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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.

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Unfortunately, very few banana genes have been isolated and characterized to date and the banana–*Foc* interaction has not yet been studied extensively at the molecular level. Our current understanding of the genetics of resistance against Fusarium wilt diseases therefore is largely limited to the interaction between *F. oxysporum* and crops such as tomato (Beckman *et al.*, 1982, 1989; Brammall and Higgins, 1988) and carnation (Baayen, 1987; Baayen *et al.*, 1989). A lack of knowledge pertaining to disease resistance mechanisms in banana complicates the matter of transforming susceptible bananas with genes conferring resistance. An analysis of pathogen-induced genes may lead to a better understanding of the molecular processes involved in resistance, and may contribute to the development of biotechnological strategies to combat the disease.

In this study, pathogenicity tests revealed that the Cavendish selection GCTCV-218 was tolerant against *Foc* 'subtropical' race 4 in the greenhouse and the field. Gene fragments associated with tolerance in GCTCV-218 to *Foc* 'subtropical' race 4 were isolated by suppression subtractive hybridization (SSH) and 79 clones were shown to be differentially up-regulated 6 h post-inoculation in GCTCV-218 compared with cv. Williams using microarray technology (Van den Berg *et al.*, 2004). These clones were sequenced to determine their putative identities. The expression profiles of selected putative defence-related gene transcripts in banana roots were compared over time between the tolerant and susceptible banana selections. Phenolic assays further indicated that tolerance to *Foc* 'subtropical' race 4 may be linked to increased cell wall-associated phenolic compounds.

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 severity values of 34 and 38% in GCTCV-218 (Fig. 1E). No symptoms developed in the control plants of either GCTCV-218 or Williams.

Typical external symptoms of Fusarium wilt of banana appeared on susceptible Williams plants as a yellowing of the leaf margins of older leaves. Severely infected bananas showed the yellowing advancing from the oldest to the youngest leaves and the typical 'skirt' of dead leaves around the pseudostem. Longitudinal splitting of the outer leaf-bases of the pseudostem of susceptible Williams plants was also often observed just above soil level (Fig. 1D). In the field GCTCV-218 consistently showed better tolerance to Fusarium wilt than Williams. At the three test sites, GCTCV-218 plants had a disease incidence of 10, 34 and 14%, compared with disease incidences for Williams of 52, 76 and 72% (Fig. 1F).

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).

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* race 4 (Fig. 2).

Catalase 2 expression was higher in the tolerant GCTCV-218 than in susceptible Williams by 6 hpi (Fig. 2A), the time-point selected for SSH and microarray analysis of genes, and was weakly up-regulated at 6 and 48 hpi. *PAE* was up-regulated in GCTCV-218 at 3 hpi with *Foc*, while no up-regulation was observed in Williams. Although the level of up-regulation is reduced, *PAE* expression was higher in the tolerant GCTCV-218 than in susceptible Williams at 3, 6, 24 and 48 hpi (Fig. 2B).

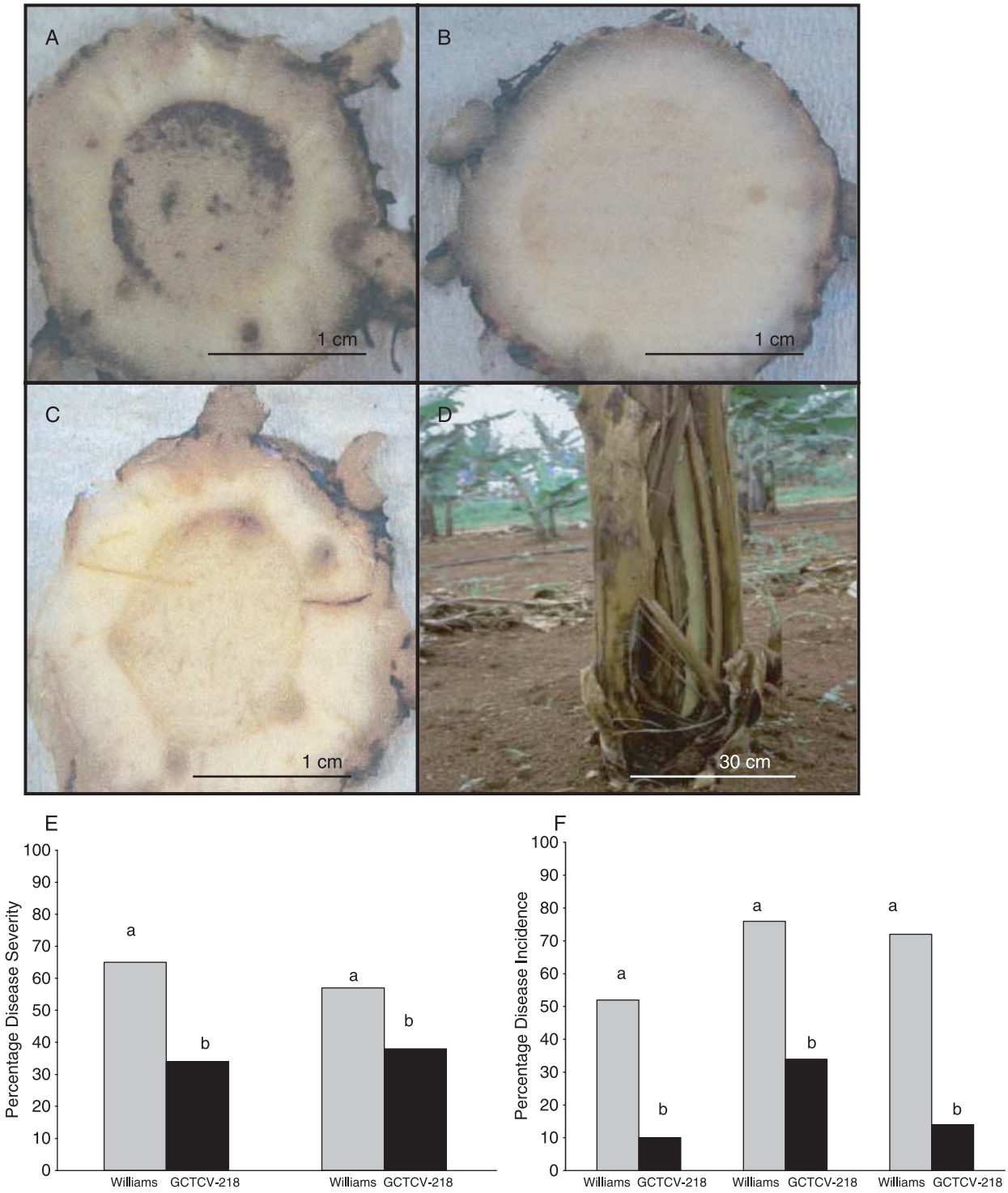

Fig. 1 Cavendish banana selection GCTCV-218 shows increased greenhouse and field tolerance to *Fusarium oxysporum* f. sp. *cubense* (Foc) 'subtropical' race 4 compared with the banana variety Williams. Disease severity score of 5 observed in Williams plants 6 weeks after infection with *Foc* in the greenhouse (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.

Putative identity	Accession no. of best BLASTX hit	Species	*E*-value	Functional category	Accession no. of SSH clone	No. of clones
Catalase 2	AAG61140.2	*Zantedeschia aethiopica*	$5e^{-50}$	Protection against oxidative burst	DQ 531612	1
Ferredoxin III	P27788	*Zea mays*	$3e^{-45}$	Energy metabolism	DQ 531614	1
Metallothionein	AAG44757.1	*Musa acuminata*	$4e^{-27}$	Cell rescue/defence	DQ 531613	2
Pectin acetylesterase	BAC 07121.1	*Oryza sativa*	$6e^{-92}$	Pectin modification	DQ 531615	2
Peroxidase	AB 013389	*Arabidopsis thaliana*	$2e^{-05}$	Secondary metabolism—Lignin biosynthesis	DQ 531609	2
Peroxidase	BAB 19339.1	*O. sativa*	$6e^{-49}$	Secondary metabolism—Lignin biosynthesis	DQ 531618	8
PR-1	AAC25629.1	*Z. mays*	$4e^{-17}$	*In vitro* antifungal activity (defence)	DQ 531622	2
Response Regulator 6	BAB 20581.1	*Z. mays*	$7e^{-56}$	Signal transduction	DQ 531611	1
Ribosomal protein S3a	CAD 56219	*Cicer arietinum*	$2e^{-42}$	Protein synthesis	DQ 531623	1
Trypsin inhibitor	CAA 29122.1	*Vigna unguiculata*	$3e^{-04}$	Protease inhibitor	DQ 531617	4
Unknown protein	CAE 02910	*O. sativa*	$9e^{-12}$	Unknown	DQ 531616	4
Unknown protein	AAL 77110	*Hordeum vulgare*	$4e^{-45}$	Unknown	DQ 531610	4
Xylanase inhibitor	CAD 27730.1	*Triticum aestivum*	$1e^{-34}$	Cell rescue/defence	DQ 531620	2

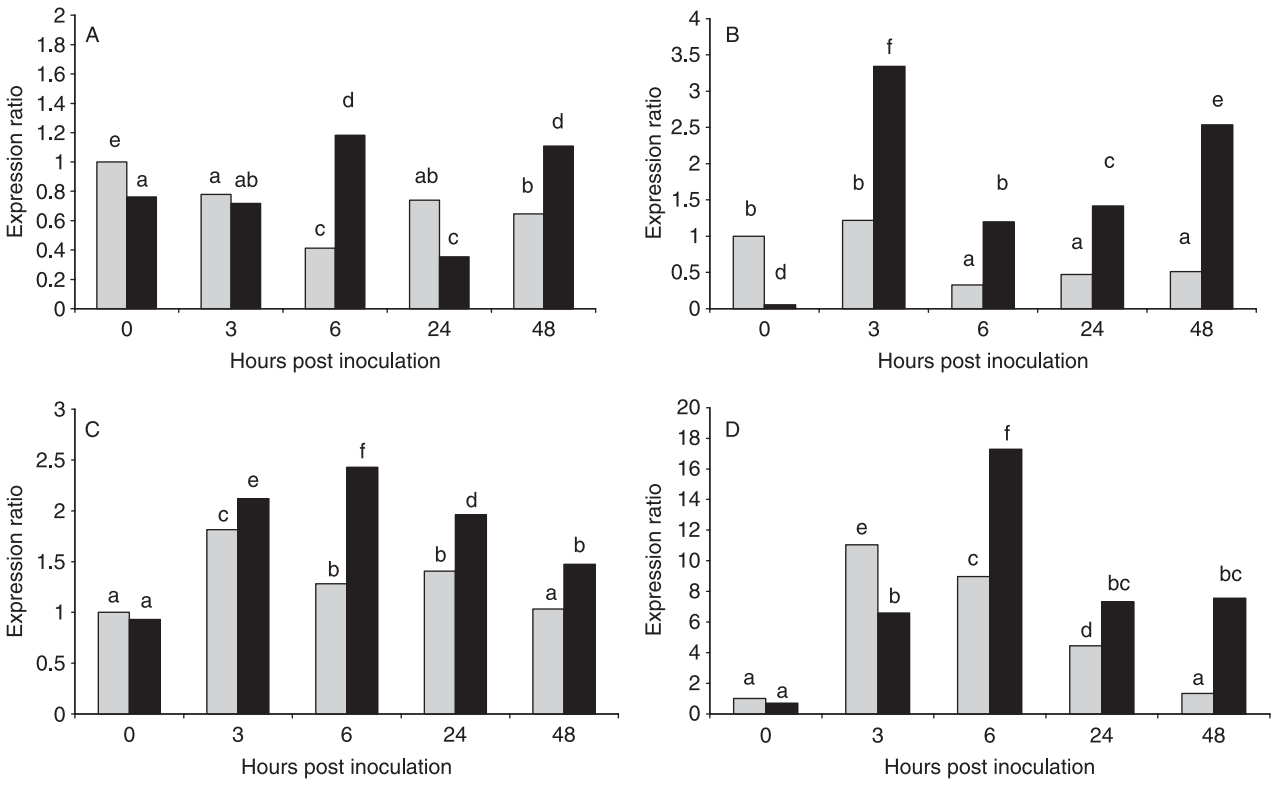

Fig. 2 Cell-wall-strengthening and defence-related genes are induced to greater extent in roots of Cavendish banana selection GCTCV-218 after infection with *Fusarium oxysporum* f. sp. *cubense* (Foc) 'subtropical' race 4 compared with variety Williams. Relative gene expression level at 0, 3, 6, 24 and 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 $P < 0.05$.

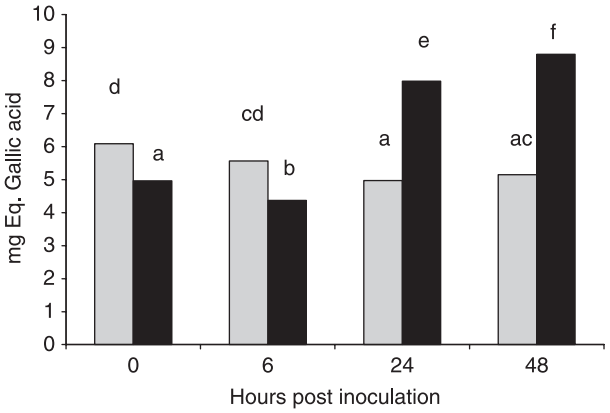

Fig. 3 Cell wall-bound phenolic compounds are significantly increased in roots of Cavendish banana selction GCTCV-218 after infection with *Fusarium oxysporum* f. sp. *cubense* (Foc) 'subtropical' race 4 compared with variety Williams. Cell wall-bound phenolics were extracted from roots of Williams (open bars) and GCTCV-218 (closed bars) were determined with the Folin reagent as milligrams of Gallic acid per gram dry weight. 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 $P < 0.05$.

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/or *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 H_2O_2, 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 H_2O_2 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 compound 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 Hondurereña de Investigacón 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 Ascensao 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 6 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 is 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 °C 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 2.5×10^3 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 (%) = [$\sum$(no. of plants in a disease scale category) × (Specific disease scale category)/(Total no. of plants in the trial) × (Maximum disease scale category)] × 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.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 Master^{PLUS} SYBR Green I (Roche Diagnostics). *PR-3* was previously identified in banana. The cycling conditions were as follows: preincubation for 10 min at 95 °C (hot start) followed by 40 cycles, each consisting of 10 s denaturing at 95 °C, 15 s annealing at 59 °C, 10 s primer extension at 72 °C and data acquisition at 80 °C. 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 °C T_m 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′-GGCTCTCCTTTCTGGATGTTC-3′; 5′-TCAGCAAGGCACTTGACTTTT-3′), *PR-1* (5′-TCCGGCCTTATTTCACATTC-3′; 5′-GCCATCTTCATCATCTGCAA-3′), *PR-3* (5′-GGCTCTGTGGTTCTGGATGA-3′; 5′-CCAACCCTCCATTGATGATG-3′), *catalase 2* (5′-AAGCATCTTGTCGTCGGAGTA-3′; 5′-CGCAACATCGACAACTTCTTC-3′) and *Musa* 25S rRNA (AY651067) (5′-ACATTGTCAGGTGGGGAGTT-3′; 5′-CCTTTTGTTCCACACGAGATT-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 (GCTCV-218 and Williams) were extracted with 1 mL of a solution containing MeOH/AC/H_2O [7 : 7 : 1 (v/v/v)]. The suspension was homogenized for 1 min before being shaken for 1 h at 200 r.p.m. 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 second supernatant 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 speedy vac (SPD111V vacuum centrifuge) (Savant, Holbrook, NY). Aliquots were made in order 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) 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 speedy vac and the precipitate was resuspended in 250 μL 50% aqueous MeOH. This solution was used to determine the cell wall-esterified phenolic acid content with Folin-Ciocalteau reagent. A blank of water was used as control. Gallic acid was used as a phenolic standard to construct a standard curve ranging from 0 to 400 μg/mL^2 ($y = 1.3527x - 0.0109$, $R^2 = 0.9986$). The concentration of phenols in the various extracts was calculated from the standard curve and expressed as μg gallic acid/g dry weight.

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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