Differential gene expression in East African highland bananas (*Musa* spp.): Interactions between non-pathogenic *Fusarium oxysporum* V5w2 and *Radopholus similis*

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Abstract

Endophytic non-pathogenic *Fusarium oxysporum* isolates are known to control *Radopholus similis* through induced resistance. Using complementary (c)DNA-amplified fragment length polymorphism (cDNA-AFLP), we identified genes induced in nematode-susceptible and -tolerant bananas following colonization by non-pathogenic endophytic *F. oxysporum* isolate V5w2. Quantitative real-time polymerase chain reactions (qRT-PCR) were then used to analyze expression profiles of selected genes. Accumulated gene transcripts included those involved in signal transduction, cell wall strengthening, Jasmonic acid (JA) pathway and transport of defense molecules. Endophyte colonization primed the expression of *β-1,3-glucan synthase* and *Coronatine insensitive 1* (*COI1*) in the susceptible cultivar, and that of *COI1* and *Lipoxygenase* (*LOX*) in the tolerant. We isolate and identify for the first time genes involved in the interaction between endophytic non-pathogenic *F. oxysporum* and banana.

Highlights

- We report identification of genes involved in fungal endophyte protection of banana.
- We report JA-induced defenses in banana- V5w2- *Radopholus similis* interaction.
- Genes were primed for expression upon nematode challenge of V5w2 inoculated plants.

Keywords : cDNA-AFLPs; *Fusarium oxysporum*; *Musa* spp; Quantitative real-time PCR; *Radopholus similis*

1. Introduction

The root burrowing nematode (*Radopholus similis* (Cobb) Thorne) is a major pest of banana in Uganda [1]. Farmers mainly control the nematode through habitat management, biological control, use of host plant resistance, use of clean planting materials and chemical control [2]. Though not yet used by farmers, non-pathogenic *Fusarium oxysporum* Schlecht. Fries endophytes of banana are reported to effectively reduce nematode numbers [3]. They can be established within tissue culture plants before field planting, and their use

to control the nematode is an attractive option because the endophytes occur within the roots where the destructive stage of the nematode also exists. Additionally, the use of microbial antagonists that occur within the plant might offer a better control option than others because they are less exposed to environmental influences [4].

Fungal endophytes are organisms which, at some stage in their life cycle, colonize living plant tissues without causing any visible symptoms [5]. They have been isolated from almost every plant species studied [6], including banana [7]. Interactions between an endophyte and its host may be neutral, parasitic or mutually beneficial. Mutualistic plant–endophyte interactions involve defense of host plants against pests and diseases, increased tolerance to abiotic stresses, and production of growth-promoting hormones such as auxins and gibberellins [8] and [9]. In return the plant provides the endophyte with nutrition and a suitable environment to survive [10].

Non-pathogenic *F. oxysporum* endophytes have been shown to kill *R. similis* juveniles *in vitro*, and have been successfully introduced into tissue culture banana [3] and [7]. *In-vivo* nematode control by the endophyte was first reported by Niere [3]. Later, Athman [11] demonstrated induced resistance as the most likely mode of action of *F. oxysporum* endophytes against *R. similis*. Her study reported increased phenolic deposition in endophyte-inoculated and *R. similis*-challenged roots of banana. In a related study, Paparu et al. [12] reported reduced nematode numbers in plants inoculated singly with isolate V5w2, and dually with isolates V5w2 and Emb2.4o.

During colonization, fungal endophytes are believed to trigger defense reactions within intact cell walls, leading to wall reinforcement [13]. This phenomenon is often deduced from the limited ingress of endophytic fungi into the cortex and vascular bundle of plants [14] and [15]. Plant defense responses induced at the point of colonization can also spread systemically throughout the plant and protect parts that have not been inoculated [11] and [16]. Biochemical changes associated with induced resistance include the accumulation of secondary metabolites such as phytoalexins [11] and [17], production of PR proteins such as chitinases and *β-1,3-glucanases* [13], [16], [18] and [19] and increased activity of enzymes involved in the phenylpropanoid pathway (lignin synthesis) [19].

The phenotypic and biochemical response of banana plants to fungal endophyte infection has been shown to vary among banana cultivars and endophyte isolates [11]. Similarly, Paparu et al. [20] reported differences in defense gene up-regulation between cultivars susceptible and tolerant to *R. similis* following challenge of endophyte-inoculated plants with the nematode. To confirm induced resistance as a mode of action for endophytic isolate V5W2, the objectives of our study were to a) use cDNA-AFLP analysis to isolate and identify gene transcripts accumulated during colonization of nematode-susceptible and –tolerant bananas by endophytic isolate V5w2, b) to use qRT-PCR to determine if endophyte colonization primes banana plants for stronger up-regulation of selected genes following nematode challenge.

2. Materials and methods

2.1. Fungal endophyte and nematode culture conditions

Non-pathogenic *F. oxysporum* endophyte V5w2 was used in this study because of its ability to suppress *R. similis* in screenhouse experiments [11]. V5w2 was originally isolated from healthy banana roots in Uganda [7] and is currently maintained in soil, on filter paper and in 15% glycerol at the facilities of the International Institute of Tropical Agriculture (IITA) in Kampala, Uganda. Spore suspensions were prepared after culturing the isolate on half strength potato dextrose agar (PDA) (19 g PDA and 19 g agar l^{-1} distilled water) according to the method by Paparu et al. [21].

R. similis population was initiated using nematodes obtained from infested roots at IITA banana fields in Namulonge, Uganda, and cultured according to the protocol by Speijer and De Waele [22]. To obtain nematodes from infested banana roots, roots were macerated in a blender at low speed for 15 s and extracted overnight using the modified Baermann funnel method [23]. Female *R. similis* were then hand-picked from the nematode suspension and surface sterilized with a 600-ppm streptomycin sulfate solution. To multiply nematodes for use in the current study, sterile females were inoculated on surface-disinfected 0.5-cm-diameter carrot (*Daucus carota* L.) discs in 30-mm-diameter Petri dishes, sealed with parafilm, and incubated at 27 °C for 3–4 weeks. During inoculations, a nematode suspension was prepared by washing nematodes from the carrot discs and edge of Petri dishes. Nematode concentration at plant inoculation was maintained at 178 females and juveniles ml^{-1}.

2.2. Production and inoculation of banana plants

Tissue culture banana plants of the EAHB cv Nabusa (highly susceptible to *R. similis*) and cv Kayinja (tolerant to *R. similis*) were propagated using a standard shoot-tip culture protocol [24]. Four weeks after rooting, plants were removed from the rooting medium and their roots and rhizomes rinsed in tap water. The plants were then grown hydroponically in 250-ml plastic cups according to the method by Paparu et al. [21].

Plants were inoculated with endophytic *F. oxysporum* isolates by dipping their roots and rhizomes in spore suspensions for 4 h. Non-inoculated plants were dipped in SDW for the same duration. Plants for cDNA-AFLP analysis were planted in cups as described above, and those for qRT-PCR were planted in sterile (steam-sterilized) soil in pots (120 mm wide and 90 mm deep). Root challenge with *R. similis* was done according to the method by Paparu et al. [12]. All experiments were conducted in a plant growth room with a photoperiod of 12/12 h light/dark routine and a temperature of approximately 25 °C, and plants in soil were watered twice per week. At harvest, root samples were collected for RNA extraction, endophyte re-isolation and nematode extraction. Endophyte re-isolations from roots were undertaken using the protocol by Paparu et al. [21], and nematodes were extracted using the modified Baermann funnel method [23].

2.3. RNA extraction and cDNA synthesis

Plants were removed from the nutrient solution and their roots washed in distilled water. Total RNA was extracted from banana roots and rhizomes using the RNeasy Plant Mini Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. RNA yield was determined by measuring absorbency at 260 nm and 280 nm, using a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, Montchanin, USA).

Total RNA was digested with RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) for 30 min at 37 °C, and column-purified using the QIAGEN RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) according to manufacturer's instructions. Poly A^+ RNA was isolated using the Oligotex mRNA Mini Kit (QIAGEN) according to the manufacturer's instructions. Double-stranded (ds)-cDNA synthesis was carried out from 150 ng of purified poly A^+ RNA using the cDNA Synthesis kit from Roche. The first strand was synthesized in a reaction volume of 21 µl, containing 2 µl oligo dT_{15} primer (200 µM), 9.5 µl RNase-free water, and 9.5 µl poly A^+ RNA. Samples were then incubated at 70 °C for 10 min and placed on ice, followed by the addition of 8 µl 5 × Reverse Transcriptase buffer, 1 µl Avian Myeloblastis Virus (AMV), 4 µl 0.1 M dithiothreitol (DTT), 1 µl RNase inhibitor (25 U/µl) and 4 µl 10 mM dNTP-mix. The samples were incubated at 42 °C for 60 min, and the reaction stopped by placing it on ice.

The second strand was synthesized from the first by adding 30 µl 5 × second strand buffer, 1.5 µl 10 mM dNTP-mix, 6.5 µl s strand enzyme blend (DNA polymerase 1, *Escherichia coli* ligase and RNase H) and 72 µl SDW to the tube containing first strand cDNA. The mixture was incubated at 16 °C for 2 h, after which 20 µl

(20 U) of T4 DNA polymerase was added and the mixture incubated for 5 more min. The reaction was stopped by the addition of 17 µl 0.2 M EDTA (pH 8.0). The ds-cDNA was purified using the High Pure Product Purification Kit from Roche. All cDNAs were assayed for genomic DNA contamination by PCR using the actin-specific primer set actinF (5′ACCGAAGCCCCTCTTAACCC-3′) and actinR (5′-GTATGGCTGACACCATCACC- 3′), and PCR products separated by electrophoresis through a 2% (w/v) agarose gel containing ethidium bromide.

2.4. Isolation and identification of defense-related genes

cDNA-AFLP analysis: Differential gene expression in susceptible and tolerant EAHB plants upon endophyte colonization was determined by complementary (c)DNA-amplified fragment length polymorphism (cDNA-AFLPs). Roots were harvested from both cultivars immediately before endophyte inoculation (0 h), and 2, 7 and 30 days after inoculation (dpi). cDNA-AFLP analysis was performed according to the protocol described by Bachem et al. [25], using the AFLP Expression Analysis Kit of LI-COR (LI-COR Biosciences, Lincoln, NE). Ds-cDNA was used as template in the generation of TaqI+0/MseI+0 pre-amplification PCR products. Pre-amplification products were generated in three steps; restriction digestion of cDNA, adapter ligation, and pre-amplification PCR.

Restriction digestion: Restriction digestion was done in two steps. In the first step, a *Taq1* restriction digestion reaction mixture of 20 µl was prepared that consisted of 10 ng of ds-cDNA, 4 µl of 5 × RL buffer, 0.5 µl *Taq1* enzyme and 5.5 µl of SDW. The mixture was incubated at 62 °C for 2 h, and placed on ice. This was immediately followed by *Mse1* restriction digestion. This step commenced by adding 1 µl 5 × RL buffer, 0.5 µl *Mse*1 enzyme and 3.5 µl SDW to the *Taq1* mixture. The mixture was incubated in two steps: first at 37 °C for 2 h and then at 80 °C for 20 min.

Adapter ligation: Adapter ligation mix (4.5 µl) and T4 DNA ligase (0.5 µl) were added to the 25 µl *Taq1*/*Mse1* restriction digest mix, gently mixed and incubated at 20 °C for 2 h.

Pre-amplification: The ligation mixture was diluted with 1 × TE buffer at a ratio of 1:10. One µl of the diluted ligation mixture was then mixed with 10 µl of a pre-amp primer mix (containing EcoR 1 and Mse 1 primers), 1.25 µl 10× amplification buffer, and 0.25 µl Taq DNA (Roche Diagnostics). This was followed by 25 cycles at 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min. Pre-amplification products were assayed for quality and quantity by electrophoresis on 1% agarose gels.

Pre-amplification was followed by selective amplification which was done as follows. The diluted pre-amplification products (1:300 in SABAX water) provided a template for selective amplification of all 64 + 2/+2 primer combinations afforded by the eight *Taq*I+2 primers and eight *Mse*I+2 primers (+GA, +GT, +TC, +TG, +CT, +CA, +AG and +AC on both adapter primers) available in the AFLP Expression Analysis Kit. The amplification reaction mixture consisted of 3 µl *Taq* DNA polymerase working mix, 1 µl diluted pre-amplification DNA, 1 µl *Mse1* primer containing dNTP's and 0.25 µl IRDyeTM 700-labeled *Taq1* primer. A touchdown PCR which consisted of one cycle at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min; 12 cycles where the annealing temperature was lowered by 0.7 °C per cycle, denaturation maintained at 94 °C for 30 s and amplification at 72 °C for 1 min; 23 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min and held at 4 °C, was used. Infrared dye (IRDye700, LI-COR)-labeled *Taq*I+2 primers in this kit are fluorescent and aid in fragment visualization. Selective PCR products were resolved on 8% denaturing polyacrylamide gels in a model 4200S LI-COR DNA Analyzers [26], and cDNA-AFLP images were saved in 16-bit TIFF format for image analysis.

Image analysis and transcript derived fragment (TDF) quantification: LI-COR TIFF images were cropped using Quantity One 1-D Analysis Software (Bio-Rad Laboratories Inc., Hercules, CA, USA) before cDNA-AFLP band sizes and intensities were determined using the AFLP-QuantarPro software (KeyGene products B.V., Wageningen, The Netherlands). Lane finding, band finding and sizing were performed as described in the AFLP-QuantarPro user manual, with band finding and scoring parameters previously described for LI-COR gels [26]. In this study a TDF is used to refer to a polymorphic band (transcripts accumulated in endophyte-inoculated plants, but not control). Based on visual analysis, differentially expressed TDFs were quantified for all primer combinations.

TDF isolation and identification: Polyacrylamide gels containing fragments of interest (TDFs up-regulated relative to non-inoculated plants) (Fig. 1) after partial electrophoresis on LI-COR DNA Analyzers were scanned using the Odyssey Infrared Imager (LI-COR). TDFs ranging in size from 140 to 469 bp were excised using a scalpel blade, and the PCR products suspended in 20 μl SDW. Elution was achieved by 10–15 cycles of freezing (−70 °C) and thawing at room temperature. Eluted PCR products were re-amplified with the same primer combinations used in the final amplification reaction. The final amplification step, however, included an elongation step of 20 min. Re-amplified fragments were confirmed by visualization on a 2% (w/v) agarose gel.

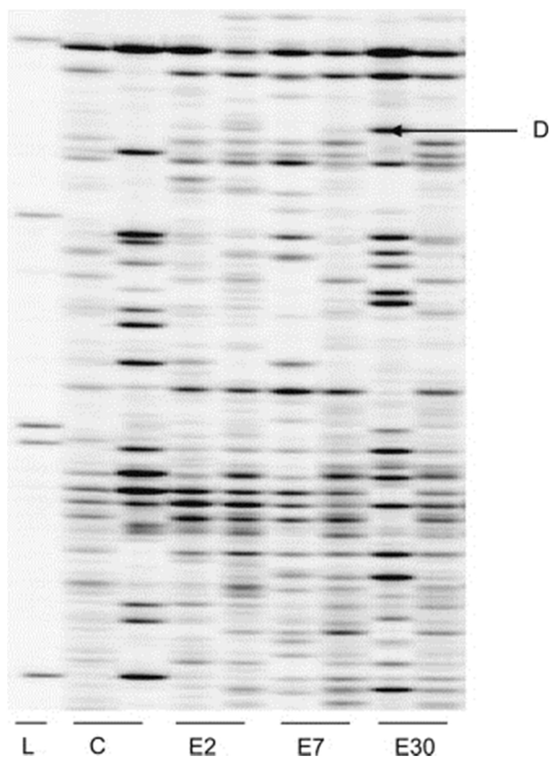

Fig. 1. Example of banding patterns observed in cDNA-AFLPs. Selective amplification with cDNA-AFLP primer combination CA/GA. L = DNA ladder, C = endophyte-free plants of the susceptible cultivar Nabusa, E2 = endophyte-inoculated plants of the susceptible cultivar Nabusa 2 days after inoculation, E7 = endophyte-inoculated plants of the susceptible cultivar Nabusa 7 days after inoculation, E30 = endophyte-inoculated plants of the susceptible cultivar Nabusa 30 days after inoculation and D = differentially expressed gene transcript.

Re-amplified TDFs were cloned into competent *E. coli* cells using the InsTAclone Cloning Kit (MBI Fermentas, Hanover, MD) according to the manufacturer's instructions. The transformed cells were then plated on Luria-Bertani (LB) agar containing 250 μg ml^{-1} ampicillin, 60 μg ml^{-1} X-gal and 60 μg ml^{-1} isopropanol-β-thiogalactopyranoside (IPTG), and the plates incubated overnight at 37 °C. Transformed cells developed white

colonies, while non-transformed ones were blue. Each transformant was separately transferred to 700 µl LB broth amended with 100 µg ml^{-1} ampicillin, incubated overnight 37 °C, and shaken at 200 rpm. Glycerol (300 µl) was added to 1.5-ml Eppendorf tubes containing the transformants, and the latter stored at −80 °C until sequencing.

The presence or absence of TDF inserts was determined with a colony PCR using M13F-pUC(-40) (5′-GTTTTCCCAGTCACGAC-3′) and M13R-pUC(-26) (5′-CAGGAAACAGCTATGAC-3′) universal primers. The reaction mixture contained 2 µl of transformed bacterial cells grown overnight in broth as template DNA (broth was put in 1.5 ml Eppendorf tubes and placed in boiling water for 10 min before adding to PCR mix), 1.5 µl $MgCl_2$, 2.5 µl NH_4^+ buffer, 2 µl 2.5 mM dNTP's, 0.4 µl of each primer (10 µM), 0.5 U *Taq* polymerase and SDW. PCR conditions were as follows: denaturation at 94 °C for 2 min, 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C, and a final elongation step of 7 min at 72 °C. PCR products were separated on 2% (w/v) agarose gel. TDF inserts were sequenced using ABI Bigdye terminator chemistry on ABI3100 instruments with the universal M13-pUC vector primers at Macrogen Corp. (Rockville, USA).

2.5. Gene regulation analysis using qRT-PCR

The regulation of defense-related genes in EAHB was determined by quantitative real-time reverse transcription (qRT)-PCR on a LightCycler version 1.2 instrument (Roche Diagnostic). After assigning putative identities to TDFs, four TDFs were chosen for regulation analysis, based on 1) similarity to defense-related genes, 2) percentage identity of more than 50% and 3) significance of the alignment (e-value). The TDFs selected for qRT-PCR included those containing fragments with similarity to *ABC transporter*, *coronatine insensitive 1* (*COI1*), *lipoxygenase* (*LOX*) and *β-1,3-glucan synthase*. Primer pairs were designed as balanced pairs between 55.1 and 64.1 °C Tm for the four TDFs with the objective to amplify fragments of between 150 and 283 bp using DNAMAN (Lynnon Biosoft, Quebec, Canada) (Table 1). An endogenous gene, *Musa* 25S rRNA (AY651067) (5′-ACATTGTCAGGTGGGGAGTT-3′; 5′-CCTTTGTTCCACACGAGATT-3′) [27], was used as a control gene since its expression remains relatively constant. All primers were synthesized by Inqaba Biotechnical Industries (Pty) Ltd (Hatfield, South Africa).

Table 1
Oligonucleotide primers designed for putative defence-related genes up-regulated in roots and rhizomes of East African Highland bananas.

Genbank accession	Putative identity	Primer sequence (5′-3′)	T_m °C	Amplicon size	Annealing temperature °C
GR972516	*Lipoxygenase*	AGACTGCGTACCGACAGGCT	62.3 °C	201	63 °C
		TGTCTGCCGAGCGAATTCA	64.1 °C		
GR972511	*Coronatine insensitive 1*	TGTAGACTGCGTACCGACTC	56.5 °C	283	63 °C
		CTCGCCAATGTAACCAAG	55.1 °C		
GR972507	*β-1,3-glucan synthase*	TGTAGACTGCGTACCGACA	56.3 °C	163	63 °C
		CCATGGGAAGGATAAGGA	55.8 °C		
GR972514	*ABC transporter*	GTAGACTGCGTACCGACAAG	56.0 °C	150	63 °C
		GTGGAGGAAACAAGAGGAAG	56.4 °C		

For gene regulation studies roots of both cultivars were harvested as follows: 1) immediately before inoculation (0 h), 2) 2 and 33 dpi, 3) 3 days after endophyte-inoculated plants (inoculated for 30 days) were challenged with *R. similis* and 4) 3 days after endophyte-free plants were challenged with *R. similis*. Total RNA was extracted from banana roots using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. One µg RNA was DNAse I treated (Fermentas Life Sciences, Hanover, USA) and first strand cDNA synthesized by random hexamer priming using Power Script TM Reverse Transcriptase (BD Biosciences, Erembodegem, Belgium) according to the method by Lacomme et al. [28]. cDNAs were assayed for genomic DNA contamination using the actin-specific primer set actinF (5′-

ACCGAAGCCCCTCTTAACCC-3′) and actinR (5′-GTATGGCTGACACCATCACC-3′), and PCR products separated by electrophoresis through on 2% agarose gel.

Dilution series and standard curves were performed to examine the linearity of amplification over the dynamic range. A serial dilution (1:10, 1:100 and 1:1000) was performed and used to draw standard curves for all genes. A 10-µl reaction for PCR amplification contained 5 µl FastStart DNA Master^{PLUS} SYBR Green I master mix, 2 µl of forward and reverse primer (10 µM), 1 µl cDNA template and 2 µl PCR grade water (Roche). Control treatments contained water instead of the cDNA template. All PCR reactions were performed in triplicate. The cycling conditions were as follows: pre-incubation for 10 min at 95 °C, followed by 55 cycles, each consisting of 10 s denaturing at 95 °C, 10 s annealing at 63 °C, 10 s primer extension at 72 °C, and data acquisition at 95 °C. For PCR amplification of all experimental samples, 1:10 cDNA template dilutions were used.

2.6. Data analysis

For RNA isolation, each treatment had three biological replicates, each consisting of three plants pooled together at harvest. TDF sequences were assigned putative identities by translating BLAST sequences (BLASTX) [29] against the non-redundant protein database in GenBank. The degree of sequence similarity between a TDF and a known sequence was represented by the E-value. For gene expression analysis, standard regression curves were calculated using crossing-points from amplification data from the serial dilutions [30]. Expression data was normalized using the amplification data for the specific target gene and the endogenous control gene, *Musa* 25S rRNA. Lightcycler data was subjected to analysis of variance and multiple mean comparisons performed using Tukey's studentized range test. Comparisons between treatments were made using a pooled *t*-test in SAS Institute [31].

3. Results

3.1. Isolation and identification of defense-related genes in EAHB

A total of 24 TDFs were successfully isolated from roots and rhizomes of endophyte-inoculated plants, and assigned putative identities (Table 2). TDFs were broadly classified into six functional categories: defense/stress-related, primary metabolism, transport, signal transduction, cell wall biosynthesis, and cell differentiation and development. Seven TDFs were identified to be involved in primary metabolism, 6 in cell differentiation and development, 4 in transport, 5 in defense, and 1 each in signal transduction and cell wall biosynthesis. Six unknown TDFs with unknown functions were also isolated.

Four TDFs with similarity to defense-related genes were isolated. *Coronatine insensitive 1*, *calmodulin-Ca^{2+}* and *lipoxygenase*, were up-regulated in both the susceptible and tolerant cvs, while, *beta-N-acetyl hexosaminidase* was up-regulated in the susceptible cv Nabusa only (Table 2). One TDF with similarity to *β-1,3-glucan synthase* was up-regulated in the susceptible cv Nabusa (Table 2). *β-1,3-glucan synthase* is involved in cell wall strengthening. Three unknown TDFs with unknown functions were also isolated (Not shown on Table 2).

Of the 24 TDFs from banana roots (Table 2), 13 were isolated from the susceptible cv Nabusa only, three from the tolerant cv. Kayinja only, and eight from both cultivars. Differences were also observed between roots and rhizomes in TDF up-regulation. The biggest numbers of TDFs were isolated from the roots alone, accounting for 18 out of 24. Three were isolated from the rhizomes only, and three were isolated from both roots and rhizomes (Table 2).

Table 2
Putative identities of transcript derived cDNA fragments differentially up-regulated in roots and rhizomes of a susceptible (cv Nabusa, AAA-EA) and tolerant (cv Kayinja, ABB) banana following inoculation with endophytic non-pathogenic *Fusarium oxysporum* isolate V5w2.

Genbank accession	Sequence homology	% ID/E-value[a]	Function[b]	Time of up-regulation (dpi)[c]	Tissue	Cultivar
GR972497	Chlorophyll A-B binding protein, *Nicotiana tabacum* (AAR 85970.1)	83%/2e^{-20}	Photosynthesis	2	Root	Nabusa
GR972498	Ribosomal protein, *Hyacinthus orientalis* (AAT 08714.1)	89%/7e^{-28}	Protein synthesis	2,7,30	Root	Nabusa
GR972520	Protein kinase, *Dictyostelium discoideum* (XP 646197.1)	29%/3.5	Signal transduction	2,7,30	Rhizome	Nabusa
GR972521	Senescence protein, *Pisum sativum* (BAB 33421.1)	60%/4e^{-15}	Cell differentiation and development	2	Rhizome	Nabusa
GR972511	*Coronatine insensitive 1*, *N. attenuate* (ABK 27928.1)	85%/3e^{-36}	Jasmonate response (Defense)	2,7,30	Root/Rhizome	Nabusa/Kayinja
GR972499	Kinesin, *Zea mays* (AAK 91819.1)	68%/7e^{-17}	Transport	2,7,30	Root/Rhizome	Nabusa/Kayinja
GR972500	Phosphatidylcholine transfer protein, *O. sativa* (BAD 08712.1)	62%/2e^{-18}	Transport	2,7,30	Root/Rhizome	Nabusa/Kayinja
GR972512	Ribosomal protein, *Picea abies* (CAC 27142.1)	90%/8e^{-08}	Protein synthesis	7,30	Root	Nabusa/Kayinja
GR972503	Actin, *M. acuminata* (AAQ 14245.1)	100%/5e^{-17}	Cell differentiation and development	2,7,30	Root	Nabusa
GR972517	CAT 2, *A. thaliana* (NP 849822.1)	82%/2e^{-23}	Transport	2,7,30	Root	Kayinja
GR972518	Alcohol dehydrogenase 1, *Hordeum vulgaris* (AAO 24249.1)	55%/0.001	Metabolism	2,7,30	Root	Kayinja
GR972519	Actin, *M. acuminata* (AAQ 14245.1)	100%/5e^{-17}	Cell differentiation	2,7,30	Root	Kayinja
GR972486	Actin, *O. sativa* (CAA 33873.1)	100%/6e^{-37}	Cell differentiation and development	2,7,30	Root	Nabusa
GR972513	Calmodulin-Ca^{2+}, *T. sativum* (P 04464)	98%/2e^{-23}	Defense	2,7,30	Root	Nabusa/Kayinja
GR972514	ABC transporter, *A. thaliana* (AAM14842.1)	94%/1e^{-19}	Transport	2	Root	Nabusa/Kayinja
GR972504	Ripening regulated protein, *O. sativa* (AAN 64140.1)	88%/5e^{-15}	Cell differentiation and development	2,7,30	Root	Nabusa
GR972515	Glutamate decarboxylase, *Populus tremula* (ABA 18652.1)	93%/2e^{-46}	Metabolism	2,7,30	Root	Nabusa/Kayinja
GR972522	Hydrolase, *O. sativa* (BAD 87632.1)	66%/2e^{-13}	Metabolism	30	Rhizome	Nabusa
GR972505	Ribosomal protein, *O. sativa* (BAB 93221.1)	89%/5e^{-16}	Protein synthesis	2,7,30	Root	Nabusa
GR972507	*β-1,3-glucan synthase*, *Lolium multiflorum* (AAQ 17229.1)	94%/1e^{-23}	Callose synthesis	2,7,30	Root	Nabusa
GR972508	Synaptobrevin-like protein, *O. sativa* (AA 072389.1)	100%/5e^{-20}	Transport	2,7,30	Root	Nabusa
GR972509	Beta-tubulin, *N. attenuata* (AAR 37366.1)	100%/7e^{-28}	Cell differentiation and development	7	Root	Nabusa
GR972510	Beta-N-acetyl hexosaminidase, *Yersinia pestis antique* (YP 652374.1)	37%/7.8	Defense	2,7,30	Root	Nabusa
GR972516	Lipoxygenase, *Zantedeschia aethiopica* (AAG 18376.1)	51%/3e^{-14}	Defense (Jasmonate response)	2,7,30	Root	Nabusa/Kayinja

[a] E-value = sequence alignment significance (Karlin and Altschul, 1990).
[b] Proteins of unknown function not included in table.
[c] dpi = days post inoculation.

3.2. Gene regulation analysis using qRT-PCR

The expression of the *ABC transporter* gene was significantly up-regulated in both the susceptible ($t = 25.6$) and the tolerant banana cultivars ($t = 28.1$, $P < 0.0001$) 2 days post inoculation (dpi) with endophytic *F. oxysporum* isolate V5w2 (Fig. 2A). Gene expression, however, reduced significantly 33 dpi in both cultivars ($t = 38.44$ for the susceptible cv Nabusa, and $t = 59.04$ for the tolerant cv Kayinja; $P < 0.0001$), and was significantly lower than at 0 h ($t = 12.83$ for the susceptible cv Nabusa and $t = 30.97$ for the tolerant cv Kayinja; $P < 0.0001$). Interestingly, when endophyte-inoculated plants were challenged with *R. similis*, the expression of the *ABC transporter* gene was again up-regulated significantly in both cultivars compared with nematode non-challenged plants or endophyte-free plants challenged with nematodes. In both cultivars, *R. similis* challenge of endophyte-free plants resulted in reduced expression of *ABC transporter* gene, compared with the expression at 0 h ($t = 9.13$ for the susceptible cv Nabusa, and $t = 31.29$ for the tolerant cv Kayinja; $P < 0.0001$) (Fig. 2A).

Two dpi, the expression of the *COI1* gene was increased from 0.01 to 0.23 ηg ($t = 3.98$, $P = 0.014$) in the susceptible cv Nabusa. *COI1* expression did not change significantly until 33 dpi (Fig. 2B). However, when endophyte-inoculated plants were challenged with *R. similis*, the expression of the *COI1* gene was significantly up-regulated compared with endophyte non-inoculated and *R. similis*-challenged plants ($t = 3.79$, $P = 0.0026$). The expression of *COI1* was significantly up-regulated in the tolerant cv Kayinja only at 33 dpi ($t = 10.89$, $P < 0.0001$), and even more significantly following *R. similis* challenge of plants previously inoculated with the endophyte ($t = 22.04$, $P < 0.0001$). *R. similis* challenge of endophyte-free plants of both the susceptible and tolerant cv Kayinja did not result in up-regulation of the *COI1* gene. *β-1,3-glucan synthase* activity was significantly up-regulated in both the susceptible and tolerant cultivars 2 dpi ($t = 8.31$ and $t = 19.13$, respectively; $P < 0.0001$), and remained high in both cultivars until 33 dpi (Fig. 2C). When endophyte-inoculated plants of the susceptible cv Nabusa were challenged with *R. similis*, a significant up-regulation of *β-1,3-glucan synthase* (68.2%) was noted compared with endophyte-treated plants at 33 dpi ($t = 6.64$, $P < 0.0001$). However, *R. similis* challenge of endophyte-inoculated plants of the tolerant cv Kayinja resulted in reduced expression of *β-1,3-glucan synthase* (35.7%), compared with endophyte-inoculated and *R. similis* non-challenged plants ($t = 6.32$, $P < 0.0001$) of the same cv.

Expression of the *LOX* gene was not significantly up-regulated in both cultivars 2 dpi inoculation. (Fig. 2D). However, its expression was significantly increased 33 dpi, but only in the susceptible cv Nabusa ($t = 10.30$, $P < 0.0001$). Following *R. similis* challenge, the increased up-regulation mentioned above was significantly reduced ($t = 8.79$, $P < 0.0001$). On the contrary, *R. similis* challenge of endophyte-inoculated tolerant cv Kayinja plants resulted in 88.8% up-regulation of *LOX* activity, compared with endophyte-inoculated and *R. similis* non-challenged plants ($t = 26.48$, $P < 0.0001$); and a 77.8% up-regulation compared with endophyte non-inoculated and *R. similis*-challenged plants ($t = 22.53$, $P < 0.0001$).

Non-pathogenic *F. oxysporum* endophytes and *R. similis* were recovered readily from inoculated banana roots at harvest, thus validating results obtained for the inoculated treatments. The first strand cDNA synthesized from banana root RNA was of high quality, and its amplification with actin gene primers indicated no genomic DNA contamination (results not shown).

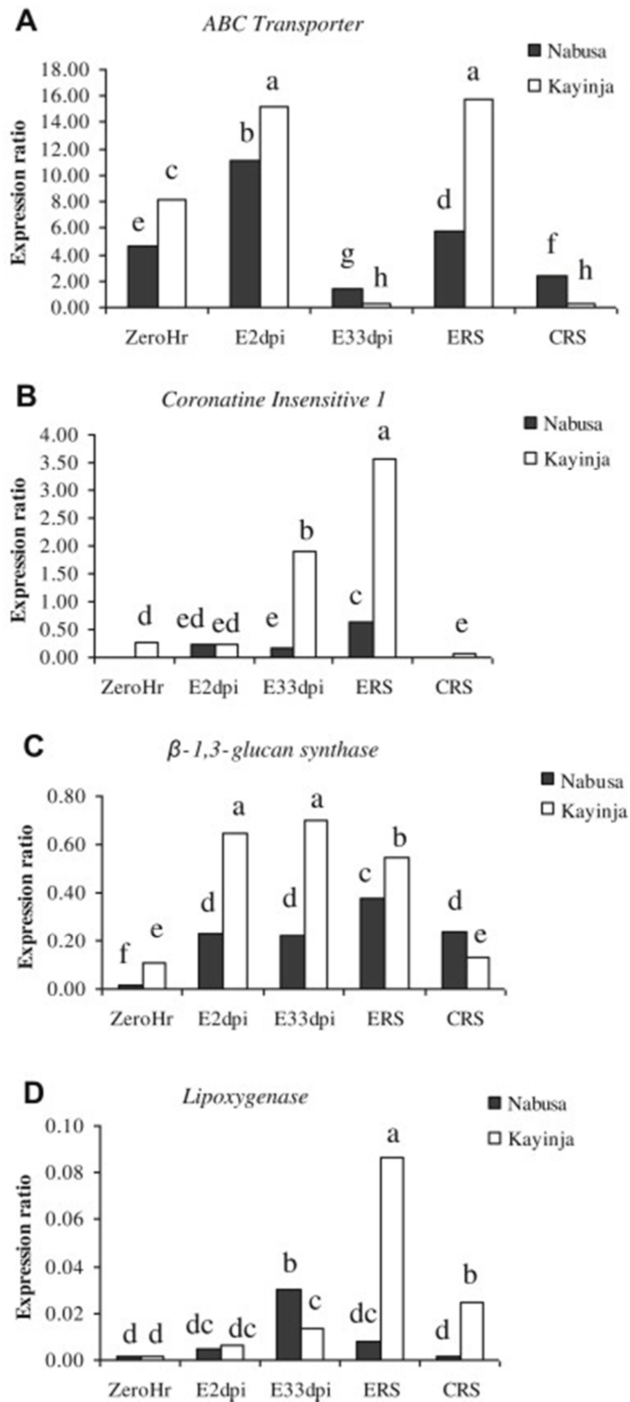

Fig. 2. Confirmation of the expression patterns of four TDFs by relative quantification of transcript abundance using qRT-PCR in roots of banana cvs susceptible (Nabusa, AAA-EA) and tolerant (Kayinja, ABB) to *Radopholus similis*. Zero hr = non-inoculated plants, E2dpi = plants inoculated with isolate V5w2 at 2 days post inoculation (dpi), E33dai = plants inoculated with isolate V5w2 at 33 dpi, ERS = plants inoculated with isolate V5w2 and challenged with *R. similis* 30 dpi and harvested 3 days after nematode challenge, and CRS = endophyte-free plants challenged with *R. similis* on day 30 and harvested 3 days later. (A) *ABC transporter*, (B) *Coronatine insensitive 1*, (C) *β-1,3-glucan synthase*, and (D) *Lipoxygenase* genes. Bars carrying different letters are significantly different at $P \leq 0.05$ (Tukey's studentized range test).

4. Discussion

Endophytic *F. oxysporum* isolate V5w2 was able to activate genes in banana plants involved in plant defense/stress, primary metabolism, transport, signal transduction, cell wall biosynthesis and cell differentiation and development. This suggests that banana plants respond by means of a diverse range of regulatory processes following endophyte colonization. Six unknown genes were also identified that may have a novel functional role in the endophyte–banana interaction. Our study presents the first report of defense gene expression in banana following colonization by a fungal endophyte and challenge with *R. similis*. We also report for the first time plant gene identification in a fungal endophyte–banana interaction. In 2009, Harish et al. [18] reported the up-regulation of defense genes *β-1,3-glucanase* and *chitinase* in banana plants following viral challenge of plants previously inoculated with the biocontrol microbe *Pseudomonas fluorescens* Pf1. Similarly, Molitor et al. [32] reported up-regulation of defense-related genes (PR1, PR2 and PR5) in barley following infection of *Piriformospora indica* colonized plants by *Blumeria graminis* f.sp. *hordei* (Powdery Mildew pathogen).

The greatest number of genes induced by endophytic *F. oxysporum*, surprisingly, was found in the roots of the *R. similis*-susceptible cv Nabusa, and not in the tolerant cv Kayinja. Four TDFs with similarity to defense-related genes were up-regulated in susceptible cv Nabusa, and three in the tolerant cv Kayinja. Similarly, a TDF related to cell wall strengthening was up-regulated in only cv Nabusa following endophyte colonization. When cv Nabusa was inoculated with the endophyte, however, gene expression was strongly up-regulated. The lesser up-regulation of these genes in the tolerant cv Kayinja may be explained by the fact that these genes were already constitutively expressed, resulting in little or no differences in their expression between non-inoculated controls and endophyte-inoculated plants. This finding is not entirely unique. When resistance was induced systemically in susceptible and tolerant cucumber (*Cucumis sativus* L.) plants by *Pseudomonas putida* Trevisan against Fusarium wilt caused by *F. oxysporum* f. sp. *cucumerinum* J.H. Owen, plant defense responses were expressed more pronouncedly in susceptible than tolerant plants [33].

The up-regulation of genes involved in defense signaling (*Calmodulin-Ca*$^{2+}$), jasmonic acid pathway (*COI1* and *LOX*), cell wall strengthening (*β-1,3-glucan synthase*) and transport of defense molecules (*ABC transporter*) in this study is of great significance. This is because these findings validate earlier speculations that inoculating banana plants with endophytic isolate V5w2 protects them against the root burrowing nematode *R. similis*. Our results further support the finding by Athman [11] that induced resistance is the primary means of *R. similis* control by endophytic isolate V5w2. Cellular signaling is initiated soon after the invader has been recognized by higher plants [34]. Calmodulin is involved in the JA-dependent and -independent wound signal transduction pathways [35]. The expression of *Calmodulin-Ca*$^{2+}$ was up-regulated from 2 to 30 days post endophyte inoculation in both cultivars, indicating the initiation of defense signaling.

Expression of *COI1* was up-regulated in the roots and rhizomes, and *LOX* in the roots of both cultivars. Both genes are involved in the JA signaling pathway that regulates plant responses to abiotic stress, defenses against herbivores and pathogens, and wound healing [36]. The specific up-regulation of genes involved in the JA signaling pathway during the interaction between

endophyte-inoculated banana and *R. similis* supports the notion that induced defenses triggered by beneficial microorganisms is predominantly regulated by JA [37].

Following endophyte colonization, expression of the gene encoding *β-1,3-glucan synthase* was up-regulated in roots of both the susceptible and tolerant cultivars. The above protein is involved in cell wall strengthening. Cell wall strengthening following endophyte inoculation has often been inferred from the fact that endophytic colonization is limited to the epidermal and outer cortical cells [14] and [15].

Similarly, expression of the ABC transporter gene was up-regulated in both the tolerant and susceptible cultivars 2 days post endophyte inoculation. ABC transporter proteins in eukaryotic cells help to catalyze the efflux of various compounds out of the cell. They have been implicated in herbicide detoxification, xenobiotic transport, pigment transport, alleviation of oxidative damage and the transport of antimicrobial compounds [38].

qRT-PCR results confirmed high up-regulation of *ABC transporter* gene activity in both the susceptible and tolerant banana cvs following root colonization by endophytic isolate V5w2. However, the gene was down-regulated in *R. similis* non-challenged plants 33 days after endophyte inoculation. Interestingly, when endophyte-inoculated plants of both cultivars were challenged with *R. similis*, expression of the *ABC transporter* gene increased significantly compared with nematode non-challenged plants or endophyte-free plants challenged with the nematode. This implies that the fungal endophyte primes banana root tissues for greater expression of the *ABC transporter* gene upon *R. similis* infestation. In a related study, Johnson et al. [39] reported up-regulation of a gene encoding ABC transporters of the group PDR-5 in tall fescue (*Lolium arundinaceum* Schreb.) plants infected with the fungal endophyte *Neotyphodium coenophialum* Morgan-Jones and Gams. The induction of the same gene was reported earlier in rice after infection by the rice blast fungus *Magnaporthe oryzae* (T.T. Hebert) M.E. Barr and following treatment with benzothiadiazole [40].

Endophyte colonization resulted in a significant up-regulation of *β-1,3-glucan synthase* in both cultivars 2 days post endophyte inoculation, with gene expression in the tolerant cv Kayinja twice as much as that in the susceptible cv Nabusa. *β-1,3-glucan* (called callose in plants) constitutes the cell walls of higher plants and is reportedly deposited in cell walls in response to plant cell wall penetration by pathogens or biocontrol organisms [13]. The fact that *β-1,3-glucan synthase* activity increased in endophyte-inoculated cv Kayinja plants until 33 days post endophyte inoculation indicates that cell wall strengthening occurs as a strong and lasting response to hyphal penetration of walls. In cv Nabusa, endophyte colonization resulted in the priming of wall strengthening enzymes for greater expression upon nematode challenge.

The expression profiles of *COI1* and *LOX* following endophyte inoculation and *R. similis* challenge implies the likely involvement of JA-induced defenses in fungal endophyte-induced resistance against *R. similis* in banana. *COI1* activity was up-regulated in the tolerant cv Kayinja at 33 days post inoculation in endophyte-inoculated plants, but not in cv Nabusa. However, when endophyte-inoculated plants of both cultivars were challenged with *R. similis*, *COI1* activity was significantly up-regulated compared with endophyte non-inoculated and *R. similis*-challenged plants at 33 days post inoculation. *COI1* is believed to be involved in jasmonate responses by

regulating the expression of jasmonate responsive genes [41], [42] and [43]. Devoto et al. [42] showed that *COI1* was required for the expression of 84% of 212 genes induced by JA and 44% of 153 genes induced by wounding in *Arabidopsis*.

The expression of *LOX* was significantly and non-significantly up-regulated in the susceptible and tolerant cultivars 33 days post endophyte inoculation, respectively. However, expression of *LOX* was later up-regulated significantly in the tolerant cultivar after *R. similis* challenge. *LOX*, like *COI1*, has been shown to play a central role in the induction of JA-induced genes such as *proteinase inhibitor* (*PI*) and *polyphenol oxidase* (*PPO*) [44]. Heitz et al. [45] reported that the activation of *LOX* preceded that of *PI* in the wound-signaling pathway, confirming their regulatory role in the production of defense genes. *PI* is involved in the defense of host plants against insect herbivores [46], while PPO is one of the enzymes involved in the phenylpropanoid pathway (lignin synthesis) and has been reported following wounding [47].

Our study shows the involvement of JA-induced defense responses in the protection of banana against *R. similis* by endophytic non-pathogenic *F. oxysporum*. JA dependent genes whose expressions were analyzed showed primed expression patterns following *R. similis* challenge. This observation clearly follows the pattern of induced systemic resistance by beneficial microorganisms in plants. The resistance induced by a *F. oxysporum* endophyte in banana against *R. similis* has been phenotypically demonstrated in the greenhouse [12]. If similar protection occurs in the field, *F. oxysporum* endophytes will contribute to the protection of banana plants against pests such as the nematode. In the Lake Victoria basin region of Eastern Africa where banana is an important staple crop, use of tissue culture banana plantlets is being promoted. The use of banana plantlets as opposed to field suckers is believed to delay infestation of newly established fields. If these plantlets can be bio-enhanced before field planting, the battle against one of the most destructive banana pests in the region would be greatly strengthened.

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