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

Abubaker Muwonge [a,b,d,*], Jaindra Tripathi [c], Karl Kunert [b], Leena Tripathi [c]

[a]International Institute of Tropical Agriculture, Kampala, Uganda

[b]Forestry and Agricultural Biotechnology Institute (FABI), University Of Pretoria, Pretoria 0002, South Africa

[c]International Institute of Tropical Agriculture, ILRI Nairobi Kenya

[d]National Crops Resources Research Institute, National Agricultural Research Organization, Uganda

*Corresponding author: Muwonge Abubaker. E-mail: muwonge@kari.go.ug

Highlights

• Stacked *HRAP* and *PFLP* transgenic plants had higher resistance to *Xcm* than non-transgenic plants.
• Cumulative transcripts of both *HRAP* and *PFLP* in stacked transgenic did not correlate with resistance.
• Transgenic plants accumulated more H_2O_2 than non-transgenic plants in response to *Xcm* infection.
• The response of transgenic plants to *Xcm* infection was characterized by up-regulation of genes encoding *PR3* and *GST*.
• Stacked *HRAP* and *PFLP* had no higher resistance against *Xcm* in comparison to individual transgenic plants.

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.

Key words: Transgenic banana; *HRAP*; *PFLP*; *Xanthomonas* wilt resistance; Gene stacking

Abbreviation :

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

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* (Tripathi et al., 2009). The pathogen, infecting all banana varieties including both East African Highland bananas and also 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 have been previously applied to improve resistance against the BXW disease in banana including the use of transgenic plants constitutively expressing either the hypersensitive response assisting protein (*Hrap*) or the plant ferredoxin-like protein (*Pflp*) gene (Tripathi et al., 2010 and 2014; Namukwaya et al., 2012). Expression of the *Hrap* or *Pflp* transgene has also been reported enhancing pathogen resistance in transgenic tobacco and *Arabidopsis* plants (Ger et al., 2002; Pandey et al., 2005) as well as in rice plants (Tang et al., 2001). Transgenic *Arabidopsis* plants with apoplast localized *Pflp* further exhibited higher bacterial pathogen resistance 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 first observable sign of the HR occurs a few hours after the pathogen enters the cell and production of reactive oxygen (ROS), with hydrogen peroxide (H_2O_2) the most stable ROS, is one of the earliest events during HR (Sutherland, 1991). Hydrogen peroxide has, however, a dual role being toxic in high concentrations and acting as a signaling molecule at low concentrations for adjusting cells to changed environmental conditions (Petrov and Van Breusegem 2012; Noctor et al., 2014). The PFLP protein, as a plant ferredoxin-like 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 (Su et al., 2014, Noctor et al., 2014). In contrast, the HRAP protein acts on harpin proteins, which are produced by many pathogens and sensed by plants triggering plant 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 *Xanthomonas* (Chen et al., 2000; Ger et al., 2002).

Although individual *Hrap* and *Pflp* transgenes provided resistance in banana against the BXW disease, an approach to stack the *Hrap* and *Pflp* transgenes to further amplify HR and improve resistance has not been carried out yet. Such gene stacking approaches have been previously applied to either target different pathogens or to avoid early resistance breakdown (Chan et al., 2005; Zhu et al., 2012; Storer et al., 2012). Recently, trans-plastomic Nicotiana benthamiana plants expressing important defense proteins in three-stacking combinations to obtain multiple resistance (insects, phytopathogens and abiotic stress) had synergistic and enhanced effects 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; Zhao et al., 2003; Datta et al., 2002).

The particular objective of this study was therefore to determine whether *Hrap* and *Pflp* transgene stacking will result in higher resistance against the BXW disease when compared to individual transgene expression. We therefore constitutively expressed either the two transgenes, *Hrap* and *Pflp* and both derived from sweet pepper (*Capsicum annuum*) (Liau et al., 2003; Chen et al., 1998), individually or stacked in transgenic banana generated from embryogenic cell suspensions (cultivar *Gonja manjaya*) of and measured resistance parameters such as time for symptom development and plant death after challenging plants with the bacterial pathogen *Xanthomonas campestris* pv. *musacearum*. We were further interested in determining if stacked transgenes will amplify to a greater extent the oxidative burst and applied histochemical staining with 2,2′-diaminobenzidine (DAB) for hydrogen peroxide production as an immediate response to *Xanthomonas* infection (Šnyrychová et al., 2009). 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*), *PR* genes (*PR-1* and *PR-3*) encoding antimicrobial proteins and of a gene encoding glutathione S-transferase (GST). NPR1 is a

defense response co-transcriptor conferring resistance to pathogens and banana contains several *NPR1s* with *NPR1A* and *NPR1B* 78% identical (Endah *et al*., 2008). *NPR1* gene expression induces PR protein production and these PR proteins accumulate after pathogen infection (van Loon et al. 2006). *NPR1* mutations cause failure of induction of these PR proteins with plants having increased susceptibility to pathogen infection (Kinkema et al., 2000). Finally we also measured transcription of the gene encoding GST and GSTs are among the most responsive genes to stress and chemical signaling treatments (Glombitza et al., 2004). Overall, we found in our study that transgenic lines with both transgenes stacked were not more resistant to *Xanthomonas* infection than lines expressing alone one of the two transgenes.

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).

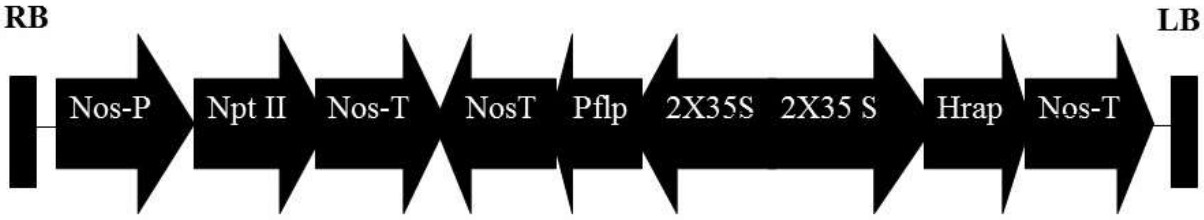

Figure 1. Schematic representation of T-DNA region of plasmid *pBIHrap-Pflp* used for banana transformation. RB and LB represents right and left border, respectively; NOS-P and NOS-T the promoter (P) and terminator region (T) of the *Agrobacterium tumefaciens* nopaline synthase gene; 2X35SP the double 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.

2.2.*Generation of transgenic plants*

Embryonic cell suspensions (ECS) of banana cultivar Gonja manjaya developed from scalps were used for transformation. Four days after sub-culturing in cell suspension media, ECSs were transformed with *Agrobacterium* strain AGL1 harboring the different plasmid constructs and regenerated on selective medium according to Tripathi et al. (2010) and Namukwaya et al. (2012). Transformed embryonic cells were regenerated on selective medium into putative transgenic shoots from about 16 ml settled cell volume of ECS. Developed 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-transformed control plantlets were produced on medium without any antibiotics for selection added. Individual shoots were finally transferred to rooting medium and rooted plantlets were weaned in sterile soil in plastic pots in a containment facility.

2.3 Molecular analysis of transgenic plants

Genomic DNA was extracted from 21 randomly selected putative transgenic banana lines applying the protocol of Untergasser (2008) but with the modification of using 0.1% sodium meta bisulfite instead of 1 % beta-mercaptoethanol and also using a chloroform:

Table 1: Primers sequences used for PCR and qRTPCR

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

(a) Primer sequences designed based on Hrap coding sequence (accession no. AF168415); and Pflp coding sequence (accession no. AF039662); primer sequences for NPR1A, NPR1B, PR-1 and PR-3 as previously reported by Endah et al. (2010); primer sequences designed using GST based on NCBI deposited coding sequence (accession #).

isoamylalcohol (24:1) mixture instead of chloroform as well as removing RNA with RNase A instead by LiCl treatment. Presence of *Hrap* and *Pflp* coding sequences 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 applied as positive controls and non-transformed plant DNA as 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 *Musa25 srRNA* 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 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).

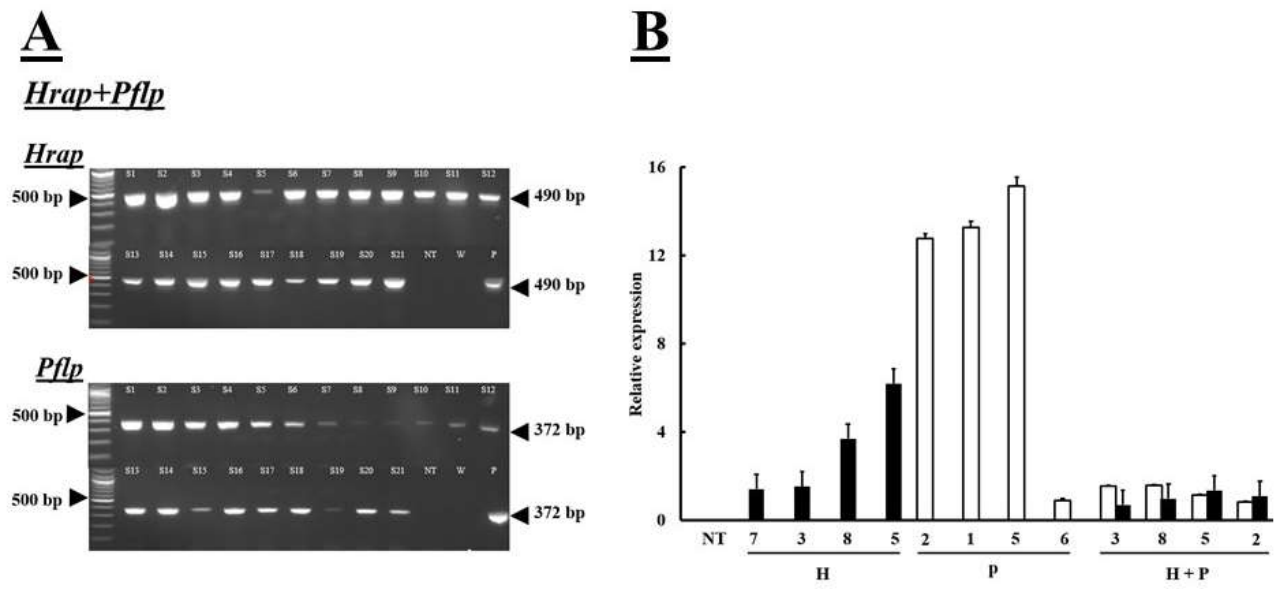

Figure 2. (**A**) PCR analysis of genomic DNA from different 21 selected transgenic lines for detection of insertion of the *Hrap* and *Pflp* coding sequence into transgenic banana carrying the stacked coding sequences. Lane NT represents a genomic DNA from a non-transformed plant; lane W a control where genomic DNA was replaced with water and lane P amplification of *Hrap* or *Pflp* from the stacked plasmid *pBIHrap-Pflp*.

(B) Relative expression of *Hrap* or *Pflp* in four selected transgenic plants carrying either the coding sequence for *Hrap, Pflp* or both coding sequences stacked. Relative expression of transgenes was calculated by comparison with expression of the *Musa* 25s rRNA coding sequence as an endogenous control.

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).

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

Transgenic line	Mean number of days for disease symptoms† appearance	Mean number of days for complete wilting	Disease severity (%)
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

Transgenic line	Mean□ number of days for disease symptoms† appearance	Mean□ number of days for complete wilting	Disease severity (%)
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.

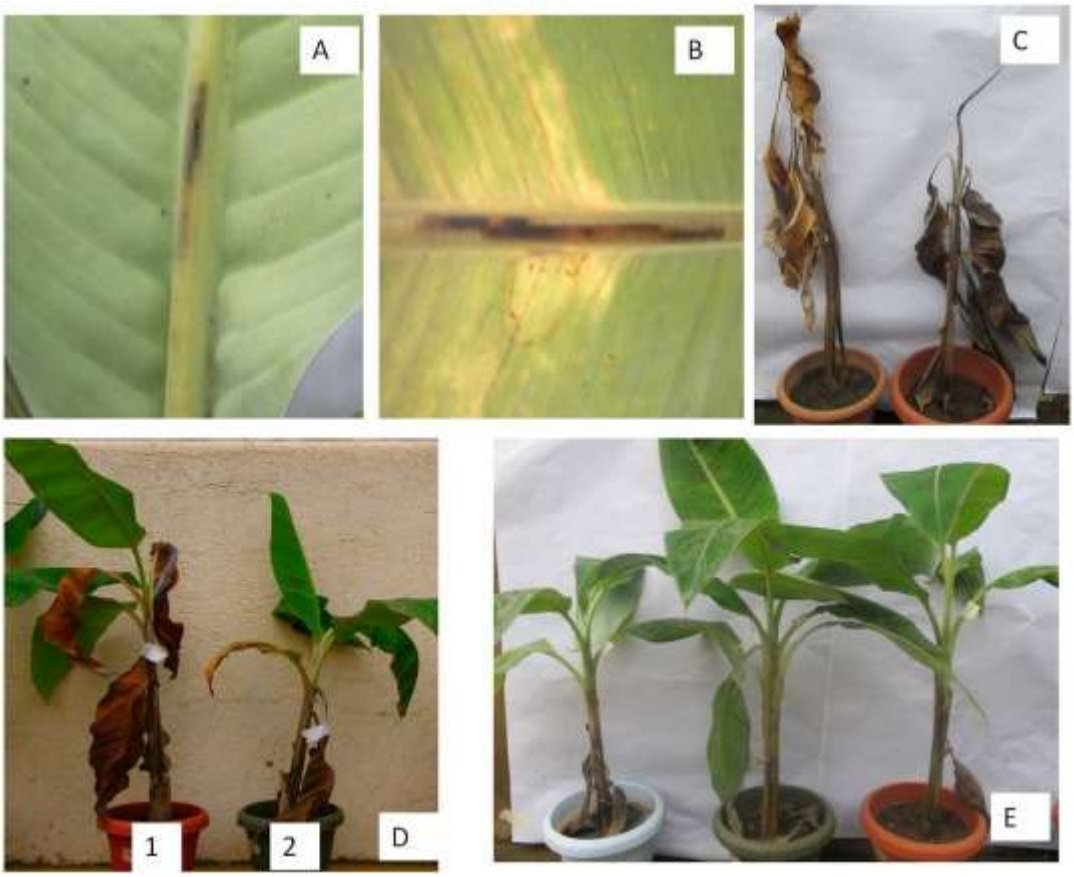

Fig. 3. (**A**) Enhanced resistance to *Xanthomonas campestris* pv. *musacearum* in transgenic banana plants with stacked genes. (**A**) Cell death at point of inoculation in leaf petiole of a transgenic plant. (**B**) Chlorosis progressing from dead tissues. (**C**) Non-transgenic control plants showing 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.

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 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).

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.

3.3. Hydrogen peroxide formation 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).

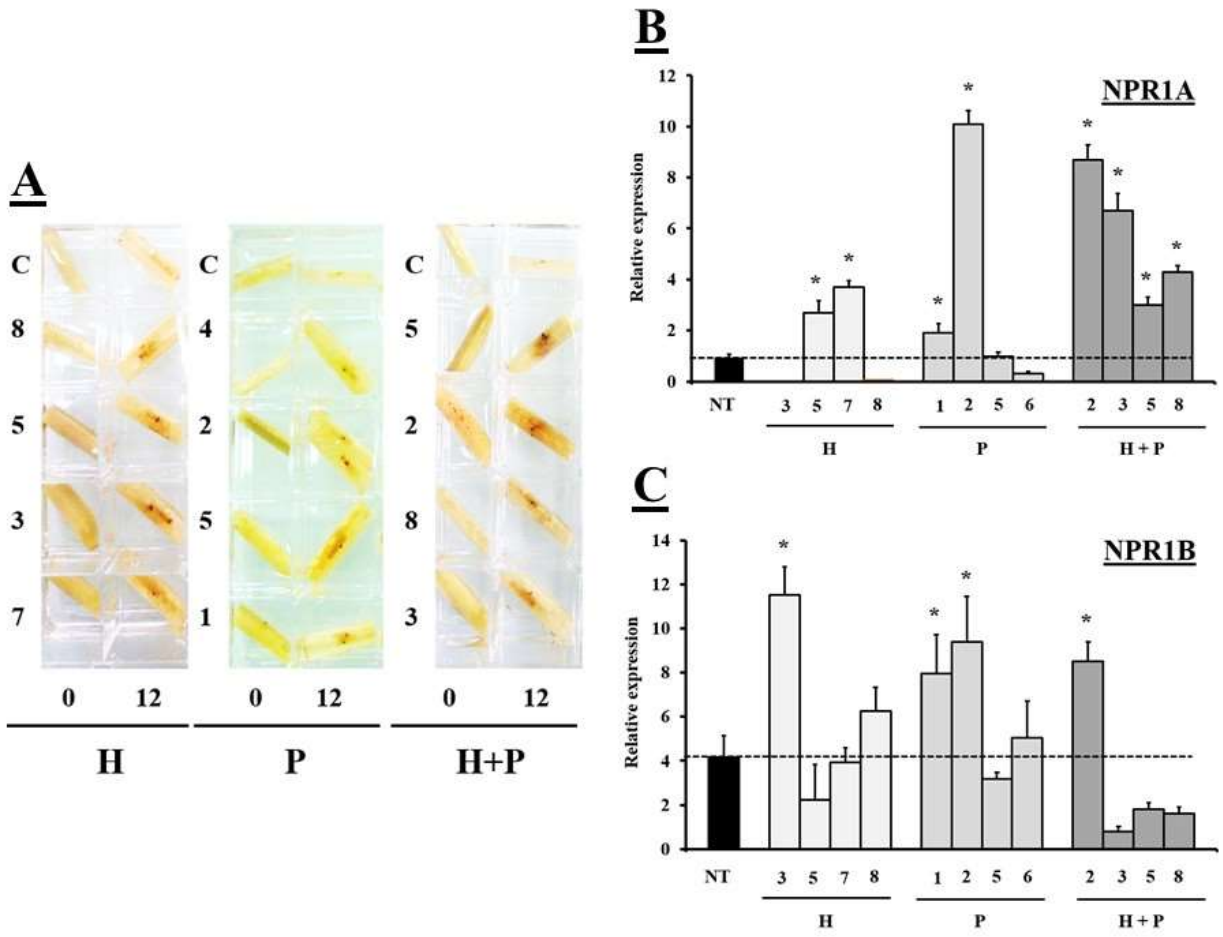

Figure 4. Histochemical staining of produced hydrogen peroxide due to *Xanthomonas* infection and water-inoculated leaves (**A**) and transcription of *NPR1A* and *NPR1B* (**B**) at 12 hrs post inoculation in four different lines selected four each transgene construct. Transgenic lines used contained either stacked transgenes (H+P) or a single *Hrap* (H) or *Pflp* (P) transgene. WT represents a non-transgenic wild-type line and dotted line indicates transcription of either the *NPR1A* or *NPR1B* gene in a non-transgenic wild-type line. 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.

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 (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).

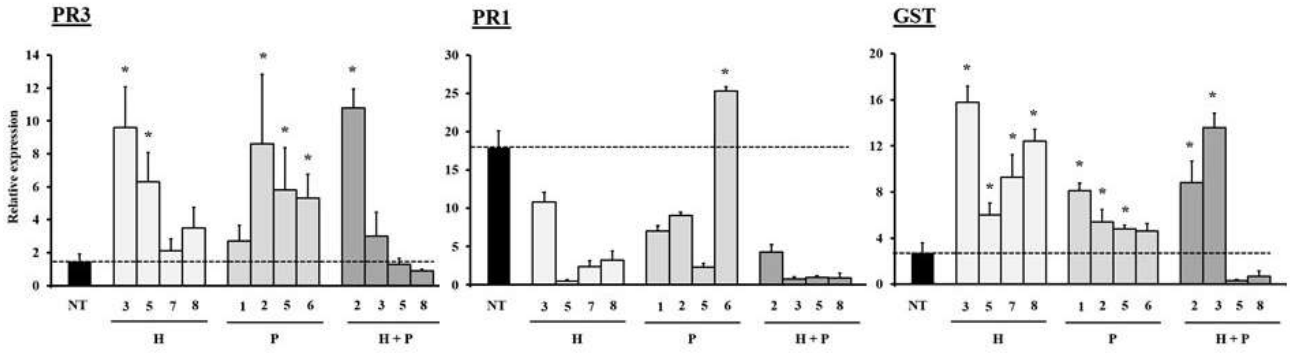

Figure 5. Relative increase in expression of the *PR-3*, *PR-1* and a gene encoding GST 12 hrs after *Xanthomonas* infection in transgenic banana lines carrying either *Hrap*, *Pflp* or stacked transgenes when compared to a non-transgenic line. Increase was calculated when compared to transcription of different genes at 0 hr. Dotted line indicates transcription of either *NPR1A* or *NPR1B* gene in a non-transgenic wild-type line. 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.

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 and 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 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 and 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 *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 and 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).

Xanthomonas 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 *Xanthomonas* resistance in transgenic banana. Both measured banana *NPR1* genes (*NPR1A* and *NPR1B*) were transcribed following infection, but there was no higher *NPR1* transcription in lines with transgenes stacked. The NPR1 protein is redox-sensitive

during pathogen attack and 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 involves ROS (Chern 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 *Xanthomona*s 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 not higher than in our non-transgenic control. Since all transgenic lines with stacked transgenes had higher transcription than the non-transgenic control, transgene stacking might, however, provide 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 line. 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 our 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 *Xanthomonas* (Robert-Seilaniantz et al., 2007). In contrast, *PR-3* gene expression, with the *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 our transgenic lines despite that higher *PR1*-gene expression should be expected after infection with a biotrophic pathogen like *Xanthomonas*. In contrast, *PR-3* gene expression, indicative for infection with a necrotrophic pathogen, was higher in all our transgenic plants than in the non-transgenic control, except for two lines (H+P lines 5 and 8). This results, higher *PR-3* gene expression, suggests that amplifying the oxidative burst in transgenic lines has possibly shifted the response normally found against a biotrophic pathogen (*Xanthomona*s) to a response more related to a necrotizing pathogen. Future studies have, however, to demonstrate if such a shift generally occurs in transgenic lines with stacked transgenes by testing more lines and also more evidence has to provide 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.

Acknowledgement

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.

Authors' contributions

All work presented in this paper emerges from the PhD's research by Muwonge Abubaker. Prof Karl Kunert supervised and Dr. Leena Tripathi co-supervised this work and Dr Tripathi also provided funding for the project. Jaindra Tripathi generated banana cells that were used for banana transformation.

References

Leena Tripathi, Jaindra Nath Tripathi, Andrew Kiggundu, Sam Korie, Frank Shotkoski, Wilberforce Kateera Tushemereirwe Field trial of Xanthomonas wilt disease-resistant bananas in East Africa Nature Biotechnology 32, 868–870 (2014)

Pandey A.K., Ger M.J., Huang H.E., Yip M.K., Zeng J.P., and Feng T.Y., 2005, Expression of the hypersensitive response-assisting protein in Arabidopsis results in harpin-dependent hypersensitive cell death in response to Erwinia carotovora, Plant Molecular Biology, 59: 771-780

Van Loon, L.C., Rep, M. and Pieterse, C.M. (2006) Significance of inducible defense-related proteins in infected plants. Annu. Rev. Phytopathol. 44, 135–162.

Dayakar BV, Lin HJ, Chen CH, Ger MJ, Lee BH, Pai CH, Chow D, Huang HE, Hwang SY, Chung MC, Feng TY (2003) Ferredoxin from sweet pepper (Capsicum annuum L.) intensifying harpinpss-mediated hypersensitive response shows an enhanced production of active oxygen species (AOS). Plant Mol Biol 51:913–924

V D Petrov and F Van Breusegem Hydrogen peroxide—a central hub for information flow in plant cells AoB Plants. 2012; 2012: pls014. Published online Jun 4, 2012.

Cheng-hsien Chen, Hao-jan Lin, Mang-jye Ger, David Chow and Teng-yung Feng (2000) cDNA cloning and characterization of a plant protein that may be associated with the harpinPSS-mediated hypersensitive response. *Plant Molecular Biology* **43:** 429–438, 2000

Tripathi L, Mwangi M, Abele S, Aritua V, Tushemereirwe WK, Bandyopadhyay R (2009) Xanthomonas wilt: a threat to banana production in east and central Africa. Plant Disease 93:440–451

Chan YL, Lin KH, Sanjaya, Liao LJ, Chen WH, Chan MT (2005) Gene stacking in Phalaenopsis orchid enhances dual tolerance to pathogen attack. Transgenic Res 14: 279–288

Suxian Zhu, Ying Li, Jack H. Vossen, Richard G. F. Visser, Evert Jacobsen Functional stacking of three resistance genes against Phytophthora infestans in potato Transgenic Res. 2012 Feb;21(1):89-99

Ssekiwoko F, Taligoola HK, Tushemereirwe WK (2006) Xanthomonas campestris pv musacearum host range in Uganda. African Crop Science J 14:111–120

Mang-jye Ger, Cheng-hsien Chen, Shaw-yhi Hwang, et al., (2002). Constitutive Expression of *hrap* Gene in Transgenic Tobacco Plant Enhances Resistance Against Virulent Bacterial Pathogens by Induction of a Hypersensitive Response. MPMI Vol. 15, No. 8, 2002, pp. 764–773

Tang K, Sun X, Hu Q, Wu A, Lin CH, Lin HJ, Twyman RM, Christou P, Feng TY (2001) Transgenic rice plants expressing the ferredoxin-like protein (AP1) from sweet pepper show enhanced resistance to Xanthomonas oryzae pv. oryzae. Plant Sci 160:1035–1042

C.-H. Chen, H.-J. Lin and T.-Y. Feng (1998). An amphipathic protein from sweet pepper can dissociate harpinPss multimeric forms and intensify the harpinPss-mediated hypersensitive response. Physiological and Molecular Plant Pathology **52**, 139-149.

H.-J. Lin, H.-Y. Cheng, C.-H. Chen, H.-C. Huang and T.-Y. Feng (1997). Plant amphipathic proteins delay the hypersensitive response caused by harpinPss and Pseudomonas syringae pv. Syringae. Physiological and Molecular Plant Pathology **51**, 367-376

Collinge DB, Lund OS, Thordal-Christensen H (2008). What are the prospects for genetically engineered, disease resistant plants? European Journal of Plant Pathology 121, 217 - 231

Zhao JZ, Cao J, Li Y, Collins HL, Roush RT, Earle ED, Shelton AM (2003) Transgenic plants expressing two Bacillus thuringiensis toxins delay insect resistance evolution. Nat Biotechnology 21:1493–1487.

Datta K., Baisakh N., Thet K.M., Tu J. and Datta S.K. 2002. Pyramiding transgenes for multiple resistance in rice against bacterial blight, yellow stem borer and sheath blight. Theor. Appl. Genet. 106: 1–8.

Šnyrychová I., Ayaydin F., Hideg É. (2009) Detecting hydrogen peroxide in leaves in vivo – a comparison of methods. Physiol. Plantarum 135:1-18)

B. Namukwaya • L. Tripathi J. N. Tripathi • G. Arinaitwe • S. B. Mukasa • W. K. Tushemereirwe Transgenic banana expressing Pflp gene confers enhanced resistance to Xanthomonas wilt disease Transgenic Res (2012) 21:855–865

Tripathi L, Mwaka H, Tripathi JN, Tushemereirwe WK (2010) Expression of sweet pepper Hrap gene in banana enhances resistance to Xanthomonas campestris pv. musacearum. Mol Plant Pathol 11:721–731

Glombitza S., Dubuis P.-H., Thulke O., Welzl G., Bovet L., Götz M., Affenzeller M., Geist B., Hehn A., Asnaghi C., Ernst D., Seidlitz H., Gundlach H., Mayer K., Martinoia E., Werck-Reichhart D., Mauch F., Schäffner A. (2004) Crosstalk and differential response to abiotic and biotic stressors reflected at the transcriptional level of effector genes from secondary metabolism. Plant Molecular Biology 54, 817-835.

Weigel D, Glazebrook J. 2006c. Transformation of Agrobacterium using the freeze-thaw method. Cold Spring Harb Protoc doi:10.1101/pdb.prot4666.

L Tripathi, JN Tripathi, WK Tushemereirwe Rapid and efficient production of transgenic East African Highland Banana (Musa spp.) using intercalary meristematic tissues African Journal of Biotechnology Vol. 7 (10), pp. 1438-1445, 16 May, 2008

Untergasser A. "DNA Miniprep using CTAB" Untergasser's Lab. Summer 2008. (Accessed 25. October 2014)). <http://www.untergasser.de/lab/protocols/miniprep_dna_ctab_v1_0.htm>.

Kenneth J. Livak and Thomas D. Schmittgen Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method. Methods 25, 402–408 (2001)

Mou Z, Fan W, Dong X Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell. 2003 Jun 27;113 (7):935-44.

Glazebrook J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol. 2005; 43:205-27.

E. Douglas, C. Halpin Gene Stacking Molecular Techniques in Crop Improvement 2009, pp 613-629

Naqvi, S., Farre, G., Sanahuja, G., Capell, T., Zhu, C. and Christou, P. (2009) When more is better: multigene engineering in plants. Trends in Plant Science 15, 48-56

Mang-Jye Ger, Guan-Yu Louh, Yi-Hsien Lin, Teng-Yung Feng and Hsiang-En Huang. Ectopically expressed sweet pepper ferredoxin PFLP enhances disease resistance to Pectobacterium carotovorum subsp. carotovorum affected by harpin and protease-mediated hypersensitive response in Arabidopsis. Molecular Plant Pathology Article first published online: 30 JUN 2014 DOI: 10.1111/mpp.12150

Peng-Jen Chen, Rajendran Senthilkumar, Wann-Neng Jane, Yong He, Zhihong Tian, and Kai-Wun Yeh Transplastomic Nicotiana benthamiana plants expressing multiple defence genes encoding protease inhibitors and chitinase display broad-spectrum resistance against insects, pathogens and abiotic stresses Plant Biotechnology Journal (2014) 12, pp. 503–515

Yu-Han Su & Chuan-Yu Hong & Yi-Hsien Lin Plant ferredoxin-like protein enhances resistance to bacterial soft rot disease through PAMP-triggered immunity in Arabidopsis thaliana Eur J Plant Pathol (2014) 140:377–384

Smadar Peleg-Grossman, Naomi Melamed-Book, Gil Cohen, and Alex Levine Cytoplasmic H_2O_2 prevents translocation of NPR1 to the nucleus and inhibits the induction of PR genes in Arabidopsis Plant Signal Behav. Nov 2010; 5(11): 1401–1406.

Storer, N. P., Thompson, G. D. And Head, G. P. (2012) Application of pyramided traits against Lepidoptera in insect resistance management for Bt crops. GM Crops and Food 3:3, 154-162.

Thordal-Cristensen H, Zhang Z, Wei Y, Collinge DB. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. The Plant Journal1997;11:1187-1194.

G Queval, J Hager, B Gakiere and G Noctor Journal of Experimental Botany, Vol. 59, No. 2, pp. 135–146, 2008 Why are literature data for H2O2 contents so variable? A discussion of potential difficulties in the quantitative assay of leaf extracts

Endah-Yocga, R. Creissen, G., Kunert, K., Chikwamba, R. (2012). Two different banana NPR1-like coding sequences confer similat protection against pathogens in *Arabidopsis*. Tropical Plant Biology 5, 309-316.

Cumming RC, Andon NL, Haynes PA, Park M, Fischer WH, Schubert D Protein disulfide bond formation in the cytoplasm during oxidative stress. J Biol Chem. 2004 May 21; 279(21):21749-58.

Chern MS, Fitzgerald HA, Yadav RC, Canlas PE, Dong X, Ronald PC. Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in Arabidopsis. Plant J 2001 27:101-113.

Wally O, Jayataj J and Punja ZK Broad-spectrum disease resistance to necrotrophic and biotrophic pathogens in transgenic carrots (Daucus carota L.) expressing an Arabidopsis NPR1 gene. Planta. 2009; 231(1):131-41

A. Ros Barceló Planta Xylem parenchyma cells deliver the H_2O_2 necessary for lignification in differentiating xylem vessels March 2005, Volume 220, Issue 5, pp 747-756

Chisholm ST, Coaker G, Day B, Staskawicz BJ **(**2006) Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* **124:** 803–814

M Kinkema, W Fan, and X Dong The Plant Cell, Vol. 12, 2339–2350, December 2000, www.plantcell.org © 2000 American Society of Plant Physiologists Nuclear Localization of NPR1 Is Required for Activation of PR Gene Expression

Robert-Seilaniantz A, Navarro L, Bari R, Jones JD: Pathological hormone imbalances. Curr Opin Plant Bio*l* 2007, 10(4)**:**372-379.].

G Noctor, A Mhamdi, and C H. Foyer The Roles of Reactive Oxygen Metabolism in Drought: Not So Cut and Dried Plant Physiology, April 2014, Vol. 164, pp. 1636–1648,

Dixon DP, Hawkins T, Hussey PJ, Edwards R (2009) Enzyme activities and subcellular localization of members of the Arabidopsis glutathione transferase superfamily. J Exp Bot 60: 1207–1218

Yi-Hsien Lin, Hsiang-En Huang, Fang-Sheng Wu, Mang-Jye Ger, Pei-Luan Liao, Yen-Ru Chen, Kuo-Chin Tzeng, Teng-Yung Feng, Plant ferredoxin-like protein (PFLP) outside

chloroplast in Arabidopsis enhances disease resistance against bacterial pathogens Plant Science Volume 179, Issue 5, November 2010, Pages 450–458

MW. Sutherland The generation of oxygen radicals during host plant responses to infection Physiological and Molecular Plant Pathology Volume 39, Issue 2, August 1991, Pages 79–93

Chia-Hui Liau, Jian-Cheng Lu, Venkatesh Prasad, Hsin-hao Hsiao, Su-Juan You, Jent-turn Lee, Ning-Sun Yang, Hsiang-En Huang, Teng-Yung Feng, Wen-Huei Chen, Ming-Tsair Chan The Sweet Pepper Ferredoxin-Like Protein (pflp) Conferred Resistance Against Soft Rot Disease in Oncidium Orchid Transgenic Research June 2003, Volume 12, Issue 3, pp 329-336

Chen C.H., Lin H.J., and Feng T.Y., 1998, An amphipathic protein from sweet pepper can dissociate harpinPss multimeric forms and intensify the harpinPss-mediated hypersensitive response, Physiological and Molecular Plant Pathology, 52(3): 139-149