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

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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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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

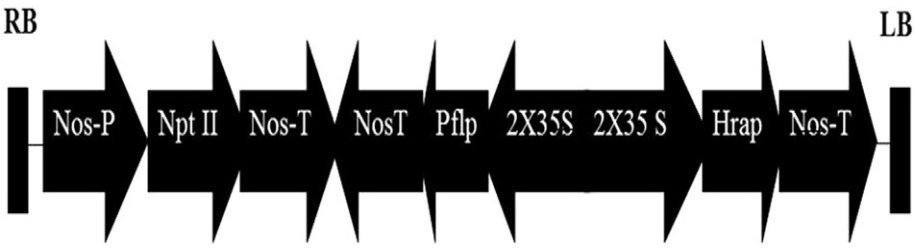

Fig. 1. Schematic representation of the T-DNA region of *pBI-HRAP-PFLP* construct used for banana transformation. RB and LB represents right and left border, respectively; NOS-P and NOS-T the promoter (P) and terminator region (T) of the *Agrobacterium tumefaciens* nopaline synthase gene; 2X35SP the 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.