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ORIGINAL PAPER

Transgenic banana expressing *Pflp* gene confers enhanced resistance to Xanthomonas wilt disease

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Abstract Banana Xanthomonas wilt (BXW), caused by *Xanthomonas campestris* pv. *musacearum*, is one of the most important diseases of banana (*Musa* sp.) and currently considered as the biggest threat to banana production in Great Lakes region of East and Central Africa. The pathogen is highly contagious and its spread has endangered the livelihood of millions of farmers who rely on banana for food and income. The development of disease resistant banana cultivars remains a high priority since farmers are reluctant to employ labor-intensive disease control measures and there is no host plant resistance among banana cultivars. In this study, we demonstrate that BXW can be efficiently controlled using transgenic technology. Transgenic bananas expressing the plant ferredoxin-like protein (*Pflp*) gene under the regulation of the constitutive CaMV35S promoter were generated using embryogenic cell suspensions of banana. These transgenic lines were characterized by molecular analysis. After challenge with *X. campestris* pv. *musacearum* transgenic lines showed high resistance. About 67% of transgenic lines evaluated were completely resistant to BXW. These transgenic lines did not show any disease symptoms after artificial inoculation of in vitro plants under laboratory conditions as well as potted plants in the screen-house, whereas non-transgenic control plants showed severe symptoms resulting in complete wilting. This study confirms that expression of the *Pflp* gene in banana results in enhanced resistance to BXW. This transgenic technology can provide a timely solution to the BXW pandemic.

Keywords Transgenic banana · *Pflp* · Disease resistance · Banana Xanthomonas wilt

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Introduction

Banana (*Musa* sp.) is one of the most important world food crops grown in more than 120 tropical and subtropical countries of the world (Jones 2000). Annual banana production in the world is estimated at 1.3×10^{11} kg, of which less than 15% enters the international commercial market, indicating that the crop is far more important for local or domestic consumption than for export (FAO 2008). Nearly a

third of the bananas produced globally are grown in sub-Saharan Africa where the crop provides more than 25% of 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 (FAO 2008). Food security studies reveal that in Burundi, Rwanda and Uganda, bananas constitute more than 30% of the daily per capita caloric intake, rising to 60% in some regions (Abele et al. 2007). Despite the importance of bananas, the crop is threatened by various constraints such as biotic and abiotic factors (Ortiz et al. 2002).

Banana Xanthomonas wilt (BXW), caused by the bacterium *X. campestris* pv. *musacearum* (Xcm), is considered as the biggest threat to banana production in East and Central Africa (Tripathi et al. 2009). It endangers the livelihood of millions of farmers, who rely on banana for staple food and cash. 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 disease is very destructive, infecting all banana varieties, including dessert, cooking, roasting and beer types (Ssekiwoko et al. 2006). The impacts of BXW are both extreme and rapid, unlike those of other diseases which cause gradually increasing losses over years. The economic impact of BXW is due to absolute yield loss and death of the mother plant that would otherwise contribute to the ratoon plant production cycles (Tripathi et al. 2009). Overall economic losses were estimated at $2 billion over a decade, arising from price increases and significant reductions in production (Abele and Pillay 2007).

The affected banana plants develop symptoms characterized by a progressive yellowing and wilting of the leaves, and uneven and premature ripening of the fruits with sections showing unique yellowish blotches and dark brown scars in the pulp (Tripathi et al. 2009). Eventually, infected plants wither and rot. The disease spreads through insect vectors, infected suckers and contaminated farming tools (Tripathi et al. 2009). BXW has similarities to other bacterial wilts of banana caused by *Ralstonia solanacearum*, including Moko, blood, and bugtok diseases (Thwaites et al. 2000). Once these pathogens have become established, disease control is very difficult (Eden-Green 2004). There are currently no commercial chemicals, biocontrol agents or resistant cultivars available to control *X. campestris* pv. *musacearum* (Tripathi et al. 2009). Although BXW can be managed by following phytosanitary practices including cutting and burying infected plants, restricting the movement of banana materials from BXW affected areas, decapitating male buds and use of "clean" tools, but the adoption of such practices has been inconsistent as they are labour intensive and also farmers belief that de-budding affects the fruit quality (Tripathi et al. 2009). The lack of natural resistance against *X. campestris* pv. *musacearum*, labor intensive and unsustainable cultural practices, and the difficulties of conventional breeding with this highly sterile crop favor a transgenic approach.

The aim of this study was to determine whether the expression of sweet pepper (*Capsicum annuum*) plant ferredoxin-like protein (*Pflp)* gene in transgenic banana provides resistance to BXW. PFLP is a member of protein Ferredoxin-I (Fd-I), an important member of photosynthesis proteins. Fd-I is involved in several important metabolic pathways such as photosynthesis, nitrate reduction and lipid synthesis (Curdt et al. 2000; Geigenberger et al. 2005; Meyer 2001). The *Pflp* gene is widely distributed in all green tissues of plants and shows little diversity in different plant species.

The *Pflp* has shown resistance against various bacterial pathogens like *Erwinia, Pseudomonas, Ralstonia,* and *Xanthomonas* spp. in transgenic tobacco, tomato, orchids, calla lily and rice (Huang et al. 2004, 2007, Liau et al. 2003; Tang et al. 2001, Yip et al. 2007). The PFLP-mediated resistance raised by over-expression of the *Pflp* gene in transgenic plants is due to intensified production of active oxygen species (AOS) and activation of the hypersensitive response (HR) when plants were challenged with bacterial pathogens or elicitors of defense responses (Dayakar et al. 2003; Haung et al. 2004). In this study, the sweet pepper *Pflp* gene was transformed into banana plants in order to assess the effect of its over-expression on resistance against the bacterial pathogen *X. campestris* pv. *musacearum.*

Materials and methods

Agrobacterium and plasmid

The pBI-PFLP vector containing the *Pflp* 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-PFLP vector is shown in Fig. 2a. The vector was transformed into *Agrobacterium tumefaciens* super virulent strain EHA 105 through electroporation. The *Agrobacterium* strain EHA105 harbouring pBI-PFLP was maintained on YEB (0.1% yeast extract, 0.5% beef extract, 0.5% peptone, 0.5% sucrose and 0.04% MgSO4) 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 rpm) at 28°C until the optical density at 600 nm (OD600 nm) reached 0.8. The bacterial cells were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C and re-suspended 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 10 (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 rpm. OD600 nm of the culture was checked and adjusted to 0.6–0.8 with TMA1 medium.

Transformation, selection and regeneration

Embryogenic cell suspensions (ECSs) of banana cultivars 'Sukali Ndiizi' and 'Nakinyika' were subcultured in MA2 medium [Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog 1962) supplemented with 100 mg/l glutamine, 100 mg/l malt extract, 1 mg/l 2,4-dichlorophenoxyaceticacid (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 transformation competence and efficiency. Five days after sub-culturing, ECSs were transformed with *Agrobacterium* strain EHA105 harbouring pBI-PFLP and regenerated on selective medium as described by Tripathi et al. (2010). The regenerated shoots 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) for multiplication. 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 pots in the containment facility. The plants were further used for molecular analysis and for the evaluation of resistance against *X. campestris* pv. *musacearum*.

PCR analysis

Genomic DNA was isolated from 12 randomly selected putative transformed plantlets using DNeasy plant mini kit (Qiagen, GmbH, Germany). The presence of *Pflp* and *npt* II gene in the plant genome of the randomly selected putatively transgenic lines was confirmed by PCR analysis using specific primers. The primer sequences were *Pflp* gene: forward 5′-CAAGAAAACCAGCTGTGACAAGCCTTAAAC-3′ and reverse 5′-CGAGTTCTGCCTCTTTGTGAGTCTCAATAG-3′; *npt*II gene: forward 5′ CCTTATCCGCAACTTCTTTACCTA 3′ and reverse 5′ ACACCCAGCCGGCCACAGTCG 3′. Plasmid DNA of pBI-PFLP was used as positive control and non-transgenic plant DNA as negative control. The PCR (25 µl) contained 1× buffer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 0.5 U of Taq DNA polymerase and 1 µg of template DNA. The PCR were started with initial denaturation at 94°C for 3 min followed by 35 cycles of amplification as denaturing for 1 min at 94°C, annealing for 1 min at 55°C, and extension of 1 min at 72°C. The final extension phase was 10 min at 72°C. The PCR products were separated by electrophoresis in a 1.0% agarose gel and visualized under UV transilluminator after ethidium bromide staining.

Bioassay of transgenic banana for resistance to BXW

Twelve transgenic lines (T1–T12) showing PCR-positive results were evaluated for resistance to *X. campestris* pv. *musacearum* using a rapid screening technique (Tripathi et al. 2008a). The bacterial suspension was prepared as described by Tripathi

et al. (2008a). The OD600 nm of the bacterial suspension was checked and the bacterial concentration was adjusted to 10^8 colony-forming units per ml (cfu/ml) with sterile water. Fresh inoculum was used for all experiments in order to maintain a high virulent potential of the pathogen. Three plantlets of each transgenic line and non-transgenic control were inoculated with 100 µl of bacterial suspension (10^8 cfu/ml) into pseudostem of in vitro plantlets, as described by Tripathi et al. (2008a). Plants were assessed every day for 60 days 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). The identity of the *X. campestris* pv. *musacearum* isolates was confirmed using morphological characteristics (yellowish, mucoid and circular colonies on YTSA–CC semiselective medium) and by PCR using *X. campestris* pv. *musacearum*-specific primers (Adikini et al. 2011).

Southern blot analysis

The integration of the *Pflp* gene was analysed using Southern hybridization. The pBI-PFLP plasmid (10 pg) and genomic DNA (10 µg) from eight transgenic plants were digested with *Hin*dIII, which cuts the plasmid vector DNA only at one site as shown in Fig. 2a. 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 a positively charged nylon membrane (Roche Diagnostics, UK). The blots were hybridized with digoxigenin (DIG)-labelled *Pflp* 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, UK) according to the manufacturer's instructions.

RNA extraction and RT-PCR

RNA was extracted from leaf tissue of transgenic plants using RNeasy plant mini kit (Qiagen, GmbH, Germany) and RT-PCR was performed using Onestep RT-PCR Kit (Qiagen, GmbH, Germany) according to manufacturer's instructions. RT-PCR was performed with *Pflp* gene: forward 5′-CAAGAAAACCAGC TGTGACAAGCCTTAAAC-3′ and reverse 5′-CGAG TTCTGCCTCTTTGTGAGTCTCAATAG-3′. *Actin* gene amplification was performed using specific primers: forward primer *Actin* 5′-ACCGAAGCCCCTCTTA ACCC-3′ and reverse primer *Actin* R: 5′-GTATGGC TGACACCATCACC -3′ to check the quality of RNA. In both cases, PCR conditions were: 45°C for 35 min for synthesis of cDNA followed by 95°C for 5 min for denaturation and 35 cycles of amplification of cDNA as denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and 72°C for 10 min for extension of amplified product. The PCR products were separated by electrophoresis in a 1.0% agarose gel and visualized under UV transilluminator after ethidium bromide staining.

Western blot analysis

The eight independently derived transgenic banana lines subjected to Southern blot analysis were also analyzed for *Pflp* expression by western blot analysis. Total protein was extracted from transgenic banana lines by homogenizing 200 mg of leaf tissue with a mortar and pestle prior to suspension in 0.15 M NaCl, 10 mM HEPES, 10 mM EDTA pH 7.4. Protein samples were denatured by boiling in SDS-PAGE loading buffer (6× is 15% 2-mercaptoethanol, 15% SDS, 1.5% bromophenol blue, 50% glycerol) prior to electrophoresis. Twenty micrograms of total protein was loaded on a precast gel (Invitrogen, NuPAGE® Novex 4–12% Bis–Tris Gel), fractioned and blotted on polyvinylidene floride (PVDF) membrane (Biorad). The PFLP protein (60 ng) was loaded as standard. The protein expression was analyzed using anti-PFLP polyclonal antibody raised in rabbit (Harlan, UK) and anti-rabbit IgG alkaline phosphatase conjugate (Sigma-Aldrich, UK), with BCIP/NBT alkaline phosphatase substrate (Sigma-Aldrich, UK).

Western blots were scanned and intensities of PFLP bands were compared visually with standard to determine PFLP amounts in each sample. The percent of total soluble protein represented by PFLP in leaf tissue was then calculated

$$[\%\ \text{PFLP in leaf tissue} = (\text{ng PFLP in sample}/20,000\ \text{ng total soluble protein loaded in gel}) \times 100].$$

Evaluation of transgenic banana using potted plants in the screen-house

The plants of nine transgenic lines (T1, T2, T3, T4, T5, T7, T8, T11, T12) showing promising results with in vitro screening bioassay were further evaluated using potted plants in the screen house. Three plants each of these transgenic and non-transgenic 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 14 days, transferred to an open environment in the screen-house for another 7 days and then transferred to bigger plastic pots (30 cm). The 3-month-old plants were artificially inoculated by injecting 100 µl of bacterial suspension (10^8 cfu/ml) into the midrib of the first fully opened leaf using a syringe fitted with a 28-gauge needle. These plants were assessed every day for 60 days 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 at 60 dpi on the basis of the reduction in wilting in comparison with control non-transgenic plants.

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

Three replicates of eight promising transgenic lines were planted in confined field trial for testing the growth and flowering in comparison to non-transgenic banana plants.

Results

Transformation, selection and regeneration of transgenic banana

Banana embryogenic cell suspension cultures were transformed with *Agrobacterium tumefaciens* strain EHA105 containing pBI-PFLP plasmid construct in which the *Pflp* gene was expressed under control of CaMV35S constitutive promoter. The transformed cells multiplied and proliferated on kanamycin-selective medium, whereas control untransformed cells turned black (Fig. 1a). The embryogenic cells were regenerated on selective medium and several putative transgenic shoots were generated (Fig. 1b, c). The regenerated transgenic shoots were multiplied and transferred to rooting medium. All the shoots developed roots within 3–4 weeks. The well rooted plants of several independent transgenic lines of banana cultivars 'Sukali Ndiizi' and 'Nakinyika' were transferred to pots in the containment facility. No phenotypic differences were observed in the transgenic plants in comparison to non-transgenic plants during the vegetative growth under screen-house conditions (Fig. 1d).

Polymerase chain reaction (PCR) analysis

The presence of the *Pflp* gene was confirmed in 12 randomly selected putative transgenic lines using PCR with gene specific primers. The amplified product of about 450 bp was observed from the genomic DNA of all putative transgenic plants tested using *Pflp* specific primers, confirming the presence of the transgene in all transgenic lines (Fig. 2b). PCR was also performed using primers specific to neomycin phosphotransferase II (*npt*II) gene. An amplified fragment of about 500 bp was observed for all tested transgenic plants, confirming the presence of both *Pflp* and *npt*II genes.

Bioassay of transgenic banana for resistance to BXW

The transgenic banana plantlets containing the *Pflp* gene were tested for BXW resistance by artificial inoculation of in vitro plantlets under controlled laboratory conditions. Twelve PCR-positive transgenic lines (T1–T12) were artificially inoculated with *X. campestris* pv. *musacearum*. Eight transgenic lines (T1, T3, T4, T5, T7, T8, T11 and T12) did not show any symptoms through the duration of the experiment (60 days after inoculation), indicating that the *Pflp* gene could provide resistance to BXW (Figs. 3a, 4a). However, four other transgenic lines (T2, T6, T9 and T10) showed delayed appearance of the symptoms. The T2 line had partially wilted by the termination of the experiment at 60 dpi (days post inoculation); whereas remaining three lines (T6, T9 and T10) showed complete wilting in about 42–53 dpi (Fig. 4a). The control non-transgenic plantlets developed symptoms in about 15 dpi and were completely wilted within 37 dpi. The pathogenic bacteria isolated from wilted plants was identified as *X. campestris* pv.

Fig. 1 Regeneration of transgenic banana plants. **a** *Agro*-infected cells proliferating on selective medium supplemented with kanamycin. **b** Embryo maturation and germination on selective medium. **c** Putative transgenic shoots regenerated on selective medium. **d** Transgenic plants transferred to soil in pots in containment facility

musacearum on the basis of their characteristic morphology (yellowish, mucoid and circular colonies on YTSA–CC semiselective medium) and further confirmed by PCR using *X. campestris* pv. *musacearum*-specific primers.

Molecular characterization of transgenic lines

Genomic DNA of eight PCR positive transgenic lines (T1, T3, T4, T5, T7, T8, T11 and T12) showing complete resistance to BXW in bioassay were analyzed by Southern blot, after restriction with *Hind*III (Fig. 2a), to confirm integration of *Pflp* gene in banana genome. Each of these line contained *Pflp*-specific hybridizing bands (Fig. 2d) and the unique hybridization patterns observed indicated that each line resulted from an independent transformation event. Multiple bands of different molecular weights were detected by a *Pflp* gene specific probe (Fig. 2d) in all the transgenic lines except for line T11, showing that multiple copies of the plasmid had been integrated into the banana genome. Only transgenic line T11 seemed to have single copy. The smallest band detected in all the lines was bigger than 1.5 kb (total size of promoter, *Pflp* gene and terminator) indicating that the complete *Pflp* gene cassette with promoter and terminator was integrated in all the transgenic lines tested.

These transgenic lines were further tested with reverse transcriptase PCR (RT-PCR) using RNA from leaf tissue in order to verify *Pflp* transgene expression. The *Pflp* transcript amplification of expected fragment size ($\sim$450 bp) was observed from samples of all tested transgenic lines (Fig. 2c). 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 DNA contamination was below PCR detection levels in RNA samples.

Western blot analysis of these eight transgenic lines showed significant amounts of PFLP protein expressed in transgenic banana plants, whereas there was no detectable amount of PFLP protein in the non-transgenic control plants (Fig. 2e). Levels of PFLP expression were estimated semi-quantitatively from the western blots; leaf tissue from positive transgenic

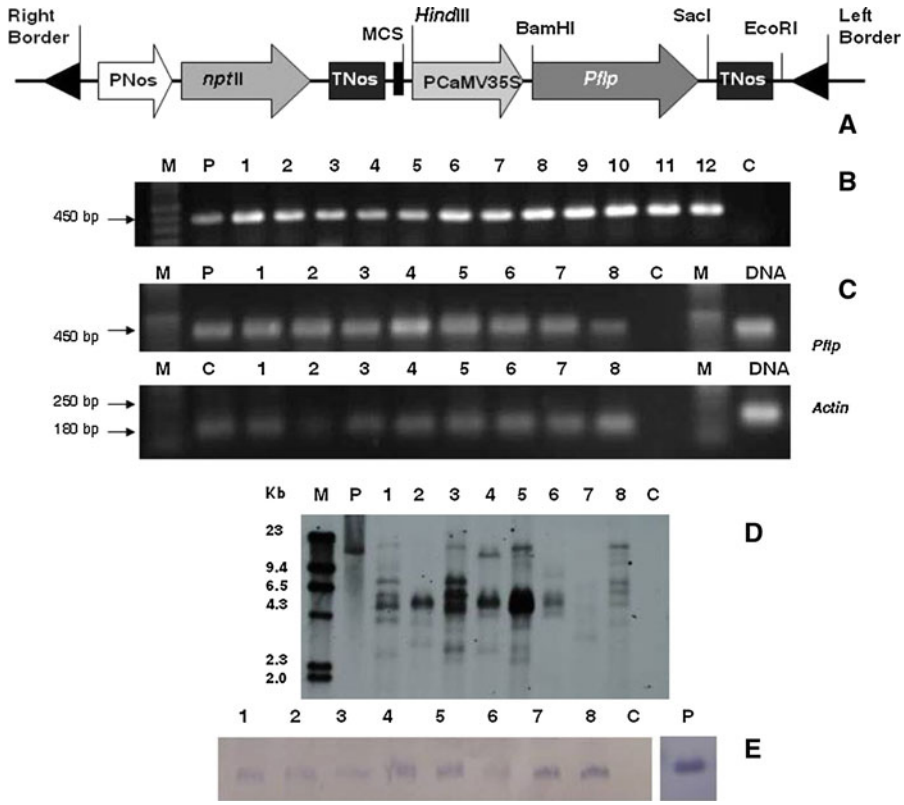

Fig. 2 **a** Schematic representation of T-DNA region of construct pBI-PFLP used for plant transformation. Pnos- nopaline synthase promoter; *npt*II, neomycin phosphotransferase II gene; Tnos- nopaline synthase terminator; PCaMV35S- CaMV35S promoter; *Pflp*, cDNA clone of plant ferredoxin like protein isolated from sweet pepper. **b** PCR analysis of genomic DNA from different transgenic lines and non-transgenic plant using *Pflp* specific primers; M, molecular weight marker, P- pBI-PFLP vector DNA; 1–12- transgenic lines (T1–T12, respectively), C- control non-transgenic plant. **c** RT-PCR analysis of RNA isolated from leaf tissue using *Pflp* 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; 1–8- transgenic plants (T1, T3, T4, T5, T7, T8, T11 and T12 respectively); C, non-transgenic plants; DNA- control reaction with genomic DNA. **d** Southern blot analysis of genomic DNA digested with *Hind*III. M-DIG labeled molecular weight marker, P- plasmid construct DNA digested with *Hind*III, 1–8- transgenic plant (T1, T3, T4, T5, T7, T8, T11 and T12 respectively) and C- control non-transgenic plant. **e** Western blot analysis of protein isolated from leaf tissue of transgenic lines and non-transgenic plants using anti-PFLP antibody

plants contained PFLP in the range 0.06–0.24% of total soluble protein. These results showed successful transcription and translation of the *Pflp* gene in the transgenic banana plants.

Evaluation of transgenic lines for resistance to BXW using potted plants in the screen-house

The nine promising transgenic lines (T1, T2, T3, T4, T5, T7, T8, T11 and T12) that showed either no symptoms or delayed symptoms with no complete wilting in the in vitro bioassay were further analyzed by inoculating potted plants in screen-house. Three plants of each transgenic line and non-transgenic control were artificially inoculated with *X. campestris* pv. *musacearum*. All the transgenic lines except T2 line did not show any symptoms up to 60 dpi (Fig. 3b), indicating absolute resistance (Fig. 4b) where as the non-transgenic control plants started developing symptoms (chlorosis or necrosis) after about 16 dpi; all leaves wilted completely and all plants died within 38 dpi (Fig. 3c). The transgenic line T2 started developing symptoms in about 33 dpi, and the disease progressed to other plant parts but plants were partially wilted within 60 dpi showing about 19% resistance (Fig. 4b).

Fig. 3 Enhanced resistance of transgenic banana lines to *X. campestris* pv. *musacearum.* **a** Rapid screening of transgenic lines using in vitro plantlets; *1* inoculated control non-transgenic plant, *2–6* inoculated transgenic plants. **b** Transgenic plants showing no BXW symptoms after 60 days post-inoculation of potted plants in screen house. **c** Non-transgenic plants showing complete wilting after artificial inoculation. All photographs were taken 8 weeks after artificial inoculation

All the eight transgenic plants looked normal with no phenotypic differences in comparison to non-transgenic plants during the growth under field condition. All the transgenic plants started flowering in about 9 months and set fruits similar to non-transgenic plants.

Discussion

The transgenic banana lines with constitutive over-expression of sweet pepper (*C. annuum*) *Pflp* gene were regenerated from embryogenic cell suspensions. Genetic engineering of banana plants has recently proven to be a useful alternative method for the introduction of new desirable traits and offer numerous advantages to circumvent the natural bottlenecks for banana breeding (Tripathi et al. 2008b; Tripathi 2011). In previous studies the over-expression of *Pflp* in transgenic plants, such as tobacco, tomato, calla lilly, rice and orchid, has been shown to offer effective resistance against related *Xanthomonas* strains and other bacterial pathogens (Huang et al. 2004, 2007; Liau et al. 2003; Tang et al. 2001; Yip et al. 2007). It is therefore hypothesized that expressing *Pflp* in banana will enhance resistance to bacterial diseases.

Variation in resistance levels to *X. campestris* pv. *musacearum* were observed in different transgenic lines. These may be due to location of the transgene insertion, different copy number and expression of gene. About 67% of transgenic lines (8/12) evaluated showed complete resistant to BXW. All these transgenic lines showed detected level of PFLP protein in the range of 0.06–0.24% of total soluble protein. It is interesting to note that line T11 having single copy insert contained PFLP protein in about 0.24% of total soluble protein and showed complete resistance to BXW. Some of the other lines with multiple copy number also showed similar amount of PFLP protein. So there is no co-relation between the copy number of transgene and expression of gene. All the eight lines with varied level of PFLP showed high resistance under screen house conditions confirming that only low level of PFLP protein is enough for providing

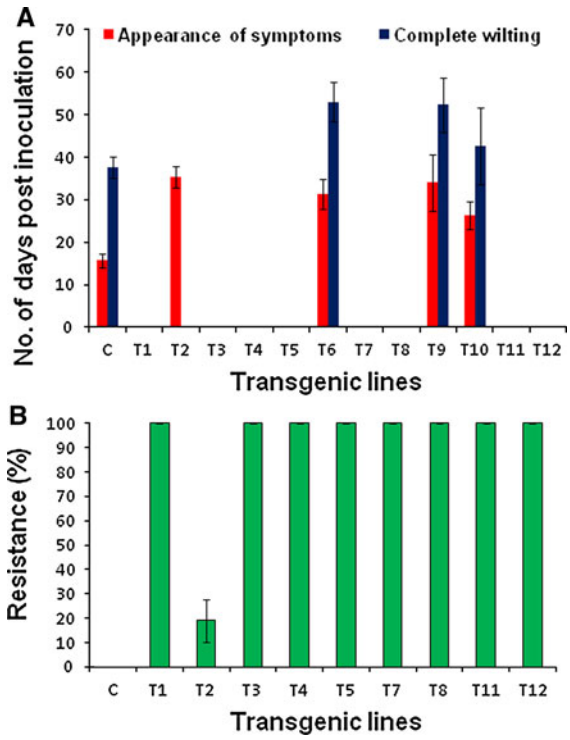

Fig. 4 **a** Graph showing time interval between inoculation and appearance of first BXW disease symptom and complete wilting of transgenic lines in comparison to non-transgenic plants after inoculation of in vitro plantlets. *Transgenic lines* showing no bar indicate no disease symptom appearance. **b** *Graph* showing resistance level of various transgenic lines against BXW after artificial inoculation of potted plants; *C* control non-transgenic plants; T1–T12 transgenic lines

resistance against BXW. In fact, high level of *Pflp* gene in transgenic plants might result in strong HR necrosis and even death, whereas expression at low level may be useful to generate plant disease resistance without causing significant damage to plant growth.

We observed that the transgenic banana plants showed disease resistance through the formation of HR-like necrosis upon challenging them with artificial inoculation of *X. campestris* pv. *musacearum* cultures. The HR is a rapid localized death of plant cells at the region of attempted invasion by an incompatible pathogen and concurrent with other defense responses including the oxidative burst (Levine et al. 1994), K+ efflux (Atkinson et al. 1990) and production of anti-microbial compounds (Dixon et al. 1994). Dayakar et al. (2003) demonstrated that enhanced generation of AOS is necessary to cause enhanced hypersensitive cell death in *Pflp* transgenic tobacco cells. Oxidative burst is considered one of the earliest events during plant HR (Alvarez et al. 1998; Lamb and Dixon 1997). AOS have direct antimicrobial activities and can therefore reduce pathogen viability. They have also been implicated in the destruction of the challenged plant cells, either through lipid peroxidation or function as a key factor mediating programmed cell death (Greenberg 1997). The HR cell death forms a physical barrier to prevent further pathogen infection (Mehdy 1994). In addition, a local HR is often associated with activation of plant defense responses in the surrounding and even distal uninfected parts of the plants leading to the development of systemic acquired resistance (SAR) (Xie and Chen 2000).

In this study, the transgenic lines were first evaluated for resistance against *X. campestris* pv. *musacearum* using a rapid bioassay protocol with in vitro plantlets followed by artificial inoculation of potted plants in screen house. This is a quick method of screening through which response to *X. campestris* pv. *musacearum* can be demonstrated within 60 days (Tripathi et al. 2008a). The method of artificial inoculation used in this study was similar to the common infection route through injury by contaminated tools. The most probable means of infection in banana field under natural conditions are insect transmission through wounds left by abscising male flowers, infected planting material and use of contaminated tools (Eden-Green 2004). The promising resistant lines identified were planted in confined field at National Agriculture Research Laboratories, Kawanda (Nordling 2010) and showed normal growth and fruit development suggesting that the over-expression of the *Pflp* gene does not seem to alter plant physiology.

Based on our results, *Pflp* appear to be good candidate gene for enhanced resistance against *X. campestris* pv. *musacearum* in banana. There is always the danger that susceptible pathogens will evolve resistance to the transgene used against them. To delay or avoid this outcome, it is necessary to identify and characterize additional resistance genes for use in pyramiding strategies with existing resistance genes. The data from this study suggest that *Pflp* could be used in combination with *Hrap,* which showed enhanced resistance to BXW in previous report (Tripathi et al. 2010), or/and other genes to provide enhanced and durable resistance against Xanthomonas wilt.

In summary, our results confirm that expression of the sweet pepper *Pflp* gene in transgenic banana leads to enhanced resistance to BXW. Transgenic bananas

expressing *Pflp* gene appear to have significant potential to overcome the BXW pandemic, which will boost the available arsenal to fight this epidemic disease and save livelihoods in Africa. As this transgenic approach has shown resistance against several bacterial pathogens, it may also provide effective control of other bacterial diseases of banana such as moko or blood disease in other parts of the world.

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