrophy followed by a necrotrophic phase (Perfect et al. 2001). Biotrophic pathogens have the following characteristics defined from Oliver and Ipcho (2004): obligate parasites, fungal pathogens often form haustoria, produce very little or no cell-wall degrading enzymes, typically cause minimal direct damage to the host plant, typically are host specific, incompatible host reactions undergo hypersensitive response (HR), complete immunity can be obtained through *R*-gene-mediated resistance, and defence responses are controlled through SA-dependent pathways. In stark contrast, necrotrophs have a

broad host range, secrete large amounts of lytic enzymes, in many instances produce toxins, little or no *R*-gene-mediated resistance, and necrotrophic resistance is controlled through JA-ET dependent signalling mechanisms (Oliver et al. 2004).

Based on these two classification schemes, several pathogens can be easily placed into a category. Biotrophs include the powdery or downy mildews, from the genus *Erysiphe* and *Hyaloperonospora,* or plant rusts from the *Uredinales* are highly host specific (Mendgen et al. 2002; Oliver et al. 2004). Less clearly defined biotrophs include the plant smuts (*Ustilaginales*), and a large number of plant pathogenic bacteria. The bacterial pathogens *Pseudomonas syringae* (pv *tomato* or pv *maculicola (Pst and Psm))* and *Xanthomonas spp* exhibit many characteristics of biotrophic pathogens, including most notably, incompatible interactions resulting in HR. *Pseudomonas spp* and *Xanthomonas* spp also have several characteristics of necrotrophs, which include being able to survive on decaying tissues, causing tissue necrosis and being responsive to JA-dependent signalling pathways (Thaler et al. 2004).

A significant amount of experimental investigation has been conducted over the past 10 years on necrotrophic pathogens, in particular those that infect Arabidopsis. Several of the well-studied necrotrophs include *Alternaria spp. (*particularly *A. brassicicola),* grey mould *(Botrytis cinerea)* and white mould *(Sclerotinia sclerotiorum). B. cinerea* and *S. sclerotiorum* have very large host ranges, and produce large amounts of lytic enzymes to aid in their infection, with no known *R*-gene mediated resistance (van Kan 2006). Despite this fact, there is

still some signalling overlap in SA signalling for all of these pathogens (reviewed in Glazebrook 2005). Within the necrotrophs, there are pathogens that are controlled via *R*-gene mediated resistance including, *Fusarium oxysporum* in tomato and cucumber among others (reviewed in Michielse et al. 2009). The level of *R*-gene-mediated resistance in *F. oxysporum may* in large part explain the high level of host-specificity among the individual strains.

There is some confusion regarding classification of pathogens which exhibit potential hemi-biotrophic infection. The pathogen *Phytophthora infestans* has been classified as a hemibiotroph, although several others have placed it in all 3 infection classes (Oliver et al. 2004). Much of the confusion arises since P. infestans resistance is mediated by SA-dependent signalling and *R*-gene resistance, however, once the infection is well established, it causes severe necrosis and tissue damage (reviewed in Foolad et al. 2008). Most pathogenic species of *Colletotrichum* are classified as hemibiotrophs, where following an initial period of biotrophic growth within the cell walls, they proliferate, generating necrotrophic secondary hyphae that results in extensive damage to the plants (reviewed in Mendgen et al. 2002). There is a distinct “switch” that occurs in several of the Colletotrichum infections, where large amounts of lytic enzymes are released from the secondary hyphae (Mendgen et al. 2002). *R*-gene-mediated resistance followed by HR results in immunity towards specific *Colletotrichum* strains in some plants (Mahuku et al. 2002). Many different pathogens, including *Septoria lycopersici* among others, have been classified as hemibiotrophs that undergo an initial asymptomatic infection phase, followed by

the release of lytic enzymes and the development of large scale tissue necrosis (Oliver et al. 2004). Fungi exhibiting infection similar to *S. lycopersici* are not truly hemibiotrophs since they do not exhibit distinct changes in infection behaviour; rather the disease symptoms are initially slow to develop. Many other pathogens have been misclassified as hemibiotrophs, indicating some disagreement or confusion among researchers.

1.3 Systemic plant defence responses

1.3.1 Systemic acquired resistance

Systemic acquired resistance (SAR) was originally discovered when tobacco plants infected with tobacco mosaic virus (TMV) were subsequently resistant to secondary infection, or additional infections with TMV (Ross 1961). Resistance was obtained in both local and systemic tissues, following a local hypersensitivity response (HR). Responses were found to be broad spectrum, inhibiting a range of pathogens as well as very long lasting, with certain responses lasting for the plant's lifetime (Ryals et al. 1996). SAR or SAR-like responses are present in many plant species, in response to compatible or incompatible interactions with a pathogen (Durrant et al. 2004; Vasyukova et al. 2007).

SAR can be determined by examining the extent of accumulation of pathogenesis-related (PR) proteins or transcript levels in both local and systemic tissues, especially the *PR-1* protein which is highly abundant during SAR (Durrant et al. 2004). PR proteins are a diverse group of proteins that are

typically present at low constitutive levels; however, protein accumulation is dramatically increased during interaction with a pathogen (Van Loon et al. 1999). There are currently 14 classes of PR-proteins, although the precise role of these proteins in plant pathogen resistance is not currently understood (Sels et al. 2008; Van Loon et al. 1999).

The phytohormone SA accumulates at relatively high levels in both local and systemic tissues within plants undergoing SAR (Metraux et al. 1990). SA also elicits SAR, and exogenous application results in PR gene expression. However, SA is phytotoxic when applied exogenously and causes local lesions prior to induction of SAR, whereas the relatively non-phytotoxic functional analogues benzothiadiazole S-methyl ester (BTH) and 2, 6-dichloroisonicotinic acid (INA) were found to induce a very similar response (Friedrich et al. 1996; Gorlach et al. 1996). SA was found to be crucial for the induced resistance to occur, since transgenic plants expressing bacterial salicylate hydroxylase (*nahG*), that rapidly degrades SA to non-SAR inducing catechol, did not accumulate SA when infected by incompatible pathogens. In addition there was no induction of SAR (Gaffney et al. 1993).

1.3.1.1 SAR mobile signal

Originally, SA itself was considered the mobile signal that was transported from locally infected tissue to the systemic tissues, since SA is crucial for SAR induction and phloem exudates contain relatively high levels of SA. However, tobacco grafting experiments with *nahG* expressing root stock, still transferred the SAR signal to wild-type systemic scions (Vernooij et al. 1994). The use of

nahG systemic scions resulted in complete removal of SAR perception (Vernooij et al. 1994). Alternatively, the SA-derivative methyl salicylate (MeSA) has been identified as a potential long range SAR signalling molecule (Park et al. 2007). MeSA itself does not initiate SAR, although it is readily converted to SA through the action of SA-binding protein 2, which exhibits MeSA esterase activity. MeSA produced during a TMV infection is transferred through the phloem, where it is converted to SA and initiates SAR (Park et al. 2007). Similarly, partial knockout mutations of *MeSA demethylase* genes resulted in complete removal of the ability to initiate SAR in Arabidopsis (Park et al. 2009; Vlot et al. 2008). However, recently the knock-out mutation *bsmt1-1*, which completely removes the MeSA generated during avirulent *Pseudomonas syringe* (*Psm avrPRm1*) challenge, was able to undergo a normal wild-type SAR response (Attaran et al. 2009). These conflicting reports indicate that the identification of the mobile SAR signalling molecule is very difficult.

Alternatively, there has been evidence that a lipid derived molecule is the functional mobile SAR signal. The Arabidopsis mutant *dir1* (*Defective in Induced Resistance 1*) exhibited normal local resistance to pathogens while systemic tissues did not accumulate *PR*-genes nor did they undergo SAR (Maldonado et al. 2002). The dir1 protein exhibits a high degree of similarity to lipid transfer proteins. Chaturvedi et al. (2008) suggested that a galactololipid molecule interacts with DIR1, since loss of function mutations in chloroplastic galactolipid metabolism (*fad7*, *sfd1*) similarly eliminated SAR without altering local resistance. Furthermore, application of infected wild-type DIR1 leaf exudates restores SAR

in *fad7* or *sfd1* mutants (Chaturvedi et al. 2008). Jasmonic acid (JA) has also been implicated as another potential lipid-derived SAR signalling molecule (Truman et al. 2007). Several JA signalling mutants have reduced SAR response towards *Psm avrRpm1*, exogenous JA application was found to enhance SAR, and there were high systemic levels of JA observed in leaves inoculated with high concentrations of inoculum (Truman et al. 2007). However, there is ample evidence indicating that JA has little to no effect on SAR, as the SAR-inducing activity collected from *Psm avrRpm1* infected petioles did not co-purify with JA or its methyl-ester Methyl-Jasmonic acid (MeJA) (Chaturvedi et al. 2008). Also, JA biosynthesis mutants (*dde2, opr3*) and certain downstream JA signalling mutants (*coi1, jar1*) actually had enhanced SAR responses when inoculated with *Psm* (Attaran et al. 2009; Cui et al. 2005). Recently azelaic acid has also received consideration as a possible mobile SAR signal, since mutation of the *AZELAIC ACID INDUCED 1* (*AZI1*) gene, which is induced by azelaic acid, results in the specific loss of systemic immunity triggered by pathogen or azelaic acid and of the priming of SA induction in plants, while maintaining local resistance to the pathogens (Jung et al 2009). Whether or not there is a single, or multiple redundant mobile SAR signals remains to be determined.

1.3.2 Induced systemic resistance

The interaction and colonization of plant roots by beneficial bacterial and some fungal species can also result in broad-spectrum pathogen disease resistance. The phenomenon of induced systemic resistance (ISR) resembles

that of SAR in most plants, although ISR results in either low levels or no increase in induction of *PR*-genes (Harman et al. 2004; Van Wees et al. 2008). Rather the plants undergoing ISR are primed to respond rapidly, inducing a wide range of PR-proteins in response to elicitation (pathogen or chemical/biological elicitor) (Pieterse et al. 1998; Pieterse et al. 1999). Additionally, ISR does not require the presence of SA; rather ISR signalling is through the action of JA and ethylene (ET) (Pieterse et al. 1999).

In Arabidopsis, ISR is associated with the expression of JA/ET responsive genes and an increase in secondary metabolites, callose and lignin deposition in the local tissues (Ahn et al. 2007). ISR responses are completely removed in *npr1* and transcription factor MYB2 knock out mutations (*myb2*) (Van der Ent et al. 2008). The cytosolic form of *NPR1* modulates JA pathways, allowing priming of the JA response factors, through removal of inhibitors (Pieterse et al. 2009). *MYB2* activation is primarily through ET, associated with several crucial ET-response factors (Liu et al. 2007). There has been far less research conducted to determine the mobile signal for ISR, but there is significant support that MeJA and ET act as the mobile signals, travelling both as volatile airborne signal and through the vascular tissue (Heil et al. 2008).

1.4 Hormones in plant defence

1.4.1 Salicylic Acid

SA is a β-hydroxy acid synthesized in plants from shikimic acid (Vasyukova et al. 2007). SA in plants is primarily synthesized in the chloroplasts

through the isochorismate pathway, with the final reaction converting isochorismate to SA through the action of *isochorismate synthase* (*SID2*) and *pyruvate lyase* (Mauch et al. 2001). Several mutations in the isochorismate pathways block the majority of the accumulation of SA, and these plants subsequently exhibit enhanced susceptibility to several pathogens (Durrant et al. 2004). Alternatively, relatively small amounts of SA can be synthesized in the cytosol through the conversion of phenylalanine in a reaction catalyzed by the enzyme phenylalanine ammonium lyase (PAL) (Lee et al. 1995). Near total elimination of SA can be accomplished through constitutive expression of the bacterial *NahG* gene; however, there are concerns that *NahG* plants exhibit different phenotypes compared to *sid2* mutants, which may indicate unknown effects of *NahG* expression (Glazebrook 2005).

There is a range of constitutive and induced SA accumulation that occurs across plant species and within varieties of the same plant. Several plants including rice, soybean and tomato and potato have very high constitutive levels of SA compared to tobacco and Arabidopsis (Vasyukova et al. 2007). Induced accumulation of SA can further increase the differences in levels. On the other hand, even plants with very low constitutive and induced SA accumulation still exhibit similar SA signalling responses.

SA typically accumulates at high levels following HR in plants, and oxidizes *NPR1* oligomers translocating it towards the nucleus, where *NPR1* binds to TGA factors to activate many SA-responsive genes (Durrant et al. 2004). However, there are some SA-dependant defence responses that act

independently of *NPR1* (Uquillas et al. 2004), including the transcription factor *AtWhy1* (Desveaux et al. 2004). *AtWhy1* is induced during biotrophic fungal infection and exogenous SA treatment. *AtWhy1* is also required for *PR-1* accumulation; however, *AtWhy1* is completely functional and maintains *PR-1* induction in *npr1* background (Desveaux et al. 2004). There is apparently significant overlap between *NPR1* dependent and independent SA responsive pathways

1.4.2 Jasmonic Acid

Jasmonic acids (JAs) are involved in a large number of diverse physiological and developmental processes including: leaf senescence, fruit ripening, seed germination, pollen development, root growth and tuber formation. However, the major functions are involved in defence responses towards herbivorous predation and microbial pathogens. JA is a lipid-based octadeconoid compound that exists in all land plants. JA originates through multi-stage oxidation of α-linolenic acid, through the action of various enzymes, which occurs primarily in the chloroplasts (Turner et al. 2002). JA is often converted to the volatile methyl-ester, methyl-JA (MeJA), through the action of JA-carboxyl-methyl transferase. JA accumulates in the local tissue during pathogen infection and tissue disruption. JA is perceived by one or more yet undetermined signal receptors, which result in the activation of at least two different signalling pathways (Pieterse et al. 2009). Early studies from Arabidopsis have indicated that there are two main pathways that responded to JA. One pathway results in the expression of vegetative storage protein which is responsible for

developmental functions, while the second pathway results in the production of defensins *PDF1.2* involved in pathogen and herbivore defence (Lorenzo et al. 2005).

There are many genes that are crucial for controlling signalling along the JA pathways in Arabidopsis. Several of these genes encode for JA biosynthesis, including those in fatty acid oxidation. However, JA-signalling can be restored in these mutants through application of exogenous JA or MeJA (Lorenzo et al. 2005). Control of pathogen induced JA signalling has been determined to involve 3 main signalling components identified from Arabidopsis, including: a. *Coronantine insensitive 1* (*COI1*), indentified where the loss of function mutant *coi1* was insensitive to the JA analogue coronantine, and was unable to resist infection by typically incompatible *Alternaria brassicola.* Responses towards exogenously applied JA in *coi1* plants were also completely removed (Xie et al. 1998). b. *Jasmonate resistant* (*JAR1*) was indentified through loss of function mutants, to be involved in forming the biological active JA-amino acid complexes, primarily JA-isoleucine (JA-Ile) involved for subsequent downstream signalling(Thines et al. 2007). c. *Jasmonate insensitive 1* (*JIN1 or MYC2*) discovered from a dominant mutation and determined to be an important transcription factor activating many important JA responsive genes including *PDF1.2* (Lorenzo et al. 2005). These 3 signalling pathways are linked via the function of jasmonate ZIM-domain (JAZ) proteins (Chico et al. 2008; Katsir et al. 2008; Thines et al. 2007). JAZ functions as a repressor of JA signalling, through binding and inactivating MYC2 transcription factors; JAZ is degraded through the

action of COI1 SCF ubiquitin ligase (Chico et al. 2008). Degradation of JAZ allows for activation of JA responsive genes and perception of JA signals (Staswick 2008; Thines et al. 2007).

JA often has some functional overlap with ET and they often regulate similar and synergistic responses in plants. Typically, in response to plant pathogens, both JA and ET activate genes in a redundant and synergistic manner (Lorenzo et al. 2005). Mutants that are affected in signalling or biosynthesis of either phytohormone typically exhibit an impaired response in JA/ET responsive genes, and are often more susceptible to the infecting pathogen (Thomma et al. 1998; Thomma et al. 1999). Whereas physiological and developmental responses initiated by JA/ET are often antagonistic (Lorenzo et al. 2005).

1.4.3 SA-JA cross-talk

Antagonism between JA and SA was clearly demonstrated in Arabidopsis infected with the virulent biotrophic pathogen *Psm,* as it rendered the infected plant more susceptible to *A. brassicola*. This susceptibility was detected in the local tissues only, with systemic leaves being unaffected (Spoel et al. 2007). In addition, the increased susceptibility was only detected with virulent *Psm* incompatible strains, where the plant exhibited HR and had unaltered susceptibility (Spoel et al. 2007). *NPR1* is the most studied and potentially the most important regulator of SA responsive genes. It has also been implicated as being a key regulator of the cross-talk between SA/JA signalling pathways in

plants (Durrant et al. 2004). The cytosolic multimeric form of *NPR1* modulates and stimulates downstream JA-responsive genes, whereas the nuclear *NPR1* monomer interacts with downstream SA-responsive promoter elements (Pieterse et al. 2004; Spoel et al. 2003). Many of the SA responsive elements downstream of *NPR1*, including WRKY70 transcription factors, antagonize JA-transcription factors and block the transcription of JA-responsive genes including *PDF1.2*. Over-expression of Arabidopsis *WRKY70* led to constitutive up-regulation of the SA-responsive gene *PR-1*, resulting in enhanced resistance towards biotrophic pathogens, whereas these transgenic plants had impaired *PDF1.2* production and were susceptible to incompatible necrotrophic pathogens (Li et al. 2004; Li et al. 2006). Following these findings, suppression of *WRKY70* resulted in increased *PDF1.2* accumulation, increased resistance towards necrotrophic pathogens, and increased susceptibility towards biotrophs (Li et al. 2006). Further antagonism was observed with *Mitogen activated protein kinase 4* (*MPK4*), where loss of function mutants *mpk4* had increased SA accumulation and constitutive expression of *PR-1*, accompanied by increased tolerance to *Pst* (Petersen et al. 2000). *PDF1.2* transcript expression in *mpk4* was greatly reduced, resulting in increased susceptibility towards *A. brassicola* (Brodersen et al. 2006), indicating that *MPK4* is a positive regulator of JA signalling and antagonistic to SA signalling pathways. The key JA-regulator *JIN1* acts as a repressor in SA signalling, with *jin1* Arabidopsis mutants having increased SA accumulation, higher *PR-1* transcripts and increased tolerance to *Pst* DC3000 (Laurie-Berry et al. 2006).These along with other JA/SA antagonistic

relationships have been reported (reviewed in Bari et al. 2009), indicating a very deep and complex interaction.

The notion of strict antagonism between the SA/JA-ET pathogen signalling pathways, with SA being involved only with biotrophic responses and JA-ET involved only with necrotrophic responses, is a gross oversimplification. Application of exogenous JA in Arabidopsis can result in reduced susceptibility towards powdery mildew, dependent on *COI1* (Zimmerli et al. 2004). Several studies conducted using Arabidopsis mutants have revealed additive or potentially synergistic interactions between the signalling pathways. The *cev1* mutant exhibits constitutive induction of JA responsive genes and exhibits reduced responses towards SA (Ellis et al. 2002b). The *cev1* mutants exhibited heightened resistance towards necrotrophic pathogens, as well as towards the biotrophs *Erysiphe chichoracearum* and *Psm* (Ellis et al. 2002a; Ellis et al. 2002b). There is implication that SA accumulation is required for defence responses against *B. cinerea* (Ferrari et al. 2003). Despite no enhanced susceptibility in the isochorismate synthase pathway mutants, pharmacological inhibition of PAL resulted in increased susceptibility towards *B. cinerea* (Ferrari et al. 2003). Treatment with exogenous BTH or SA also has been shown to increase local resistance to *B. cinerea* (Ferrari et al. 2003) and *S. sclerotiorum* (Guo et al. 2007).This indicates a possible role of SA generated in the cytoplasm as crucial for local defence responses against necrotrophic pathogens (Glazebrook 2005).

1.5 *Genetic engineering to enhance disease resistance*

Plant pathogens and diseases are a major limitation to crop production worldwide. While plants are naturally immune to the vast majority of potential bacterial and fungal pathogens, the few that manage to establish disease can be extremely problematic and cause economic losses. Control of pathogens has primarily been achieved using breeding programs for either qualitative or quantitative resistance to particular pathogens, or through the use of pesticides. Breeding has been extremely effective in generating plants with qualitative resistance to a specific pathogen. The use of race-specific resistance through the incorporation of naturally occurring *R*-genes has yielded excellent resistance in many crops to important pathogens, mainly biotrophs (Campbell et al. 2002). However, the common and widespread use of monocultured crops with little to no genetic diversity can easily result in pathogenic bacterial and fungal strains that overcome plants defences. Use of pesticides to control pathogens has similar problems of resistance development, in addition to being harmful to beneficial microorganisms. Genetic engineering has the potential to overcome many of the problems associated with traditional breeding, using a broader and more diverse range of genes in a carefully targeted manner (Gurr et al. 2005b). Additionally, genetic engineering has the potential to increase disease tolerance to a range of pathogens, with minimal effect on beneficial soil microbes (Liu et al. 2005).

Development of transgenic plants with enhanced resistance to fungal and bacterial pathogens has merited limited success (reviewed in Punja 2006). Many

strategies using a variety of genes have been used in transgenic plants that include: expressing *R*-genes, *pathogenesis-related* (*PR*) and antimicrobial genes, detoxification of pathogen virulence factors, increasing structural barriers, RNAi, and the modification of defence signalling pathways.

1.5.1 *R*-genes

There has been extensive research using *R*-genes for engineering resistance in plants due to the high levels of qualitative resistance from traditional breeding programs. *R*-gene products directly or indirectly recognize an individual pathogen and trigger a signalling cascade of different response pathways. Recognition of a particular avirulence (*avr*) protein from the pathogen by a plant *Resistance* (*R*) protein results in an incompatible reaction. Both the *R*-gene and *avr* gene are needed or else there is a compatible reaction, leading to the concept of gene-for-gene resistance (reviewed in Kiraly et al. 2007). There are a range of responses activated by *R*-genes; including an oxidative burst, HR, accumulation of inhibitory secondary metabolites and activation of signalling pathways (primarily the SA pathway) (Kiraly et al. 2007). Genetic engineering allows for introduction of *R*-genes from unrelated plant species, which often remain functional in the new host plant (Collinge et al. 2008). The *R*-gene *Rxo1* from maize was successfully introduced into rice and conferred resistance against bacterial streak disease caused by *Xanthomonas oryzae* pv. *oryzicola* (Zhao et al. 2005). Additional examples of this strategy involve the *R*-gene *RCT1* from *Medicago truncatula* that was expressed in alfalfa and conferred resistance to *Colletotrichum trifolii* (Yang et al. 2008), and RPI-BLB2 from wild potato

Solanum bulbocastanum conferring resistance to *Phytophtohora infestans* in cultivated potato (van der Vossen et al. 2005). Limitations to transgenic *R*-gene expression are similar to that of breeding in that resistance is conferred only against a single pathogen. The use of multiple *R*-genes (pyramiding) expressed in a single plant through a combination of genetic engineering has been successful in producing rice resistant to multiple pathogenic bacteria isolates (Zhou et al. 2009). Problems involved with *R*-genes include the potential for spontaneous activation leading to cell death via HR-like response (Tian et al. 2003), reduced overall fitness and the potential hypersensitivity to necrotrophic pathogens that intentionally activate the genes (Collinge et al. 2008).

An interesting exploitation of *R*-gene response was proposed by Dewit (Dewit 1992), where a plant was designed to express an active avr protein under the control of a pathogen-responsive promoter in which the plant has the *R*-gene for. This induced *avr* product will activate the induced responses of the plant, resulting in incompatible reactions to a wide range of biotrophic pathogens. This strategy has successfully expressed an HR responsive product cryptogein under the control of the pathogen-inducible promoter *hsr203J* in tobacco (Keller et al. 1999). While the non-transgenic tobacco plants were sensitive to *Phytophthora parasitica*, the transgenic plants when challenged by the pathogen underwent HR and were resistant, while growing normally when un-induced (Keller et al. 1999). The main limitation to this type of strategy is the requirement for the promoter to be completely inactive in the absence of pathogens, with spontaneous HR

inducting resulting from promoter leakage occurring in similar subsequent experiments (Belbahri et al. 2001; Takakura et al. 2004).

1.5.2 Antimicrobial proteins

The most commonly used approach for engineering fungal and bacterial resistance in plants is through the expression of antimicrobial peptides and PR-proteins. PR-proteins have a variety of functions, including degrading the fungal cell walls, membranes, RNA or are involved in generating secondary metabolites or increasing cell physical barriers.

Chitinases (PR-3, 4, 8 and 11) and β, 1-3 glucanases (*PR-2*) have been investigated extensively since they are hydrolytic enzymes that serve to break down the main structural components of fungal cell walls, chitin and laminarin. These naturally occurring PR proteins are constitutively expressed at low levels and are induced to high levels during pathogen challenge or application of either SA or JA (Ferreira et al. 2007). Over-expression of both chitinases and glucanases from a wide range of donor organisms has been examined in a variety of plant species. Chitinase over-expression has been moderately successful in increasing tolerance to diseases caused by both biotrophic and necrotrophic fungal pathogens (reviewed in Punja 2001; Punja 2006). However, this chitinase-derived resistance was rarely at a level high enough to pursue commercial development. There has been limited success reported from over-expression of β-1, 3 glucanases, with little to no increased disease resistance reported in nearly all cases (reviewed in Punja 2001; Punja 2006). However, combined expression of a chitinase and β-1, 3 glucanase often resulted in a

synergistic effect, further enhancing the resistance in several plant species (Anand et al. 2003; Jach et al. 1995; Jongedijk et al. 1995; Zhu et al. 1994).

Over-expression of genes encoding for other PR-proteins or antimicrobial peptides in a variety of plants has met with some success. The use of ribosomal inactivating proteins has been successful in tobacco (Corrado et al. 2005), lipid transfer proteins and thaumatin-like proteins (TLP) have been successful in carrot (Chen et al. 2002; Jayaraj et al. 2007) in addition to others (reviewed in Punja 2006).

Synthetic modifications have further enhanced the effectiveness of several PR proteins. One approach involved linking a single chain antibody gene against a *Fusarium graminearum* cell wall protein to different antimicrobial genes including; defensins (*PR-14*) and chitinases (Peschen et al. 2004). These antibody-linked antimicrobial proteins were targeted to the cell wall, where the antibodies would attach to the invading pathogen's cell wall and the action of the antimicrobial proteins would effectively degrade the fungi. This approach was highly effective in Arabidopsis against nine different pathogens consisting of different species of the *Fusarium* genus, while being ineffective against distantly related pathogens (Peschen et al. 2004). Successful implementation of antibody-linked PR proteins in transgenic wheat resulted in reduced disease symptoms due to Fusarium head blight (Li et al. 2008), one of the most important wheat diseases worldwide.

1.5.3 Detoxification of pathogenicity factors

Necrotrophic pathogens produce copious amounts of pathogenicity factors, including toxins and cell wall degrading enzymes, as a means of successfully establishing infections. Mutants lacking these pathogenicity factors often have reduced virulence or in some instances are completely avirulent (Collinge et al. 2008). Polygalacturonase-inhibitory proteins (PGIPs) serve to inhibit the activity of the fungal cell wall degrading polygalacturonases (De Lorenzo et al. 2001). Over-expression of PGIPs in transgenic plants has successfully reduced disease symptoms due to *B. cinerea* (Joubert et al. 2007; Manfredini et al. 2005).

Oxalic acid was previously discussed as a potent pathogenicity factor produced by several species of fungi. Proteins that can degrade oxalic acid include wheat oxalate oxidase and oxalate decarboxylase, converting oxalic acid to CO_2 and hydrogen peroxide or CO_2 and formate, respectively. Over-expression of these enzymes in sunflower (Hu et al. 2003), soybean (Cober et al. 2003), rape seed (Dong et al. 2008), tomato (Walz et al. 2008a) and tobacco (Walz et al. 2008b), all exhibited at least partial resistance to *S. sclerotiorum*. Additional resistance against *B. cinerea* (Walz et al. 2008a) and *Septoria musiva* (Liang et al. 2001) in tomato and poplar, respectively, was achieved.

1.5.4 Modification of signalling pathways

Over-expression of a single or combination of a small number of individual genes is generally unlikely to provide high levels of resistance against a broad range of pathogens. Modifications of existing innate signalling pathways,

including SAR and ISR, can activate a number of transcription factors, increasing the expression of a large number of defence genes. The major drawback to activating entire signalling pathways is the high fitness cost and potential yield reduction associated with constitutive expression of a large number of genes. Therefore, genes that activate partial pathways or augment pathways are ideal candidates. Potential candidate genes for genetic engineering include transcription factors like *WRKY*, *ERF1* or Whirly factors, *mitogen activated protein kinases* (*MAPK*), or key signalling nodes most notably *NPR1*.

WRKY transcription factors are involved in SA mediated defence pathways. Several WRKYs have the potential for increasing disease resistance, with *WRKY70* from Arabidopsis being the most studied. Arabidopsis constitutively over-expressing *WRKY70* had increased resistance to powdery mildew, *Pseudomonas syringe* and *Pectobacterium carotovora* (Li et al. 2004; Li et al. 2006). These plants did not have any reduced growth or fitness; however, they did develop hypersensitivity to the necrotrophic pathogen *A. brassicicola* (Li et al. 2006).

The potential for signalling cascades controlled by MAPKs have been extensively examined as potential for increasing disease resistance in plants. Difficulties using MAPKs arise, due to the complexity involved in the host-defence pathways and redundancies in MAPK gene families (reviewed in Pedley et al. 2005). Over-expression of a SA activated MAPK in tobacco resulted in complete downstream activation of the entire SA complex (Zhang et al. 2001). Enhanced resistance towards bacterial blight (Jeong et al. 2008) and *Alternaria*

alternata (Song et al. 2006) using MAPK over-expression was observed in addition to bacterial infection in tobacco (Takahashi et al. 2007).

1.5.5 *NPR1*

Potentially the most promising candidate for increasing pathogen resistance through signalling modifications is *NPR1*. The loss of function Arabidopsis mutant *npr1* (*non-expressor of pr genes*) was originally identified from mutant screens where *npr1* plants were unable to neither mount a SAR response, nor accumulate PR transcripts and were hypersensitive to biotrophic pathogens (Cao et al. 1994; Delaney et al. 1995). To date, *NPR1*-like orthologues have since been characterized from over 15 different plant species. The functional sequences of the NPR1 protein are a BTB/POZ domain and ankyrin repeat domains. These domains are known to mediate protein-protein interactions and are often found in transcription factors (Cao et al. 1998; Malnoy et al. 2007). *NPR1* has been identified as the key master control switch of SAR, with implications in other JA/ethylene controlled signalling pathways (reviewed in Pieterse et al. 2004).

NPR1 is constitutively expressed at low levels and transcript accumulation increases up to 2-fold during pathogen challenge or when treated with SA (Cao et al. 1998). The gene product is located as an inactive multimeric protein in the cytosol (Mou et al. 2003). During pathogen infection, or application of an elicitor, the *NPR1* complex is reduced and the structure is changed to the monomeric form that is transported to the nucleus. Nuclear *NPR1* interacts with TGA family

of basic leucine zipper transcription factors, which in turn induces expression of several PR genes (Fan et al. 2002; Subramaniam et al. 2001). However, TGAs are not crucial for *NPR1* function. Several TGA mutants including triple mutants exhibited decreased induced PR gene expression, while not being hypersensitive to pathogens as in *npr1* (Zhang et al. 2003b). *NPR1* also regulates the SA-mediated expression of proteins that lead to suppression of JA-dependent signalling events that include the WRK70 transcription factors (Li et al. 2004; Ndamukong et al. 2007). Direct regulation of JA-pathways does not require nuclear localization of *NPR1*, with translocation deletions maintaining interaction both in Arabidopsis (Glazebrook et al. 2003; Spoel et al. 2003) and rice (Yuan et al. 2007), indicating a dual function between the cytosolic and nuclear located *NPR1*.

NPR1 appears to be downstream of *R*-gene mediated resistance, with *snc1 R*-gene mutants exhibiting *NPR1* independent phenotypes (Zhang et al. 2003a). However, there is conflicting information from tomato indicating that *NPR1* silencing resulted in reduced responses to the *pto R*-gene, responses for PstDC3000 having susceptibility to the normally avirulent pathogen (Ekengren et al. 2003). One possible explanation for this discrepancy could be the loss of basal resistance associated with suppression of *NPR1* leading to opportunistic infections by typically incompatible pathogens (Dong 2004).

Since *NPR1* can control both SA and JA dependent pathways, it has been a logical target for genetic engineering of broad spectrum disease resistance. Over-expression of Arabidopsis *NPR1* (*AtNPR1*) or an endogenous *NPR1*

orthologue has resulted in increased resistance to biotrophic (Cao et al. 1998; Friedrich et al. 2001; Lin et al. 2004; Malnoy et al. 2007) and necrotrophic (Lin et al. 2004; Makandar et al. 2006) pathogens in a variety of different plant species, in addition it has resulted enhanced insect resistance in tobacco plants (Meur et al. 2008). The majority of the transgenic studies resulted in little or no constitutive induction of native *PR*-genes. Rather, the transgenic plants exhibited a phenotype that was “primed” to respond and express PR proteins more quickly, at higher intensity and for a longer duration. This resulted in a heightened capacity to undergo SAR when challenged with a pathogen or treated with a SA analogue. *AtNPR1* remained functional when expressed in tomato (Lin et al. 2004), rice (Fitzgerald et al. 2004), wheat (Makandar et al. 2006) and tobacco (Meur et al. 2008), indicating conserved functionality between *NPR1*-like proteins and signalling systems. All of the transgenic plants were phenotypically normal with the exception of transgenic rice expressing either *AtNPR1* or the rice orthologue *OsNH1*, which exhibited constitutive induction of *PR*-genes (Chern et al. 2005; Chern et al. 2001; Fitzgerald et al. 2004). In addition to increased pathogen resistance, these transgenic rice plants had increased sensitivity to light, developed spontaneous lesions, and had severely stunted growth (Chern et al. 2005). This deleterious phenotype was likely due to high native levels of salicylates present in rice, which naturally has levels 5-fold greater than that of wheat (Makandar et al. 2006). The increased salicylate levels activate the over-expressed *NPR1*s and constitutively activate many of the down-stream pathways.

1.5.6 Peroxidases

Peroxidases (POX) are a protein super family that consist of three distinct classes of peroxidase enzymes. The three classes have low similarity at the amino acid level, while they share a common haem group and overall have a somewhat similar three-dimensional structure (Smulevich et al. 2006). Class I POXs are believed to be the common ancestor for all the peroxidase classes, being present in all kingdoms with the exception of animals (Passardi et al. 2007). Class I POXs are intracellular with their main function being the detoxification of ROS (Cosio et al. 2009). In plants, class I POX consist of ascorbate, cytochrome c and catalase peroxidases, which are typically sub-divided by their organelle localization. The Class II POXs are found only in fungi, where their main function is the degradation of soil debris, including lignin (Martinez et al. 2005).

Class III POX, which will be discussed exclusively (referred to here after simply as POX), exist as a large multi-gene family present in all land plants. POX catalyzes the oxidation of a broad range of organic substrates using H_2O_2 (Passardi et al. 2005). Additionally, POX can generate ROS through a distinct hydroxylic cycle (Passardi et al. 2004b). POX have a great range of physiological functions in plants involved in wound healing, auxin metabolism, scavenging of ROS, root elongation, and they are involved in abiotic and biotic stress (reviewed in Cosio et al. 2009). In addition, by controlling the levels of H_2O_2 in the plant cell, POX's can indirectly adjust downstream signalling pathways influencing a large number of genes. Ascertaining the precise functions of POXs is very difficult in

plants, since there is a large degree of redundancy with over 130 POXs in rice (Passardi et al. 2004a) and more than 70 discovered in Arabidopsis (Tognolli et al. 2002).

POXs play an important role in plant defence against pathogens, mainly through the formation of lignin, generation of phenolic compounds, and modifying ROS concentrations. A large number of POX transcripts are upregulated during pathogen attack, with 10 POXs genes increased in rice challenged with *Magnaporthe grisea* (Sasaki et al. 2004), in Arabidopsis, 7 and 3 POX transcripts were increased when challenged with *Psedumonas* spp. (Mohr et al. 2007) and *B. cinerea* (Chassot et al. 2007) respectively. The differences in the number of genes upregulated indicate potentially distinct POX responses towards necrotrophic and biotrophic pathogens.

Lignin is a strong amorphous heteropolymer that is synthesized through the oxidative coupling of hydroxycinnamoyl alcohols, with the final cross-linking through the action of POX using H_2O_2 as an oxidant (reviewed in Almagro et al. 2009; Marjamaa et al. 2009). Lignin is very difficult for a potential pathogen to penetrate with synthesis increasing during pathogen challenge and in wound responses (Lagrimini 1991). Lignin is an important factor involved in non-specific basal defences against pathogens (Kawasaki et al. 2006; Quiroga et al. 2000). Over-expression of POXs in transgenic tobacco has led to significant increases in total lignin levels (Kim et al. 2008b; Lagrimini 1991).

While the main function of POXs is the oxidation of organic substrates, which results in the overall lowering of H_2O_2, POXs' play a significant role in

direct scavenging and generation of H_2O_2. Cell wall bound POXs produce the majority of all H_2O_2 produced during the oxidative burst following pathogen infection in a number of plant species including Arabidopsis (Bindschedler et al. 2006), whereas other species rely mainly on the function of NAD(P)H (Bolwell et al. 1997; Bolwell et al. 1998; Torres et al. 2006). Production of ROS through POXs involves a complicated hydroxylic cycle, which is an alternative to the normal peroxidative cycle (Passardi et al. 2004b). The generation of H_2O_2 through POXs and the subsequent downstream events including HR and *PR*-gene induction, is assumed to lead to resistance against biotrophic pathogens (Choi et al. 2007). POXs can also regulate H_2O_2 production through the direct scavenging with the production of molecular oxygen with no reductants needed (Baker et al. 2000). However, direct scavenging has been reported infrequently and often cited “scavenging” refers more to removal of H_2O_2 through substrate oxidation.

Transgenic over-expression and silencing experiments with POX resulted in a range of different results. Sweet potato POX (*Swpa4*) was identified as a highly induced stress related POX (Kim et al. 2008b). Over-expression of *Swpa4* in transgenic tobacco resulted in constitutive increases in H_2O_2, lignin, total phenolics and heightened *PR*-gene expression. These plants also exhibited enhanced resistance to the biotrophic pathogen *Phytophthora parasitica* var *nicotinae* (Kim et al. 2008b). In bell pepper (*Capsicum annuum*), the POX *CaPO2* was identified and involved in increased H_2O_2 accumulation during *Xanthomonas campestris pv vesicatoria* attack (Choi et al. 2007). When silenced, the pepper

plants exhibited reduced H_2O_2 accumulation and HR; these plants were also hypersensitive towards the *Xanthomonas*. Over-expression of *CaPO2* in Arabidopsis resulted in increased H_2O_2 accumulation, HR and *PR*-gene expression, resulting in increased resistance to virulent *Pseudomonas* (Choi et al. 2007). These findings indicate that increased accumulation of H_2O_2 induced downstream effects, including HR that significantly enhanced resistance towards biotrophic pathogens.

Similarly, in transgenic wheat and barley, the wheat POX *TaPERO* was over-expressed in the leaf epidermis, resulting in resistance to several powdery mildew pathogens (Altpeter et al. 2005; Johrde et al. 2008; Schweizer 2008). Over-expression led to increased H_2O_2 accumulation and HR. Silencing of *TaPERO* led to increased sensitivity to the powdery mildews; however, the H_2O_2 levels were not significantly altered (Johrde et al. 2008). Based on the H_2O_2 findings, Johrde and Schweizer (2008) suggested that the increased POX levels resulted in enhanced basal resistance. However, with no analysis of lignin or assessment against necrotrophic pathogens, it is unclear if basal resistance was actually increased.

In direct contrast, the POX *Ep5C* in tomato is correlated with susceptibility to *Pseudomonas syringe pv tomato* (*Pst*) (Coego et al. 2005a). Silencing of *Ep5C* resulted in increased H_2O_2 accumulation, increased HR and was correlated with enhanced resistance to *Pst*. Alternatively; over-expression resulted in reduced H_2O_2 and hypersensitivity to towards *Pst* (Coego et al. 2005a). A subsequent screen in Arabidopsis discovered mutants which the *Ep5C* promoter was

constitutively activated, the mutant was labeled *overexpressor of cationic peroxidase 3* (*ocp3*) (Coego et al. 2005b). The mutant *ocp3* plants exhibited heightened resistance towards necrotrophic pathogens including *B. cinerea*, whereas there was no effect on sensitivity to biotrophic pathogens including *Pst* (Coego et al. 2005b).

While there seems to be little consensus with regard to the overall trends of increased POX expression of disease resistance, much of the conflicting reports are due to the multiple cellular functions that can be conducted by POXs. From the literature there seems to be at least three main mechanisms that POXs can perform; 1.) Increased basal resistance through generation of lignin, or phenolic compounds, 2.) Increased induced resistance through the generation of H_2O_2, thereby increasing resistance against biotrophs and 3.) Removal of H_2O_2 resulting in increased resistance towards necrotrophic pathogens.

1.6 Oxidative burst

One of the earliest responses in plants to pathogen attack is the generation of copious amounts of ROS, primarily $O_2^{\bullet -}$ and H_2O_2 (Lamb et al. 1997). This rapid generation of ROS is considered a standard response to pathogens termed oxidative burst, which has been reported to occur in dozens of plant species (Bindschedler et al. 2006). The recognition of the pathogen or elicitor through an unknown receptor stimulates Ca^{++} efflux into the cytosol and activates a MAPK signalling cascade resulting in the production of ROS (Shetty et al. 2008).The increase in ROS leads to extracellular alkalization, making the

cell inhospitable to the invading pathogen and downstream signalling events (Bolwell et al. 2002).

The $O_2^{\bullet-}$ is released as the initial ROS, since treatments with the superoxide dismutase inhibitor N, N diethyldithiocarbamate resulted in accumulation of high $O_2^{\bullet-}$ levels (Auh et al. 1995). The ROS are generated through the function of either cell wall bound POXs or a mechanism similar to mammalian cells using a membrane bound NAD(P)H oxidase (Lamb et al. 1997). The hydroxylic cycle that POX uses to generate ROS has been previously discussed, whereas the NAD (P) H oxidases catalyze a direct oxidation using O_2, producing NAD (P) $^+$, $O_2^{\bullet-}$ and H^+ (Bolwell et al. 1997). The alkalization of the apoplast occurs during the conversion of unstable $O_2^{\bullet-}$ to stable H_2O_2 (Lamb et al. 1997). In rose, oxidative burst is controlled nearly completely through the action of NADPH oxidases, since addition of the specific NAD(P)H oxidase inhibitor diphenylene iodinum (DPI) completely eliminated the production of H_2O_2 (Bolwell et al. 1998). Whereas, the oxidative burst in Arabidopsis is largely unaffected by DPI but is highly sensitive to POX inhibitors such as KCN or NaN_3 (Bindschedler et al. 2006; Davies et al. 2006). Plants such as French bean have oxidative bursts that are dependent approximately 50:50 on both NAD(P)H oxidases and POXs, as addition of either POX inhibitors or DPI results in a 50% reduction in H_2O_2 accumulation (Bindschedler et al. 2001; Bindschedler et al. 2006; Bolwell et al. 1998).

1.7 Promoters used in enhancing disease resistance

Regulation of transgene expression at the proper levels in the desired tissues is crucial in genetic engineering, including for pathogen resistance. The most commonly used strategy was and currently is to express a single transgene under the control of a constitutive promoter, resulting in gene expression in all or nearly all tissue types. The use of constitutive promoters has rarely resulted in adequate resistance towards fungal and bacterial pathogens nor has it been widely accepted, unlike the use of successful herbicide, viral and insect resistant crops. Much of the problem is due to the choice of genes to be expressed; however, the promoters used to express the transgenes have potential for improvement.

There are several promoter based strategies that can improve or at least reduce several of the problems associated with transgenic pathogen resistant plants. The choices of promoters include constitutive over/under-expression of genes, pathogen or chemical induced expression and tissue/organ enhanced gene expression. These strategies can be implemented using naturally occurring endogenous, or exogenous promoters in addition to using synthetic promoters.

1.7.1 Gene transcription

Initiation of transcription requires the binding of RNA polymerase II to the genomic DNA sequences that contain the coding regions. The RNA polymerase II binding increases through the action of a number of protein transcription factors, with the overall promoter referred to as the 5’ up-stream non-transcribed region (Potenza et al. 2004).The fine-tuning and control over transcription is

through the action of transcription factors. Transcription factors bind to DNA cis-elements (enhancers) that are present within the gene promoters (Potenza et al. 2004). Cis-elements can be located near the initiation site, downstream of the gene or even within the gene itself, with some cis-elements acting thousands of base pairs away from the coding region (Alberts et al. 2002).

Specific gene regulation at the transcriptional level is complex and relies on the type, number, position and exact combination of different cis-elements. In addition, activation of the promoter through RNA polymerase II binding is dependent on the activation of transcription factors which require a cellular signal to become activated (Alberts et al. 2002).

1.7.2 Constitutive promoters

Constitutive promoters are the primary choice for plant transformation for increased pathogen resistance. Typically, the Cauliflower Mosaic virus *35S* (*35S*) promoter is used in dicots and the maize ubiquitin or rice actin promoters in monocots. The main benefits to constitutive promoters, especially *35S*, is that they deliver relatively high levels of transgene expression in virtually all tissue types and are readily available in a range of plant transformation vectors (Potenza et al. 2004).

General limitations associated with constitutive promoters include; detrimental effects on non-target organisms and the development of resistant pathogens (Gurr et al. 2005a). Limitations of *35S* further consist of the publically perceived notion of human/livestock health risks associated with consumption of

viral genes. Additionally, *35S* promoters are subject to high levels of transcriptional gene silencing, as plants perceive the promoter as foreign and intentionally silence it through a variety of mechanisms (Scheid et al. 2002). Using plant derived constitutive promoters are less prone to silencing and there is greater public acceptance of them (Potenza et al. 2004). Constitutive expression of transcription factors such as *WRKY70* in Arabidopsis can result in deleterious phenotypes from constitutive activation of a large number of genes (Li et al. 2004). Therefore, it is crucial to determine if there are downstream problems before utilizing a constitutive promoter. Despite some limitations, constitutive promoters are ideal for expression of genes, which become activated during pathogen challenge, such as *R*-gene over-expression or gene master control switches including *NPR1* (Makandar et al. 2006; van der Vossen et al. 2005; Zhou et al. 2009).

1.7.3 Tissue enhanced promoters

Tissue/organ enhanced promoters express genes in an enhanced fashion in a particular tissue or organ type. These promoters are potentially very useful in controlling pathogens that infect particular organs or tissues. The epidermal fusion promoter from wheat *GstA1::WIR1a* was used to express a POX in transgenic wheat and barley, leading to enhanced basal resistance to powdery mildew (Altpeter et al. 2005). Similar basal resistance to post harvest pathogens could be increased using periderm specific promoters in sweet potato, carrot and beets (Scott et al. 1998). Additionally, transgenes could be targeted away from the edible portion of the plant to alleviate perceived health risks associated with

consumption of transgenes. The use of a leaf specific promoter such as Rubisco small subunit promoter (Gilmartin et al. 1990), could potentially lead to high levels of foliar disease resistance in plants with edible tubers and roots. Alternatively, root specific promoters could be used in leafy vegetable crops to reduce root diseases (Leach et al. 1991).

Tissue enhanced promoters have limitations since they continue to rely on endogenous transcription factors to activate the promoter. Heterologous tissue specific promoters often are not recognized across plant genera or even species, resulting in low levels of gene expression (Gurr et al. 2005a).

1.7.4 Inducible promoters

Inducible promoters offer several advantages over tissue specific promoters, due to higher evolutionary conservation within hormonal signalling pathways (Potenza et al. 2004). Many inducible promoters can be used. However, pathogen induced or phytohormone induced promoters have the greatest potential for increasing pathogen resistance. The promoters of *PR1* from Arabidopsis exhibit high levels of induction in the presence of pathogen or the phytohormone SA (Rushton et al. 1996). Expressing an antifungal protein with *PR1* promoter could potentially reduce the disease symptoms. Similarly, a pathogen induced *win3.12*T promoter from poplar drove expression of 2 exogenous amphibian antifungal proteins in transgenic tobacco. These tobacco plants exhibited enhanced resistance to several necrotrophic pathogens, with no detectable constitutive transgene expression (Yevtushenko et al. 2007).

However, the *win3.12*T promoter was an exception as most pathogen induced promoters are insufficient for use in increasing disease resistance (Gurr et al. 2005a). The problem lies with the pathogen needing to be recognized by the host cell; typically, this occurs following the establishment of the disease. One potential solution is the additional application of an elicitor in combination with a transgene under the control of a *PR1* promoter. Chemical elicitors such as BTH (non phytotoxic SA analogue), or an oligosaccharide elicitor such as chitosan are known to induce *PR1* expression (Durrant et al. 2004; Jayaraj et al. 2009). Elicitor applications could be made when infection risk is at the highest, with the added benefit of inducing several innate defence responses.

The core promoter element of the *35S* promoter does not alone drive the expression of any genes; however, it does contain all of sequences necessary for the initiation of transcription. Ligation of specific cis-elements to the *35S* backbone has the potential to generate a large number of promoters for precise expression of transgenes (Venter 2007). Rushton et al (2002) identified a large number of pathogen inducible cis-elements in Arabidopsis. When combined with up to eight of these elements, expression levels were highly induced during *Pseudomonas* infection (Rushton et al. 2002). Spacing and precise arrangement of these cis-elements further increased the expression (Rushton et al. 2002).

1.8 Objectives of this study

Diseases caused by fungal and bacterial pathogens are among the most important limitations to carrot production throughout the world. These diseases afflict the carrots not only during the growing season, but also during the long

post-harvest storage period. There currently is not a sufficient supply of genetic resources for which the use of traditional breeding programs to be successfully implemented at a large scale. Therefore, transgenic expression of genes that can confer resistance towards pathogens represents an ideal solution for improving disease tolerance in carrot.

The main aim of this study was to identify candidate genes and promoters for the development of transgenic carrot lines, which exhibit commercially desirable levels of disease resistance against a range of pathogens. The emphasis will focus on pathogens that infect either the foliar tissues or the taproot post-harvest.

Specific objective include:

1. Determination of optimal promoters to drive gene expression in carrot
2. Investigation of efficacy and comparison of traditionally used *PR* genes (wheat chitinase and β-1,3, glucanases) in controlling carrot diseases
3. Determine the efficacy and mechanisms of resistance of a more rarely used *PR* gene (rice peroxidase)
4. Investigation the use of a transcription master control gene *NPR1* and determine the efficacy in carrot

2: COMPARATIVE EXPRESSION OF β-GLUCURONIDASE WITH FIVE DIFFERENT PROMOTERS IN TRANSGENIC CARROT (*DAUCUS CAROTA* L.) ROOT AND LEAF TISSUES

Results from this chapter were published: Wally, O., Jayaraj, J., and Punja, Z.K. (2008). Plant Cell Reports. 27: 279-287

2.1 Introduction:

Carrot (*Daucus carota* L. subsp. *sativa*), a member of the family Apiaceae, is grown for its edible taproot, which contains high levels of β-carotene (provitamin A), vitamins B_1 and C, and provides a good source of dietary fibre (Ammirato 1986). Commercial cultivars of carrot have been developed using traditional breeding methods for improved root growth, root shape and colour, β-carotene levels and smooth skin (Ammirato 1986). Carrot is a model system for tissue culture studies and previous research has demonstrated the utility of somatic embryogenesis, bioreactor scale-up of suspension cultures and protoplast culture and fusion for carrot improvement (Ammirato 1986; Komamine et al. 2005; Zimmerman 1993). In addition, transgenic technology has been used to enhance fungal disease resistance in carrot (Chen et al. 2002; Jayaraj et al. 2007; Melchers et al. 2000; Takaichi et al. 2000), to create herbicide-resistant plants (Chen et al. 2002), or for metabolic engineering of designer medical products (Bouche et al. 2003; Kumar et al. 2004; Marquet-Blouin et al. 2003) and novel antioxidant compounds (Jayaraj et al. 2008a). In the majority of these

studies, the *Cauliflower Mosaic Virus 35S* (CaMV *35S*) (Odell et al. 1985) was used, while the maize ubiquitin promoter (Christensen et al. 1992) was also utilized to drive the constitutive expression of transgenes in carrot (Chen et al. 2002). The regulation of transgene expression is crucial for successful commercial genetic engineering to ensure expression levels are high and in the desired tissues. A comparative assessment of promoter tissue specificity and strength in different tissues has not been previously conducted in carrot as they have in other plants (Gandhi et al. 1999; Holtorf et al. 1995; Kamo et al. 1999; Ni et al. 1995; Samac et al. 2004; Schledzewski et al. 1994). In particular, the utility of promoters to provide expression in carrot taproots has not been previously assessed.

The objective of this study was to characterize β- glucuronidase (*uidA*) expression in transgenic carrot tissues under control of three constitutive promoters: the *Arabidopsis* Ubiquitin 3 promoter (*UBQ3*) (Norris et al. 1993), the CaMV *35S* promoter (Odell et al. 1985) and domain B duplicated CaMV *35S* (*D35S*) (Kay et al. 1987). The promoters *mannopine synthase* (*mas2*) (Feltkamp et al. 1995) from *Agrobacterium tumefaciens* and the rooting loci gene promoter *rolD* (Leach et al. 1991) from *A. rhizogenes* were also evaluated, since previous reports indicated that these promoters had enhanced root activity. Relative strengths of these promoters were measured in the leaves, lateral roots and taproots of mature greenhouse-grown carrot plants as well as *in vitro* grown calli, leaves and roots of five independently-derived transgenic lines for each promoter.

2.2 Materials and Methods:

2.2.1 Plasmid DNA and plant transformation

The p*CAMBIA* 1391 Z plant transformation vector containing the hygromycin resistance gene under control of the CaMV *35S* promoter and the 2 exons of *uid*A with the catalase intron in front of the pUC9 multi-cloning site (MCS) with a *nopaline synthase* terminator (CAMBIA, Australia) was used. Plasmid DNA was isolated from 2 ml of overnight grown cultures of *E. coli* using the Qiagen Qiaquick spin column isolation kit (Qiagen, Maryland, USA). The CAMV *35S*, *D35S* (Dr. Shawn Mansfield, University of British Columbia), *UBQ3* (Syngenta, Canada), *mas2* (Dr. Stephane Garberk, INRA, France) and *rolD* (Francesca Leach, INRA, France) promoters were cloned into the *Hind*III and *Bam*HI sites of the MCS (Fig. 2.1). The ligated plasmids were transformed by electroporation into electromax LBA4404 competent *A. tumefaciens* cells (Invitrogen, Carlsbad, CA, USA) using established methods (Wally et al. 2006). Sterile 'Nantes Coreless' carrot petiole explants were transformed and regenerated as described by Wally et al. (2006). The transgenic callus was maintained on full-strength Murashige and Skoog (MS) medium (Murashige et al. 1962) supplemented with 100 mg/L hygromycin and 0.5 mg/L 2,4-D. Regenerated plantlets were maintained on hormone-free half-strength MS with 100 mg/L hygromycin, and transferred to soil and grown in the greenhouse (Appendix A1; Wally et al. 2006). Root and leaf tissues from 4-5 month old greenhouse-grown plants were

harvested and used for analysis of GUS expression and molecular analysis. In addition, lateral root and leaf tissues from 4-8 week old tissue-culture derived plantlets were also included in the analysis.

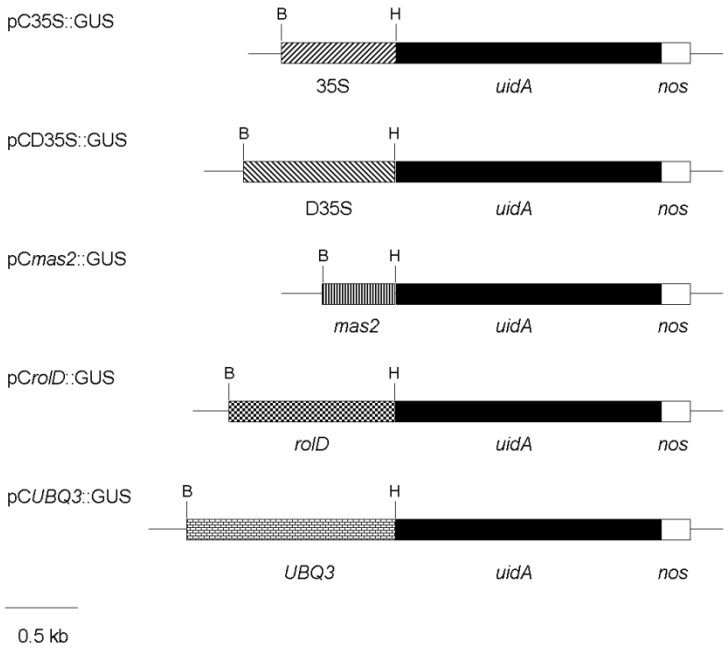

Figure 2.1 Plasmid DNA constructs used for *Agrobacterium* transformation of carrot. From top to bottom: the *uidA* fusion constructs in pCambia1391Z plasmid, under the control of CaMV *35S* promoter (pC35S::GUS), enhanced *35S* promoter (pCD35S::GUS), the *A. tumefaciens mannopine synthase* (*mas2*) promoter (pC*mas2*::GUS), the *A. rhizogenes* rooting loci gene (*rolD*) promoter (pC*rolD*::GUS) and the *Arabidopsis* ubiquitin (*UBQ3*) promoter (pC*UBQ3*::GUS

2.2.2 Confirmation of gene integration

The presence of the *hygromycin phosphotransferase* (*hph*) gene using PCR was used as the first step for confirmation of transformation. Total genomic DNA was isolated from 200 mg (fresh weight) of leaf tissue that was lyophilized prior to being extracted using established protocols (Wally et al. 2006). The primers used to amplify a 1025 bp fragment of the *hph* gene were hph-F2 (5'-CTA TTT CTT TGC CCT CGG AC-3') and hph-R2 (5'-AAG CCT GAA CTC ACC GCG AC-3'). Each reaction (25 µl) contained 50 ng carrot DNA, 50 p*M* hph-F2 and hph-R2, 0.5 units *Taq* polymerase (Invitrogen) and 1.5 mM $MgCl_2$. The PCR conditions included a 55 °C annealing temperature and proceeded for 35 cycles using a PTC-200 thermocylcer (MJ Research, Waltham, MA, USA). Subsequently, Southern analysis was used to demonstrate integration of the *uidA* gene. Total genomic DNA (12 µg) was digested for 16 h at 37° C with 80 units of either *Bam*HI or *Hind*III (Promega, Madison, WI, USA), to cut at sites that are both unique in the transgene construct. The digested DNA was run on a 1.2% agarose TBE gel for 20 h and transferred to a nylon membrane under alkaline conditions using established protocols (Sambrook et al. 1989). The blots were probed with a ^{32}P labelled 740 bp fragment of the *uidA* gene generated by PCR using the primers GUSA-F1 (5'- TGA AGA TGC GGA CTT ACG TG -3') and GUSA-R1 (5'-CCA GCC ATG CAC ACT GAT AC-3') under the above PCR conditions with a 58 °C annealing temperature. Random primers were used to label the probe

using [α-^{32}P] dCTP and Prime-A-gene kit (Promega) following the manufacturers' protocols. The blots were hybridized with the probes for 16 h at 65 °C following a 2 h pre-hybridization at 55°C using Ekono hybridization buffer (Research Products International Corp, Mt. Prospect, IL, USA). Following hybridization, the blots were washed 3 times at room temperature with 2X SSC, 0.1% (w/v) SDS for 5 min each, followed by 2 washes with 1X SSC, 0.1% (w/v) SDS at 60 and 65°C, respectively, for 20 min (Sambrook et al. 1989). The blots were exposed to X-ray film at -80°C for 3-7 days with an intensifying screen.GUS copy number was estimated by counting the number of intact fragments, as well at estimation of hybridization intensity using Image J, software.

2.2.3 GUS expression

Histochemical staining of petioles, leaves, lateral and taproots was performed according to Jefferson et al. (1987). The plant tissues were placed in fixative (10 mM MES, pH 5.6, 0.3 M mannitol and 0.3% formaldehyde) on ice for 30 min, followed by washing in 50 mM NaH_2PO_4, pH 7.0. The samples were vacuum infiltrated (20 mBar) for 15 min and stained overnight at 37°C in 50 mM NaH_2PO_4 , 0.5% (v/v) Triton X-100 and 1mM 5-bromo-3-chloro-3-indoyl-β-D-glucuronide cyclohexamide sodium salt (X-gluc, Inalco Pharmaceuticals, Italy) dissolved in dimethyl-formamide. Petioles, leaves and orange roots were destained by repeated washings with 70% ethanol for 24-48 h, or until all the coloured pigments were removed. Sections were visualized using 40 X

magnification under a light microscope with white light (Zeiss, Axioskop, Germany).

Specific expression of GUS for each promoter was determined using 5 independent lines derived from different transformation events and confirmed to be transformed by Southern analysis. Tissue samples were taken a minimum of three times at 1-week intervals from mature greenhouse-grown shoots and roots, callus and tissue-cultured shoots and roots. GUS activity was determined according to Jefferson et al. (1987) by measuring the accumulation of the fluorescent substrate 4-methylumbelliferone (4-MU) cleaved from 4-methylumbelliferone-glucuronide (4-MUG). Samples of 250 -750 mg fresh weight of the various carrot tissues were macerated using an ice-cold mortar and pestle with a pinch of sea sand with a 2:1 (v/w) buffer: tissue ratio. The extraction buffer contained 50 mM NaH_2PO_4, pH 7.0, 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium lauryl sarcosine, 4% (w/v) polyvinylpolypropadine (PVPP) and 5 mM dithioretitol (DTT) (Jefferson et al. 1987). Macerated samples were centrifuged twice at 4°C for 15 min at 14 000 g, and the supernatant was transferred to a new 1.5 ml micro centrifuge tube. Clarified protein extracts were quantified using the Bradford reagent (Sigma, USA) using a Bio-Tek 1200 microplate reader (Fisher, USA). Five µg of protein extract was assayed in a total volume of 50 µl in a 1 mM MUG solution (50 mM NaH_2PO_4, 10 mM EDTA, 0.1% (v/v) Triton X-100 and 5 mM DTT) on a 96 well micro-titre plate. The reactions were stopped at 1.5, 3 and 4.5 hours by adding 250 µl of 0.2 M sodium carbonate. Fluorescence was measured using a 96-well fluorometer (Isoplate-96

Perkin-Elmer Corporation, Norwalk, CT, USA) set at 365 nm for excitation and 455 nm for emission. Non-transformed 'Nantes Coreless' carrot roots and leaves served as negative controls/blanks for both histochemical and fluorometric assays.

2.3 Results

2.3.1 Molecular analysis

Of the 76 total lines confirmed to be positive for the presence of the *hph* gene by PCR, 70 contained one to three copies of the *uid*A gene, as determined by *HindIII* and *Bam*HI digestion and Southern blot hybridization (Fig. 2.2). Up to 10 independent lines, with an estimated 1-5 *uidA* copies, from each of the 5 promoter constructs were then analyzed for GUS activity by histochemical staining (Fig. 2.3). The majority (65 of 70) had detectable levels of X-gluc staining, and the 5 visually strongest expressing lines from each promoter were selected for further analysis. The percentage of regenerated plantlets that grew on MS medium with 100 mg/l hygromycin and showed *uid*A expression was 100% for *UBQ3* and *mas2*, 80% for *rolD*, 75 % for *35S* and 50% for *D35S*.

2.3.2 Visualization of GUS expression

There were significant differences in the intensity of staining of carrot tissues with X-gluc in plantlets containing the different constitutive promoter constructs. Strong GUS activity was found in the leaves of plants expressing

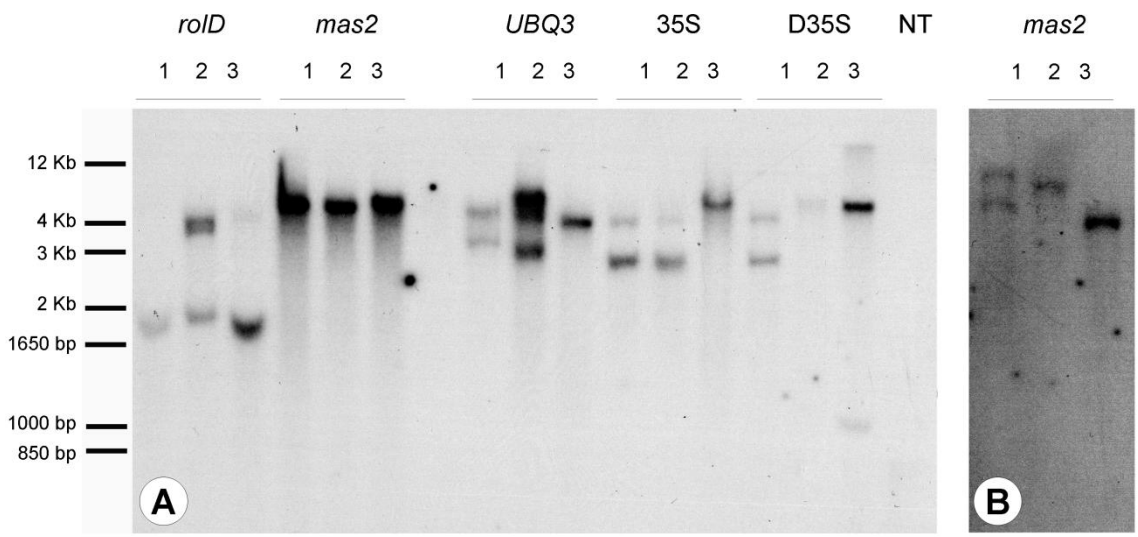

Figure 2.2 Southern hybridization analysis of transgenic carrot lines containing the *uidA* fusion gene under control of either the *rolD*, *mas2* , *UBQ3,* CaMV *35S* or *D35S* promoters. Genomic DNA was digested with *Bam*HI (A) or *HindIII* (B); the DNA blot was hybridized with the 740 bp *uidA* gene fragment. Three different lines were shown for each promoter. Size markers from the 1 Kb+ (Invitrogen) ladder are shown on the left. NT, non transgenic control plant.

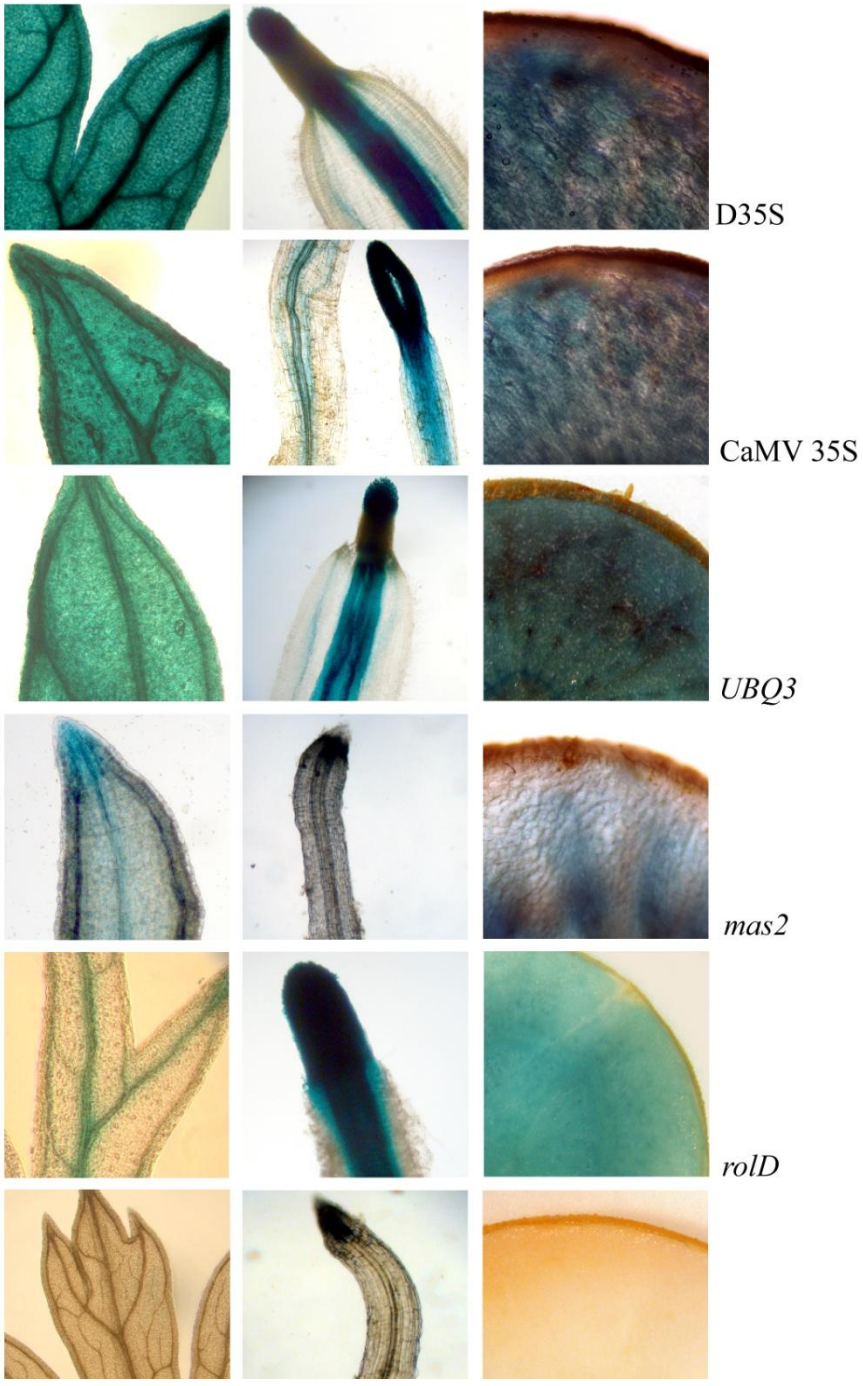

Figure 2.3 Histochemical assay for GUS activity shown from left to right, leaves, seedling roots and taproots of carrot. The various promoters used in this study are indicated.

UidA under control of the *D35S*, *UBQ3* and *35S* promoters. All leaf tissues stained very darkly, including the trichomes, mesophyll and vascular tissues (Fig. 2. 3). There were only slight visually observable differences between the promoters, with the *D35S* lines appearing stronger overall. When the lateral roots were stained, these three promoters provided expression throughout the length of the root, including the root tip, root hairs and the vascular tissues (Fig. 2.3). Cross-sections of the taproots revealed similar patterns of staining; however, the *35S* promoter provided slightly less intense and non-uniform staining throughout the length of the root compared to *UBQ3* and *D35S* (Fig. 2.3). There was intense GUS staining in the root parenchyma cells, phloem rays, xylem and cambium in plants containing the *35S*, *D35S* and *UBQ3* promoters (Fig. 2.3).

Plantlets expressing *uidA* under control of the *mas2* and *rolD* promoters showed significant differences in staining intensities in different tissue types of greenhouse-grown plants compared to the smaller tissue culture-grown plants. Only slight staining was visible in the veins of leaves of *rolD* plants (Fig. 2.3), while the *mas2* promoter showed weak staining throughout the leaves, with more staining near the tip of the leaf (Fig. 2.3). In *mas2* lines, the cotyledons and hypocotyl tissues exhibited GUS activity, while no activity was seen in these tissues in the *rolD* lines (not shown). Lateral roots of *mas2* and *rolD* lines stained darkly, with enhanced staining observed at root tips, vascular bundles and root hairs. The *rolD* roots stained slightly darker than roots of the *mas2* lines; however, expression was still significantly lower when compared to the constitutive promoters. The taproots of plants with *mas2* and *rolD* promoters

were also stained throughout the different tissue types, with the weakest staining observed in the periderm and more intense staining in the phloem and cambium (Fig. 2.3).

2.3.3 Quantification of promoter strength

GUS enzyme activity assays were performed on the 5 lines selected for each of the different promoter constructs. For the CaMV *35S* promoter, GUS activity was lowest in callus tissue and highest in leaves of tissue-cultured plants (Fig. 2.4). High GUS activity was observed in the roots and leaves of mature greenhouse-grown plants, which was similar to the levels measured in the roots of tissue-cultured plants. There was more variation among individual *35S* promoter plant lines in leaf expression, compared to root or callus tissue.

With the *D35S* promoter, there was very high GUS activity in all tissue types examined, with highest activities in mature greenhouse-grown leaves and in leaves and roots of tissue culture-grown plants (Fig. 2.4). The lowest activity was found in callus tissue, which had approximately 50% of the GUS activity compared to the leaves. The *D35S* promoter had higher GUS activity than the *35S* promoter in all tissues examined, ranging from an average of 1.5-fold higher activity in tissue-culture grown leaves to an average of over 3-fold higher activity in callus tissues (Table 2.1). With the *UBQ3* promoter, the highest GUS activity was observed in the roots of mature greenhouse-grown plants, with an average of 2.5-fold higher activity than the *35S* promoter. The GUS activity was also significantly higher compared to the *35S* promoter in the callus tissue, with nearly

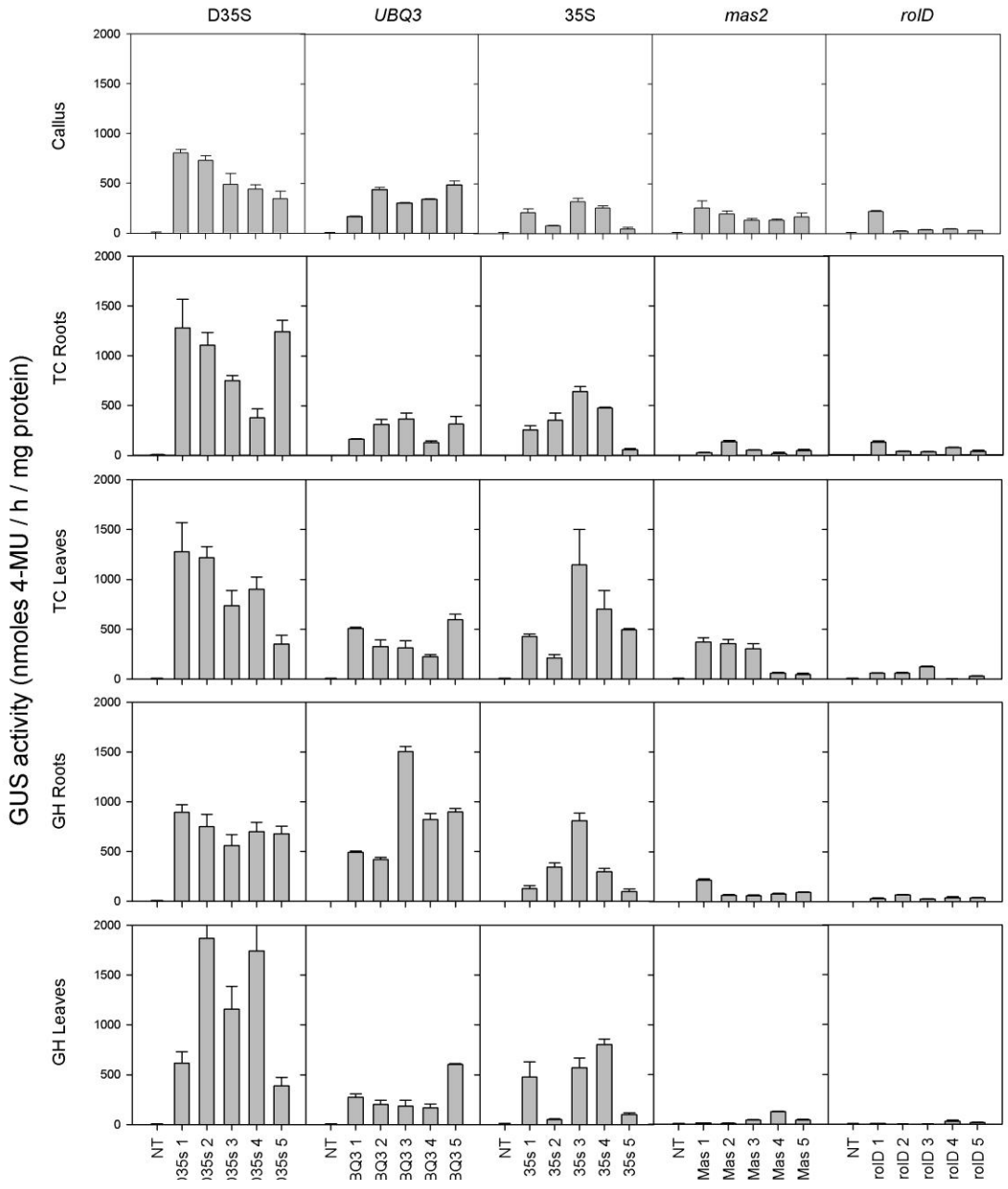

Figure 2.4 Specific activity of GUS in callus, tissue-cultured (TC) roots, tissue-cultured leaves, greenhouse-grown (GH) roots and leaves of transgenic carrot plants expressing the *uidA* gene under control of five promoters (*D35S*, *UBQ3*, *35S*, *mas2* or *rolD*). Values represent the mean specific activity in 5 individual lines for each promoter, with a minimum of 3 replicates +/- standard error for each transgenic line.

Table 2.1 Average GUS expression in callus, leaves, and roots of carrot plants expressing the *uidA* gene under control of CaMV *35S*, *D35S*, *UBQ3*, *mas2* or *rolD* promoters.

Promoter		Callus	Tissue culture-grown plants		Greenhouse-grown plants	
			Leaves	Roots	Leaves	Roots
D35S	Mean[a]	561.7 +/- 48.9 (3.08)	896.8 +/- 94.7 (1.51)	950.7 +/- 95.6 (2.70)	1154.3 +/- 164.4 (2.89)	715.6 +/- 30.2 (2.13)
	Range[b]	345-804	350-1280	377-1278	390-1870	560-891
UBQ3	Mean	350.5 +/- 30.9(1.92)	391.9 +/- 38.5 (0.66)	350.6 +/- 26.1 (0.98)	286.3 +/- 45.5 (0.72)	827.1 +/- 107.5 (2.47)
	Range	171-487	223-600	129-365	166-603	420-1500
35S	Mean	182 +/- 29.1 (1.00)[c]	595 +/- 88.3 (1.00)	355.9 +/- 55.24 (1.00)	399.2 +/- 80.2 (1.00)	335.3 +/- 16.2 (1.00)
	Range	45-320	210-1145	55-640	99-801	100-800
mas2	Mean	178.1 +/- 12.8 (0.98)	227.0 +/- 40.3 (0.38)	57.3 +/- 11.6 (0.16)	48.2 +/- 12.1 (0.12)	101.1 +/- 16.2 (0.30)
	Range	135-257	47-372	20-136	10-129	56-214
rolD	Mean	71.4 +/- 21.1 (0.39)	54.1 +/- 11.0 (0.09)	61.2 +/- 10.2 (0.17)	11.1 +/-2.5 (0.03)	39.2 +/- 4.1 (0.12)
	Range	32-221	3-120	31-127	3-27	25-66

[a] Values are the means of specific activity (nmoles 4-MU/h/mg protein) +/- standard error of five independently transformed lines for each promoter. The assays were repeated 3-6 times.
[b] Range of GUS activity (nmoles 4-MU/h/mg protein), with highest and lowest values
[c]Value in parenthesis indicates the relative GUS expression level compared to the CaMV *35S* level for the particular tissues.

an average increase of 2-fold. The *UBQ3* promoter GUS activity was significantly lower than *35S* promoter in the leaves of tissue-cultured plants, and was similar to that in roots of tissue culture-grown plants. However, in greenhouse-grown plants, the roots had an average of nearly 3-fold higher GUS activity compared to the leaves.

With the *mas2* promoter, the average GUS activity was highest in leaves of tissue culture-grown plants (Fig. 2.4). There was also fairly high GUS activity in the callus tissue with levels similar to that of the *35S* promoter (Table 2.1). There was an average of 4-fold higher activity in tissue-culture grown leaves as compared to roots of the *mas2* lines examined. However, in mature greenhouse-grown plants, there was 50 % less GUS activity in leaves compared to roots. There was an average of 2-fold higher activity in mature roots compared to young tissue-culture grown roots. There was approximately 4-fold higher activity in young tissue-culture grown leaves compared to mature greenhouse-grown leaves.

With the *rolD* promoter, GUS activity was highest in callus tissues (Fig.2. 4). The activity was relatively low in all of the other tissues tested, with GUS levels at only 3-12% of that in the roots and leaves of *35S* promoter plants (Table 2.1). There were no significant differences in GUS activity between tissue-culture grown roots and leaves. However, in mature greenhouse-grown roots, there was nearly a 4-fold increase in GUS activity compared to the leaves. GUS activity was similar in greenhouse-grown roots and tissue-cultured roots.

2.4 Discussion

The CaMV *35S* promoter is the most widely used promoter for transgene expression in plants, and provides very high constitutive levels of expression in dicotyledonous species and slightly weaker expression in monocotyledon species (Gandhi et al. 1999). The enhanced *D35S* promoter has a duplication of the -343 to -90 domain B which has been shown to enhance transgene expression by up to 10-fold when compared to the *35S* promoter (Kay et al. 1987). As with previous reports from other dicotyledonous plants (Comai et al. 1990; Holtorf et al. 1995), the CaMV *35S* and enhanced *D35S* promoters were found to be the strongest promoters overall in carrot tissues. The duplication of the domain B increased the overall expression levels in carrot by an average of 3.1-fold in callus, 2.7 and 2.1-fold in roots, and 1.5 and 2.9-fold in leaves under different growing conditions. These increases are within the ranges reported for other transgenic plants (Potenza et al. 2004). Both *35S* and *D35S* promoters provided strong GUS expression in all carrot tissues examined, including leaves, petioles, cotyledons, lateral roots and taproots. Both promoters had highest activity in leaves, followed by roots and callus tissue. These findings are consistent with those from *Arabidopsis* and tobacco, where petiole and leaf expression levels were significantly higher than in roots (Holtorf et al. 1995; Malik et al. 2002) but differs from the expression levels reported in gladiolus and alfalfa (Kamo 2003; Samac et al. 2004), in which the *35S* promoter had the highest activity in roots. Overall, the *D35S* promoter provided the highest level of gene expression in all carrot tissue types tested, except for mature roots.

Ubiquitin is highly conserved across plant species, is highly abundant in the cytoplasm and is involved in many crucial cellular processes. Many of the Ubiquitin genes are constitutively expressed, including the maize *ubi-1* and *Arabidopsis UBQ3* genes (Christensen et al. 1992; Norris et al. 1993). Typically, monocotyledon plants have highest constitutive levels of transient gene expression with monocot-derived actin or ubiquitin promoters, while dicots typically have highest expression with viral promoters or dicot-derived ubiquitin promoters (Gandhi et al. 1999; Holtorf et al. 1995; Kamo 2003). In this study, the *Arabidopsis UBQ3* promoter provided significantly higher expression levels compared to the CaMV *35S* promoter in callus (2.0-fold higher) and mature greenhouse-grown roots (2.5-fold higher). The *UBQ3* driven GUS expression levels were very similar to the *35S* promoter in tissue-cultured young roots and slightly lower in both tissue-cultured (0.65 of the *35S*) and mature greenhouse leaves (0.7 of the *35S*). These findings are similar to those made in transiently-expressing *Arabidopsis*, where comparable expression was observed in the leaves with genes driven by the *35S* and *UBQ3* promoters (Norris et al. 1993). The *UBQ3* driven GUS expression was very prominent in carrot root tissues, with enhanced activity when compared to the *35S* and *D35S* promoters. When quantified, the GUS activity provided by the *UBQ3* promoter in mature roots was significantly higher than that of the *D35S* promoter. The heightened overall root activity indicates that *UBQ3* is ideal for expressing proteins in taproots, and for post-harvest roles, including suppression of post-harvest diseases by over-expression of pathogenesis-related proteins.

The *mannopine synthase* gene is a bi-directional gene from *Agrobacterium tumefaciens*, which requires the activity of a *mannopine conjugase* (*mas2*) and *reductase* (*mas1*). The *mas2* promoter has been analyzed in detail, and contains 2 enhancer sequences and is reported to provide significantly higher levels of gene expression than the *mas1* promoter, which contains only a single enhancer sequence (Guevara-Garcia et al. 1999). In this study, the *mas2* promoter drove high levels of GUS expression in callus tissue cultured *in vitro*, with GUS expression levels approaching that of the *35S* promoter. The *mas* promoters are sensitive to auxin and activity increases when the auxin: cytokinin ratio is increased (Langridge et al. 1989). The carrot calli were maintained on medium containing 2, 4-D (0.5 mg/l); therefore, enhanced expression in the callus tissue was expected. Similar to transgenic potato and tobacco transformed with *mas2*::*uidA* fusion constructs, there was higher GUS activity in mature greenhouse-grown carrot roots compared to the shoot or leaf tissues (Feltkamp et al. 1995). However, these levels were substantially less than in transgenic tobacco, where root activity levels exceeded those of the *35S* promoter (Comai et al. 1990). Our results also differed from the *mas2* activity reported in transgenic rapeseed varieties, which exhibited high *mas2* driven GUS activities in the leaves, with reduced activity in roots, substantially lower than for the *35S* promoter (Pauk et al. 1995). In carrot, the *mas2* promoter is potentially useful for use in suspension culture bio-reactors, reflecting the high GUS expression levels observed in callus tissues, and for expression of transgenic proteins in mature roots.

The rooting loci gene (*rolD*) isolated from the root-inducing plasmid of *Agrobacterium rhizogenes* has been reported to drive high levels of expression in both the leaves and roots of young transgenic tobacco seedlings (Leach et al. 1991) and transgenic pea (Fei et al. 2003). The transgenic carrots examined had very low levels of GUS expression in all of the tissues tested. Strongest expression was seen in the callus, roots and leaves of tissue-cultured plants. In mature plants, there was 4-fold higher activity in the roots compared to the leaves. Conversely, mature transgenic tobacco containing the *rolD*::*uidA* construct had 30-fold higher GUS expression in roots compared to the shoots (Elmayan et al. 1995). In carrot, the overall strength of *rolD* in mature root tissues was substantially lower than that reported from other plants. Transgenic *Gladiolus* plants exhibited strong GUS root expression with *rolD*, and expression levels were comparable to that of the *35S* promoter (Kamo 2003). In transgenic *N. plumbaginifolia*, a 3-5 fold higher root expression was seen with *rolD* compared to that of the *35S* promoter (Fraisier et al. 2000). Despite lower GUS activity, histochemical staining of GUS was still evident with the *rolD* promoter in carrot taproots. However, these findings indicate that the *rolD* promoter will likely not be very useful for expressing transgenic proteins in carrots.

In conclusion, the *D35S* promoter provided highest levels of GUS activity in carrot leaves followed by the *35S* promoter, while the *UBQ3* promoter from *Arabidopsis* provided high levels of GUS activity in all tissues, especially in the taproots. The previously reported root enhanced promoter's *mas2* and *rolD* provided proportionally lower levels of GUS activity in mature carrot roots.

Understanding the GUS expression profiles of the different promoters will allow for more precise control of expression levels and organ targeting in both *in vitro* and field grown transgenic carrots.

3: COMPARATIVE RESISTANCE TO FOLIAR FUNGAL PATHOGENS IN TRANSGENIC CARROT PLANTS EXPRESSING GENES CODING FOR CHITINASE, B-1,3-GLUCANASE AND PEROXIDASE

Results from this chapter were published: Wally, O., Jayaraj, J., and Punja, Z.K. (2009) European Journal of Plant Pathology 123: 331-342.

3.1 Introduction

Carrot (*Daucus carota L*. subsp. *sativa*) is a biennial plant which is grown worldwide for its edible taproot that contains pro-vitamin A and is an excellent source of dietary fibre (Ammirato 1986). A wide range of fungal pathogens infect the foliage and roots of carrot, causing reductions in yield and quality. Additionally, some of these pathogens can infect the roots post-harvest during storage (Kora et al. 2003). Many commercially-grown carrot cultivars lack adequate genetic resistance to these fungal pathogens, which currently constrain carrot production worldwide (Davis et al. 2002).

Genetic engineering approaches to increase resistance to fungal pathogens have proven to be successful in a number of agriculturally important crops, including carrot (Melchers et al. 2000; Punja 2001). The most widely-used approach is the over-expression of pathogenesis-related (PR) proteins, such as chitinases and thaumatin-like proteins (reviewed in Punja 2001; Punja 2006). PR proteins represent a large group of proteins which are strongly induced during pathogen infection, as well as by other physiological stresses (reviewed in Ferreira et al. 2007). Several of the PR proteins

function by inhibiting the growth of invading fungal pathogens directly, while others may be involved in signal transduction (Melchers et al. 2000).

In the present study, we compared the effects of three PR-protein genes (chitinase, β-1, 3-glucanase and peroxidase) for their ability to enhance disease resistance in transgenic carrots, either when expressed singly or in combination. Plants containing the transgenes were assessed for foliar resistance to the necrotrophic pathogens *Botrytis cinerea* and *Sclerotinia sclerotiorum*, both of which are known to cause losses during carrot production and for which there is no genetic resistance available (Kora et al. 2003).

3.2 Materials and Methods

3.2.1 Gene selection and transformation

Chitinase gene 383 isolated from 'scab' (*Fusarium graminearum*)-infected 'Sumai-3' wheat encodes a class IV PR-3 acidic exo-chitinase of 1088 bp in length (Gi-4741847) (Li et al. 2001). The β-1, 3-glucanase gene 638 isolated from wheat encodes for a *PR-2* acidic exo β-1, 3-glucanase of 1350 bp in length (Gi-4741849) (Li et al. 2001). The peroxidase gene *POC1* was isolated from 'blast' (*Magnaporthe grisea*)-infected rice and encodes for a unique highly pathogen-induced peroxidase of 311 amino acids (Gi-8901179) (Hilaire et al. 2001; Young et al. 1995).

The plasmids pCambia1300: ubi- 383 and pCambia1300: ubi- 638 were provided courtesy of Dr. S. Muthukrishnan (Department of Biochemistry, Kansas State University, Kansas, USA) and generated through ligation of the *HindIII* fragments containing the chitinase 383 or β-1, 3-glucanase 638 gene along with the 2.0 kb maize *ubiquitin*

promoter-intron and the 0.25 kb fragment containing the CaMV polyA transcription terminator (Anand et al. 2003). The pCambia 1300: ubi-*POC1* was created using the 2.0 kb maize *ubiquitin* promoter-intron and an endogenous 0.2 kb rice poly-A terminator. These fragments were then ligated into an open *HindIII* site of pCambia1300 containing the phosphinothricin N-acetyltransferase gene (*bar*) (Deblock et al. 1987). This confers resistance to phosphinothricin (PPT), the active ingredient of the herbicide glufosinate (Liberty™), under control of the CaMV *35S* promoter utilizing a *nos* terminator (Deblock et al. 1987). Confirmation of the transformation vectors was achieved through sequencing of the ligation sites. The plasmids were transformed by electroporation into electromax competent *A. tumefaciens* strain LBA4404 cells (Invitrogen, Carlsbad, CA, USA) using established methods (Wally et al. 2006). Co-transformations with each of the three constructs in different combinations were conducted by mixing equal concentrations of the appropriate *A. tumefaciens* strains and exposing sterile 'Nantes Coreless' carrot petiole explants to the bacteria solution as previously described . The tissue culture selection and plant regeneration conditions were followed as described previously (Wally et al. 2006).

3.2.2 Characterization of regenerated carrots

The putatively transgenic plants after tissue culture selection were tested for resistance to the herbicide Liberty. Leaves were painted with a 0.4% (v/v) Liberty solution using a cotton swab and visually assessed for phytotoxicity to rule out the possibility of escapes (Chen et al. 2002). Surviving plants were tested for the presence of the *bar* gene using PCR as the first step for confirmation of gene integration. Total genomic DNA was isolated from 200 mg

(fresh weight) of leaf tissue that was lyophilized prior to being extracted using established protocols (Wally et al. 2006). The primers used to amplify a 392 bp fragment of the *bar* gene were inBar-F1 (5'-AAG CAC GGT CAA CCG TA-3') and inBar-R1 (5'-GAA GTC CAG CTG CCA GAA AC -3'). Each reaction (10 µl) contained 10 ng carrot DNA, 20 p*M* primers, 0.1 units *Taq* polymerase (Invitrogen) and 1.5 mM $MgCl_2$. The PCR conditions included a 53 °C annealing temperature and proceeded for 35 cycles using a PTC-200 thermocylcer (MJ Research, Waltham, MA, USA). For detection of the chitinase 383 and β-1, 3-glucanase 638 genes, the primers Ubi A (forward primer in the intron of the maize ubiquitin primer) and PolyA-R (reverse primer in the CaMV polyA terminator) (Anand et al. 2003) were used following the above conditions but with a 62°C annealing temperature, which produced a 1.1 and 1.4 kb fragment for the chitinase 383 and β-1, 3-glucanase 638 transgenes, respectively. For detection of *POC1*, the internal primers POC-EX-F2 (5'-ATG GCC AAA GCA ACT TGC -3') and POC-EX-R2 (5'-CTT CGA GTT CAC CTT GGA GC -3') were used following the above conditions, but with a 55°C annealing temperature, to produce a 935 bp fragment.

Following PCR, Southern analysis was used to confirm integration of the chitinase 383, β-1, 3-glucanase 638 and *POC1* genes. Total genomic DNA was digested with *HindIII* using previously established protocols for carrot (Chapter 2). Random primers were used to label the different PCR products, using [α-^{32}P] dCTP and Prime-A-gene kit (Promega, Madison, WI, USA) following the manufacturers' protocols, and used as radioactive DNA probes. RNA blot

hybridization was conducted on total RNA extracted from young healthy leaf tissue with the TRIZOL® reagent (Invitrogen) according to the supplied protocols. Ten micrograms of total RNA of each line was separated by electrophoresis in a 1.2 % formaldehyde agarose gel and blotted onto Hybond XL (Amersham, Uppsala, Sweden) nylon membranes. The RNA blots were pre-hybridized for 3 h at 55 °C using Ekono hybridization buffer (Research Products International Corp, Mt. Prospect, IL, USA). Hybridization and washing of the membranes was carried using the protocol outlined for Southern blotting. Quantification of signal intensity was carried out with software Image J (NIH, USA) and normalized to that of 28S RNA.

3.2.3 Measurement of enzyme activity in transgenic carrot

For chitinase, β-1, 3-glucanase and peroxidase measurement, protein was extracted from 200 mg of fresh carrot leaves and ground in a mortar and pestle under liquid nitrogen. For chitinase and β-1,3-glucanase measurements the material was suspended in cold phosphate buffer (10 mM, pH 6.0) and ground again with silica sand in micro centrifuge tubes using a polypropylene pestle (Sigma). For peroxidase measurements the soluble protein fraction was extracted from ground leaf tissue in cold Tris buffer (100 mM Tris-HCl pH 7.2, 5 mM dithiothreitol, 250 mM sucrose and 5% (w/v) polyvinylpyrrolidone) (Bindschedler et al. 2006). The resulting suspensions were centrifuged twice at 10,000 g at 4^{o}C and the supernatant was stored at -80^{o}C, and used in enzyme assays. Ionically bound peroxidase was extracted from the pellet using a high salt Tris buffer (20 mM Tris-HCl, 1M NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$), incubated

at 4 ºC for 30 min while constantly agitated. The ionically bound fraction was cleared by centrifugation at 10 000 g at 4 ºC (Bindschedler et al. 2006). Clarified protein extracts were quantified using the Bradford reagent (Sigma, USA) using bovine serum albumin (Sigma) as the standard. Five replicate leaf samples taken from individual plants for each transgenic line were assayed.

Chitinase activity was assayed by monitoring the release of N-acetyl glucosamine from colloidal chitin according to the method of Reissig et al. (Reissig et al. 1955). Chitinase specific activity was expressed as micromoles of N-acetyl glucosamine per mg protein per h at 37^{o}C, and was standardized as a ratio of the activity in non-transformed plants. β-1, 3-glucanase activity was measured using the glucose oxidase reagent (Sigma) according to the manufacturer's recommendations. Specific activity of β-1, 3-glucanase was expressed as micromoles of glucose released from laminarin per mg of protein per h at 40^{o}C. Peroxidase activity was determined from both the soluble and ionically bound protein fraction according to Mika and Luthje (Mika et al. 2003), using o-diansidine (Sigma) as a substrate. Peroxidase enzyme activity was expressed as the change in absorbance/min/mg protein, and standardized against the control extracts.

3.2.4 Assessment of disease resistance

Plantlets of the regenerated lines containing each of the different genes individually and combinations thereof were maintained under greenhouse conditions for up to 8 months as previously described (Appendix A1). Control

plants were tissue culture-derived ‘Nantes Coreless’ carrots, grown under the same conditions as the transgenic plants. Additional controls included low-expressing transgenic lines derived from tissue culture selection and identified by Northern analysis. The compound leaves (25-30 cm in length) were taken from the outer rosette of 4-6 month old transgenic and control plants and inoculated with either *Botrytis cinerea* or *Sclerotinia sclerotiorum*. Both pathogens were isolated from naturally infected carrot leaves, and grown on potato dextrose agar under ambient laboratory conditions (cool-white fluorescent lamps, 12 h photoperiod, and temperature range of 21-24 °C). For inoculations, 2-week old cultures were used and mycelial plugs of 0.5 cm diameter were taken from the colony edge. Leaves of 6 transgenic lines each expressing *POC1*, chitinase 383, or β-1, 3-glucanase 638 individually, and 2 lines each expressing a combination of either chitinase 383 and β-1, 3-glucanase 638, or chitinase 383 and *POC1*, were selected for inoculation. The detached carrot leaves were placed over a wire mesh screen inside a large plastic container lined with moistened paper towels. An average of 3-4 leaves was used per container. The agar plugs were placed mycelial side down on the petiole at the position of the first internode of the detached leaves. The containers were then sealed and placed under ambient laboratory conditions (21-24 °C) for 7 days. The extent of leaf necrosis was determined by counting the number of individual lobed segments per leaflet which were diseased and expressing this as a percentage of total leaf segments on the detached leaf (Punja 2005).

Each experiment was conducted a minimum of three times, with at least three replicates per treatment. Treatments were pooled if there were no differences between individual trials. Controls included non-transformed carrot leaves and non-inoculated leaves. Significant differences between lines were determined by analysis of variance (ANOVA) with means separation using the least significant difference (LSD) (P = 0.05) in Fisher's protected LSD test, employing statistical analysis system (SAS) computer program (SAS Institute, NC, USA). Percentage values were analyzed after arcsine-transformation of the raw data.

3.2.5 Lignin extraction and quantification

Total lignin was extracted using the thiogylcolic acid method (Doster et al. 1988) from 0.5 g of tissue from mature petioles that were trimmed to remove leaves and placed under infection conditions for 3 days, with or without the presence of 4 *S*. *sclerotiorum* plugs. Lignin was quantified as A280 readings relative to the reading for the control plants (Doster et al. 1988).The experiments were conducted twice with 9 replicates for each of the treatments described. Lignin data was analyzed for significant differences using ANOVA as described above.

3.2.6 Tissue fixation and microscopy

Small petiole sections (5-10 mm long) from *S. sclerotiorum*-infected leaves of transgenic line P23 and non-transformed control line were cut out with a sharp scalpel from the margin of the diseased area and immediately fixed in freshly

prepared aqueous FAA (63% [v/v] ethanol, 5% [v/v] acetic acid, and 2% [v/v] formalin) at 4 ºC for 72h. The samples were then rinsed in several changes of buffer before dehydration through an ethanol series (70%, 95% and 100%; 2 h in each). The samples were preinfiltrated with a 100% ethanol-2-hydroxyethylmethacrylate mixture (Technovit® 7100) (1:1, v/v) for 2 h, and infiltrated overnight in Technovit® 7100 according to the manufacturer's instructions (Marivac Ltd., Halifax, Nova Scotia). Serial sections (3-µm thick) were cut through the tissues, in cross-section, and stained with Toluidine Blue O (0.05%) for 5 min. The sections were dried at 50 °C for 10 min and mounted in Permount® on slides and examined under a light microscope (Carl Zeiss, Oberkochen, Germany). A minimum of three sections of each sample was examined and photographed using a PENTAX D camera (Tokyo, Japan).

3.3 Results

3.3.1 Development and analysis of transgenic plants

A total 115 independently derived transgenic plants, each originating from different explants following *Agrobacterium* transformation, were established in soil in the greenhouse after selection on medium containing PPT. Leaf painting of the established plants resulted in 95 of the plants showing high levels of resistance to Liberty herbicide (0.4%) (Fig. 3.1). Molecular analysis using PCR confirmed 89 of the plants to be positive for the *bar* gene (Fig. 3.2A). PCR analysis for the presence of the transgenes revealed that there were 50 lines containing *POC1* (Fig. 3.2B), 25 lines containing β-1, 3-glucanase 638, and 10

lines containing chitinase 383 (Fig.3.2C). There were also 2 lines containing *POC1* plus chitinase 383 (*POC1*/383) and 2 lines containing chitinase 383 plus

Figure 3.1 Response of transgenic (left) and non-transgenic (right) 'Nantes Coreless' carrot plants to the herbicide Liberty (0.4% v/v). Symptoms were visualized 3 days after foliar application of the herbicide.

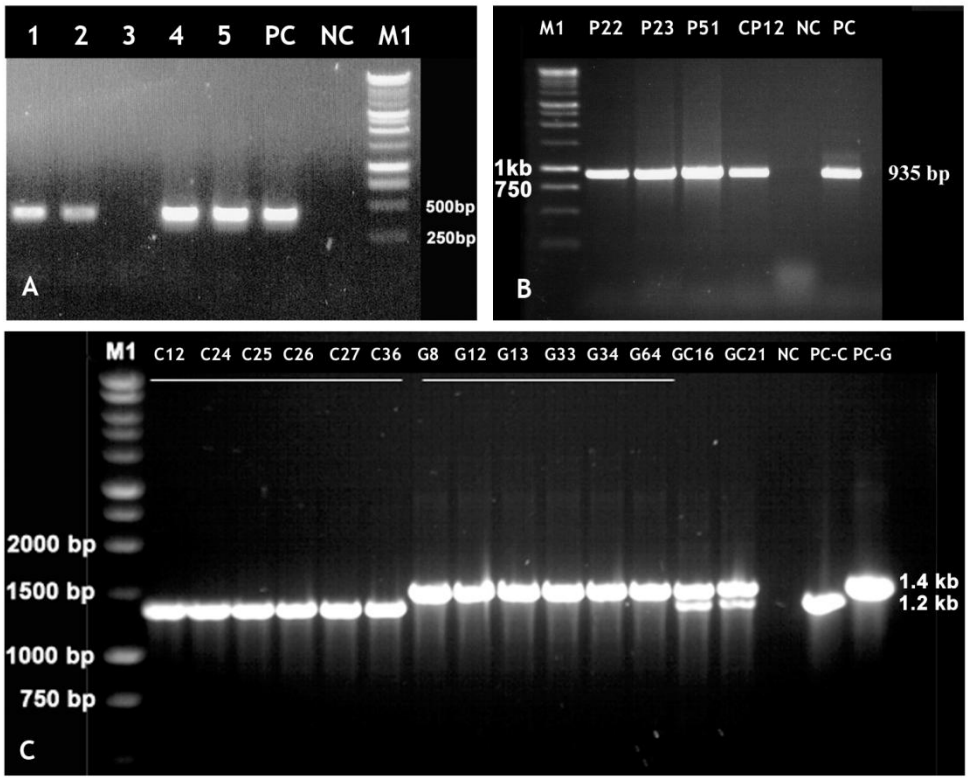

Figure 3.2 PCR detection of transgenes in carrot. (A) PCR detection of the *bar* gene. Lanes 1, 2, 4 and 5 were positive for *bar*, while lane 3 is an escape plant. PC is plasmid control and NC is non transformed carrot control. M1 is Benchtop 1 Kb ladder (Promega). (B) Detection of the peroxidase (*POC1*) transgene. Transgenic lines expressing *POC1* alone (P22, P23 and P51) along with a line co-expressing *POC1* and chitinase 383 (CP12), with plasmid and negative non transformed controls (PC, NC) shown. (C) PCR detection of chitinase 383 and β-1,3-glucanase 638 genes. Lines expressing chitinase383 alone (lanes C12-C36), with lines expressing β-1,3-glucanase 638 alone (lanes G8-G64), and lines co-expressing both genes (lanes GC16 and GC21) are shown. NC is non-transformed control, PC-C and PC-G are the positive plasmid controls for the different genes.

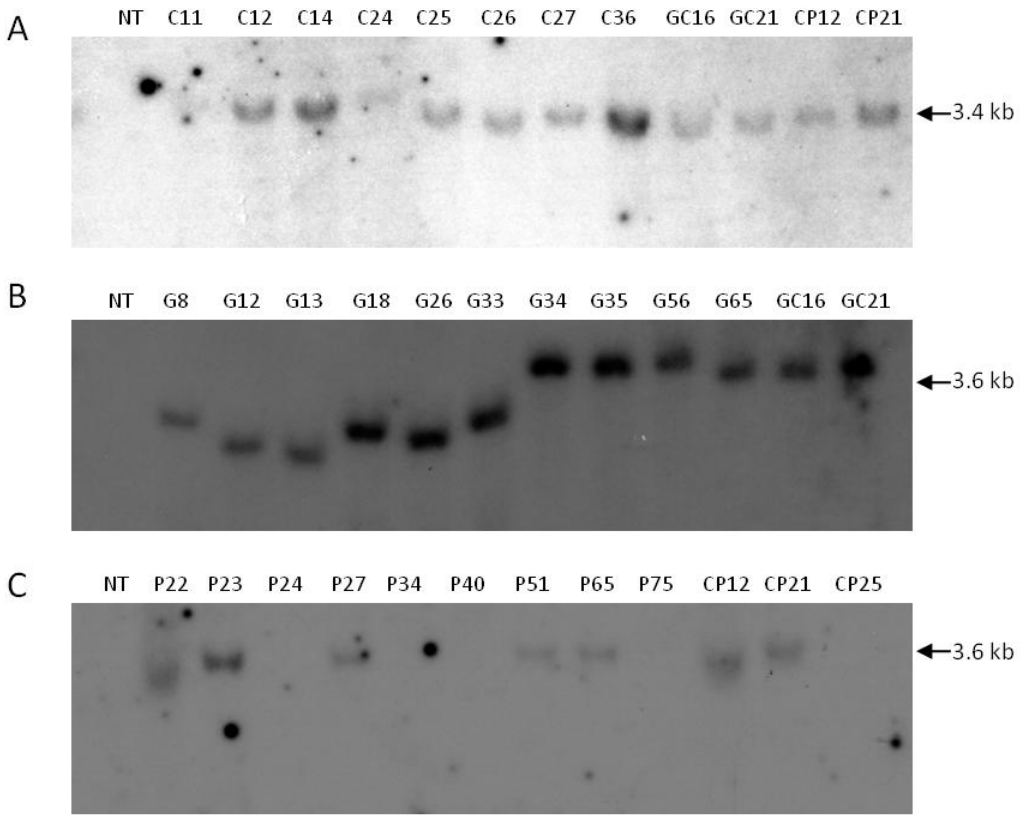

Figure 3.3 Southern blot detection of PR genes. (A) Southern blot hybridized with ^{32}P labelled 383 probe, lines indicated above with non-transformed carrot (NT). (B) Blot hybridized with 638 probe, (C) hybridized with *POC1* probe.

β-1, 3-glucanase 638 (383/638) (Fig. 3.2C). Southern blot analysis confirmed the PCR results, since all of the lines released the entire cassette of the appropriate size when digested with *HindIII* (Fig. 3.3). Northern blot hybridization was conducted on the PCR-positive lines, and a range of transgene transcript expression levels was observed among the lines (Fig. 3.4). The 6 lines which displayed high levels of transcript expression for the single transgenes, along with the 2 lines expressing *POC1*/383 and the 2 lines expressing 383/638, were used for pathogen inoculation. The gene expression levels were also correlated with an increase in enzyme activity. The β-1,3-glucanase 638 lines had up to 2.5-fold increase in β-1,3-glucanase activity compared to the control levels (Fig. 3.5A), whereas the chitinase 383 lines had up to an 8-fold increase in chitinase activity compared to controls (Fig 3.5B). The soluble peroxidase activity levels were unchanged in the transgenic lines; however, significantly increased activity was observed in the cell-wall bound ionic protein fraction, with peroxidase levels up to 3.5-fold higher compared to the control (Fig 3.5C).

3.3.2 Assessment of disease resistance

In non-transformed 'Nantes Coreless' excised leaves, disease symptoms due to *B. cinerea* developed slowly. Relatively small necrotic areas began to appear 4 days after inoculation (dai) and necrosis and sporulation continued to 7 dai, covering approximately 35% of the leaf area (Fig. 3.6A). The lines expressing *POC1* alone showed very high levels of resistance. There were no visible lesions on 3 of the 6 lines at 4 dai, with a significant reduction in the

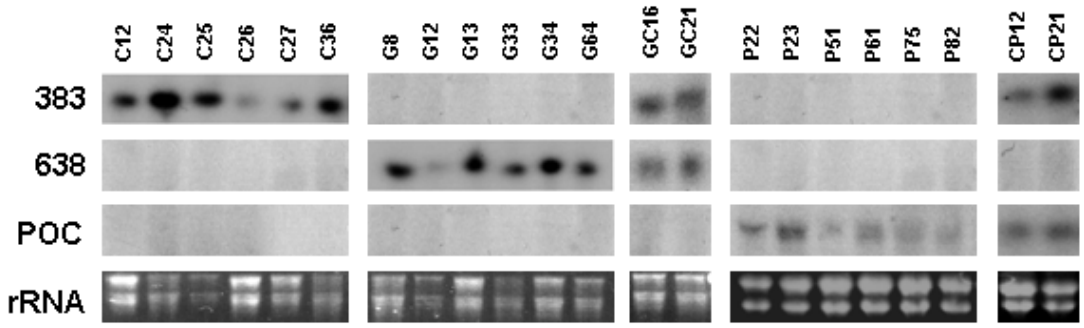

Figure 3.4 Detection of expression of wheat chitinase 383, β-1,3-glucanase 638 and rice peroxidase *POC1* in transgenic carrot by Northern hybridization. Carrot lines transformed with chitinase 383 (C12- C36), β-1,3-glucanase 638 (G8-G64), co-transformed 383/638 (GC16 and GC21), *POC1* (P22-P82), and POC/383 (CP12 and CP21) are shown. The genes used to probe the blot (383, 638 and POC), are shown on the left; rRNA served as the loading control.

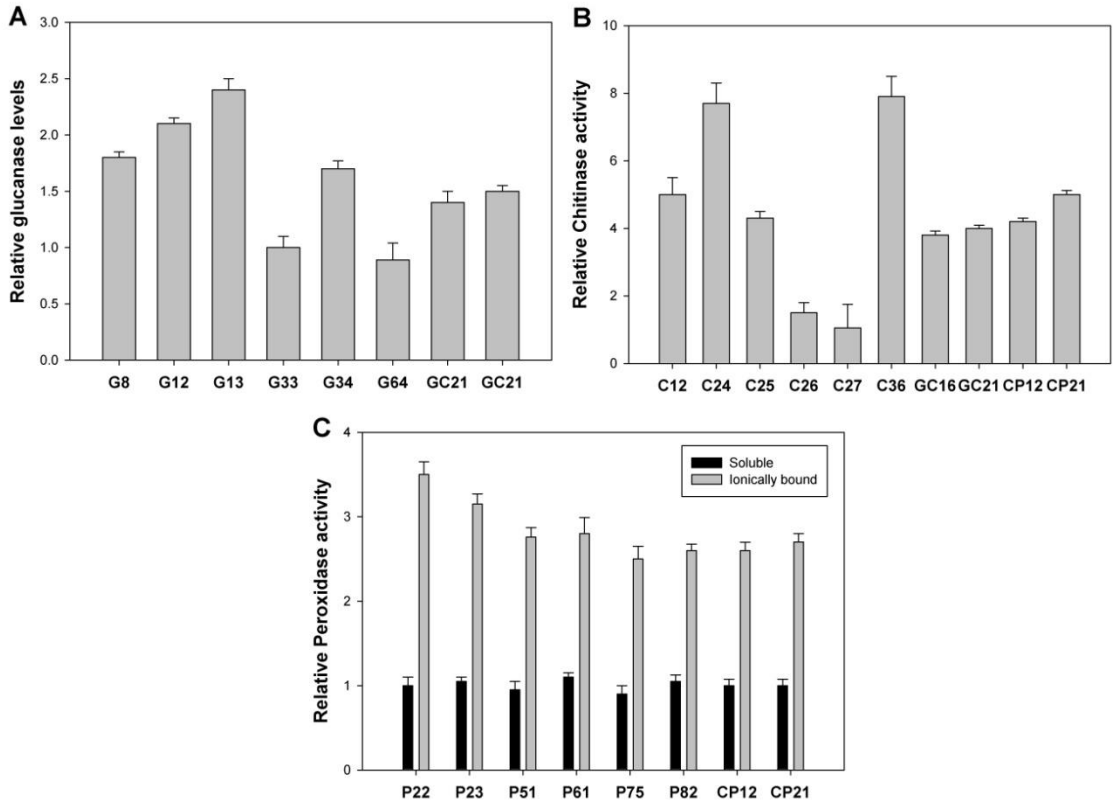

Figure 3.5. **Enzyme activity in transgenic carrot lines, assayed for (A) β-1,3-glucanase , (B) chitinase and (C)soluble and ionically bound peroxidase fractions . Enzyme activity is expressed as a ratio of the activity in non-transformed control plants. Lines expressing β-1,3-glucanase 638 (G8-G64), chitinase 383 (C12-C36), 383/638 (GC-16 and GC-21), peroxidase *POC1* (P22-P82) and POC/383(CP12 and CP-21). Vertical error bars indicate standard error of the mean for five replicate plants for each line.**

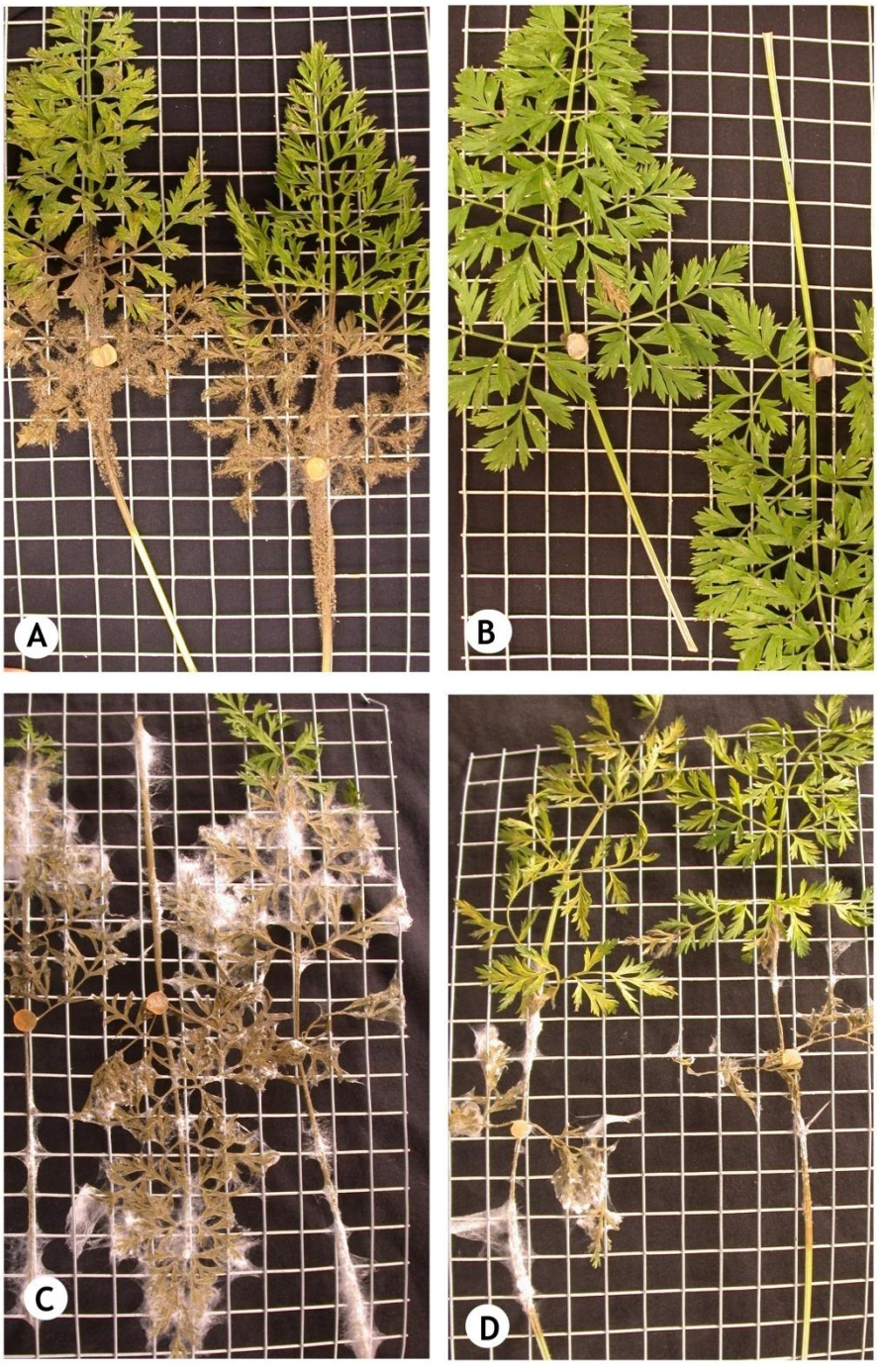

Figure 3.6 Disease symptoms due to *B. cinerea* (A,B) and *S. sclerotiorum* (C,D) on non-transgenic 'Nantes Coreless' (A,C) and on highly resistant peroxidase *POC1* expressing line P22 (B,D) 7 days after inoculation.

remaining 3 *POC1* lines. At 7 dai, 4 of the lines (P-22, P-23, P-51, P-75) had less than 10% of the leaf area diseased (Fig. 3.6B) while the remaining 2 lines (P-61, P-82) had less than 25% leaf area diseased (Fig. 3.7A). By comparison, only two lines (C-24, C-36) expressing the chitinase 383 gene showed a significant reduction in diseased leaf area. The other four chitinase 383 expressing lines had similar disease levels to that of the non-transformed control plants at both 4 and 7 dai (Fig. 3.7B). None of the six lines expressing the β-1, 3-glucanase 638 gene had any reduction in diseased leaf area compared to the controls at either 4 or 7 dai (Fig. 3.7B). The two lines expressing a combination of *POC1*/ 383 had significant reductions in diseased leaf area compared to the non-transformed control line (Fig. 3.7A). These lines had less disease compared to the best chitinase 383 singly-expressing line, but more diseased area than the average singly-expressing *POC1* lines. Both of the lines co-expressing 383/638 had only slight reductions in lesion area due to *B. cinerea* (Fig. 3.7B).

In contrast to *B. cinerea*, disease symptoms due to *S. sclerotiorum* were very severe on the non-transgenic control plants, and many of the tissues were completely covered by watery necrotic lesions at 7 dai, with the average diseased area being 80 % (Fig. 3.6C). When examined microscopically, cells in the petiole section had lost integrity (Fig. 3.8A), and mycelia were visible within the vascular bundles and collenchyma ribs (Fig. 3.8B). The lines over-expressing *POC1* had significantly reduced diseased areas at both 4 and 7 dai, ranging from 2.5-15 % and 21-53 % at the two time points, respectively (Fig. 3.6D, Fig. 3.7A). The reduction in tissue necrosis was apparent when examined microscopically,

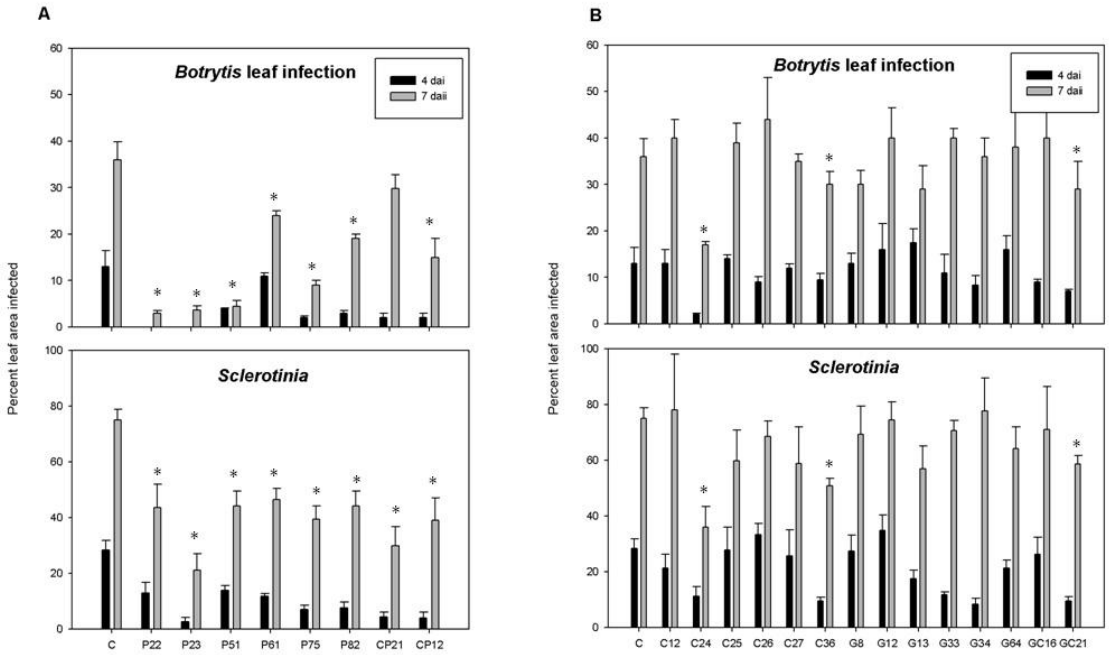

Figure 3.7 Mean percent leaf area diseased in transgenic carrots compared to controls. (A) Peroxidase *POC1* over-expressing lines (P22-P82), *POC1* and chitinase 383 co-expressing lines (CP21 and CP12) inoculated with *B. cinerea* (top) and *S. sclerotiorum* (bottom). (B) Carrot lines expressing chitinase 383 (C12-C36), β-1,3-glucanase 683 (lines G8-G64) and co-expression of 383/638 (lines GC16 and GC21) inoculated with the different pathogens. * indicate significant differences from the control LSD with α <0.05. Vertical error bars indicate standard errors for a minimum of 3 replicate trials for each transgenic line, with trials consisting of a minimum of 3 leaves.

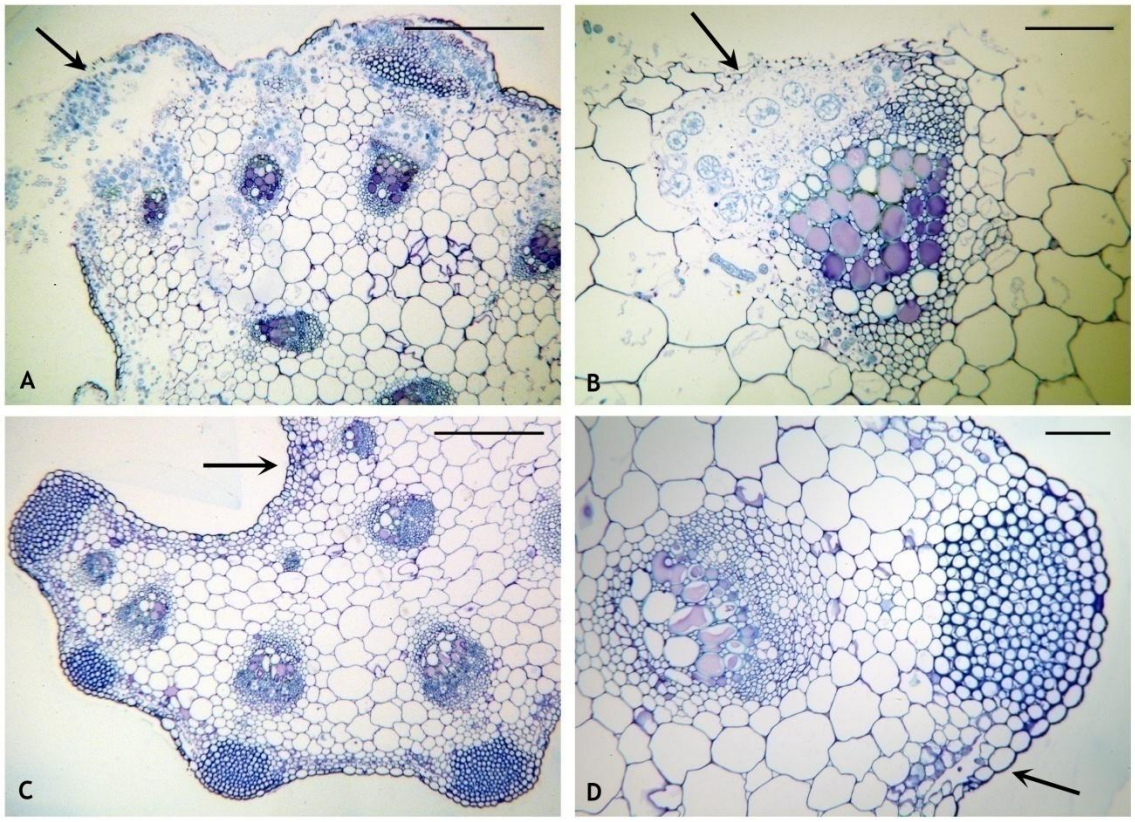

Figure 3.8 Histochemical examination of *S. sclerotiorum*-inoculated petioles, 7 dai. (A-B) Control petiole sections, showing the breakdown of cuticle, epidermis and mycelial colonization of vascular bundles and surrounding cells (arrows). (C-D) Petiole sections from carrot line P23 expressing peroxidase, showing the intact cuticle, collenchyma rib and mycelia-free vascular bundle; arrows indicate point of inoculation. Scale bars = 0.5 mm for A, C and 100 µm for B, D

as the entire petiole section had retained integrity (Fig. 3.8C), and there was no mycelia visible in the sections (Fig. 3.8D). Similar to that for *B. cinerea* infections, two lines (C-24 and C-36) expressing chitinase 383 had a significant reduction in diseased leaf area (Fig. 3.7B). Among the lines over-expressing chitinase 383, these resistant lines had the highest level of gene expression (Fig. 3.4), and this correlated with the highest level of resistance to both *S. sclerotiorum* and *B. cinerea*. Several of the β-1, 3-glucanase 638 over-expressing lines (G13, G33, G34 and G64) had reductions in disease severity due to *S. sclerotiorum* at 4 dai; however, this reduction was not seen at 7 dai (Fig. 3.7B). The two lines expressing a combination of *POC1*/383 had reduced diseased areas after 7 dai. Line CP-21 had the second lowest diseased area of any of the lines, and line CP-12 had diseased area slightly above the average of the singly-expressed *POC1* lines (Fig. 3.7A). One of the lines co-expressing 383/ 638 (GC-21) had a significant reduction in diseased area compared to the control plants; however, the other line (GC-16) showed no significant reduction in disease (Fig. 3.6B).

3.3.3 Lignin levels

Line P23 was assessed for total lignin as it had high relative resistance to both pathogens tested. Non-inoculated P23 petioles had lignin levels that were

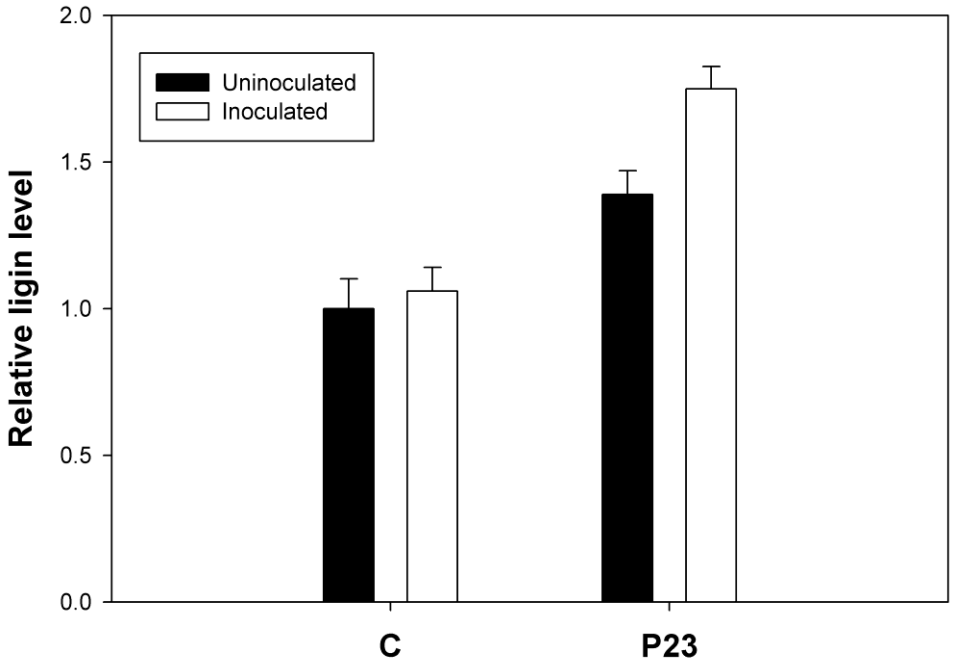

Figure 3.2 Total lignin content in control and peroxidase over-expressing carrot line P23 petioles from uninoculated (black bars) and inoculated petioles (white bars) 3 days after inoculation. Vertical error bars indicate standard errors from 9 replicates for each treatment.

1.39-fold higher than the control plants (Fig. 3.9). The lignin levels increased in the presence of *S. sclerotiorum* , being 1.79 -fold higher than in the control plants (Fig. 3.9). There was no significant difference between uninoculated or *S. sclerotiorum* -infected control petioles. The data was similar in both experimental trials and was pooled for data analysis.

3.4 Discussion

Transgenic carrot plants expressing a number of different anti-fungal proteins with enhanced resistance to fungal pathogens have been previously described (reviewed in Punja et al. 2007). These plants were generally found to have low levels of resistance following fungal inoculation, which seldom exceeded 50% disease reduction (Punja et al. 2007). In this study, we examined the efficacy of three different PR proteins, alone and in combination, to attempt to increase the overall levels of disease resistance attained in transgenic carrots.

There was limited resistance to *B. cinerea* and *S. sclerotiorum* in carrot leaves expressing the wheat class IV acidic chitinase 383 gene, and only the highest expressing lines (8-fold increase in chitinase activity) exhibited enhanced resistance to either pathogen. Our data showed greater disease reduction compared to transgenic wheat expressing the same gene when inoculated with *Fusarium graminearum* under greenhouse conditions (Anand et al. 2003), where there was no reduction in disease symptoms. The level of resistance observed on carrot in response to *S. sclerotiorum* was similar to that reported previously in carrot expressing a fungal chitinase (Baranski et al. 2008), a basic barley chitinase (Jayaraj et al. 2007), and a basic tobacco chitinase (Punja et al. 1996)

when infected with *B. cinerea*. However, the resistance obtained was much lower than that seen on detached wheat leaves over-expressing a barley chitinase when infected by powdery mildew (*Blumeria graminis*), where a 50-94% reduction in lesion numbers compared to the controls was reported (Bliffeld et al. 1999). Chitinases are proposed to have a role in plant defence by degrading the fungal cell walls, catalyzing the hydrolytic cleavage of the β-1,4-glycoside bond present in the biopolymer of *N*-acetyl glucosamine (chitin) found in fungal cell walls. The amount of chitinase enzyme produced and the proportion of chitin present in different fungal cell walls may account for the different disease reduction values reported in the literature. In addition, acidic chitinases, which are targeted to the cell wall, may be less effective at reducing disease caused by necrotrophic pathogens than intracellular basic chitinase isoforms (Ferreira et al. 2007; Punja et al. 1996).

There was no enhanced resistance in any of the carrot lines expressing the acidic wheat β-1, 3-glucanase 638 gene. These results are similar to that for greenhouse-grown transgenic wheat, where β-1, 3-glucanase 638 over-expression did not reduce disease caused by *F. graminearum* (Anand et al. 2003). In contrast to previous reports (Anand et al. 2003; Jach et al. 1995; Jongedijk et al. 1995; Zhu et al. 1994), there was no synergy observed when the chitinase and β-1,3-glucanase genes were co-expressed in carrot. In one line (GC21), there was a slight decrease in *S. sclerotiorum* lesion development, while a second line (GC16) showed no reduction in disease by either pathogen. The lack of a resistant phenotype may be due to the lower overall level of chitinase

expression in the 383/638 lines (a 4-fold increase in enzyme activity) compared to the controls. The overall frequency of recovery of co-transformed 383/638 lines was low (~1%); hence, only two lines were available for testing in this study. Evaluation of a larger number of transgenic lines may identify some that express both chitinase and β-1,3-glucanase at higher levels, potentially leading to heightened disease resistance as reported by other investigators (Anand et al. 2003; Ferreira et al. 2007)

All six transgenic lines expressing *POC1* had significantly reduced diseased leaf area compared to non-transformed controls. The rice *POC1* gene used in this study encodes for a pathogen-inducible cationic class III (PR-9) peroxidase, which was induced to high levels in rice inoculated with avirulent strains of *Xanthomonas oryzae* (Young et al. 1995). The *POC1* gene plays a crucial role in the early defence responses in resistant rice varieties (Hilaire et al. 2001). Plant class III peroxidases have different biochemical functions in plant pathogen defence, including roles in lignin formation (Quiroga et al. 2000), xylem wall thickening (Hilaire et al. 2001), generation of reactive oxygen species (Bestwick et al. 1998), H_2O_2 scavenging (Kawaoka et al. 2003) and phytoalexin biosynthesis (Kristensen et al. 1999). Two peroxidases from *Arabidopsis* were shown to be essential for resistance to fungal and bacterial pathogens (Bindschedler et al. 2006). However, previous reports of plants over-expressing peroxidases have not described enhanced resistance to necrotrophic fungal pathogens (Lagrimini 1991; Ray et al. 1998). Partial resistance to the biotrophic pathogen *B. graminis* was observed in wheat plants over-expressing an endogenous peroxidase gene (Altpeter et al. 2005).

Over-expression of peroxidases in transgenic tobacco was reported to increase lignin production and increase cell wall thickness (Lagrimini 1991). Lignin is a strong structural polymer which is very difficult for pathogens to penetrate or degrade (Quiroga et al. 2000). The most resistant *POC1* carrot line (P23) had a significantly higher level of lignin when compared to the control, which was increased further in the presence of the pathogen *S. sclerotiorum.* The lignin levels detected in transgenic carrot petioles (with the maize ubiquitin promoter) were similar to the levels reported in peroxidase over-expressing tobacco (with the CaMV *35S* promoter), which also had stronger initial lignification in response to wounding compared to non-transformed plants (Lagrimini 1991). Lignin-forming peroxidases may be a rate limiting step in pathogen-challenged responses in plant cells, similar to that seen in wound responses (Lagrimini 1991). The presence of defence-induced lignin precursors in *S. sclerotiorum*-infected transgenic carrots may be rapidly converted to lignin by *POC1*, thereby enhancing disease resistance. However, further analysis of lignin precursors before and during pathogen challenge is required to elucidate the role of *POC1* in disease resistance in transgenic carrot tissues.

This is the first report of peroxidase over-expression leading to a significant reduction in disease caused by necrotrophic fungal pathogens. Whether or not heightened physical barriers through lignification of the cell walls are the main mechanism of pathogen resistance or not requires further investigation, since plant peroxidases exhibit a wide range of functions in response to pathogen attack (Bindschedler et al. 2006).

4: MECHANISMS OF DISEASE RESISTANCE IN TRANSGENIC CARROT (DAUCUS CAROTA L.) PLANTS OVER-EXPRESSING A RICE CATIONIC PEROXIDASE

Results from this chapter were accepted for publication in Molecular Plant-Microbe Interactions.

4.1 Introduction

Plant class III peroxidases are present in all higher plants and are encoded by a large gene family which can consist of over 70 genes (Passardi et al. 2005). A large number of peroxidase genes are induced during pathogen challenge, with 10 peroxidase genes up-regulated in *Magnaporthe* infected rice (Sasaki et al. 2004), with Arabidopsis having 7 and 3 peroxidase genes upregulated when challenged with *Pseudomonas* spp (Mohr et al. 2007) and *Botrytis cinerea* (Chassot et al. 2007), respectively. Plant class III peroxidases catalyze the oxidation of a range of organic substrates using hydrogen peroxide (H_2O_2) as an oxidant (Hiraga et al. 2001). As well, many different biochemical functions in plant pathogen defence are associated with peroxidases, including lignin formation (El Mansouri et al. 1999; Lagrimini 1991; Quiroga et al. 2000), xylem wall thickening (Hilaire et al. 2001) and phytoalexin biosynthesis (Kristensen et al. 1999). Peroxidases have also been shown to be involved in the generation of reactive oxygen species (Bestwick et al. 1997; Bindschedler et al. 2006) and scavenging of H_2O_2 (Baker et al. 2000; Kawaoka et al. 2003). These

reports indicate that peroxidases have multiple functions in plant defences against both biotic and abiotic stresses (Passardi et al. 2005). Since the role of peroxidases is broad and the physiological reactions they are involved with are complex, it has been difficult to demonstrate the mechanisms by which, peroxidases may be involved in plant-pathogen interactions.

In previous reports, a reduction of peroxidase activity in Arabidopsis (Bindschedler et al. 2006) and bell pepper (Choi et al. 2007) resulted in reduced H_2O_2 accumulation and heightened susceptibility towards fungal and bacterial pathogens. Similarly, overexpression of peroxidases resulted in increased H_2O_2 accumulation and enhanced tolerance to biotrophic pathogens in Arabidopsis (Choi et al. 2007), and tobacco (Kim et al. 2008b). In stark contrast, over-expression of a tomato peroxidase *Ep5C* in Arabidopsis resulted in a reduction in H_2O_2 accumulation and hypersensitivity to *Pseudomonas syringe* pv *tomato* (Coego et al. 2005a). These reports indicate that different peroxidases function in distinct manners, with certain peroxidases involved with defence against biotrophs while others function in necrotrophic interactions.

We previously generated transgenic carrot plants constitutively over-expressing the rice *POC1* gene (Chapter 3), which encodes a pathogen-inducible cationic class III (PR-9) peroxidase that was induced to high levels in rice inoculated with avirulent strains of *Xanthomonas oryzae* (Young et al. 1995). POC1 encodes a 311 amino acid peptide with a putative N- terminus extracellular localization signal, extracellular transport was confirmed by immune-localization of inoculated rice as well as enhanced cell wall bound enzyme

fractions in transgenic carrots (Hillaire et al. 2001, chapter 3). Currently there are no known carrot peroxidase sequences available for comparison, with the highest degree of amino acid similarity to POC1 found in wheat peroxidases TaPrx103 (Altpeter et al. 2005, Schweizer 2008) and the barley peroxidase HvPrx08 (Johrde and Schweizer 2008). The *POC1* gene plays an important role in the early defence responses in resistant rice cultivars and was proposed to be a putative lignin-forming enzyme (Hilaire et al. 2001). This was the first report of peroxidase over-expression in plants resulting in disease resistance against necrotrophic pathogens. In the transgenic carrot line P23, peroxidase enzyme activity was increased by 3.5-fold in the ionically bound protein fraction. This line was highly resistant to the foliar necrotrophic pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Chapter 3).

In the present study, we explored the possible mechanisms by which *POC1* expression enhanced resistance to necrotrophic fungal pathogens in root and foliar tissues by measuring pathogenesis related (PR) gene expression, H_2O_2 production, lignin and phenolic levels.

4.2 Materials and methods

4.2.1 Transgenic carrot plants

Transgenic ‘Nantes Coreless’ carrots constitutively over-expressing the rice cationic peroxidase gene *POC1* under control of the maize ubiquitin promoter, or control plants containing the *35S*:: GUS chimeric construct

developed in previous chapters, were grown in the greenhouse or maintained in suspension cultures (Chen et al. 2002).

4.2.2 Chemicals

All chemicals were obtained from Sigma (Oakville, Ontario, Canada) unless otherwise noted.

4.2.3 Effect of *POC1* on defence gene transcript levels

Total RNA was extracted from lyophilized carrot suspension cultures (~250 mg fresh weight) from the *POC1* line P23 and the control line using the monophasic RNA extraction method (Chomczynski et al. 1987). The suspension cultures were treated with 2,6-dichloroisonicotinic acid (INA) (500 µM), methyl-jasmonic acid (JA) (100 µM), H_2O_2 (500 µM) or *S. sclerotiorum* cell walls (SS-walls) (100 µg ml^{-1} dw) from 7 day old *S. sclerotiorum* cultures grown on potato dextrose broth at room temperature according to published procedures (Tweddell et al. 1994). The lyophilized *S. sclerotiorum* cell walls were suspended in H_2O (10 mg ml^{-1} dw) and sterilized by autoclaving. Radioactive probes of the following defence genes in carrot: phenylalanine ammonia lyase (*DcPAL*), *DcPR1*, *DcPR2*, *DcPR3, DcPR5, DcAct* and *Dc18S* were generated using RT-PCR on non-transformed carrot cDNA using specific primers (Table 5.1). Each sample was replicated a minimum of three times. Quantitative expression of carrot PR genes was assessed by exposing filters to phospho-storage screens (GE) and by subsequently scanning with the phospho-imaging system Si 445 (Molecular

Dynamics, CA, USA). The signal intensities were normalized against both actin and 18S rRNA and standardized to the time zero points for the control lines.

4.2.4 Detection of hydrogen peroxide

Qualitative *in vivo* assessment of H_2O_2 production in carrot leaves was conducted by placing samples in 3',3-diaminobenzidine (DAB) solution (1 mg ml^{-1}) overnight (ThordalChristensen et al. 1997). The chlorophyll was cleared by boiling the leaves in 3:1:1 solution of 95% ethanol: glycerol: glacial acetic acid for up to 45 min.

Xylenol orange was used to specifically monitor the production of H_2O_2 during the oxidative burst in carrot suspension cell cultures. The hydroperoxides are reduced by ferrous ions in the acid solution forming a ferric–xylenol orange complex that was detected spectrophotometrically at 560 nm (Bindschedler et al. 2001; Gay et al. 1999). The H_2O_2 measurements were conducted at early time points up until 3 hours after elicitation. Inhibitors of the oxidative burst KCN (1 mM), NaN_3 (1 mM), L-cysteine (2.5 mM) and diphenylene iodonium (DPI 50 µM), were added 30 min prior to elicitation. Scavenging potential was measured by spiking the cultures with exogenous H_2O_2 up to 500 µM, and monitoring the rate of H_2O_2 reduction.

4.2.5 Quantification of lignin and soluble phenolics

Total lignin was extracted from carrot tissue from the alcohol insoluble residue (AIR) and preferential solubilization through derivatization of lignin with a modified thiogylcolic acid method (Doster et al. 1988). Taproots were examined

for lignin content in the outer 2 mm peel comprised mainly of periderm and phloem tissues, using 0.5 g fresh weight of each tissue type. For suspension cultures, lignin was extracted from 50 mg (dw) of cells. All tissues were homogenized with a tissue polytron in absolute methanol, the cell material was pelleted and re-suspended with 5 washes of methanol. The AIR was dried under vacuum overnight. Approximately 50 mg of AIR was incubated in 5 ml of 10% thiogylcolic acid in 2N HCl at 95 °C for 6 h. The soluble ligno-thioglycolic acid derivative (LTGA) product was precipitated by centrifugation for 10 min in a clinical centrifuge at 800g, and washed with water. The LTGA was suspended in 2 ml of 0.5 N NaOH through gentle shaking for 16h at room temperature, the extract was cleared by centrifugation and the supernatant was acidified with 0.5 ml of concentrated HCl. The LTGA was precipitated following 4 h incubation at 4 °C followed by 10 min centrifugation. The LTGA pellet was washed twice with water before being resuspended in 0.5 ml of 0.5N NaOH. The LTGA was quantified as A280 readings relative to the reading for the control plants (Doster et al. 1988).The experiments were conducted three times with 9 replicates for each of the treatments described. Lignin data was analyzed for significant differences using ANOVA.

Total soluble phenolics were measured from 100 µl of the methanol soluble fraction. The extract was incubated with 1 ml of freshly prepared 2% (w/v) $NaCO_3$ for 5 min, to which 25 µl of undiluted Folin-Ciocalteu reagent was added (Vermerris et al. 2006) . The sample was mixed thoroughly and incubated for 30

min at 25 °C. The absorbance at 750 nm was measured spectrophotometrically. Guaiacol was used to generate a standard curve.

4.2.6 Assessment of disease tolerance

Greenhouse grown carrot roots (20-24 weeks of age) were washed with water and placed in sealed plastic trays lined with moistened paper towels. Mycelial plugs of the fungal pathogen *Alternaria radicina* (provided by Dr. Barry M. Pryor, University of Arizona, Tucson, USA) from V8 agar cultures (2 weeks old) were placed evenly along the root (2-3 plugs per root). Lesion area was measured at 10 days after inoculation (dai) and compared to control lines. Three replicates consisting of 6 roots each were conducted.

For assessment of powdery mildew resistance (*Erysiphe heraclei*), heavily infected leaves from non-transgenic plants were used as inoculum. Leaves from transgenic and non-transgenic control plants were harvested, washed, and cut into 3-4 cm segments and placed adaxial side up on water agar (6 g l^{-1}) containing benzimidazole (0.1g l^{-1}), until 90% of the agar surface was covered. Spores were blown into a settling tower, allowed to settle over the tissues for 5 min, and the plates were sealed and placed on the laboratory bench 22-25º C for 10 days. Newly formed sporulating colonies were counted per plate at 7 and 10 days, using a stereo dissecting microscope. Three replicates consisting of 10 plates each were conducted.

4.3 Results:

4.3.1 Effect of *POC1* on defence gene transcript levels

Transcript levels of genes *DcPR1*, *DcPR2, DcPR3* and *DcPR5* in line P23 were detected at elevated levels compared to the *35S::GUS* controls at time 0 (Table 4.1). There was a measurable increase in the transcript levels of *DcPR1, DcPR2, DcPR3* and *DcPR5* in cell cultures treated with purified cell wall extracts of *Sclerotinia sclerotiorum* (SS-walls), with no significant increase in *DcPAL* levels, after elicitation (Figure 4.1); the maximum expression was observed 12-24 h after elicitation (Figure 4.2). The *PR* genes were induced to varying degrees, with much greater induction in the P23 cultures, with up to 30-fold increases in *DcPR*1, 45-fold increase in *DcPR2*, *DcPR3* and 20-fold increases with *DcPR5* in comparison to 18, 8, 15 and 4.5 –fold maximal induction for DcPR1-DcPR5 respectively (Figure 4.2). Application of the salicylic acid analogue INA (500 µM) or the phytohormone JA (100 µM) enhanced defence gene expression in both P23 and *35S::GUS* lines, although the P23 line was more responsive, with more rapid expression of *DcPR1, DcPR2, DcPR3* and *DcPR5* compared to the *35S::GUS*. However, the degree of induction due to INA and JA was much lower than with the SS-walls treatment (Figure 4.3, 4.4).

Two non-specific peroxidase inhibitors, NaN_3 (1 mM) or KCN (1 mM), were added to the cell cultures to determine if peroxidase was responsible for the increase in defence gene transcript levels in transgenic tissues. Overall, defence gene transcript levels after addition of SS-walls were greatly reduced by addition of both NaN_3 and KCN (figure 4.5) in the P23 line.

Table 4.1 **Fold transcript induction of *PR-1*, *PR-2, PR-3, PR-5* and *PAL* in *POC1* line P23 relative to control values normalized to both actin and 18s rRNA. Data represented is the mean of a minimum of 4 independent northern blots. Standard error of the mean is presented. * indicates significance α of <0.05, ** indicates α of <0.01.**

	PR-1	*PR-2*	*PR-3*	*PR-5*	*PAL*
P23	**1.98***	**2.25****	**2.44***	**3.13****	**1.23**
Control	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**
St. error	**0.26**	**0.23**	**0.45**	**0.59**	**0.18**

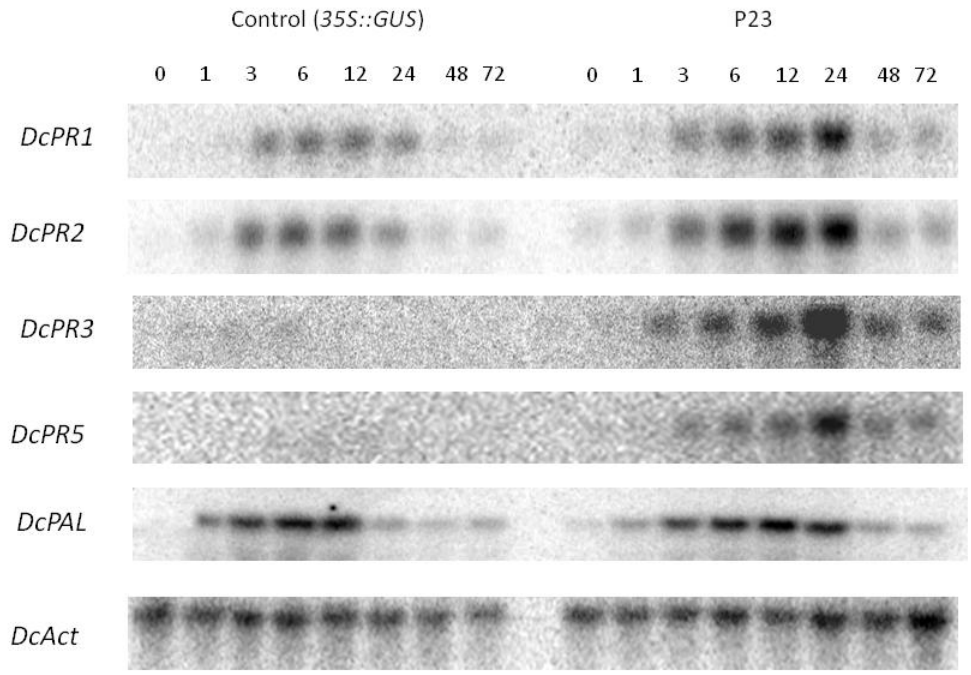

Figure 4.1 Time course induction of *PR* genes in transgenic carrot lines elicited by *S. sclerotiorum* cell wall fragments. Total RNA from transgenic *POC1* expressing line P23 or 35S::GUS-transformed control suspension cultures was extracted at 0,1,3,6,12,24,48 and 72 h after treatment with *S. sclerotiorum* cell walls (100 µg ml^{-1} dw). Northern blots were probed with the radio-labelled cDNA probes listed. Shown is a representative blot, from five separate replicates.

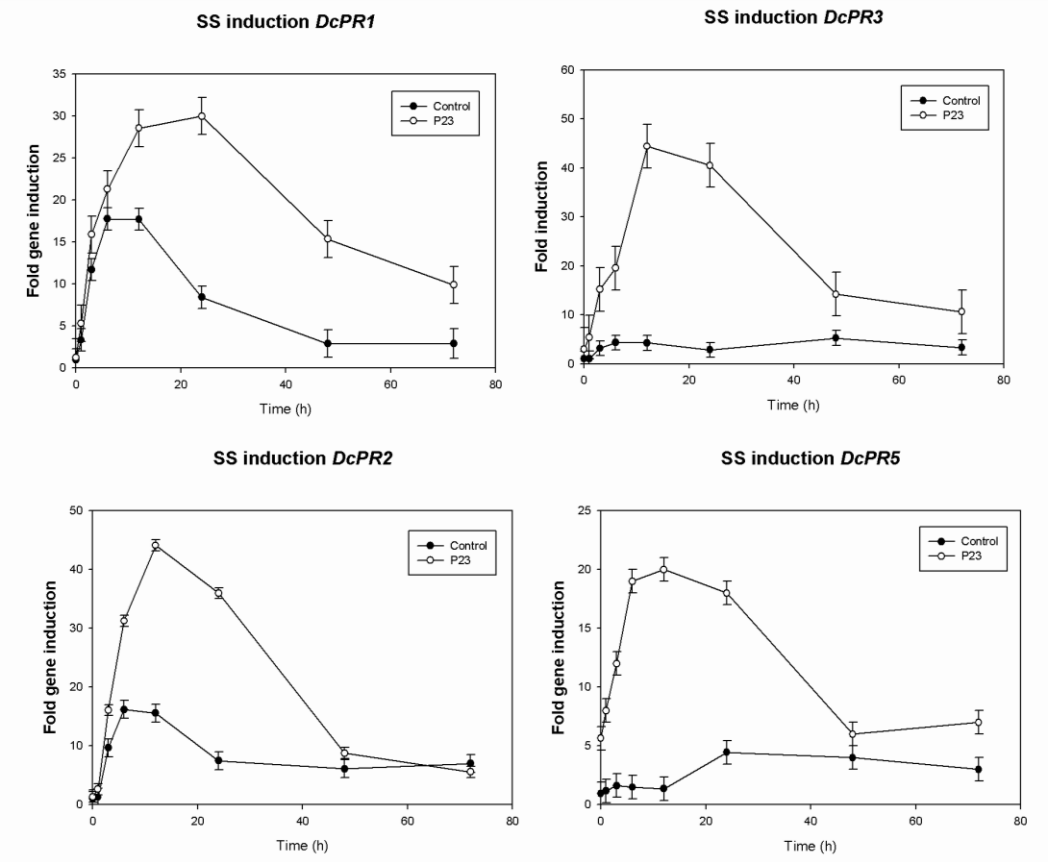

Figure 4.2 Increased induction of various carrot pathogenesis related (DcPR) genes following culture induction using purified S. sclerotiorum cell wall fragments (SS- walls) at various time points, comparing *POC1* over-expressing P23 lines against *35S::GUS* control lines. Induction was quantified from northern blots relative to 0 h transcript level for the control lines. Transcripts were standardized to both 18S and Actin levels. Experiment was repeated 5 times, with the vertical error bars representing the standard error of the mean.

To determine if changes in gene induction were due to increases in extracellular H_2O_2, we investigated the effect of adding exogenous H_2O_2 on gene expression. Control lines responded weakly to 250 µM, with slight increases in *PR-1*, *PR-2* and *PR-5* expression. Much larger increases in expression of these genes were observed in P23 (Figure 4.5, Table 4.2).

4.3.2 Detection of H_2O_2

There were no qualitative differences in the accumulation of H_2O_2 in the leaves of line P23 compared to the control (Figure 4.7). Similar low levels of polymerized DAB were found near the wound sites of both control and P23 lines.

Table 4.2 Fold transcript induction of *DcPR1*, *DcPR3* and *DcPR5* in OsPrx114 expressing line P23 relative to *35S::GUS* values induced with SS-walls or H_2O_2 (500 µM), normalized to both actin and 18s rRNA. Data represented is the mean of a minimum of 6 independent northern blots. Standard error of the mean is presented.

Time (h)	Treatment											
	Control (SS-walls)			P23 (SS-walls)			Control (H_2O_2)			P23 (H_2O_2)		
	PR1	*PR3*	*PR5*	*PR1*	*PR3*	*PR5*	*PR1*	*PR3*	*PR5*	*PR1*	*PR3*	*PR5*
6	17.8	6.4	1.6	21.3	19.1	12.1	1.2	1	1.0	3.3	1.8	4.8
12	17.9	5.2	1.5	28.2	44.8	19.0	1.9	1.4	1.11	3.9	3.0	3.9
24	8.8	7.67	4.5	30.1	40.2	20.3	2.9	1.3	1.10	13.7	15.0	5.99
48	2.8	6.71	4.4	15.4	14.6	6.2	1.7	1.5	1.2	7.9	9.2	4.06
SE	1.32	1.51	1.02	2.25	4.45	1.7	0.67	0.42	0.39	1.4	1.52	1.1

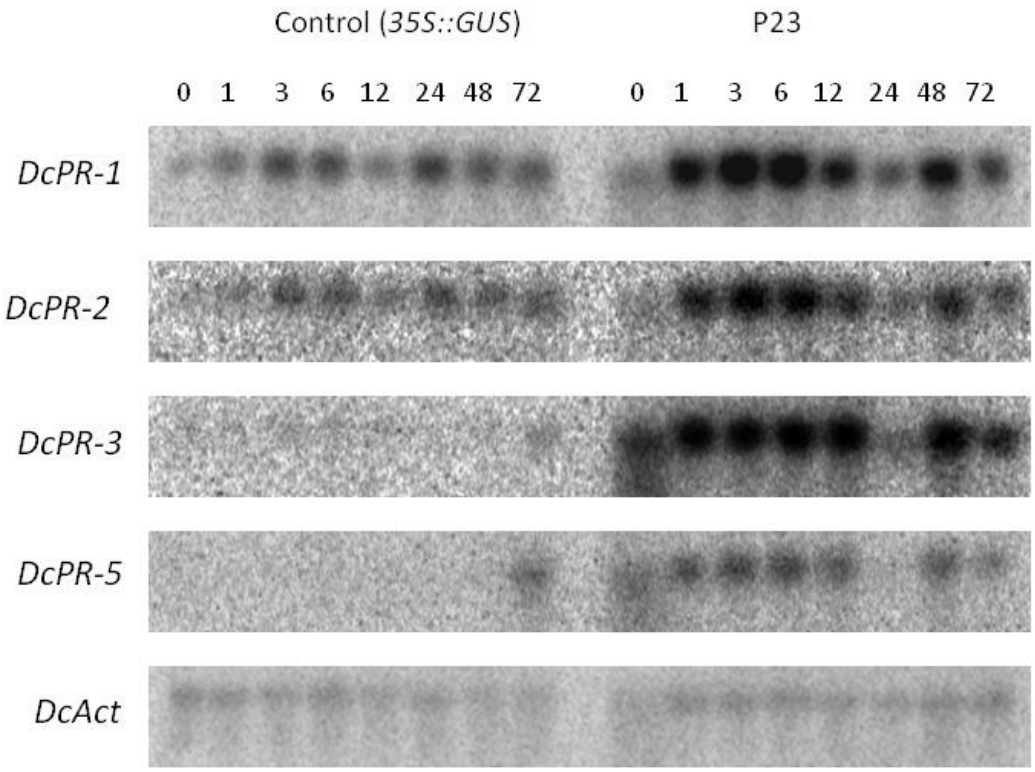

Figure 4.3 Time course induction of PR genes in transgenic carrot lines elicited by jasmonic acid. Total RNA from transgenic *POC1* expressing line P23 and control (*35S::GUS*) suspension cultures was extracted at 0,1,3,6,12,24,48 and 72 h after treatment with jasmonic acid (100 µM). Northern blots were probed with the radio-labelled cDNA probes listed. Shown is a representative blot.

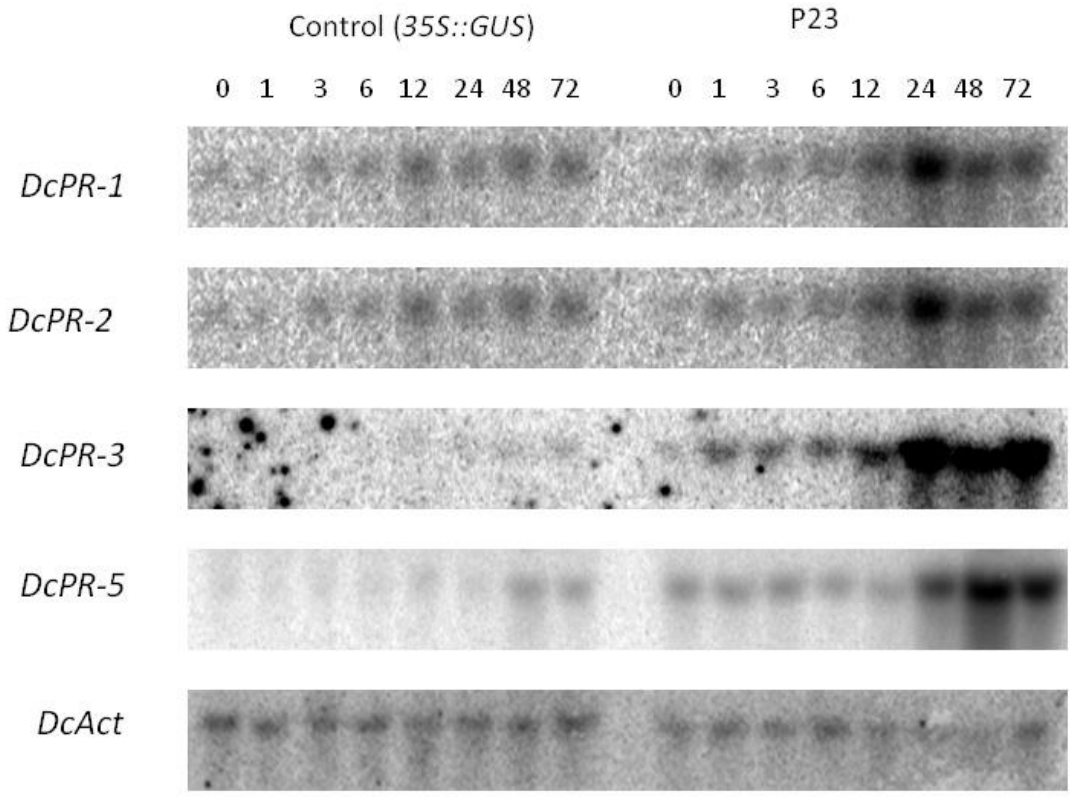

Figure 4.4 Time course induction of PR genes in transgenic carrot lines elicited by 2,6-dichloroisonicotinic acid (INA). Total RNA from transgenic *POC1* expressing line P23 and control (*35S::GUS*) suspension cultures was extracted at 0,1,3,6,12,24,48 and 72 h after treatment with 2,6-dichloroisonicotinic acid (500 µM). Northern blots were probed with the radio-labelled cDNA probes listed. Shown is a representative blot.

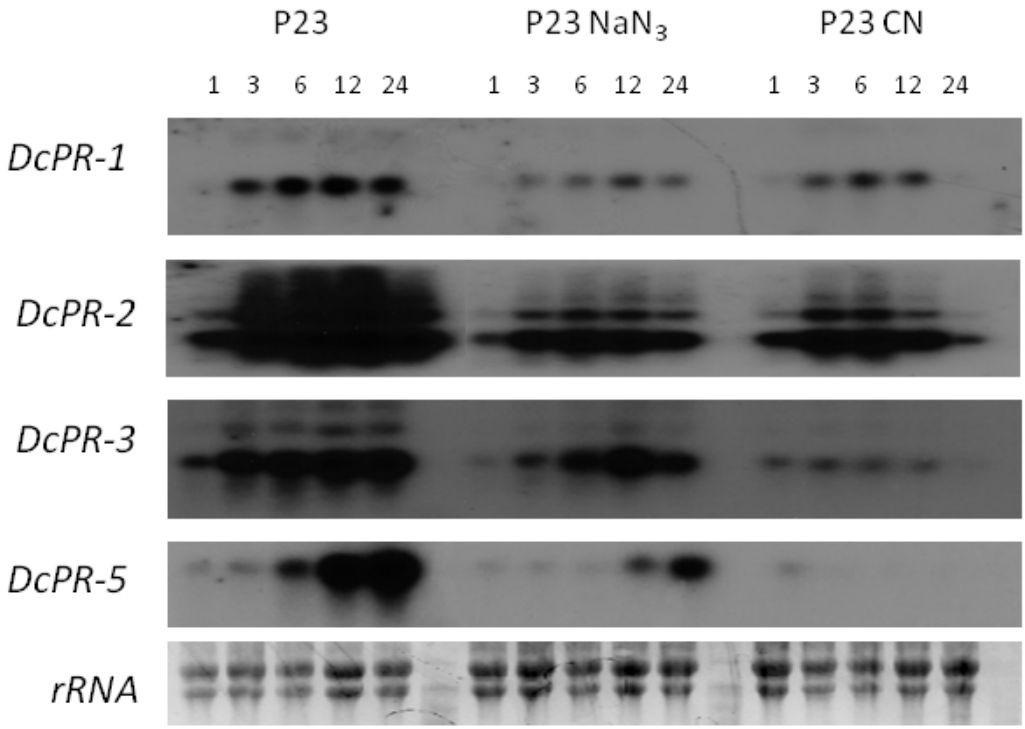

Figure 4.5 Time course induction of PR genes in transgenic P23 carrot lines elicited by *S. sclerotiorum* cell wall fragments, comparing inhibitors. Total RNA from transgenic *POC1* expressing line P23, P23 with 1 mM NaN_3 and P23 with 1 mM KCN suspension cultures was extracted at 0,1,3,6,12 and 24 h after treatment with *S. sclerotiorum* cell walls (100 µg ml^{-1} dw). Northern blots were probed with the radio-labelled cDNA probes listed.

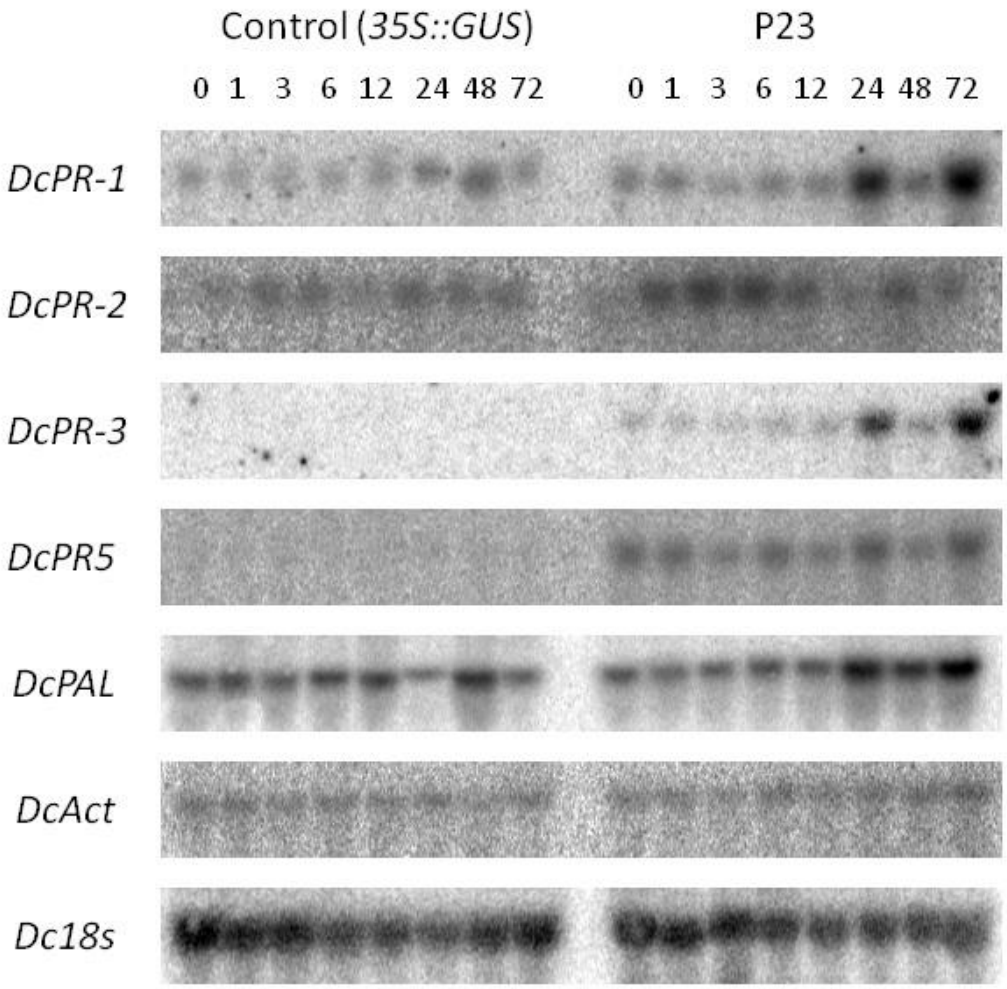

Figure 4.6 Time course induction of PR genes in transgenic carrot lines when induced with H_2O_2. Total RNA from transgenic *POC1* line P23, and non-transformed control suspension cultures, extracted at 0,1,3,6,12,24,48 and 72 h after treatment with H_2O_2 (500 µM). Northern blots were probed with the radio-labelled cDNA probes listed. These experiments were confirmed using 3 separate replicates.

Figure 4.7 Qualitative assessment of H_2O_2 levels in leaves of P23 and control carrots. Visualization of H_2O_2 using DAB comparing P23 (top) and control (bottom) leaves.

Control cells treated with SS-walls at a concentration of 100 mg l^{-1} exhibited a rapid and repeatable oxidative burst when assayed with xylenol orange (Figure 4.8A). In contrast, when the P23 cells were treated with SS-walls, the oxidative burst was negligible with no apparent increase in H_2O_2 levels (Figure 4.8A).

To determine if the H_2O_2 generated through the oxidative burst was due to activity of peroxidases or NADH-oxidases, the NADH-oxidase specific inhibitor DPI and the non-specific peroxidase inhibitors NaN_3 and KCN, which do not affect NADH-oxidases, were tested. The oxidative bust was lowered slightly by DPI treatment, with the control cells showing lower extracellular H_2O_2 slightly across all time points (Figure 4.8B). The addition of either NaN_3 (Figure 4.8B) or KCN (Figure 4.9 A) resulted in a near abolition of the oxidative burst, with the levels in both control and P23 lines at the basal levels. Since both KCN and NaN_3 are highly oxidized and non-specific inhibitors of peroxidase, we also examined L-cysteine as an alternative inhibitor (1 mM). L-cysteine reduced the oxidative burst by nearly 80 %, in the control lines and did not alter the levels found in P23 (figure 4.9B). These findings indicate that the extracellular oxidative burst in carrot was controlled mainly through peroxidase activity and not NADH-oxidases.

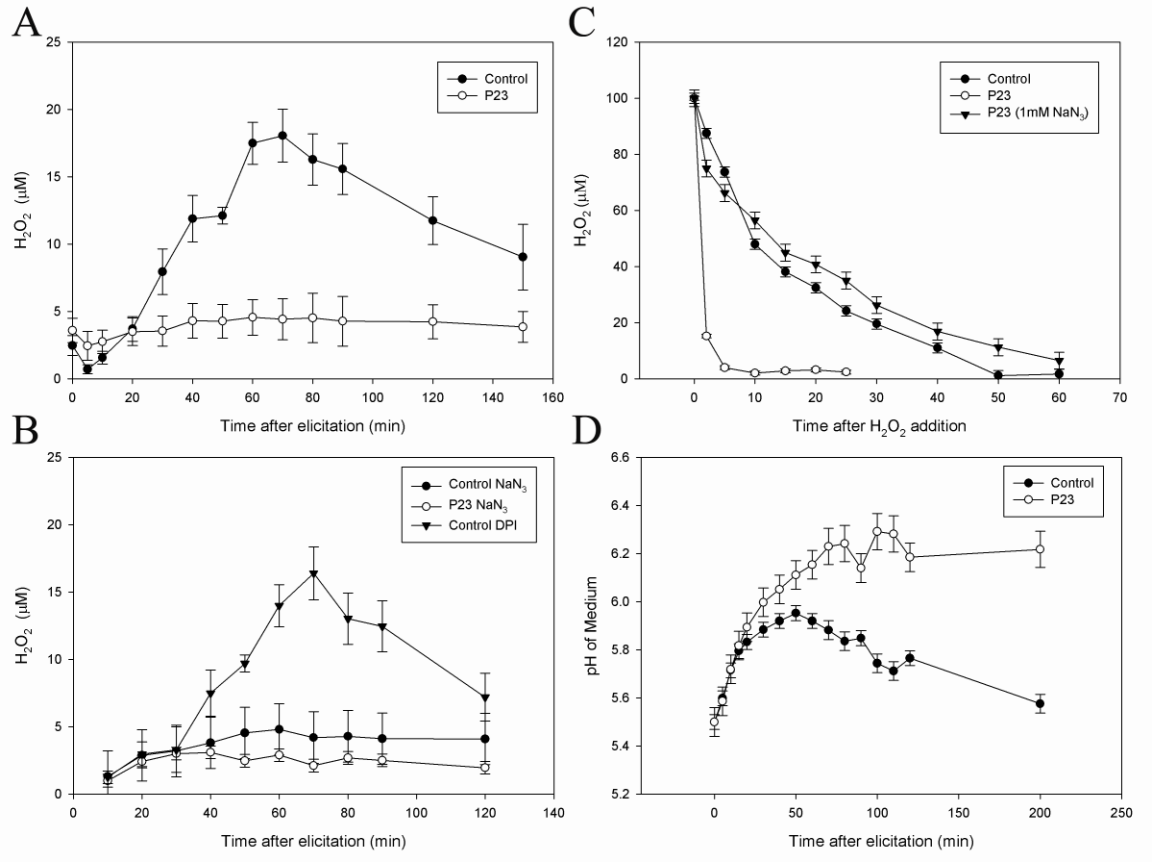

Figure 4.8 Elicitation of an oxidative burst in carrot tissue cultures. A**, Comparative level of the oxidative burst in *POC1* expressing P23 carrot suspension cultures (open circles) or controls (closed circles) treated with 100 µg ml^{-1} glucose equivalents of *S. sclerotiorum* cell wall elicitor. Production of H_2O_2 was measured using a xylenol orange assay.** B**, Inhibition of the oxidative burst in P23 with 1 mM NaN_3, compared to controls treated with both NaN_3 and diphenylene iodonium (DPI) 50 µM (closed triangles).** C**, Rate of H_2O_2 consumption in suspension cultures *POC1* expressing carrot line P23 (open circles) and control line (closed circle), supplemented with 100 µM H_2O_2, and 1 mM NaN_3 pre-applied to P23 (triangles).** D**, Changes in pH of culture media following elicitation with *S. sclerotiorum* cell wall fragments (100 µg ml^{-1}), of P23 transgenic (open circles) and control (closed) circles. Vertical error bars represent the standard error of the mean from 5 independent replicates. Vertical error bars represent the standard error of the means from a minimum of 4 independent experiments.**

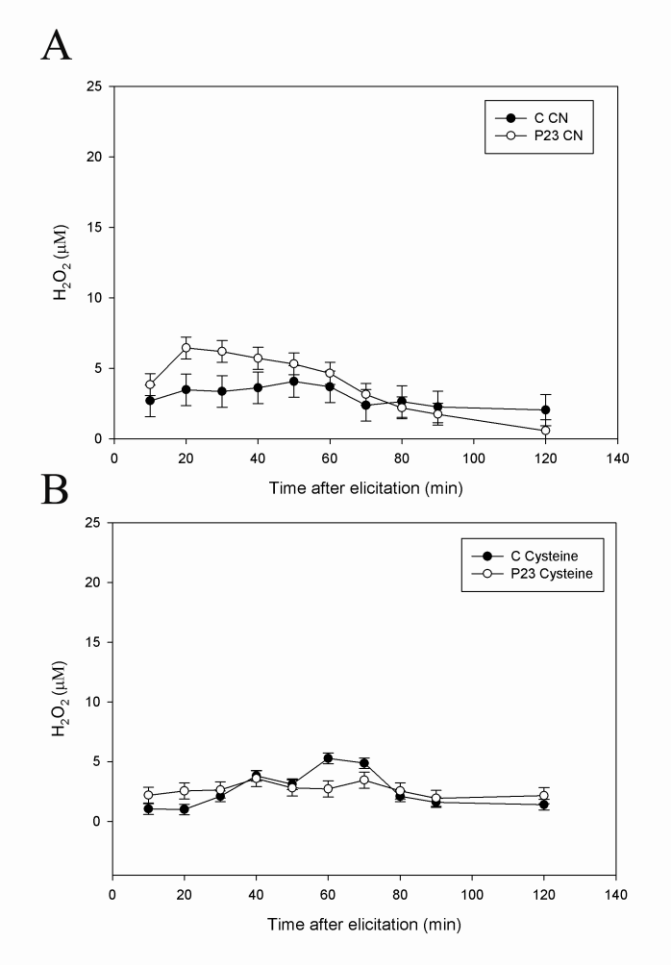

Figure 4.9 Effect of KCN and L-cysteine inhibitors on oxidative burst. Measurement of H_2O_2 levels using xylenol orange assay of P23 (open circles) or control (closed circles) when treated with SS-walls with the addition of (A) 1mM KCN or (B) 2.5 mM L-cysteine.

4.3.3 H_2O_2 Scavenging potential

When suspension cultures were initially spiked with 25 µM H_2O_2, levels of H_2O_2 were undetectable after 30s in the P23 lines, indicating extremely rapid H_2O_2 consumption. Additional H_2O_2 supplementation was conducted with higher concentrations of 100 and 250 µM. When 100 µM of H_2O_2 was applied to the P23 line, the levels were reduced by 80% within 2 min and were at basal levels by 5 min (Figure 4.8C). In contrast, the controls exhibited a more gradual reduction in H_2O_2, showing an 80% reduction by 40 min and a return to basal levels at 50 min. To confirm that peroxidase levels were responsible for the increased scavenging, NaN_3 was applied to the cells prior to H_2O_2 application. This resulted in P23 lines scavenging H_2O_2 at levels very similar to the controls (Figure 4.8C), and similar results were obtained using KCN (not shown). The controls' ability to consume H_2O_2 was largely unaffected by the presence of either NaN_3 or KCN.

To determine if the oxidative burst is reduced in the P23 cells and not a consequence of the heightened scavenging ability, we examined the effect of extracellular akalinization in suspension cultures when elicited with SS-walls. The response for P23 was identical to controls during the initial 30 min of the oxidative burst, with a rapid increase in the pH of the growth media from 5.3 to over 5.8 (Figure 4.8D). After the initial phase the rate of akalinization slows, peaking at 60 min before gradually decreasing. In contrast the pH continued to increase in the P23 suspension cultures peaking at a pH of 6.3 at 120 minutes and maintaining a pH higher than 6.0 in excess of 200 min (Figure 4.8D).

4.3.4 Assessment of disease tolerance

The rice *POC1* over-expressing line P23 was highly resistant to the foliar necrotrophic pathogens *Botrytis cinerea* and *Sclerotinia sclerotiorum* (chapter 3). The resistance of P23 line to the root pathogen *A. radicina* and the biotrophic foliar pathogen *E*. *heraclei* was further investigated. The taproots of P23 were highly resistant to infection by *A. radicina* (Figure 4.10A), with the total lesion area reduced by 66% compared to the control roots (Figure 4.11A). Furthermore, the lesions were largely superficial on the P23 lines, with *A. radicina* producing mycelia only on the outermost surface of the root (Figure 4.10A, B). The level of fungal penetration was greatly reduced in the P23 lines (Figure 4.10B). These differences were very apparent when sections stained for the presence of lignin were examined microscopically (Figure 4.10 C,D). The control roots had very low detectable levels of lignin, detected with phloroglucinol, with tissue degradation and tissue infection proceeding beyond the barrier (Figure 4.10C). The P23 taproots produced a thick lignin layer directly underneath the infection site, preventing further penetration and infection of the tissues (Figure 4.10D). Contrary to *A. radicina* root infection the P23 leaves and petioles exhibited control level susceptibility towards the biotrophic pathogen *E*. *heraclei* (Figure 4.11B).

4.3.5 Determination of lignin levels

The margins surrounding and just under the lesion on tap roots infected by *A. radicina* were also highly lignified, this resulting physical barrier may have been a major limitation to the progression of the fungal deep into the taproot

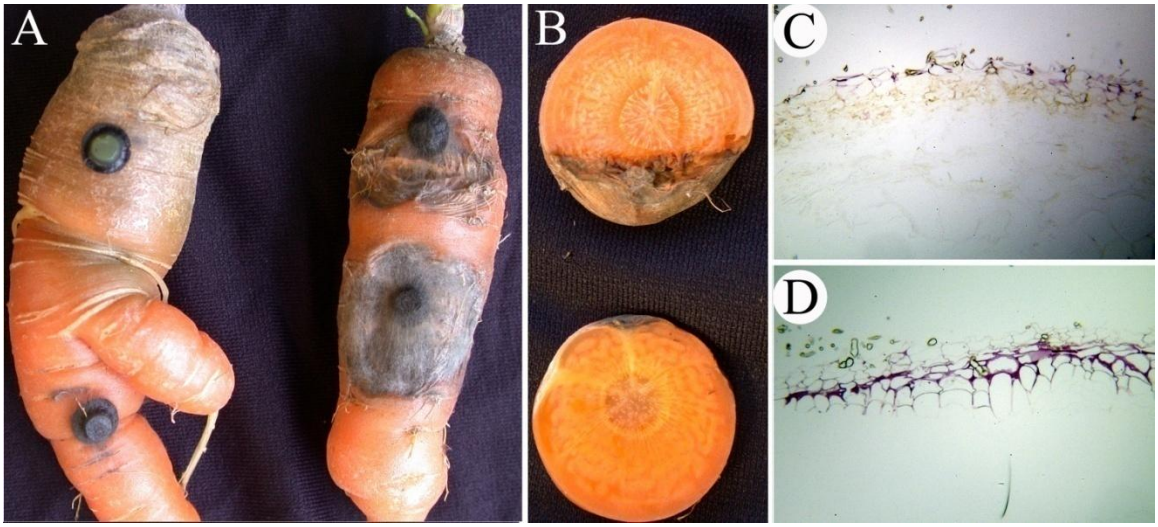

Figure 4.10 Root resistance of P23 carrot infected with *A. radicina* 10 days after inoculation, compared to controls. A**, Typical large developing lesions in the controls, right, compared to relatively lesion free P23 root, left.** B**, Cross section through the lesion area, the deep necrotic lesion is apparent in the control, top, while the symptoms are fairly superficial on P23 root, bottom. Low magnification micrograph of the control (C) and P23 root (D), taken from the margin of the infection and stained with phloroglucinol to stain for the presence of lignin.**

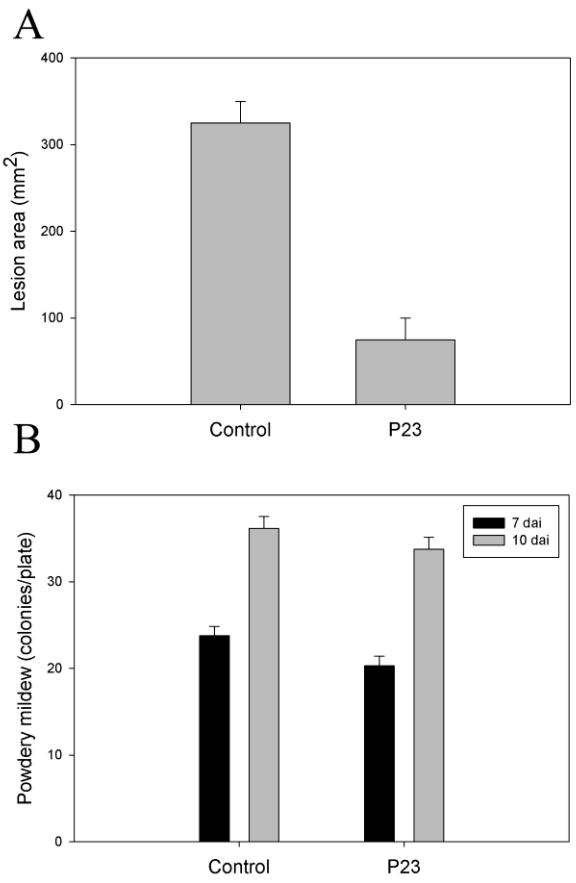

Figure 4.11 Response of P23 transgenic carrots to black rot and powdery mildew. (A),Resistance to black rot (*A. radicina*) of harvested taproots10 dai measured as the average total area of each lesion. (B), Disease resistance of the transgenic lines compared to controls on excised leaflets, inoculated with *E heraclei* spores on water agar plates, based on the number of newly formed sporulating colonies at 7 and 10 days after inoculation. Three replicates of 10 plates were counted for each line. Error bars represent standard error of the means.

(Figure 4.10D). There was no visibly detectable fungal mycelium through the lignin layer of P23, whereas there was a high level of tissue necrosis with visible mycelia present beneath the thin lignin layer of the control. Interestingly the peridermal zone of high lignin did not appear to be constitutive, areas away from the lesion did not have detectable lignin using phloroglucinol (Figure 4.12). Lignin levels were assessed in the taproot tissue and in suspension cultures, under control conditions or in the presence of *S. sclerotiorum*. The lignin was measured in the outermost (~2mm) peel, comprised mainly of periderm and inner layer of vascular tissue comprised mainly of the phloem rays. The P23 line had a ~20% increase in the total lignin levels of the peel under normal growing conditions, with no increase of lignin in the phloem tissues (Figure 4.13). Lignin levels were increased when challenged with *A. radicina*, for both the peel and phloem of the P23 roots (Figure 4.13). The level of lignin increase was greater for P23 relative to *35S::GUS* controls, increasing the lignin level in the peel by 30% and by 50% in the phloem tissue, the controls had no significant increase in lignin due to infection (Figure 4.13).

Since the level of lignification was enhanced in the presence of a pathogen, we examined the level of soluble phenolic compounds to determine if there was also enhancement or if the levels were limiting to lignin deposition during infection. Total soluble phenolics were measured from both control and P23 taproots and leaf tissue, with or without the presence of the pathogen *S. sclerotiorum*. There was some overall increase in the phenolic levels during the infection, however the variation was very high and no differences were

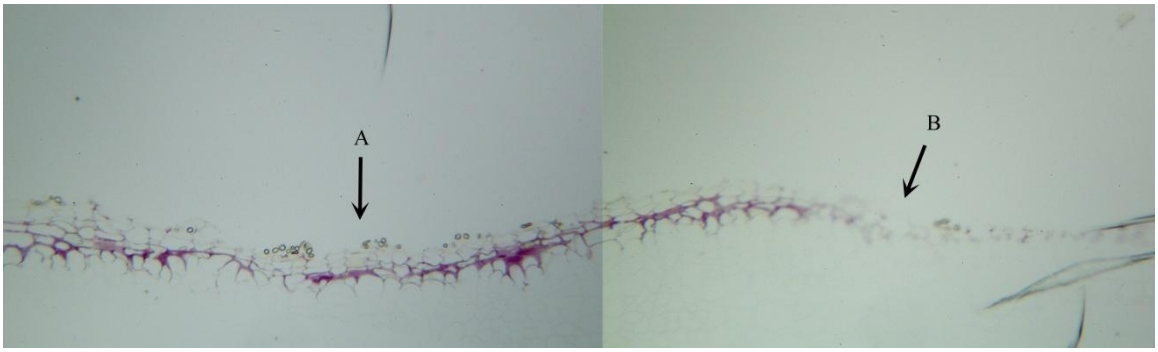

Figure 4.12 Evidence of increased lignification at the site of infection. Low magnification micrograph of P23 root taken along the margin of A. radicina infection. Arrow A is within the margin and highly lignified, while arrow B is outside the margin and has less stained lignin.

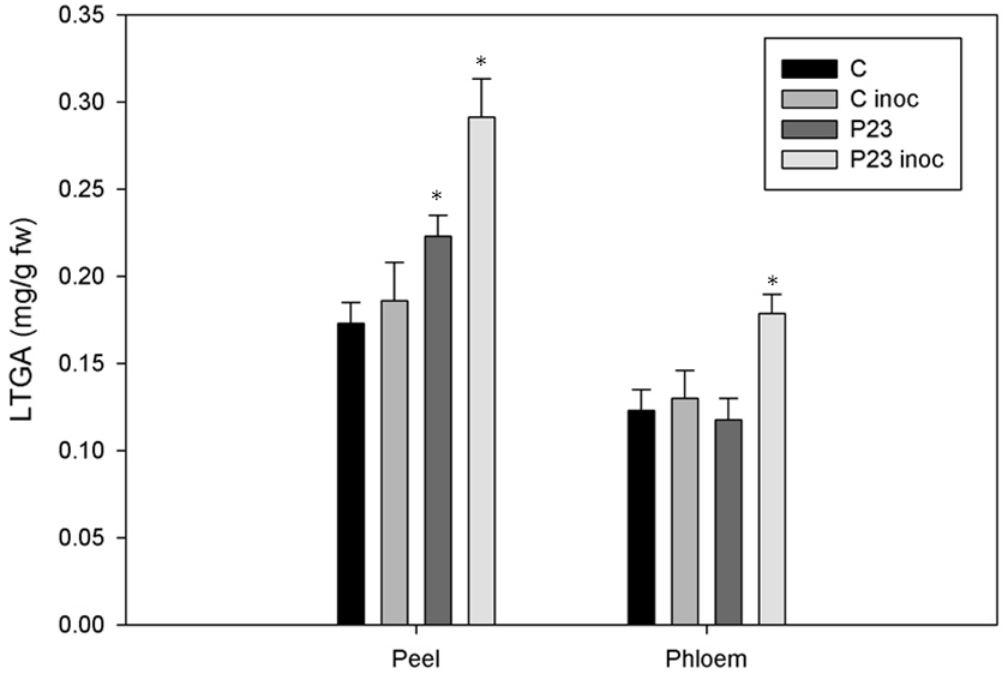

Figure 4.13 Total derivatized lignin-thiogylcolic acid complex content in control and peroxidase over-expressing carrot line P23 roots from the outer 2 mm peel or from the secondary phloem uninoculated and inoculated roots 3 days after inoculation. Vertical error bars indicate standard error of the mean from 3 treatments with 9 replicate samples taken for each treatment. * indicate significant differences from the control α <0.05.

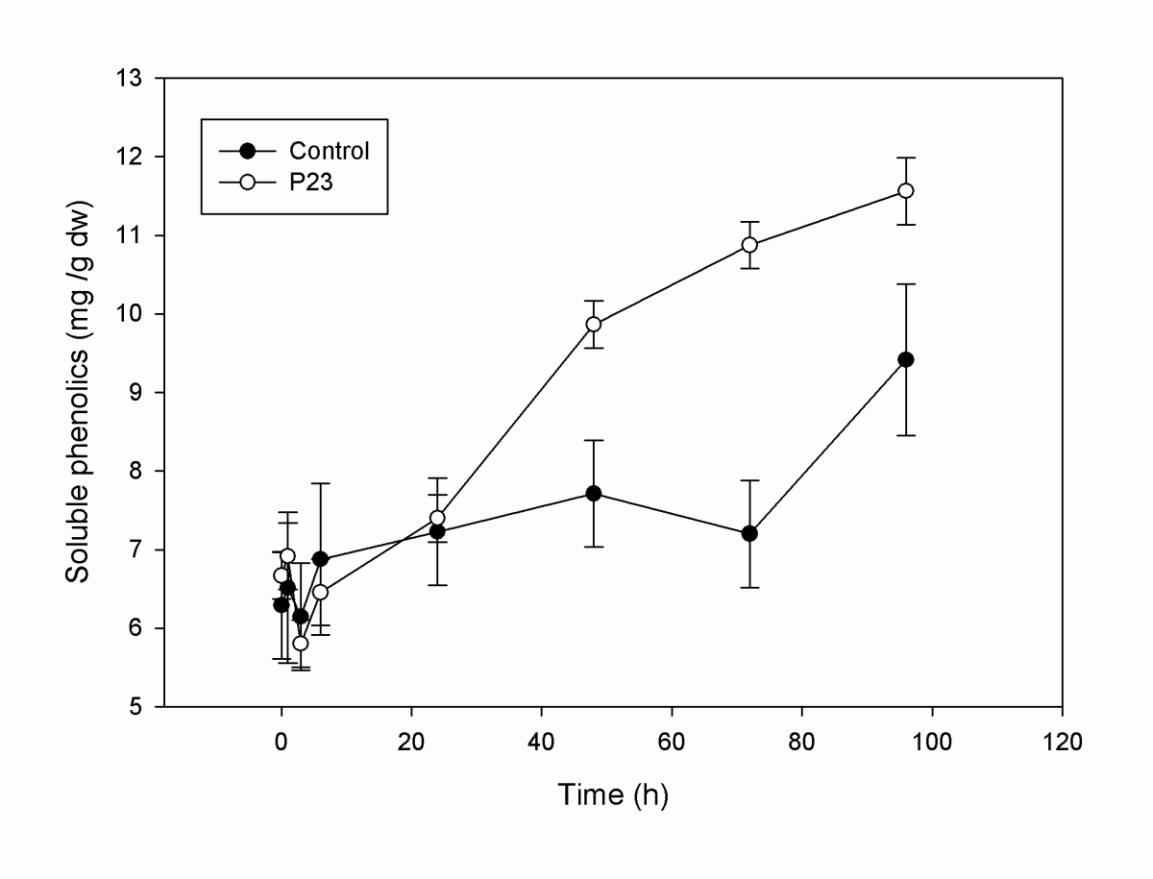

Figure 4.14 Soluble phenolic compounds extracted from suspension cultures of control or P23 peroxidase expressing carrots following inoculation with *S. sclerotiorum* cell wall fragments. Error bars indicate the standard error of the mean for each time point, experiments were conducted 3 times.

determined between the control and P23 lines. A more sensitive suspension culture system was used, using a defence response elicited with *S. sclerotiorum* cell walls, measuring the phenolic levels over a 4 day period. There was no constitutive increase in the phenolic levels for P23 cells compared to *35S::GUS controls*, up until after 24h. However, there was a significant increase in the production of phenolics after 48h, continuing to increase to 96h (Figure 4.14).

4.4 Discussion:

Peroxidases have been shown to have important roles in a plant's ability to resist abiotic and biotic stresses, as well as participating in various aspects of plant growth and development, including generation and utilization of H_2O_2 (Cosio et al. 2009; Johrde et al. 2008; Passardi et al. 2005).H_2O_2 is involved in multiple ways in plant defence responses to pathogens. At low concentrations, H_2O_2 can function as a signalling molecule, involved in gene regulation and activation of secondary defence pathways (Bolwell 1999; Neill et al. 2002a; Neill et al. 2002b). At higher concentrations, H_2O_2 has been associated with cell wall modification, the hypersensitive response, or even directly inhibiting pathogens (Lamb et al. 1997). However, the role of H_2O_2 in pathogen defence is complex, since there is evidence to suggest that H_2O_2 accumulation can be beneficial for colonization of plant tissue by necrotrophic pathogens (Govrin et al. 2002), while being suppressive to biotrophic pathogens. In contrast, the local production of H_2O_2 has been associated with enhanced resistance towards the necrotrophic

fungi *B. cinerea* in tomato (Asselbergh et al. 2007). While tobacco over-expressing a sweet potato peroxidase had up to 5-fold the basal H_2O_2 levels in leaves (Kim et al. 2008b), P23 carrots had no detectable increase in H_2O_2 (Figure 4.7). Apoplastic oxidative burst is typically controlled either through cell wall bound peroxidases as in French bean (Bindschedler et al. 2001) and Arabidopsis (Bindschedler et al. 2006; Davies et al. 2006) or through the function of NADH-oxidases as in rose cells (Bolwell et al. 1998). The oxidative burst in carrot elicited by SS-walls and was found to be controlled mainly by cell wall bound peroxidases, since the burst was effectively eliminated following addition of NaN_3, KCN or L-cysteine, and only marginally reduced by adding DPI. Interestingly, the P23 line had no detectable accumulation of H_2O_2 in culture media. However, in tissue cultured cells of line P23, alkalinization of the medium continued at a longer and slightly stronger rate than the controls, indicating the oxidative burst was not inhibited. Additionally, cells of P23 were able to rapidly remove high levels of exogenous H_2O_2; this ability was effectively eliminated by adding a peroxidase inhibitor. These findings indicate that *POC1* over-expression in the P23 line rapidly utilizes or scavenges H_2O_2 generated during the oxidative burst, reducing H_2O_2 accumulation. *POC1* appears to be functioning more as a H_2O_2 scavenging peroxidase than as a H_2O_2 generating peroxidase, similar in function to the EP5C peroxidase from tomato (Coego et al. 2005a).

The signal transduction pathway downstream of H_2O_2 may be modulated directly or indirectly through the activity of peroxidases, potentially through interactions with extracellular signalling recognition via protein kinases (Lamb et

al. 1997). Greatly enhanced constitutive expression of many defence-related genes was observed in transgenic tobacco over-expressing peroxidase (Kim et al. 2008b). The level of expression was reduced significantly by application of either NaN_3 or KCN, which was attributed to a reduction in the overall level of H_2O_2 present in the tobacco cells (Kim et al. 2008b). There was slight constitutive enhancement of defence gene expression in P23 carrot cells; but the level of expression was dramatically increased when the cells were elicited with SS-walls, indicating that these plants were primed for a more rapid and intense response to pathogens. While the addition of NaN_3 or KCN lowered the enhanced response, it did not bring the defence gene expression back to control levels. The application of exogenous phytohormones INA and JA resulted in marginal increases in both control and P23 lines, however, the degree of gene induction was a fraction of that observed with the SS-walls. The use of complex oligosaccharides has been shown to induce multi-factorial signalling events in plants, generally resulting in much stronger levels of gene induction than a single hormone (Jayaraj et al. 2009; Leitner et al. 2008) and ultimately providing an induced resistance response.

Lignin is a strong structural polymer which is very difficult for pathogens to penetrate or degrade (Quiroga et al. 2000). *POC1* has been identified as a putative lignin-forming peroxidase (Hilaire et al. 2001). Previously it was found that *POC1* expressing carrot petioles constitutively accumulated 40% more lignin than controls and this increased to 70% during pathogen challenge (Chapter 3). Since excess lignin can potentially reduce the palatability of edible tissue, the

lignin levels of mature taproots of P23 were measured relative to controls. Lignin levels in the peel of the root (consisting mainly of periderm) were consistent with those observed in the petiole, both constitutively and during pathogen challenge. However, there was no constitutive increase in the lignin levels in the phloem tissues, with increases only observed during pathogen challenge. Since the peel is generally removed before carrot consumption, the increased lignin in the root peel and petiole should not affect the palatability. There were also no increases in the lignin levels in P23 suspension cultures constitutively or induced with SS-walls, possibly indicating a lack of lignin pre-cursors. Increases in soluble phenolic compounds has been associated with peroxidase over-expression in tobacco (Kim et al. 2008b; Lagrimini 1991), however, the mechanism of this induction remains unknown. P23 carrots did not exhibit heightened phenolic content in roots, leaves or suspension cultures. There was a gradual increase in the total phenolic content of P23 suspension cultures after elicitation with SS-walls after 24 h and continuing past 96 h compared to controls. This increase in phenolics is indicative that peroxidase over-expression may allow for more rapid responses of the phenylpropanoid pathways, rather than constitutive induction.

The harvested taproots of P23 carrots had greatly enhanced resistance towards the necrotrophic pathogen *A. radicina*, exhibiting greater than a 66% reduction in lesion area. It was difficult to compare the levels of resistance observed in the taproots since to previous published over-expression experiments since transgenic plants with increased tolerance towards root pathogens has rarely been conducted in literature, those few reports available

have mainly focused on forest and fruit trees, and there are no previous reports of pathogen resistance in transgenic taproot post harvest. The level of taproot resistance observed in this study was high, in line with foliar resistance observed for P23 when challenged with *S. sclerotiorum* or *B. cinerea* (Chapter 3). The lack of increase in the resistance toward powdery mildew observed, is in contrast to wheat (Altpeter et al. 2005; Johrde et al. 2008), barley (Johrde et al. 2008) and tobacco (Kim et al. 2008b) that over-expressed a peroxidase gene and led to enhanced resistance towards powdery or downy mildews. The P23 lines did not have a constitutive increase in H_2O_2, nor the high levels of *PR* gene expression as in the transgenic tobacco (Kim et al. 2008b). The level of peroxidase expression were also much higher in barley and tobacco, in excess of 40-fold (Johrde et al. 2008; Kim et al. 2008b), constitutive expression at such high levels may have led to decreased infection. While the enhanced epidermal expression in wheat could result in specific resistance to epidermal infecting powdery mildew (Altpeter et al. 2005). The increased lignin and low levels of constitutive *PR-1* transcripts in P23 carrot leaves may be insufficient to increase basal resistance in P23, being unable to reduce the colonization by *E*. *heraclei*.

In summary, we have shown that line P23 expressing *POC1* had reduced accumulation of H_2O_2 when undergoing an oxidative burst response. In addition, heightened induced expression of defence-related gene transcripts and taproot lignin levels were observed. These findings suggest that constitutive *POC1* expression is involved in multiple signalling and defence responses in carrot tissues.

5: BROAD SPECTRUM DISEASE RESISTANCE TO NECROTROPHIC AND BIOTROPHIC PATHOGENS IN TRANSGENIC CARROTS (*DAUCUS CAROTA L*.) EXPRESSING AN ARABIDOPSIS *NPR1* GENE

Results from this chapter are published: Wally O, Jayaraj J and Punja ZK. 2009. *Planta* 231:131-141.

5.1 Introduction:

Genetic engineering for enhanced disease resistance in carrot has been previously shown to be successful through expression of a single pathogenesis-related (PR) protein, such as chitinase, β-1,3 glucanase, thaumatin-like protein or peroxidase (Baranski et al. 2008; Chen et al. 2002; Jayaraj et al. 2007; Melchers et al. 2000; Punja et al. 1996; Punja 2005), or using a combination of two genes (Melchers et al. 2000; Jayaraj et al. 2007). These approaches have not, however, provided broad-spectrum resistance towards a wide a range of pathogens. Manipulation of a plant's innate defence signalling pathways offers an alternative approach by controlling a large number of induced genes either directly or indirectly. The gene *NPR1* (also known as *NIM1* and *SAI*) was identified as a loss-of-function *npr1* mutant in Arabidopsis that was unable to undergo systemic acquired resistance (SAR) in the presence of the signalling molecule salicylic acid (SA) or SA analogues (Cao et al. 1994; Delaney et al. 1995; Shah et al. 1997). *NPR1* was identified as a key regulatory gene of the SA-mediated SAR in Arabidopsis (Cao et al. 1997; Shah 2003), and has also been identified to play a key role in ethylene and jasmonic acid signalling pathways involved in induced systemic resistance (ISR) (Pieterse et al. 1998). Arabidopsis plants over-expressing *NPR1*

exhibited enhanced resistance towards *Pseudomonas syringae* and *Peronospora parasitica* (Cao et al. 1998). Further evidence from Arabidopsis indicated a crucial role of *NPR1* in the local and systemic plant response to the broad host-range necrotrophic pathogens *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Ferrari et al. 2003; Guo et al. 2007), which do not initiate SAR responses (Govrin et al. 2002).

Studies from Arabidopsis have revealed that *NPR1* is constitutively expressed at low levels and the gene product is located as an inactive multimeric protein in the cytosol (Mou et al. 2003). During pathogen infection, or application of an elicitor, the *NPR1* complex is reduced and the structure is changed to the monomeric form which is transported to the nucleus. Nuclear *NPR1* interacts with TGA family of basic leucine zipper transcription factors, which in turn induces expression of several PR genes (Fan et al. 2002; Subramaniam et al. 2001). Functionality of *AtNPR1* remains active in different plant species and has led to pathogen and SA-induced increases in PR gene expression in transgenic rice (Fitzgerald et al. 2004; Quilis et al. 2008), wheat (Makandar et al. 2006), tobacco (Meur et al. 2008) and tomato (Lin et al. 2004). This indicates that *AtNPR1* when expressed in a heterologous system reacts in a similar fashion to the endogenous *NPR1*-like proteins. The plants constitutively expressing *AtNPR1* have shown resistance to both necrotrophic (Lin et al. 2004; Makandar et al. 2006) and biotrophic fungal, viral, and bacterial pathogens (Lin et al. 2004) in addition to insect pests (Meur et al. 2008).

Carrot (*Daucus carota L*. subsp. *sativa*) is a biennial plant which is grown worldwide and major economic losses occur due to reduction in yield and carrot quality following infection by a wide range of foliar pathogens, especially fungi, resulting in

reduced photosynthate accumulation (Davis et al. 2002). In addition, further weakening of the carrot tops greatly reduces the effectiveness of mechanical harvesters (Farrar et al. 2004). Some of these fungi can also infect the roots during long-term post-harvest storage (Davis et al. 2002). Many commercially-grown carrot cultivars lack adequate genetic resistance to these fungal pathogens (Kora et al. 2003; Punja et al. 2007). Resistance to multiple pathogens with different infection and growth characteristics is desirable in conventionally bred or transgenic carrot. Since carrot plants can undergo SAR and respond to SA in a similar manner to that reported in tomato and Arabidopsis (Jayaraj et al. 2008b), we explored the potential of enhancing the innate SAR pathway in carrot for broad spectrum disease resistance. We describe the use of *AtNPR1* over-expression as a means to provide pathogen resistance in carrot leaves and taproots challenged with necrotrophic and biotrophic pathogens. These transgenic plants may be incorporated in a breeding program to enhance disease resistance in carrots.

5.2 Materials and methods:

5.2.1 Genetic transformation of carrot with *AtNPR1*

The *NPR1* cDNA was amplified from total mRNA extracted from leaves of *Arabidopsis thaliana* by reverse transcriptase-PCR (RT-PCR) using SuperScript II RT (Invitrogen, Carlsbad, CA, USA). First-strand synthesis was conducted using the reverse primer *AtNPR1*-RC 5' (Table 1) using 500 ng of RNA. The cDNA was PCR amplified using the above primer along with the forward primer, *AtNPR1*-FC (Table 1). The resulting 1818 bp DNA fragment was cloned into pCR2.1-TOPO plasmid (Invitrogen) and was verified through sequencing. The

AtNPR1 cDNA was released using *Eco*RI and *Pst*I. The CaMV-*35S* promoter was obtained from the plasmid pBSK-CaMV (Chapter 2) that was digested with *Hind*III and *Eco*RI and the resulting CaMV promoter fragment was subcloned into pCAMBIA1300 at *Hind*III and *Eco*RI sites. The NOS terminator fragment was prepared by digestion with *Pst*I and *Eco*RI (Figure 5.1). The At*NPR1* cDNA and NOS terminator fragments were ligated into the *Eco*RI-linearized pCAMBIA-CaMV plasmid by three-fragment ligation (Figure 5.1). The gene orientation of the construct was verified by restriction digestion and sequencing of the promoter-gene junction. The plasmid sequence was verified and used to transform electrocompetent *Agrobacterium tumefaciens* LBA4404 (Invitrogen). Sterile 'Nantes Coreless' carrot petioles were transformed, callus regenerated and plantlets grown using previously established protocols (Wally et al. 2006).

5.2.2 Molecular characterization of regenerated carrots

The putatively transgenic plants growing on tissue-culture medium containing hygromycin (100 mg l^{-1}) were assayed for the presence of *AtNPR1*. Total genomic DNA was isolated from 200 mg (fresh weight) of leaf tissue that was lyophilized prior to being extracted using established protocols (Wally et al. 2006). The primers used to amplify a 750 bp fragment of *AtNPR1* gene were *AtNPR1*-F1 and *AtNPR1*-R5 (Table 5.1)(Makandar et al. 2006). The PCR reactions were carried out with a 56 ºC annealing temperature in 10 µl volume (Chapter 3). RT-PCR analysis was performed using Superscript II one step RT-PCR kit (Invitrogen) on 1 µg of total RNA extracted from carrot leaves using Trizol reagent (Invitrogen). The primers *AtNPR1*-F1 and *AtNPR1*-R5 were

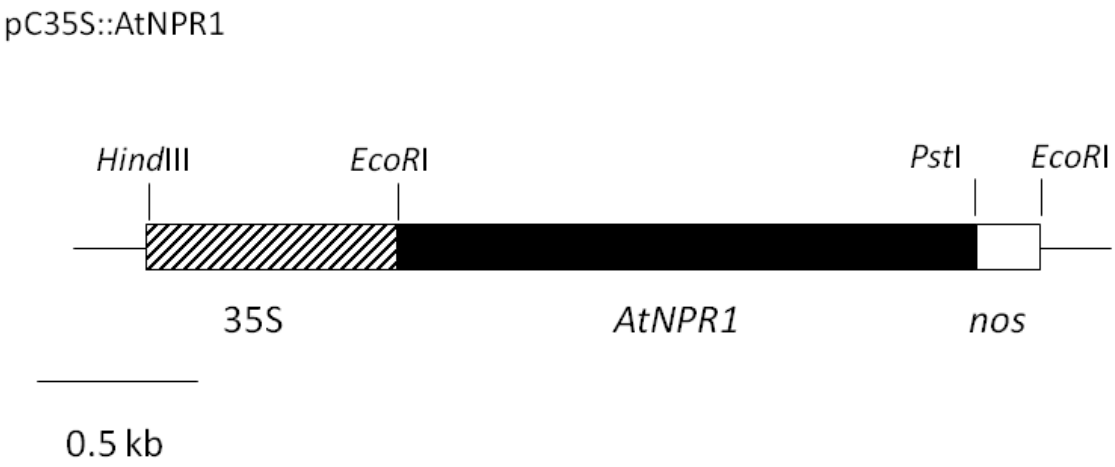

Figure 5.1 pCambia 1300 *35S*::*AtNPR1* chimeric construct. The *Arabidopsis thaliana NPR1*(*AtNPR1*) gene cDNA was cloned into pCambia 1300 under the control of the CaMV *35S* promoter. The 3' nontranscribed region of the *A. tumefaciens nopaline synthase (nos)* gene was used as terminator

Table 5.1. Primer sets used for cloning and detecting *AtNPR1*, and for generation of cDNA probes for Northern blot analysis.

Gene	Primer (set)	Sequence	Genbank
AtNPR1	*AtNPR1*-FC	5′-GGAATTCCTTTAATTTGTGAATTTCAA -3′	NM105102.
	AtNPR1-RC	5′-CCTGCAGTCACCGACGACGATGAGA-3′	
AtNPR1	*AtNPR1*-F1	5′-GAGGACACATTGGTTATACTC-3′	NM105102.
	AtNPR1-R5	5′-CAAGATCGAGCAGCGTCATCTT-3′	
DcAct	DcACT-F1	5′-ACACTGGTGTGATGGTTGGA-3′	X17525
	DcACT-R1	5′-TGGTGATAACTTGCCCATCA-3′	
DcPR1	DcPR1-F	5′-GCGATCCATCGCATCAGTCTCACAG-3′	AB127984.
	DcPR1-R	5′-CCTGCAGATTGAAACATCTTGCATCG-3′	
DcPR2	DcPR2-F	5′-CACAACGCTAACCAGCTTGA-3′	AB127960.
	DcPR-R	5′-TCAACGGCCTTGAATACTGA-3′	
DcPR3	DcPR3-F	5′-GGGATCCATGAAAACATTTTTCATTTT-3′	U52848
	DcPR3-R	5′-CGAGCTCCTAGCATCGCTGATTATCACCA-3′	
DcPR5	DcPR5-F	5′-TAGACAACTGCCCGAACACA-3′	AY065642
	DcPR5-R	5′-GGAAGAAGCTGATGGTTTGC-3′	
Dc18s	Dc18s-F	5′-CTACGTCCCTGCCCTTTGTA-3′	AY552527.
	Dc18s-R	5′-GATCCTTCCGCAGGTTCAC-5′	
DcPAL	DcPAL-F	5′-TGGAACGATCACTGCTTCTG-3′	D85850.1
	DcPAL-R	5′-GCCTCAGGTCTACAGCTTGG-3′	

used along with the primers for actin, DcACT-F1 and DcACT-R1 (Table 5.1) as a control.

Following PCR, Southern analysis was used to confirm integration of the *AtNPR1* gene in the carrot genome. Total genomic DNA was digested with *HindIII* or *EcoRI* following previously established protocols for carrot (Chapter 2). Random primers were used to label the *AtNPR1* PCR products using [α-^{32}P] dCTP and Prime-A-gene kit (Promega) following the manufacturers protocols and used as radioactive DNA probes. RNA blot hybridization was conducted on total RNA extracted from young healthy leaf tissue (~100 mg) or 2 week old suspension cultures (~250 mg) using the TRIZOL® reagent (Invitrogen) according to the supplied protocols. Twelve µg of total RNA of each line was separated by electrophoresis in a 1.2 % formaldehyde agarose gel and blotted onto Hybond XL (Amersham, Uppsala, Sweden) nylon membranes, and analyzed using described protocols (Chapter 3). Quantification of signal intensity was carried out with software Imagequant version 5.2 (Molecular Dynamics, USA) and normalized to actin and 18s RNA.

5.2.3 Effect of *AtNPR1* on gene expression:

Total RNA was extracted from carrot suspension cultures (~250 mg) and leaves (~100 mg) from the *AtNPR1* expressing lines and CaMV 35 GUS expressing lines (Chapter 2) which served as controls. Suspension cultures of the lines were established using described protocols (Chen et al. 2002). Samples were taken at 0,1,3,6,12,24,48 and 72 hours after induction for suspension cultures and at 0, 3, 24, 72 and 120 hours for leaves. Suspension cultures were

treated with *S. sclerotiorum* cell walls (SS-walls), (100 µg ml^{-1} dw), 2, 6-dichloroisonicotinic acid (INA) (500 µM), SA (500 µM), methyl jasomonic acid (MeJA) (100 µM), or a mock treatment of NaCl (1 mM) while leaves were treated with SS-walls SA, and a mock treatment. SS-walls were prepared from 7 day old *S. sclerotiorum* cultures grown in potato dextrose broth at room temperature according to published procedures (Tweddell et al. 1994). The lyophilized cell walls were suspended in water (10 mg dw ml^{-1}) and sterilized by autoclaving. To monitor gene expression, radioactive probes of carrot phenylalanine ammonia lyase (*DcPAL*), *DcPR1*, *DcPR2, DcPR3, DcPR5, DcAct* and *Dc18S* were generated using RT-PCR on non-transformed carrot cDNA using various primers (Table 5.1). Each sample was replicated a minimum of three times. Quantitative evaluation of carrot PR gene expression was obtained by exposing filters to phospho-storage screens (GE) and by subsequently scanning with the phospho-imaging system Si 445 (Molecular Dynamics, CA, USA). The signal intensities were normalized against both actin and 18S rRNA and standardized to the time zero points for the control lines.

5.2.4 Assessment of disease resistance

To evaluate resistance of the transgenic carrot lines, we selected the following three necrotrophic foliar pathogens; *S. sclerotiorum*, *B. cinerea*, and *A. radicina*; a root pathogen: *A. radicina*; a foliar infecting bacterium: *Xanthomonas hortorum pv. carotae*; and a biotrophic fungus: *E. heraclei*. Two primary regenerated lines (I and XI) were grown in the greenhouse for up to 8 months

under previously described conditions (Wally et al. 2006) and compared to non-transformed ‘Nantes Coreless’ controls.

Inoculation with *B. cinerea* and *S. sclerotiorum* was conducted as previously described (chapter 3). The extent of leaf necrosis was determined by counting the number of individual lobed segments per leaflet which were diseased and expressing this as a percentage of total leaf segments on detached leaves (Punja 2005). Each experiment was conducted 5 times with a minimum of 9 compound leaves per treatment.

For assessment of foliar resistance to *A. radicina*, pure cultures (provided by Dr. Barry M. Pryor, University of Arizona, Tucson, USA) were maintained on V8 agar for 2 weeks. Carrot plants were inoculated with a conidial suspension (1×10^6 conidia ml^{-1}) using an atomizer until run-off and incubated in a humid chamber for 72 h and then transferred to the greenhouse. Disease severity was assessed 12 days after inoculation (dai), using a 6-point disease rating scale based on the percentage of leaf area infected (1=0%; 2=1–10%; 3=11–25%; 4=26–40%; 5=41–55%; 6=>56%) calculated as the sum of disease ratings of individual leaves/total number of leaves. The experiment was conducted five times with 9 replicates per carrot line. Root resistance to *A. radicina* was assessed on 4-6 month old roots from greenhouse-grown plants, which were washed prior to use. Two actively growing mycelial plugs from 2-week old cultures were placed on the root and lesion area was measured at 10 dai and compared to control lines. Sixteen roots were assessed for each line.

For *E. heraclei*, heavily-infected leaves of non-transgenic carrots were used as inoculum. Leaves from transgenic and non-transgenic control plants were harvested, washed, and cut into 3-4 cm segments and placed adaxial side up on water agar (6 g l^{-1}) containing benzimidazole (0.1g l^{-1})(Sigma), until 90% of the agar surface was covered. Condida were dislodged from infected tissues using low pressure airflow and blown into a settling tower, where they were allowed to settle over the leaves for 5 min. The petri plates containing the infected leaves were sealed and placed on the laboratory bench 22-25º C for 10 days. Newly formed sporulating colonies were counted per plate at 7 and 10 days using a stereo dissecting microscope. Powdery mildew experiments were conducted three times with 10 plates per replicate.

For bacterial leaf blight, *X. hortorum* pv. *carotae* (provided by Dr. Lindsey du Toit, Washington State University, Mount Vernon, USA), a 50 ml culture of liquid nutrient broth inoculated with a single bacterial colony was grown overnight at 22 ºC with constant agitation. The culture was centrifuged for 10 min at 800 g at 22 ºC, the pellet washed with sterile water and resuspended in 10 mM $MgCl_2$ and 0.1% (v/v) Tween 20 to an OD 600 of 0.5, corresponding to ~10^9 CFU ml^{-1}. Carrot leaves (from 4-6 month old plants) were inoculated by dipping sterile scissors into the *X. hortorum* solution and making a single cut across the upper leaves at an angle to increase infection area (Kauffman et al. 1973). The plants were enclosed in a humid chamber for 2 days, returned to the greenhouse and evaluated for disease after 10 days, using a 0-6 DSI.

(number of lightly diseased leaves *2) + (number of heavily diseased leaves*4)/ total number of leaves)

Bacterial leaf blight experiments were replicated 4 times with a minimum of 5 plants per replicate.

5.2.5 Statistical analysis

Treatments were analyzed for significant differences using one-way ANOVA, followed by Tukey-Kramer HSD test and subsequently compared to controls using Dunnett's control test using the JMP version 7 software (SAS institute, 2008). LSD values at $\alpha < 0.05$ were used to determine significance.

5.3 Results

5.3.1 Genetic transformation of carrot with *AtNPR1* and molecular characterization

The carrot variety 'Nantes Coreless' was used to generate the CaMV *35S*::*AtNPR1* transgenic plants. The *hph1* gene, which confers resistance to hygromycin, was used to select the primary transformants on ½ MS medium containing 100 mg l^{-1} hygromycin. Following selection, two distinct lines (I and XI) were characterized further from a total of 18 lines regenerated. The *AtNPR1* gene was detected in leaves of these two lines, using PCR (Figure 5.2A) and Southern blot hybridization (Figure 5.2B); while gene expression was confirmed using RT-PCR (Figure 5.2C) and Northern blot hybridization (Figure 5.2D). Line XI has 2 fold greater *AtNPR1* gene expression than line I (quantified from Northern hybridization standardized to actin), while control plants had no detectable level of the gene. With low stringency washes of Northern blots, there was no cross-hybridization between the carrot orthologue of *NPR1* and the *AtNPR1* probe used (Figure 5.2C, D).

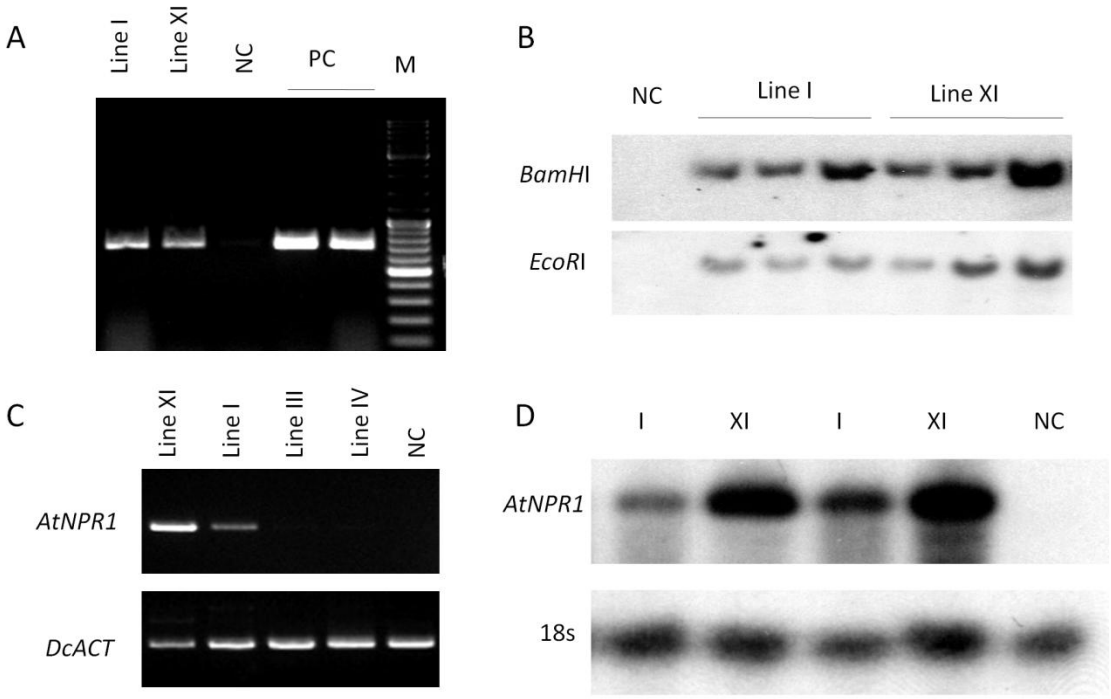

Figure 5.2 Figure 2. Integration and expression of *AtNPR1* gene in transgenic carrot. A, **DNA isolated from leaves of transgenic carrot lines I and XI, non- transgenic 'Nantes coreless' (NC), plasmid control (PC) were used to amplify a portion of the *AtNPR1* fragment by PCR, with molecular weight markers (M) Fermentas gene ladder.** B, **DNA extracted from leaves of non-transgenic and transgenic lines I and XI were digested with *Bam*HI (top) *and Eco*RI (bottom), resolved on an agarose gel, and probed with an *AtNPR1* radio-labelled probe by southern hybridization. Both *Bam*HI and *Eco*RI released the entire *AtNPR1* cassette.** C, **RNA extracted from leaf tissue of *AtNPR1* transgenic lines and 'Nantes coreless' was used to amplify the *AtNPR1* gene using reverse transcriptase (RT)-PCR. Expression of the carrot actin (*DcAct*) served as a control for the RT-PCR.** D, ***AtNPR1* gene expression was confirmed from RNA of the RT-PCR positive plants was subjected to northern hybridization, using a radio-labelled *AtNPR1* probe. Loading control was conducted by probing with a radio-labelled carrot 18s rRNA probe.**

5.3.2 Induction of PR genes after elicitor treatment

Gene expression levels of carrot pathogen-responsive genes were determined in greenhouse-grown plants and in suspension cell cultures. There was little to no increase in constitutive expression of the defence-related genes examined (time 0) (Figure 5.3 and 5.4A) with low expression levels seen in both control and transgenic plants. There was some apparent induction of *PR-1* and *PR-2* in the *AtNPR1* lines; however, these increases were not statistically significant (Table 5.2). A much stronger and longer-lasting *PR* gene response was observed in cell cultures treated with SS-walls. A greater than 10-fold increase in *PR-1* gene expression was observed in line XI by 3 h (Table 5.2), compared to a 6-fold increase in the controls. The *PR-1* expression levels continued to increase in the *AtNPR1* lines, peaking at 12 h, representing a 22-fold increase, and was maintained at a high level past 72 h (Table 5.2). The controls also peaked at 12 h; however, the levels of expression were lower (~33% of the *AtNPR1* line) and decreased very rapidly to basal levels by 48h (Figure 5.3). *PR-2* gene expression patterns were similar but the levels of induction were higher than *PR-1* (Figure 5.3). *PR-5* expression followed a similar but slower trend, increasing approximately 15-fold by 24 h in the *AtNPR1* lines while the controls had a very low increase in *PR-5* with no significant increase occurring until 48h (Figure 5.3). There were also significantly higher increases in *PAL* and *PR3* transcript levels; however, these increases were lower than for the other genes examined (Figure 5.3). Similar patterns but significantly weaker gene

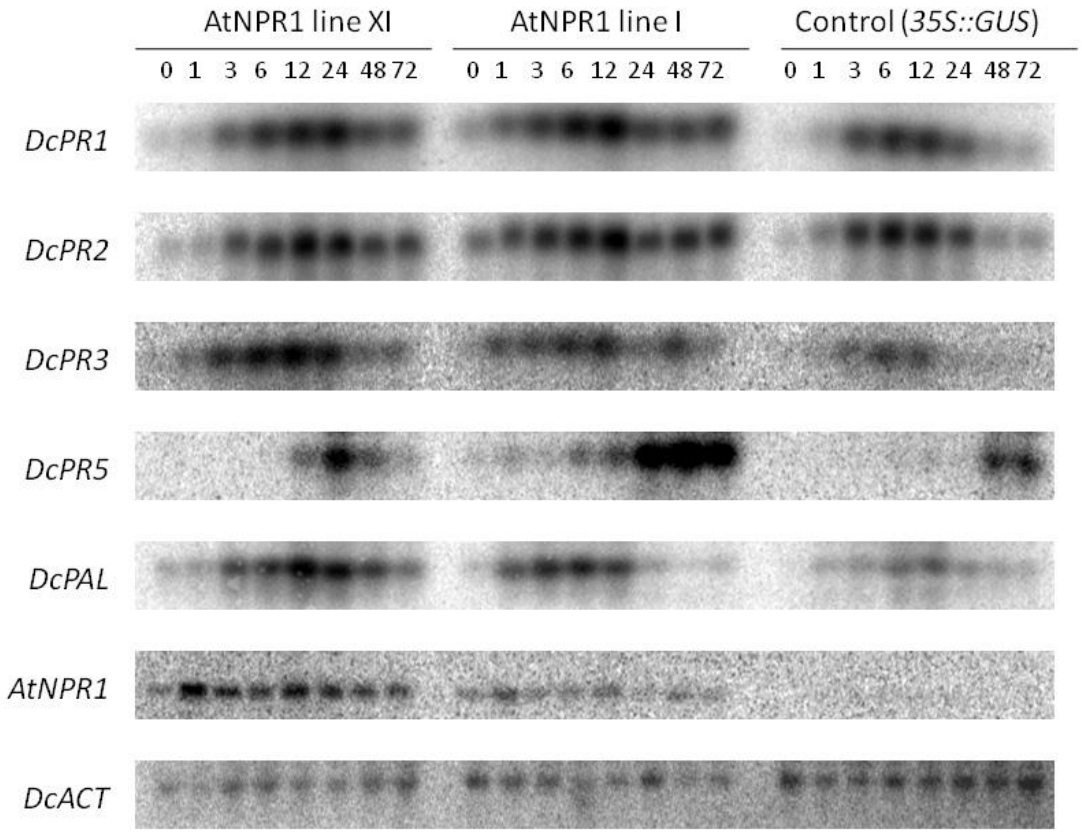

Figure 5.3 Time course induction of PR genes in transgenic carrot lines when induced with *S. sclerotiorum* cell wall fragments. Total RNA from transgenic *AtNPR1* lines XI and I, and *35S::GUS*-transformed control suspension cultures, extracted at 0,1,3,6,12,24,48 and 72 h after treatment with *S. sclerotiorum* cell walls (100 µg ml^{-1} dw). Northern blots were probed with the radio-labelled cDNA probes listed. These experiments were confirmed using 3 separate replicates.

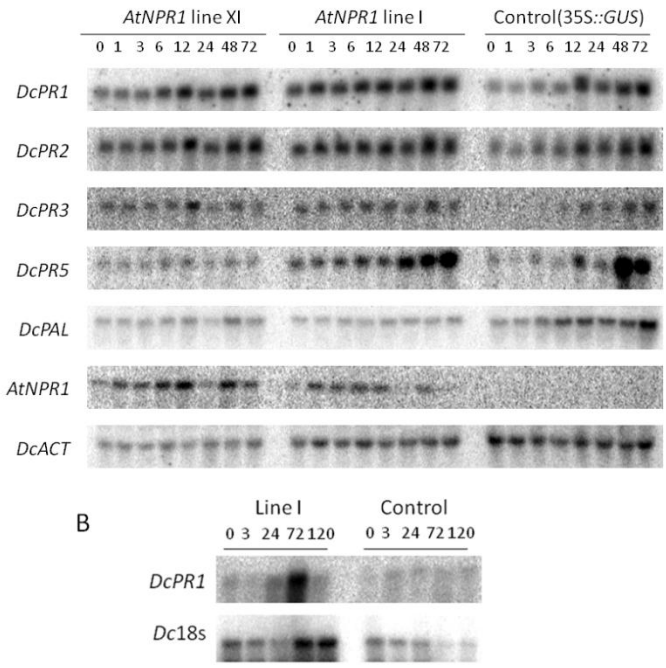

Figure 5.4 Time course induction of *PR* genes in transgenic carrot lines when induced with Salicylic acid or SA analogue 2,6-dichloroisonicotinic acid. A. Total RNA from transgenic *AtNPR1* lines XI and I, and *35S::GUS* transformed control suspension cultures, extracted at 0,1,3,6,12,24,48 and 72 h after treatment with INA (500 µM). Northern blots were probed with the radio-labelled cDNA probes listed. B. Total RNA from leaf tissues treated with SA (100 µM) until run off. The blot was probed with radio-labelled *PR-1* and 18s rRNA probes.

Table 5.2 Fold transcript induction of *PR-1*, *PR-2* and *PR-5* in *AtNPR1* line XI and control suspension cultures, from 0h-72h after treatment. Transcript level was normalized to both actin and 18s rRNA and standardized to the basal level of the control lines at T0. Data presented is the mean of 3 separate replicates. Standard error of the means is indicated.

Time (h)	Treatment											
	Control (SS-walls)			XI (SS-walls)			Control (INA)			XI (INA)		
	PR-1	*PR-2*	*PR-5*	*PR-1*	*PR-2*	*PR-5*	*PR-1*	*PR-2*	*PR-5*	*PR-1*	*PR-2*	*PR-5*
0	1.0	1.0	1.0	2.3	2.7	1.9	1.0	1.0	1.0	1.9	1.9	1.7
3	6.3	16.3	1.2	12.7	28.9	2.4	0.9	1.1	0.9	2.2	3.3	1.8
6	8.8	21.3	1.6	16.	34.8	3.4	1.2	1.4	1.0	3.2	3.1	1.6
12	8.9	21.9	1.5	22.4	57.5	6.1	2.6	2.4	1.2	5.5	6.3	1.9
24	4.8	10.7	1.4	10.4	19.0	13.1	1.7	1.7	1.1	4.7	5.9	2.3
48	1.8	1.9	4.4	9.1	23.5	16.1	2.6	2.5	4.0	4.9	4.9	2.5
72	0.9	1.2	4.1	7.8	18.1	12.7	2.6	2.9	2.2	4.5	4.6	3.5
St error	0.75	0.89	0.96	0.75	0.89	0.96	0.47	0.52	0.49	0.47	0.52	0.49

induction responses were also observed in the suspension cells treated with INA for both *PR-1* and *PR-2* gene expression with no significant changes in PAL or *PR-5* transcripts levels and slight increases in *PR-3* levels by 4-fold at 12h (Figure 5.4A). Control lines responded similarly to INA treatment, with a gradual increase in *PR-1*, *PR-2* and *PR-3* gene expression up to 2.5 fold basal levels at 72h; there were no significant changes in PR5 or PAL expression (Figure 5.4A). In leaf tissues, there was a strong induction of *PR-1* in the *AtNPR1* line in response to SA, peaking at 72h, while the control plant had no change in *PR-1* gene expression (Figure 5.4B). Similarly, there was also a strong long lasting induction in *PR-2* transcript in the *AtNPR1* transgenic lines when treated with MeJA with levels 5-10 fold higher than the controls (Figure 5.5). Otherwise MeJA had minimal effect relative to controls for the other defence related genes.

5.3.3 Assessment of resistance to pathogens

For *B. cinerea*, small necrotic areas began to appear 4 days after inoculation (dai) on control plants and necrosis and sporulation continued to 7 dai, covering approximately 30% of the leaf area. Lesion area was reduced by more than 50% in lines I and XI at 7 dai compared to the controls (Figure 5.6A). Necrosis due to *S. sclerotiorum* was very severe on the non-transgenic control plants, and most of the tissues were completely necrotic by 7 dai, with an average diseased area of nearly 80 % (Figure 5.6B). Lines I and XI displayed significant resistance, with reductions in diseased area of more than 60% at 4 dai

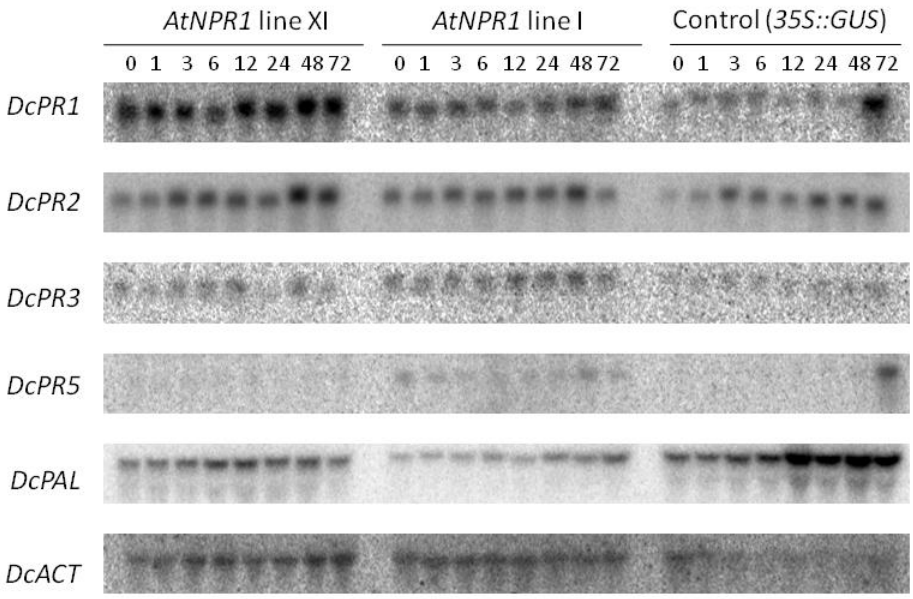

Figure 5.5 Time course induction of *PR* genes in transgenic carrot lines when induced with Methyl-jasmonic acid. Total RNA from transgenic *AtNPR1* lines XI and I, and *35S::GUS* transformed control suspension cultures, extracted at 0,1,3,6,12,24,48 and 72 h after treatment with MeJA (100 µM). Northern blots were probed with the radio-labelled cDNA probes listed

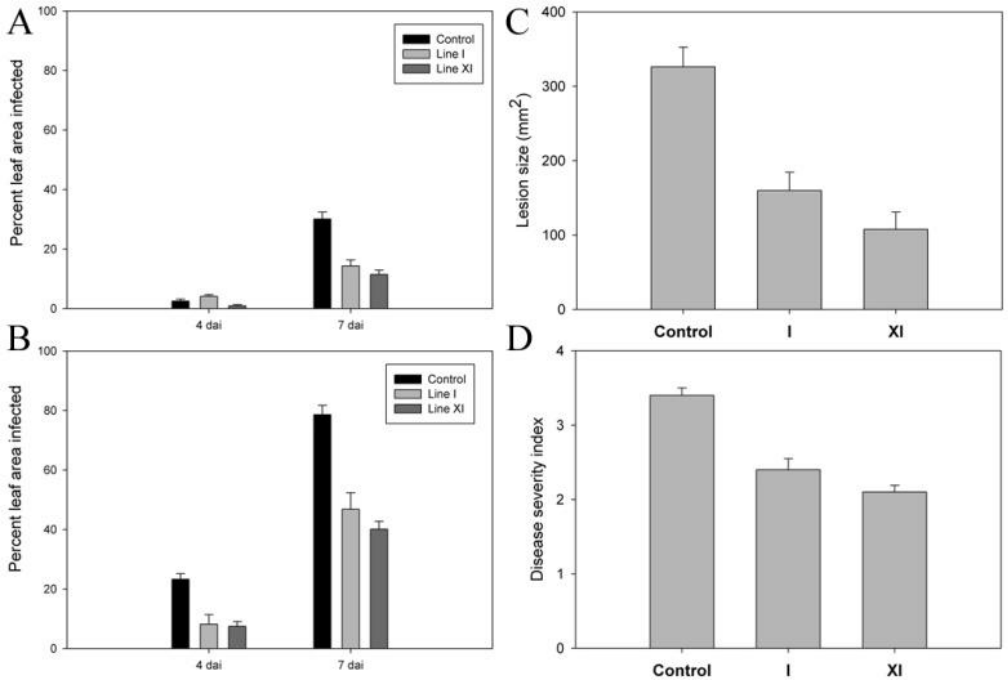

Figure 5.6 Diseased area and severity in *AtNPR1* transgenic plants compared to controls. (A), *B.cinerea* resistance was measured as percentage of total leaf area infected by the fungus, in non-transgenic control plants and *AtNPR1* lines I and XI. (B), . *S. sclerotiorum* resistance was measured as percentage of total leaf area infected. (C), and (D), Resistance to black rot (*A. radicina*) of *AtNPR1* transgenic carrot plants and harvested taproots. (C), *A. radicina* resistance was measured as the mean of the area of individual root lesions infected by the fungus. (D), Disease resistance based on a 6 point disease severity index of foliar lesions on the transgenic lines compared to controls inoculated with *A. radicina* spores. The mean of 5 replicates consisting of 9 individual compound leaves at 4 and 7 days after inoculation are plotted, for A and B, 16 roots for each line were used for C, and 5 replicates of 9 plants each were used for D. Error bars represent standard error of the means.

and more than 40% at 7 dai (Figure 5.7A,B). There was no significant difference in disease tolerance between the two transgenic lines at 7 dai, while line XI exhibited higher resistance to *B. cinerea* at 4 dai.

The transgenic lines were also resistant to *A. radicina* infection, both on the foliage and on taproots. Mycelial plugs placed on the roots of both control and transgenic carrots produced typical black rot symptoms, with dry, black sunken lesions (Figure 5.7C). Lesions for both lines I and XI were reduced in diameter by more than 50% at 10 dai (Figure 5.6C). Both lines I and XI had a reduced number of necrotic lesions on the leaf tissue, with greater than 33% reduction in DSI for both lines. Additionally, there was a much greater reduction in the chlorotic zones surrounding the foliar lesions for lines I and XI compared to controls (data not shown).

Routine inspection of greenhouse-grown plants indicated that the *AtNPR1* expressing lines developed powdery mildew symptoms much less frequently and with lower severity through natural infection (Figure 5.7D). When lines I and XI were tested for their resistance to powdery mildew infection, the number of newly formed sporulating colonies was significantly reduced (by 90%) in both lines at 7 and 10 dai (Figure 5.8A). Colonies developed on control leaves after 3 days, while no colonies were detected until 6 dai on either of the transgenic lines. The size and degree of sporulation was much greater on the control leaves as compared to the *AtNPR1* lines.

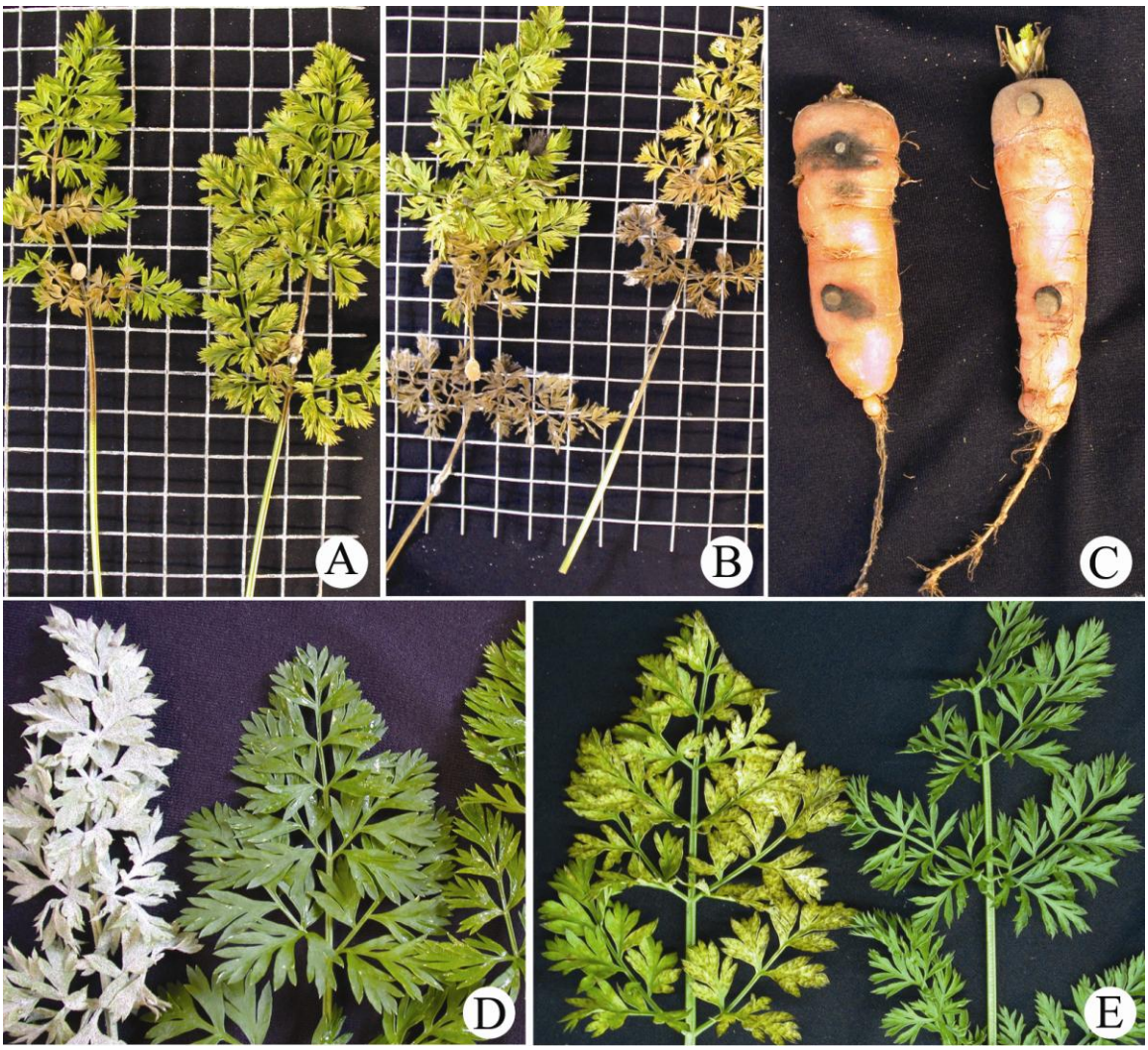

Figure 5.7 Evaluation of resistance to pathogens in *AtNPR1* transgenic carrot plants. (A,B) Symptoms of *S. sclerotiorum* on *AtNPR1* line I (A) and non-transgenic 'Nantes Coreless' (B) at 7 days after inoculation. C, typical black rot symptoms on taproots of non-transformed 'Nantes coreless'(left) carrot and *AtNPR1* expressing transgenic (right) 10 days after inoculation. D, comparison of natural powdery mildew infection of control leaf (left) grown next to *AtNPR1* transgenic plant (right) in the greenhouse. E, bacterial leaf blight infection of carrot leaves, control plant (left) is largely chlorotic, with necrosis beginning to form on the leaf tips whereas the *AtNPR1* expression plant (right) is nearly symptomless.

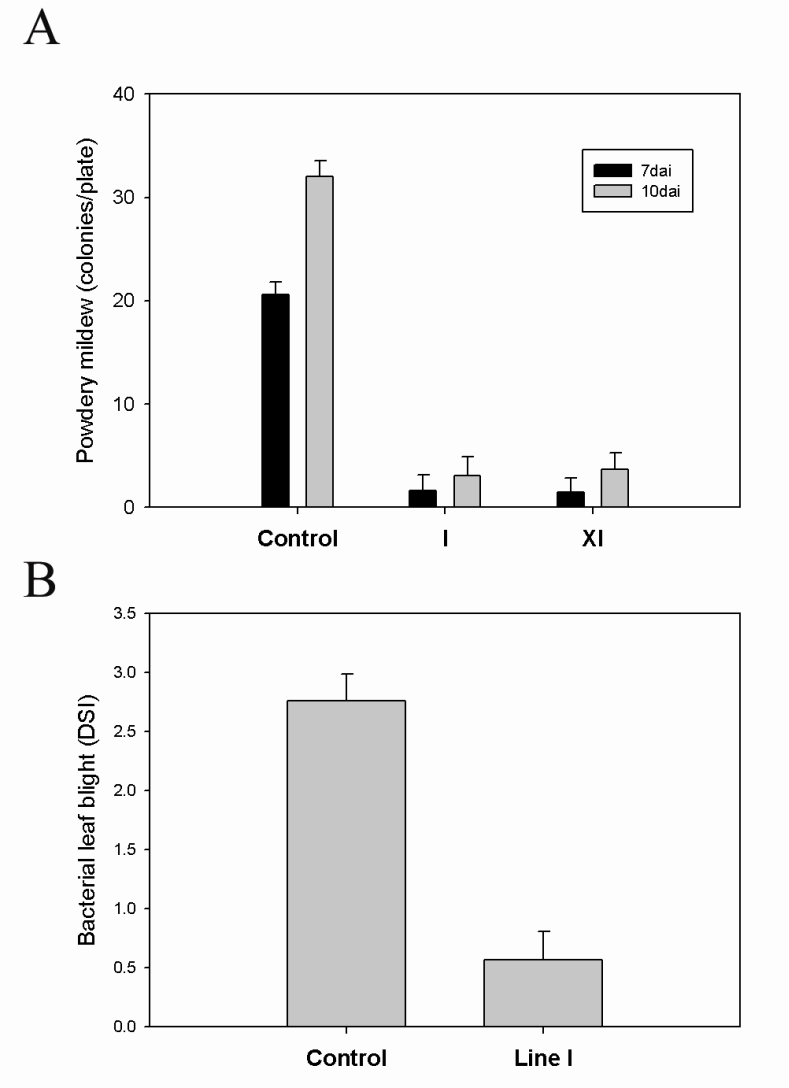

Figure 5.8 Resistance to powdery mildew (*Erysiphe heraclei*) and bacterial leaf blight (*Xanthomonas hortorum* pv. *carotae*) of *AtNPR1* transgenic carrot plants. A, **Disease resistance of the transgenic lines compared to controls on excised leaflets, inoculated with *E heraclei* spores on water agar plates, based on the number of newly formed sporulating colonies at 7 and 10 dai. B, Leaf disease severity index in response to *X. hortorum* 10 dai on greenhouse grown carrots. Vertical bars represent standard error of the mean.**

AtNPR1 expressing lines also exhibited a high level of resistance to bacterial leaf blight. Line I exhibited ~80% reduction in DSI towards *X. hortorum* compared to the controls at 10 dai (Figure 5.8B). Many of the transgenic lines were completely asymptomatic, whereas control leaves were completely chlorotic with early stages of necrosis occurring on the leaf tips (Figure 5.7E).

5.4 Discussion

In transgenic carrots constitutively over-expressing *AtNPR1*, the enhanced resistance was not correlated with a constitutive induction of SAR but rather with a more intense and longer induction of specific defence genes in the presence of phytohormones or fungal cell wall extracts. The enhanced defence gene response was similar to that seen in Arabidopsis plants over-expressing *AtNPR1*, where the transgenic plants became increasingly sensitive to the SA analogue BTH, resulting in higher production of *PR-1*(Friedrich et al. 2001). Heterologous *AtNPR1* can maintain functionality by interacting with PR gene transcription factors in other plant species (Chern et al. 2001; Lin et al. 2004; Makandar et al. 2006; Meur et al. 2008), indicating a relatively high level of evolutionary conservation within the gene. The protein similarities are seen in the characterized *NPR1*-like orthologues identified from apple (Malnoy et al. 2007), *Brassica juncea* (Meur et al. 2006), rice (Chern et al. 2005; Yuan et al. 2007) and banana (Endah et al. 2008), indicating a large degree of functional over-lap. We report for the first time the existence of a putative *NPR1*-like orthologue in roots and leaves of carrot plants.

Overexpression of *AtNPR1* in Arabidopsis, tomato and rice led to enhanced resistance to pathogens in largely a dose-dependent fashion (Cao et al. 1998; Chern et al. 2001; Friedrich et al. 2001; Lin et al. 2004). In the transgenic carrot lines I and XI, there was no significant difference in disease resistance despite a 75% higher *AtNPR1* transcript level in line XI compared to line I. The level of *AtNPR1* in line I may exceed the threshold level required to observed the enhanced resistance (Cao et al. 1998).

Depending on the plant species, over-expression of endogenous *NPR1*-like genes or *AtNPR1* resulted in alteration towards SAR responses. In rice, over-expression of *AtNPR1* or the rice orthologue *osNH1* enhanced resistance to bacterial blight (Chern et al. 2005; Chern et al. 2001). However, these transgenic plants developed spontaneous chlorotic lesions, they were hypersensitive to light, accumulated high levels of reactive oxygen species, underwent cell death and the constitutive induction of the SAR pathway (Chern et al. 2005; Fitzgerald et al. 2004). The application of exogenous phytohormones further magnified the deleterious phenotypes in these rice plants (Chern et al. 2005). Similarly, tomato plants expressing *AtNPR1* exhibited constitutive induction of many PR genes, including *PR-1*, *PR-2* and *PR-3* in a dose dependent manner relative to the level of *AtNPR1* present in the transgenic line (Lin et al. 2004). Despite the fact that there were several *PR* genes that were constitutively upregulated in the transgenic tomato plants, there were no deleterious phenotypes or reduction in plant vigour observed (Lin et al. 2004). Wheat plants expressing *AtNPR1* had no constitutive increase in *PR-1* gene expression (Makandar et al. 2006); while

tobacco plants exhibited similar increases in *PR-1* induction to that of the tomato plants (Meur et al. 2008). There were no reported deleterious phenotypes in either the transgenic wheat or tobacco, with the wheat exhibiting in a significant increase in seed yield (Makandar et al. 2006). Transgenic apple constitutively over-expressing the *NPR1* orthologue *mpNPR1* had slight increases in some PR genes, with no deleterious phenotypes (Malnoy et al. 2007). Our study showed that transgenic carrot lines had no significant constitutive increases in PR gene transcript levels, and the foliage and roots appeared phenotypically normal. Rice accumulates basal SA at levels much higher than in most other plants (Silverman et al. 1995). These high levels of endogenous SA appear to be responsible for the much higher constitutive induction of PR genes, and the deleterious phenotypes that is associated with constitutive SAR induction (Clarke et al. 1998; Heil et al. 2000).

The highest degree of resistance towards the pathogens tested in carrot lines expressing *AtNPR1* was against the obligate parasite *E*. *heraclei*, with greater than 90% reduction in the number of powdery mildew colonies (Figure 5.8A). The increase in *E*. *heraclei* resistance is higher than in a previous report of transgenic carrot expressing human lysozyme (Takaichi et al. 2000). Powdery mildews are true biotrophic pathogens (Oliver et al. 2004), which are strongly linked to SA signalling, with *NahG* and *npr1* Arabidopsis mutants being hypersensitive (Glazebrook 2005). Many bacterial pathogens are considered biotrophs (Oliver et al. 2004) since they may show *R*-gene mediated responses (Glazebrook 2005). A high level of *AtNPR1* based resistance was seen in carrot

leaves inoculated with *X. hortorum* (Figure 5.8B). Enhanced *NPR1* expression and SAR intensity could enhance the resistance towards biotrophic fungi, as shown in Arabidopsis (Cao et al. 1998; Clarke et al. 1998; Friedrich et al. 2001) and apple (Malnoy et al. 2007) or towards bacterial pathogens as seen in Arabidopsis (Cao et al. 1998), tomato (Lin et al. 2004) and rice (Chern et al. 2001) . The high level of powdery mildew and bacterial leaf blight resistance in *AtNPR1* expressing carrot plants is likely due to the more than 5 fold increase in *PR1* and PR2 transcript when treated with the SA analogue INA (Figure 5.4).

Resistance towards necrotrophic pathogens was traditionally thought to be completely independent of the SA-signalling pathways, since the *NahG* or *npr1* Arabidopsis maintained resistance to *A. brassicicola* and had no increased sensitivity to *B. cinerea* (Thomma et al. 1998). However, *NPR1* has since been identified to play a key role in the cross-talk between the SA dependent and independent pathways (Pieterse et al. 2004). The majority of the *PR*-genes examined in the *AtNPR1* expressing carrot lines were largely unaffected by MeJA treatment with the exception of PR2 transcripts that were increased up to 11-fold after MeJA treatment (Figure 5.5). The MeJA response in the transgenic lines may indicate there is functional over-lap, mediated by an *NPR1* in carrot. Necrotrophic fungal resistance has also been observed in transgenic wheat (Makandar et al. 2006), tomato (Lin et al. 2004) and apple (Malnoy et al. 2007) expressing *NPR1* genes. The degree of resistance in the *AtNPR1* carrots ranged from 30-50%, depending on the pathogen and tissue type tested. These levels of resistance are similar to those observed in tomato in response to pathogens

causing Fusarium wilt and gray leaf spot (Lin et al. 2004), and significantly lower than the observed resistance to Fusarium head blight in wheat (Makandar et al. 2006). It is difficult to compare the differences in disease reductions due to different tissue types examined and infection behaviour of the various fungal pathogens. *AtNPR1* expression in carrot did not provide complete resistance but it did significantly slow the progression of three important necrotrophic fungal pathogens, including the first observation of pathogen resistance in tap-roots of transgenic plants.

The level of *PR* gene expression, especially *PR-1* and *PR-2*, was dramatically increased in the *AtNPR1* lines compared to the controls when treated with SS-walls. Levels of *PR-2* increased to greater than 15-fold the basal transcript levels by 3 hpi and greater than 50-fold by 12 hpi, with somewhat lower fold increases in *PR-1* transcript levels (Table 5.2). The increased transcripts due to SS-walls were much stronger than with INA (Table 5.2). Application of purified and modified oligosaccharide derivatives to carrot foliage revealed stronger expression of *PR* genes than SA application alone (Jayaraj et al. 2009). Apple expressing *MpNPR1* inoculated with *Erwinia amylovora* had significant increases in *PR-2* transcript levels, by more than 5-fold basal levels with peak expression being 2.5 times greater than the peak control expression (Malnoy et al. 2007). While there have been few reports of quantification of PR gene expression between elicitor and pathogen treatments, qualitative assessment of *AtNPR1* wheat and Arabidopsis indicated a higher level of *PR-1* gene induction from fungal inoculation compared to SA analogue application (Friedrich et al. 2001;

Makandar et al. 2006). These findings further support the role that *NPR1* is involved in multiple hormonal signalling pathways involved in carrot responses towards pathogens.

The development of broad spectrum disease resistance in crop species is a major challenge using biotechnology or traditional breeding. Over-expression of *AtNPR1* led to a more intense and longer lasting defence gene response, which resulted in carrots becoming resistant towards several pathogenic fungi. Applications of chemical elicitors or fungicides have the potential to further enhance transgenic *AtNPR1* expressed based disease resistance (Friedrich et al. 2001). *AtNPR1* expressing carrots could become important breeding material for development of disease resistant carrot cultivars.

6: GENERAL DISCUSSION AND CONCLUSIONS

Increasing genetic resistance of carrots towards pathogens has the potential for alleviating one of the most limiting factors to carrot production. The use of genetic engineering to increase pathogen resistance has great potential for improving yields of carrot and many other agriculturally important plants. Distinct methods of transgene expression using a variety of promoters and range of different transgenes were investigated in the context of this thesis. Data gathered indicate that different choices of transgenes can result in vast differences in controlling pathogens exhibiting different lifestyles in transgenic carrots. The findings presented in this thesis may be useful to increase the utility of genetically engineered carrots.

In order to maximize the efficiency of transgene expression in carrots, including for enhanced pathogen resistance, a detailed comparison of promoters was required. Detailed promoter comparisons have been conducted in many different plant species, including Arabidopsis (Holtorf et al. 1995; Schledzewski et al. 1994), gladiolus (Kamo 2003) and alfalfa (Samac et al. 2004), while not in carrot. The use of GUS reporter system allowed for testing two root enhanced (*mas*2, *rol*D) and constitutive promoters (Ubiquitin, *35S*, *D35S*) in different tissues using quantitative and qualitative assays. The level of GUS expression was found to be highest in all tissue types with the constitutive promoters, despite the fact that there was considerable expression in roots and callus tissue

using both *mas*2 and *rolD* promoters. Therefore, the subsequent expression of transgenes was conducted using the ubi or *35S* promoters. While this investigation did not reveal high levels of tissue enhanced promoter activity, it did provide a quantitative assessment of several commonly available promoters used for carrot transformation. Future use of novel taproot specific promoters, isolated from carrot or similar to those found in beet (Oltmanns et al. 2006) would be useful for control of post-harvest carrot diseases. Alternatively, investigation of the promoters for leaf or chloroplast specific expression could be useful for potential control of important foliar pathogens. Discovery of novel native root or periderm specific promoters from carrot could be identified using enhancer-trapping constructs. Further modification of native promoters could be achieved through stacking the native enhancer elements or adding elements from promoters including CaMV *35S* (Venter 2007).

While there have been several reports of transgenic plants over-expressing chitinases and glucanases with varying degrees of success, we co-expressed a putatively highly antifungal chitinase (383) and glucanases (638) from wheat (Anand et al. 2003; Li et al. 2001) to determine if synergistic inhibition of fungal growth occurred in carrot. Carrot lines expressing 383 exhibited enhanced resistance to *B. cinerea* and *S. sclerotiorum* in a dose dependent manner, whereas 638 expression maintained susceptibility. When co-expressed. the plants exhibited resistance similar to that of 383 expression alone, indicating either insufficient glucanase expression or lack of functionality against the pathogens tested. Results obtained using the 383 and 638 genes were largely

unsuccessful, as they did not achieve agriculturally significant levels of pathogen resistance. The use of endogenous chitinases and glucanases from carrot may lead to improved resistance, as they can further augment natural basal defences.

In addition, we investigated the effect of over-expression of a putative lignin peroxidase from rice (*POC1*) (Young et al. 1995). *POC1* transgenic lines exhibited high levels of resistance towards necrotrophic fungal pathogens and exhibited heightened apoplastic peroxidase activity. No increased resistance towards powdery mildew was observed, indicating alternative mechanisms of resistance initiated through POX between biotrophs and necrotrophs. When co-expressed with 383, the resistance did not exceed that of *POC1* when it was expressed alone. Increased lignin levels alone may have contributed to basal resistance. Interestingly, *POC1* lines exhibited enhanced activation of defence responsive genes, while exhibiting only low basal increases of these *PR*-genes. Further testing of lines in the field, examining foliar and root expression of defence genes, under various conditions including pathogen stress or application of elicitors (such as alexin, chitosan or BTH) could provide further information towards understanding the mechanisms of the observed enhanced resistance. Inhibitor application (NaN_3, KCN or L-cysteine) resulted in decreased defence gene responses, although the levels of response were still elevated compared to the control lines. Unfortunately, most available peroxidase inhibitors including, NaN_3 are non-specific, altering the function of many other proteins and could lead to some secondary effects. A feasible solution involves using a reverse genetics approach, be it in carrot or a model plant system, to create lines with a

variety of peroxidase levels to ascertain the effect of peroxidases on lignin levels and pathogen susceptibility.

Much of the increased resistance towards necrotrophic pathogens may be attributed to the modification of the oxidative burst. These *POC1* expressing lines accumulated far less H_2O_2 following inoculation with *S. sclerotiorum* cell walls, in addition to rapid removal of exogenously applied H_2O_2. These findings were relatively unique, since most studies involving POX over-expression lead to enhanced accumulation of H_2O_2 (Bindschedler et al. 2006). Potentially, the heightened levels of extracellular POX counteract for necrotrophic pathogen's virulence mechanism of POX suppression as proposed by von Tiedemann (1997). Different types of POXs function in vastly different mechanisms, with little to do with the physical structure of the enzyme. POXs that operate primarily in the hydroxylic cycle serve to generate ROS, and when over expressed will lead to heightened ROS accumulation, HR and enhanced resistance to biotrophic pathogens (Bindschedler et al. 2006; Choi et al. 2007; Kim et al. 2008b). Whereas, POXs involved primarily in the peroxidative cycle will tend to remove ROS, decrease HR and have increased resistance towards necrotrophic pathogens (Chassot et al. 2007; Coego et al. 2005a). Logic would dictate antagonistic relationships with over-expression of hydroxylic POXs having heightened susceptibility to necrotrophs and peroxidative POXs with hypersensitivity to biotrophs. Despite this obvious statement, relatively little research has been conducted on this relationship. The data presented here represents the first examination of both necrotrophic and biotrophic pathogen

resistance in transgenic plants over-expressing a POX. The lack of hypersensitivity can be attributed to powdery mildew growing mainly on the surface with only moderate cellular penetration; testing of additional biotrophic pathogens would further solidify this hypothesis. Further research to understanding the relationship between POXs and ROS in respect to both necrotrophic and biotrophic pathogens could shed new insight into the complicated infection mechanisms which exist.

Expression of master control switches, such as *NPR1*-like genes that serve to augment the plants innate defence responses, are a logical target to enhance disease resistance (Gurr et al. 2005b). To test this hypothesis in carrot, we constitutively over-expressed *AtNPR1*, resulting in two independent lines. These plants responded with a more intense and longer lasting defence response when inoculated with SS-walls or with the SA analogue INA. Data collected indicate evolutionary similarity between the *AtNPR1* and a yet undiscovered carrot *NPR1*-like gene. Observed responses were consistent with "priming" through accumulation of cytosolic *NPR1*, and activation when contacted with SA (Cao et al. 1998). Interestingly, these lines were highly resistant to necrotrophic and biotrophic fungal pathogens on both the taproot and the foliage, as well as highly resistant to bacterial blight. This broad-spectrum resistance is rarely observed, confirming functionality of *NPR1* in necrotrophic as well as classic biotrophic interactions. Further investigation is needed to determine if the enhanced *AtNPR1* necrotrophic resistance also occurs in systemic tissue, or if it is simply involved in SA-mediated local resistance similar to that reported in

Arabidopsis (Ferrari et al. 2003). Additional research is required to identify and characterize the carrot *NPR1*-like gene, to determine functional overlap between *AtNPR1* or determine if carrot *NPR1* is a better target gene for over-expression. Friedrich et al. (2001) demonstrated that application of fungicides or elicitors (both chemical and oligosaccharide) could further augment the effectiveness of *NPR1* over-expression. Further investigation with elicitors could potentially result in extremely disease tolerant carrot lines.

Development of a transgenic-based strategy to control fungal and bacterial diseases in carrot was investigated throughout the context of this thesis. Results indicated that the use of constitutive promoters was required to drive high levels of gene expression in the tissues of interest. The over-expression of chitinase and β-1, 3 glucanase proteins was relatively ineffective alone or in combination at reducing fungal diseases. Over-expression of a rice peroxidase gene led to high levels of necrotrophic fungal resistance, through a combination of enhanced PR gene expression, increased lignin accumulation and detoxifying ROS. Expression of a master control switch *NPR1*, resulted in high levels of broad-spectrum resistance not previously seen in carrots. The ultimate goal of this study was to investigate potential target genes and promoters to increase disease resistance in carrot, with the aim of leading to commercialized carrot cultivars. Results from *POC1* over-expression still require additional determination of precise mechanisms of pathogen resistance. However, *NPR1* expression appears to be an excellent candidate gene for control against a wide range of carrot pathogens.

APPENDICES

Appendix A Carrot transformation protocols

This appendix was published in: Wally O., Jayaraj J, Punja ZK. (2006) Carrot (*Daucus carota. L*). In: K.Wang, ed *Agrobacterium* protocols Volume 2. Humana Press Totowa, NJ.:,pp. 3-12

1. Introduction

Carrot (*Daucus carota* L. subsp. datives), a member of the family *Apiaceae*, is grown worldwide for its edible taproot, which provides a source of vitamin A and fibre in the diet. One of the greatest challenges to carrot production is the management of fungal diseases. There are a number of widespread pathogens that destroy the foliage and roots, thereby reducing quality and yield. Genetic resistance to fungal pathogens is lacking in most commercially grown carrot cultivars in use today.

The utility of genetic engineering approaches to enhance resistance of plants to a range of fungal pathogens has been reviewed (Punja 2001), and can provide an opportunity to enhance disease resistance in carrot. Our current research involves the expression of a number of pathogenesis-related (PR) proteins with anti-fungal activity (chitinases, thaumatin-like proteins (TLP), peroxidases and β-1, 3 glucanases) in carrot tissues and assessing their effects on fungal pathogen development in vivo. We describe the protocols for engineering carrot plants to express TLP and the use of the bar gene, encoding

for phosphinothricin acetyltransferase for herbicide resistance, as a selectable marker (Chen and Punja 2002). The methods used for assessing transgenic plants for resistance to fungal pathogens are described elsewhere (Punja 2005).

2. Materials

2.1. Media and hormone solutions

1. Murashige and Skoog (MS) medium(Murashige and Skoog 1962) (containing 332.16 mg/l $CaCl_2$, 0.025 mg/l $CoCl_2 \cdot 6H_2O$, 0.025 mg/l $CuSO_4 \cdot 5H_2O$, 27.8 mg/l $FeSO_4 \cdot 7H_2O$, 6.2 mg/l H_3BO_3, 170 mg/l KH_2PO_4, 0.83 mg/l KI, 1,900 mg/l KNO_3, 370 mg/l $MgSO_4 \cdot 7H_2O$, 16.9 mg/l $MnSO_4 \cdot H_2O$, 37.3 mg/l $Na_2 \cdot EDTA$, 0.25 mg/l $Na_2MoO_4 \cdot 2H_2O$, 1,650 mg/l NH_4NO_3 and 8.6 mg/l $ZnSO_4 \cdot 7H_2O$. Supplemented with 2 mg/l Glycine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine-HCl, 0.1 mg/l thiamine-HCl.), full and half- strength, in liquid and solid form, supplemented with various concentrations of 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 0.3 % w/v sucrose. MS1D is supplemented with 1 mg/l 2, 4-D, MS (1/2) D is supplemented with 0.5 mg/l 2, 4-D and MS (1/4) D is supplemented with 0.25 mg/l 2, and 4-D. Media are used for tissue culture, plant transformation, and plantlet regeneration. The pH is adjusted to 5.8 with 1 M KOH; phytagel (2.5 g/L) is added prior to autoclaving (*see* **Notes 1-4**).
2. Yeast extract-mannitol (YM) medium (for growth of *Agrobacterium tumefaciens*): 0.04% yeast extract, 1% mannitol, 1.7 mM NaCl, 0.8 mM

$MgSO_4$ and 2.2 mM K_2HPO_4. The pH is adjusted to 7.2 with 1 M NaOH prior to autoclaving. Used as a liquid or agar medium (15 g/L Bacto-agar).

3. 2,4-D stock solution 0.5 mg/ml: 50 mg of 2,4-D dissolved in 5 ml ethanol and gradually diluted to 100 ml with H_2O and stored at 4 ºC in dark for up to 3 months. 2,4-D is added to media prior to autoclaving.
4. Acetosyringone stock solution (0.1M): 1 g acetosyringone is dissolved into 50 ml DMSO and stored at 4ºC in foil-wrapped containers. It crystallizes at this temperature but will turn to liquid again once returned to room temperature; 2 µl of this stock added to 1 ml of bacterial medium or 2 ml stock to l L of co-cultivation medium would give a final concentration of 200 µM.
5. Antibiotic stock solutions: Kanamycin sulphate (Sigma) (100 mg/ml), streptomycin sulphate (Sigma) (200 mg/ml) and timentin (30:1 ticarcillin: clavulanic acid 300mg/ml, SmithKline Beecham). All antibiotics are dissolved in H_2O and filter-sterilized through 0.22 µm filters, and stored at –20ºC.
6. DL- phosphinothricin stock solution: 250 mg of DL- phosphinothricin (RPI Research Products International Corp. IL) is dissolved in 25 ml of H_2O, filter-sterilized through 0.22 µm filters, and stored at –20 ºC.
7. Liberty Solution: The herbicide Liberty (Aventis, Saskatoon, Saskatchewan) is diluted in H_2O to 0.2- 0.4% (w/v) and used as an aerosol or painted on the leaf surface using a cotton swab.

8. Potting medium: Soil mix 4 (Sunshine, Surrey, British Columbia), containing 55-60% Canadian Sphagnum peat moss, perlite, dolomitic limestone (for pH adjustment) and gypsum.

2.2. Carrot cultivars

1. Open-pollinated carrot cultivars evaluated: Nantes Coreless, Danvers Half-long, Nanco, Golden State, Scarlet Nantes, and the experimental high beta-carotene producing HCM line. HCM seeds were donated by Dr. Phil Simon (USDA, University of Wisconsin, Madison, WI), while the other lines were purchased at a local wholesale seed supplier.

2.3. **Agrobacterium tumefaciens** ***strain and binary vector***

1. *A. tumefaciens* LBA 4404 Electromax cells (Invitrogen): The strain contains the disarmed Ti plasmid pAL 4404 which contains only the *vir* and ori region of the Ti plasmid.
2. Binary vector pCambia-bar-TLP: The construct contains 2 selectable marker genes, bar and hpt, driven by the CaMV 35 S promoter, and a rice tlp gene (1 kb) (***7***) driven by the maize ubiquitin promoter (2 kb). The ubiquitin-tlp fragment (3 kb) can be released through digestion of the entire vector with *Hind*III.

2.4. DNA extraction specifically for carrot tissue

Mature carrots may have high levels of phenolic and other compounds that exist at sufficient levels to inhibit PCR amplifications of the DNA samples. Therefore, specialized DNA extraction techniques have been developed, to

minimize the levels of potential inhibitors while still maintaining high yield and quality of genomic DNA.

1. Extraction buffer: 2 % (w/v) cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 20 mM ethylene diaminetetra-acetic acid (EDTA), 100 mM Tris-HCl and 5 % (w/v) polyvinylpyrrolidone (PVPP), pH 8.0, stored at room temperature.
2. Phenol:chloroform:isoamyl alcohol: buffer saturated phenol, pH 8.0 (Fisher) is mixed with 0.1% (w/v) hydroxyquinoline and mixed with an equal volume of chloroform: isoamylalcohol (24:1), stored away from light at 4º C.
3. TE buffer: 10 mM Tris-HCl and 1 mM EDTA, pH 8.0, autoclaved at 15 psi for 15 min.

2.5. Polymerase chain reaction

1. 10X buffer: 200 mM Tris-HCl (pH 8.4), 500 mM KCl,
2. 50 mM $MgCl_2$ (Invitrogen)
3. 10 mM dNTPs (equal volumes of individual 100 mM dNTP (Invitrogen) stocks are mixed and diluted 10 fold with H_2O. Diluted stocks are aliquated into small volumes and stored a -20 °C) ,
4. Primers for amplifying the TLP gene sequence:
    a. Forward primer: tlpF 5’-AACAGGTGCCAGTACACGGTGT-3’
    b. Reverse primer: tlpR 5’-CACGGTTACATCCACACATGCA-3’
5. Primers for amplifying the bar gene sequence:
    a. Forward primer: BarF 5’-ACTGGGCTCCACGCTCTAC-3’

b. Reverse primer: BarR 5'- GAAGTCCAGCTGCCAGAAAC-3'

6. Recombinant Taq DNA polymerase (5U/µl, Invitrogen)
7. The PCR machine used was a GeneAmp PCR system 9700 (Perkin-Elmer Corporation, Norwalk, Conn.).

3. Methods

3.1. Sterile carrot tissue

1. Seeds should be soaked overnight in H_2O at 4ºC, and then washed twice with H_2O and once for 5 min with 70% ethanol. The seeds are then soaked in 1% (v/v) NaOCl with a drop of Tween 20 for 15 minutes and washed 4 times with sterile H_2O.
2. Sterilized seeds are blotted dry on sterile filter paper and approximately 10-15 seeds are placed in Magenta boxes containing 50 ml of half-strength MS medium supplemented with 0.3% sucrose and incubated for 4-6 weeks at room temperature (22^oC) under cool white fluorescent lights (450 µmol/m^2/s, 16 h day). Once the plants reach a size of 15-20 cm (6 weeks) they are used as source material for transformations.

3.2 Agrobacterium *cultures*

1. LBA 4404 electrocompetent cells are thawed on ice and 20 µl added to 100 ng of pCambia- bar-TLP binary vector and mixed by swirling with the pipette tip. The mixture is added to a 0.1 cm cuvette and electroporated at 2 kV, 200 Ω and 25 µF.

2. Following electroporation, 1 ml of YM broth is added, and the mixture removed and transferred to a round-bottomed 15 ml Falcon tube.
3. The contents are agitated on a rotary shaker at 225 rpm and 30°C for 3 hours, the cells are then diluted 1:10 with fresh YM and are plated on solid YM medium containing 100 mg/l streptomycin and 50 mg/l kanamycin.
4. Plates are incubated for 56 h at 30°C. Single colonies are isolated and inoculated to fresh YM media.
5. The presence of the binary vector in *Agrobacterium* is confirmed by plasmid mini-preparation of *Agrobacterium* followed by restriction enzyme digestion and gel electrophoresis.
6. For carrot transformation, a single colony of LBA 4404 containing the plasmid pCambia-bar/TLP is used to inoculate 50 ml liquid YM supplemented with 100 mg/l streptomycin and 50 mg/l kanamycin, and is grown overnight on a rotary shaker (250 rpm) at 30°C.
7. Cultures are centrifuged at 3,000 g at room temperature for 15 min. The supernatant is removed and the pellet is resuspended to a density of OD_{600} = 0.3 (corresponding to approximately 1 x 10^8 cells/ml) in 1/10^{th} MS media supplemented with 200 µM acetosyringone, 2 % sucrose and 1% glucose.

3.3 Transformation

1. Sterile petioles are cut into 5-10 mm long segments using a scalpel and incubated in the *Agrobacterium* suspension in an empty petri plate for 10 to 30 min with gentle shaking. The suspension is then drained and the

carrot explants are blotted dry on a sterile filter paper and placed onto MS1D. Approximately 20-30 explants are placed on a 9 cm Petri plate and co-cultivated in the dark for 2-3 days (*see* **Note 5**).

2. Infected explants are subsequently rinsed in sterile H_2O, blotted dry and placed on selective MS1D medium containing 1 mg/L PPT and 300 mg/L

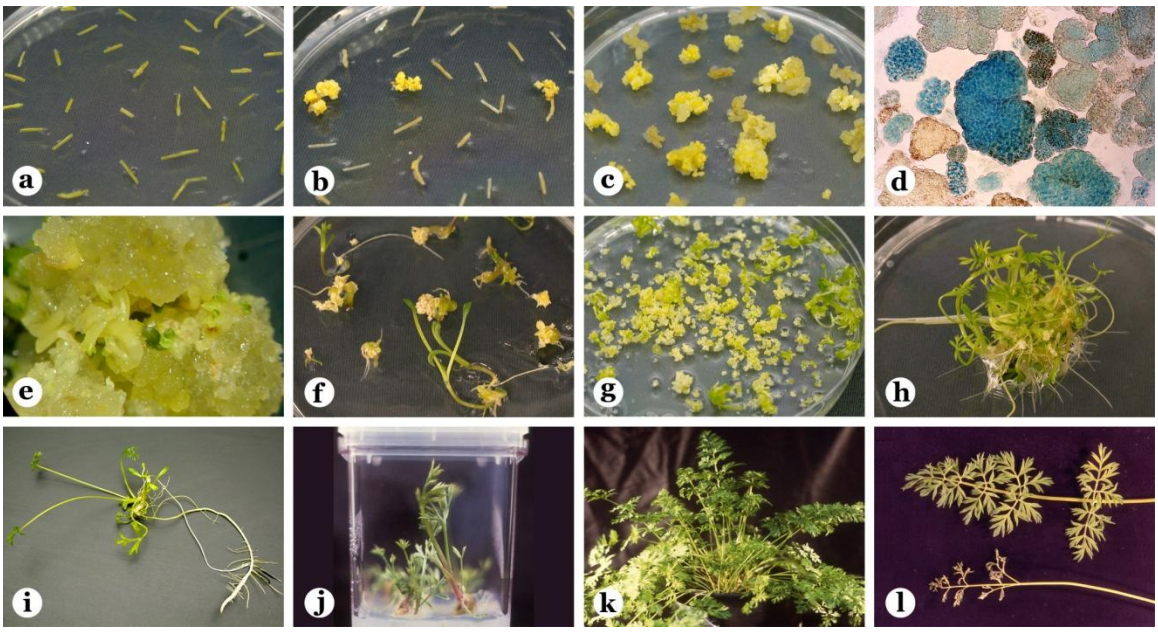

Figure A1: Transformation procedure and development of transgenic carrots. (A.) Petiole explants following infection with A. tumefaciens and transfer to MS1D supplemented with 1 mg/l PPT. (B.) Callus development from explants on MS (1/2)D medium supplemented with 10 mg/l PPT, 6 weeks post infection with Agrobacterium. (C.) Callus growth and early formation of somatic embryos on MS(1/2)D supplemented with 10mg/l PPT, 12 weeks post infection. (D.) Callus fixed and displaying GUS positive phenotype. (E.) Somatic embryo formation and the start of plantlet regeneration on MS with 10 mg/l PPT, 14 weeks post infection. (F.) Plantlet regeneration from somatic embryos on MS with 10 mg/l PPT, 18 weeks post infection (G.) Plating of suspension cultures containing somatic embryos on MS with 10mg/l PPT, 3 weeks after plating. (H.) Cluster of carrot plantlets on MS with 10 mg/l PPT. (I.) Plantlet removed from a Magenta box. (J.) Plantlet showing root development growing in a Magenta box on MS with 10 mg/l PPT. (K.) Mature carrot plant, potted in soil and growing vigorously. (L.) Transgenic carrot leaf treated with 0.4% Liberty solution (top) and control plant leaf (bottom). Photographs are of 9 cm diameter Petri dishes used for tissue culture.

timentin (Fig. A1a). Positive and negative controls are included. Positive controls are placed on medium lacking PPT while negative controls are non-infected with bacteria and placed on selective medium. Incubated at room temperature (22^{o}C) under cool white fluorescent lights (450 µmol/m^{2}/s, 16 h day), all subsequent steps are incubated under these same conditions.

3. Explants are transferred to higher selection after 2 weeks, using MS(1/2)D medium containing 10 mg/l PPT and 300 mg/l timentin. Explants are maintained on this medium, with transfers every 4 weeks, until calli develop (Fig. A1b). Somatic embryos typically begin to appear 8 to 12 weeks following infection. Non-embryogenic callus are maintained for up to 20 weeks and discarded at that time if embryos have not developed (Fig. A1c).
4. Somatic embryos (Fig. A1e) are transferred to hormone-free MS medium containing 10 mg/L PPT and 300 mg/L timentin for regeneration (Fig A1f). Alternatively, the embryogenic calli are transferred into conical flasks (250 ml) containing 50 ml of liquid MS1/4D (50 ml) with 5 mg/L PPT and 150 mg/L timentin for initiating suspension cultures.
5. The embryos are subcultured to fresh medium every 2 weeks. The embryos are transferred every month onto hormone-free MS with selection for regeneration (Fig. A1g).
6. The tiny plantlets or small shoots are transferred to Magenta boxes containing hormone-free MS medium plus 10 mg/L PPT and 300 mg/L

timentin (Fig. A1h). Roots are induced quickly after transfer of shoots to larger containers, if they are not present prior to the initial transfer (Fig. A1i).

7. The fully rooted plantlets are transferred to potting medium in 9cm X 9cm pots and placed in a growth chamber maintained at 26° C, 85% relative humidity and a 16h photoperiod **(**450 µmol/m^2/s) where they grow into full plants (Fig. A1k).
8. Once the plants reached a size greater than 15 cm, they are tested for resistance to the herbicide Liberty. Leaves of carrot plants are painted with a 0.2 or 0.4% (w/v) Liberty solution and visually assessed for resistance (Fig. A1l) (*see* Note 6). Visually healthy leaf tissue was collected from plants at this stage for PCR, Southern, Northern and Western analysis.
9. Effect of carrot cultivar on transformation frequency was measured as a proportion of infected explants that developed into individual Southern positive plants that grew on herbicide selection medium (*see* Note 7).
10. Once the transgenic carrots are larger than 20 cm in height, they are transferred to 18 cm diameter pots and grown to maturity under the same conditions. The plants are watered 2 times weekly (or as needed) and fertilized once a week with a 20:20:20 fertilizer (500 mg/l). Pest problems are primarily from western flower thrips (*Frankliniella occidentalis* (Perg.)), which are difficult to control (*see* Note 8).
11. Carrots are a biennial plant and flower after their second growing season. To induce vernalization, the carrot plants at the 7-8 leaf stage are placed

at 8°C for 10 weeks (Dugdale et al. 2000). The dead and dying foliage is removed and the plants are transferred back to the previous growing conditions. When flowering shoots are produced, the individual heads are encased in a glassine bag with ‘blow flies’ and tied off to prevent insect escape . Seeds are then removed after 3 -6 weeks and stored desiccated at room temperature (Dugdale et al. 2000).

3.4. Molecular analysis of transformants

1. Total genomic DNA is isolated from rooted plantlets using a protocol modified from Zang et al. (1998). A single plantlet or a leaflet, approximately 50 mg fresh weight, is ground with a mortar and pestle under liquid nitrogen with a pinch of sea sand.
2. The macerated plant tissue is transferred to a 1.5 ml microcentrifuge tube to which 500 µl of pre-warmed (60°C) extraction buffer and 5 µl of DTT stock are added. The mixture is vortexed for 30s and incubated at 60°C for 60 min.
3. Following incubation, 500 µl of cold phenol:chloroform:isoamylalcohol is added and mixed by inversion and centrifuged at 7,500 g for 15 min.
4. The aqueous phase is removed and transferred to a fresh 1.5 ml tube, to which 500 µl of chloroform:isoamylalcohol is added, mixed and centrifuged at 7,500 g for 10 min.
5. The aqueous phase is transferred to another fresh 1.5 ml tube to which 250 µl of cold isopropanol is added, mixed and incubated at -20° C for no more than 30 min.

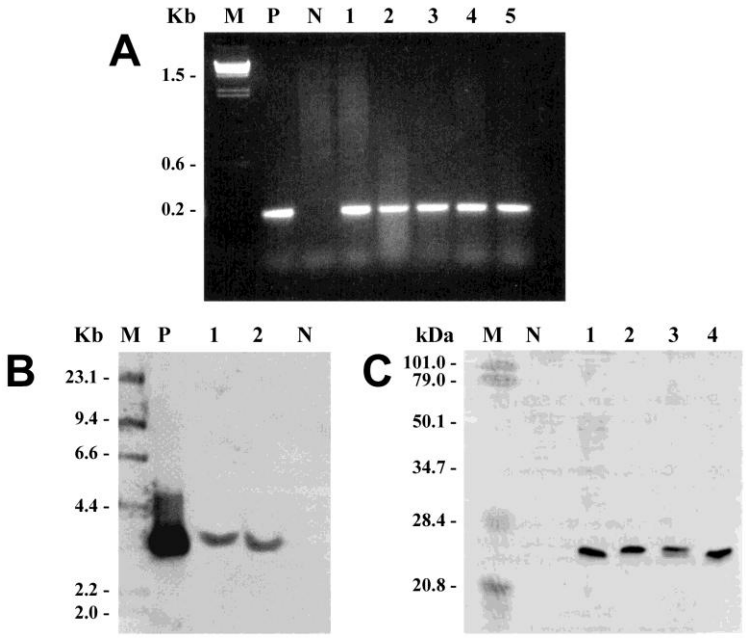

Figure A2. Detection of transgenes in calli and plantlets by PCR analysis and Southern analysis and detection of TLP protein by Western analysis. (A) PCR analysis for the bar gene in transgenic tissues produced a band of 202 bp, M is molecular weight markers, P is positive control plasmid DNA, N is non-transformed control carrot DNA, lanes 1-5 are sample lanes. (B) Southern analysis. Plant DNA extracts and plasmid controls were digested with Hind III and probed with PCR-amplified TLP coding region. (C) Western blot. Each lane was loaded with 25 μg protein, and probed with anti-TLP antibody. M, molecular weight markers; N, non-transformed tissue protein, transformed tissues showing a 23 kDA band.

6. The mixture is then centrifuged at 18,000 g for 15 min and the supernatant drained, the pellet washed with 500 µl of 70% ethanol and re-centrifuged at 18,000 g for 5 min.
7. The pellet is then drained and allowed to air dry before being resuspended in 50 µl of TE.
8. The DNA is quantified after treating the sample with 5 µl RNAse A and incubating at room temperature for 1 hour, at which point the OD_{260} is measured using a spectrophotometer. Once quantified, the remaining DNA can be stored at -20° C.
9. PCR: each reaction in 25 µl contains 100 ng of carrot DNA, 50 pM primers, 1 unit of Taq polymerase, and 1.5 mM $MgCl_2$ in 1 X PCR buffer. The annealing temperature is 66°C for TLP and 35 cycles of 94°C for 30s, 66°C for 50s and 72°C for 90s followed by a 5 min 72°C final extension.
10. 10 µl of the PCR product is run on a 1% agarose TAE gel and stained with ethidium bromide. The tlp PCR using the primers tlpF/tlpR produces a fragment of 691 bp, while the bar PCR using the primers barF/barR produces a fragment of 202 bp (Fig A2.a).
11. Southern blotting is performed to identify unique transformation events; using 10 µg of total genomic DNA digested with Hind III, run on 1% agarose gel, transferred to a nylon membrane and hybridized using

standard protocols with specifically designed bar and TLP DNA probe (Fig A2.b).

12. Western blotting is performed to ensure recombinant protein expression; using 200 μg of total proteins extracted, run on polyacrylamide gel, transferred a nylon membrane and probed with anti-bar and anti-TLP rabbit antibodies using standard protocols (Velazhahan et al 1998) (Fig A2.c).

4. Notes

1. For sterilization, all water, media, and instruments are autoclaved at 15 psi for 15 min. A bead sterilizer is used for sterilization of instruments used in transformations and for callus transfer.
2. All water used in these experiments has a resistance of greater than 18 MΩ -cm and minimal organic content, referred to as H_2O.
3. Media performed the best when allowed to dry in the laminar flow hood for at least 24 h after pouring. This drying step reduced the amount of condensation formed on the plates and resulted in reduced fungal contamination.
4. Antibiotics and DL- PPT solutions are added to media after they are cooled to approximately 50 °C, and swirled to mix. 2,4-D can be added prior to sterilization as it is thermo-stable.
5. Carrot petioles are dissected on top of sterile filter paper and cut into the appropriate sizes. This alleviated some of the difficulties associated with

the petioles dehydrating and sticking to smooth surfaces, such as a petri plate. Following dissection of each petiole (10-25 explants), they are immersed in the *Agrobacterium* solution. This caused some explants to have longer exposure to the inoculum; however, it was necessary to avoid the suberization of wound sites.

6. Some of the PPT-resistant plants still show some susceptibility to the 0.4% Liberty application; however, it much less than the control plants. The minimum lethal concentration of Liberty to each plant needs to be ascertained by a preliminary experiment. The concentrations range between 0.1 to 0.4%. Cotton swabs are used to gently paint the herbicide solution over the leaf surface pre-marked with a water-proof ink or marker pen. Typical phytotoxicity symptoms developed after 7 days.
7. There are significant differences in the transformation frequency depending on the cultivar selected. Danvers Half-long and Nantes Coreless have efficiencies greater than 3%, Nanco and HCM cultivars have efficiencies of less than 1% (Chen and Punja 2002). These efficiencies are the number of independent Southern-positive events from 100 explants.
8. Insect pests on carrot plants can be mimized with weekly applications of “Safer” insecticidal soap, according manufacturer’s instructions. In severe cases where thrips are visually detected, applications of active *Amblyseius cucumeris*, a predatory mite grown on a bran flake medium, are applied directly to infested carrot plants.

REFERENCE LIST

Ahn IP, Lee, S. W.,Suh, S. C. (2007). Rhizobacteria-induced priming in Arabidopsis is dependent on ethylene, jasmonic acid, and *NPR1*. Mol Plant Microbe Interact 20: 759-768

Alberts,B., Johnson,A., Lewis,J., Raff,M., Robers,K., and Walter,P. (2002) Molecular biology of the cell.Garland Science New York .

Almagro L, Ros, L. V. G., Belchi-Navarro, S., Bru, R., Barcelo, A. R.,Pedreno, M. A. (2009). Class III peroxidases in plant defence reactions. J Exp Bot 60: 377-390

Altpeter F, Varshney, A., Abderhalden, O., Douchkov, D., Sautter, C., Kumlehn, J., Dudler, R.,Schweizer, P. (2005). Stable expression of a defence-related gene in wheat epidermis under transcriptional control of a novel promoter confers pathogen resistance. Plant Mol Biol 57: 271-283

Ammirato PV (1986) Carrot. In: D.A.Evans, W.R.Sharp, and P.V.Ammirato, eds Handbook of plant cell culture. MacMillian New York:,pp. 457-499

Anand A, Zhou, T., Trick, H. N., Gill, B. S., Bockus, W. W.,Muthukrishnan, S. (2003). Greenhouse and field testing of transgenic wheat plants stably expressing genes for thaumatin-like protein, chitinase and glucanase against *Fusarium graminearum*. J Exp Bot 54: 1101-1111

Asselbergh B, Curvers, K., Franca, S. C., Audenaert, K., Vuylsteke, M., Van Breusegem, F.,Hofte, M. (2007). Resistance to *Botrytis cinerea in sitiens*, an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis. Plant Physiol 144: 1863-1877

Attaran E, Zeier, T. E., Griebel, T.,Zeier, J. (2009). Methyl Salicylate Production and Jasmonate Signalling Are Not Essential for Systemic Acquired Resistance in Arabidopsis. Plant Cell 21: 954-971

Auh CK,Murphy, TM. (1995). Plasma-Membrane Redox Enzyme Is Involved in the Synthesis of O_2- and H_2O_2 by Phytophthora Elicitor-Stimulated Rose Cells. Plant Physiol 107: 1241-1247

Baker CJ, Deahl, K., Domek, J.,Orlandi, E. W. (2000). Scavenging of H_2O_2 and production of oxygen by horseradish peroxidase. Arc Bioch Biophys 382: 232-237

Baranski R, Klocke, E.,Nothnagel, T. (2007). Enhancing resistance of transgenic carrot to fungal pathogens by the expression of *Pseudomonas fluorescence* microbial factor 3 (MF3) gene. Physiol Molec Plant Pathol 71: 88-95

Baranski R, Klocke, E.,Nothnagel, T. (2008). Chitinase CHIT36 from *Trichoderma harzianum* enhances resistance of transgenic carrot to fungal pathogens. J Phytopathol 156: 513-521

Bari R,Jones, J. (2009). Role of plant hormones in plant defence responses. Plant Mol Biol 69: 473-488

Belbahri L, Boucher, C., Candresse, T., Nicole, M., Ricci, P.,Keller, H. (2001). A local accumulation of the *Ralstonia solanacearum* PopA protein in transgenic tobacco renders a compatible plant-pathogen interaction incompatible. Plant J 28: 419-430

Bestwick CS, Brown, I. R., Bennett, M. H. R.,Mansfield, J. W. (1997). Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae pv phaseolicola*. Plant Cell 9: 209-221

Bestwick CS, Brown, I. R.,Mansfield, J. W. (1998). Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce. Plant Physiol 118: 1067-1078

Bindschedler LV, Dewdney, J., Blee, K. A., Stone, J. M., Asai, T., Plotnikov, J., Denoux, C., Hayes, T., Gerrish, C., Davies, D. R., Ausubel, F. M.,Bolwell, G. P. (2006). Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. Plant Journal 47: 851-863

Bindschedler LV, Minibayeva, F., Gardner, S. L., Gerrish, C., Davies, D. R.,Bolwell, G. P. (2001). Early signalling events in the apoplastic oxidative burst in suspension cultured French bean cells involve cAMP and Ca^{2+}. New Phytologist 151: 185-194

Bliffeld M., Mundy, J., Potrykus, I.,Futterer, J. (1999). Genetic engineering of wheat for increased resistance to powdery mildew disease. Theor App Gene 98: 1079-1086

Bolton MD., Thomma, BPHJ.,Nelson, BD. (2006). *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. Molec Plant Pathol 7: 1-16

Bolwell GP. (1999). Role of active oxygen species and NO in plant defence responses. Curr Opin Plant Biol 2: 287-294

Bolwell GP., Bindschedler, LV., Blee, KA., Butt, VS., Davies, DR., Gardner, SL., Gerrish, C.,Minibayeva, F. (2002). The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. J Exp Bot 53: 1367-1376

Bolwell GP., Davies, DR., Gerrish, C., Auh, CK.,Murphy, TM. (1998). Comparative biochemistry of the oxidative burst produced by rose and French bean cells reveals two distinct mechanisms. Plant Physiol 116: 1379-1385

Bolwell GP.,Wojtaszek, P. (1997). Mechanisms for the generation of reactive oxygen species in plant defence - a broad perspective. Physiol Molec Plant Pathol 51: 347-366

Bouche FB., Marquet-Blouin, E., Yanagi, Y., Steinmetz, A.,Muller, CP. (2003). Neutralising immunogenicity of a polyepitope antigen expressed in a transgenic food plant: a novel antigen to protect against measles. Vaccine 21: 2065-2072

Brodersen P., Petersen, M., Nielsen, HB., Zhu, SJ., Newman, MA., Shokat, KM., Rietz, S., Parker, J.,Mundy, J. (2006). Arabidopsis MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via *EDS1* and *PAD4*. Plant J 47: 532-546

Campbell MA., Fitzgerald, HA.,Ronald, PC. (2002). Engineering pathogen resistance in crop plants. Transgenic Res 11: 599-613

Cao H., Bowling, SA., Gordon, AS.,Dong, XN. (1994). Characterization of an arabidopsis mutant that is nonresponsive to inducers of systemic acquired-resistance. Plant Cell 6: 1583-1592

Cao H., Glazebrook, J., Clarke, JD., Volko, S.,Dong, XN. (1997). The Arabidopsis *NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell 88: 57-63

Cao H., Li, X.,Dong, XN. (1998). Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. Proc Nat Acad Sci USA 95: 6531-6536

Cessna SG., Sears, VE., Dickman, MB.,Low, PS. (2000). Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. Plant Cell 12: 2191-2199

Chassot C., Nawrath, C.,Metraux, JP. (2007). Cuticular defects lead to full immunity to a major plant pathogen. Plant J 49: 972-980

Chaturvedi R., Krothapalli, K., Makandar, R., Nandi, A., Sparks, AA., Roth, MR., Welti, R.,Shah, J. (2008). Plastid omega 3-fatty acid desaturase-dependent

accumulation of a systemic acquired resistance inducing activity in petiole exudates of *Arabidopsis thaliana* is independent of jasmonic acid. Plant J 54: 106-117

Chen WP.,Punja, ZK. (2002). Transgenic herbicide- and disease-tolerant carrot (*Daucus carota L.*) plants obtained through Agrobacterium-mediated transformation. Plant Cell Reports 20: 929-935

Chern M., Fitzgerald, HA., Canlas, PE., Navarre, DA.,Ronald, PC. (2005). Overexpression of a rice *NPR1* homolog leads to constitutive activation of defence response and hypersensitivity to light. Molec Plant-Microbe Inter 18: 511-520

Chern MS., Fitzgerald, HA., Yadav, RC., Canlas, PE., Dong, XN.,Ronald, PC. (2001). Evidence for a disease-resistance pathway in rice similar to the *NPR1*-mediated signalling pathway in Arabidopsis. Plant Journal 27: 101-113

Chico JM., Chini, A., Fonseca, S.,Solano, R. (2008). JAZ repressors set the rhythm in jasmonate signalling. Curr Opin Plant Biol 11: 486-494

Choi HW., Kim, YJ., Lee, SC., Hong, JK.,Hwang, BK. (2007). Hydrogen peroxide generation by the pepper extracellular peroxidase CaPO2 activates local and systemic cell death and defence response to bacterial pathogens. Plant Physiol 145: 890-904

Chomczynski P.,Sacchi, N. (1987). Single-Step Method of Rna Isolation by Acid Guanidinium Thiocyanate Phenol Chloroform Extraction. Anal Biochem 162: 156-159

Choquer M., Fournier, E., Kunz, C., Levis, C., Pradier, JM., Simon, A.,Viaud, M. (2007). *Botrytis cinerea* virulence factors: new insights into a necrotrophic and polyphageous pathogen. Fems Microbiol Let 277: 1-10

Christensen AH., Sharrock, R. A.,Quail, P. H. (1992). Maize Polyubiquitin Genes - Structure, Thermal Perturbation of Expression and Transcript Splicing, and Promoter Activity Following Transfer to Protoplasts by Electroporation. Plant Mol Biol 18: 675-689

Clarke JD., Liu, YD., Klessig, DF.,Dong, XN. (1998). Uncoupling PR gene expression from *NPR1* and bacterial resistance: Characterization of the dominant Arabidopsis *cpr6-1* mutant. Plant Cell 10: 557-569

Cober ER., Rioux, S., Rajcan, I., Donaldson, PA.,Simmonds, DH. (2003). Partial resistance to white mold in a transgenic soybean line. Crop Sci 43: 92-95

Coego A., Ramirez, V., Ellul, P., Mayda, E.,Vera, P. (2005a). The H_2O_2-regulated *Ep5C* gene encodes a peroxidase required for bacterial speck susceptibility in tomato. Plant J 42: 283-293

Coego A., Ramirez, V., Gil, MJ., Flors, V., Mauch-Mani, B.,Vera, P. (2005b). An Arabidopsis homeodomain transcription factor, *OVEREXPRESSOR OF CATIONIC PEROXIDASE 3*, mediates resistance to infection by necrotrophic pathogens. Plant Cell 17: 2123-2137

Collinge DB., Lund, OS.,Thordal-Christensen, H. (2008). What are the prospects for genetically engineered, disease resistant plants? Eur J Plant Pathol 121: 217-231

Colmenares AJ., Aleu, J., Duran-Patron, R., Collado, IG.,Hernandez-Galan, R. (2002). The putative role of botrydial and related metabolites in the infection mechanism of *Botrytis cinerea*. J Chem Ecology 28: 997-1005

Comai L., Moran, P.,Maslyar, D. (1990). Novel and useful properties of a chimeric plant promoter combining Camv-*35S* and Mas elements. Plant Mol Biol 15: 373-381

Cornell Univesity,Vegetable MD online (2009). Carrot: Disease resistance table. Cornell university

Corrado G., Bovi, PD., Ciliento, R., Gaudio, L., Di Maro, A., Aceto, S., Lorito, M.,Rao, R. (2005). Inducible expression of a *Phytolacca heterotepala* ribosome-inactivating protein leads to enhanced resistance against major fungal pathogens in tobacco. Phytopathol 95: 206-215

Cosio C., Dunand, C. (2009). Specific functions of individual class III peroxidase genes. J Exp Bot 60: 391-408

Cui J., Bahrami, AK., Pringle, EG., Hernandez-Guzman, G., Bender, CL., Pierce, NE.,Ausubel, FM. (2005). *Pseudomonas syringae* manipulates systemic plant defenses against pathogens and herbivores. Proc Natl Acad Sci U S A 102: 1791-1796

Davies DR., Bindschedler, LV., Strickland, TS.,Bolwell, GP. (2006). Production of reactive oxygen species in Arabidopsis thaliana cell suspension cultures in response to an elicitor from Fusarium oxysporum: implications for basal resistance. J Exp Bot 57: 1817-1827

Davis RM. (2004) Carrot diseases and their management. In: Naqvi and S.A.M.H., eds Diseases of fruits and vegetables. Kluwer Academic Publishers The Netherlands:,pp. 397-439

Davis,RM., Raid,R.N. (2002) Compendium of umbelliferous crop diseases. American phytopathological society press St. Paul, Minn .

De Lorenzo G., D'Ovidio, R.,Cervone, F. (2001). The role of polygalacturonase-inhibiting proteins (PGIPS) in defence against pathogenic fungi. Annu Rev Phytopathol 39: 313-335

Deblock M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gossele, V., Movva, NR., Thompson, C., Vanmontagu, M.,Leemans, J. (1987). Engineering herbicide resistance in plants by expression of a detoxifying enzyme. Embo J 6: 2513-2518

Delaney TP, Friedrich, L.,Ryals, JA. (1995). Arabidopsis signal-transduction mutant defective in chemically and biologically induced disease resistance. Proc Natl Acad Sci U S A 92: 6602-6606

Desveaux D, Subramaniam, R., Despres, C., Mess, JN., Levesque, C., Fobert, PR., Dangl, JL.,Brisson, N. (2004). A "whirly" transcription factor is required for salicylic acid-dependent disease resistance in Arabidopsis. Develo Cell 6: 229-240

Dewit PJGM. (1992). Molecular characterization of gene-for-gene systems in plant-fungus interactions and the application of avirulence genes in control of plant-pathogens. Annu Rev Phytopathol 30: 391-418

Dong XB., Ji, RQ., Guo, XL., Foster, SJ., Chen, H., Dong, CH., Liu, YY., Hu, Q.,Liu, SY. (2008). Expressing a gene encoding wheat oxalate oxidase enhances resistance to *Sclerotinia sclerotiorum* in oilseed rape (*Brassica napus*). Planta 228: 331-340

Dong XN (2004). *NPR1*, all things considered. Curr Opin Plant Biol 7: 547-552

Doster MA.,Bostock, RM. (1988). Quantification of lignin formation in almond bark in response to wounding and infection by phytophthora species. Phytopathol 78: 473-477

Dugdale LJ., Mortimer AM., Isaac S., Collin HA. (2000) Disease response of carrot and carrot somaclones to *Alternaria dauci*. Plant Path 49: 57-67

Durrant WE.,Dong, XN. (2004). Systemic acquired resistance. Ann Rev Phytopathol 42: 185-209

Ekengren SK., Liu, YL., Schiff, M., Dinesh-Kumar, SP.,Martin, GB. (2003). Two MAPK cascades, *NPR1*, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato. Plant J 36: 905-917

El Mansouri I., Mercado, JA., Santiago-Domenech, N., Pliego-Alfaro, F., Valpuesta, V.,Quesada, MA. (1999). Biochemical and phenotypical characterization of transgenic tomato plants overexpressing a basic peroxidase. Physiologia Plantarum 106: 355-362

Ellis C., Karafyllidis, I., Wasternack, C.,Turner, JG. (2002a). The Arabidopsis mutant *cev1* links cell wall signalling to jasmonate and ethylene responses. Plant Cell 14: 1557-1566

Ellis C., Karafyllidis, L.,Turner, JG. (2002b). Constitutive activation of jasmonate signalling in an Arabidopsis mutant correlates with enhanced resistance to Erysiphe cichoracearum, Pseudomonas syringae, and Myzus persicae. Molec Plant-Microbe Interact 15: 1025-1030

Elmayan T.,Tepfer, M. (1995). Evaluation in Tobacco of the Organ Specificity and Strength of the *Rold* Promoter, Domain-A of the *35S* Promoter and the *35S*(2) Promoter. Transgenic Res 4: 388-396

Endah R, Beyene, G., Kiggundu, A., van den Berg, N., Schluter, U., Kunert, K.,Chikwamba, R. (2008). Elicitor and Fusarium-induced expression of *NPR1*-like genes in banana. Plant Physiol Biochem 46: 1007-1014

Fan WH., Dong, XN. (2002). In vivo interaction between *NPR1* and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. Plant Cell 14: 1377-1389

Farrar JJ., Pryor, BA.,Davis, RM. (2004). Alternaria diseases of carrot. Plant Disease 88: 776-784

Fei HM., Chaillou, S., Hirel, B., Mahon, J. D.,Vessey, J. K. (2003). Overexpression of a soybean cytosolic *glutamine synthetase* gene linked to organ-specific promoters in pea plants grown in different concentrations of nitrate. Planta 216: 467-474

Feltkamp D., Baumann, E., Schmalenbach, W., Masterson, R.,Rosahl, S. (1995). Expression of the *Mannopine synthase* promoter in roots is dependent on the *mas* elements and correlates with high transcript levels of mas-binding factor. Plant Sci 109: 57-65

Ferrari S., Plotnikova, JM., De Lorenzo, G.,Ausubel, FM. (2003). Arabidopsis local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires *EDS4* and *PAD2*, but not *SID2*, *EDS5* or *PAD4*. Plant J 35: 193-205

Ferreira RB., Monteiro, S., Freitas, R., Santos, CN., Chen, Z., Batista, LM., Duarte, J., Borges, A.,Teixeira, AR. (2007). The role of plant defence proteins in fungal pathogenesis. Molec Plant Pathol 8: 677-700

Fitzgerald HA., Chern, MS., Navarre, R., Ronald, PC. (2004). Overexpression of *(At)NPR1* in rice leads to a BTH- and environment-induced lesion-mimic/cell death phenotype. Molec Plant-Microbe Interact 17: 140-151

Foolad MR., Merk, H. L.,Ashrafi, H. (2008). Genetics, genomics and breeding of late blight and early blight resistance in tomato. Crit Rev Plant Sci 27: 75-107

Fraisier V., Gojon, A., Tillard, P.,Daniel-Vedele, F. (2000). Constitutive expression of a putative high-affinity nitrate transporter in *Nicotiana*

plumbaginifolia: evidence for post-transcriptional regulation by a reduced nitrogen source. Plant J 23: 489-496

Friedrich L., Lawton, K., Dietrich, R., Willits, M., Cade, R.,Ryals, J. (2001). NIM1 overexpression in arabidopsis potentiates plant disease resistance and results in enhanced effectiveness of fungicides. Molec Plant-Microbe Inter 14: 1114-1124

Friedrich L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, MG., Meier, B., Dincher, S., Staub, T., Uknes, S., Metraux, JP., Kessmann, H.,Ryals, J. (1996). A benzothiadiazole derivative induces systemic acquired resistance in tobacco. Plant J 10: 61-70

Gaffney T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H.,Ryals, J. (1993). Requirement of salicylic-acid for the induction of systemic acquired-resistance. Science 261: 754-756

Gandhi R., Maheshwari, SC.,Khurana, P. (1999). Transient gene expression and influence of promoters on foreign gene expression in *Arabidopsis thaliana*. *In vitro* Cell Develop Biol Plant 35: 232-237

Gay C., Collins, J.,Gebicki, JM. (1999). Hydroperoxide assay with the ferric-xylenol orange complex. Anal Biochem 273: 149-155

Gilmartin PM.,Chua, NH. (1990). Spacing between Gt-1 binding-sites within a light-responsive element is critical for transcriptional activity. Plant Cell 2: 447-455

Glazebrook J. (2005). Contrasting mechanisms of defence against biotrophic and necrotrophic pathogens. Ann Rev Phytopathol 43: 205-227

Glazebrook J., Chen, WJ., Estes, B., Chang, HS., Nawrath, C., Metraux, JP., Zhu, T.,Katagiri, F. (2003). Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. Plant J 34: 217-228

Gorlach J., Volrath, S., KnaufBeiter, G., Hengy, G., Beckhove, U., Kogel, K. H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H.,Ryals, J. (1996). Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell 8: 629-643

Govrin EM.,Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Curr Biolo 10: 751-757

Govrin EM.,Levine, A. (2002). Infection of Arabidopsis with a necrotrophic pathogen, *Botrytis cinerea*, elicits various defence responses but does not induce systemic acquired resistance (SAR). Plant Molec Biol 48: 267-276

Guevara-Garcia A., Lopez-Bucio, J.,Herrera-Estrella, L. (1999). The mannopine synthase promoter contains vectorial cis-regulatory elements that act as enhancers and silencers. Molec Gen Genet 262: 608-617

Guo XM.,Stotz, HU. (2007). Defence against *Sclerotinia sclerotiorum* in Arabidopsis is dependent on jasmonic acid, salicylic acid, and ethylene signalling. Molec Plant Microbe Interac 20: 1384-1395

Gurr SJ.,Rushton, PJ. (2005a). Engineering plants with increased disease resistance: how are we going to express it? Trends Biotechnol 23: 283-290

Gurr SJ.,Rushton, PJ. (2005b). Engineering plants with increased disease resistance: what are we going to express? Trends Biotechnol 23: 275-282

Han Y., Joosten, HJ., Niu, WL., Zhao, ZM., Mariano, PS., McCalman, M., van Kan, J., Schaap, PJ.,Dunaway-Mariano, D. (2007). Oxaloacetate hydrolase, the C-C bond lyase of oxalate secreting fungi. J Biol Chem 282: 9581-9590

Harman GE., Howell, CR., Viterbo, A., Chet, I.,Lorito, M. (2004). Trichoderma species - opportunistic, avirulent plant symbionts. Nature Rev Microbiol 2: 43-56

Heil M., Hilpert, A., Kaiser, W.,Linsenmair, KE. (2000). Reduced growth and seed set following chemical induction of pathogen defence: does systemic acquired resistance (SAR) incur allocation costs? J Ecology 88: 645-654

Heil M.,Ton, J. (2008). Long-distance signalling in plant defence. Trends Plant Sci 13: 264-272

Hilaire E., Young, S. A., Willard, LH., Mcgee, JD., Sweat, T., Chittoor, JM., Guikema, JA.,Leach, JE. (2001). Vascular defence responses in rice: Peroxidase accumulation in xylem parenchyma cells and xylem wall thickening. Molec Plant-Microbe Interac 14: 1411-1419

Hiraga S., Sasaki, K., Ito, H., Ohashi, Y.,Matsui, H. (2001). A large family of class III plant peroxidases. Plant cell Physiol 42: 462-468

Holtorf S., Apel, K.,Bohlmann, H. (1995). Comparison of different constitutive and inducible promoters for the overexpression of transgenes in *Arabidopsis thaliana*. Plant Mol Biol 29: 637-646

Howard,RJ., Garland,AJ., and Seaman,LW. (1994) Diseases and pests of vegetable crops in Canada.Canadian phytopathological society .

Hu X., Bidney, DL., Yalpani, N., Duvick, JP., Crasta, O., Folkerts, O.,Lu, GH. (2003). Overexpression of a gene encoding hydrogen peroxide-generating oxalate oxidase evokes defence responses in sunflower. Plant Physiol 133: 170-181

Jach G., Gornhardt, B., Mundy, J., Logemann, J., Pinsdorf, P., Leah, R., Schell, J.,Maas, C. (1995). Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J 8: 97-109

Jayaraj J., Devlin, R.,Punja, ZK. (2008a). Metabolic engineering of novel ketocarotenoid production in carrot plants. Transgenic Res 17: 489-501

Jayaraj J.,Punja, ZK. (2007). Combined expression of chitinase and lipid transfer protein genes in transgenic carrot plants enhances resistance to foliar fungal pathogens. Plant Cell Rep 26: 1539-1546

Jayaraj J., Rahman, M., Wan, A.,Punja, ZK. (2009). Enhanced resistance to foliar fungal pathogens in carrot by application of elicitors. Annals Appl Biol 155:71–80

Jayaraj J., Wan, A., Rahman, M.,Punja, ZK. (2008b). Seaweed extract reduces foliar fungal diseases on carrot. Crop Prot 27: 1360-1366

Jefferson RA., Kavanagh, TA.,Bevan, MW. (1987). GUS fusions - *Beta-glucuronidase* as a sensitive and versatile gene fusion marker in higher-plants. Embo J 6: 3901-3907

Jeong JA., Yoo, SA., Yang, DH., Shin, SH., Lee, MC., Cho, BH.,Yang, KY. (2008). Transgenic rice plants expressing an active tobacco mitogen-activated protein kinase kinase induce multiple defence responses. Plant Pathol J 24: 375-383

Johrde A.,Schweizer, P. (2008). A class III peroxidase specifically expressed in pathogen-attacked barley epidermis contributes to basal resistance. Molec Plant Pathol 9: 687-696

Jongedijk E., Tigelaar, H., Vanroekel, JSC., Bresvloemans, SA., Dekker, I., Vandenelzen, PJM., Cornelissen, BJC.,Melchers, LS. (1995). Synergistic activity of chitinases and beta-1,3-Glucanases enhances fungal resistance in transgenic tomato plants. Euphytica 85: 173-180

Joubert DA., Kars, I., Wagemakers, L., Bergmann, C., Kemp, G., Vivier, M. A.,van Kan, JAL. (2007). A polygalacturonase-inhibiting protein from grapevine reduces the symptoms of the endopolygalacturonase BcPG2 from *Botrytis cinerea* in *Nicotiana benthamiana* leaves without any evidence for *in vitro* interaction. Mol Plant Microbe Interact 20: 392-402

Jung HW., Tschaplinski, TJ., Wang, L., Glazebrook, J., Greenberg, JT. (2009) Priming in systemic plant immunity. Science 324: 89-91

Kamo K., Blowers, A. (1999). Tissue specificity and expression level of gusA under *rolD*, *mannopine synthase* and *translation elongation factor* 1 subunit alpha promoters in transgenic Gladiolus plants. Plant Cell Rep 18: 809-815

Kamo KK. (2003). Long-term expression of the uidA gene in Gladiolus plants under control of either the ubiquitin, rolD, mannopine synthase, or cauliflower mosaic virus promoters following three seasons of dormancy. Plant Cell Rep 21: 797-803

Katsir L., Chung, HS., Koo, AJK.,Howe, GA. (2008). Jasmonate signalling: a conserved mechanism of hormone sensing. Curr Opin Plant Biol 11: 428-435

Kauffman HE., Reddy, APK., Hsieh, SPY.,Merca, SD. (1973). Improved technique for evaluating resistance of rice varieties to *Xanthomonas-Oryzae*. Plant Dis Reptr 57: 537-541

Kawaoka A., Matsunaga, E., Endo, S., Kondo, S., Yoshida, K., Shinmyo, A.,Ebinuma, H. (2003). Ectopic expression of a horseradish peroxidase enhances growth rate and increases oxidative stress resistance in hybrid aspen. Plant Physiol 132: 1177-1185

Kawasaki T., Koita, H., Nakatsubo, T., Hasegawa, K., Wakabayashi, K., Takahashi, H., Urnemura, K., Urnezawa, T.,Shimamoto, K. (2006). Cinnamoyl-CoA reductase, a key enzyme in lignin biosynthesis, is an effector of small GTPase Rac in defence signalling in rice. Proc Natl Acad Sci U S A 103: 230-235

Kay R., Chan, A., Daly, M.,Mcpherson, J. (1987). Duplication of Camv-*35S* promoter sequences creates a strong enhancer for plant genes. Science 236: 1299-1302

Keller H., Pamboukdjian, N., Ponchet, M., Poupet, A., Delon, R., Verrier, JL., Roby, D.,Ricci, P. (1999). Pathogen-induced elicitin production in transgenic tobacco generates a hypersensitive response and nonspecific disease resistance. Plant Cell 11: 223-235

Kim KS., Min, JY.,Dickman, MB. (2008a). Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. Mol Plant Microbe Interact 21: 605-612

Kim YH., Kim, CY., Song, WK., Park, DS., Kwon, SY., Lee, HS., Bang, JW.,Kwak, SS. (2008b). Overexpression of sweetpotato *swpa4* peroxidase results in increased hydrogen peroxide production and enhances stress tolerance in tobacco. Planta 227: 867-881

Kiraly L., Barnaz, B.,Kiralyz, Z. (2007). Plant resistance to pathogen infection: Forms and mechanisms of innate and acquired resistance. J Phytopathol 155: 385-396

Komamine A., Murata, N.,Nomura, K. (2005). 2004 SIVB Congress Symposium Proceeding: Mechanisms of somatic embryogenesis in carrot suspension cultures - Morphology, physiology, biochemistry, and molecular biology. *In vitro* Cell Develop Biol Plant 41: 6-10

Kora C., McDonald, MR.,Boland, GJ. (2003). Sclerotinia rot of carrot - An example of phenological adaptation and bicyclic development by *Sclerotinia sclerotiorum*. Plant Disease 87: 456-470

Kora C., McDonald, MR.,Boland, GJ. (2005). Epidemiology of sclerotinia rot of carrot caused by *Sclerotinia sclerotiorum*. Canadian J Plant Pathol 27: 245-258

Kristensen BK., Bloch, H., Rasmussen, SK. (1999). Barley coleoptile peroxidases. Purification, molecular cloning, and induction by pathogens. Plant Physiol 120: 501-512

Kumar S., Dhingra, A.,Daniell, H. (2004). Plastid-expressed betaine aldehyde dehydrogenase gene in carrot cultured cells, roots, and leaves confers enhanced salt tolerance. Plant Physiol 136: 2843-2854

Lagrimini LM. (1991). Wound-induced deposition of polyphenols in transgenic plants overexpressing peroxidase. Plant Physiol 96: 577-583

Lamb C., Dixon, RA. (1997). The oxidative burst in plant disease resistance. Ann Rev Plant Physiol Plant Molec Biol 48: 251-275

Langridge WHR., Fitzgerald, KJ., Koncz, C., Schell, J.,Szalay, AA. (1989). Dual promoter of *Agrobacterium tumefaciens mannopine synthase genes* is regulated by plant-growth hormones. Proc Natl Acad Sci U S A 86: 3219-3223

Laurie-Berry N., Joardar, V., Street, IH.,Kunkel, BN. (2006). The *Arabidopsis thaliana jasmonate insensitive 1* gene is required for suppression of salicylic acid-dependent defenses during infection by *Pseudomonas syringae*. Mol Plant Microbe Interact 19: 789-800

Leach F., Aoyagi, K. (1991). Promoter analysis of the highly expressed *Rolc* and *Rold* root-inducing genes of *Agrobacterium rhizogenes* - enhancer and tissue-specific DNA determinants are dissociated. Plant Sci 79: 69-76

Lee HI., Leon, J., Raskin, I. (1995). Biosynthesis and metabolism of salicylic-acid. Proc Natl Acad Sci U S A 92: 4076-4079

Leitner M., Kaiser, R., Rasmussen, MO., Driguez, H., Boland, W.,Mithofer, A. (2008). Microbial oligosaccharides differentially induce volatiles and signalling components in *Medicago truncatula*. Phytochem 69: 2029-2040

Li HP., Zhang, JB., Shi, RP., Huang, T., Fischer, R.,Liao, YC. (2008). Engineering fusarium head blight resistance in wheat by expression of a fusion protein containing a fusarium-specific antibody and an antifungal peptide. Mol Plant Microbe Interact 21: 1242-1248

Li J., Brader, G., Kariola, T.,Palva, ET. (2006). WRKY70 modulates the selection of signalling pathways in plant defence. Plant J 46: 477-491

Li J., Brader, G.,Palva, ET. (2004). The WRKY70 transcription factor: A node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defence. Plant Cell 16: 319-331

Li WL., Faris, JD., Muthukrishnan, S., Liu, DJ., Chen, PD.,Gill, BS. (2001). Isolation and characterization of novel cDNA clones of acidic chitinases and beta-1,3-glucanases from wheat spikes infected by *Fusarium graminearum*. Theor Appl Genet 102: 353-362

Liang HY., Maynard, CA., Allen, RD.,Powell, WA. (2001). Increased *Septoria musiva* resistance in transgenic hybrid poplar leaves expressing a wheat oxalate oxidase gene. Plant Mol Biol 45: 619-629

Lin WC., Lu, CF., Wu, JW., Cheng, ML., Lin, YM., Yang, NS., Black, L., Green, SK., Wang, JF.,Cheng, CP. (2004). Transgenic tomato plants expressing the Arabidopsis *NPR1* gene display enhanced resistance to a spectrum of fungal and bacterial diseases. Transgenic Res 13: 567-581

Liu B., Zeng, Q., Yan, FM., Xu, HG.,Xu, CR. (2005). Effects of transgenic plants on soil microorganisms. Plant Soil 271: 1-13

Liu JY.., Maldonado-Mendoza, I., Lopez-Meyer, M., Cheung, F., Town, CD.,Harrison, MJ. (2007). Arbuscular mycorrhizal symbiosis is accompanied by local and systemic alterations in gene expression and an increase in disease resistance in the shoots. Plant J 50: 529-544

Lorenzo O.,Solano, R. (2005). Molecular players regulating the jasmonate signalling network. Curr Opin Plant Biol 8: 532-540

Mahuku GS., Jara, CE., Cajiao, C.,Beebe, S. (2002). Sources of resistance to *Colletotrichum lindemuthianum* in the secondary gene pool of *Phaseolus vulgaris* and in crosses of primary and secondary gene pools. Plant Dis 86: 1383-1387

Makandar R., Essig, JS., Schapaugh, MA., Trick, HN.,Shah, J. (2006). Genetically engineered resistance to Fusarium head blight in wheat by expression of Arabidopsis *NPR1*. Molec Plant Microbe Interac 19: 123-129

Maldonado AM., Doerner, P., Dixon, RA., Lamb, CJ.,Cameron, RK. (2002). A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. Nature 419: 399-403

Malik K., Wu, K., Li, XQ., Martin-Heller, T., Hu, M., Foster, E., Tian, L., Wang, C., Ward, K., Jordan, M., Brown, D., Gleddie, S., Simmonds, D., Zheng, S., Simmonds, J.,Miki, B. (2002). A constitutive gene expression system derived from the tCUP cryptic promoter elements. Theor Appl Genet 105: 505-514

Malnoy M., Jin, Q., Borejsza-Wysocka, EE., He, SY.,Aldwinckle, HS. (2007). Overexpression of the apple Mp*NPR1* gene confers increased disease resistance in malus x domestica. Molec Plant Microbe Interac 20: 1568-1580

Manfredini C., Sicilia, F., Ferrari, S., Pontiggia, D., Salvi, G., Caprari, C., Lorito, M.,De Lorenzo, G. (2005). Polygalacturonase-inhibiting protein 2 of *Phaseolus vulgaris* inhibits BcPG1, a polygalacturonase of *Botrytis cinerea* important for pathogenicity, and protects transgenic plants from infection. Physiol Molec Plant Pathol 67: 108-115

Marjamaa K., Kukkola, EM.,Fagerstedt, KV. (2009). The role of xylem class III peroxidases in lignification. J Exp Bot 60: 367-376

Marquet-Blouin E., Bouche, FB., Steinmetz, A.,Muller, CP. (2003). Neutralizing immunogenicity of transgenic carrot (*Daucus carota L.*)-derived measles virus hemagglutinin. Plant Mol Biol 51: 459-469

Martinez AT., Speranza, M., Ruiz-Duenas, FJ., Ferreira, P., Camarero, S., Guillen, F., Martinez, MJ., Gutierrez, A.,del Rio, JC. (2005). Biodegradation of lignocellulosics: microbial chemical, and enzymatic aspects of the fungal attack of lignin. Int Microbiol 8: 195-204

Mauch F., Mauch-Mani, B., Gaille, C., Kull, B., Haas, D.,Reimmann, C. (2001). Manipulation of salicylate content in *Arabidopsis thaliana* by the expression of an engineered bacterial salicylate synthase. Plant J 25: 67-77

Melchers LS.,Stuiver, MH. (2000). Novel genes for disease-resistance breeding. Curr Opin Plant Biol 3: 147-152

Mendgen K.,Hahn, M. (2002). Plant infection and the establishment of fungal biotrophy. Trends Plant Sci 7: 352-356

Metraux J., Signer, H., Ryals, J., Ward, E., Wyssbenz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W.,Inverardi, B. (1990). Increase in salicylic-acid at the onset of systemic acquired-resistance in cucumber. Science 250: 1004-1006

Meur G., Budatha, M., Gupta, AD., Prakash, S.,Kirti, PB. (2006). Differential induction of *NPR1* during defence responses in Brassica juncea. Physiol Molec Plant Pathol 68: 128-137

Meur G., Budatha, M., Srinivasan, T., Kumar, KRR., Gupta, AD.,Kirti, PB. (2008). Constitutive expression of Arabidopsis *NPR1* confers enhanced resistance to the early instars of *Spodoptera litura* in transgenic tobacco. Physiologia Plantarum 133: 765-775

Michielse CB.,Rep, M. (2009). Pathogen profile update: *Fusarium oxysporum*. Molec Plant Pathol 10: 311-324

Mika A.,Luthje, S. (2003). Properties of guaiacol peroxidase activities isolated from corn root plasma membranes. Plant Physiol 132: 1489-1498

Mohr PG.,Cahill, DM. (2007). Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in Arabidopsis infected with *Pseudomonas syringae* pv. tomato. Funct Integr Genomics 7: 181-191

Mou Z., Fan, WH.,Dong, XN. (2003). Inducers of plant systemic acquired resistance regulate *NPR1* function through redox changes. Cell 113: 935-944

Murashige T.,Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Plant Physiol 15: 473-&

Ndamukong I., Al Abdallat, A., Thurow, C., Fode, B., Zander, M., Weigel, R.,Gatz, C. (2007). SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive PDF1.2 transcription. Plant J 50: 128-139

Neill S., Desikan, R.,Hancock, J. (2002a). Hydrogen peroxide signalling. Curr Opin Plant Biol 5: 388-395

Neill SJ., Desikan, R., Clarke, A., Hurst, RD.,Hancock, JT. (2002b). Hydrogen peroxide and nitric oxide as signalling molecules in plants. J Exp Bot 53: 1237-1247

Ni M., Cui, D., Einstein, J., Narasimhulu, S., Vergara, CE.,Gelvin, SB. (1995). Strength and tissue-specificity of chimeric promoters derived from the *octopine* and *mannopine synthase* genes. Plant J 7: 661-676

Norris SR., Meyer, SE.,Callis, J. (1993). The intron of *Arabidopsis thaliana* polyubiquitin genes is conserved in location and is a quantitative determinant of chimeric gene-expression. Plant Mol Biol 21: 895-906

Odell JT., Nagy, F.,Chua, NH. (1985). Identification of DNA-sequences required for activity of the Cauliflower Mosaic Virus-*35S* Promoter. Nature 313: 810-812

Oliver RP.,Ipcho, SVS. (2004). Arabidopsis pathology breathes new life into the necrotrophs-vs.-biotrophs classification of fungal pathogens. Molec Plant Pathol 5: 347-352

Oltmanns H., Kloos, DU., Briess, W., Pflugmacher, M., Stahl, DJ.,Hehl, R. (2006). Taproot promoters cause tissue specific gene expression within the storage root of sugar beet. Planta 224: 485-495

Park SW., Kaimoyo, E., Kumar, D., Mosher, S.,Klessig, DF. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. Science 318: 113-116

Park SW., Liu, PP., Forouhar, F., Vlot, AC., Tong, L., Tietjen, K.,Klessig, DF. (2009). Use of a synthetic salicylic acid analog to investigate the roles of methyl salicylate and Its esterases in plant disease resistance. J Biol Chem 284: 7307-7317

Passardi F., Bakalovic, N., Teixeira, FK., Margis-Pinheiro, M., Penel, C.,Dunand, C. (2007). Prokaryotic origins of the non-animal peroxidase superfamily and organelle-mediated transmission to eukaryotes. Genomics 89: 567-579

Passardi F., Cosio, C., Penel, C.,Dunand, C. (2005). Peroxidases have more functions than a Swiss army knife. Plant Cell Rep 24: 255-265

Passardi F., Longet, D., Penel, C.,Dunand, C. (2004a). The class III peroxidase multigenic in land plants family in rice and its evolution. Phytochem 65: 1879-1893

Passardi F., Penel, C.,Dunand, C. (2004b). Performing the paradoxical: how plant peroxidases modify the cell wall. Trends Plant Sci 9: 534-540

Patel RM., van Kan, JAL., Bailey, AM.,Foster, GD. (2008). RNA-Mediated Gene Silencing of *Superoxide Dismutase* (*bcsod1*) in *Botrytis cinerea*. Phytopathol 98: 1334-1339

Pauk J, Stefanov, I., Fekete, S., Bogre, L., Karsai, I., Feher, A.,Dudits, D. (1995). A Study of Different (Camv *35S* and Mas) Promoter Activities and Risk Assessment of Field Use in Transgenic Rapeseed Plants. Euphytica 85: 411-416

Pedley KF.,Martin, GB. (2005). Role of mitogen-activated protein kinases in plant immunity. Curr Opin Plant Biol 8: 541-547

Perfect SE., Green, JR.,O'Connell, RJ. (2001). Surface characteristics of necrotrophic secondary hyphae produced by the bean anthracnose fungus, *Colletotrichum lindemuthianum*. Eur J Plant Pathol 107: 813-819

Peschen D., Li, HP., Fischer, R., Kreuzaler, F.,Liao, YC. (2004). Fusion proteins comprising a Fusarium-specific antibody linked to antifungal peptides protect plants against a fungal pathogen. Nature Biotechnol 22: 732-738

Petersen M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H. B., Lacy, M., Austin, M. J., Parker, J. E., Sharma, S. B., Klessig, D. F., Martienssen, R., Mattsson, O., Jensen, A. B.,Mundy, J. (2000). Arabidopsis *MAP kinase 4* negatively regulates systemic acquired resistance. Cell 103: 1111-1120

Pieterse CM.,Van Loon, L. (2004). *NPR1*: the spider in the web of induced resistance signalling pathways. Curr Opin Plant Biol 7: 456-464

Pieterse CMJ., Leon-Reyes, A., Van der Ent, S.,Van Wees, SCM. (2009). Networking by small-molecule hormones in plant immunity. Nature Chemical Biol 5: 308-316

Pieterse CMJ.,Van Loon, L.C. (1999). Salicylic acid-independent plant defence pathways. Trends Plant Sci 4: 52-58

Pieterse CMJ., Van Wees, SCM., Van Pelt, JA., Knoester, M., Laan, R., Gerrits, N., Weisbeek, PJ.,Van Loon, LC. (1998). A novel signalling pathway controlling induced systemic resistance in Arabidopsis. Plant Cell 10: 1571-1580

Potenza C., Aleman, L.,Sengupta-Gopalan, C. (2004). Targeting transgene expression in research, agricultural, and environmental applications: Promoters used in plant transformation. *In vitro* Cellular & Developmental Biology-Plant 40: 1-22

Punja ZK. (2001). Genetic engineering of plants to enhance resistance to fungal pathogens - a review of progress and future prospects. Can J Plant Pathol 23: 216-235

Punja ZK. (2005). Transgenic carrots expressing a thaumatin-like protein display enhanced resistance to several fungal pathogens. Can J Plant Pathol 27: 291-296

Punja ZK. (2006). Recent developments toward achieving fungal disease resistance in transgenic plants. Can J Plant Pathol 28: S298-S308

Punja ZK., Jayaraj J, Wally O. (2007) Carrot. In: E.C.Pua and D.R.Davies, eds Transgenic crops. Springer Berlin:,pp. 277-294

Punja ZK.,Raharjo, SHT. (1996). Response of transgenic cucumber and carrot plants expressing different chitinase enzymes to inoculation with fungal pathogens. Plant Disease 80: 999-1005

Quilis J., Penas, G., Messeguer, J., Brugidou, C.,Segundo, BS. (2008). The Arabidopsis At*NPR1* inversely modulates defence responses against fungal, bacterial, or viral pathogens while conferring hypersensitivity to abiotic stresses in transgenic rice. Molec Plant Microbe Interac 21: 1215-1231

Quiroga M., Guerrero, C., Botella, MA., Barcelo, A., Amaya, I., Medina, MI., Alonso, FJ., de Forchetti, SM., Tigier, H.,Valpuesta, V. (2000). A tomato peroxidase involved in the synthesis of lignin and suberin. Plant Physiology 122: 1119-1127

Ray H., Douches, DS.,Hammerschmidt, R. (1998). Transformation of potato with cucumber peroxidase: expression and disease response. Physiol Molec Plant Pathol 53: 93-103

Reissig JL., Strominger, JL.,Leloir, LF. (1955). A modified colorimetric method for the estimation of N-Acetylamino sugars. J Biological Chem 217: 959-966

Rolke Y., Liu, SJ., Quidde, T., Williamson, B., Schouten, A., Weltring, K. M., Siewers, V., Tenberge, KB., Tudzynski, B.,Tudzynski, P. (2004). Functional analysis of H_2O_2-generating systems in *Botrytis cinerea*: the major Cu-Zn-*superoxide dismutase* (BCSOD1) contributes to virulence on French bean, whereas a *glucose oxidase* (BCGOD1) is dispensable. Molec Plant Pathol 5: 17-27

Rollins JA.,Dickman, MB. (2001). PH signalling in *Sclerotinia sclerotiorum*: Identification of a pacC/RIM1 Homolog. Appl Environ Microbiol 67: 75-81

Ross AF. (1961). Systemic Acquired Resistance Induced by Localized Virus Infections in Plants. Virology 14: 340-&

Rosslenbroich HJ.,Stuebler, D. (2000). *Botrytis cinerea* - history of chemical control and novel fungicides for its management. Crop Protect 19: 557-561

Rushton PJ., Reinstadler, A., Lipka, V., Lippok, B.,Somssich, I. E. (2002). Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen- and wound-induced signalling. Plant Cell 14: 749-762

Rushton PJ., Torres, JT., Parniske, M., Wernert, P., Hahlbrock, K.,Somssich, I. E. (1996). Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley *PR1* genes. Embo J 15: 5690-5700

Ryals JA., Neuenschwander, UH., Willits, MG., Molina, A., Steiner, HY.,Hunt, MD. (1996). Systemic acquired resistance. Plant Cell 8: 1809-1819

Samac DA., Tesfaye, M., Dornbusch, M., Saruul, P.,Temple, S. J. (2004). A comparison of constitutive promoters for expression of transgenes in alfalfa (*Medicago sativa*). Transgenic Res 13: 349-361

Sambrook,J., Fritsch,EF., Maniatis,T. (1989) Molecular cloning: A laboratory manual.Cold Spring Harbor Laboratory Press Cold Spring Harbor, NY .

Sasaki K., Iwai, T., Hiraga, S., Kuroda, K., Seo, S., Mitsuhara, I., Miyasaka, A., Iwano, M., Ito, H., Matsui, H.,Ohashi, Y. (2004). Ten rice peroxidases redundantly respond to multiple stresses including infection with rice blast fungus. Plant Cell Physiol 45: 1442-1452

Scheid OM., Probst, AV., Afsar, K.,Paszkowski, J. (2002). Two regulatory levels of transcriptional gene silencing in Arabidopsis. Proc Natl Acad Sci U S A 99: 13659-13662

Schledzewski K.,Mendel, RR. (1994). Quantitative transient gene-expression - comparison of the promoters for Maize Polyubiquitin1, Rice Actin1, Maize-Derived Emu and Camv *35S* in Cells of Barley, Maize and Tobacco. Transgenic Res 3: 249-255

Schweizer P. (2008). Tissue-specific expression of a defence-related peroxidase in transgenic wheat potentiates cell death in pathogen-attacked leaf epidermis. Molecular Plant Pathology 9: 45-57

Scott DL., Clark, CW., Deahl, KL.,Prakash, CS. (1998). Isolation of functional RNA from periderm tissue of potato tubers and sweet potato storage roots. Plant Molec Biol Rep 16: 3-8

Sels J., Mathys, J., De Coninck, BMA., Cammue, BPA.,De Bolle, MFC. (2008). Plant pathogenesis-related (PR) proteins: A focus on PR peptides. Plant Physiol Biochem 46: 941-950

Shah J. (2003). The salicylic acid loop in plant defence. Curr Opin Plant Biol 6: 365-371

Shah J., Tsui, F.,Klessig, DF. (1997). Characterization of a salicylic acid-insensitive mutant (sai1) of Arabidopsis thaliana, identified in a selective screen utilizing the SA-inducible expression of the tms2 gene. Molec Plant Microbe Inter 10: 69-78

Shetty NP., Jorgensen, HJL., Jensen, J.D., Collinge, DB.,Shetty, HS. (2008). Roles of reactive oxygen species in interactions between plants and pathogens. Eur J Plant Pathol 121: 267-280

Siewers V., Viaud, M., Jimenez-Teja, D., Collado, IG., Gronover, CS., Pradier, JM., Tudzynski, B.,Tudzynski, P. (2005). Functional analysis of the cytochrome P450 monooxygenase gene bcbot1 of *Botrytis cinerea* indicates that botrydial is a strain-specific virulence factor. Mol Plant Microbe Interact 18: 602-612

Silverman P., Seskar, M., Kanter, D., Schweizer, P., Metraux, J. P.,Raskin, I. (1995). Salicylic-acid in rice - biosynthesis, conjugation, and possible role. Plant Physiol 108: 633-639

Smulevich G., Jakopitsch, C., Droghetti, E.,Obinger, C. (2006). Probing the structure and bifunctionality of catalase-peroxidase (KatG). J Inorganic Biochem 100: 568-585

Song D., Chen, J., Song, F.,Zheng, Z. (2006). A novel rice MAPK gene, *OsBIMK2*, is involved in disease-resistance responses. Plant Biol 8: 587-596

Spoel SH., Johnson, JS.,Dong, XN. (2007). Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. Proc Natl Acad Sci U S A 104: 18842-18847

Spoel SH., Koornneef, A., Claessens, SMC., Korzelius, JP., Van Pelt, JA., Mueller, MJ., Buchala, AJ., Metraux, JP., Brown, R., Kazan, K., Van Loon, LC., Dong, XN.,Pieterse, CMJ. (2003). *NPR1* modulates cross-talk between salicylate- and jasmonate-dependent defence pathways through a novel function in the cytosol. Plant Cell 15: 760-770

Staats M., van Baarlen, P.,van Kan, JAL. (2005). Molecular phylogeny of the plant pathogenic genus Botrytis and the evolution of host specificity. Molec Biol Evol 22: 333-346

Staswick PE. (2008). JAZing up jasmonate signalling. Trends Plant Sci 13: 66-71

Subramaniam R., Desveaux, D., Spickler, C., Michnick, SW.,Brisson, N. (2001). Direct visualization of protein interactions in plant cells. Nature Biotech 19: 769-772

Takahashi Y, Bin Nasir, KH., Ito, A., Kanzaki, H., Matsumura, H., Saitoh, H., Fujisawa, S., Kamoun, S.,Terauchi, R. (2007). A high-throughput screen of cell-death-inducing factors in Nicotiana benthamiana identifies a novel MAPKK that mediates INF1-induced cell death signalling and non-host resistance to Pseudomonas cichorii. Plant J 49: 1030-1040

Takaichi M.,Oeda, K. (2000). Transgenic carrots with enhanced resistance against two major pathogens, Erysiphe heraclei and Alternaria dauci. Plant Sci 153: 135-144

Takakura Y., Ishida, Y., Inoue, Y., Tsutsumi, F.,Kuwata, S. (2004). Induction of a hypersensitive response-like reaction by powdery mildew in transgenic tobacco expressing harpin(pss). Physiol Molec Plant Pathol 64: 83-89

Thaler JS., Owen, B.,Higgins, VJ. (2004). The role of the jasmonate response in plant susceptibility to diverse pathogens with a range of lifestyles. Plant Physiol 135: 530-538

Thines B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, GH., Nomura, K., He, SY., Howe, GA.,Browse, J. (2007). JAZ repressor proteins are targets of the SCFCO11 complex during jasmonate signalling. Nature 448: 661-6U2

Thomma BPHJ (2003). Alternaria spp.: from general saprophyte to specific parasite. Molecular Plant Pathology 4: 225-236

Thomma BPHJ., Eggermont, K., Penninckx, IAMA., Mauch-Mani, B., Vogelsang, R., Cammue, BPA.,Broekaert, WF. (1998). Separate jasmonate-dependent and salicylate-dependent defence-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proc Nat Acad Sci USA 95: 15107-15111

Thomma BPHJ., Eggermont, K., Tierens, KFMJ.,Broekaert, WF. (1999). Requirement of functional *ethylene-insensitive 2* gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea*. Plant Physiol 121: 1093-1101

ThordalChristensen H., Zhang, ZG., Wei, YD.,Collinge, DB. (1997). Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J 11: 1187-1194

Tian D., Traw, MB., Chen, JQ., Kreitman, M.,Bergelson, J. (2003). Fitness costs of R-gene-mediated resistance in Arabidopsis thaliana. Nature 423: 74-77

Tognolli M., Penel, C., Greppin, H.,Simon, P. (2002). Analysis and expression of the class III peroxidase large gene family in *Arabidopsis thaliana*. Gene 288: 129-138

Torres MA., Jones, J. D. G.,Dangl, J. L. (2006). Reactive oxygen species signalling in response to pathogens. Plant Physiology 141: 373-378

Truman W., Bennettt, MH., Kubigsteltig, I., Turnbull, C.,Grant, M. (2007). Arabidopsis systemic immunity uses conserved defence signalling pathways and is mediated by jasmonates. Proc Natl Acad Sci U S A 104: 1075-1080

Turner JG., Ellis, C.,Devoto, A. (2002). The jasmonate signal pathway. Plant Cell 14: S153-S164

Tweddell RJ., Jabajihare, SH.,Charest, PM. (1994). Production of chitinases and beta-1,3-glucanases by *Stachybotrys elegans*, a mycoparasite of *Rhizoctonia solani*. Appl Environ Microbiol 60: 489-495

Uquillas C, Letelier, I., Blanco, F., Jordana, X.,Holuigue, L. (2004). *NPR1*-independent activation of immediate early salicylic acid-responsive genes in Arabidopsis. Mol Plant Microbe Interact 17: 34-42

Van der Ent S., Verhagen, BWM., van Doorn, R., Bakker, D., Verlaan, MG., Pel, MJC., Joosten, RG., Proveniers, MCG., Van Loon, LC., Ton, J.,Pieterse, CMJ. (2008). MYB72 is required in early signalling steps of rhizobacteria-induced systemic resistance in arabidopsis. Plant Physiol 146: 1293-1304

van der Vossen EAG., Gros, J., Sikkema, A., Muskens, M., Wouters, D., Wolters, P., Pereira, A.,Allefs, S. (2005). The Rpi-blb2 gene from Solanum bulbocastanum is an Mi-1 gene homolog conferring broad-spectrum late blight resistance in potato. Plant J 44: 208-222

van Kan JAL. (2006). Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci 11: 247-253

Van Loon LC.,Van Strien, EA. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of *PR-1* type proteins. Physiol Molec Plant Pathol 55: 85-97

Van Wees SCM., Van der Ent, S.,Pieterse, CMJ. (2008). Plant immune responses triggered by beneficial microbes. Curr Opin Plant Biol 11: 443-448

Vasyukova NI.,Ozeretskovskaya, OL. (2007). Induced plant resistance and salicylic acid: A review. App Biochem Microbiol 43: 367-373

Velazhahan R., Chen-Cole K., Anuratha CS., MuthukrishnanS. (1998) Induction of thaumatin-like proteins (TLPs) in Rhizoctonia solani-infected rice and characterization of two new cDNA clones. Plant Physiol 100: 1503-1507.

Venter M. (2007). Synthetic promoters: genetic control through cis engineering. Trends Plant Sci 12: 118-124

Vermerris W.,Nicholson R. (2006) Isolation and identification of phenolic compounds, a practical guide. In: W.Vermerris and R.Nicholson, eds Phenolic compound biochemistry. Springer Netherlands Amsterdan:,pp. 151-196

Vernooij B., Friedrich, L., Morse, A., Reist, R., Kolditzjawhar, R., Ward, E., Uknes, S., Kessmann, H.,Ryals, J. (1994). Salicylic acid is not the translocated signal responsible for inducing systemic acquired-resistance but is required in signal transduction. Plant Cell 6: 959-965

Vlot AC., Liu, PP., Cameron, RK., Park, SW., Yang, Y., Kumar, D., Zhou, FS., Padukkavidana, T., Gustafsson, C., Pichersky, E.,Klessig, DF. (2008). Identification of likely orthologs of tobacco salicylic acid-binding protein 2 and their role in systemic acquired resistance in *Arabidopsis thaliana*. Plant J 56: 445-456

Wally O., Jayaraj J, Punja ZK. (2006) Carrot (*Daucus carota. L*). In: K.Wang, ed *Agrobacterium* protocols Volume 2. Humana Press Totowa, NJ.:,pp. 3-12

Walz A., Zingen-Sell, I., Loeffler, M.,Sauer, M. (2008a). Expression of an oxalate oxidase gene in tomato and severity of disease caused by *Botrytis cinerea* and *Sclerotinia sclerotiorum*. Plant Pathol 57: 453-458

Walz A., Zingen-Sell, I., Theisen, S.,Kortekamp, A. (2008b). Reactive oxygen intermediates and oxalic acid in the pathogenesis of the necrotrophic fungus *Sclerotinia sclerotiorum*. Eur J Plant Pathol 120: 317-330

Williamson B., Tudzynsk, B., Tudzynski, P.,van Kan, JAL. (2007). *Botrytis cinerea*: the cause of grey mould disease. Molec Plant Pathol 8: 561-580

Xie DX., Feys, BF., James, S., Nieto-Rostro, M.,Turner, JG. (1998). *COI1*: An Arabidopsis gene required for jasmonate-regulated defence and fertility. Science 280: 1091-1094

Yang SM., Gao, MQ., Xu, CW., Gao, JC., Deshpande, S., Lin, SP., Roe, BA.,Zhu, HY. (2008). Alfalfa benefits from *Medicago truncatula*: The RCT1 gene from M-truncatula confers broad-spectrum resistance to anthracnose in alfalfa. Proc Natl Acad Sci U S A 105: 12164-12169

Yevtushenko DP.,Misra, S. (2007). Comparison of pathogen-induced expression and efficacy of two amphibian antimicrobial peptides, *MsrA2* and *temporin A*, for engineering wide-spectrum disease resistance in tobacco. Plant Biotechnol J 5: 720-734

Young SA., Guo, A., Guikema, JA., White, FF.,Leach, JE. (1995). Rice cationic peroxidase accumulates in xylem vessels during incompatible interactions with *Xanthomonas oryzae Pv Oryzae*. Plant Physiol 107: 1333-1341

Yuan YX., Zhong, SH., Li, Q., Zhu, ZR., Lou, YG., Wang, LY., Wang, JJ., Wang, MY., Li, QL., Yang, DL.,He, ZH. (2007). Functional analysis of rice *NPR1*-like genes reveals that *OsNPR1/NH1* is the rice orthologue conferring disease resistance with enhanced herbivore susceptibility. Plant Biotech J 5: 313-324

Zhang SQ.,Liu, YD. (2001). Activation of salicylic acid-induced protein kinase, a mitogen-activated protein kinase, induces multiple defence responses in tobacco. Plant Cell 13: 1877-1889

Zhang YL., Goritschnig, S., Dong, XN.,Li, X. (2003a). A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in *suppressor of npr1-1, constitutive 1*. Plant Cell 15: 2636-2646

Zhang YL., Tessaro, MJ., Lassner, M.,Li, X. (2003b). Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. Plant Cell 15: 2647-2653

Zhang YP., Uyemoto JK., KirkpatrickBC. (1998) A small-scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay. J Virol Methods 71: 45-50

Zhao BY., Lin, XH., Poland, J., Trick, H., Leach, J.,Hulbert, S. (2005). A maize resistance gene functions against bacterial streak disease in rice. Proc Natl Acad Sci U S A 102: 15383-15388

Zhao JW., Wang, JL., An, LL., Doerge, RW., Chen, ZJ., Grau, CR., Meng, JL.,Osborn, TC. (2007). Analysis of gene expression profiles in response to Sclerotinia sclerotiorum in Brassica napus. Planta 227: 13-24

Zhou YL., Xu, JL., Zhou, SC., Yu, J., Xie, XW., Xu, MR., Sun, Y., Zhu, LH., Fu, BY., Gao, YM.,Li, ZK. (2009). Pyramiding Xa23 and Rxo1 for resistance to two bacterial diseases into an elite indica rice variety using molecular approaches. Molec Breeding 23: 279-287

Zhu Q., Maher, EA., Masoud, S., Dixon, RA.,Lamb, CJ. (1994). Enhanced protection against fungal attack by constitutive coexpression of chitinase and glucanase genes in transgenic tobacco. Bio-Technology 12: 807-812

Zimmerli L., Stein, M., Lipka, V., Schulze-Lefert, P.,Somerville, S. (2004). Host and non-host pathogens elicit different jasmonate/ethylene responses in Arabidopsis. Plant J 40: 633-646

Zimmerman JL. (1993). Somatic embryogenesis - a model for early development in higher-plants. Plant Cell 5: 1411-1423