Tolerance in banana to Fusarium wilt is associated with early up-regulation of cell wall-strengthening genes in the roots

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SUMMARY

Fusarium wilt, caused by the fungal pathogen Fusarium oxysporum f. sp. cubense (Foc), is one of the most destructive diseases of bananas. In the tropics and subtropics, Cavendish banana varieties are highly susceptible to Foc race 4 (VCG 0120). Cavendish selection GCTCV-218 was shown to have significantly lower disease severity and incidence compared with susceptible cultivar Williams in replicated greenhouse and field trials. Suppression subtractive hybridization (SSH) was previously carried out to identify genes induced in roots of GCTCV-218, but not in Williams, after infection with Foc 'subtropical' race 4. Seventy-nine SSH clones were sequenced and revealed 13 non-redundant gene fragments, several of which showed homology to defence-associated genes, including cell wall-strengthening genes. Quantitative RT-PCR was used to confirm up-regulation and differential expression of a number of genes throughout a time-course, following Foc infection in the tolerant GCTCV-218 when compared with susceptible cv. Williams. Tolerance of GCTCV-218 was linked to significantly increased induction of cell wall-associated phenolic compounds.

INTRODUCTION

Musa acuminata (banana) is one of the most important food crops in the world and provides a staple food and source of income in many households especially in Africa (Jones, 2000). Banana production world-wide is under serious threat due to Fusarium wilt caused by Fusarium oxysporum f. sp. cubense (Foc) (Ploetz, 2005). Fusarium wilt of banana has been one of the most devastating agricultural diseases of the past century, after it destroyed thousands of virgin orchards in Central America (Stover, 1962). In the early 1960s, the international banana export trade in Central America was rescued by the timely replacement of Foc race 1-susceptible Gros Michel bananas with resistant Cavendish varieties. Losses of Cavendish bananas to Foc race 4, first in the subtropics (Ploetz, 1990), and then in the tropics (Ploetz, 1994), have raised fears that the world trade in banana might again be threatened. Fusarium wilt has destroyed many thousands of hectares of Cavendish bananas in tropical countries such as Indonesia and Malaysia (Hwang and Ko, 2004). Likewise in South Africa, where bananas are planted in the subtropics, the disease has already been reported from four of the six banana-producing areas (Viljoen, 2002). Moreover, there is also no variety with resistance to Foc race 4 available to replace the seedless, sweet Cavendish banana.

No sustainable control strategy exists for Fusarium wilt of banana, other than replacing susceptible varieties with those resistant to the disease. Conventional breeding efforts to find a resistant replacement for Cavendish bananas have had limited success, often because of the reluctance by consumers to accept the new hybrids (Daniells et al., 1995; Rowe and Rosales, 1993; Stover and Buddenhagen, 1986). Hwang and Tang (1996) therefore initiated a Fusarium wilt screening programme for Cavendish bananas in Taiwan, using the unconventional improvement method for generating and screening somaclonal variants. Two clones, GCTCV-215-1 and 217, with good resistance to Foc ‘tropical’ race 4 (VCG 0121) were found (Hwang, 1999). However, a field selection from Giant Cavendish, known as GCTCV-218, eventually rescued the banana industry in Taiwan from destruction by Fusarium wilt (Hwang and Ko, 2004).

Natural disease resistance exists in wild-type bananas and a few hybrids (Jeger et al., 1995), but these bananas are not acceptable to the Cavendish market and the search for a new tolerant or resistant Cavendish banana is actively being pursued. Conventional breeding strategies are, however, hindered by the fact that Cavendish bananas are sterile and do not produce seed (Robinson, 1996). Therefore, non-conventional strategies such as transformation are more realistic and could be more successful.
RESULTS

Pathogenicity trial

Yellowing of banana leaves and wilting appeared 4–5 weeks after inoculation with *Foc* in the greenhouse. After 6 weeks, Williams plants developed more severe internal symptoms than GCTCV-218. Many of the Williams plants scored the maximum of 5 compared with 1 (few) or 0 (no) internal symptoms in GCTCV-218. Infected Williams plants with a disease score of 5 showed dark-purple vascular discoloration of the entire corm (Fig. 1A), while tolerant GCTCV-218 plants, with a disease score of 0 or 1, had no symptoms (Fig. 1B) or had only isolated reddish-brown speckles within the corm (Fig. 1C). Disease severity values for Williams were 65 and 57% for the two trials, compared with disease incidences for Williams of 52, 76 and 72% (Fig. 1F).

Quantitative RT-PCR

To confirm further the up-regulation of the genes in Table 1 shown previously at 6 hpi using a microarray (Van den Berg et al., 2004), expression levels of four selected defence-associated genes [*catalase 2, PAE, PR-1* and *PR-3* (previously identified in banana)] were tested by quantitative RT-PCR (qRT-PCR) utilizing a Light cycler platform with independent biological samples. The results from independent experiments confirmed the microarray data and revealed a significant, differential induction (compared with cv. Williams) of these genes in the tolerant Cavendish banana, GCTCV-218, in response to *Foc* infection (Table 1). They encoded banana proteins with similarities to two putative peroxidases, two proteins of unknown function, a trypsin inhibitor, *PR-1*, a pectin acetyl esterase (PAE), xylanase inhibitor, metallothionein, ribosomal protein S3a, response regulator 6 (putative role in two component signal transduction), catalase 2 and ferredoxin III (Table 1).

Generation, screening and sequence analysis of subtracted cDNA

A cDNA library of 736 banana clones enriched for genes differentially expressed in GCTCV-218 at 6 h post-inoculation (hpi) of *Foc* race 4 was generated using SSH (Van den Berg et al., 2004). Subsequent high-throughput DNA microarray screening resulted in the identification of 79 clones that were differentially expressed in GCTCV-218 in response to *Foc* (Van den Berg et al., 2004) and these were sequenced.

Fifty-five of the 79 sequences showed significant homology to plant gene sequences and 24 had no significant homology to any sequences in public databases. There were 13 non-redundant differentially expressed gene fragments isolated from the tolerant GCTCV-218 banana cultivar after *Foc* infection (Table 1). They encoded banana proteins with similarities to two putative peroxidases, two proteins of unknown function, a trypsin inhibitor, *PR-1*, a pectin acetyl esterase (PAE), xylanase inhibitor, metallothionein, ribosomal protein S3a, response regulator 6 (putative role in two component signal transduction), catalase 2 and ferredoxin III (Table 1).
Defence-associated genes in bananas against *Foc*  

**Fig. 1** Cavendish banana selection GCTCV-218 shows increased greenhouse and field tolerance to *Fusarium oxysporum* f. sp. *cubense* (Foc) ‘subtropical’ race 4 compared with the banana variety Williams. Disease severity score of 5 observed in Williams plants 6 weeks after infection with *Foc* in the greenhouse (A), compared with disease severity scores of 0 (B) and 1 (C) observed in GCTCV-218 plants after the same treatment. Scale bars = 1 cm. Typical longitudinal splitting of the pseudostem observed in Williams plants infected by *Foc* in the field trials (D). Scale bar = 30 cm. Percentage disease severity of Williams (open bars) and GCTCV-218 (closed bars) bananas infected with *Fusarium oxysporum* f. sp. *cubense* during two independent greenhouse trials (E) and percentage disease incidence during three independent field trials in *Foc*-infected areas in South Africa (F) were calculated according to the formula of Sherwood and Hagedorn (1958). Data were analysed using one-way analysis of variance (**ANOVA**) and the Tukey Honest Significant Difference (HSD) test. Bars presented with the same letter are not significantly different at $P < 0.05$. 

Table 1 BLASTX identities of non-redundant clones derived from the banana suppression subtractive hybridization (SSH) library enriched for transcripts induced in roots of Cavendish banana selection GCTCV-218 after infection with *Fusarium oxysporum* f. sp. *cubense* (Foc) subtropical race 4 compared with the Williams banana variety.

<table>
<thead>
<tr>
<th>Putative identity</th>
<th>Accession no. of best BLASTX hit</th>
<th>Species</th>
<th>E-value</th>
<th>Functional category</th>
<th>Accession no. of SSH clone</th>
<th>No. of clones</th>
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<tr>
<td>Catalase 2</td>
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<tr>
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<td>Cell rescue/defence</td>
<td>DQ 531620</td>
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Fig. 2 Cell-wall-strengthening and defence-related genes are induced to greater extent in roots of Cavendish banana selection GCTCV-218 after infection with *Fusarium oxysporum* f. sp. *cubense* (Foc) ‘subtropical’ race 4 compared with variety Williams. Relative gene expression level at 0, 3, 6, 24 and 48 hpi of catalase 2 (A), pectin acetyl esterase (B), PR-1 (C) and PR-3 (D) in roots of Williams (open bars) and GCTCV-218 (closed bars) bananas after infection with *Fusarium oxysporum* f. sp. *cubense* race 4. Expression ratios were determined by quantitative RT-PCR and are expressed relative to a ‘calibrator’, the expression level for the particular transcript in Williams roots at 0 hpi. Data were analysed using *anova* and the Duncan’s Multiple Range Test. Bars presented with the same letter are not significantly different at *P* < 0.05.
Defence-associated genes in bananas against Foc

Up-regulation of PR-1 occurred in both GCTCV-218 and Williams (Fig. 2C). However, the expression ratio of PR-1 was more substantial after 3 and 6 hpi in GCTCV-218. PR-1 expression was highest at 6 hpi in GCTCV-218, after which it was reduced. PR-3 was up-regulated in both GCTCV-218 and Williams following inoculation with Foc. Maximal expression in Williams occurred at 3 hpi, after which expression was reduced. However, in GCTCV-218, PR-3 induction was highest at 6 hpi, and showed up-regulation throughout the time-course following inoculation (Fig. 2D). PR-3 expression was higher in the tolerant GCTCV-218 than in susceptible Williams at 6, 24 and 48 hpi.

Phenolic assays

The identification of genes involved in cell wall modification, such as those including peroxidase and PAE, prompted an investigation of cell wall-bound phenolic content. GCTCV-218 responded to pathogen infection at 24 and 48 hpi with a significant increase in cell-wall-bound phenolics as well as a significantly higher phenolic content when compared with Williams. By contrast, whilst containing a greater basal level (compared with GCTCV-218) of phenolics prior to inoculation, phenolic content in Williams decreased by 24 hpi (Fig. 3).

DISCUSSION

Pathogenicity trials in the greenhouse and field showed that GCTCV-218 developed significantly less Fusarium wilt than Williams bananas in South Africa. It could thus be considered tolerant to Foc ‘subtropical’ race 4 (VCG 0120). Susceptible Williams plants developed typical Fusarium wilt symptoms both in the greenhouse and in the field. Internal disease symptoms clearly distinguished between susceptible and tolerant bananas in the greenhouse. Susceptible Williams plants in the greenhouse with the highest disease scores showed 100% vascular discoloration. In the field, severely infected Williams plants were entirely chlorotic and in some cases large numbers of plants succumbed to the disease. This result has important implications for the continued cultivation of Cavendish bananas in countries affected by Foc race 4. Hybrids with high levels of resistance to the pathogen, such as FHIA-01 (‘Goldfinger’) (Jones, 2000; Moore et al., 1995) and SH-3640/10 (‘High Noon’) (De Beer, 1997; Eckstein et al., 1996), are not always acceptable to the Cavendish-dominated markets. In these situations, GCTCV-218 could be considered a good replacement for susceptible Cavendish varieties in countries affected by Foc race 4. The evaluation and confirmation of disease tolerance in GCTCV-218 further provided an opportunity to study resistance mechanisms in Cavendish bananas against Foc race 4.

In this study, 13 non-redundant gene fragments associated with tolerance to Foc ‘subtropical’ race 4 were identified in the tolerant Cavendish banana selection GCTCV-218. Nine of the 13 clones showed significant similarities to defence-associated genes, indicating that the tolerant GCTCV-218 banana recognizes either Foc or virulence factors secreted by the pathogen and is able to respond at the transcriptional level, through the induction of defence genes. Four defence-related genes (catalase 2, PAE, PR-1 and PR-3) investigated in this study were significantly up-regulated in GCTCV-218, validating the previous SSH and microarray strategy adopted to investigate genes up-regulated specifically in this variety (Van den Berg et al., 2004).

The induction of PR-1 and PR-3 (endochitinase) in GCTCV-218 following Foc infection, and the marked increase of PR-1 over time, suggests that proteins encoded by these genes are associated with plant defence in banana roots. The induction and greater accumulation of PR-1 in GCTCV-218 after Foc infection could play a role in the successful containment of the pathogen. This is consistent with the study of Pegg and Young (1982), who reported that the release of β-1–3 glucanase and chitinase might serve to inhibit Foc in banana. Van Hemelrijck et al. (2006) reported that F. oxysporum f. sp. cubense for which wild-type Arabidopsis thaliana shows non-host resistance, caused enhanced lesion formation on esa1 as compared with wild-type plants, suggesting that esa1 is more sensitive to Foc. In addition they were able to show that the A. thaliana wild-type resistance phenotype towards Foc could be partially restored by expression of the pathogenesis-related proteins PR1 or PR5 from tobacco in esa1, suggesting that PR1 and PR5 expression may be useful.

traits to obtain enhanced resistance to *F. oxysporum* f. sp. *cubense* in banana.

Catalase gene expression was significantly increased in GCTCV-218 at 6 and 48 hpi. This is consistent with the findings of García-Limones *et al.* (2002), who showed that catalase activities are enhanced in the incompatible interaction between chickpeas and *F. oxysporum* f. sp. *ciceri*. Consequently, they suggested that the expression of catalases in the roots is an early response to *Fusarium* infection. Catalases are known to decrease the levels of *H₂O₂*, which acts as a second messenger for the activation of plant defence responses and has, in addition, been linked to cell wall cross-linking and as a potential toxin against invading pathogens (Clark *et al.*, 2000). Increased catalase activity may be a consequence of increased levels of *H₂O₂* to protect the plant cells against the oxidative burst.

The up-regulation of PAE in GCTCV-218 found in this study is most probably related to the modification of the pectin component in root cell walls, which in turn may affect cell wall strengthening. PAEs catalyse the deacetylation of pectin, which is a major component of plant primary cell walls (Vercauteren *et al.*, 2002). More specifically, PAE hydrolyses acetyl esters in the homogalacturonan regions of pectin, thereby modifying cell walls during root development and pathogen interactions (Savary *et al.*, 2003). PAE has also been demonstrated to be up-regulated in *A. thaliana* roots shortly after nematode infection (Vercauteren *et al.*, 2002).

In addition to PAE, other genes identified in this study imply a role for cell wall modification in resistance to *Foc* race 4. Two of the 13 non-redundant banana cDNA clones isolated from GCTCV-218, 6 h after *Foc* infection, showed significant homology to two class III peroxidases. Peroxidases have been implicated in protecting banana against infection by root pathogens (Ploetz, 1992). Peroxidases are important in the formation of phenolic compounds that lignify host cell walls and vascular gels (Beckman, 1987; Pegg, 1985). High constitutive levels of peroxidase have previously been reported in the *Foc*-resistant banana hybrid SH-3362. For example, a resistant synthetic AA hybrid produced at the breeding programme of the Fundación Hondureña de Investigación Agrícola (FHIA) in Honduras had peroxidase levels ten-fold higher than in Pisang Mas, a susceptible AA cultivar (Novak, 1992). The relative abundance of peroxidase cDNA clones [ten out of 79 identified following SSH and microarray analyses (Van den Berg *et al.*, 2004)] in the tolerant banana GCTCV-218 as early as 6 hpi could indicate that the banana disease response involves lignin production and cell wall strengthening through the incorporation of phenolic compounds into host cell walls. Metallothionein was also isolated 6 h after *Foc* infection from the tolerant GCTCV-218 banana. Metallothionein has been implicated to play a role in the assembly and turnover of cellulose synthase complexes. Jacob-Wilk *et al.* (2006) have proposed a model wherein active cellulose synthase complexes contain CesA proteins in dimerized form, and that the turnover and degradation of the complexes are mediated through reductive zinc insertion by metallothionein and subsequent proteolysis involving a cysteine protease.

Based on the fact that some differentially expressed genes isolated from GCTCV-218 (including PAE and peroxidase genes; Table 1) are implicated in cell wall strengthening, we investigated the cell wall-bound phenolic content in GCTCV-218 and Williams in response to *Foc* and a significant increase in cell wall-bound esterified phenolics was apparent in GCTCV-218 in response to *Foc*. The role of phenolics in defence responses in banana has been illustrated previously. De Ascenso and Dubery (2000) reported a substantial increase in total soluble phenolics in FHIA banana roots 8 h after treatment with elicitors from *Foc* race 4. This is in contrast to Williams, which only responded after 12 h and did not show the same prominent increase in phenolics that were found in the tolerant hybrid. Phenolics are precursors of several secondary metabolites involved in disease resistance, such as phytoalexins and lignin (Matern *et al.*, 1995). Moreover, they may also possibly contribute to the effective and timely production of papillae and gels in response to *Foc*. GCTCV-218 is therefore able actively to induce a structural and biochemical defence response against *Foc*. Apart from simply inducing strong defence responses, GCTCV-218 appears to be able to induce them early enough to contain *Foc* and to prevent further spread.

Plant cell wall-degrading enzymes such as xylanases have been isolated from many *Fusarium* spp. (Gómez-Gómez *et al.*, 2001, 2002), including *Foc* (S. Groenewald *et al.*, unpublished data). The presence of a xylanase inhibitor in the tolerant GCTCV-218 cultivar may therefore play an important role in the plant’s ability to protect itself against pathogen invasion. Moreover, the protection of xylan could imply the role of structural barriers, in the form of cell wall reinforcement and protection of the middle lamella, in resisting *Foc* race 4.

An effective resistance response against *Fusarium* wilt diseases depends on the rate and extent of recognition and activation of the defence mechanisms (Beckman, 1987, 1990). GCTCV-218 showed that it is able to respond rapidly to *Foc* infection by inducing genes involved in biochemical and structural defence mechanisms. Two genes in this study, *PR-1* and *PAE*, were induced very early (3 hpi) in the tolerant defence response, while *PR-3* and *catalase 2* followed with a significant induction at 12 hpi. In Williams, *PR-1* was induced by 3 hpi, showing that *PR-1* is also induced in this variety, but more slowly and to a lesser degree than in the case in GCTCV-218.

Results of this study have provided information on a tolerant plant–pathogen interaction and a soil-borne root pathogen. Both areas of study are relatively poorly explored. The results shed light on genes involved in defence and provide a step towards understanding *Fusarium* wilt of banana and thereby developing an effective disease management strategy.
EXPERIMENTAL PROCEDURES

Plant material and fungal treatment

Micropropagated Cavendish banana plantlets of the variety Williams and selection GCTCV-218 were transferred into plastic cups containing water and maintained in a greenhouse at 18–25 ºC with a 16-h light/8-h dark photoperiod. For pathogenicity tests and quantitative RT-PCR, the roots of the plantlets were wounded by gently crushing the root system, prior to inoculation. Plantlets in cups were inoculated by adding 2.5 mL of the spore suspension (10^6 conidia/mL of each isolate, CAV 045, 092 and 105) to each cup in order to achieve a final inoculum concentration of 2.5 x 10^3 conidia/mL. Sterile distilled water was added to cups containing the control plants in the pathogenicity trial. Plants were kept in the greenhouse until sampling (0, 3, 6, 24 and 48 h) or for a further 6 weeks. Plantlets in the pathogenicity trial were watered only when approximately 50 mL of water was left in the cups. Five replicates with six plants in each replicate were inoculated for both GCTCV-218 and Williams, and the entire experiment was repeated.

Three separate field trials were conducted and monitored over a period of 2 years in the Kiepersol area, Mpumalanga, South Africa. Tissue-cultured banana plantlets approximately 40 cm high were planted in three different fields infested with Foc ‘subtropical’ race 4 (VCG 0120) in January 2002. Experimental plots consisted of a completely randomized block design, with 15 or 20 plants of either GCTCV-218 or Williams per block, dependent on the trial site, and five replicate blocks randomized in the plantation. Standard banana cultivation methods were applied to the trial.

Disease rating

Disease development in the greenhouse was evaluated 4–6 weeks after inoculation using a modified version of the disease severity rating scale for Fusarium wilt of banana (Carlier et al., 2002). The rating scale ranged from 0 to 5, with plants showing no internal symptoms scoring a 0 and plants showing 100% vascular discoloration scoring 5. In the field disease incidence was scored according to the presence or absence of external disease symptoms. Healthy plants were given a value of 0, while diseased plants were scored as 1. Percentage disease severity for greenhouse and percentage disease incidence for field trials were calculated using the formula of Sherwood and Hagedorn (1958): Disease severity (%) = [Σ(no. of plants in a disease scale category)] x (Specific disease scale category)/(Total no. of plants in the trial)] x (Maximum disease scale category)] x 100.

Statistical analysis for the data was conducted using the General Linear Models (GLM) procedure of STATISTICA, version 7 (StatSoft Inc., 2004). Experiments were analysed using one-way analysis of variance (ANOVA) and the Tukey Honest Significant Difference (HSD) test. Significance was evaluated at P < 0.05.

Generation, screening and sequence analysis of subtracted cDNA

The banana SSH library was previously constructed and screened using a high-throughput DNA microarray method (Van den Berg et al., 2004). This quantitative approach allowed us to identify and exclude clones that were not derived from truly up-regulated transcripts.

Nucleotide sequences of the 79 selected SSH cDNA clones were determined on an ABI PRISM 377 DNA analyser (Perkin Elmer, Applied Biosystems, Ontario, Canada) using the BigDye termination Cycle Sequencing Ready Reaction kit (V3) (Perkin Elmer, Applied Biosystems). Vector and SSH adaptor sequences were removed manually using Vector NTI® Suite V.6 (InforMax®, North Bethesda, MD). Sequence homologies were determined with BLAST programs (Altschul et al., 1990) at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST).

Quantitative RT-PCR

Total RNA was extracted from Cavendish banana varieties GCTCV-218 and Williams using the Qiagen RNeasy plant mini kit (Qiagen, Valencia, CA). DNaseI treatment (Fermentas Life Sciences, Hanover, MD) and first-strand cDNA synthesis by random hexamer priming using Power Script TM Reverse Transcriptase (BD, Biosciences, Belgium) were as described previously (Lacomme et al., 2003).

The expression profiles of the four putative defence related genes, PR-1, PR-3, PAE and catalase 2, in GCTCV-218 and Williams bananas were assessed using LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics). PR-3 was previously identified in banana. The cycling conditions were as follows: preincubation for 10 min at 95 ºC (hot start) followed by 40 cycles, each consisting of 10 s denaturing at 95 ºC, 15 s annealing at 59 ºC, 10 s primer extension at 72 ºC and data acquisition at 80 ºC. Each PCR was conducted in triplicate and normalized to Musa 25S rRNA. An independent biological replicate produced similar results.

Primers were designed as balanced pairs of between 58 and 61 ºC Tm to amplify fragments of between 75 and 154 bp using Primer3 (Whitehead Institute, MIT, Cambridge, MA) and Netprimer (Premier Biosoft, Palo Alto, CA). Primer sequences were: PAE (5’-GGCTCTCCTTCTCGATGTTGC-3’; 5’-TCAGCAAG-GCACTTGACCTTT-3’), PR-1 (5’-TCCGGGCTTATTTCCATT-3’; 5’-GCCCATTTCATATGCAAAAGA-3’), PR-3 (5’-GGCTCTGTGTCTTGGAGATG-3’; 5’-CCAACCCCTACATGATGTG-3’), catalase 2 (5’-AACAGCATCTGTGATGGAAGATG-3’; 5’-CGCAGCACTGAAAGATGCGT-3’; 5’-CACTGGTGAATCTTTT-3’; 5’-CTTGTGGTCCACGAGATT-3’).
Expression data were normalized making use of the standard curve for the specific target gene and the endogenous control gene, *Musa* 25S rRNA as previously described (Applied Biosystems, User Bulletin No. 2, 2001).

**Phenolic assays**

*Extraction of phenolics*

Phenolics were extracted using a modification to the method described by De Ascensao and Dubery (2003). Phenolics from the root material (0.05 g) of the control and treated plants (GCTV-218 and Williams) were extracted with 1 mL of a solution containing MeOH/AC/H$_2$O [7 : 7 : 1 (v/v/v)]. The suspension was homogenized for 1 min before being shaken for 1 h at 200 rpm and centrifuged for 5 min at 12,000 g. After centrifugation, the supernatant was saved. The remaining precipitate was re-homogenized and centrifuged as above. The four combined supernatants were concentrated to 1 mL by evaporation in a speedivac (SPD111V vacuum centrifuge) (Savant, Holbrook, NY). Aliquots were weighed (0.01 g) and resuspended in 0.5 M NaOH (1 mL for 10 mg) for 1 h at 96 °C. The resulting alcohol insoluble residue (AIR) yielded the cell wall material that was used to extract the ester-bound cell wall phenolic acids.

*Cell wall-bound phenolics*

The ester-bound phenols incorporated into the cell wall were extracted following alkaline hydrolysis (Campbell and Ellis, 1992). Dry cell wall material (AIR) was weighed (0.01 g) and resuspended in 0.5 M NaOH (1 mL for 10 mg) for 1 h at 96 °C. Cell wall-esterified hydroxycinnamic acid derivatives were selectively released under these mild saponification conditions. The supernatant was acidified to pH 2 with HCl, centrifuged at 12,000 g for 10 min and then extracted with 1 mL diethyl ether (Saarchem, Merck Laboratories). The extract was dried in a speedivac and the precipitate was resuspended in 250 µL 50% aqueous MeOH. This solution was used to determine total soluble phenolic acids, free phenolic acids, MeOH soluble ester-bound phenolic acids and MeOH soluble glycoside-bound phenolic acids. The remaining precipitate was dried at 70 °C for 24 h. The resulting alcohol insoluble residue (AIR) was combined with the first and the procedure was repeated twice further. The four combined supernatants were concentrated to 1 mL by evaporation in a speedivac (SPD111V vacuum centrifuge) (Savant, Holbrook, NY). Aliquots were weighed (0.01 g) and resuspended in 0.5 M NaOH (1 mL for 10 mg) for 1 h at 96 °C. The resulting alcohol insoluble residue (AIR) yielded the cell wall material that was used to extract the ester-bound cell wall phenolic acids.

**Statistical analysis**

Statistical analysis was conducted using the GLM procedure of STATISTICA, version 7 (StatSoft Inc., 2004). Data from the disease rating and phenolic assays were analysed using ANOVA and the Tukey HSD test. Data from the qRT-PCR were analysed using ANOVA and the Duncan’s Multiple Range Test. In all cases significance was evaluated at $P < 0.05$.

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