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Expression of sweet pepper *Hrap* gene in banana enhances resistance to *Xanthomonas campestris* pv. *musacearum*

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SUMMARY

Banana *Xanthomonas* wilt (BXW), caused by the bacterium *Xanthomonas campestris* pv. *musacearum*, is the most devastating disease of banana in the Great Lakes region of Africa. The pathogen's rapid spread has threatened the livelihood of millions of Africans who rely on banana fruit for food security and income. The disease is very destructive, infecting all banana varieties, including both East African Highland bananas and exotic types of banana. In the absence of natural host plant resistance among banana cultivars, the constitutive expression of the hypersensitivity response-assisting protein (*Hrap*) gene from sweet pepper (*Capsicum annuum*) was evaluated for its ability to confer resistance to BXW. Transgenic lines expressing the *Hrap* gene under the regulation of the constitutive CaMV35S promoter were generated using embryogenic cell suspensions of two banana cultivars: 'Sukali Ndiizi' and 'Mpologoma'. These lines were characterized by molecular analysis, and were challenged with *Xanthomonas campestris* pv. *musacearum* to analyse the efficacy of the *Hrap* gene against BXW. The majority of transgenic lines (six of eight) expressing *Hrap* did not show any symptoms of infection after artificial inoculation of potted plants in the screenhouse, whereas control nontransgenic plants showed severe symptoms resulting in complete wilting. This study demonstrates that the constitutive expression of the sweet pepper *Hrap* gene in banana results in enhanced resistance to BXW. We describe the development of transgenic banana varieties resistant to BXW, which will boost the arsenal available to fight this epidemic disease and save livelihoods in the Great Lakes region of East and Central Africa.

INTRODUCTION

Banana *Xanthomonas* wilt (BXW), caused by the bacterium *Xanthomonas campestris* pv. *musacearum*, is the most devastating disease of banana in the Great Lakes region of Africa, including Uganda, the Democratic Republic of Congo, Kenya, Tanzania, Rwanda and Burundi (Tripathi *et al*., 2009). The disease was first reported about 40 years ago in Ethiopia on *Ensete*, which is closely related to banana (Yirgou and Bradbury, 1968), and then on banana (Yirgou and Bradbury, 1974). Outside Ethiopia, BXW was first identified in Uganda in 2001 (Tushemereirwe *et al*., 2004), and subsequently in the Democratic Republic of Congo (Ndungo *et al*., 2006), Rwanda (Reeder *et al*., 2007), Kenya, Tanzania and Burundi (Carter *et al*., 2009). The rapid spread of the disease has endangered the livelihoods of millions of farmers who rely on banana for staple food and income. Bananas (together with plantains) represent one of the most important world food crops after maize, rice, wheat and cassava. The annual world banana production is estimated at 1.3×10^{11} kg, less than 15% of which enters the international commercial market, indicating that the crop is far more important for local or domestic consumption than for export (Food and Agricultural Organization, 2008). Nearly one-third of the bananas produced globally are grown in sub-Saharan Africa, where the crop provides more than 25% of the food energy requirements for over 100 million people (Robinson, 1996). East Africa is the largest banana-producing and -consuming region in Africa. Uganda is the world's second largest producer after India, with a total of about 1×10^{10} kg (Food and Agricultural Organization, 2008).

The disease is very destructive, infecting all banana varieties, including both East African Highland bananas and exotic types (dessert, roasting and beer) of banana (Ssekiwoko *et al*., 2006; Tushemereirwe *et al*., 2003), and causing annual losses of over 500 million dollars across East and Central Africa (Bafana, 2008). The economic impact of the disease is potentially disastrous, because it destroys whole plants, leading to complete yield loss of banana, and farmers do not have the option of relocating to new planting sites that are infection free.

The most common symptoms of the disease are yellowing and wilting of the leaves, and uneven and premature ripening of the fruit with sections showing unique yellowish blotches and dark brown scars in the pulp (Tripathi *et al*., 2009). Eventually,

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infected plants wither and the plant rots. There are currently no commercial chemicals, biocontrol agents or resistant cultivars available to control the pathogen (Tripathi *et al*., 2009). The use of disease-resistant cultivars has been an effective and economically viable strategy for the integrated management of other major diseases in numerous crops. High levels of cell-mediated resistance to *X. campestris* pv. *musacearum* have not been identified in any banana cultivar. Even if resistant germplasm sources are identified, conventional breeding of banana is a difficult and lengthy process because of the sterility of most cultivars, coupled with long generation times. To circumvent these difficulties, transgenic technologies may provide a cost-effective alternative solution to the BXW pandemic. Some successes in the genetic engineering of banana have been achieved, enabling the transfer of foreign genes (Becker *et al*., 2000; Ganapathi *et al*., 2001; Khanna *et al*., 2004; May *et al*., 1995; Sagi *et al*., 1995; Tripathi *et al*., 2005, 2008b).

Plants employ a wide array of defence mechanisms against pathogen attack. Of these, the hypersensitive response (HR) is an induced resistance mechanism, characterized by rapid, localized cell death on encounter with a microbial pathogen (Dangl *et al*., 1996; Goodman and Novacky, 1994). Cell death resulting from HR forms a physical barrier to prevent further pathogen infection. In addition, a local HR is often associated with the activation of plant defence responses in surrounding and even distal uninfected parts of the plants, leading to the development of systemic acquired resistance (SAR). HR, a plant defence mechanism against invading pathogens often found in disease-resistant plants, commonly precedes a slower systemic (whole-plant) response, which ultimately leads to SAR (Freeman, 2003).

The hypersensitive response-assisting protein (HRAP), isolated from sweet pepper (*Capsicum annuum*), is a novel plant protein that can intensify the harpinPSS (harpin derived from *Pseudomonas syringae* pv. *syringae*)-mediated HR (Chen *et al*., 2000). The constitutive expression of the *Hrap* gene in transgenic tobacco and *Arabidopsis* plants confers enhanced resistance against virulent pathogens (Ger *et al*., 2002; Pandey *et al*., 2005). In this study, the sweet pepper *Hrap* gene was transformed into banana plants in order to assess the effect of its expression on resistance against the bacterial pathogen *X. campestris* pv. *musacearum*.

RESULTS

Transformation, selection and regeneration of transgenic banana

Agrobacterium tumefaciens-infected cells multiplied and proliferated on kanamycin-selective medium, whereas control untransformed cells turned black (Fig. 1A). Embryogenic cells were regenerated on RD1-selective medium. The regenerated transgenic shoots were proliferated and transferred to rooting medium (Fig. 1B). All the shoots developed roots within 2–3 weeks. More than 100 independent kanamycin-resistant transformed lines of banana cultivars 'Sukali Ndiizi' and 'Mpologoma' were generated. The rooted plantlets were transferred to the soil in pots in the containment facility. There were no apparent phenotypic alterations observed during the vegetative growth of plants (Fig. 1C).

Polymerase chain reaction (PCR) analysis

The presence of the *Hrap* gene was confirmed in 12 randomly selected kanamycin-resistant banana lines using PCR with specific primers. The amplified product of about 800 bp was observed from the DNA of all tested transgenic plants using *Hrap*-specific primers, confirming the presence of the transgene in all tested kanamycin-resistant transgenic banana lines (Fig. 2A). PCR was also performed using neomycin phosphotransferase II (*npt*II) gene-specific primers. An amplified fragment of about 500 bp was observed for all tested transgenic plants, confirming the co-integration of both *Hrap* and *npt*II genes (Fig. 2B). The amplification of the *Actin* gene, the internal control for DNA quality, was observed for all plants, including nontransgenic controls (Fig. 2C).

Evaluation of transgenic lines for enhanced resistance to BXW using *in vitro* plants

The transgenic banana plantlets containing the *Hrap* gene were tested for BXW resistance by artificial inoculation of *in vitro* plantlets under controlled laboratory conditions (Tripathi *et al*., 2008a). Twelve PCR-positive transgenic lines (T1–T12) were artificially inoculated with *X. campestris* pv. *musacearum* culture. Four transgenic lines (T1, T6, T8 and T10) did not show any symptoms through the duration of the experiment (8 weeks after inoculation), demonstrating that the *Hrap* gene can provide resistance to BXW (Fig. 3A; Table 1). However, four other transgenic lines (T3, T4, T5 and T9) showed the delayed appearance of symptoms. They started to develop symptoms at 28 days post-inoculation (dpi), but had not completely wilted by the termination of the experiment at 60 dpi; in contrast, control plantlets developed symptoms at about 18 dpi and were completely wilted within 39 dpi. The remaining transgenic lines (T2, T7, T11 and T12) developed symptoms within 18–26 dpi and became completely wilted like nontransgenic control plants (Table 1). The transgenic lines showing no symptoms or delayed symptoms and surviving beyond 60 dpi (Fig. 3B) were further evaluated as potted plants in the screenhouse.

Bacteria were isolated from each diseased plant and plated onto semiselective medium (Tripathi *et al*., 2007). In each case, colonies were identified as *X. campestris* pv. *musacearum* on the basis of their morphological characteristics (i.e. yellowish,