Metabolomic, Biochemical, and Gene Expression Analyses Reveal the Underlying Responses of Resistant and Susceptible Banana Species during Early Infection with *Fusarium oxysporum* f. sp. *cubense*

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Abstract

Banana (*Musa* spp.) is an important staple and economic fruit crop, especially in Africa, Southeast Asia, and Latin America. The wilt disease caused by *Fusarium oxysporum* f. sp. *cubense*, especially *F. oxysporum* f. sp. *cubense* strain TR4, is disastrous for banana production. Banana plants infected by *F. oxysporum* f. sp. *cubense* TR4 gradually die from leaf blight or vascular rot. There is no efficient method to control this disease, and the underlying response of banana plants to *F. oxysporum* f. sp. *cubense* remains unknown. In this study, the responses of an economically important banana cultivar, the *F. oxysporum* f. sp. *cubense*-susceptible ‘BX’, and a wild banana relative, the *F. oxysporum* f. sp. *cubense*-resistant *Musa yunnanensis* (‘YN’), to *F. oxysporum* f. sp. *cubense* infection were investigated using metabolomic, biochemical, and molecular biological methods. Numerous metabolomic compounds, including defense-responsive signaling molecules, phytohormones, phenolics, and antioxidants, were identified through metabolomic

analysis. Changes in salicylic acid (SA), methyl-jasmonic acid, abscisic acid (ABA), cytokinin, 3-indoleacetic acid, gibberellic acid, and total phenolic levels were detected using liquid chromatography-mass spectrometry and the Folin-Ciocalteu method. The expression levels of genes involved in the biosynthesis of some defense-responsive compounds were studied through quantitative real-time polymerase chain reaction. The results revealed that the resistant YN had a larger change in SA content and a lower ABA level throughout the early infection period, compared with the levels in BX. The susceptible BX had a lower phenolic content. The resistant YN also expressed pathogenesis-related (*PR*) genes, especially *PR1*, *PR4*, *PR5-1*, and *PDF2.2*, at higher levels than the susceptible BX. These dynamic metabolic and gene-expression profiles from susceptible and resistant banana during the early stage of *F. oxysporum* f. sp. *cubense* infection increase our understanding of the complex interaction response between this crop and its pathogen.

Banana (*Musa* spp.) serves as a staple food, due to its high nutritional and carbohydrate contents, to people in areas such as Africa, Southeast Asia, and Latin America, and is a favored fruit around the world. According to Food and Agriculture Organization estimates, more than 100 million tons of banana fruit were produced worldwide in 2013, 54.6% of which were in Asia (<http://www.fao.org/faostat/en/#data/QC/visualize>). However, in recent decades, this crop has been seriously threatened by Fusarium wilt disease caused by the soilborne fungus *Fusarium oxysporum* f. sp. *cubense*. The virulent strain *F. oxysporum* f. sp. *cubense* tropical race 4 (TR4) is particularly lethal to the main commercial cultivar, ‘Cavendish Brazilian’ or ‘BaXi’ (‘BX’), accounting for approximately 45% of all banana production in the world (Ploetz 2015). *F. oxysporum* f. sp. *cubense* TR4 causes substantial yield loss, because it usually breaks out during the fruiting period. It chokes the xylem vessels, eventually killing the plant. In addition, the fungus spreads rapidly from infected fields through rain, irrigation, and foot traffic. There is still no effective method to control this disease in infected banana fields (Ploetz 2006, 2015).

Recent research has shown that many wild banana relatives are highly resistant to TR4 (Chen et al. 2006; Huang et al. 2005; Li et al. 2015c). The wild species *Musa yunnanensis* (‘YN’) from Yunnan Province, China, can tolerate seasonal frosts (Häkkinen and Wang 2007) and exhibits more resistance in the field than in greenhouse conditions (Li et al. 2015c). Wild resistant banana species are an essential natural resource for research on the response of banana plants to TR4 infection, the susceptible mechanism of BX, and the underlying pathogen control methods.

Extensive work in model plants has shown that metabolites such as the hypersensitive response signal molecules salicylic acid (SA) and

jasmonic acid (JA) (Alvarez 2000); the growth-regulating phytohormones abscisic acid (ABA) (Adie et al. 2007; Xie et al. 2011), 3-indoleacetic acid (IAA) (Park 2007), cytokinin (CTK), and gibberellic acid (GA) (Ha et al. 2012; Novak et al. 2013); the antioxidants glutathione and ascorbic acid (Bao et al. 2016; Kovacs et al. 2015; Sinha et al. 2015; Xu et al. 2013); the defense compounds polyphenol, phenolic acids, and flavonoids (García-Sánchez et al. 2012; Joshi et al. 2015); and the organic acids syringic acid, gallic acid, and sinapic acid (Barding et al. 2013) are induced to respond to pathogens and active plant defense. During plant–pathogen interactions, signal molecules and phytohormones induce the expression of pathogenesis-related (*PR*) genes. For instance, *PR-1*, *PR-2*, and *PR-5* are SA induced whereas *PR-3*, *PR-4*, and *PR-12*, as well as polyphenol oxidase (PPO) activity, are regulated by JA (Bosch et al. 2014; Shah et al. 2001; Thomma et al. 1998).

In banana, plant resistance depends on the rate and extent of phenolic production and lignin deposition, as well as an increase in cell-wall strengthening-related enzymes (Beckman 1990; De Ascensao and Dubery 2000). Some resistant banana plants exhibit significantly increased cell-wall-associated phenolic compounds (van den Berg et al. 2007) in addition to an increase in soluble and wall-bound phenolics and phenolic polymers in banana roots as part of anti-*F. oxysporum* f. sp. *cubense* defense activation (de Ascensao and Dubery 2003).

Using high-throughput sequencing, numerous genes involved in defense response pathways, including SA, JA, ethylene (ET), and *PR* genes, have been identified in the response of banana plants to *F. oxysporum* f. sp. *cubense* infection (Li et al. 2012; Li et al. 2013a; Li et al. 2013b; Wang et al. 2012). Exogenous SA contributed to the resistance of BX to *F. oxysporum* f. sp. *cubense* TR4 (Wang et al. 2015). Research also shows that thaumatin-like protein (*TLP* or *PR-5*) and β -1,3-glucanase had higher expression levels in resistant species compared with the susceptible BX (Li et al. 2015a), and the transgenic banana plants with rice *TLP* gene shows an enhanced resistance to TR4 (Mahdavi et al. 2012). The levels of expression of *PR* genes (for instance, *PR-5*), phenylalanine ammonia-lyase 3 (*PAL3*), and allene oxide synthase 2 (*AOS2*) are greatly increased in BX to TR4 infection compared with *F. oxysporum* f. sp. *cubense* strain TR1 (nonvirulent to BX) infection (Li et al. 2013a).

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Although the defense response has been well studied in model plants, little information is available on the metabolic changes, transcriptional regulation, or physiological responses of bioactive and signaling compounds during infection of banana with *F. oxysporum* f. sp. *cubense* TR4. In this study, we investigated changes in metabolic compounds and gene expression in susceptible and resistant banana infected with *F. oxysporum* f. sp. *cubense* through metabolomic, biochemical, and molecular biological methods. The results showed that the response differences between two banana species during TR4 infection included signal molecules, phytohormones, antioxidants, and phenolics. The comparative analysis also illustrated the dynamic profiles of metabolites and gene expression during the early stage of the interaction between *Fusarium* spp. and banana.

Materials and Methods

Plant materials. Susceptible BX banana is a Cavendish subgroup and the resistant species *M. yunnanensis* (YN) is a wild banana relative belonging to the *M. acuminata* subspecies (Häkkinen and Wang 2007). Banana plantlets were propagated by tissue culture under sterile conditions. Suckers were used for multiplication and rooting by placing them in plastic pots filled with soil and vermiculite (1:1). The plantlets were placed in a growth room at 28°C with a period of 16 h of light and 8 h of darkness and a light intensity of 5,000 lux for 90 days.

***Fusarium* strain and generation of the green fluorescent protein line.** The strain TR4 VCG01213/16 was isolated from the Hainan Island of China by Dr. Junsheng Huang. The strain, transformed with green fluorescent protein (GFP), was constructed and kept in our research laboratory (Li et al. 2013a). Protoplasts of TR4 were transformed through a method of polyethylene glycol/CaCl₂-mediated transformation, as described previously (Lorang et al. 2001; Xiao et al. 2009).

Pathogen preparation, inoculation, and microscopic observation of the infection process. The GFP-expressing strain was used for all infection processes of 90-day old plantlets. A small block of TR4 was transferred to potato dextrose agar (PDA) and grown at 28°C for 72 h in darkness. For inoculation, three wounds were artificially made into the root using a sterile dissecting needle and covered with a freshly prepared TR4 agar block of about 0.5 cm² (Supplementary Fig. S1). For mock inoculation, the roots were wrapped with a PDA block without *F. oxysporum* f. sp. *cubense*. For microscopic examination, banana roots were washed with sterile, distilled water before being prepared for observation under a laser confocal microscope (Olympus FV10ASW) equipped for GFP detection (488 nm) and root autofluorescence detection (543 and 595 nm). Roots were collected at 3, 27, and 51 h after inoculation for metabolomic analysis and RNA extraction. The roots were frozen in liquid nitrogen and stored at -80°C until use.

Metabolomic analysis. Six treated and six mock-inoculated banana plantlets, including three to five roots per plant, were collected at the same time. Roots (100 mg) were ground in liquid nitrogen and extracted with 800 µl of 70% methanol (vol/vol) in a sonication bath for 10 min. The mixture was centrifuged at 12,000 × g for 10 min. The supernatant was transferred to a new tube for analysis.

Metabolomic analysis was conducted by Shanghai Senschip Info-tech Co. through reversed-phase high-performance liquid chromatography (HPLC) (Agilent 1290) quadrupole time-of-flight mass spectrometry (Q-TOF-MS) with an electrospray ionization source (Agilent 6500). Separation was achieved with a C18 column (100 by 2.1 mm, interior diameter = 1.8 µm; Waters) at 40°C using A, ultrapure water with 0.1% (vol/vol) formic acid and B, acetonitrile with 0.1% (vol/vol) formic acid. The gradient program was 0 to 1 min 5% B, 1 to 13 min linear gradient from 5 to 95% B, and 13 to 15 min 95% B.

Typical total ion chromatograms for each ionization technique were conducted in both positive and negative ion modes. MS parameters were 4.0 kV capillary voltage and 35 and 50 kV sampling cone for positive and negative modes, respectively, at 100°C. Desolvation temperature was 350 and 300°C and the desolvation gas flow was 600 and 700 liter/h for positive and negative modes, respectively. The extraction cone voltage was set at 4 V. The system was equipped with 0.03 s scan time, 0.02 s interscan time, and 50 to 1,000 m/z scan range.

Metabolic data processing and analysis. The raw liquid chromatography (LC) data were processed using Mass-Hunter Qualitative Analysis software (version B.03.01) and the MS identification through the METLIN metabolite database (<http://metlin.scripps.edu>). Principal components analysis and orthogonal projection to latent structure discriminant analysis (OPLS-DA) were conducted by SIMCA-P+ software (version 11.0). A *t* test (*P* < 0.05) was applied to determine statistically significant differences between groups.

Determination of phytohormone and phenolics content in banana plants. SA and methyl (Me)-JA were directly measured using a previous method, with modifications (Yang et al. 2005). Briefly, 5 g of ground fresh banana roots were extracted with 15 ml of 80% (vol/vol) methanol containing 1% acetic acid (vol/vol) for 12 h at 4°C. After centrifugation, the supernatant was extracted using a 3:1 mixture of 0.2 M Na₂HPO₄/H₃PO₄ (vol/vol) and 2 ml of petroleum ether at 4°C for three times. The water phase was adjusted to pH = 8.0 by Na₂HPO₄ and was twice extracted with an equal volume of ethyl acetate. The ester phase was evaporated at 20°C and dissolved in 50% methanol (vol/vol) to 1 ml for LC-MS analysis.

ABA, CTK, IAA, and GA were directly measured as described previously (Li et al. 2015b). SA and Me-JA were also analyzed by HPLC using a methanol/H₂O/phosphate mixture at 55:44.9:0.1 (vol/vol/vol) and methanol/H₂O/acetic acid at 54:43.5:2.5 (vol/vol/vol), respectively, at 220 to 280 nm. The chromatographic peaks were identified by a combination of LC (Ultimate 3000) and tandem MS (AmaZon SL) (LC-MS). The authentic standards were IAA (MW = 175.18), (+)-ABA (MW = 264.32), transzeatin (CTK; MW = 219.28), GA (MW = 346.37), Me-JA (MW = 224.30), and SA (MW = 138.12) (Sigma-Aldrich).

For total phenolics measurement, fresh TR4-inoculated and mock-inoculated roots (1 g) were ground and treated with 3 ml of 60% methanol (vol/vol) using a slightly modified method (Pérez-Jiménez and Saura-Calixto 2005). Extraction was carried out in a shaking water bath at 80°C for 15 min under 380 rpm for three times. Supernatant was collected by centrifuging at 12,000 × g for 30 min and kept at 4°C for analysis. Total phenolic content was determined using the Folin-Ciocalteu method at 765 nm (Singleton and Rossi 1965).

RNA extraction and quantitative real-time polymerase chain reaction analysis. Total RNA was extracted from TR4-inoculated and mock-inoculated roots using the modified cetyltrimethylammonium bromide method (Ruan et al. 2011). First-strand cDNA synthesis was performed with 1.5 µg of total RNA using the FastQuant RT kit according to the manufacturer's instruction (KR106; Tiangen). Reactions were run on an Applied Biosystems StepOne Real-Time polymerase chain reaction (PCR) system with a 96-well plate. Gene-specific primers were designed based on the gene sequence information (<http://banana-genome-hub.southgreen.fr/>). Specific genes and primers for quantitative real-time (RT-q)PCR are listed in Supplementary Table S1.

The PCR was 95°C for 30 s, followed by 40 cycles of 5 s at 95°C and 30 s at 58 to 60°C. At the end of each experiment, a melt-curve analysis was performed using the default parameters (15 s at 95°C, 1 m at 55°C to 95°C in 0.3°C increments, and 15 s at 95°C). The relative expression levels of the target genes were calculated by the 2^{-ΔΔC_t} method (Livak and Schmittgen 2001). The β-actin gene and glyceraldehydes-3-phosphate dehydrogenase 2 were employed as internal references to normalize the transcriptional levels of target genes.

Results

***F. oxysporum* f. sp. *cubense* TR4 infection process in BX and YN banana plants.** *F. oxysporum* f. sp. *cubense* usually invades the entire vascular system of pseudostems through damaged roots. GFP-expressing TR4 was used to monitor the pathogen infection processes between YN and BX, in tissues with similar fluorescence intensity, under a confocal microscope. Spores and hyphae of TR4 had attached to the epidermal cells of the banana roots 3 h after infection (Fig. 1A and E), and had penetrated into the cortex parenchyma at 27 h (Fig. 1B and F). At 51 h, a greater amount of hyphae and spores were in the cells of the banana roots and into the endodermis of the vascular tissues in both BX (Fig. 1C) and YN (Fig. 1G). Hyphae were also observed in the endodermis of the vascular tissues

5 mm above the infection sites of both BX (Fig. 1D) and YN (Fig. 1H) after 51 h. No apparent difference in infection process between BX and YN was observed in our experiment.

Molecular signaling and phytohormone metabolite profiles. A series of pairwise OPLS-DA were applied to maximize the discrimination between experimental samples and to focus on metabolic variations significantly contributing to the resulting classifications. The differences between the control and infected groups in the OPLS-DA suggested that significant biochemical perturbation occurred in these samples (Supplementary Fig. S2). All significantly changed compounds ($P < 0.05$, with the variable importance in the projection > 1.0) between the TR4-treated and mock-inoculated banana plants are listed in Supplementary Table S2.

Investigation of the signal molecules and phytohormone metabolites showed that compounds related to SA and JA seemed to be inhibited at 3 h after inoculation in the susceptible BX (Table 1). In BX, some SA- and JA-related compounds were generally higher at 27 h than at 3 h after inoculation, such as SA, formylsalicylic acid, β -D-glucopyranosyl-11-hydroxy jasmonic acid, and Me-JA. However, SA and formyl-SA decreased to 0.74- and 0.52-fold higher than mock-inoculated, respectively, at 51 h in BX. In the resistant YN, SA and formyl-SA were induced at 3 h and remained at higher levels throughout the infection process (Table 1). Formyl-SA showed a

larger fold change than SA at 51 h after being inoculated in YN. β -D-glucopyranosyl-11-hydroxyjasmonic acid and Me-JA were up slightly at 3 h but decreased at 27 h after inoculation.

Some plant growth regulators also exhibited different profiles during *F. oxysporum* f. sp. *cubense* infection, depending on the plant material (Table 1). For example, ABA had a slight decrease at 3 h but increased from 27 to 51 h after inoculation in BX, while the levels of ABA glucose ester increased significantly to 1.51-fold in the BX cultivar throughout the early stages of infection. In contrast, the levels of ABA and ABA glucose ester all decreased in YN compared with the mock-inoculated YN. The content of zeatin (transzeatin) showed a small increase in BX but considerable upregulation in YN 27 h after inoculation. The levels of tryptophan involved in the biosynthesis of IAA were also significantly upregulated in the infected BX at both the beginning of inoculation and 51 h after inoculation. The noteworthy difference was that tryptophan decreased in BX but increased in YN (up 3.76-fold) 27 h after inoculation, whereas it increased in BX but decreased in YN at 3 and 51 h after inoculation. In contrast, levels of compounds related to GA were not significantly different under our criteria.

Concentrations of signal molecules and phytohormones in banana plants during *F. oxysporum* f. sp. *cubense* infection. The directly measured concentrations of SA, Me-JA, ABA, CTK, IAA, and GA fluctuated throughout the interaction between banana plants and

Fig. 1. Examination of the infection process using green fluorescent protein-expressing *Fusarium oxysporum* f. sp. *cubense* TR4. **A to D**, BaXi and **E to H**, *Musa yunnanensis*. **A**, **C**, **E**, and **G** were longitudinal sections and **B**, **D**, **F**, and **H** were transverse sections of banana plant roots.

Table 1. Phytohormone metabolites detected by liquid chromatography quadrupole time-of-flight mass spectrometry in banana infected by *Fusarium oxysporum* f. sp. *cubense* strain TR4^a

VIP ^b	RT (min) ^c	Mass	Name	Fold changes (BX)			Fold changes (YN)		
				3 h	27 h	51 h	3 h	27 h	51 h
1.713	4.710	138.032	Salicylic acid	0.62↓	1.82↑	0.74↓	3.01↑	2.13↑	1.20↑
2.049	4.442	166.027	Formylsalicylic acid	0.65↓	1.32↑	0.52↓	1.61↑	1.26↑	1.43↑
1.889	5.020	388.173	β -D-glucopyranosyl-11-hydroxyjasmonic acid	0.60↓	2.09↑	2.06↑	1.33	0.56↓	1.47↑
1.208	10.130	224.142	Methyl jasmonate	0.91↓	1.21↑	1.12	1.24	0.83↓	1.89↑
1.578	4.520	264.137	Absciscic acid	0.85↓	1.11↑	1.02	0.95↓	0.73↓	0.81↓
1.710	5.930	426.189	Absciscic acid glucose ester	1.84↑	1.85↑	2.70↑	0.48	0.38↓	0.22↓
1.394	2.950	219.111	Zeatin (transzeatin)	1.17	1.27	1.04	0.86↓	1.38↑	0.91
1.812	4.431	204.090	Tryptophan	1.48↑	0.62↓	1.66↑	0.75↓	3.76↑	0.46↓

^a Fold change was the peak area ratio of the *F. oxysporum* f. sp. *cubense*-inoculated banana over the mock-inoculated banana according to the results of high-performance liquid chromatography. BX = BaXi and YN = *Musa yunnanensis*. Up and down arrows indicate significant increasing and decreasing, respectively ($P < 0.05$).

^b VIP = variable importance in the projection (> 1).

^c Retention time.

TR4 (Fig. 2). The levels of SA increased in the infected YN over the mock-inoculated YN (Fig. 2A, circles). The increase in SA content in the infected YN was as high as 2.6-fold over that in the mock-inoculated YN. In the susceptible BX, the concentration of SA did not significantly change between the infected and mock-inoculated BX at the beginning of inoculation. Interestingly, SA was lower in the infected BX compared with the mock-inoculated BX at 27 and 51 h after inoculation (Fig. 2A, squares).

Me-JA showed a peak increase in the infected BX plants at 27 h after inoculation, while remaining flat in the infected YN plants from 3 to 27 h after inoculation and increasing greatly at 51 h (Fig. 2B). The content of Me-JA increased in the mock-inoculated YN at 27 h after inoculation (1.14-fold over the infected YN). However, the level of Me-JA (119.13 ng g fresh weight [fwt]⁻¹) in the infected YN greatly increased over the mock-inoculated YN at 51 h after inoculation and was higher than that of SA. Furthermore, the levels of Me-JA were higher in the infected YN than in the infected BX (80.12 ng g fwt⁻¹) at 51 h after inoculation.

The concentration of ABA in BX was clearly higher than that in YN 3 to 51 h after inoculation (Fig. 2C). The infected BX had higher ABA content than the mock-inoculated BX at 27 h after inoculation, while the infected YN had lower levels of ABA than the mock-inoculated YN. Furthermore, the peak content of ABA (242.13 ng g fwt⁻¹) in the infected BX at 27 h was twofold higher than in the infected YN (95.89 ng g fwt⁻¹). This measurement was consistent with the metabolomic data that showed that YN had lower fold changes in ABA levels than did BX from 27 to 51 h after inoculation. The lower ABA concentration in the resistant plants and the smaller increase in ABA levels after infection in resistant plants compared with the mock-inoculated banana might be important in the banana mechanisms of resistance to *F. oxysporum* f. sp. *cubense*.

In contrast, IAA in the infected YN was higher than that in the infected BX throughout the time course. IAA reached its maximum (241.45 ng g fwt⁻¹) at 27 h in the infected YN. The response was the opposite in the susceptible cultivar, in which IAA in the infected BX decreased to levels lower than in the mock-inoculated BX. At 51 h, the two cultivars showed change similar to those at 3 h (Fig. 2D).

The concentrations of CTK and GA₃ in banana were lower in the resistant YN than in the susceptible BX during the whole infection.

The level of CTK in the infected YN was higher than that in the mock-inoculated YN only at 27 h, whereas it was higher in the infected BX than in the mock-inoculated BX from 3 to 51 h after inoculation (Fig. 2E). As for GA₃, it seemed that all infected banana plants had lower levels than the mock-inoculated banana plants. Furthermore, the resistant YN had lower levels of GA₃ than the susceptible BX during the whole infection (Fig. 2F).

Profiles of phenolic compounds and antioxidants in banana roots responding to *F. oxysporum* f. sp. *cubense* infection. The polyphenols syringic acid, gallagic acid, and sinapic acid were all induced in the infected BX at 3 h (Table 2) but syringic and gallagic acids were downregulated at 27 h. In the resistant YN, these compounds remained higher in the infected YN than in the mock-inoculated from 3 to 51 h. Gallagic acid was increased 1.43- and 1.54-fold at 27 and 51 h, respectively, and syringic acid was up 1.78-fold at 3 h after inoculation.

Flavonoids and catechins were also regulated by *F. oxysporum* f. sp. *cubense* infection in banana plants. These compounds were greatly increased 3 h after BX was inoculated with TR4 but only two compounds (trihydroxy-flavone-glucoside and pyrocatechol) were increased at 27 h, and three were significantly decreased at 51 h after inoculation (Table 3). These compounds were significantly upregulated in the infected YN at 27 h after inoculation. In particular, in the infected YN, trihydroxyflavone glucoside was increased 2.59-fold at 27 h and 1.74-fold at 51 h, glucopyranosylcatechin was up 2.72-fold at 27 h, and pyrocatechol was up 1.66-fold at 51 h. Furthermore, phyllofavanine and pyrocatechol were not detected in the mock-inoculated YN. The profile of these compounds implied that the resistant banana cultivar YN upregulated these compounds in response to *F. oxysporum* f. sp. *cubense* infection.

The concentration of total phenolics in banana plants during infection by TR4 was also measured. Total phenolic content in the infected banana roots (Fig. 2, filled shapes) was higher than in the mock-inoculated roots in both varieties. Furthermore, the phenolics were higher in the infected YN than in the infected BX from 3 to 51 h after inoculation (Fig. 3). The concentration of total phenolics in the infected YN was 1.4-fold higher than in the infected BX at 27 h and 1.5-fold higher at 51 h after inoculation. It was interesting that the concentration of total phenolics increased gradually in the

Fig. 2. Concentration of **A**, salicylic acid (SA); **B**, methyl-jasmonic acid (Me-JA); **C**, absciscic acid (ABA); **D**, cytokinin (CTK); **E**, 3-indoleacetic acid (IAA); and **F**, gibberellic acid (GA) during banana infection by *Fusarium oxysporum* f. sp. *cubense* through high-performance liquid chromatography mass spectrometry. Error bars represent standard error from six samples and fwt = fresh weight. Infected BaXi (BX, filled square) and mock-inoculated BX (open square). Infected *Musa yunnanensis* (YN, filled circle) and the mock-inoculated YN (open circle).

infected YN but decreased gradually in the infected BX. The concentration of total phenolics in the mock-inoculated YN was also higher than in the mock-inoculated BX from 27 to 51 h. The levels in the mock-inoculated YN (51 h) were also higher than in the infected BX, indicating that the response may be mediated through wounding. The trends detected via Folin-Ciocalteu for total phenolics were almost consistent with the results of the metabolomic analyses in Table 2.

We also found that glutathione and ascorbic acid showed significant changes in our metabolomic data during the infection process. For instance, in the infected YN banana plant, oxidized glutathione was significantly upregulated 1.34-fold at 3 h and 1.59-fold at 27 h after inoculation. Ascorbic acid increased 1.18- and 1.62-fold in BX at 3 and 27 h after inoculation, respectively, compared with mock-inoculated susceptible plants. The data implied that different antioxidants play different roles in banana plants susceptible or resistant to *F. oxysporum* f. sp. *cubense* infection (Table 3).

The relationship between gene expression and metabolite levels.

The expression levels of the genes encoding PAL, isochlorismate synthase (ICS), and nonexpressor of PR protein 1 (NPR1), all involved in the biosynthesis and regulation of SA, were investigated 3, 27, and 51 h after infection with *F. oxysporum* f. sp. *cubense* (Fig. 4). Of the nine genes analyzed, expression of *PAL1* showed the highest increase in the infected plants compared with the mock-inoculated plants (nearly 20-fold). *PAL2* was also induced 3 h after inoculation in both YN (4.6-fold) and BX (1.3-fold). All *PAL* gene expression levels increased more in the infected YN than in the infected BX at the beginning of infection, which was in accordance with the higher content of SA in the infected YN banana plants at 3 h (Table 1). Most SA biosynthesis genes, except *PAL1* and *PAL2*, had decreased expression levels at 51 h after inoculation in the infected YN. Relatively small changes (<2.0-fold) in *ICS* and *NPR1* expression levels were found in the infected plants compared with their mock-inoculated counterparts at 3 h but significant increases were found at 27 h, especially in the infected YN.

Genes involved in the biosynthesis of JA were also analyzed, including lipoxygenases (*LOX*), acyl-CoA oxidase (*ACO*), AOS, allene

oxide cyclases (*AOC*), and 12-oxophytodienoate reductases (*12-OPR*) (Fig. 5). Most of the genes, except *LOX1.1-1*, *LOX1.1-2*, and *LOX1.1-4*, were more highly induced in the infected YN than in the infected BX at the early stage of infection (3 h), which was consistent with the change of Me-JA shown in Figure 2B. At 27 h, the expression levels of *LOX1.1-1*, *LOX1.1-2*, and *LOX1.1-4* increased over twofold in the infected BX compared with the mock-inoculated BX, a higher change than in YN. *ACO-2*, *AOS-5*, *AOC3-1*, and *12-OPR-3* also had greater fold changes in the infected BX, implying that they also influence the higher level of Me-JA in BX at 27 h (Fig. 2B). The expression levels of *AOS-2*, *AOC3-1*, and *AOC3-2* genes were higher at 51 h than at 3 or 27 h in the infected BX, while *12-OPR-4* and *AOS-5* showed stable expression levels at 27 to 51 h. In the YN cultivar, it was notable that *LOX1.1-3*, *2.3*, *AOC3-1*, *12-OPR-1*, and *12-OPR-2* had high fold changes in the infected YN at the early (3 h) and late (51 h) time points, while *AOS-3* and *AOS-4* had the highest fold change at 27 h. The expression levels of *ACO-1*, *ACO-2*, *AOS-1*, *AOS-5*, and *12-OPR-3* gradually decreased in the infected YN over the time course, whereas *AOS-1* and *12-OPR-4* showed stable expression levels between the infected and mock-inoculated YN.

The expression levels of genes encoding xanthoxin dehydrogenase (*ABA2*) and aldehyde oxidase (*AAO*), catalyzing the last two steps of ABA biosynthesis, were analyzed (Fig. 6). The fold change of *ABA2* and *AAO* over mock was higher in BX than in YN. The lower fold change (<1) of these genes in the infected YN during the whole infection was in accordance with the changes in ABA levels in Figure 2C. The flavin monooxygenase genes (*YUC*; functioning downstream in IAA production) *YUC5* and *YUC6* both showed the highest change at 3 h after infection in BX and YN, and then decreased, especially *YUC5* (Fig. 6). In the infected banana plants, isopentenyltransferase (*IPT*) and 2-oxoglutarate 3-dioxygenase (*GA3ox*) both showed lower expression levels compared with the mock-inoculated banana plants (<1). The changes in *IPT* expression in the infected YN were, for the most part, greater than in the infected BX, whereas those of *GA3ox* were less than in the infected BX (Fig. 6). The expression profile of these genes

Table 2. Phenolic compounds detected by liquid chromatography quadrupole time-of-flight mass spectrometry in banana infected by *Fusarium oxysporum* f. sp. *cubense* strain TR4^a

VIP ^b	RT (min) ^c	Mass	Name	Fold changes (BX)			Fold changes (YN)		
				3 h	27 h	51 h	3 h	27 h	51 h
1.903	3.45	198.05	Syringic acid	1.82↑	0.97	0.88	1.78	1.28	1.38↑
1.727	0.85	604.011	Gallagic acid	1.14↑	0.88	1.10	1.57↑	1.43↑	1.54↑
1.602	4.057	224.062	Sinapic acid	1.61↑	1.57↑	1.06	0.41↓	0.72	0.21↓
1.982	16.5	168.04	Vanillic acid	0.96	1.25↑	1.04	1.05	0.99	1.20↑
1.533	5.3	346.11	Dihydroxy-trimethoxyflavanone	1.50↑	1.04	0.41↓	1.06	2.12↑	1.60↑
2.281	0.745	432.113	Trihydroxyflavone-glucoside	1.84↑	1.49↑	1.04	3.03↑	2.59↑	1.74↑
1.756	8.13	660.185	Phylloflavanine	1.12	0.71	0.76	0.19↓	ND	0.86
1.847	6.74	110.037	Pyrocatechol	1.00	1.72↑	1.23	1.31↑	ND	1.66↑
1.649	5.83	452.131	Glucopyranosylcatechin	2.00↑	0.46↓	0.68	1.62↑	2.72↑	1.06

^a Fold change was the peak area ratio of the *F. oxysporum* f. sp. *cubense*-inoculated banana over the mock-inoculated banana according to the results of high-performance liquid chromatography. BX = BaXi and YN = *Musa yunnanensis*. Up and down arrows indicate significant increasing and decreasing, respectively. ND means not detected in the mock-inoculated YN.

^b VIP = variable importance in the projection (>1).

^c Retention time.

Table 3. Antioxidants detected by liquid chromatography quadrupole time-of-flight mass spectrometry in banana infected by *Fusarium oxysporum* f. sp. *cubense* strain TR4^a

VIP ^b	RT ^c	Mass	Name	Fold changes (BX)			Fold changes (YN)		
				6 h	24 h	48 h	6 h	24 h	48 h
1.093	1.05	612.152	Glutathione, oxidized	1.12	0.94	1.20	1.34↑	1.59↑	0.67↓
2.447	0.98	338.09	L-ascorbic acid-2-glucoside	1.18↑	1.62↑	0.77↓	0.74↓	0.96	0.52↓

^a Fold change was the peak area ratio of the *F. oxysporum* f. sp. *cubense*-inoculated banana over the mock-inoculated banana according to the result of high-performance liquid chromatography. BX = BaXi and YN = *Musa yunnanensis*. Up and down arrows indicate significant increasing and decreasing, respectively ($P < 0.05$).

^b VIP = variable importance in the projection (>1).

^c Retention time in minutes.

was in accordance with the concentration changes seen for CTK and GA₃ (Fig. 2E and F).

We also investigated the expression levels of dehydroascorbate reductases (*DHAR*) and monodehydroascorbate reductases (*MDHAR*) (Fig. 7). *DHAR* genes all showed lower expression levels in the infected BX compared with the mock-inoculated BX but increased expression levels in the infected YN, especially from 3 to 27 h after

Fig. 3. Concentration of total phenolics during banana infection by *Fusarium oxysporum* f. sp. *cubense* through Folin-Ciocalteu method. Error bars represent standard error from six samples. Infected BaXi (BX, filled square) and mock-inoculated BX (open square). Infected *Musa yunnanensis* (YN, filled circle) and the mock-inoculated YN (open circle).

infection. It was intriguing that the change in expression levels of the *MDHAR* genes showed opposite trends compared with those of the *DHAR* genes in the infected BX and YN cultivars.

Expression levels of PR genes. The expression of some well-known PR genes was investigated in the *F. oxysporum* f. sp. *cubense*-susceptible BX and -resistant YN banana plants (Fig. 8). *PR1*, an SA-dependent gene, was upregulated, especially in the infected YN. It was obvious that *PR2*, encoding endo-(1, 4)- β -glucanase; *PR5-1* and *PR5-2*, encoding thaumatin-like proteins; and *PR9*, encoding peroxidase were strongly induced by TR4, especially 27 h after infection. Furthermore, the increases in the expression levels of these PR genes were higher in the infected YN than in the infected BX. *PR2* and *PR5* reached peak expression at 27 h, while *PR4*, encoding endochitinase, and *PR9* showed further increases in expression over the course of the inoculation. Analysis of the expression levels of *PR12* genes encoding defensin-like protein (PDF) showed that *PDF2.2* was also higher in the infected YN, whereas *PDF2.3* was lower in the infected banana plants compared with the mock-inoculated plants.

PPO genes encoding polyphenoloxidase were also greatly induced in the infected YN. For instance, 51 h after infection, *PPO-1* was found to be upregulated nearly 20-fold; *PPO-2* was up to 20- and 35-fold at 27 h and 51 h, respectively; and *PPO-3* increased over 8-fold at 27 and 51 h during the infection. In the infected BX cultivar, these genes had lower fold changes in their expression levels.

Discussion

Fusarium wilt caused by *F. oxysporum* f. sp. *cubense* is an extremely serious disease that limits the yield of banana fruit. Management of

Fig. 4. Expression fold changes of genes involved in the biosynthesis of salicylic acid during banana infection by *Fusarium oxysporum* f. sp. *cubense*. Error bars represent standard error from six samples. BaXi (filled column) and *Musa yunnanensis* (open column). The value was the ratio of the infected over the mock-inoculated banana plants. PAL = phenylalanine ammonia lyase, ICS = isochlorogenic acid synthase, and NPR1 = nonpathogenesis-related protein 1.

Fusarium wilt, including soil solarization and fumigation (Herbert and Marx 1990; Liu et al. 2012), fungicides (Nel et al. 2007), crop rotation (Su et al. 1986; Xin et al. 2014), and antagonistic microbes (Wang et al. 2013; Zhang et al. 2011), has been widely employed. Research on the response of banana plants to *F. oxysporum* f. sp. *cubense* should contribute to the control of Fusarium wilt in banana planting.

Our results indicate that numerous metabolites were activated to coordinate the banana defense response to *F. oxysporum* f. sp. *cubense*, especially signal transduction molecules. The role of the signaling pathways mediated by SA, JA, and ET in the *Arabidopsis* innate

immune response has been well established (Glazebrook 2005). Treatment with SA enhances the resistance to *F. oxysporum* in tomato (Mandal et al. 2009). SA, ET, and JA signaling pathways interact in a positive way for *Arabidopsis* to resist banana *F. oxysporum* f. sp. *cubense* (Radhajealakshmi et al. 2014). In our study, the resistant species YN responded to TR4 infection with higher SA levels than did the susceptible BX during the entire infection process. Because there was no obvious difference in the number of hyphae and spores attached to the roots during early infection (the first 2 days) between *F. oxysporum* f. sp. *cubense*-susceptible BX and -resistant YN, the defense pathways

Fig. 5. Expression fold changes of genes involved in the biosynthesis of jasmonic acid during banana infection by *Fusarium oxysporum* f. sp. *cubense* strain TR4. Error bars represent standard error from six samples. The value was the ratio of the infected over the mock-inoculated banana plants. BaXi (filled column) and *Musa yunnanensis* (open column). LOX = lipoxygenase, ACO = acyl-CoA oxidase, AOS = allene oxide synthase, AOC = allene oxide cyclase, and 12-OPR = 12-oxophytodienoate reductase.

induced by SA likely play an important positive role in banana against TR4. The JA pathway might involve the defense response in the banana–pathogen interaction, especially in the early stage, due to its higher level at 51 h after inoculation in YN (up 2.04-fold over the mock-inoculated YN).

Many hormones work together to modulate disease resistance (Robert-Seilanianitz et al. 2011). Our results also revealed the complex cross-talk between signal molecules and phytohormones during this plant–pathogen interaction. The concentrations of ABA were much lower in the infected YN than in the infected BX. ABA usually

inhibits the expression of defense genes induced by JA (Anderson et al. 2004) and interacts antagonistically with the SA signaling pathway (Jiang et al. 2010; Meguro and Sato 2014; Xu et al. 2013). The higher ABA seemed to be related to the lower level of SA and lower resistance of BX to *F. oxysporum* f. sp. *cubense* in our study. The lower concentration of IAA in the infected BX than in the infected YN, especially at the later infection time, might be related to the level change of SA and ABA signals (Park 2007). The continuous decline of CTK in infected BX compared with the infected YN during the infection process implied that cell division activity in BX was greatly

Fig. 6. Expression fold changes of genes committed to phytohormone biosynthesis during banana infection by *Fusarium oxysporum* f. sp. *cubense* strain TR4. Error bars represent standard error from six samples. The value was the ratio of the infected over the mock-inoculated banana plants. BaXi (filled column) and *Musa yunnanensis* (open column). ABA2 = xanthoxin dehydrogenase, AAO = abscisic aldehyde oxidase, YUC = flavin monooxygenase, IPT = isopentenyltransferase, and GA3ox = 2-oxoglutarate-dependent dioxygenase.

Fig. 7. Expression level changes of genes involved in ascorbic acid metabolism during banana infection by *Fusarium oxysporum* f. sp. *cubense* strain TR4. Error bars represent standard error from six samples. The value was the ratio of the infected over the mock-inoculated banana plants. BaXi (filled column) and *Musa yunnanensis* (open column). DHAR = glutathione S-transferase dehydroascorbate reductase and MDHAR = monodehydroascorbate reductase.

Fig. 8. Expression level changes of pathogenesis-related genes during banana infection by *Fusarium oxysporum* f. sp. *cubense* strain TR4. Error bars represent standard error from six samples. The value was the ratio of the infected over the mock-inoculated banana plants. BaXi (filled column) and *Musa yunnanensis* (open column). PR1 = pathogenesis-related gene 1, PR2 = endo-(1,4)- β -glucanase, PR4 = endochitinase, PR5 = thaumatin, PR9 = peroxidase, PPO = polyphenol oxidase, and PDF2.2 and 2.3 = defensin-like.

impaired due to the prolonged stress (Ghanem et al. 2008; Kudoyarova et al. 2007) and that the orchestrated CTK and SA signaling in response to plant pathogen attack would be disrupted (Choi et al. 2010; Sano et al. 1996). Although GA₃ showed lower levels in the infected banana plants compared with the mock-inoculated plants, it still was higher in the infected BX than in the infected YN, which was coincident with the lower level of JA. As reported, JA was antagonistic with GA in regulating plant growth and resistance to pathogens through the regulation of DELLAs on JAZ1 (Hou et al. 2010; Wild et al. 2012).

Our investigation into the expression level of genes indicates that the levels of some biosynthetic genes might determine the concentration of metabolites. In the infected YN, the higher level of ICS resulted in a higher concentration of SA. The biosynthetic genes *LOX1.1-3*, *ACO-1*, *AOS-1*, *AOS-3*, *AOS-4*, *AOC3-1*, and *12-OPR-2* were more likely responsible for the JA biosynthesis in *F. oxysporum* f. sp. *cubense*-infected YN. Our study revealed direct relationships between the expression levels of PR genes and the underlying induction signal molecules. For instance, the higher level of SA was consistent with the high expression levels of PR1, PR2, and PR5 in the resistant YN, especially 27 h after inoculation, which may indicate how the resistant species responds to TR4 infection. The high level of JA (1.89-fold higher than in mock-inoculated plants) in YN might be intimately related to the expression of PR4 and PDF2.2.

The resistance response in the wild banana (*M. yunnanensis*) to *F. oxysporum* f. sp. *cubense*, especially strain TR4, involves higher levels of SA and CTK, lower levels of ABA, a higher concentration of phenolics, and an increase in PR genes induced by SA. In contrast, the response of these compounds are opposite in the susceptible BX cultivar during the early stage of infection by TR4. These responses in BX might be explored to produce methods to control the pathogen, after more intense investigation in the future. Most importantly, understanding of the underlying response in the resistant banana species will allow for selective integration of these traits into commercially useful cultivars.

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