South African Journal of Botany 104 (2016) 125–133

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Expressing stacked *HRAP* and *PFLP* genes in transgenic banana has no synergistic effect on resistance to *Xanthomonas* wilt disease

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ARTICLE INFO

Article history:
Received 20 January 2015
Received in revised form 3 September 2015
Accepted 22 September 2015
Available online 3 February 2016

Edited by Z-H Chen

Keywords:
Transgenic banana
HRAP
PFLP
Xanthomonas wilt resistance
Gene stacking

ABSTRACT

Banana production in Africa's great lakes region is threatened by the Banana *Xanthomonas* wilt (BXW) disease caused by *Xanthomonas campestris* pv. *musacearum*, a biotrophic pathogen. Transgenic banana plants, cv. "Gonja manjaya," expressing stacked hypersensitive response-assisting protein gene (*HRAP*) and the plant ferredoxin-like protein gene (*PFLP*) were evaluated for resistance against BXW in comparison to transgenic lines having single gene. Transgenic lines with stacked gene as well as single gene had higher resistance to the pathogen than non-transgenic control plants indicated by either no symptom development or delayed symptoms for completely and partially resistant plants, respectively. Transgenic lines also produced more hydrogen peroxide due to pathogen infection and also had higher transcription of stress response genes encoding *NPR1*, a defense response co-transcriptor, the antimicrobial *PR-3* and glutathione S-transferase. However, transcription of *PR-1*, an indicator for infection with a biotrophic pathogen, was not increased in both stacked and single transgenic lines, indicating a possible shift to infection with a necrotrophic pathogen in plants due to transgenes expression.

Expression of stacked *HRAP* and *PFLP* genes in transgenic banana lines did not show higher or additive resistance levels against pathogen in comparison to individual genes; however, stacking might provide the benefit of durable resistance in case one transgene function is lost.

1. Introduction

Bananas, an important staple food crop in countries in the great lakes region of Africa (Biruma et al. 2007), are threatened by the banana *Xanthomonas* wilt (BXW) disease caused by the bacterium *Xanthomonas campestris* pv. *musacearum* (*Xcm*) (Tripathi et al. 2009). The pathogen, infecting all banana varieties including East African Highland Bananas and exotic types of banana, causes progressive wilting, yellowing, and eventual death of the infected plants, with so far no source of resistance (Ssekiwoko et al. 2006; Tripathi et al. 2009).

Strategies previously applied to improve resistance against the BXW disease in banana included the use of transgenic plants constitutively expressing either the hypersensitive response-assisting protein (*HRAP*) or the plant ferredoxin-like protein (*PFLP*) gene (Namukwaya et al. 2012; Tripathi et al. 2010, 2014). Expression of the *HRAP* or *PFLP* transgene has also been reported to enhance resistance against bacterial pathogens in transgenic tobacco, *Arabidopsis* plants, calla lily, and rice (Tang et al. 2001; Ger et al. 2002; Liau et al. 2003; Pandey et al. 2005; Huang et al. 2004, 2007). Transgenic *Arabidopsis* plants, with apoplast-localized *PFLP*, further exhibited higher resistance against bacterial pathogen than transgenic plants with cytoplasm-localized *PFLP* transgenic plants (Lin et al. 2010).

The pathogen-induced plant hypersensitive response (HR) is a rapid and highly localized cell death required for neutralizing any invading pathogen. The earliest event during HR, which occurs a few hours after the pathogen enters the cell, is production of reactive oxygen (ROS), with hydrogen peroxide (H_2O_2) the most stable ROS (Sutherland 1991). Hydrogen peroxide has, however, a dual role being toxic at high concentrations and acting as a signaling molecule at low concentrations for adjusting cells to changed environmental conditions (Noctor et al. 2014; Petrov and Van Breusegem 2012). The PFLP protein is involved in redox reactions associated with enhanced production of ROS (Dayakar et al. 2003). Expression of *PFLP* further changes cytosolic calcium levels and also activates membrane-bound NADPH oxidase involved in ROS production (Noctor et al. 2014; Su et al. 2014). In contrast,

Abbreviation: *PFLP*, Plant ferredoxin-like protein; *HRAP*, Hypersensitive response-assisting protein; *Xcm*, *Xanthomonas campestris* pv. *musacearum*; BXW, Banana *Xanthomonas* wilt.

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http://dx.doi.org/10.1016/j.sajb.2015.09.017
0254-6299/

the *HRAP* protein acts on harpin proteins, which are produced by many pathogens and sensed by plants triggering defense responses. HRAP dissociates harpin multimeric protein forms into monomers and dimers and these forms elevate HR and prevent propagation of a bacterial pathogen like *Xcm* (Chen et al. 2000; Ger et al. 2002).

Although individual *HRAP* and *PFLP* transgenes provided resistance in banana to the BXW disease, an approach to stack the *HRAP* and *PFLP* transgenes to further amplify HR, and whereby improve durability of resistance, has not been carried out yet. Such gene stacking approaches have been previously applied to either target different pathogens or to avoid breakdown of resistance (Chan et al. 2005; Storer et al. 2012; Zhu et al. 2012). Recently, trans-plastomic *Nicotiana benthamiana* plants expressing sporamin, cystatin, and chitinase genes in three-stacked combinations to obtain multiple resistance (insects, phytopathogens, and abiotic stress) had synergistic and enhanced resistance against larva of *Spodoptera litura* and *Spodoptera exigua*, and phyto-pathogens *Alternaria alternata* and *Pectobacterium carotovorum* subsp. *carotovorum* in comparison to plants expressing such genes individually (Chen et al. 2014). Single genes expression is particularly prone to rapid breakdown, necessitating strategies, such as transgene stacking, to obtain not only higher but also more durable resistance (Collinge et al. 2008; Datta et al. 2002; Zhao et al. 2003).

The particular objective of this study was therefore to determine whether stacking *HRAP* and *PFLP* transgenes will result in higher resistance to the BXW disease when compared to individual transgene expression. We, therefore, generated transgenic banana plants of cultivar "Gonja manjaya" constitutively expressing the *HRAP* and *PFLP* transgene, both derived from sweet pepper (*Capsicum annuum*) individually or stacked, and evaluated them for resistance against the bacterial pathogen *Xcm*. This study also determined if stacked transgenes will amplify to a greater extent the oxidative burst in response to pathogen infection. In addition, we also investigated if transgene stacking, compared to individual transgenes, enhances transcription of stress responsive genes, and we measured transcription of the two banana *NPR1* genes (*NRR1A* and *NPR1B*) and *PR* genes (*PR-1* and *PR-3*) encoding antimicrobial proteins. NPR1 is a defense response co-transcriptor conferring resistance to pathogens and banana contains several *NPR1s* with *NPR1A* and *NPR1B* that are 78% identical (Endah-Yocga et al. 2008). *NPR1* gene expression induces PR protein production that accumulates after pathogen infection (Van Loon et al. 2006). *NPR1* mutations further cause failure of induction of these *PR* genes with plants having increased susceptibility to pathogen infection (Kinkema et al. 2000). Finally, we also measured transcription of the gene encoding glutathione S-transferase (*GST*), which is among the most responsive genes to stress and chemical signaling treatments (Glombitza et al. 2004). Overall, this study reports the generation of transgenic banana lines with stacked genes and evaluation for enhanced resistance against *Xcm* in comparison to single gene transgenic lines.

2. Materials and methods

2.1. Plasmid construction

The pBI-*HRAP* and pBI-*PFLP* plasmids carrying the *HRAP* and *PFLP* coding sequence, respectively, each under the control of the CaMV35S promoter, were acquired from Academia Sinica, Taiwan. Both *HRAP* and *PFLP* genes were used to construct the vector pBI- *HRAP-PFLP* with both genes stacked (Fig. 1) The full coding sequence of *PFLP* together with its promoter and terminator sequences was amplified from pBI-*PFLP* plasmid using the primers 35SH: 5′-ACAAAGCTTGCATGCC TGC AGGTC-3′ and NosH: 5′-TGTTTCGAACCGA TCTAG TAAC ATA G-3′. The PCR reaction mixture consisted of 1X *Pfu* buffer, 0.2 mM dNTPs, 3.0 mM $MgSO_4$, 0.5 mM of each primer, and 1 U of *Pfu* DNA polymerase (Fermentas, UK), and 100 nanograms of template DNA in a reaction volume of 50 μl. The following program: 95 °C for 5 min; 35 cycles of 95 °C for 40 s, 65 °C for 1 min, 72 °C for 2 min, and finally 72 °C for 10 min was used for DNA amplification. To the purified PCR product, Taq DNA was used to add adenosine (A) overhangs and then amplified product cloned into the pCR 2.1-TOPO vector. Using *Hind III*, the *Pflp* fragment was removed from the TOPO vector and then ligated into pBI-*HRAP* vector at the *Hind III* restriction site. All plasmids (pBI-*HRAP*, pBI-*PFLP*, and pBI-*HRAP-PFLP*) were validated by PCR, restriction analysis, in addition to sequencing the T-DNA region for pBI-*HRAP-PFLP* before transferring into cells of *Agrobacterium tumefaciens* strain AGL1 by electroporation as described by Weigel and Glazebrook (2006).

2.2. Generation of transgenic plants

Embryonic cell suspensions (ECS) of banana cultivar "Gonja manjaya" developed from scalps were used for transformation. The ECSs were transformed with *Agrobacterium* strain AGL1 harboring the different plasmid constructs and regenerated on selective medium according to Tripathi et al. (2010). Regenerated shoots were transferred onto proliferation medium (MS salts and vitamins, 10 mg/l ascorbic acid, 5 mg/l BAP, 30 g/l sucrose, 2.4 g/l gelrite, pH 5.8) supplemented with 50 mg/l geneticin for 30 days to ensure that only transgenic shoots survived. Shoots were then transferred to fresh proliferation medium without antibiotic selection for shoot multiplication. Non-transgenic control plantlets were regenerated on medium without any antibiotics selection. Individual transgenic and non-transgenic plants were finally transferred to rooting medium and fully developed rooted plantlets were weaned in sterile soil in plastic pots to the containment facility.

2.3. Molecular analysis of transgenic plants

Genomic DNA was extracted from 63 randomly selected putative transgenic banana lines (21 lines with each construct) according to

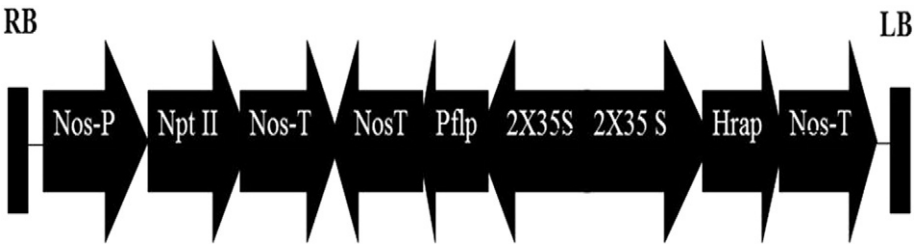

Fig. 1. Schematic representation of the T-DNA region of *pBI-HRAP-PFLP* construct used for banana transformation. RB and LB represents right and left border, respectively; NOS-P and NOS-T the promoter (P) and terminator region (T) of the *Agrobacterium tumefaciens* nopaline synthase gene; 2X35SP the double cauliflower mosaic virus 35S promoter sequence; n*ptII* the coding sequence of the neomycin phosphotransferase II gene; *PFLP* coding sequence of plant ferredoxin-like protein derived from sweet pepper and *HRAP* coding sequence of the hypersensitive response-assisting protein from sweet pepper.

the protocol of Untergasser (2008), with slight modifications, 0.1% sodium meta bisulfite was applied instead of 1% beta-mercaptoethanol, using a chloroform: isoamylalcohol (24:1) mixture instead of chloroform as well as removing RNA with RNase A instead by a LiCl treatment. Presence of *HRAP* and *PFLP* genes in putative transgenic plants was confirmed by PCR analysis with gene-specific primers (Table 1). Plasmid DNA of pBI-*HRAP-PFLP*, pBI-*HRAP*, and pBI-*PFLP* were used as positive controls and non-transformed plant DNA as a negative control.

2.4. Glasshouse evaluation of transgenic plants for BXW resistance

A pure bacterial culture of the *Xcm* isolate was obtained from the National Agricultural Research Laboratories (NARL), Pathology Laboratory, on YPGA (yeast extract 5 gl^{-1}, peptone 10 gl^{-1}, glucose 20 gl^{-1}, agar 40 mgl^{-1}, pH 7.0) plate. Bacterial culture identity was first verified by screening for morphological characteristics as well as by PCR analysis using *Xcm* specific primers. A bacterial culture was initiated from a single colony in YPG broth and then incubated at 28 °C for 48 h under continuous shaking at 150 rpm. The bacterial pellet was re-suspended in sterile water and its optical density adjusted to 10^8 colony-forming units/ml (cfu/ml). This bacterial suspension was used for artificial inoculation of experimental plants.

Three-month-old PCR-positive transgenic lines carrying either stacked or individual *HRAP* or *PFLP* coding sequences (21 lines for each construct) were randomly selected and evaluated for resistance to *Xcm* with the leaf petiole assay technique. In the assay, the first fully opened leaf of potted plants was inoculated with 100 μl (10^8 cfu/ml) of a bacterial suspension according to Tripathi et al. (2008). Three plants of each line were inoculated and plants reaction to pathogen evaluated in a glasshouse at 27–30 °C under natural illumination with daily watering. Plants were monitored every day over 60 days for number of days to BXW disease symptom development including wilting, leaf chlorosis development, number of leaves showing symptoms as well as number of days for complete chlorosis and complete wilting of plants. Transgenic lines were further evaluated by measuring chlorotic areas and lesion sizes using the leaf blade assay.

2.5. Gene expression analysis

Transcription of *HRAP* and *PFLP* transgenes was determined from four representative lines, selected randomly from 21 transgenic lines, carrying either stacked or individual *HRAP* or *PFLP* transgenes. The same four lines of each construct were also used for further analysis of expression of stress responsive genes. For analysis, the youngest leaf was sampled from three biological replicates of each line and then pooled for total RNA extraction. Total RNA was extracted according to Yang et al. (2008). Synthesized cDNA was diluted 10-fold for quantitative real-time PCR (qRT-PCR). Transgenes were amplified with gene-specific primers designed with primer3 software (Table 1). The *Musa* 25 s rRNA coding sequence was applied as an endogenous control.

qRT-PCR was carried with the 7900HT Fast Real-Time PCR system (Applied Biosystems, USA) with a Maxima SYBR-Green/ROX qPCR Master Mix for detection (Thermo Scientific, UK). The reaction mixture contained 2 μl of diluted template (1/10), 0.6 μl of 10 μM of forward and reverse primers, 5 μl SYBR-Green I master mix, and 5 μl nuclease-free water was added into respective wells in a 96-well qRT-PCR micro-titer plate. Non-template control reactions, containing water instead of cDNA, were also included into the analysis. Real-time PCR conditions were as follows: an initial 95 °C denaturation step for 15 min was followed by 40 cycles consisting of a denaturation step at 94 °C for 10 s, primer annealing at 60 °C for 20 s, and extension of DNA sequence at 72 °C for 20 s. The reaction was finally subjected to a temperature of 65 °C for 10 s and the plate was incubated at 95 °C for the fluorescence signal of samples to be assessed. A standard curve for *HRAP*, *PFLP*, and *Musa 25 s rRNA* genes was done to a final arbitrary concentration of 1 (stock) and sequential dilutions (factor 2) to 0.5, 0.25, 0.125, and 0.0625. Specificity of PCR amplification was confirmed by melting curve analysis (75 °C–95 °C).

Transcription of stress responsive genes was assayed in four lines each of individual and stacked transgenes, by sampling the inoculated leaf as stated above using *NPR1*, *PR1*, *PR3*, and *GST* primers (Table 1). For final analysis of changes in transcription of stress responsive genes, the comparative CT method to determine $2^{-\Delta\Delta CT}$ was applied (Livak and Schmittgen 2001).

2.6. Measurement of hydrogen peroxide in tissues

Production of hydrogen peroxide after artificial infection with *Xcm* was determined by a histochemical staining assay with DAB (3,3-diaminobenzidine) (Šnyrychová et al. 2009). Plant leaves were inoculated with the pathogen and then inoculated tissues were collected at 12 h post-inoculation for DAB staining. Explants from four lines for each construct were immersed in a 1 mg/ml DAB solution (Sigma, UK), vacuum-infiltrated for 5 min, then incubated for 6–8 h at room temperature. After staining and removal of the DAB solution, chlorophyll was removed from the leaf explants by boiling in an ethanol-acetic acid (3:1) solution for 5 min.

2.7. Data analysis

All collected data on resistance evaluation of resistance and gene expression analysis were analyzed for means and level of significance among the means using GENSTAT (Version 12.1).

3. Results

3.1. Generation and molecular characterization of transgenic lines

The embryonic cell suspensions of banana cultivar "Gonja manjaya" was transformed with either individual *HRAP* or *PFLP* or stacked transgenes (Fig. 1). Transgenes were each under the control of the CaMV35S promoter and the Nos-terminator sequences. In total, 150 independent transgenic lines having stacked transgenes and 140 lines with individual transgenes (70 independent transgenic lines for each individual transgene) were generated. No visible phenotypic differences in the regeneration of transformed cells and transgenic lines with stacked transgenes were observed. The presence of transgenes in 21 randomly selected stacked transgenic lines and 21 lines each with individual transgene was confirmed by PCR analysis using gene-specific primers for both the *HRAP* and *PFLP* transgene (Table 1). For all tested transgenic plants, expected amplified products of 490 bp for *HRAP* and 372 bp for *PFLP* were observed, confirming the presence of the

Table 1
Primers sequences used for PCR and qR-TPCR.

Target gene	Forward primer sequence	Reverse primer sequence
Hrap[a]	GAGCTCACAGCATTTTGGCCATCCC	TGGAGTTGGAGGACGAGGAAC
Pflp[a]	GAGCTCCCAAACGTTGGGGAAGC	ACGAGTTCTGCCTCTTTGTGAGT
qRT-PCR		
Hrap	CCAACACAATACTTCAATAGGG	AGCAGAATCACAAGGGACAAT
Pflp	CCTGACGGACCAATAGAAT	CACAAGATGAGCAAGAACCT
MNPR1A[b]	GTCGGCATTGTACCAACACA	CAGTGCAGGAGTCAGCAAAA
MNPR1B[b]	AGGTTTGCCCGAACAAGAAG	TGAGAGGCAACAACTCAGAGAG
PR-1[b]	TCCGGCCTTATTTCACATTC	GCCATCTTCATCATCTGCAA
PR-3[b]	GGCTCTGTGGTTCTGGATGA	CCAACCCTCCATTGATGATG
GST[c]	TAAGAAGCGCTTTGGGATTG	AATCTAGGCCAACGGTTCCT
Musa 25s[b] rRNA	ACATTGTCAGGTGGGGAGTT	CCTTTTGTTCCACACGAGATT

[a] Primer sequences designed based on *HRAP* coding sequence (accession no. AF168415); and *PFLP* coding sequence (accession no. AF039662) for amplification of genomic DNA.
[b] Primer sequences previously reported by Endah-Yocga et al. (2010).
[c] Primer sequences designed based on NCBI deposited coding sequences.

transgenes. The amplification products of both the *HRAP* and *PFLP* transgenes in representative transgenic lines transformed with stacked transgenes is shown in Fig. 2A. These 21 PCR-positive lines of each construct were randomly selected lines and transferred to sterile soil in pots for 3 months in the glasshouse for further analysis.

The relative expression of *HRAP* and *PFLP* transgenes in four representative lines, randomly selected from 21 transgenic lines with stacked or individual genes, by applying qRT-PCR. Transgene expression was measured relative to expression of the *Musa* 25 s rRNA coding sequence. No transgene transcription was detected in the non-transgenic control line but all four transgenic lines selected with stacked or each individual transgene transcribed the transgenes at various degrees. We found, however, much higher (at least more than one-fold) *HRAP* and *PFLP* transcriptions in lines with individual transgenes in comparison to lines with stacked transgenes (Fig. 2B).

3.2. Glasshouse evaluation of transgenic plants for BXW resistance

Twenty-one PCR-positive transgenic lines carrying stacked *HRAP* and *PFLP* transgenes were evaluated in a glasshouse for resistance against *Xcm* and their resistance compared with transgenic lines carrying individual transgene (21 transgenic lines of each individual transgene). Using the leaf petiole inoculation assay, symptoms of leaf wilting followed by chlorosis were observed. These disease symptoms and disease severity varied among transgenic lines tested (Table 2). Most of the transgenic lines with stacked genes showed localized death at the point of inoculation without further symptom development (Fig. 3A), whereas in some transgenic lines, disease symptom developed and progressed from inoculated dead yellow tissues (Fig. 3B). Symptom appearance in stacked lines was on average at 34 dpi in contrast to 27.4 and 30.2 dpi in lines with individual *PFLP* and *HRAP*, respectively. In contrast, disease symptoms in the non-transgenic control plants were observed at 15–17 days post-inoculation (dpi) (Table 2). All plants representing the non-transgenic control plants had completely wilted and died within 30–35 dpi due to pathogen infection (Fig. 3C). Furthermore, six transgenic lines with stacked genes (S-3, S-9, S-11, S-15, S-19, and S-32) in contrast to seven transgenic lines with individual *PFLP* gene (P-1, P-3, P-19, P-11, P-13, P-20, and P-21) and six transgenic lines with individual *HRAP* gene (H-11, H-14, H-16, H-17, H-21, and H-25) showed symptoms in only the inoculated leaf throughout the duration of the experiment (60 dpi) (Fig. 3D-2). Symptoms development in this one inoculated leaf ranged from 41 to 52 dpi in stacked transgenic lines in contrast to 26–40 dpi in transgenic lines with individual *PFLP* and 24–48 dpi in transgenic lines with individual *HRAP* gene (Table 2), showing that stacked lines exhibited delayed symptom development than in transgenic lines with individual genes. These transgenic lines showing symptoms in the inoculated leaf only were scored a DSI = 1 and were considered partially resistant, having a lower disease severity in comparison to non-transgenic control plants (Table 2). A few stacked transgenic lines (S-4, S-8, S-10, and S-25) had 2–3 leaves showing symptoms at 60 dpi (Fig. 3D-1).

Seven transgenic lines with stacked genes (S-1, S-2, S-5, S-7, S-14, S-16, and S-21; representing 33.1% of tested lines) did not show any disease symptoms in comparison to four transgenic lines with individual *PFLP* (P-5, P-6, P-16, and P-36, representing 19.1% of the tested lines), and six transgenic lines with individual *HRAP* gene (H-4, H-5, H-8, H-15, H-18, and H-24, representing 28.6% of the tested lines) at 60 dpi (Table 2).These lines were scored a DSI = 0 and were considered completely resistant (Fig. 3E).

One transgenic line with stacked genes (S-22) developed symptoms and wilted completely in contrast to three transgenic lines with individual *PFLP* (P-4, P-10, and P-25) and one transgenic line with individual *HRAP* (H-10) (Table 2). However, complete wilting and eventual death in the stacked transgenic line was delayed to 46 dpi in contrast to transgenic lines with individual gens, which was 42 dpi, and disease severity in these lines was the same as for the non-transgenic control plants (Table 2).

Further comparison of resistance levels using the leaf blade inoculation assay showed no significant difference in lesion length and chlorotic areas in transgenic lines with stacked genes and individual genes (data not shown). However, all non-transgenic control lines had developed significant lesions and chlorosis at 28 dpi than in all transgenic lines.

To determine whether transgene expressions correlated to resistance levels to *Xcm*, we further quantified transgenes transcripts in four lines of individual *HRAP* (H7, 3, 8, 5), *PFLP* (P2, 1, 5, 6), and stacked transgenes (S3, 8, 5, 2) using qRT-PCR. Transgenic lines H5, H8, P6, P5, S2, and S5 that showed absolute resistance (no symptoms development) and lines H3, H7, P2, P1, S8, and S3, partial resistance (delayed symptoms development) compared to susceptible non-transgenic control plants were tested (Fig. 2B; Table 3). Results confirmed transgenes expressions in transgenic lines with stacked or individual transgenes unlike in the non-transgenic control plants (Table 3). There was no significant difference in relative expression of *HRAP* and *PFLP* in stacked

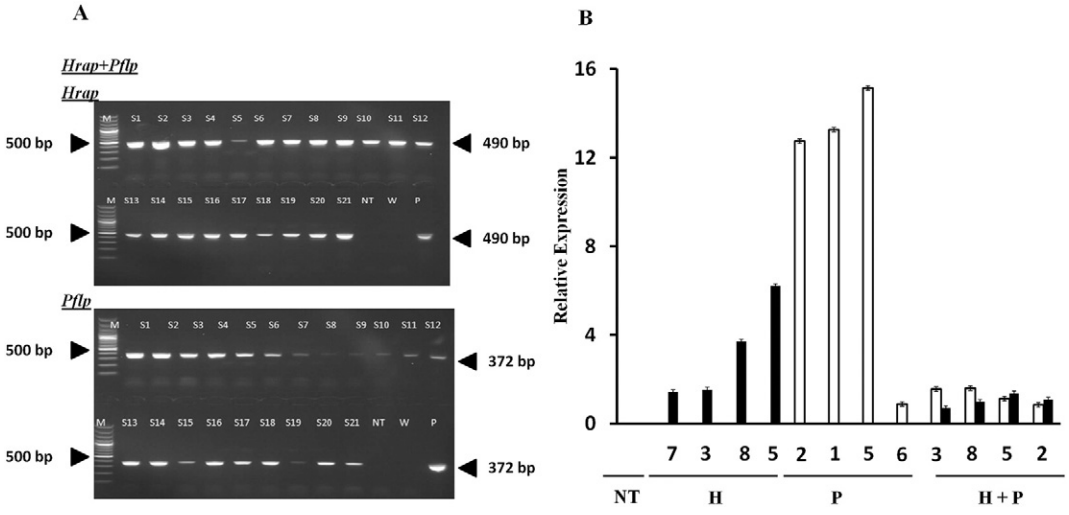

Fig. 2. (A) PCR analysis of genomic DNA from stacked transgenic lines for the presence of both *HRAP* and *PFLP* genes. Lane NT represents a genomic DNA from a non-transgenic control plant; lane W a control where genomic DNA was replaced with water; S1–S21: transgenic plants and lane P plasmid pBI-*HRAP-PFLP*. (B) Relative expression of *HRAP* or *PFLP* in transgenic plants carrying either single (H, P) or stacked transgenes (H + P). Relative expression of transgenes was calculated by comparison with expression of the *Musa* 25 s rRNA coding sequence as an endogenous control.

Table 2
Evaluation of transgenic lines with stacked and individual genes for enhanced resistance to *Xanthomonas campestris* pv. *msacearum* in artificially inoculated soil potted plants.

Transgenic line	Mean* number of days for disease symptoms† appearance	Mean* number of days for complete wilting	Disease severity (%)
Control	17.4	35.9	100
S-1	–	–	0
S-3	41.2‡	–	25‡
S-4	21.8	–	88.9
S-2	–	–	0
S-5	–	–	0
S-6	34.5‡	–	56.7‡
S-7	–	–	0
S-8	26.7	–	32.2‡
S-9	42.3‡	–	13.9‡
S-10	22.3	–	53.3‡
S-11	52.6‡	–	12.2‡
S-14	–	–	0
S-15	49.3‡	–	18.9‡
S-16	–	–	0
S-17	24.0	–	93
S-19	43.2‡	–	23.3‡
S-20	24.2	–	83.3
S-21	–	–	0
S-22	21.5	46‡	100
S-25	22.3	–	73.3‡
S-32	51.0‡	–	13.3‡
H-3	21.7	–	87.8
H-4	–	–	0
H-5	–	–	0
H-6	21.9	–	36.7‡
H-7	22.2	–	76.7‡
H-8	–	–	0
H-9	32.3‡	–	42.2‡
H-10	22.8	42.6‡	100
H-11	48.0‡	–	13.3‡
H-12	30.2‡	–	47.6‡
H-13	26.2	–	46.7‡
H-14	33.0‡	–	25.5‡
H-15	–	–	0
H-16	24.0	–	28.9‡
H-17	47.8‡	–	13.3‡
H-18	–	–	0
H-20	23.7	–	51.6‡
H-21	34.5‡	–	26.7‡
H-22	19.2	–	50‡
H-24	–	–	0
H-25	46.3‡	–	21.7‡
P-1	34.8‡	–	27.8‡
P-2	29.2	–	41.1‡
P-3	27.2	–	25.6‡
P-19	31.0‡	–	28‡
P-4	17.3	44‡	100
P-5	–	–	0
P-6	–	–	0
P-7	37.0‡	–	41.1‡
P-8	26.2	–	53.3‡
P-9	26.0	–	43‡
P-10	20.2	38.3	100
P-11	35.0‡	–	27.2‡
P-13	26.3	–	30‡
P-12	20.0	–	68‡
P-14	21.5	–	78.3
P-16	–	–	0
P-18	22.0	–	56.1‡
P-20	34.3‡	–	20.6‡
P-21	40.0‡	–	23.3‡
P-25	18.5	43‡	100
P-36	–	–	0

Disease severity (%) = (number of leaves with symptoms/total number of leaves on a plant) × 100.
* Mean of three replicates.
† The disease symptoms were chlorosis or necrosis on the leaves of inoculated plants.
‡ Significant differences ($P < 0.05$) in transgenic lines in comparison to non-transgenic control plants.

lines, and compared to transgenic lines with individual transgenes, all stacked transgenic lines had significantly lower transcripts of *HRAP* and *PFLP*. Total transcripts of *HRAP* and *PFLP* in each transgenic stacked line whether absolutely resistant (S-2 and S-5) or partially resistant (S-3 and S-8) were in the same range (1.9–2.5). These results suggest that enhanced resistance to *Xcm* may not be associated with increased transcripts of both or either *HRAP* or *PFLP* in stacked transgenic lines. Transgenic lines with individual *PFLP* gene except for P6 showed higher expression of the transgene compared to transgenic lines with individual *HRAP* and stacked genes. However, we could not find any direct relationship between relative transcription levels and disease phenotype in tested transgenic lines with stacked as well as individual transgenes (Table 3).

3.3. Hydrogen peroxide production and NPR1 transcription

Histochemical staining with DAB 12 h after pathogen infection confirmed production of hydrogen peroxide as a consequence of *Xcm* infection in leaves of all tested transgenic plants identifiable by dark-brown stains (Fig. 4A). However, no dark-brown stains were detectable at time of inoculation (0 h) and also in leaves of the non-transgenic control (Fig. 4A).

We further investigated if hydrogen peroxide production relates to increase in transcription of the two banana *NPR1* homologues, *NPR1A* and *NPR1B*. We found that transcription of the banana *NPR1A* gene was generally higher in all tested transgenic lines carrying the stacked transgenes than in the non-transgenic control at 12 h after *Xcm* infection, which correlated with hydrogen peroxide production. Two *HRAP*, two *PFLP*, and all four lines with stacked transgenes had higher *NPR1A* transcription than the control plant (Fig. 4B). Higher transcription in transgenic lines was, however, less prominent for *NPR1B* and only one *HRAP*, two *PFLP*, and one line with stacked transgenes had higher *NPR1B* transcription than the control (Fig. 4C).

3.4. Transcription of PR and GST

The *PR* (*PR-3* and *PR-1*) gene transcription after *Xcm* infection was also measured. *PR-3* transcription was found to be higher at 12 h after *Xcm* infection in transgenic lines in comparison to the non-transgenic control. Two *HRAP*, three *PFLP*, and one line with stacked transgenes had higher *PR3* transcription than the non-transgenic control (Fig. 5). In contrast, *PR-1* transcription increased as expected in the non-transgenic control. However, *PR-1* transcription was lower in all transgenic lines in comparison to the non-transgenic control, except for one *PFLP* line. This line had significantly higher *PR-1* transcription than the non-transgenic line (Fig. 5). Transgenic lines had also higher transcription of the gene for *GST* than the non-transgenic control after *Xcm* infection. All *HRAP*, three *PFLP*, and two line with stacked transgenes had higher transcription of the gene coding for *GST* than the non-transgenic control (Fig. 5).

4. Discussion

This study confirmed previous results by Namukwaya et al. (2012) and Tripathi et al. (2010) that transgenic banana lines engineered with the *HRAP* and *PFLP* transgene, either individually or both stacked, provide resistance against *Xcm* infection with delayed, or no, symptom development after artificial infection. In general, a transgene stacking approach, as applied in this study, has the potential to either delay rapid breakdown of single gene action by providing higher and more durable resistance (Storer et al. 2012) or to simultaneously overcome different stress factors limiting plant performance (Douglas and Halpin 2010; Naqvi et al. 2009). A similar example has been reported for enhanced nematode resistance through ectopic overexpression of a protease inhibitor together with a chitinase in transgenic tomato (Chen et al. 2014). However, in this study, transgenic lines with stacked

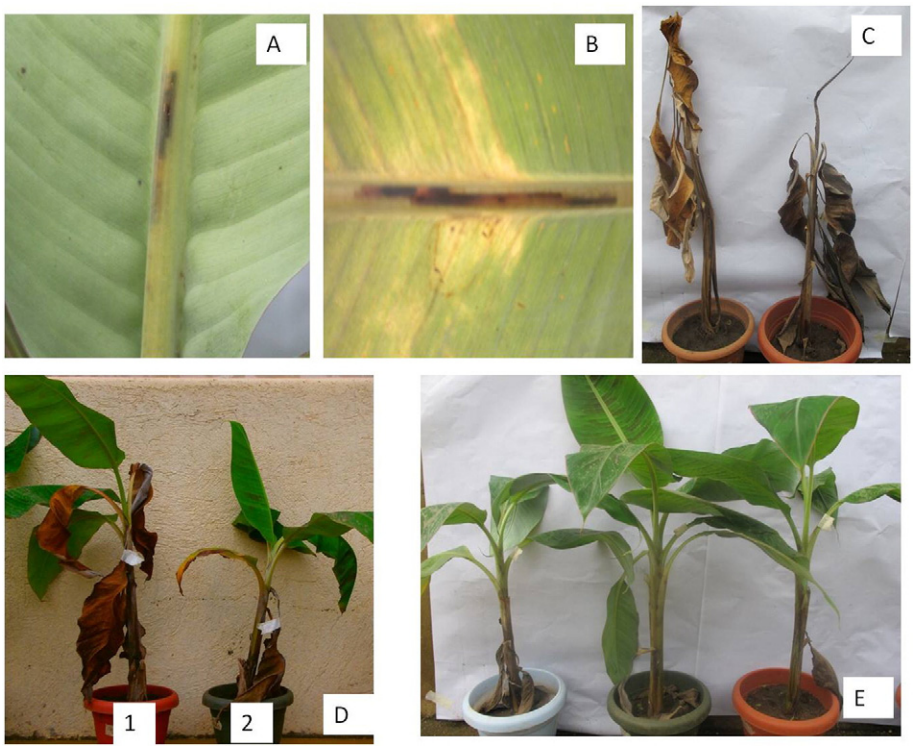

Fig. 3. (A) Enhanced resistance to *Xanthomonas campestris* pv. *musacearum* in transgenic banana plants with stacked genes. (A) Cell death at point of inoculation in leaf petiole of a transgenic plant. (B) Chlorosis progressing from dead tissues. (C) Non-transgenic control plants showing complete wilting. (D) Transgenic plants showing with partial resistance: 1, inoculated leaf (with a white tag) and two other leaves showing symptoms; 2, only inoculated leaf showing wilting symptom. (E) Stacked transgenic plants showing no BXW symptoms after 60 days after artificial inoculation in the screen house.

transgenes, *HRAP* and *PFLP*, both amplifying the HR but with different individual action, did not have higher levels of resistance against *Xcm* compared to transgenic lines engineered only with a single transgene. The observed stacked (7 lines out of 21) and individual transgene (4–6 lines out of 21) transgenic lines with complete resistance virtually showed no significant difference. Lesion length and time to wilting have been used as traits for measuring resistance in plants (Elvira-Recuenco et al., 2014). Although symptom development was delayed in stacked in comparison to individual transgenic lines, the mean number of days to complete wilting and average days to symptom development in stacked (44.8 dpi) and individual *HRAP* (38.9 dpi) or individual *PFLP* (35 dpi) transgenic lines was not significantly different. Likewise, the lesion length in stacked and individual transgenic lines was not found significantly different. These results showed that the level of resistance against *Xcm* in transgenic lines with stacked *HRAP* and *PFLP* genes were comparable to transgenic lines with individual genes. As observed in previous reports, expression of a single *HRAP* or *PFLP* transgene (Namukwaya et al. 2012; Tripathi et al. 2010) is sufficient to provide complete protection against *Xcm*. The stacked transgene strategy might, however, provide durable resistance, but we still need to test this by evaluating promising lines under field conditions.

Significantly, lower amount of transcripts for both *HRAP* and *PFLP* transgenes were observed in stacked compared to individual *HRAP* and *PFLP* transgenic banana plants. The lower transcripts of *HRAP* and

Table 3
BXW disease phenotype and transgene expression.

Transgenic line	Mean number of days for appearance of symptoms	Mean number of days for complete wilting	Disease reaction phenotype	Relative expression (fold increase)
Control[a]	15.7 ± 2.3	34.3 ± 8.5	Susceptible	No *HRAP* or *PFLP* expression
P1[b]	20.0 ± 2.5	44.3 ± 3.3	Partial resistance	13.3 ± 0.3[†]
P2[b]	20.0 ± 1.7	44.6 ± 5.5	Partial resistance	12.8 ± 0.2[†]
P5[b]	–	–	Absolute resistance	15.1 ± 0.4[†]
P6[b]	–	–	Absolute resistance	0.9 ± 0.1
H3[c]	20.0 ± 1.8	45.0 ± 4.1	Partial resistance	1.5 ± 0.1
H7[c]	19.7 ± 2.5	48.3 ± 5.8	Partial resistance	1.4 ± 0.2
H5[c]	–	–	Absolute resistance	6.2 ± 0.2[†]
H8[c]	–	–	Absolute resistance	3.7 ± 0.2[†]
				Pflp + *Hrap*
S3[d]	19.3 ± 2.2	44.3 ± 2.1	Partial resistance	1.55 ± 0.02 0.75 ± 0.01
S8[d]	17.0 ± 2.4	45.7 ± 8.4	Partial resistance	1.58 ± 0.04 0.97 ± 0.02
S2[d]	–	–	Absolute resistance	0.84 ± 0.03 1.08 ± 0.02
S5[d]	–	–	Absolute resistance	1.13 ± 0.03 1.36 ± 0.03

[a] Non-transgenic plants.
[b] Transgenic lines expressing individual *PFLP* gene.
[c] Transgenic lines expressing individual *HRAP* gene.
[d] Transgenic lines expressing stacked *HRAP* and *PFLP* genes.
[†] Significant differences ($P < 0.05$) in relative expression in comparison to transgenic lines with stacked *HRAP* and *PFLP* genes.

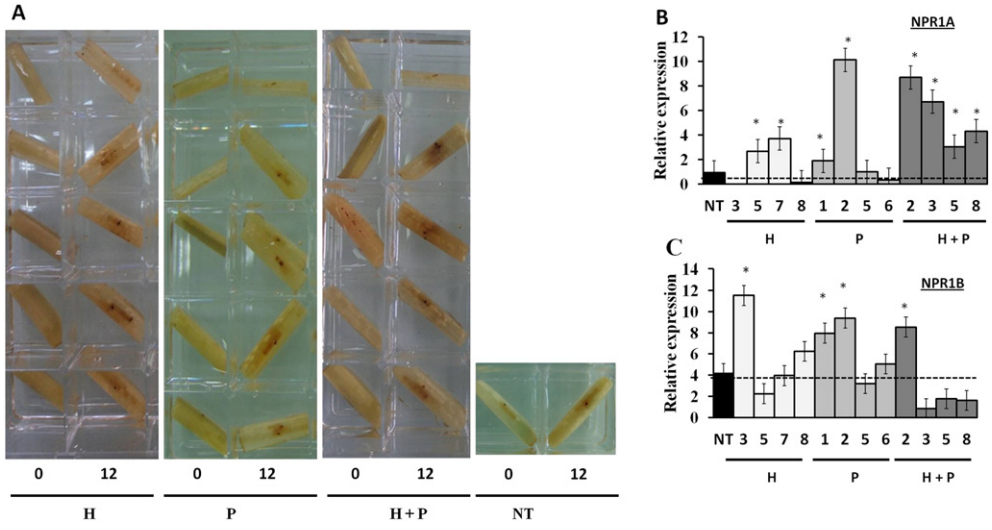

Fig. 4. (A) Histochemical staining of hydrogen peroxide produced due to *Xanthomonas campestris* pv. *musacearum* infection. (B) Transcription of *NPR1A* and *NPR1B* at 12 h post-inoculation in four different lines selected from each transgene construct. Transgenic lines used contained either stacked transgenes (H + P) or a single *HRAP* (H) or *PFLP* (P) transgene. NT: non-transgenic control plant and dotted line indicates transcription of either the *NPR1A* or *NPR1B* gene in a non-transgenic control plant. Data shown are the mean ± SE of three biological replicates from each line with three technical replicates. Asterisk indicates significant difference at $P < 0.05$ when compared to the non-transgenic control plant.

PFLP in all stacked lines could possibly be related to the high energy demands of the cell. In this study, homologous constitutive dual CaMV-35S promoters were used to drive both *HRAP* and *PFLP* genes in stacked transgenic lines. There are reports demonstrating that constitutive promoters involve a high energetic cost in transgenic plant (Park et al. 1996). As only a few transgenic lines were tested by qRT-PCR, further studies with more number of lines need to be performed in order to confirm this. Also, there is no enough information on correlation between mRNA accumulation and transgenic protein levels. Therefore, further studies should be performed in order to investigate if reduced accumulation of transgene transcripts corresponds to reduced levels of *HRAP* and *PFLP* protein in stacked lines.

We further clearly demonstrated by tissue staining that more hydrogen peroxide was produced in all transgenic lines following *Xcm* infection. Enhanced production of hydrogen peroxide, due to *HRAP* and *PFLP* transgene expression in transgenic lines, has previously also been reported for *Arabidopsis* and tobacco after challenge with a bacterial pathogen (Ger et al. 2014; Pandey et al. 2005). Hydrogen peroxide, almost non-detectable as an immediate response to *Xcm* infection in our non-transgenic line, can be involved in rapid lignification of cell walls (Ros Barceló 2005). Callose deposition strengthens the cell wall at sites of pathogen infection (Chisholm et al. 2006) whereby provides better protection against a pathogen. *In situ* staining of hydrogen peroxide with DAB, as done in our study provides, however, only an indication of hydrogen peroxide produced rather than measurement of an exact amount. Since DAB oxidation further relies on *in vivo* peroxidases, whose activity could be different between compartments or conditions, the staining technique also provides no indication about the exact cellular location of hydrogen peroxide production (Thordal-Christensen et al. 1997). We also tried to quantify hydrogen peroxide production after *Xcm* infection. Our preliminary results have shown that indeed majority transgenic plants produced more hydrogen peroxide over a period of 48 h than a non-transgenic control. However, we found that the different transgenic lines produced significantly different amounts of hydrogen peroxide. This did not permit to establish any direct relation between amount of hydrogen peroxide produced and resistance to *Xcm* (Abubaker, unpublished results). We are also very cautious about this type of quantitative data, because assays have the potential problem of accurate hydrogen peroxide extraction and artifactual assay effects (Queval et al. 2008).

Xcm infection was further associated with expression of the *NPR1* gene(s), a defense response co-transcriptor conferring resistance to pathogens, which very likely also contributes to *Xcm* resistance in transgenic banana. Both banana *NPR1* genes (*NPR1A* and *NPR1B*) were transcribed following infection, but *NPR1* transcription was not higher in lines with stacked transgenes in comparison to lines with a single

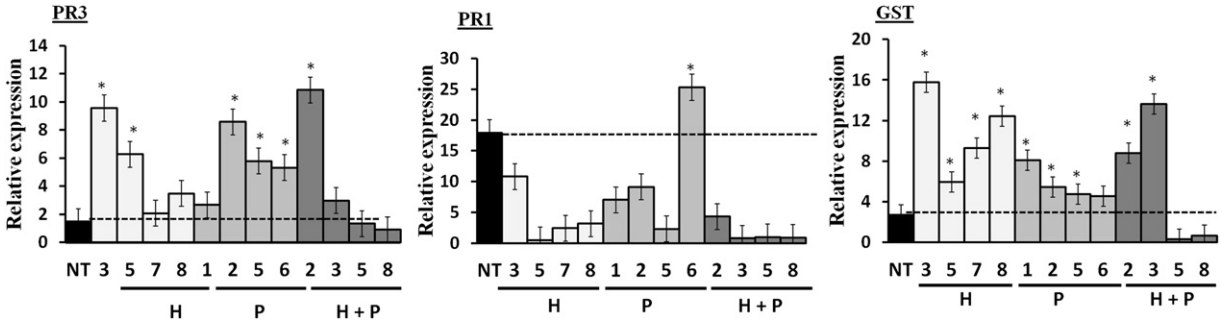

Fig. 5. Relative expression of the *PR-3*, *PR-1*, and GST genes in transgenic lines at 12 h post-inoculation with *Xanthomonas campestris* pv. *musacearum*. NT: non-transgenic control plant; H: transgenic lines with *HRAP* gene; P: transgenic lines with *PFLP*; H + P: transgenic lines with stacked *HRAP* and *PFLP* genes. Increase was calculated when compared to transcription of different genes at 0 h. Dotted line indicates transcription of either *PR-1*, *PR-3*, or *GST* gene in a non-transgenic control plant. Data shown are the mean ± SE of three biological replicates from each line with three technical replicates. Asterisk indicates significant difference at $P < 0.05$ when compared to the non-transgenic line.

transgene. The NPR1 protein is redox-sensitive during pathogen attack. The NPR1 protein is reduced to its monomeric form before translocation to the nucleus for establishment of systemic acquired resistance (SAR). The required dissipation of disulfide bonds in the NPR1 protein further involves ROS (Chen et al. 2001; Cumming et al. 2004). The potential of the NPR1 protein protecting against a pathogen has been previously demonstrated with *Arabidopsis NPR1*-overexpressing carrots where transgenic plants provided a broad-spectrum disease resistance to both necrotrophic and also biotrophic pathogens (Wally et al. 2009). *NPR1* gene transcription, due to *Xcm* infection, varied in our study. All transgenic lines had either increased transcription of both *NPR1* genes, or higher transcription of at least one of the two *NPR1* genes. Line (P5) was the only exception, where transcription of both *NPR1* genes was lower than in non-transgenic control. Since all transgenic lines with stacked transgenes had higher transcription than the non-transgenic control, transgene stacking might, however, trigger a more durable hydrogen production in contrast to lines transcribing only an individual transgene. However, stronger evidence has to be provided to support this idea.

We also found a direct relation between *NPR1* gene transcription and induction of *PR* gene expression. Particularly, the *PR-3* gene was more expressed in transgenic lines than in the non-transgenic control. Transgene stacking provided, however, no advantage regarding amounts of *PR* gene transcripts (*PR-3* and *PR-1*) produced. Both banana *NPR1* genes have been found to be equally active in *Arabidopsis* mutants to induce *PR-1* gene expression and restoring a pathogen-resistant phenotype (Endah-Yocga et al. 2012). In the non-transgenic line, transcription of the *PR-1* gene was, as expected, more induced than transcription of the *PR-3* gene. The *PR-1* gene encodes a glucanase and is indicative for the salicylic pathway and establishment of SAR when a plant is infected with a biotrophic pathogen such as *Xcm* (Robert-Seilaniantz et al. 2007). In contrast, expression of *PR-3* gene, encoding a chitinase, is indicative for the jasmonic acid signaling pathway. The *PR-3* gene is particularly induced in response to necrotizing pathogens (Glazebrook 2005). *PR-1* gene transcription was even suppressed in most of the transgenic lines tested, despite that higher *PR1*-gene expression should be expected after infection with a biotrophic pathogen like *Xcm*. In contrast, *PR-3* gene expression, indicative for infection with a necrotrophic pathogen, was higher in all our transgenic lines than in the non-transgenic control, except for two lines (H + P lines 5 and 8). These results suggests that amplifying the oxidative burst in transgenic lines might possibly shifted the response, normally found against a biotrophic pathogen (*Xcm*), to a response more related to a necrotizing pathogen. However, further studies are required, to demonstrate if such a shift generally occurs in transgenic lines with stacked transgenes by testing more lines and also more evidence has to be provided if such shift would offer the advantage of improved protection against a biotrophic pathogen.

The majority of transgenic lines tested had also higher transcription for the gene encoding GST than the non-transgenic control. Genes for GSTs are among the most responsive genes to biotic stress (Dixon et al. 2010) and chemical signaling treatments (Glombitza et al. 2004). This also includes response to oxidative stress due to ROS production (Noctor et al. 2014). Increased ROS production was indeed related to higher transcription of a gene encoding GST. The exact functions of GSTs remain, however, still elusive and it is for example not clear whether the primary GST function is acting as a conjugase or peroxidase, or both (Dixon et al. 2010).

In conclusion, this study demonstrates that transgenic banana lines, either expressing the *HRAP* or *PFLP* transgene individually or stacked, were resistant to *Xcm* infection. Transgenic lines with transgenes stacked, however, did not show enhanced resistance against *Xcm* infection than lines expressing the transgenes alone. Furthermore, no correlation between amount of transgenes expressed and *Xcm* resistance was found. Simply amplifying the oxidative burst by stacking transgenes might also be problematic because pathogen-triggered nuclear translocation of the NPR1 protein can be prevented by strong accumulation of hydrogen peroxide in the cytosol. This can result in inhibition of *NPR1*-dependent gene expression ultimately causing less PR protein production required for protection against a pathogen (Peleg-Grossman et al. 2010). Further, amplifying the oxidative burst, due to transgene expression, possibly shifts the general response from expected biotrophic pathogen more to a necrotrophic pathogen with more *PR-3* gene expression. Future more detailed research is, however, required to provide stronger evidence that transgene stacking results in a more durable resistance by avoiding rapid loss of single transgene action due to gene silencing or earlier breakdown of resistance.

Authors' contributions

All work presented in this paper emerges from the PhD's research by Abubaker Muwonge. Prof K Kunert and Dr. L Tripathi supervised this work. Dr. Tripathi also provided funding for the project. Dr. J Tripathi generated banana cell suspension that was used for generating transgenic plants. All authors contributed in writing and reviewing the manuscript.

Acknowledgment

We thank Dr. T.Y. Feng (Academia Sinca, Taiwan) for providing the pBI-*PFLP* and pBI-*HRAP* constructs and African Agricultural Technology Foundation (AATF) for negotiating a royalty-free license for the *PFLP* and *HRAP* gene from the patent holder, Academia Sinica, Taiwan. The first author acknowledges the receipt of a fellowship from AATF. This research was supported by USAID.

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