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

LEENA TRIPATHI[1,*], HENRY MWAKA[2,3], JAINDRA NATH TRIPATHI[1] AND WILBERFORCE KATEERA TUSHEMEREIRWE[2]

[1]*International Institute of Tropical Agriculture, PO Box 7878, Kampala, Uganda*
[2]*National Agriculture Research Organisation, PO Box 7065, Kampala, Uganda*
[3]*Makerere University, PO Box 7062, Kampala, Uganda*

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,

**Correspondence*: Email: l.tripathi@cgiar.org

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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,

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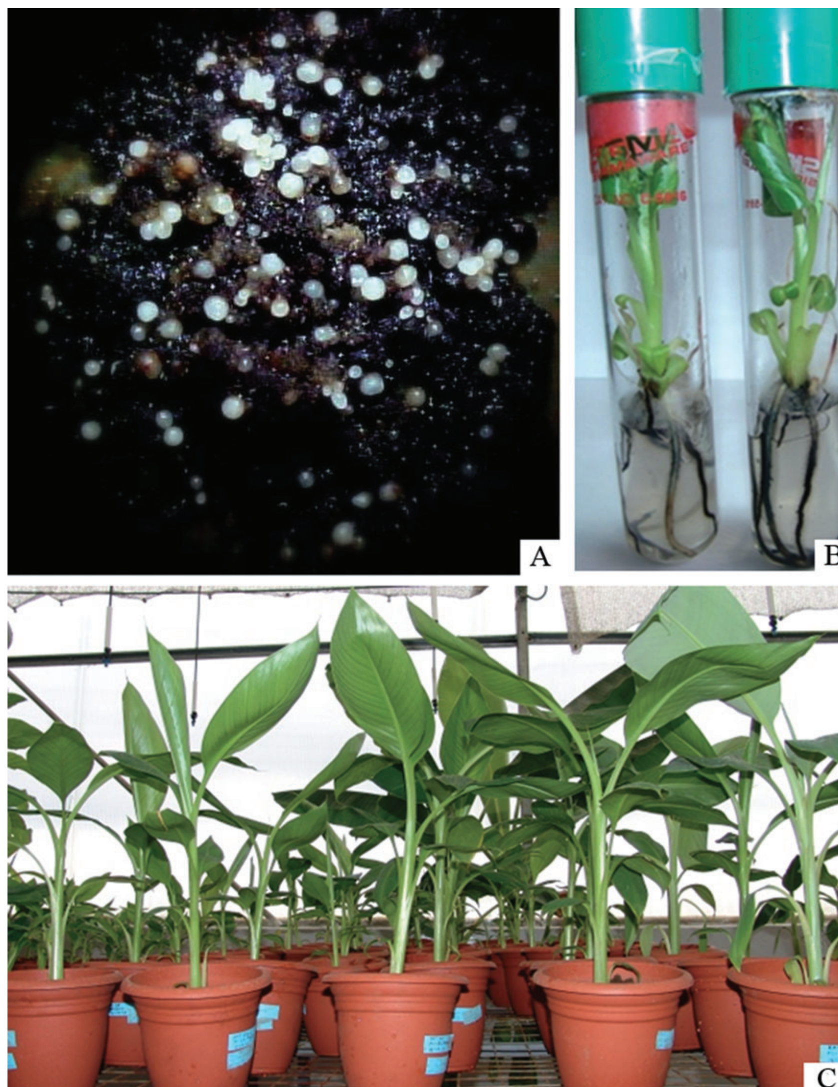

Fig. 1 Regeneration of transgenic banana plants. (A) *Agrobacterium*-infected cells proliferating on selective medium supplemented with kanamycin. (B) Transformed plantlets regenerated on selective medium. (C) Transformed plants transferred to soil in pots in containment facility.

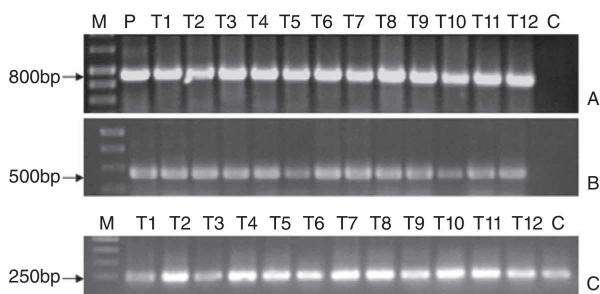

Fig. 2 Polymerase chain reaction (PCR) analysis of genomic DNA from different transgenic lines and nontransgenic plants using gene-specific primers. (A) Hypersensitivity response-assisting protein (*Hrap*) gene-specific primers. (B) Neomycin phosphotransferase II (*npt*II) gene-specific primers. (C) *Actin*-specific primers. M, molecular weight marker; P, pBI-HRAP vector DNA; T1–T12, transgenic plants; C, control nontransgenic plant.

mucoid and circular colonies on YTSA–CC (1% yeast extract, 1% tryptone, 1% sucrose, 1.5% agar, 150 mg/L cycloheximide and 50 mg/L cephalexin) semiselective medium), confirming that the symptoms were caused by the bacteria used as inoculum. The identification of isolated bacteria was also confirmed by PCR with *X. campestris* pv. *musacearum*-specific primers. No pathogenic bacteria were successfully recovered from healthy transgenic plantlets.

Evaluation of transgenic lines for enhanced resistance to BXW using potted plants in the screenhouse

As the integration of the *Hrap* gene caused resistance to *X. campestris* pv. *musacearum* in *in vitro* plantlets, the eight promising transgenic lines (T1, T3, T4, T5, T6, T8, T9 and T10) that showed either no symptoms or delayed symptoms in the *in vitro*

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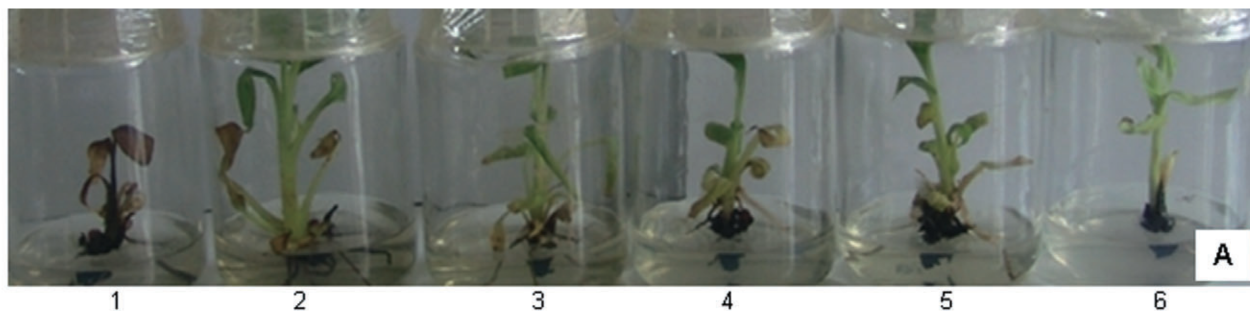
A
1
2
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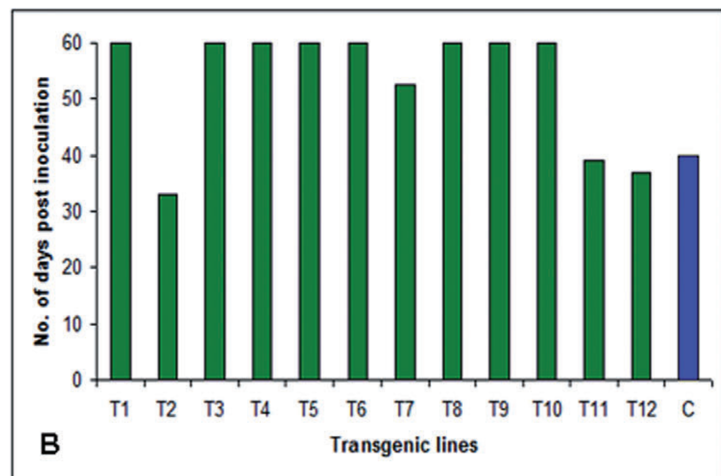
No. of days post inoculation
60
50
40
30
20
10
0
T1
T2
T3
T4
T5
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T7
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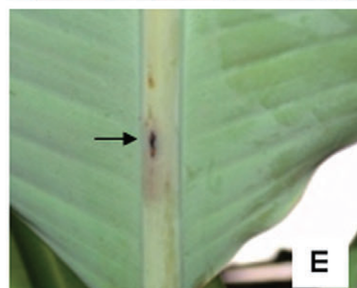
T9
T10
T11
T12
C
B
Transgenic lines

C

D


E

F

Fig. 3 Enhanced resistance of transgenic banana lines to *Xanthomonas campestris* pv. *musacearum*. (A) Evaluation of transgenic lines using *in vitro* plantlets: 1, inoculated nontransgenic plant; 2–6, inoculated transgenic plants. (B) Graph showing survival of transgenic lines in comparison with nontransgenic plants after inoculation of *in vitro* plantlets. (C) Nontransgenic plants showing complete wilting after artificial inoculation. (D) Transgenic plants showing wilting of only inoculated leaf. (E) Photograph showing browning at the point of inoculation in the leaf of transgenic plants. (F) Transgenic plants showing no banana *Xanthomonas* wilt (BXW) symptoms after 60 days following the inoculation of potted plants in the screenhouse. All photographs were taken 8 weeks after artificial inoculation.

Table 1 Evaluation of transgenic lines for enhanced resistance to *Xanthomonas campestris* pv. *musacearum* using *in vitro* plants.

Transgenic line	Mean* number of days for appearance of disease symptoms†	Mean* number of days for complete wilting	Wilting incidence (%)
Control	18.3	39.3	100
T1	—	—	0
T2	18.6	35.3	100
T3	30.0‡	—	0
T4	53.0‡	—	0
T5	40.3‡	—	0
T6	—	—	0
T7	23.0	53.3‡	100
T8	—	—	0
T9	32.0‡	—	0
T10	—	—	0
T11	23.0	39.6	100
T12	26.0	37	33.3

*Mean of three replicates.

†The disease symptoms were chlorosis or necrosis on the leaves of inoculated plants.

‡Significant differences ($P < 0.05$) in symptoms in a comparison of transgenic lines with nontransgenic control plants.

Wilting incidence (%) = (Number of plants wilted completely/Total number of plants inoculated) × 100.

assay were further analysed by inoculating potted plants in a secure screenhouse. Three plants of each transgenic line and the nontransgenic control were artificially inoculated with *X. campestris* pv. *musacearum*. The nontransgenic control plants started to develop symptoms (chlorosis or necrosis) after about 14 dpi; all leaves wilted completely and all plants died within 38 dpi (Fig. 3C; Table 2). The transgenic line T5 started to develop symptoms in inoculated leaves at 31 dpi, and the affected leaf wilted completely. However, the disease symptoms did not progress to other parts of the plant (Fig. 3D). One other transgenic line, T4, also developed symptoms at about 40 dpi, and the disease progressed to other plant parts, resulting in wilting of almost all leaves after 60 dpi (Table 2). However, six of the eight transgenic lines (T1, T3, T6, T8, T9 and T10) did not show any symptoms up to 60 dpi (Fig. 3F), indicating absolute resistance. The inoculation site of all inoculated transgenic lines turned brown but without the development of other symptoms (Fig. 3E). These results demonstrate that over-expression of the *Hrap* gene in banana provides high resistance to BXW.

We attempted to isolate *X. campestris* pv. *musacearum* from the pseudostem and inoculated leaf of all inoculated plants to check whether the bacteria were contained at the point of inoculation in the leaf or whether they had spread systemically to other parts of the transgenic plants. No pathogenic bacteria were recovered from the pseudostem or inoculated leaf tissues of seven of the eight transgenic lines (T1, T3, T5, T6, T8, T9 and T10), showing that bacteria were contained and there was no systemic spread or multiplication of bacteria. However, bacteria were recovered from the inoculation sites and pseudostems of wilted control plants and transgenic line T4. Isolated colonies were identified as *X. campestris* pv. *musacearum* on the basis of their morphological characteristics (i.e. yellowish, mucoid and circular colonies on YTSA–CC semiselective medium) and specific PCR.

Molecular characterization of transgenic lines

Southern blot analysis was performed with *Hin*dIII-digested DNA from transgenic and nontransgenic control plants in order to confirm the integration and to reveal the number of inserted copies. This enzyme has a single restriction site in the pBI-HRAP construct used in plant transformation (Fig. 4A). Positive bands were observed from all eight transgenic lines tested, whereas no band was observed from the nontransgenic control (Fig. 4B). The copy number of the integrated transgenes was estimated to be between one and three for most samples.

The transgenic lines were further tested using reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blot analysis. We performed RT-PCR using RNA from leaf tissue in order to verify *Hrap* transgene expression. *Hrap* transcript amplification of the expected fragment size (~800 bp) was observed from samples of all tested transgenic lines (Fig. 5A). Specific *Actin* transcript amplification was detected from all plants as an internal control for cDNA synthesis. A gDNA control was included in the assay with *Actin* primers and showed the larger unspliced fragments, indicating that DNA contamination was below PCR detection levels in RNA samples (Fig. 5A).

To analyse *Hrap* mRNA accumulation, Northern blot analyses were performed using RNA extracted from leaves; positive bands were observed in samples from all transgenic lines (Fig. 5B). Transgenic lines T1 and T6 accumulated high levels of *Hrap* mRNA compared with the other transgenic lines. Ethidium

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Table 2 Evaluation of transgenic lines for enhanced resistance to *Xanthomonas campestris* pv. *musacearum* using potted plants.

Transgenic line	Mean* number of days for appearance of disease symptoms†	Mean* number of days for complete wilting	Degree of wilting (grade)	Mean* number of leaves wilted	Reduction in wilting	Resistance (%)
Control	14	38.6	5	6.6	0	0
T1	—	—	0	0	6.6	100
T3	—	—	0	0	6.6	100
T4	46‡	—	3	5.3	1.3	19.6
T5	31.3‡	—	1	1	5.6	84.8
T6	—	—	0	0	6.6	100
T8	—	—	0	0	6.6	100
T9	—	—	0	0	6.6	100
T10	—	—	0	0	6.6	100

*Mean of three replicates.

†The disease symptoms were chlorosis or necrosis on the leaves of inoculated plants.

‡Significant differences ($P < 0.05$) in symptoms in a comparison of transgenic lines with nontransgenic control plants.

Degree of wilting (grade): 0, no symptoms; 1, only the leaf inoculated wilted; 2, two to three leaves wilted; 3, four to five leaves wilted; 4, all leaves wilted but plant still alive; 5, whole plant died.

Resistance (%) = (Reduction in wilting/Total number of leaves wilted in control) × 100.

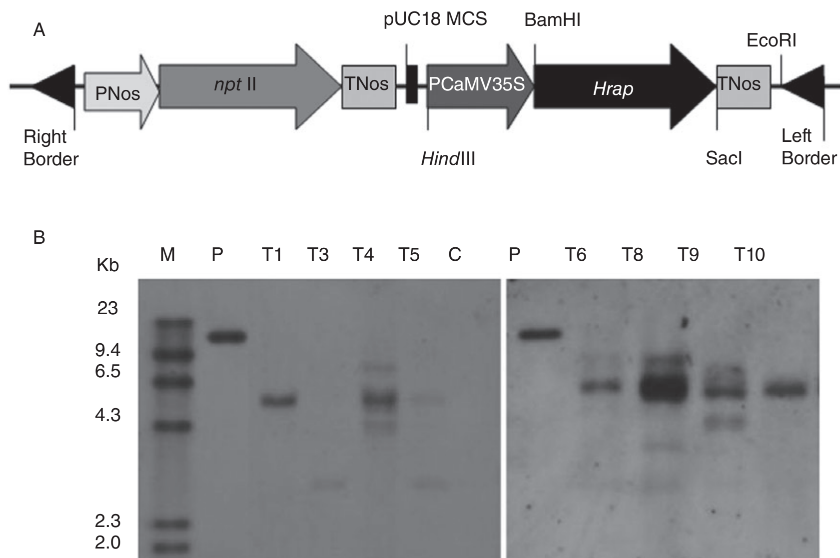

Fig. 4 (A) Schematic representation of the T-DNA region of the pBI-HRAP construct used for plant transformation. Pnos, nopaline synthase promoter; *npt*II, neomycin phosphotransferase II gene; Tnos, nopaline synthase terminator; PCaMV35S, CaMV35S promoter; *Hrap*, cDNA clone (834 bp) of hypersensitive response-assisting protein isolated from sweet pepper. (B) Southern blot analysis of genomic DNA digested with *Hin*dIII. M, digoxigenin (DIG)-labelled molecular weight marker; P, plasmid construct DNA digested with *Hin*dIII; T1–T10, transgenic plants; C, control nontransgenic plant.

bromide staining of RNA was used to verify the loaded amount of total RNA.

DISCUSSION

Banana is an important food and cash crop in the Great Lakes region. Food security studies have revealed that, in Uganda, Rwanda and Burundi, bananas constitute more than 30% of the daily per capita calorie intake, rising to 60% in some regions (Abele *et al*., 2007). BXW, caused by the Gram-negative bacterium *X. campestris* pv. *musacearum*, is a devastating disease of banana in East and Central Africa. The disease can be contained in fields in which debudding is effectively practised and tools are effectively decontaminated. However, the adoption of these practices has been inconsistent among farming communities (Kagezi *et al*., 2006). Farmers feel that debudding affects the quality of the banana, especially for the juice cultivars (Bagamba *et al*., 2006), and the decontamination of tools after use with every plant is laborious.

Experience with bacterial wilt diseases in other crops has shown that resistant varieties are often the most sustainable method of disease management. In this study, we have generated transgenic banana plants containing and expressing the *Hrap* gene from sweet pepper. This is one of the most important hypersensitive cell death (HCD)-associated genes that can be utilized to protect plants from bacterial pathogen attack (Chen *et al*., 2000). Disease resistance resulting from enhanced HCD is an important mechanism of plant defence. HRAP has been

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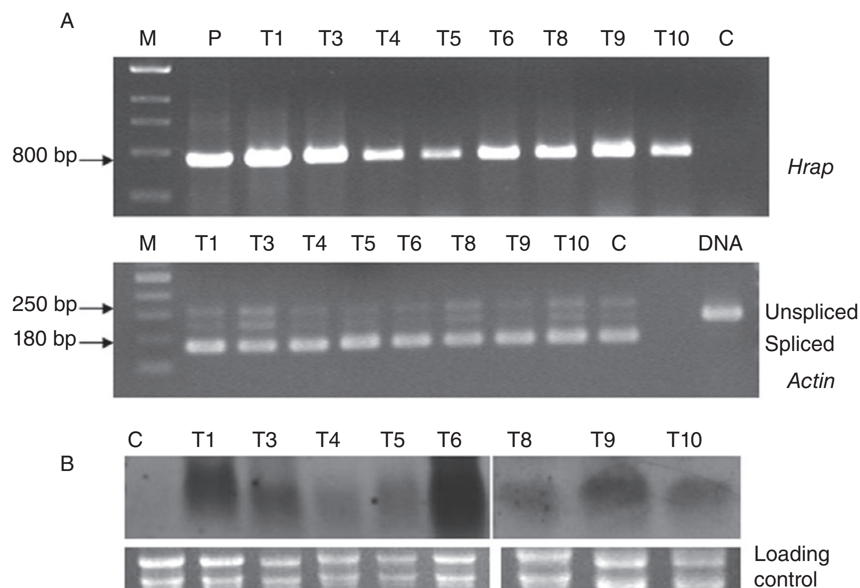

Fig. 5 Expression of hypersensitive response-assisting protein gene (*Hrap*) in transgenic banana plants. (A) Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of RNA isolated from leaf tissue using *Hrap*- or *Actin*-specific primers. Amplified PCR product designations are shown on the right and product sizes are shown on the left; P, plasmid construct DNA; T1–T10, transgenic plants; C, nontransgenic plants; DNA, control reaction with genomic DNA. (B) Northern blot analysis performed with RNA isolated from leaf tissue from transgenic lines and nontransgenic plants. Ethidium bromide staining of RNA was used to verify the loaded amount of total RNA (bottom panel).

reported to intensify the harpinPSS-mediated HCD in sweet pepper plants (Chen *et al*., 2000). The *Hrap* gene is widely distributed throughout a broad range of plant species, including tobacco, *Arabidopsis* and rice. However, it has no appreciable similarity to any known sequences. Sequence comparisons of HRAP have shown that it contains a typical signal peptide leading to secretion into extracellular matrices (Chen *et al*., 1998). In addition, the transcriptional activity of the *Hrap* gene is induced early during the incompatible interaction of sweet pepper with *P. syringae*, indicating that HRAP is closely involved in the plant defence mechanism (Chen *et al*., 2000).

HarpinPSS, a proteinaceous elicitor secreted from pathogenic Gram-negative bacteria, is necessary for bacteria to gain entry into the cell. It interacts with certain plant molecules and is recognized by its receptors in the extracellular matrix of the plant cell wall to mediate HR. HRAP has the ability to dissociate harpinPSS multimeric forms into dimers and monomers that trigger a stronger HCD necrosis in sweet pepper (Chen *et al*., 2000). The elicitor-induced resistance is not specific against particular pathogens, and therefore it may be a very useful strategy for the development of broad spectrum resistance.

The presence and integration of the *Hrap* gene in the genome of transgenic banana plants were confirmed by PCR and Southern blot analysis. Southern blots confirmed transformation events and indicated a low copy number (one to three) for most events. The expression of the *Hrap* gene was verified by RT-PCR and Northern blot analysis. The accumulation of *Hrap* mRNA in the leaves was not uniform across transgenic plants; several transgenic lines had more *Hrap* mRNA in Northern blots, after standardization for transcripts of the housekeeping gene, *Actin*.

The transgenic lines were first evaluated for resistance against *X. campestris* pv. *musacearum* using a rapid screening protocol with *in vitro* plantlets. The response to *X. campestris* pv. *musacearum* can be demonstrated within 60 days using this technique (Tripathi *et al*., 2008a); transgenic lines showing similar susceptibility to nontransgenic control plants were identified and eliminated from further study. Eight promising lines were advanced to inoculated pot trials in the screenhouse.

These plants were inoculated with *X. campestris* pv. *musacearum* culture in the midrib of the first fully open leaf. The control nontransgenic plants started to develop symptoms (necrosis or chlorosis) at about 14 dpi and died within 38 dpi, whereas the six transgenic lines remained healthy for 8 weeks, confirming very high resistance. In these plants, slight browning was observed at the point of inoculation (Fig. 3E) as a result of the rapid, localized death of plant cells at the site of inoculation. This is characteristic of the HR response, which is thought to be an important defence response to prevent further multiplication and to restrict the spread of the pathogen to other parts of the plant (Chen *et al*., 2000). To confirm this, pathogenic bacteria were isolated from the pseudostem and inoculated leaves of all asymptomatic transgenic lines tested. The inability to recover viable bacteria from even the inoculation sites of the transgenic plants indicated a successful resistance response. One transgenic line showed delayed symptoms after 30–35 dpi, indicating partial resistance. The reduction in wilting was significantly higher in this line compared with nontransgenic plants. The bacterial population was restricted to the leaf inoculated in this line, as bacteria were not recovered from the pseudostem of these plants.

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The most probable means of infection in a field setting are insect transmission through wounds left by abscising male flowers, via the roots, through infected plant material or from the use of contaminated tools (Eden-Green, 2004). The method of artificial inoculation used in this study was similar to the common infection route through injury by contaminated tools. Disease development can also be affected by the age of the host plant (Agrios, 1997). Therefore, the promising resistant lines identified will be further evaluated for resistance in a confined field trial. An application to the National Biosafety Committee in Uganda has been filed for approval for a confined field trial of these promising transgenic lines.

This study has shown that constitutive expression of the *Hrap* gene in banana results in a high level of resistance to BXW. Our results are supported by earlier studies performed in tobacco and *Arabidopsis* (Ger *et al*., 2002; Pandey *et al*., 2005). The constitutive expression of *Hrap* genes in transgenic tobacco plants confers enhanced resistance to virulent pathogens (*Pseudomonas syringae* pv. *tabaci* and *Erwinia carotovora* ssp. *carotovora*) (Ger *et al*., 2002). Pandey *et al*. (2005) have demonstrated that constitutive expression of the *Hrap* gene in *Arabidopsis* results in enhanced disease resistance towards soft rot pathogen, *E. carotovora* ssp. *carotovora*.

In this study, BXW-resistant transgenic plants did not exhibit visual morphological differences from nontransgenic plants in screenhouse conditions, suggesting that the constitutive expression of the *Hrap* gene does not seem to alter plant physiology.

In conclusion, our data suggest that *Hrap* is an interesting candidate gene for the engineering of banana plants to confer resistance against BXW. Transgenic banana expressing the *Hrap* gene appears to have significant potential to control the BXW pandemic. Our results confirm that the constitutive expression of the *Hrap* gene in banana leads to enhanced resistance to BXW. This article describes the development of transgenic banana varieties resistant to BXW, which will boost the arsenal available to fight the disease and save livelihoods in the Great Lakes region. This transgenic approach may also provide effective control of other bacterial diseases of banana (Moko, Blood and Bugtok diseases) in other parts of the world, as these diseases are also caused by Gram-negative bacteria similar to *X. campestris* pv. *musacearum*.

EXPERIMENTAL PROCEDURES

Plant material

Embryogenic cell suspensions (ECSs) of banana cultivars 'Sukali Ndiizi' and 'Mpologoma' were obtained from the banana programme, NARO Uganda. ECSs were subcultured in MA2 medium [Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962), 100 mg/L glutamine, 100 mg/L malt extract, 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 45 g/L sucrose, pH 5.3]. A settled cell volume of 1 mL of ESC was subcultured in 50 mL of liquid MA2, and cultured at reduced density for 5 days to increase cell competence and transformation efficiency. Five days after subculturing, ECSs were collected in 50-mL Falcon tubes and used as starting material for transformation experiments.

Agrobacterium and plasmid

Agrobacterium tumefaciens supervirulent strain AGL1 (Lazo *et al*., 1991) was used in this study. The pBI-HRAP vector containing the *Hrap* gene regulated by the CaMV35S promoter and the *npt*II gene as selection marker was acquired from Academia Sinica, Taiwan. A schematic representation of the pBI-HRAP vector is shown in Fig. 4A. The vector was transformed into *Agrobacterium* through electroporation. The *Agrobacterium* strain AGL1 harbouring pBI-HRAP was maintained on YEB (0.1% yeast extract, 0.5% beef extract, 0.5% peptone, 0.5% sucrose and 0.04% $MgSO_4$) medium supplemented with kanamycin (50 mg/L). The bacterial culture was grown in liquid YEB medium supplemented with kanamycin (50 mg/L) with shaking (150 r.p.m.) at 28 °C until the optical density at 600 nm ($OD_{600\ nm}$) reached 0.8. The bacterial cells were harvested by centrifugation at 5000 ***g*** for 10 min at 4 °C and resuspended in 25 mL of TMA1 medium (MS Macro, MS Micro, MS vitamins, 1 mg/L biotin, 100 mg/L malt extract, 100 mg/L glutamine, 230 mg/L proline, 40 mg/L ascorbic acid, 5 g/L polyvinylpyrrolidone (PVP) 10, 200 mg/L cysteine, 1 mg/L indoleacetic acid (IAA), 1 mg/L naphthaleneacetic acid (NAA), 4 mg/L 2,4-D, 85.5 g/L sucrose, pH 5.3) supplemented with 100 μM acetosyringone. The bacterial suspension was incubated at 28 °C for 3 h with shaking at 150 r.p.m. Towards the end of this culture phase, $OD_{600\ nm}$ of the culture was checked and adjusted to 0.6–0.8 with TMA1 medium.

Transformation, selection and regeneration

Banana cells were transformed with *Agrobacterium* strain AGL1 harbouring pBI-HRAP using the procedure described by Khanna *et al*. (2004). Five days after the cocultivation of ECSs with *Agrobacterium* in the dark at 22 °C, *Agrobacterium*-infected ECSs were washed and then transferred onto selective embryo formation medium (MA3; SH Macro, SH Micro, MS vitamins, 100 mg/L glutamine, 100 mg/L malt extract, 1 mg/L biotin, 230 mg/L proline, 45 g/L sucrose, 10 g/L lactose, 0.05 mg/L zeatin, 0.1 mg/L kinetin, 0.2 mg/L NAA, 0.2 mg/L 6-dimethylallylamino purine, 3 g/L gelrite, pH 5.8) supplemented with timentin (200 mg/L) and kanamycin (100 mg/L) for 3 months with fortnightly subculturing. Proliferating ECS clones were regenerated as described by Cote *et al*. (1996). Putatively transformed embryos were then transferred to solidified RD1 (MS salts and

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vitamins, 10 mg/L ascorbic acid, 100 mg/L myo-inositol, 30 g/L sucrose, 3 g/L gelrite, pH 5.8) medium containing kanamycin (100 mg/L). Embryos germinated on this medium were transferred to RD2 [MS salts and vitamins, 10 mg/L ascorbic acid, 100 mg/L myo-inositol, 0.227 mg/L 6-benzylaminopurine (BAP), 30 g/L sucrose, 3 g/L gelrite, pH 5.8] medium. The shoots developed on this medium were transferred to proliferation medium (MS salts and vitamins, 10 mg/L ascorbic acid, 100 mg/L myo-inositol, 5 mg/L BAP, 30 g/L sucrose, 3 g/L gelrite, pH 5.8). The individual shoots were transferred to rooting medium [MS salts and vitamins, 10 mg/L ascorbic acid, 100 mg/L myo-inositol, 1 mg/L indole-3-butyric acid (IBA), 30 g/L sucrose, 3 g/L gelrite, pH 5.8]. Rooted plantlets were then transferred to soil in pots in the containment facility. The plants were further used for molecular analysis and for the evaluation of resistance against *X. campestris* pv. *musacearum*.

DNA extraction and PCR analysis

Genomic DNA was isolated from 12 randomly selected putative transformed plantlets using a modified CTAB (hexadecyltrimethylammonium bromide) extraction method for *Musa*, described by Gawel and Jarret (1991). PCR with *Hrap* and *npt*II gene-specific primers was used to confirm the presence of transgenes in the plant genome. The banana *Actin* gene-specific primers were used for the amplification of this housekeeping gene to check the quality of DNA. The primer sequences were as follows: *Hrap* gene: forward, 5′-AAGGGATGACGCACAATCCC ACTATCCTTC-3′; reverse, 5′-TTAAAATAGTTGACCAAGGGTCTC C-3′; *npt*II gene: forward, 5′-CCTTATCCGCAACTTCTTTACCTA-3′; reverse, 5′-ACACCCAGCCGGCCACAGTCG-3′; *Actin* gene: forward, 5′-ACCGAAGCCCCTCTTAACCC-3′; reverse, 5′-GTAT GGCTGACACCATCACC-3′. A 25-μL PCR mixture contained 1.5 mM $MgSO_4$, 1 × reaction buffer, 0.2 mM nucleotide mix, 1 μM primers, 1 U Taq DNA Polymerase and 1 μg of template DNA. The initial denaturation of DNA was performed at 94 °C for 5 min, followed by 35 cycles of amplification with denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 7 min. The PCR products were visualized by electrophoresis on a 0.8% agarose gel with ethidium bromide staining.

Evaluation of transgenic banana lines for bacterial resistance

Preparation of bacterial suspensions

For bacterial resistance screening, pure cultures of *X. campestris* pv. *musacearum* isolated from infected plants, for which Koch's postulate had been proved, were maintained on YTSA medium (1% yeast extract, 1% tryptone, 1% sucrose and 1.5% agar) at 4 °C. A single bacterial colony was inoculated into 25 mL of YTS medium (1% yeast extract, 1% tryptone and 1% sucrose) and cultured at 28 °C with shaking at 150 r.p.m. for 48 h. The bacterial culture was centrifuged at 2500 ***g*** for 5 min and the pellet was resuspended in sterile double-distilled water. $OD_{600\ nm}$ of the bacterial suspension was checked and the bacterial concentration was adjusted to 10^8 colony-forming units (cfu)/mL with sterile water. Fresh inoculum was used for all experiments in order to maintain a high virulent potential of the pathogen.

Evaluation using in vitro plantlets

Twelve transgenic lines (T1–T12) showing PCR-positive results were evaluated for resistance against *X. campestris* pv. *musacearum* using a rapid screening technique (Tripathi *et al*., 2008a). The transgenic plantlets were tested for resistance to *X. campestris* pv. *musacearum* by injecting inoculum into the pseudostem of *in vitro* plantlets, as described by Tripathi *et al*. (2008a). Three plantlets of each transgenic line and nontransgenic control were inoculated. Plants were assessed every day for 8 weeks for disease symptoms, with preliminary symptoms of chlorosis or necrosis of the leaves and final symptoms of complete wilting of the plants.

The pathogenic bacteria were re-isolated from wilted plants as described by Tripathi *et al*. (2007), and identified as *X. campestris* pv. *musacearum* on the basis of their characteristic morphology (yellowish, mucoid and circular colonies on YTSA–CC semiselective medium) and by PCR using *X. campestris* pv. *musacearum*-specific primers.

Evaluation of transgenic banana using potted plants in the screenhouse

The plants of eight transgenic lines (T1, T3, T4, T5, T6, T8, T9, T10) showing promising results with *in vitro* plantlets were further evaluated using potted plants in the screenhouse. Three plants each of these transgenic and nontransgenic (control) lines were transferred to sterile soil in plastic cups (8 cm) for acclimatization. The plants were maintained in a humid and shady environment for 12–15 days, transferred to a screenhouse for a further 2 weeks and then transferred to larger plastic pots (30 cm); 100 μL of bacterial suspension (10^8 cfu/mL) were injected into the midrib of the first fully opened leaf of 3-month-old plants using a syringe fitted with a 28-gauge needle.

These plants were assessed every day for 8 weeks for disease symptoms, with preliminary symptoms of chlorosis or necrosis of the leaves and final symptoms of complete wilting of the plants. The relative resistance of transgenic plants to BXW was evaluated 8 weeks after inoculation on the basis of the reduction in wilting in comparison with control nontransgenic plants:

$$\text{Resistance (\%)} = (\text{Reduction in wilting/Total number of leaves wilted in control}) \times 100$$

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After 8 weeks, the pathogenic bacteria were re-isolated from the pseudostem and inoculated leaf of all the inoculated plants, as described by Tripathi *et al*. (2007), and identified as *X. campestris* pv. *musacearum* on the basis of the characteristic morphology (yellowish, mucoid and circular colonies on YTSA–CC semiselective medium) and by PCR using *X. campestris* pv. *musacearum*-specific primers.

Southern blot analysis

The integration of the *Hrap* gene was analysed using Southern hybridization. The pBI-HRAP plasmid (10 pg) and genomic DNA (10 μg) from eight transgenic plants were digested with *Hin*dIII, which cut the plasmid DNA as shown in Fig. 4A. The *Hin*dIII restriction enzyme has one cleavage site in the binary vector. When integrated into genomic DNA, *Hin*dIII cut once at one end of the promoter (outside the probe region) and again in the genomic DNA beyond the T-DNA border.

The restricted DNA was resolved on 0.8% (w/v) agarose gel and blotted onto 'Zetaprobe' nylon membrane (according to the manual provided by Bio-Rad, Parklands, South Africa). The blots were hybridized with digoxigenin (DIG)-labelled *Hrap* probe generated using a PCR DIG Probe Synthesis Kit. Hybridization and detection of the probe were carried out using a DIG Luminescent Detection Kit for Nucleic Acids (Roche Diagnostics, Lewes, East Sussex, UK) according to the manufacturer's instructions.

RNA extraction, RT-PCR and Northern blot analysis

RNA was extracted from the leaf tissue of transgenic plants using an RNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) and RT-PCR was performed using a Onestep RT-PCR Kit (Qiagen GmbH), according to the manufacturer's instructions. RT-PCR was performed with *Hrap*-specific primers (forward, 5′-AAGG GATGACGCACAATCCCACTATCCTTC-3′; reverse, 5′-TTAAAATA GTTGACCAAGGGTCTCC-3′). *Actin* gene amplification was performed using specific primers (forward primer *Actin* F, 5′-ACCGAAGCCCCTCTTAACCC-3′; reverse primer *Actin* R, 5′-GTATGGCTGACACCATCACC-3′) to check the quality of RNA. In both cases, the PCR conditions were as follows: 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, for 36 cycles.

For Northern blot analysis, total RNA from leaf tissue was isolated using a Trizol commercial system extraction (Invitrogen, Paisley, UK) according to the manufacturer's instructions, repeating chloroform extractions twice. For RNA gel blot experiments, 10 μg of total RNA was electrophoretically resolved in 1.5% agarose gels using 3(*N*-morpholino)propanesulphonic acid (MOPS) buffer (Sigma, St Louis, MO, USA), and transferred to Hybond N+ Nylon Membrane™. The blots were hybridized with DIG-labelled *Hrap* probe generated using a PCR DIG Probe Synthesis Kit. Hybridization and detection of the probe were carried out using a DIG Luminescent Detection Kit for Nucleic Acids (Roche Diagnostics) according to the manufacturer's instructions.

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