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Transgenic expression of the rice *Xa21* pattern recognition receptor in banana (*Musa* sp.) confers resistance to *Xanthomonas campestris* pv. *musacearum*

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Summary

Banana Xanthomonas wilt (BXW), caused by the bacterium *Xanthomonas campestris* pv. *musacearum* (*Xcm*), is the most devastating disease of banana in east and central Africa. The spread of BXW threatens the livelihood of millions of African farmers who depend on banana for food security and income. There are no commercial chemicals, bio-control agents or resistant cultivars available to control BXW. Here we take advantage of the robust resistance conferred by the rice pattern recognition receptor (PRR), XA21, to the rice pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). We identified a set of genes required for activation of *Xa21* mediated immunity (*rax*) that were conserved in both *Xoo* and *Xcm*. Based on the conservation, we hypothesized that intergeneric transfer of *Xa21* would confer resistance to *Xcm*. We evaluated 25 transgenic lines of the banana cultivar ‘Gonja manjaya’ (AAB) using a rapid bioassay and 12 transgenic plants in the glass house for resistance against *Xcm*. About fifty percent of the transgenic lines showed complete resistance to *Xcm* in both assays. In contrast, all of the non-transgenic control plants showed severe symptoms that progressed to complete wilting. These results indicate that the constitutive expression of the rice *Xa21* gene in banana results in enhanced resistance against *Xcm.* Furthermore this work demonstrates the feasibility of PRR gene transfer between monocotyledonous species and provides a valuable new tool for controlling the BXW pandemic of banana, a staple food for 100 million people in east Africa.

Keywords

rice pattern recognition receptor; *Xa21*; transgenic banana; *Xanthomonas campestris* pv. *musacearum*; banana Xanthomonas wilt

Introduction

Banana and plantain (*Musa* sp.) is the eighth most important staple food crop in the tropics and subtropics, grown in more than 120 countries with an annual world production of

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around 133 million tons (FAOSTAT, 2011). Approximately one third of global *Musa* production is from Africa, of which more than 50% is produced in the East African Great Lakes region including Burundi, Rwanda, Democratic Republic of Congo, Uganda, Kenya and Tanzania (FAOSTAT, 2011). The banana production, however, is affected by several diseases and pests, such as black Sigatoka, Fusarium wilt, banana Xanthomonas wilt (BXW), viruses such as *Banana bunchy-top virus* (BBTV) and *Banana streak virus* (BSV), weevils and nematodes (Jones, 2000; Tushemereirwe *et al.,* 2004).

BXW caused by *Xanthomonas campestris* pv. *musac*earum (*Xcm*), is one of the most important diseases of banana, and is considered the biggest threat to banana production in the Great Lakes region of east and central Africa (Tripathi *et al.,* 2009). BXW was first reported in Ethiopia in *Ensete* species and then on banana (Yirgou and Bradbury, 1968, 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.*, 2005), Rwanda (Reeder *et al.*, 2007), Kenya, Tanzania and Burundi (Carter *et al.*, 2009). There are no commercial chemicals, bio-control agents or resistant cultivars available to control BXW (Tripathi *et al.,* 2009). The disease can, however, be managed by following cultural practices such as the cutting and burying of infected plants, restricting the movement of banana planting materials from BXW affected to disease free areas, removal of male buds and the use of sterilized tools. The adoption of such practices has been inconsistent as they are very labour intensive. The lack of known genetic resistance in banana against *Xcm*, the difficulties associated with conventional breeding of this highly sterile crop, and a dependence on labor intensive, favor a transgenic approach to control BXW.

For the last 100 years, a considerable effort has been directed at introducing genes conferring resistance to a variety of bacterial, fungal and viral diseases through conventional breeding approaches. In the 1990s, many of these disease resistance genes were isolated and characterized. These studies identified two distinct classes of receptors. The first class includes “race specific” receptors that have long been used in breeding programs. These race specific receptors typically fall into the “NBS-LRR” class and recognize specific races of the pathogen. Resistance conferred by race specific receptors is frequently ephemeral because the pathogen can evolve mechanisms to overcome the resistance. In contrast, the second class of immune receptors, the pattern recognition receptors (PRRs), confers broad-spectrum resistance that is predicted to be durable. This is because these immune receptors recognize conserved microbial products that are essential to pathogen fitness. In this case, if the microbe mutates these critical components, the microbe is impaired in virulence.

The discovery of PRRs and NBS-LRR receptors presents opportunities to transfer immune receptors and chimeric receptors across genera using genetic engineering. For example, the tomato race specific *Pto* gene was introduced in transgenic tobacco to enhance resistance to *Pseudomonas syringae* pv. *tabaci* (Thilmony *et al.*, 1995). Similarly, the pepper race specific *Bs2* gene was shown to confer resistance to bacterial spot disease when transferred to tomato (Tai *et al.*, 1999). The maize NBS-LRR, *Rxo,* was transferred to rice (Zhao *et al*., 2005).

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Over 300 immune receptors of the PRR class have been predicted in rice and 35 in *Arabidopsis* (Dardick *et al*., 2006). However, despite their importance and abundance in plant genomes, only a few plant PRRs have been isolated and characterized (Lacombe *et al*., 2010). Even fewer have been tested for their ability to confer resistance when transferred from one plant species to another. An important example is the transfer of the *Arabidopsis* EF-Tu (elongation factor thermo unstable) receptor (EFR) to other dicotyledonous genera (Zipfel *et al*., 2004; Lacombe *et al*., 2010). EFR recognizes the conserved bacterial molecule EF-Tu (or its eliciting epitope elf18). In plants, perception of EF-Tu seems to be restricted to the order *Brassicales*. Transgenic expression of EFR in the solanaceous species *Nicotiana benthamiana* and *Solanum lycopersicum* confers responsiveness to EF-Tu and makes the plants more resistant to a range of phytopathogenic bacteria from different genera (Lacombe *et al*., 2010). Other PRRs have also been successfully transferred to taxonomically diverse plant species (Mendes *et al*., 2010; Afros *et al.,* 2011). The demonstration that PRRs can be transferred between species opens the possibility of engineering resistance to devastating diseases of monocots, which provide the staple food for 80% of the world's people.

The rice XA21 pattern recognition receptor, confers broad-spectrum resistance to the Gram-negative bacterium, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). The *Xa21* was identified in the wild rice species *Oryza longistaminata* (Ikeda *et al*., 1990; Ronald *et al.*, 1992) and confers broad-spectrum resistance to the most races of *Xoo*, the causal agent of bacterial blight of rice (Ronald *et al.*, 1992, Wang *et al*., 1996). Engineered chimeric XA21 receptors confer resistance to a serious fungal pathogen when introduced into normally susceptible rice varieties (Kishimoto *et al*., 2010).

In the past several years, genetic analysis led to the identification of ten *Xoo* genes, which are required for activation of *Xa21*-mediated immunity, that fall into three functional classes. The first class consists of 3 genes (*raxA*, *raxB* and *raxC)* that encode components of a putative bacterial Type I secretion system (da Silva *et al.,* 2004). The second class includes *raxP* and *raxQ,* which encode an adenosine-5′-triphosphate (ATP) sulfurylase and adenosine-5′-phosphosulfate kinase. These proteins function in concert to produce 3′-phosphoadenosine 5′-phosphosulfate (Shen *et al*., 2002), the universal sulfuryl group donor. This class also includes *raxST,* which encodes a tyrosyl-sulfotransferase (Han *et al*., 2012). The third class includes four genes encoding two-component regulatory systems: the *RaxR/H* system (Burdman *et al*., 2004) and the *PhoP/Q* system (Lee *et al*., 2008). *RaxR/H* negatively regulates *PhoP*, which governs virulence by control of *hrp* gene expression (Lee *et al*., 2008). We found that all three functional classes are present in the recently sequenced *Xcm* genome (Studholme *et al*., 2011). These results suggest that *Xa21* receptors may recognize a microbial determinant that is conserved in *Xcm*. In this study, we tested this prediction by expressing the rice *Xa21* gene in transgenic banana and assaying for resistance against *Xcm*.

Results

RaxSTAB analysis

The *Xoo raxSTAB* operon is required for elicitation of *Xa21*-mediated immunity in rice. We hypothesized that XA21 recognizes a molecule that is secreted and/or modified by the

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raxSTAB operon. In this case, *Xanthomonas* strains that carry *raxSTAB*, or close orthologs, would no longer be able to infect XA21 plants. To gain insight into the genome of *Xcm* and to better predict the function of the XA21 receptor in banana, we carried out a comparative genomic analysis of the *raxSTAB* operon in 7 sequenced *Xanthomonas* strains: *Xcm*, *Xoo*_PXO99, *Xoo*_MAFF, *X. oryzae* pv. *oryzicola* (*Xoc*), *X. axonopodis* pv. *citrumelo* F1 (*Xac*), *X. euvesicatoria* 85-10, and *X. translucens* (Fig. S1).

We found that *Xcm* also carries the *raxSTAB* operon, and that it shares high sequence similarity with the predicted orthologous operon in *Xoo* and other *Xanthomonas* species (Fig. S1). Specifically *Xcm* and *Xoo*_PXO99 share 91, 85 and 92% sequence identity for RaxST, RaxA and RaxB, respectively. A detailed alignment of these proteins is presented in Fig. S2 (supplementary information). Based on the conservation, we hypothesized that intergeneric transfer of *Xa21* would confer resistance to *Xcm*.

Generation and validation of transgenic plants

Banana embryogenic cell suspension (ECS) cultures were successfully transformed with *A. tumefaciens* strain EHA105 containing the pCAMBIA1300-Ubi:Xa21 plasmid vector in which the *Xa21* gene was expressed under control of the maize *Ubiquitin* constitutive promoter. The transformed cells multiplied and proliferated on hygromycin selective medium, whereas untransformed cells turned black (Fig. 1). The embryogenic cells were regenerated on selective medium and approximately 30–40 transgenic plants were regenerated from 0.5 ml settle cell volume of cell suspension on selective media supplemented with hygromycin. In total 95 independent putative transgenic lines were obtained in three experiments. The regenerated independent transgenic shoots were clonally multiplied and transferred to rooting medium. All shoots developed roots within three to four weeks. The well rooted plantlets of the independent transgenic lines were transferred to pots in the contained glasshouse for disease evaluation and growth analysis (Fig. 1).

The presence of the *Xa21* gene was confirmed in all the 95 putative transgenic lines by PCR using *Xa21* specific primers. The amplified product of 410 bp was observed from the genomic DNA of all transgenic plants, confirming the presence of the transgene in all the transgenic lines tested (Fig. 2b). An amplified fragment of 415 bp was observed for all tested transgenic plants using the *hygromycin phosphotransferase (hpt)* specific primers (Fig. 2b). This confirmed the presence of both *Xa21* and *hpt* genes in all the transgenic lines tested. Twenty five lines were prioritized for further evaluation.

Evaluation of transgenic lines for disease resistance using rapid bioassay

Small *in vitro* plantlets were inoculated with *Xcm* using rapid bioassay, and the resistance responses were categorized into three categories: resistant, partially resistant and susceptible (Fig. 3, Table 1). The non-transgenic control plantlets developed symptoms (chlorosis, necrosis and wilting) at about 8 days post inoculation (dpi) and were completely wilted by 20 dpi (Fig. 3a). Out of 25 transgenic lines tested, 12 lines did not show any symptoms up to 60 dpi and were classified as resistant lines (Fig. 3b–d, Table 1). Four transgenic lines showed symptoms in the inoculated leaf, but these did not spread to other leaves and plants were never completely wilted, confirming partial resistance. The remaining nine lines

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showed symptoms and completely wilted within 30 dpi, similar to the control non-transgenic plants. These lines were classified as susceptible transgenic lines.

The pathogenic bacteria re-isolated from wilted plants were identified as *Xcm* on the basis of their characteristic morphology (yellowish, mucoid and circular colonies) (Tripathi *et al*., 2007). Their identity was further confirmed by PCR using *Xcm* specific primers (Adikini *et al*., 2011).

Evaluation of transgenic lines for resistance to BXW in glasshouse using potted plants

The 12 transgenic lines (GX3, GX5, GX8, GX15, GX26, GX36, GX37, GX46, GX57, GX62, GX74 and GX76), previously subjected to the rapid screening assay, were selected for further screening using potted plants in a glasshouse (Fig. 3; Table 2). Three plantlets of each transgenic line were grown and artificially inoculated with *Xcm*. The non-transgenic control plants developed symptoms after about 16–18 dpi and completely wilted 39 dpi (Fig. 3e). Out of the 12 transgenic lines tested, three lines (GX37, GX62 and GX74) showed symptoms such as drooping of inoculated leaves, which then turned-yellowish brown and symptoms spread to other leaves and finally plants completely wilted in 41–45 dpi. These lines were also found to be susceptible when tested using the rapid bioassay. Seven transgenic lines (GX3, GX5, GX8, GX15, GX26, GX46 and GX76) showed no symptoms at all, not even in inoculated leaves up to 60 dpi, indicating that they were completely resistant against *Xcm* (Table 2, Fig. 3f–h). These 7 lines were also found to be resistant in the rapid bioassay. The remaining two lines (GX36 and GX57) were found to be partial resistance with disease symptoms in only two to three leaves and these plants were only partially wilted at 60 dpi.

No internal symptom (yellow ooze) was observed in asymptomatic transgenic plants where as yellow ooze due to growth of *Xcm* was observed in inoculated non-transgenic plants and symptomatic transgenic plants (Fig. 3i–k).

Bacterial population analysis in transgenic and non-transgenic plants

Two transgenic lines (GX8 and GX26) along with non-transgenic plants were further evaluated for growth of bacterial populations. The bacteria isolated from inoculated transgenic lines at 0 dpi were similar to those obtained from non-transgenic plants (Fig. 4a). Over time, the bacterial population significantly ($P < 0.05$) decreased to 3.72 log cfu/cm^2 in transgenic line GX8 and 2.53 log cfu/cm^2 in transgenic line GX26 in comparison to inoculated non-transgenic banana, where the bacterial population reached 6.7 log cfu/cm^2 at 16 dpi. These results demonstrated that populations of *Xcm* were much lower in leaves of resistant transgenic lines than in those of the non-transgenic control plants. No bacteria were recovered 5 cm above and 5cm below the inoculation point in leaves of transgenic line GX8 confirming that bacteria was contained in the transgenic plants (Fig. 4b). However, slight bacterial growth was observed in transgenic line GX26 at 5 cm below the inoculation point although no bacteria were found at above the inoculation point.

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Molecular characterization of transgenic lines

Genomic DNA of all the twelve transgenic lines (GX3, GX5, GX8, GX15, GX26, GX36, GX37, GX46, GX57, GX62, GX74 and GX76) evaluated for disease resistance in the glasshouse were confirmed to contain the *Xa21* gene by Southern blot analysis (Figure S3, supplementary information). Their unique hybridization patterns further indicated that each transgenic line resulted from an independent transformation event.

The same twelve transgenic lines were further analyzed for expression levels of the *Xa*21 gene by RT-PCR and qRT-PCR using gene-specific primers. The amplified *Xa21* transcript of 110 bp was observed in all the transgenic lines tested (Fig. 5a) confirming expression of transgene in all the tested lines.

The *25S* ribosomal transcripts amplification of the internal control was also detected in all the plants tested. To evaluate *Xa21* transcripts, we then performed qRT-PCR, which clearly showed that *Xa21* transcript level varied among the different transgenic lines (Fig. 5b). In four of the transgenic lines (GX3, GX5, GX8, and GX76), *Xa21* transcript levels correlated with disease resistance (i.e. the lines with high transcript levels were also highly resistant against *Xcm)*. However, some lines did not show any correlation between transcript level and disease resistance. For example, lines GX15, GX26 and GX46 had relatively low *Xa21* transcript level, and yet showed 100% resistance against *Xcm* (Table 2). The five transgenic lines that were susceptible or partial resistance also contained varying levels of *Xa21* transcripts. No transcripts were found in the RNA isolated from non-transgenic plants.

Growth analysis of transgenic plants

The 12 transgenic lines (GX3, GX5, GX8, GX12, GX15, GX26, GX29, GX32, GX37, GX60, GX62, and GX76) evaluated for growth analysis showed no phenotypic differences when compared to the non-transgenic control plants (Fig. 6). A few transgenic lines were taller with larger leaf area than non-transgenic control plants, but these traits were not statistically significantly different from the non-transgenic control plants.

Discussion

Genetic engineering (GE) has proven to be a useful technology for incorporating beneficial traits into diverse plant species and offers a method to overcome bottlenecks associated with classical breeding. The GE crops currently on the market (primarily cotton, maize, canola and soybean) have been widely adopted by farmers; 170 million hectares, approximately 12% of the global arable land, were planted by GE crops in 2012 (Qaim and Kouser, 2013). Genetic engineering of staple food crops, such as banana and rice, for resistance to diseases, pests and abiotic stresses, is expected to substantially alleviate poverty, hunger and malnutrition (Demont and Stein, 2013). In the case of banana, a staple food for about 100 million people in Africa, genetic engineering provides a new tool to limit the spread of pathogens that are difficult to control using current conventional methods, such as *Xcm* (Tripathi *et al*., 2009).

In farmers' fields, BXW can be contained if de-budding is effectively practiced. However, adoption of de-budding has been inconsistent among farming communities (Kagezi *et al*.,

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2006). Once BXW occurs in a field, there is no remedy other than to cut down all infected plants, completely dig out the rhizomes and place the field under fallow or a prolonged crop rotation regime. A 6-month fallow period is adequate to avoid re-infection from soil-borne inoculum if all infected plant material is removed (Tripathi *et al*., 2009). The generation and growth of resistant cultivars is a cost-effective approach for managing bacterial diseases. However, resistance to *Xcm* has not yet been identified in studies of diverse banana germplasm. Even if resistant germplasm sources are identified, conventional breeding of banana is a difficult and lengthy process due to sterility of the most cultivars coupled with long generation times. Transgenic technologies present a cost-effective alternative approach for developing strategies to control the BXW pandemic.

The rice *Xa21* gene was considered a good candidate for engineering transgenic resistance to *Xcm* because of its broad-spectrum resistance against *Xoo* (Song *et al.,* 1995; Wang *et al.,* 1996). We and others previously demonstrated that the *Xa21* gene is effective in controlling resistance to diverse isolates of *Xoo* when expressed in susceptible rice cultivars (Wang *et al*., 1996), and provides protection against a range of the bacterial pathogens in transgenic citrus and tomato (Mendes *et al*., 2010; Afros *et al.,* 2011). Here, we show that the *Xoo raxSTAB* operon, which is required for the activation of *Xa21*-mediated immunity, is highly conserved in diverse *Xanthomonas* species, including *Xcm* and that the *Xa21* gene confers resistance to *Xcm*.

In the current study, 25 independent transgenic banana plants of the cultivar ‘Gonja manjaya’ (AAB) expressing the rice *Xa21* PRR were evaluated for resistance against *Xcm* using rapid bioassay. Twelve of these *in vitro* plantlets showed resistance against *Xcm.* In contrast, all of the non-transgenic control *in vitro* plantlets showed severe symptoms that progressed to complete wilting. The advantage of the initial *in vitro* plantlets screening is that the response of the transgenic plantlets to *Xcm* can be determined within six weeks. This is very efficient especially in instances where large numbers of transgenic lines have to be screened because highly susceptible lines can be quickly identified and eliminated from further study (Tripathi *et al.,* 2008). We further evaluated the 12 transgenic lines for resistance to *Xcm* in the glass house using 90 days-old potted plants. The 12 transgenic lines screened in the glasshouse conferred varying levels of resistance to *Xcm*, with 58% lines showing complete resistance.

The level of *Xa21* transcripts varied among the 12 transgenic lines, and the level of expression did not correlate with the level of the observed disease resistance. All the 12 lines tested expressed the transgene, even the susceptible lines. These results suggest that the observed variation in disease resistance is not due to transcript accumulation.

The transgenic plants displayed no measureable morphological differences (plant height, total leaf area and pseudostem thickness) compared with non-transgenic control plants in glasshouse house conditions. These results indicate that the constitutive expression of the rice *Xa21* gene in banana results in enhanced resistance to *Xcm* with no visible physiological effects.

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It is well known that pathogens can evolve and “breakdown” genetic resistance. Today, knowledge of resistance gene structure and function and the ability to sequence diverse pathogen strains provides a new opportunity to engineer plants for resistance and to predict their potential usefulness and durability. It has been hypothesized that resistance conferred by race specific receptors is not durable because the pathogen can quickly evolve mechanisms to overcome the resistance. In contrast, some PRRs confer broad-spectrum, durable, resistance. For example, the introduction of the stem rust-resistance gene *(RPG1,* a predicted PRR*)* into barley in the mid 1940’s, has conferred durable and broad-spectrum resistance to many isolates of the stem rust pathogen *Puccinia graminis* f. sp. *tritici* (Brueggeman *et al*., 2002). Preliminary results similarly suggest that *Xa21* may confer durable resistance in rice (Choi *et al*., 2003). This may be due to the recognition of a conserve microbial determinant (also known as pathogen associated molecular pattern (PAMP) that is essential to pathogen fitness. In this case, the microbe cannot easily overcome the plants resistance through mutation. Still, because it is known that PRR-mediated immunity can be overcome through suppression of the immune response by bacterial effectors (Dodds and Rathjen, 2010), it is not possible to predict if the observed *Xa21*-mediated resistance to *Xcm* will be durable. To enhance the durability of resistance, breeders have often used a stacking approach, where diverse resistance genes are combined into a single line (Zhu *et al*., 2012). The data from this study suggest that *Xa21* could be used in combination of other resistance genes such as *Hrap* and/or *Pflp,* which showed enhanced resistance against *Xcm* in previous reports (Tripathi *et al.,* 2010; 2013; Namukwaya *et al*., 2012).

The selected XA21 transgenic lines will be further evaluated for field resistance, agronomic traits and fruit quality. If proven valuable under field conditions in east and central African countries, XA21-banana will be an important tool in the battle against bacterial wilt epidemics and improve the livelihoods of more than 100 million people in Africa.

Experimental Procedures

RaxSTAB analysis

The amino acid sequence of RaxST, RaxA and RaxB of different *Xanthomonas* species, as detailed in Fig. S1 (supplementary information), were aligned using the ClustalW2 alignment tool (Larkin *et al.* 2007). Identity scores were calculated as the number of identities between two sequences, divided by the length of the alignment, and represented as a percentage.

Plasmid construct and banana transformation

The binary vector pCAMBIA1300-Ubi:Xa21 containing the rice *Xa21* gene driven by a maize *ubiquitin* promoter, and *hygromycin phosphotransferase* (*hpt*) as selection marker was used in all the genetic transformation experiments (Fig. 2a) (Park *et al*., 2010). The plasmid vector was transformed into an *Agrobacterium tumefaciens* super virulent strain EHA 105 (Hood *et al.,* 1993) through electroporation. Thereafter, EHA 105 harboring pCAMBIA1300-Ubi:Xa21 was prepared for banana transformation as described previously by Tripathi *et al.* (2012).

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ECSs of the banana cultivar 'Gonja manjaya' were transformed with *Agrobacterium* strain EHA 105 harboring pCAMBIA1300-Ubi:Xa21 using the procedure described by Tripathi *et al.* (2012). The *Agrobacterium*-infected ECSs were then cultured on selective embryo development medium (EDM; Tripathi *et al*., 2012) supplemented with cefotaxime (300 mg/L) and hygromycin (25 mg/L) for two to three months, and transferred every two weeks to fresh medium of the same type. Putatively transformed embryos that developed on EDM were transferred to selective embryo maturation medium (EMM; Tripathi *et al*., 2012) supplemented with cefotaxime (300 mg/L) and hygromycin (25 mg/L). When mature embryos developed, they were transferred to selective germination medium (GM; Tripathi *et al*., 2012) supplemented with cefotaxime (300 mg/L) and hygromycin (25 mg/L). Shoots developing on GM were transferred to proliferation medium (PM; Tripathi *et al*., 2012) and kept at 28°C for a 16 h/8 h light/dark photoperiod under fluorescent tube lights for maintenance and multiplication of shoots. Regenerated putative transgenic shoots were transferred to rooting medium (RM; Tripathi *et al*., 2012). Rooted plantlets were planted in sterile soil in pots and maintained in a containment facility (Fig. 1). Regenerated putative transgenic shoots were regularly micro-propagated and clonally multiplied to obtain 12 plantlets of each line for rapid bioassay, glasshouse screening, growth analysis and safekeeping in growth room for molecular analysis.

Genomic DNA isolation and PCR of transgenic lines

The plant genomic DNA was extracted from the regenerated putative transgenic shoots using a DNeasy kit (Qiagen, GmbH, Germany), and a PCR was performed using *Xa21* and *hpt* gene-specific primers for preliminary testing of transgenic lines. Non-transgenic control plant DNA was included as negative and plasmid DNA as positive control in each PCR reaction. The primers used for the *Xa21* gene were: forward *Xa21* 5′ TTGTTCTCTGCGCTGCTGCTCTGC 3′ and reverse *Xa21* 5′ CCAATGGCCGCGGGGATGCTC 3′, and for the *hpt* gene were: forward 5′ GATGTTGGCGACCTCGT 3′ and reverse 5′ GTGTCACGTTGCAAGACCTG 3′.

Evaluation of transgenic lines for resistance to BXW using rapid bioassay

Twenty five PCR positive transgenic lines containing the rice *Xa21* gene were randomly selected for rapid bioassay for disease resistance under growth room condition. Control non-transgenic plants were also included in each experiment. Three *in vitro* plantlets (with 3–4 leaves) of each line were artificially inoculated with 100 μL of *Xcm* culture (10^7 cfu/mL) in the pseudostem as previously reported (Tripathi *et al.*, 2008). For comparison, three non-transgenic plants were inoculated with *Xcm* and three additional plants with water as control. The inoculated plantlets were cultured into plant tissue culture vessels (Sigma, V0633) containing proliferation medium and incubated at 28°C for a 16 h/8 h light/dark photoperiod under fluorescent tube lights providing illumination of 94 μmol/m^2/second for 60 days. Plantlets incubated under *in vitro* conditions were monitored and assessed for the development of disease symptoms daily for 60 days. The disease symptoms included chlorosis, necrosis and the complete wilting and death of plants. Observations were made regularly and the data recorded for each plantlet. The relative resistance of transgenic lines to *Xcm* was evaluated 60 days after inoculation of *in vitro* plantlets. Plantlets were categorized as resistant if a plant did not show any disease symptoms, partial resistant if a

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plant showed symptoms but the symptoms did not spread to another part of the plant and was not completely wilted, and susceptible if a plant developed symptoms and completely wilted after inoculation similar to control non-transgenic plants. The wilting incidence was calculated as follows:

$$\text{Wilting incidence (\%)} = (\text{Number of plants wilted} / \text{Total number of plants inoculated per independent transgenic lines}) \times 100$$

Evaluation of transgenic lines for resistance to BXW in glasshouse using potted plants

The plants of 12 transgenic lines (GX3, GX5, GX8, GX15, GX26, GX36, GX37, GX46, GX57, GX62, GX74 and GX76) previously tested with a rapid screening method were further evaluated using potted plants in the glasshouse. Three plants of each transgenic line and the non-transgenic control were transferred to sterile soil in plastic cups (8 cm diameter) for acclimatization. The plants were maintained in a humid shaded environment for 30 days, transferred to an open environment in the glasshouse for another seven days and then transferred to bigger plastic pots (30 cm diameter). Three month old plants were artificially inoculated by injecting 100 μL of a bacterial suspension (10^8 cfu/mL) into the midrib of the second fully opened leaf as described by Tripathi *et al.* (2010). These plants were assessed daily for 60 days for disease symptoms, with preliminary symptoms of chlorosis or yellowing 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 as follows:

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

where Reduction in wilting = total number of leaves - number of wilted leaves.

Bacterial population analysis in transgenic and non-transgenic plants

Two transgenic line (GX8 and GX26) showing complete resistance against *Xcm* were further evaluated for growth of bacterial populations. Three month old plants of transgenic lines GX8 and GX26 and non-transgenic control were artificially inoculated by injecting 100 μL of a bacterial suspension (10^8 cfu/mL) into the midrib of the second fully opened leaf as described by Tripathi *et al.* (2010). One centimeter long section of midrib of the leaf of inoculated transgenic banana plants and non-transgenic control plants were collected at different time interval (0, 4, 8, 12 and 16 dpi) and ground with 1 ml of sterilized water in a microfuge tube and incubated on shaker at room temperature for 1 hr. Sample suspensions were serially diluted with sterilized water, plated on semi-selective YTSA agar medium (Yeast extract (1%), tryptone (1%), sucrose (1%), agar (1.5%), pH 7.0; Tripathi *et al*., 2007) supplemented with 50 mg/L cephelexin antibiotic, and incubated at 28°C for 48 h before counting to determine populations. At 16 dpi samples were also collected from midrib section 5 cm above and 5 cm below the point of inoculation and bacterial populations were determined. Two clones of each line were used for each treatment and three replicates were plated for each clone at each time point. The data presented is the average of 6 samples at each time point.

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Molecular characterization of transgenic lines

Southern blot analysis—The integration of the *Xa21* gene into the genome of banana was analyzed using Southern hybridization. Genomic DNA was isolated from twelve PCR positive transgenic plantlets (GX3, GX5, GX8, GX15, GX26, GX36, GX37, GX46, GX57, GX62, GX74 and GX76) which were evaluated in glasshouse using a modified hexadecyltrimethylammonium bromide (CTAB) extraction method for *Musa* described by Gawel and Jarret (1991). The pCAMBIA1300-Ubi:Xa21 plasmid (10 pg) and genomic DNA (10 μg) from the transgenic plants were digested with *BamH*I, which cuts the plasmid vector DNA only at one site (Fig. 2). When integrated into genomic DNA, *BamH*I cuts once at start of *Xa*21 gene (outside the probe region) and again in the genomic DNA beyond the T-DNA border. Genomic DNA of non-transgenic banana plant restricted with *BamH*I enzyme was used as negative control. The restricted DNA was resolved on a 0.8% (w/v) agarose gel and blotted onto the positively charged nylon membrane (Roche Diagnostics, UK). The blots were hybridized with a digoxigenin (DIG) labeled with the 410 bp *Xa21* gene specific probe that was part of the *Xa21* gene. 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, RT-PCR and qRT-PCR analysis—Total RNA was extracted from 100 mg leaf tissue of 12 transgenic banana plants (GX3, GX5, GX8, GX15, GX26, GX36, GX37, GX46, GX57, GX62, GX74 and GX76) which were evaluated in glasshouse using the RNeasy plant mini kit (Qiagen, GmbH, Hilden, Germany) and treated with DNase (RNeasy Plant Mini kit, Qiagen). The quantity and quality (A260/230 and A260/280) of total RNA were determined using the Nanodrop. RNA was checked with PCR for absence of genomic DNA. Complementary DNA (cDNA) was synthesized, starting with 1 μg of total RNA and using reverse transcriptase of the Maxima H Minus First Strand cDNA synthesis kit with oligo DT primers (Thermo scientific). Reverse transcriptase PCR (RT-PCR) was performed with 1 μl of each cDNA synthesized using primers specific to the *Xa*21 gene (forward primer: 5′ TCCCCAATTCTATCTGGAACCT 3′; reverse primer: 5′ CTCGAGGAGGTGAAGGGTTTT 3′). Amplification of the banana *25S* ribosomal transcript, used as an internal control to determine the quality of RNA, was performed using the forward primer (forward primer: 5′ ACATTGTCAGGTGGGGAGTT 3′; reverse primer: 5′ CCTTTTGTTCCACACGAGATT 3′).

Real-time RT-PCR was carried on 7900 Real Time PCR System (Applied Biosystems, USA) using Maxima SYBR green/ROX PCR kit (Thermo Scientific) according to the manufacturer's instructions. qRT-PCR was performed with 1 μl of each cDNA synthesized at 1:10 dilution, using *Xa*21 specific primers as mentioned above. Two independent biologically replicated experiments were set up with three technical replicates in each experiment. No-template controls and non-transgenic control were included. Relative expression data were normalized using the banana *25S* ribosomal gene. Non-transgenic control plant acts as calibrator to calculate relative expression level of *Xa*21 in transgenic plants. The relative levels of *Xa*21 gene were analyzed using the $2^{- Ct}$ method (Livak & Schmittgen 2001).

$$\Delta \text{Ct}\,(Xa21){=}\text{Ct}\,(Xa21){-}\text{Ct}\,(\text{internal control }25S\text{ ribosomal gene})$$
$$\Delta\Delta \text{Ct}{=}\Delta \text{Ct}\,(\text{target sample or transgenic line}){-}\Delta \text{Ct}\,(\text{calibrator or non}{-}\text{transgenic plant})$$

Growth analysis of transgenic plants

Three replicates of 12 well rooted transgenic plants (GX3, GX5, GX8, GX12, GX15, GX26, GX29, GX32, GX37, GX60, GX62, and GX76) were weaned in small disposable cups in a humid chamber. These transgenic plants were subculture and clonally multiplied from individual transgenic lines in tissue culture facility. The three non-transgenic control plants were included to compare the growth with transgenic plants. Plants were then hardened off for four weeks under low plastic tents where humidity was reduced over time by gradual opening of the side of the tent in the third week. The plants were removed from the plastic tents after four weeks and transferred to 10 liter pots in the glasshouse. Plants were irrigated manually on alternate days, and the temperature maintained at 26–28°C. After 90 days, growth characteristics such as plant height, pseudostem girth at base and middle, total number of leaves, length and width of fully opened third leaf were recorded. Total leaf area was calculated using a formula described by Kumar *et al.* (2002):

$$\text{Total leaf area}{=}0.8 * \text{L} * \text{W} * \text{N}$$

L= length of middle leaf

W= width of middle leaf

N= Total no. of leaves in a plant

Data collection and analysis

The means and standard deviation were calculated for three replicates per experiment. Analysis of variance (ANOVA) was conducted using Minitab 16 (Minitab 16 statistical software, 2012), and interaction between means was by least significant difference (LSD) at P= 0.05. Grouping was performed using the Fisher test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Adikini S, Tripathi L, Beed F, Tusiime G, Magembe EM, Kim DJ. Development of specific molecular tool for detecting *Xanthomonas campestris* pv. *musacearum*. Plant Pathol. 2011; 60:443–452.

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Afros A, Chaudhry Z, Rashid U, Ali GM, Nazir F, Iqbal J, Khan MR. Enhanced resistance against bacterial wilt in transgenic tomato (*Lycopersicon esculentum)* lines expressing the *Xa21* gene. Plant Cell Tiss Org Cult. 2010; 104:227–237.

Brueggeman R, Rostoks N, Kudrna D, Kilian A, Han F, Chen J, Druka A, Steffenson B, Kleinhofs A. The barley stem rust-resistance gene Rpg1 is a novel disease-resistance gene with homology to receptor kinases. Proc Natl Acad Sci U S A. 2002; 99:9328–33. [PubMed: 12077318]

Burdman S, Shen Y, Lee SW, Xue Q, Ronald P. axH/RaxR: A two-component regulatory system in Xanthomonas oryzae pv.oryzae required for AvrXa21 activity. Mol Plant-Micr Interact. 2004; 17:602–612.

Carter BA, Reeder R, Mgenzi SR, Kinyua ZM, Mbaka JN, Doyle K, Nakato V, Mwangi M, Beed F, Aritua V, Lewis Ivey ML, Miller SA, Smith JJ. Identification of *Xanthomonas vasicola* (formerly *X. campestris pv. musacearum*), causative organism of banana Xanthomonas wilt, in Tanzania, Kenya and Burundi. New Dis Rep. 2009; 19:25.

Choi SH, Lee SW, Han SS, Lee DK, Noh TH. Identification of Durable resistance genes against *Xanthomonas oryzae* pv. *oryzae* in Korea. Korean J Breeding. 2003; 35:283–88.

Demont M, Stein AJ. Global value of GM rice: a review of expected agronomic and consumer benefits. Nature Biotechnol. 2013; 5:426–36.

Dardick C, Ronald P. Plant and animal pathogen recognition receptors signal through non-RD kinases. PLoS Patho. 2006; 2:e2.

da Silva FG, Shen Y, Dardick C, Burdman S, Yadav RC, de Leon AL, Ronald PC. Bacterial genes involved in type I secretion and sulfation are required to elicit the riceXa21-mediated innate immune response. Mol Plant-Micr Interact. 2004; 6:593–601.

Dodds PN, Rathjen JP. Plant immunity: towards an integrated view of plant–pathogen interactions. Nature Rev Genet. 2010; 11:539–548. [PubMed: 20585331]

FAOSTAT Agriculture Data. 2011. www.fao.org/ag

Gawel NJ, Jarret RL. A modified CTAB DNA extraction procedure for *Musa* and Ipomoea. Plant Mol Biol Rep. 1991; 9:262–266.

Han SW, Lee SW, Bahar O, Schwessinger B, Robinson MR, Shaw JB, Madsen JA, Brodbelt JS, Ronald PC. Tyrosine sulfation in a Gram-negative bacterium. Nature Communi. 2012; 3:1153.

Hood EE, Gelvin SB, Melchers LS, Hoekema A. New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res. 1993; 2:208–218.

Ikeda R, Khush GS, Tabien RE, Ogawa T. A new resistance gene to bacterial blight derived from *O. longistaminata*. Japanese J Breeding. 1990; 40:280–281.

Jones, DR. History of Banana Breeding. Diseases of Banana, Abaca and Ensete. CABI Publishing; Wallingford, UK: 2000. p. 544

Kagezi GH, Kangire A, Tushemereirwe W, Bagamba F, Kikulwe E, Muhangi J, Gold CS, Ragama P. Field assessment of banana bacterial wilt incidence in Uganda. Afri Crop Sci J. 2006; 14:83–91.

Kishimoto K, Kouzai Y, Kaku H, Shibuya N, Minami E, Nishizawa Y. Perception of the chitin oligosaccharides contributes to disease resistance to blast fungus *Magnaporthe oryzae* in rice. Plant J. 2010; 2:343–354. [PubMed: 21070413]

Kumar N, Krishnamoorthy V, Nalina L, Soorianathasundharam K. A new factor for estimating total leaf area in banana. Infomusa. 2002; 11:42–43.

Lacombe S, Rougon-Cardoso A, Sherwood E, Peeters N, Dahlbeck D, Van E, Peter H, Smoker M, Rallapalli G, Thomma BPHJ, Staskawicz B, Jones JDG, Zipfel C. Interfamily transfer of a plant pattern-recognition receptor confers broad-spectrum bacterial resistance. Nature Biotechnol. 2010; 4:365–369. [PubMed: 20231819]

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigna PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompsom JD, Gibson TJ, Higgins DG. Clustalwand clustalX version 2. Bioinformatics. 2007; 23:2947–2948. [PubMed: 17846036]

Lee JS, Krause R, Schreiber J, Mollenkopf HJ, Kowall J, Stein R, Jeon BY, Kwak JY, Song MK, Patron JP, Jorg S, Roh K, Cho SN, Kaufmann SH. Mutation in the transcriptional regulator PhoP contributes to avirulence of Mycobacterium tuberculosis H37Ra strain. Cell Host Micr. 2008; 2:97–103.

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Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-DDCt method. Methods. 2001; 25:402–408. [PubMed: 11846609]

Mendes BMJ, Cardoso SC, Boscariol-Camargo RL, Cruz RB, Mourão Filho FAA, Bergamin Filho A. Reduction in susceptibility to *Xanthomonas axonopodis* pv. *citri* in transgenic *Citrus sinensis* expressing the rice *Xa21* gene. Plant Pathol. 2010; 59:68–75.

Minitab 16 Statistical software. Computer software. State College, PA: Minitab, Inc; 2012. www.minitab.com

Namukwaya B, Tripathi L, Tripathi JN, Arinaitwe G, Mukasa SB, Tushemereirwe WK. Transgenic banana expressing *Pflp* gene confers enhanced resistance to Xanthomonas Wilt Disease. Transgenic Res. 2012; 12:855–865. [PubMed: 22101927]

Ndungo V, Eden-Green S, Blomme G, Crozier J, Smith J. Presence of banana Xanthomonas wilt (*Xanthomonas campestris* pv. *musacearum*) in the Democratic Republic of Congo (DRC). Plant Pathol. 2006; 55:294.

Park CJ, Bart R, Chern M, Canlas PE, Bai W, Ronald PC. Overexpression of the endoplasmic reticulum chaperone BiP3 regulates XA21-mediated innate immunity in rice. PLoS ONE. 2010; 5:e9262. [PubMed: 20174657]

Qaim M, Kouser S. Genetically modified crops and food security. PLoS ONE. 2013; 8:e64879. [PubMed: 23755155]

Reeder R, Opolot O, Muhinyuza J, Aritua A, Crozier J, Smith J. Presence of banana bacterial wilt (*Xanthomonas campestris* pv. *musacearum*) in Rwanda. New Dis Rep. 2007; 14:52.

Ronald PC, Albano B, Tabien R, Abenes L, Wu K, McCouch S, Tanksley SD. Genetic and physical analysis of the rice bacterial blight disease resistance locus, *Xa21*. Mol Gen Genet. 1992; 236:113–120. [PubMed: 1362973]

Shen Y, Sharma P, Silva FG, Ronald P. The *Xanthomonas oryzae* pv. *oryzae* raxP and raxQ genes encode an ATP sulfurylase and APS kinase that are required for AvrXa21 avirulence activity. Mol Microbiol. 2002; 44:37–48. [PubMed: 11967067]

Song WY, Wang GL, Chen LL, Kim HS, Pi LY, Holsten T, Gardner J, Wang B, Zhai WX, Zhu LH, Ronald P. A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. Science. 1995; 270:1804–1806. [PubMed: 8525370]

Studholme DJ, Wasukira A, Paszkiewicz K, Aritua V, Thwaites R, Smith J, Grant M. Draft genome sequences of *Xanthomonas sacchari* and two banana-associated Xanthomonads reveal insights into the *Xanthomonas* Group 1 clade. Genes. 2011; 2:1050–1065. [PubMed: 24710305]

Tai TH, Dahlbeck D, Clark ET, Gajiwala P, Pasion R, Whalen MC, Stall RE, Staskawicz BJ. Expression of the *Bs2* pepper gene confers resistance to bacterial spot disease in tomato. Proc Natl Acad Sci USA. 1999; 96:14153–14158. [PubMed: 10570214]

Thilmony RL, Chen Z, Bressan RA, Martin GB. Expression of the tomato *Pto* gene in tobacco enhances resistance to *Pseudomonas syringae* pv. *tabaci* expressing *avrPto*. Plant Cell. 1995; 7:1529–1536. [PubMed: 12242354]

Tripathi J, Muwonge A, Tripathi L. Highly efficient regeneration and transformation protocol for plantain cv. 'Gonja Manjaya' (*Musa* spp. AAB) using embryogenic cell suspension. In Vitro Cell & Dev Biol. 2012; 48:216–224.

Tripathi L, Mwaka H, Tripathi JN, Tushemereirwe WK. Expression of Sweet pepper *Hrap* gene in banana enhances resistance to *Xanthomonas campestris* pv. *musacearum*. Mol Plant Pathol. 2010; 10:1–11.

Tripathi L, Mwangi M, Abele S, Aritua V, Tushemereirwe WK, Bandyopadhyay R. A threat to banana production in east and central Africa. Plant Dis. 2009; 93:440–451.

Tripathi L, Odipio J, Tripathi JN, Tusiime G. A rapid technique for screening banana cultivars for resistance to Xanthomonas wilt. Europ J Plant Pathol. 2008; 121:9–19.

Tripathi L, Tripathi JN, Tushemereirwe WK, Bandyopadhyay RB. Development of a semi-selective medium for isolation of *Xanthomonas campestris* pv. *musacearum* from banana plants. Europ J Plant Pathol. 2007; 117:177–186.

Tripathi L, Tripathi JN, Tushemereirwe WK, Arinaitwe G, Kiggundu A. Transgenic bananas with enhanced resistance against Xanthomonas wilt disease. Acta Hort. 2013; 974:81–90.

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Tushemereirwe WK, Kangire A, Ssekiwoko F. First report of *Xanthomonas campestris* pv. *musacearum* on banana in Uganda. Plant Pathol. 2004; 53:802.

Wang GL, Song WY, Ruan DL, Sideris S, Ronald PC. The cloned gene *Xa21* confers resistance to multiple *Xanthomonas oryzae* pv. *oryzae* isolates in transgenic plants. Mol Plant-Micr Interact. 1996; 9:850–855.

Yirgou D, Bradbury JF. Bacterial wilt of *Ensete* (*Ensete ventricosum*) incited by *Xanthomonas musacearum* sp. nov. Phytopathol. 1968; 58:111–112.

Yirgou D, Bradbury JF. A note on wilt of banana caused by the enset wilt organism *Xanthomonas musacearum*. East Afri Agric For J. 1974; 40:111–114.

Zhao B, Lin X, Poland J, Trick H, Leach J, Hulbet S. A maize resistance gene functions against bacterial streak disease in rice. Proc Natl Acad Sci USA. 2005; 43:15383–15388. [PubMed: 16230639]

Zhu S, Li Y, Vossen JH, Visser RGF, Jacobsen E. Functional stacking of three resistance genes against *Phytophthora infestans* in potato. Transgenic Res. 2012; 21:89–99. [PubMed: 21479829]

Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JDG, Felix G, Boller T. Bacterial disease resistance in *Arabidopsis* through flagellin perception. Nature. 2004; 428:764–767. [PubMed: 15085136]

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Figure 1.

Regeneration of transgenic banana plants. a) Co-cultivation of embryogenic cells and *Agrobacterium* in liquid callus induction medium, b) Embryo maturation, c) Germination on selective medium supplemented with hygromycin, d) Putative transgenic shoots regenerated on selective medium, e) Transgenic shoots in proliferation media and f) Transgenic plants transferred to soil in small plastic cups in glass house.

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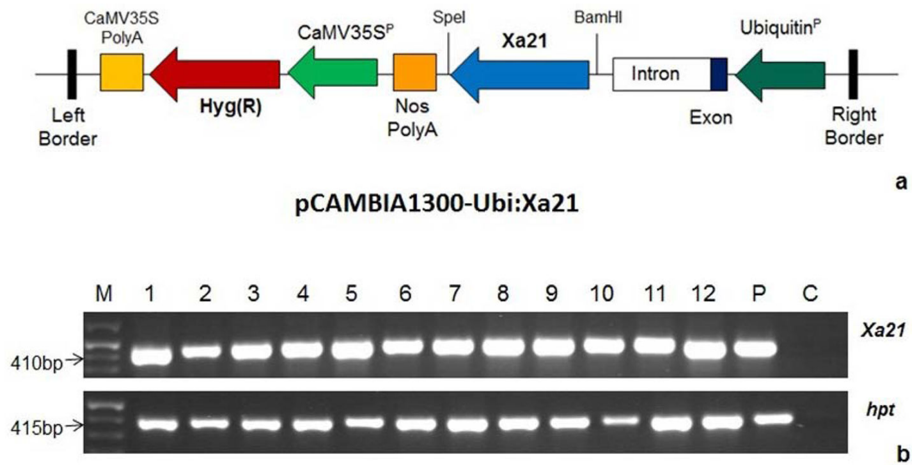

Figure 2.

a). Schematic representation of the T-DNA region of construct pCAMBIA1300-Ubi:Xa21 used for plant transformation. b) Representative picture showing PCR analysis of genomic DNA from transgenic banana lines and the non-transgenic plant using *Xa21* specific primers and *hygromycin phosphotransferase (hpt)* specific primers. Amplified PCR product designations were shown on the right and product sizes were shown on the left. M - molecular weight marker, 1–12- transgenic plants, P - plasmid DNA, C - non-transgenic control plant.

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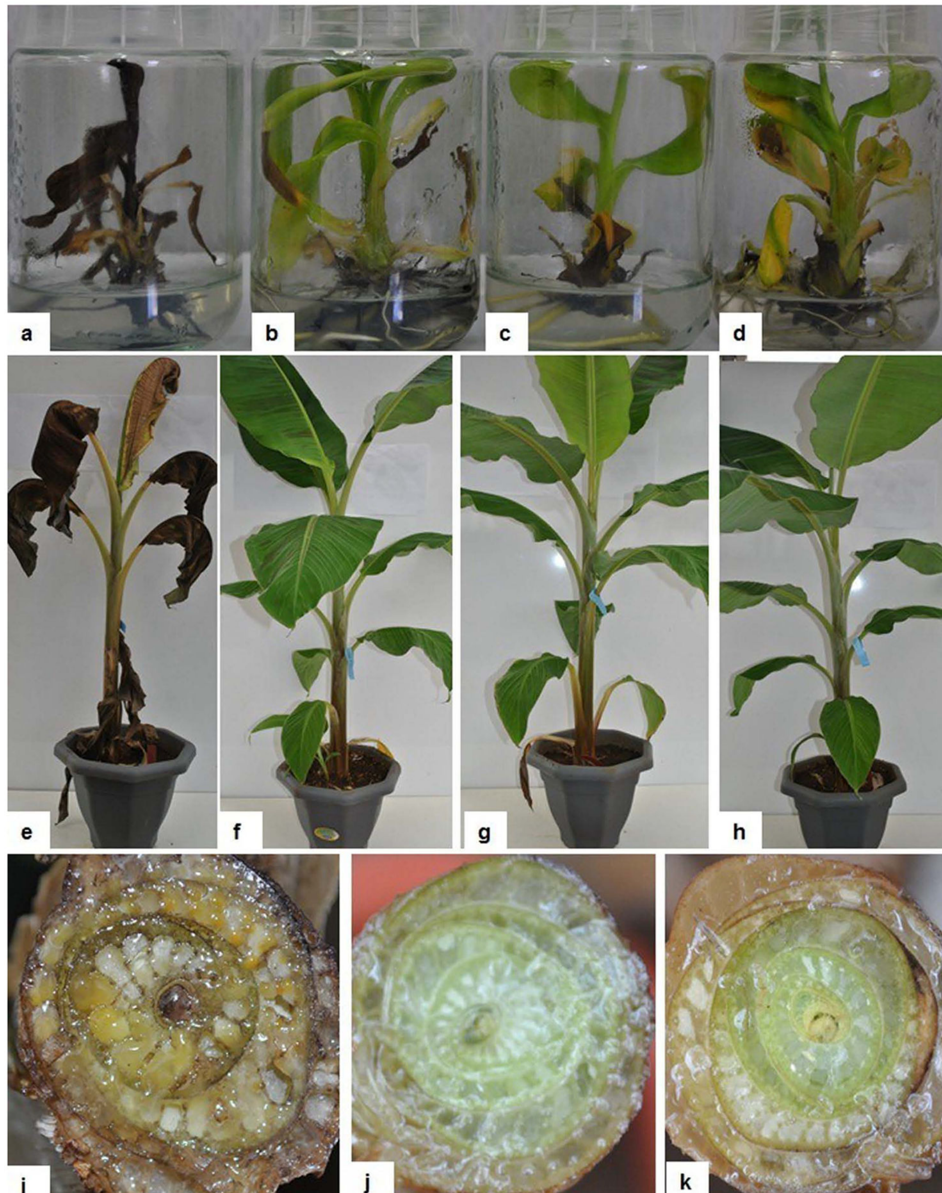

Figure 3.
Representative picture showing enhanced resistance of transgenic banana lines to *Xanthomonas campestris* pv. *musacearum*. Evaluation of transgenic lines using *in vitro* banana plantlets, a) Inoculated non-transgenic control plant, b–d) Inoculated transgenic plants (GX5, GX8 and GX26). Evaluation of transgenic banana plants in glasshouse, e) Non-transgenic control plant showing complete wilting after artificial inoculation, f-h) Transgenic plants (GX5, GX8 and GX26) showing no BXW symptoms after 60 days following inoculation of potted plants, i) Cut pseudostem of non-transgenic control plant showing yellow bacterial ooze of *Xanthomonas campestris* pv. *musacearum* after 60 days of artificial inoculation, j–k) Transgenic plants (GX8 and GX26) showing no bacterial ooze. All photographs were taken 60 days after artificial inoculation.

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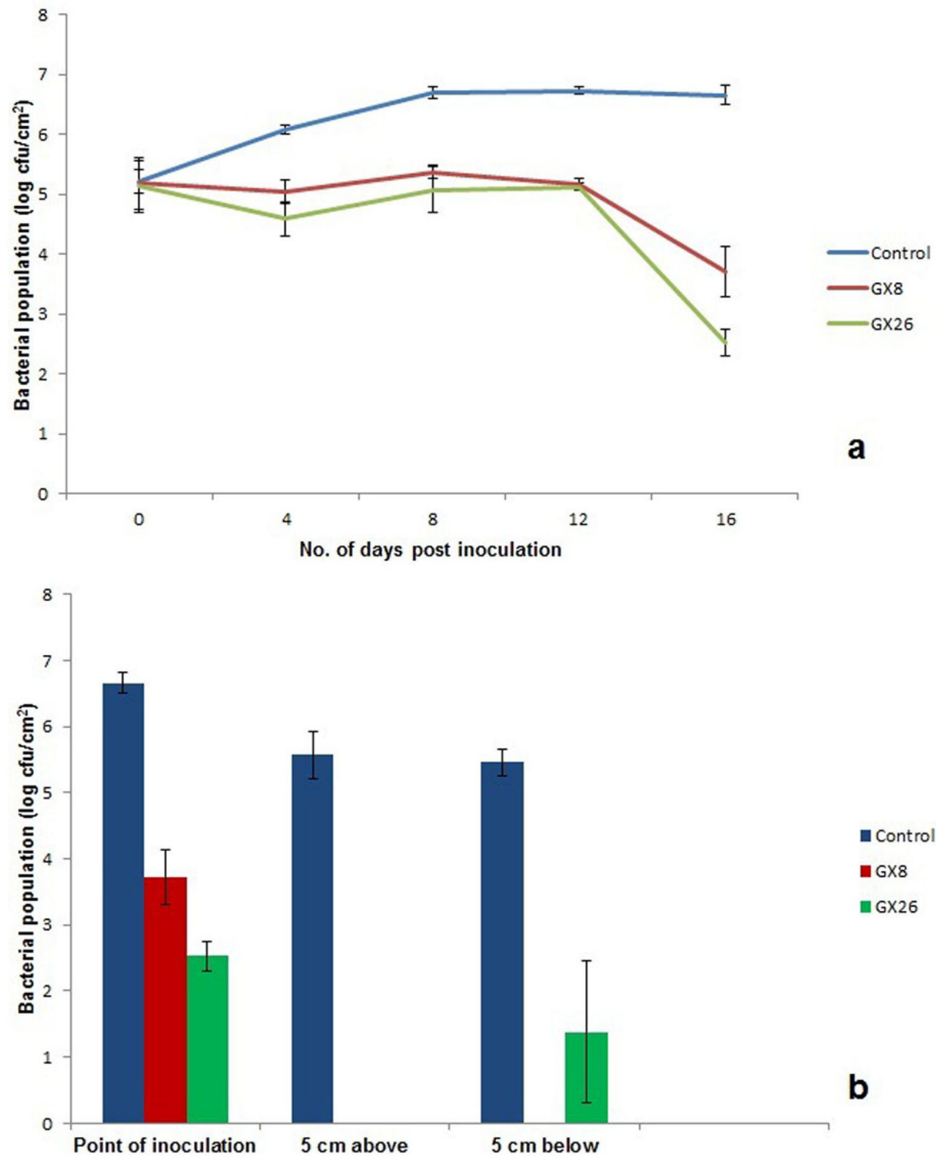

Figure 4.

Bacterial population analysis of transgenic plants (GX8 and GX26) and control non-transgenic plants. a) Bacterial population growth analysis for 16 dpi. b) Bacterial population at 16 dpi in different section (from inoculation point, 5 cm above and 5 cm below point of inoculation) of midrib of inoculated leaves. The data presented is the average of 6 samples at each time point. The error bar represents the standard deviation.

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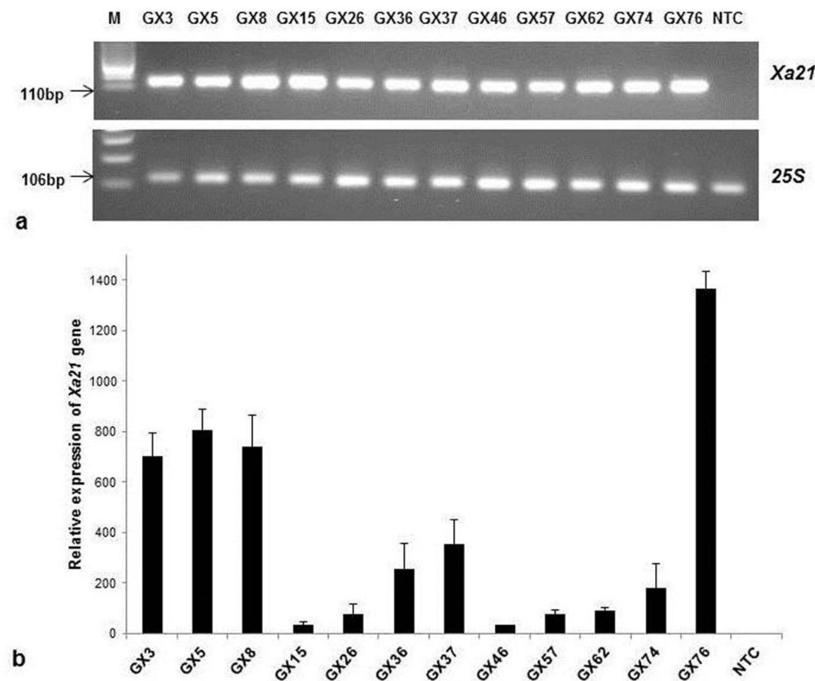

Figure 5.

RT-PCR and qRT-PCR of 12 transgenic lines (GX3, GX5, GX8, GX15, GX26, GX36, GX37, GX46, GX57, GX62, GX74 and GX76) and non-transgenic control plant. a) RT-PCR of transgenic banana lines using primers specific to *Xa21* and *25S* ribosomal gene. Amplified RT-PCR product designations were shown on the right and products sizes were shown on the left. M - molecular weight marker, NTC - non-transgenic control, b) Relative transcript levels of the *Xa*21 gene in transgenic lines in comparison to non-transgenic control plants. Expression of *Xa21* gene was normalized with banana *25S* ribosomal gene (internal control) and non-transgenic plant served as a calibrator. Relative expression was determined from replicate measurements in two independent biological replicates and three technical replicates. Data are mean ±SD.

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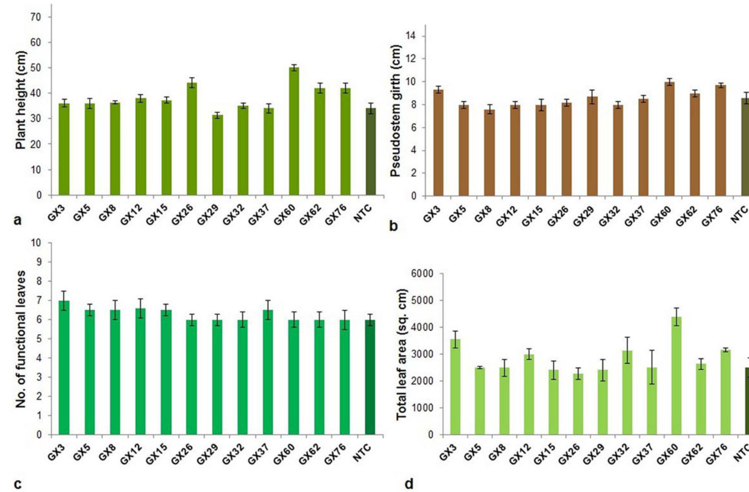

Figure 6.

Figure showing comparison of plant growth characteristics in transgenic and non-transgenic banana plants. a) Plant height, b) Pseudostem girth, c) Number of functional leaves and d) Total leaf area of transgenic lines in comparison to non-transgenic control plants. All values were mean ± standard deviation of three replicates of independent transgenic line and non-transgenic control plant. Plant growth characteristics were recorded at 90 days of planting in pots in glasshouse.

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Table 1

Evaluation of transgenic lines for enhanced resistance to *Xanthomonas campestris* pv. *musacearum* using rapid bioassay.

Transgenic lines	Mean no. of days for appearance of first symptoms[1,2,3,4]	Mean no. of days for complete wilting[1,2,3,4]	Wilting Incidence[5]	Rating
GX3	NS	NCW	0	R
GX5	NS	NCW	0	R
GX8	NS	NCW	0	R
GX11	16 ± 2.9b	NCW	0	PR
GX12	14 ± 0.6c	NCW	0	PR
GX13	16 ±1.5b	NCW	0	PR
GX14	NS	NCW	0	R
GX15	NS	NCW	0	R
GX16	14+0.6 c	23 ± 2.1b	100	S
GX26	NS	NCW	0	R
GX29	13.7 ± 0.6c	23 ± 2.1b	100	S
GX35	14.7± 0.6c	NCW	0	R
GX36	22.3 ± 2.1a	29 ± 2.1a	100	S
GX37	18 ± 2.6b	24 ± 2.6b	100	S
GX39	13 ± 1.5c	NCW	0	PR
GX41	NS	NCW	0	R
GX46	NS	NCW	0	R
GX48	16 ± 1.5b	25 ± 0.57b	100	S
GX52	19.6 ± 0.6b	27 ± 1.2a	100	S
GX53	NS	NCW	0	R
GX57	18 ± 2.6b	21 ± 1.2c	100	S
GX62	22 ± 0.6a	28 ± 1.5a	100	S
GX65	NS	NCW	0	R
GX74	20 ± 0.6a	27 ± 0.6a	100	S
GX76	NS	NCW	0	R
Control non-transgenic	7.7 ± 0.6d	20 ± 0.6c	100	S

[1]Mean and standard deviation of three clonal replicates of independent transgenic lines were calculated.

[2]Significant differences ($P < 0.05$) in rate of symptoms development

[3]Disease symptoms were recorded up to 60 days post inoculation

[4]Means followed by same letter within a column showing no significant difference

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

Abbreviations: NS - no symptoms, NCW- no complete wilting, R - resistance, PR- partial resistance, S- susceptible

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Table 2

Evaluation of transgenic banana lines for enhanced resistance to *Xanthomonas campestris* pv. *musacearum* under glasshouse conditions.

Transgenic lines	Mean number of days for appearance of first symptoms[1,2,3,4]	Mean number of days for complete wilting[1,2,3,4]	% Resistance[5]	Rating
GX3	NS	NCW	100a	R
GX5	NS	NCW	100a	R
GX8	NS	NCW	100a	R
GX15	NS	NCW	100a	R
GX26	NS	NCW	100a	R
GX36	50 ± 3a	NCW	66.5 ± 4.2b	PR
GX37	22.3 ± 2.9b	45 ± 0.57a	0c	S
GX46	NS	NCW	100a	R
GX57	56 ± 2.6a	45 ± 0.55a	67 ± 9.4b	PR
GX62	22 ± 2b	41 ± 3.6a	0c	S
GX74	23± 4.3b	42.6 ± 2.5a	0c	S
GX76	NS	NCW	100a	R
Control non-transgenic	18 ±2c	39 ± 3.6b	0c	S

[1] Mean and standard deviation of three clonal replicates of independent transgenic lines were calculated.

[2] Disease symptoms were recorded up to 60 days post inoculation

[3] Significant differences ($P < 0.05$) in symptom development when comparing transgenic lines (GX) with non-transgenic control plants

[4] Means followed by the same letter within a column showing no significant difference

[5] Resistance % = (Reduction in wilting/Total number of leaves wilted in control plant) × 100, Where reduction in wilting = Total number of leaves in a plant - number of leaves wilted at 60 days post inoculation

Abbreviations: NS - no symptoms, NCW- no complete wilting, R - resistance, PR- partial resistance, S- susceptible