Comparative resistance to foliar fungal pathogens in transgenic carrot plants expressing genes encoding for chitinase, β-1,3-glucanase and peroxidise

Owen Wally · Jayaraman Jayaraj · Zamir Punja

Abstract Genes encoding an acidic wheat class IV chitinase (383), an acidic wheat β 1,3-glucanase (638) and a rice cationic peroxidase (POC1) were introduced into 'Nantes Coreless' carrot (*Daucus carota*) by *Agrobacterium*-mediated transformation. The genes were introduced singly or in various combinations followed by selection imposed by the herbicide phosphinothricin. Regenerated plantlets were screened for presence and expression of the three transgenes using PCR, Southern and Northern hybridisations. Eighteen transgenic lines expressing a single transgene and 2 lines each co-expressing 638/ 383 and 383/POC1 were assessed for resistance to the necrotrophic fungal pathogens *Botrytis cinerea* and *Sclerotinia sclerotiorum*. Percentage leaf area diseased was measured 4 and 7 days after inoculation (dai) and compared to non-transformed control plants. Six lines expressing β-1,3-glucanase 638 alone had no enhanced resistance to *B. cinerea* at 4 dai and only slight resistance to *S. sclerotiorum*; there was no effect at 7 dai. Two out of the six lines expressing 383 alone had enhanced tolerance to both pathogens with a 20–50% reduction in disease development at 7 dai. Two lines co-expressing 638/383 had slight reductions in disease by (10–20%) similar to that of the lines expressing chitinase 383 alone. Highest levels of disease resistance were seen in transgenic lines expressing POC1, alone or in combination with chitinase 383. Disease symptoms were slower to develop and symptoms were reduced by up to 90% for *B. cinerea* and 70% for *S. sclerotiorum*. The 383/ POC1 co-expressing plants developed disease at levels similar to that of POC1 alone. Petioles of plants over-expressing POC1 had higher levels of lignin accumulation constitutively compared to control plants, which was greatly enhanced following inoculation with *S. sclerotiorum*. These results indicate that peroxidase over-expression can lead to significant disease reduction against necrotrophic pathogens in transgenic carrot plants.

Keywords *Botrytis* · Disease resistance · *Sclerotinia* · Pathogenesis-related proteins

Introduction

Carrot (*Daucus carota* 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

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currently constrain carrot production worldwide (Davis and Datnov 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 and Stuiver 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, 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 and Stuiver 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).

Materials and methods

Gene selection and transformation

Chitinase gene 383 isolated from 'scab'(*Fusarium graminearum*)-infected 'Sumai-3' wheat encodes for a class IV PR-3 acidic exo-chitinase of 1,088 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).

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 *Hind*III 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 *Hind*III site of pCambia1300 containing the phosphinothricin *N*-acetyltransferase gene (*bar*; De Block et al. 1987). This confers resistance to phosphinothricin (PPT), the active ingredient of the herbicide glufosinate (Liberty™), under control of the CaMV 35S promoter utilising a *nos* terminator (De Block 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 *Agrobacterium 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 bacterial solution as previously described (Wally et al. 2006). The tissue culture selection and plant regeneration conditions were followed as described previously (Wally et al. 2006).

Characterisation 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 and Punja 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 lyophilised 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 pM 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 *Hin*dIII using previously established protocols for carrot (Wally et al. 2008). 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 manufacturer's protocols, and used as radioactive DNA probes. RNA blot hybridisation 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-hybridised for 3 h at 55°C using Ekono hybridisation buffer (Research Products International Corp, Mt. Prospect, IL, USA). Hybridisation and washing of the membranes was carried using the protocol outlined for Southern blotting (Wally et al. 2008). Quantification of signal intensity was carried out with software Image J (NIH, USA) and normalised to that of 28S RNA.

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 pestle and mortar 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 microcentrifuge 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°C and the supernatant was stored at −80°C, and used in enzyme assays. Ionically bound peroxidase was extracted from the pellet using a high salt Tris buffer (20 mM Tris–HCl, 1 M NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$), incubated at 4°C for 30 min while constantly agitated. The ionically bound fraction was cleared by centrifugation at 10,000 g at 4°C (Bindschedler et al. 2006). Clarified protein extracts were quantified using the Bradford reagent (Sigma, USA) using bovine serum albumin (Sigma) as the standard. There were five replicate leaf samples taken from individual plants for each transgenic line, assayed.

Chitinase activity was assayed by monitoring the release of *N*-acetylglucosamine from colloidal chitin according to the method of Reissig et al. (1955). Chitinase specific activity was expressed as micromoles of *N*-acetylglucosamine mg^{-1} protein h^{-1} at 37°C, and was standardised 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 mg^{-1} protein h^{-1} at 40°C. Peroxidase activity was determined from both the soluble and ionically-bound protein fraction according to Mika and Lüthje (2003), using *o*-diansidine (Sigma) as a substrate. Peroxidase enzyme activity was expressed as the change in absorbance min^{-1} mg^{-1} protein, and standardised against the control extracts.

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 (Wally et al. 2006). 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 diam were taken from the colony edge. Leaves of six transgenic lines each expressing *POC1*, chitinase 383, or β-1, 3-glucanase 638 individually, and two 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 three to four leaves were 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 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. Controls, included non-transformed carrot leaves and non-inoculated leaves. Significant differences between lines were determine 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 programme (SAS Institute, NC, USA). Percentage values were analysed after arcsine-transformation of the raw data.

Lignin extraction and quantification

Total lignin was extracted using the thiogylcolic acid method (Doster and Bostock 1988) from 0.5 g of tissue from mature petioles trimmed to remove leaves

and placed under infection conditions for 3 days, with or without the presence of four *S. sclerotiorum* plugs. Lignin was quantified as A280 readings relative to the reading for the control plants (Doster and Bostock 1988).The experiments were conducted twice with nine replicates for each of the treatments described. Lignin data was analysed for significant differences using ANOVA as described above.

Tissue fixation and microscopy

Small petiole sections (5–10 mm long) from *S. sclerotiorum*-infected leaves of transgenic line P23 and the 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°C for 72 h. 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-hydroxyethyl-methacrylate 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).

Results

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. 1). Molecular analysis using PCR confirmed 89 of the plants to be positive for the *bar* gene (Fig. 2a). PCR

Fig. 1 Response of transgenic (*left*) and non-transgenic 'Nantes Coreless' carrot plants (*right*) to the herbicide Liberty (0.4% *v/v*). Symptoms were visualised 3 days after foliar application of the herbicide

analysis for the presence of the transgenes revealed that there were 50 lines containing *POC1* (Fig. 2b), 25 lines containing β-1, 3-glucanase 638, and ten lines containing chitinase 383 (Fig. 2c). There were also two lines containing *POC1* plus chitinase 383 (*POC1*/383) and two lines containing chitinase 383 plus β-1, 3-glucanase 638 (383/638; Fig. 2c). Southern blot analysis confirmed the PCR results, since all of the lines released the entire cassette of the appropriate size when digested with *Hin*dIII (data not shown). Northern blot hybridisation was conducted on the PCR-positive lines, and a range of transgene transcript expression levels was observed among the lines (Fig. 3). The six lines which displayed high levels of expression for the single transgenes, along with the two lines expressing *POC1*/383 and the two 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. 4a), whereas the chitinase 383 lines had up to an eight-fold increase in chitinase activity compared to controls (Fig. 4b). 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. 4c).

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. 5a). The lines expressing *POC1* alone showed very high levels of resistance. There were no visible lesions on three of the six lines at 4 dai, with a significant reduction in the remaining three *POC1* lines. At 7 dai, four of the lines (P-22, P-23, P-51, P-75) had <10% of the leaf area diseased (Fig. 5b) while the remaining two lines (P-61, P-82) had <25% leaf area diseased (Fig. 6a). By comparison, only one line (C-24) expressing the chitinase 383 gene showed a significant reduction in diseased leaf area. The other five chitinase 383-expressing lines had similar disease levels to that of the non-transformed control plants at both 4 and 7 dai (Fig. 6b). 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. 6b). The two lines expressing a combination of *POC1*/383 had significant reductions in diseased leaf area compared to the non-transformed control line (Fig. 6a). 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. 6b).

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. 5c). When examined microscopically, cells in the petiole section had lost integrity (Fig. 7a), and mycelia were visible within the vascular bundles and collenchyma ribs (Fig. 7b). 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 (Figs. 5d and 6a). The reduction in tissue necrosis was apparent when examined microscopically, as the entire petiole sec-
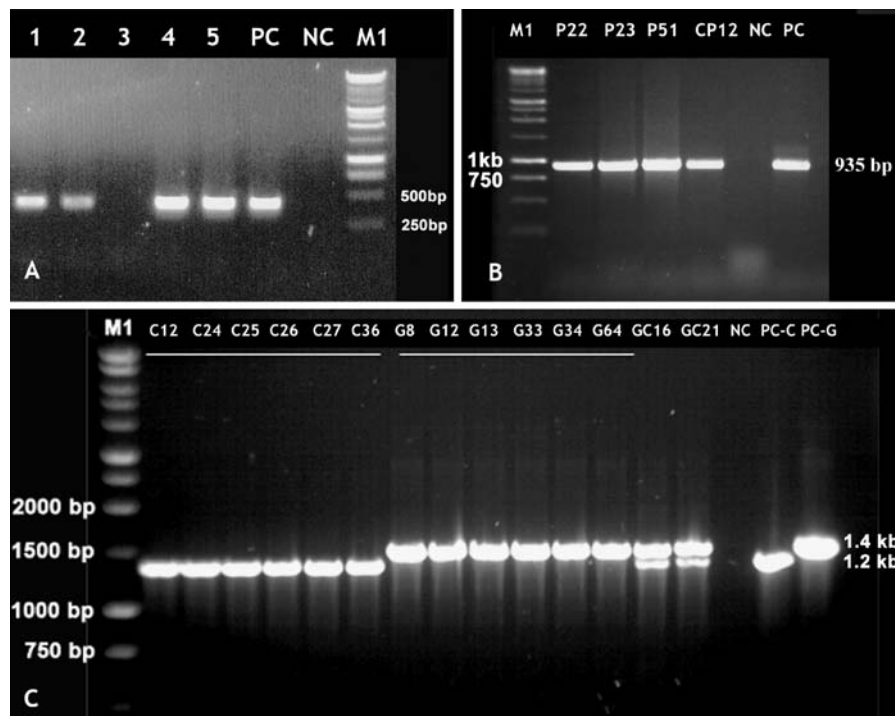
Fig. 2 PCR detection of transgenes in carrot. **a** PCR detection of the *bar* gene. *Lanes 1, 2, 4* and *5* were positive for *bar*, while *lane 3* is an escape plant. *PC* is plasmid control and *NC* is non-transformed carrot control. *M1* is Benchtop 1 Kb ladder (Promega). **b** Detection of the peroxidase (*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

tion had retained integrity (Fig. 7c), and there were no mycelia visible in the sections (Fig. 7d). Similar to that for *B. cinerea* infections, only one line (C-24) expressing chitinase 383 had a significant reduction in diseased leaf area (Fig. 6b). Among the lines over-expressing chitinase 383, line C24 had the highest

level of gene expression (Fig. 3), and this correlated with the highest level of resistance to both *S. sclerotiorum* and *B. cinerea*. The only other chitinase 383-expressing line with reduced disease symptoms to both pathogens was line C36, which expressed chitinase transcript at a slightly reduced level com-
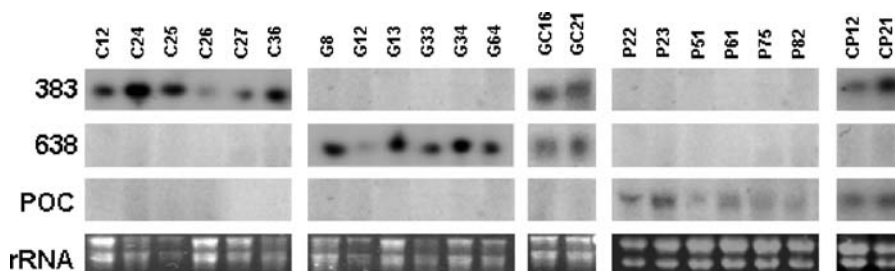
Fig. 3 Detection of expression of wheat chitinase 383, β-1,3-glucanase 638 and rice peroxidase *POC1* in transgenic carrot by Northern hybridisation. Carrot lines transformed with chitinase 383 (*C12–C36*), β-1,3-glucanase 638 (*G8–G64*), co-transformed 383/638 (*GC16* and *GC21*), *POC1* (P22–P82), 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
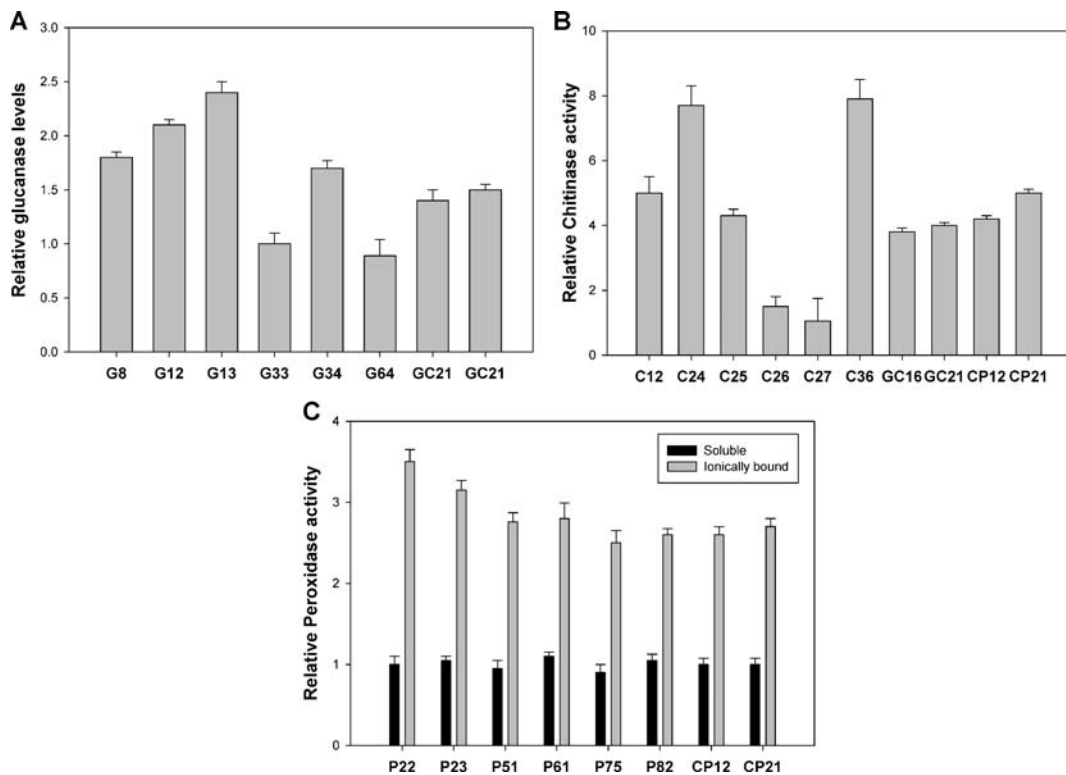
Fig. 4 Enzyme activity in transgenic carrot lines, assayed for **a** β-1,3-glucanase, **b** chitinase and **c** soluble and ionically-bound peroxidase fractions. Enzyme activity is expressed as a ratio of the activity in non-transformed control plants. Lines expressing β-1,3-glucanase 638 (*G8–G64*), 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

pared to line C24, and had a similar level of total chitinase enzyme activity (Fig. 4b). 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. 6b). 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. 6a). 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. 5b).

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 1.39-fold higher than the control plants (Fig. 8). The lignin levels increased in the presence of *S. sclerotiorum*, being 1.79-fold higher than in the control plants (Fig. 8). There was no significant difference between uninoculated or *S. sclerotiorum*-infected control petioles. The data were similar in both experimental trials and were pooled for data analysis.

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

Fig. 5 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 dai
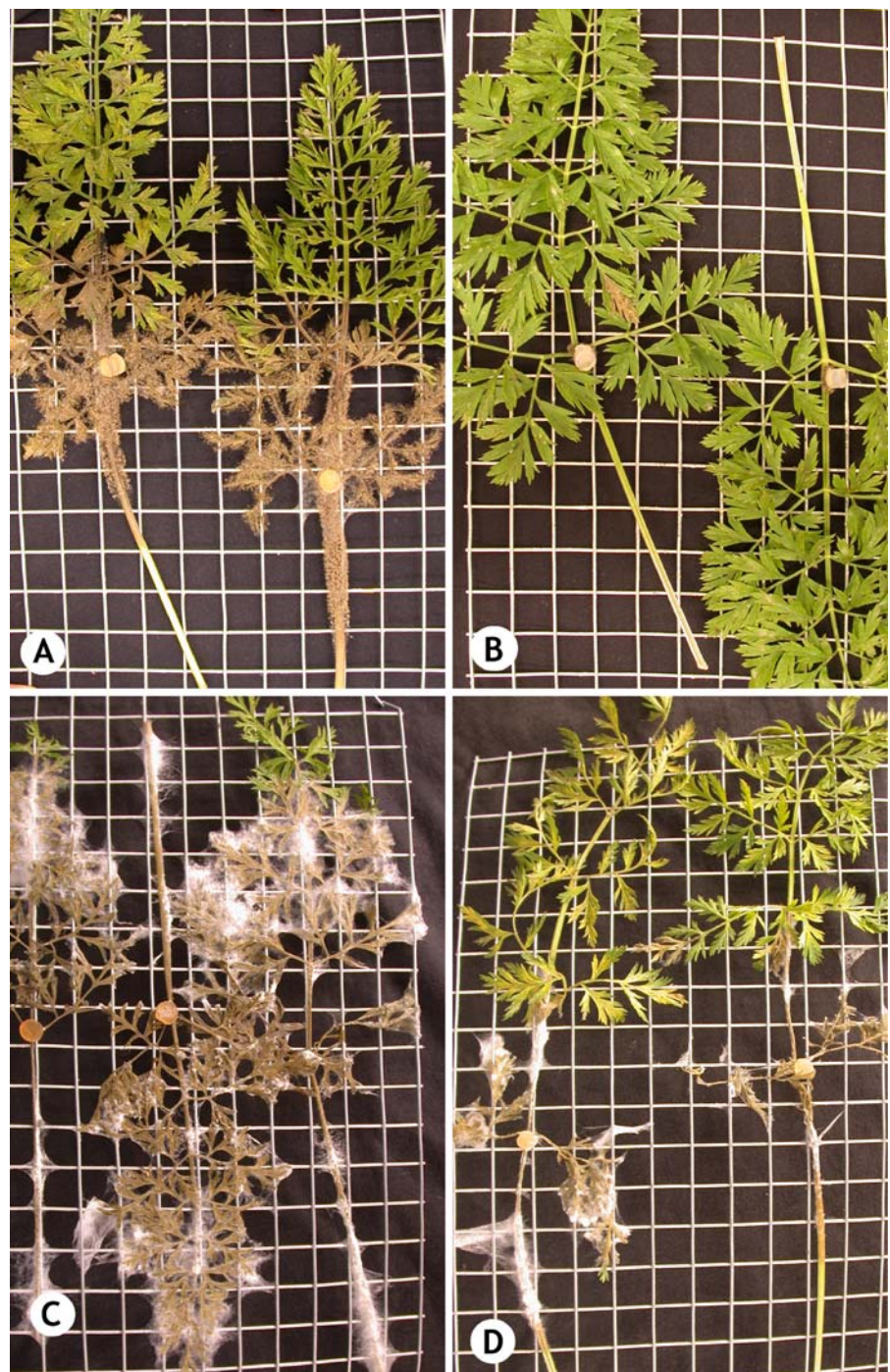
proteins, alone and in combination, to attempt to
increase the overall levels of disease resistance
attained in transgenic carrots.

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 *F. graminearum* under
greenhouse conditions (Anand et al. 2003), where
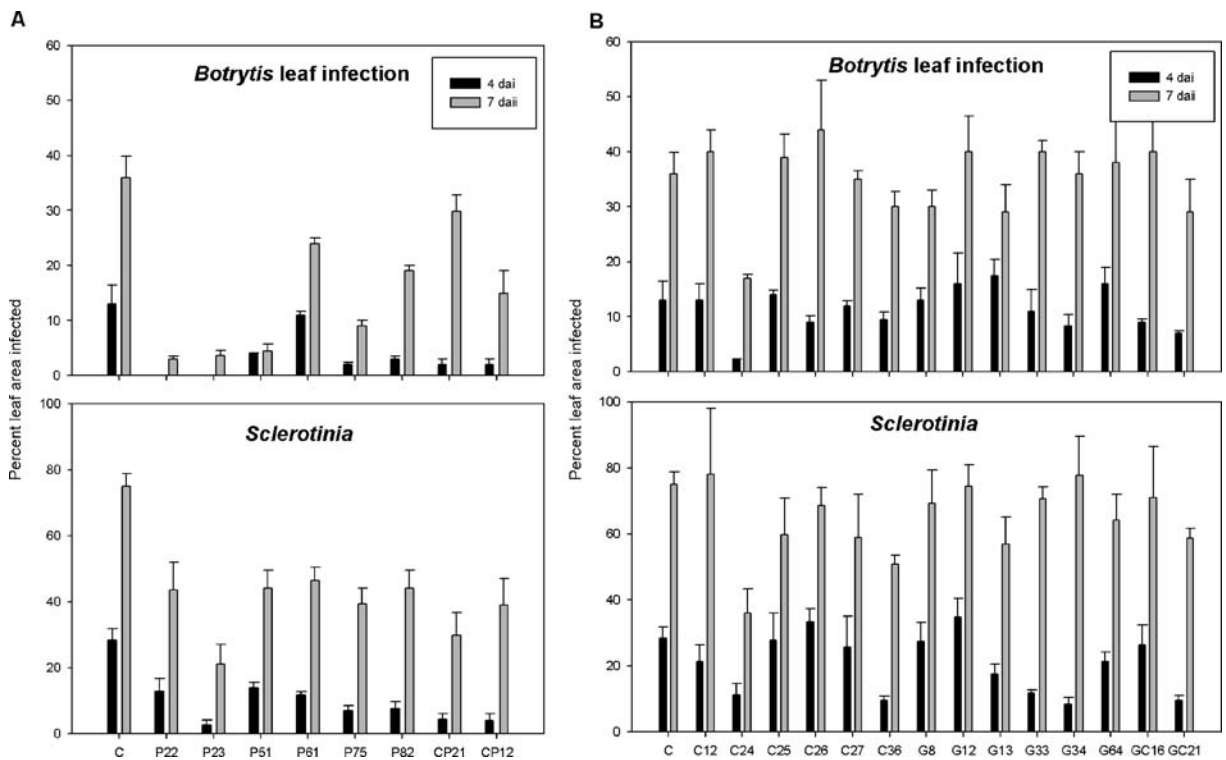
Fig. 6 Mean percent leaf area diseased in transgenic carrots compared to controls. **a** Peroxidase *POC1* over-expressing lines (*P22–P82*), *POC1* and chitinase 383 co-expressing lines (*CP21* and *CP12*) inoculated with *B. cinerea* (*top*) and *S. sclerotiorum* (*bottom*). **b** Carrot lines expressing 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. *Vertical error bars* indicate standard errors for a minimum of three replicates for each of the transgenic lines

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 and Punja 2007), and a basic tobacco chitinase (Punja and Raharjo 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, catalysing 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 (Punja and Raharjo 1996; Ferreira et al. 2007).

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 (Zhu et al. 1994; Jach et al. 1995; Jongedijk et al. 1995; Anand et al. 2003), 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 four-fold increase in enzyme
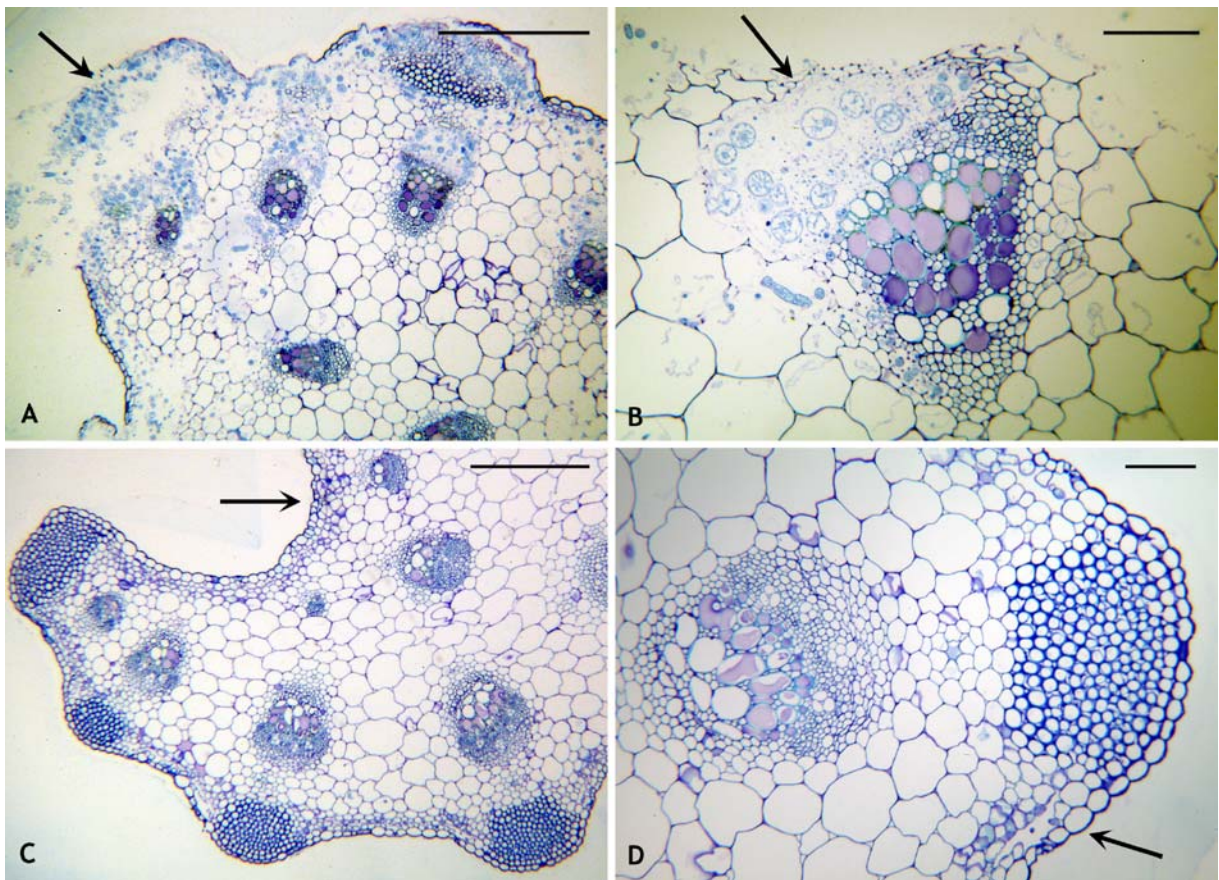
Fig. 7 Histochemical examination of *S. sclerotiorum*-inoculated petioles, 7 dai. **a**–**b** Control petiole sections, showing the breakdown of cuticle, epidermis and mycelial colonisation of vascular bundles and surrounding cells (*arrows*). **c**–**d** Petiole sections from carrot line P23 expressing 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**

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
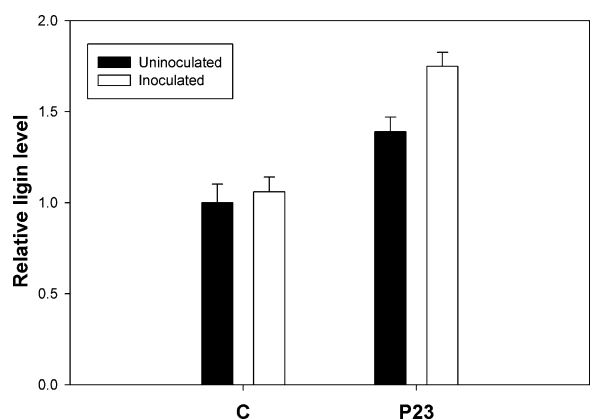
Fig. 8 Total lignin content in control and peroxidase over-expressing carrot line P23 petioles from uninoculated (*black bars*) and inoculated petioles (*white bars*) 3 dai. *Vertical error bars* indicate standard errors from nine replicates for each treatment

(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-expresssing peroxidases have not described enhanced resistance to necrotrophic fungal pathogens (Lagrimini et al. 1990; 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).

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