The antagonistic effect of *Banana bunchy top virus* multifunctional protein B4 against *Fusarium oxysporum*

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SUMMARY

The viral-induced banana bunchy top disease and the fungal-induced banana blight are two major causes of concern for industrial scale production of bananas. Banana blight is particularly troublesome, affecting ~80% of crops worldwide. Strict guidelines and protocols are in place in order to ameliorate the effects of this devastating disease, yet little success has been achieved. From the data presented here, we have found that *Banana bunchy top virus* (BBTV)-infected bananas are more resistant to *Fusarium oxysporum* f. sp. *cubense* (*Foc*). BBTV appears to be antagonistic towards *Foc*, thus improving the survivability of plants against blight. The BBTV suppressor of RNA silencing, namely protein B4, displays fungicidal properties *in vitro*. Furthermore, transgenic tomatoes expressing green fluorescent protein (GFP)-tagged protein B4 demonstrate enhanced resistance to *F. oxysporum* f. sp. *lycopersici* (*Fol*). Differential gene expression analysis indicates that increased numbers of photogenesis-related gene transcripts are present in dark-green leaves of B4-GFP-modified tomato plants relative to those found in WT plants. Conversely, the transcript abundance of immunity-related genes is substantially lower in transgenic tomatoes compared with WT plants, suggesting that plant defences may be influenced by protein B4. This viral–fungal interaction provides new insights into microbial community dynamics within a single host and has potential commercial value for the breeding of transgenic resistance to *Fusarium*-related blight/wilt.

Keywords: *Banana bunchy top virus*, *Fusarium* wilt, plant immunity, transgenic crops, viral–fungal interaction.

INTRODUCTION

Fusarium oxysporum is a common soil inhabitant and plant-pathogenic fungus. *Fusarium oxysporum* has a broad host range

and infects both monocotyledonous and dicotyledonous plants (Pietro *et al.*, 2003). Pathogenic and host-specific *F. oxysporum* lines cause severe diseases in economically important crops, such as banana, tomato, cucumber and melon (Pietro *et al.*, 2003; Gordon and Martyn, 1997). *Fusarium* wilts are characterized by the aggressive colonization of the vascular system, preventing plants from acquiring, translocating and disseminating nutrients. Concerning *Fusarium* wilts, banana blight (i.e. Panama disease), which is caused by *F. oxysporum* f. sp. *cubense* (*Foc*) poses a formidable threat to current banana production (Butler, 2013; Kema and Weise, 2013). Indeed, the popular Cavendish cultivar is susceptible to the *Foc* Tropical Race 4 (*Foc*-TR4) strain which threatens ~80% of the world's banana crops (Butler, 2013).

The administration of fungicides is no longer a suitable treatment for *Foc*-infected bananas because of the relatively short half-life of fungicides within soils and the inadvertent selection of chemically resistant fungi (Ghag *et al.*, 2012). *Foc* chlamydospores can survive for decades in the soil, remaining dormant until the opportunity arises to infect new floral hosts. Therefore, the development of cultivars resistant to *Foc* infection is urgent (Ma, 2014; Swarupa *et al.*, 2014). It is difficult to generate resistant varieties through traditional hybridization breeding, restricted by the fact that the Cavendish cultivar is a seedless triploid. Thus, banana plantations remain at risk from Panama disease.

Although there are no documented natural sources of resistance against *Foc* in cultivated bananas, active rhizosphere microbes (such as *Trichoderma harzianum*, *Pseudomonas fluorescens* and *Actinomyces*) can colonize the roots of bananas and aid host defences against *Foc* (Saravanan *et al.*, 2003; Ting *et al.*, 2008; Weindling, 1932). *Bacillus* species from soils produce antifungal agents that inhibit the growth of several fungi, including *F. oxysporum* (reviewed by Lugtenberg and Kamilova, 2009). However, the biological dynamics of antagonistic microbial communities within the field remain poorly characterized. On observing almost no visible fungal lesions in plants co-infected with *Banana bunchy top virus* (BBTV), we investigated the likelihood of BBTV antagonization of *Foc* within a single floral host. BBTV replication is commonly confined to the vasculature of bananas and *Foc* also produces conidia and

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hyphae within the vascular tissues (Pietro *et al.*, 2003). First, we considered that the occupancy of these tissues by BBTV particles may physically hinder *Foc* and, second, that the BBTV-encoded protein B4 possesses antifungal properties, thereby hindering the upward infection of *Fusarium* from the roots of the plant.

BBTV is one representative species of *Babuvirus* (family *Nanoviridae*) and has at least six circular single-stranded DNA (ssDNA) components (Burns *et al.*, 1995). The DNA4 component encodes a movement protein (B4; consisting of 116 or 117 residues) that is responsible for the systemic transport of BBTV throughout the xylem and phloem of bananas (Wanitchakorn *et al.*, 2000). According to *in silico* analyses, we found that protein B4 has a single transmembrane (TM) motif rich in hydrophobic residues and flanked by positively charged residues (adjacent to the TM). These structural configurations imply that B4 probably exists as an amphiphilic molecule, which is a common feature of antimicrobial peptides (AMPs). In the present study, we overexpressed protein B4 in *Escherichia coli* and recorded the antifungal activity of purified protein B4 against *Foc*-TR4 *in vitro*. Furthermore, B4-green fluorescent protein (GFP)-transgenic tomatoes are highly resistant to *F. oxysporum* f. sp. *lycopersici* Snyder et Hansen (*Fol*). To our knowledge, this is the first account of virus-associated antifungal resistance in bananas and tomatoes towards *Fusarium*. BBTV appears to be a naturally occurring bio-control agent against *Foc*.

RESULTS

The antimicrobial potential of protein B4

After observing a lack of fungal lesions present on the leaves of bananas with viraemia, we investigated the putative relationship between BBTV and *Foc*. Protein B4 from the BBTV Hainan isolate was expressed in *E. coli* and purified to homogeneity through Ni²⁺ affinity chromatography. Protein B4 folds into a twist-wire-like tertiary structure similar to the topology of closed circular double-stranded DNA (dsDNA), visualized using negative staining and transmission electron microscopy (TEM) (Fig. 1A). The covalently closed circular dsDNAs (cccDNAs) generally form supercoiled structures. The topological number can be described by $L = T + W$ (L, linking number; T, twisting number; W, writhing number). Structurally similar to cccDNA, the supercoiled nature of protein B4 is present in various sizes, indicating that B4 can form multimers and therefore drive the generation of extra tensile force. The unique conformation of protein B4 may be influenced by the bundle formation of hydrophobic interfaces present in α -helices within the cytoplasmic region.

Purified B4 was tested for its ability to inhibit the growth of *Foc* hyphae using radial diffusion assays (RDAs; Fig. S1, see Supporting Information). After 2 days of incubation with protein B4, the zones

of inhibition of *Foc* were measured. Fluorescein isothiocyanate (FITC)-labelled B4 appeared to interact directly with *Foc* conidia, preventing ~90% of conidia from sporulating (Figs 1B and S1). The sporulation rates of *Foc* were unaffected by dodecyl- β -D-maltopyranoside (DDM) (0.02%) or FITC (0.5 mg/mL) alone in citrate buffer (Figs 1B and S1). FITC-labelled B4 displays fungistatic activity *in vitro*. The majority of *Foc* conidia fluoresced green after treatment with FITC-labelled B4 (visualized using confocal microscopy), whereas control FITC (absent protein B4) did not lead to fluorescent *Fusarium* conidia (Fig. 1C,D), indicating that protein B4 (pI ~ 10) probably targets the negatively charged cellular surface of *Foc* conidia in a manner similar to cationic AMPs.

BBTV-encoded protein B4 is a TM protein capable of locating in the outer membrane of banana embryo suspension cells (Wanitchakorn *et al.*, 2000). B4 contains an N-terminal ectodomain with 14 residues, a single TM domain of 23 residues that range from positions 15 to 37, and a cytoplasmic tail in the C-terminal region (Fig. 2). The TM domain is capable of forming a hydrophobic α -helix of ~3.5 nm in length and can span the membrane in a perpendicular manner. Furthermore, hydrophilic and cationic residues are rich in the bilateral region of the hydrophobic TM (Fig. 2). The discrete spatial separation of hydrophilic and hydrophobic amino acid patches is consistent with the typical features of many AMPs (Nguyen *et al.*, 2011; Zhuang *et al.*, 2015). Multiple amino acid sequence alignments of B4 isolates have revealed that the amphiphilic region (including the TM domain and the bilateral region) is highly conserved when compared with the C-terminal region (Fig. 2), suggesting that these structural features may contribute to BBTV pathogenesis.

BBTV-infected bananas are more resistant to *Foc*-TR4

To determine the performance of BBTV-infected banana (*Musa acuminata*) challenged with *Foc*, the plants were monitored daily for visible symptoms under glasshouse conditions. At 4 weeks (c. 30 days) post-inoculation, control bananas suffered from typical cracking in the pseudostems and yellowing discoloration from the margin of the leaf lamina, extending towards the midrib region in senile leaves (Fig. 3A). These symptoms were followed by discoloration (browning) of leaves, necrosis and, ultimately, detachment from the pseudostem (Fig. 3B). Conversely, BBTV-infected plants did not display the typical symptoms of blight disease (Fig. 3C). The infected petioles of banana plants were sampled and tested for *Foc*-TR4 abundance using a quantitative polymerase chain reaction (qPCR) approach. Fungal biomass in control bananas was six-fold higher in comparison with that in BBTV and *Foc* co-infected bananas (Fig. 3D). The location of protein B4 in BBTV-infected bananas corresponds directly to the spatial distribution of *Foc*, indicating a plausible antagonistic role for BBTV against *Foc*-TR4.

Fig. 1 Protein B4 demonstrates antifungal activity [against *Fusarium oxysporum* f. sp. *cubense*-Tropical Race 4 (*Foc*-TR4)] *in vitro*. (A) A twisted conformation of purified protein B4 in detergent solution was visualized using transmission electron microscopy (TEM) and negative staining. Scale bar represents 50 nm. (B) Fluorescein isothiocyanate (FITC)-labelled protein B4 inhibits the sporulation rate of *Fusarium* conidia. *x*-axis labels 1, 2, 3 and 4 represent serial dilutions (using citrate buffer) of 1 : 27, 1 : 9, 1 : 3 and 1 : 2, respectively. Initially, ~150 μM of B4 was present, together with 0.02% (v/v) dodecyl-β-D-maltopyranoside (DDM) and 0.05 mg/mL FITC. (C) FITC alone did not adhere to the cellular surface of *Foc*-TR4 conidia. (D) Patches of green fluorescence indicate that FITC-labelled protein B4 interacts directly with the cell surface of *Foc*-TR4 conidia. Scale bars in (C) and (D) represent 10 and 7.5 μm, respectively. All experiments were conducted on three independent occasions.

BBTV protein B4 is located in the outer membrane of vascular tissues

Prior to investigating the antifungal activity of protein B4 in transgenic tomatoes, the location of protein B4 within *Nicotiana benthamiana* tissues was studied via agrobacterial inoculation of chimeric PVX:B4-GFP. Visible green fluorescence under UV light depicted upward and systemic expansion of B4-GFP into mesophyll cells of developing leaves from the stem vasculature

(Fig. 4A). B4-GFP accumulated specifically in the outer membranes of pavement cells and vasculature in the leaves and stems, respectively (Fig. 4B). Consistent with B4-GFP expression patterns in *N. benthamiana*, the expression of B4-GFP driven by a constitutive 35S promoter was also confined to the outer membrane of the vasculature in tomatoes (Fig. 4C,D). The manner in which protein B4 is located and accumulates within these cells provides further evidence to support an antifungal role against vasculature-degrading *Fusarium*.

Fig. 2 Multiple sequence alignments of protein B4 (117 amino acids) from 13 *Banana bunchy top virus* (BBTV) isolates. The transmembrane (TM) motif is indicated by a black box. The positively charged amino acids in the bilateral region of TM are identified using black arrows. The highly conserved residues are represented by a consensus > 80. Amino acids are coloured as follows: HKR, cyan; DE, red; STNQ, maroon; AVLIM, pink; FYW, blue; PG, orange. Sequences were aligned in CLUSTALX 2.1 and edited in ESPrpt 3.0.

Protein B4 confers resistance against *Fol* in transgenic tomato

To assess the antifungal activity of protein B4 against *Fusarium in vivo*, transgenic tomatoes infected with *Fol* were employed, as they are amenable to the stable expression of B4 and its derivatives, whilst maintaining a regular physiological profile. A B4-GFP fused gene was incorporated successfully into the genome of tomatoes, convenient for the identification of B4 expression and location within different tissues (observed directly under UV light; Fig. S2, see Supporting Information). In contrast with chlorosis of older leaves and yellowing expansion to the nascent lamina in wild-type (WT) tomatoes under *Fol* infection (Fig. 5A,B), the B4-GFP-transgenic tomato leaves showed no visible lesions or yellowing patches on *Fol* infection at 14 or 20 days post-inoculation (dpi) [Figs 5, S2 and S3 (see Supporting Information)].

To ascertain the extent of *Fusarium* inhibition in transgenic tomato expressing B4-GFP, qPCR analysis of the *Fol tubulin* gene in DNA extracted from the leaves and stems of transgenic and control plants was performed at 20 dpi. The abundance of *Fol tubulin* genes was 10-fold less in the stem of transgenic B4-GFP tomatoes compared with the WT ($***P < 0.001$; *t*-test). The biomass of *Fol* in the leaves of transgenic tomatoes was five-fold less compared with that in WT tomatoes ($**P < 0.01$; *t*-test) (Fig. 5F). The presence/abundance of *Fol* from the same tissues amongst different transgenic lines (lines 1–4) was similar. In control plants, the biomass of *Fol* in the stem was five-fold higher than that found in the leaves, supporting the premise that vascular tissues are the primary location of *Fol* infection. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting confirmed that the synthesis (and accumulation) of B4-GFP protein was highest in the roots, less in the stems and least in the leaves (Fig. 5).

Fig. 3 Co-infection of *Banana bunchy top virus* (BBTV) and *Fusarium oxysporum* f. sp. *cubense*-Tropical Race 4 (*Foc*-TR4) in bananas. (A) The senile lamina turned yellow at 30 days post-inoculation (dpi) with *Foc*-TR4 in the absence of BBTV. (B) Browning and detachment of the leaves from the pseudostem in bananas at 45 dpi with *Foc*-TR4 (absence of BBTV). (C) BBTV-infected banana showed typical symptoms of banana bunchy top disease (BBTD), despite being co-infected with *Fusarium oxysporum*. (D) The biomass of *Foc*-TR4 was measured at 35 dpi using quantitative polymerase chain reaction (qPCR) and fungal-induced gene silencing of 28S RNA. Bars represent the average means of standard deviations (SDs) in three independent experiments. In contrast with the control, the reduced *Foc* biomass from the petiole of BBTV-infected bananas is statistically significant (** $P < 0.01$; Student's *t*-test). All scale bars represent 20 cm.

It should be noted that B4-GFP-transgenic tomatoes exhibited small lateral lobes and extensive protrusion surrounding the margin of leaves relative to WT plants (Fig. 5C). This indicates that leaf morphogenesis genes (including auxin-related) respond differently at the transcriptional level in transgenic tomatoes expressing B4-GFP.

Differential expression profiling of B4-GFP-transgenic tomato versus WT tomato

Differential gene expression (DGE) profiles of WT and B4-GFP transgenic tomato plants were analysed using next-generation sequencing (Illumina Platform) in order to interrogate the host response to *Fol* infection. The DGE method produced a 50-bp read of each transcript, corresponding to cDNA. The number of reads of a transcript tag was used as a measure of individual transcript abundance in a given sample. In total, 23 309 and 24 347 unique transcripts were detected in WT and B4-GFP-transgenic tomato, respectively. Of the 3386 differentially expressed genes (DEGs), 1807 genes were up-regulated and 1579 were down-regulated in transgenic plants in comparison with WT (Fig. 6A). Differential gene ontology (GO) was categorized through the *Kyoto Encyclopedia of Genes and Genomes* (KEGG) analysis. Transcriptional levels of metabolic, photosynthesis-related and antenna protein-encoding genes in B4-GFP-transgenic tomato were significantly

up-regulated within dark-green leaves (Fig. 6B). The increased expression of glycolysis- and photosynthesis-related genes (fructose-bisphosphate aldolase and chlorophyll *a/b*-binding protein) was confirmed by qPCR (Fig. S4, see Supporting Information). Notably, the elevated level of chlorophyll *a/b*-binding protein gene was approximately 50-fold higher in transgenic tomatoes relative to WT (Fig. S4). Transcript numbers associated with plant–pathogen interactions (i.e. immunity) were down-regulated in B4-expressing tomatoes in contrast with WT when exposed to *Fol* (Fig. 6B). Confirmation of immunity-related gene expression (such as WRKY13, respiratory burst oxidase; Fig. S5, see Supporting Information) in both WT and transgenic tomatoes was achieved using qPCR (adopting a threshold value of 1.5–2.5-fold).

DISCUSSION

Banana is a staple food for tens of millions of people and serves as a rich source of carbohydrates, fibre, vitamins and minerals, such as phosphorus, calcium and potassium. The global yield of bananas is threatened continually by many diseases, especially *Fusarium* wilt and banana bunchy top disease (BBTD). However, the clonal and seed-sterile nature of triploid bananas hampers the breeding of cultivars with desirable traits (Ghag *et al.*, 2012), which could help to improve banana production and harvesting.

Fig. 4 Cytoplasmic membrane protein B4 locates within the vasculature of *Nicotiana benthamiana*. (A) B4-GFP movement to mesophyll cells via vascular tissue after agroinfiltration of PVX:B4-GFP. (B) B4 is a cytoplasmic membrane protein and locates within the stem of *N. benthamiana*, indicated by the green fluorescence. Protein B4 is also expressed in the vasculature of stem (C) and root (D) in B4-GFP-transgenic tomatoes. Scale bars: 4 cm (A), 100 μ m (B, C) and 300 μ m (D).

Currently, many guidelines exist that attempt to prevent/control the spread of *Fusarium* wilt and BBTD, particularly from soil-dwelling species. The vascular tissues in bananas are a common habitat for both pathogens, namely *Foc* and BBTB. *Foc* is a hemibiotroph, whereas BBTB is wholly dependent on a floral host. These pathogens usually require different cellular niches within banana tissues. *Foc* absorbs nutrients from dying (necrotic) tissue, whereas BBTB requires living cells to sustain replication and other biological processes. By preferentially inhabiting the same host, BBTB and *Foc* are in direct competition with each other. In this study, we have observed antifungal activity of protein B4 encoded by BBTB. Protein B4 appears to be capable of suppressing the growth of hyphae and sporulation of *Foc*-TR4 conidia *in vitro* (Figs 1 and S1) and *in vivo* (Figs 3, 5, S2 and S3).

Resulting from the systemic expression of B4-GFP, the leaves of *N. benthamiana* gradually display green islands, crimpling and hypersensitive necrosis (Fig. S5). Likewise, the symptoms induced by PVX:B4-GFP in many leaves resemble those induced by PVX:B4 (Fig. S5D). These data indicate that protein B4 may be an effector of the hypersensitive necrosis reaction (HR) in tobacco plants, similar to other viral suppressor proteins, e.g. *Cauliflower mosaic virus* (CaMV) P6 and *Tomato bushy stunt virus* (TBSV) P19 (Angel and Schoelz, 2013; Cawly *et al.*, 2005). In general, BBTB-encoded B4 might activate effector-triggered immunity (ETI) in view of host plant immune surveillance (i.e. HR) in response to viral effectors (Pumplin and Voinnet, 2013). Fortunately, B4-GFP in transgenic tomato does not trigger an HR, perhaps because B4-GFP expression in transgenic tomatoes is reduced compared with its expression in *N. benthamiana*. In addition, the leaves of transgenic tomatoes expressing B4-GFP develop dark-green lamina, similar to the typical symptoms of BBTB-infected banana plants.

The versatile protein B4 not only acts as a candidate antifungal agent, but is also capable of inhibiting the systemic silencing of GFP in 16c (GFP-transgenic line) and functions as a strong viral suppressor of RNA silencing (VSR) (Niu *et al.*, 2009; Wicczorek and Obrepalska-Stepelowska, 2015), which is a counter-defence to host RNAi-mediated antiviral immunity (Ding, 2010; Ding and Voinnet, 2007). However, the Δ NTMB4 mutant without the N-terminal domain and TM motif abolishes the major suppression activity (Figs S5 and S6, see Supporting Information), whereas synthetic peptides corresponding to the N-terminal domain (ALTTERVKLFFEWFLFIGAIFIA; pI = 6.5, ~2.7 kDa in size and 70% hydrophobicity) and TM motif (ITILYILLALLFEVPKYIKEI; pI = 6.5, ~2.5 kDa in size and 67% hydrophobicity) (Fig. 2) do not display fungistatic activity. This suggests that the VSR and antifungal properties of protein B4 may be conferred by alternative functional domains. That said, the synthetic peptide LLALLFEVPKYIKEIVRYLVEYL (pI = 6.6, ~2.8 kDa in size and 61% hydrophobicity) shows some fungicidal potential (data not shown).

With respect to the lack of natural sources of resistance against *Foc* from cultivated bananas, the likely way forward for the development of *Fusarium*-resistant cultivars is via the incorporation of novel genes from other organisms using genetic engineering. Although animal apoptosis-related gene and floral defensin genes have been transferred previously into bananas to ameliorate *Fusarium*-related pathologies (Ghag *et al.*, 2012; Paul *et al.*, 2011), these transgenic bananas show some resistance to *Foc*, but not complete immunity. Recently, host-induced gene silencing (HIGS) has been developed as an effective strategy for the control of fungal disease (Koch *et al.*, 2013), thus presenting a potentially powerful tool that could assist in improving banana plant defences against *Foc* (Ghag *et al.*, 2014). The HIGS-based strategy for the control of fungal disease is entirely different to the anti-

Fig. 5 Protein B4 confers resistance to *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) in tomatoes. (A) and (B) depict the yellowing symptoms caused by *Fol* in wild-type (WT) tomato at 14 to 20 days post-inoculation (dpi). (C) and (D) depict B4-GFP-transgenic tomatoes with dark-green leaves and no lesions, indicating enhanced resistance to *Fol*. The leaflets display hypergenesis (denoted by red arrows). The morphologies of B4-GFP-transgenic and WT tomato leaves are demarcated with broken lines. The protrusion of the lobes is indicated by a yellow arrow. (E) The B4-GFP protein is most abundant in the roots of B4-GFP-transgenic tomato relative to the stem and leaves, as detected by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). B4G-R, B4G-S and B4G-L represent the root, stem and leaves, respectively. (F) The *Fol tubulin* gene is 10-fold less abundant in the stem of transgenic B4-GFP tomato compared with the WT ($***P < 0.001$; *t*-test). The biomass of *Fol* within the leaves of transgenic tomatoes is five-fold less than in the WT ($**P < 0.01$; *t*-test). The presence of *Fol* in the stem is significantly greater than in the leaves of transgenic tomatoes, and these trends are consistent amongst all transgenic lines (1–4) used. Data are presented as mean values $\pm$ standard deviation (SD) from three independent experiments. All scale bars represent 4 cm.

fungus properties of protein B4 presented here. Experimentally, B4-GFP-transgenic tomatoes display strong resistance to *Fol*. Our data suggest that protein B4 may have broad-spectrum anti-*Fusarium* properties. Certainly, we cannot rule out the possibility that BBTV infection boosts banana defences against *Foc*. Interestingly, immune-related gene transcripts in *Fol*-infected transgenic tomatoes were significantly reduced compared with those in the infected WT (Figs 6 and S4). Perhaps the suppression of plant defence genes is caused by protein B4 in order to facilitate or sustain BBTV infection. Protein B4 probably antagonizes the primary infection stage of *F. oxysporum* in the roots and impedes the penetration of the fungus across the epidermis into the root cortex, thus providing an instant defence mode. In other words, B4-transgenic tomatoes either threatened or invaded by *F. oxysporum* do not significantly activate ETI. Therefore, protein B4 not only attacks or limits the spread of fungal rivals within tissues, but manipulates host immune defences.

BBTV-infected banana plants usually display dwarfism, narrow/bunchy leaves on the shoots, do not blossom and are sterile. Although B4-GFP-transgenic tomatoes show slight alterations in

leaf morphology and the promotion of axillary leaflet outgrowths (Fig. 5C), their breeding and productivity seem to be unaffected (Fig. S2). Thus, the incorporation of the *B4* gene into the banana genome presents a possible alternative for the development of resistance to *Foc*-TR4.

EXPERIMENTAL PROCEDURES

Sequence alignments, expression and purification of protein B4

The amino acid sequences of protein B4 from different BBTV isolates were obtained from the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>). Multiple sequence alignments were performed using CLUSTALX (Larkin *et al.*, 2007) and MEGA5 (Tamura *et al.*, 2011), and edited further with ESPrpt 3.0 (Robert and Gouet, 2014). The *B4* gene was amplified via conventional PCR (Table S1, see Supporting Information) and subcloned into pET22b (Novagen, KGaA, Darmstadt, Germany) following the instructions provided. The overexpression of B4 was induced in *E. coli* Rosetta (DE3) by 0.2 mM isopropyl- β -D-thiogalactoside (IPTG) when the cell density reached an

Fig. 6 (A) Differentially expressed genes in wild-type (WT) and transgenic tomatoes. Blue dots indicate unchanged expression and red dots indicate significant up-regulation or down-regulation. (B) Differential expression profiles of photosynthesis-related and immunity-related genes. Photosynthesis-related genes are up-regulated at the transcriptional level. Differential gene analysis was carried out using the *Kyoto Encyclopedia of Genes and Genomes* (KEGG) (<http://www.genome.jp/kegg/>).

optical density at 600 nm (OD_{600}) of ~ 1.0 . Following growth for 4–6 h at 37 °C, *E. coli* cells were harvested and the bacterial pellets were suspended in TBS buffer [50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8.0, 100 mM NaCl, 5% glycerol]. After sonication on ice, the suspension was centrifuged for 10 min at 17 500 g (JA-25 rotor, Beckman, California, USA) to remove the cell debris. The supernatants were collected and centrifuged at 150 000 g for 1 h at 4 °C. The membrane fraction was solubilized in TBS [containing 0.4% (w/v) DDM, 10 mM imidazole] and incubated at 4 °C for 1 h. Following ultracentrifugation at 150 000 g for 30 min, the supernatant was collected and loaded onto an Ni^{2+} affinity resin (Merck, Darmstadt, Germany), and rinsed three times with 20 mM Tris-HCl, pH 8 (containing 150 mM NaCl, 60 mM imidazole, 0.01% DDM). The B4 protein was eluted from the affinity resin using 25 mM Tris-HCl, pH 8 (containing

150 mM NaCl, 250 mM imidazole, 0.02% DDM) and flash frozen in liquid nitrogen.

Correspondingly, the $\Delta TMB4$ mutant was amplified and cloned into the pET-22b vector (see Table S1). The recombinant plasmid was transformed into Rosetta (DE3), and the $\Delta TMB4$ protein was purified as described above.

Potato virus X (PVX) chimeras and agroinfiltration on *N. benthamiana*

B4 mutants and B4-GFP fused genes were all amplified through conventional or overlapping PCRs. The full-length B4, B4 mutants (such as ΔN , ΔTM , ΔNTM and $\Delta CB4$) and B4-GFP were amplified using the primers listed in Table S1 and subcloned into the linearized PVX vectors (pretreated

with the restriction enzymes *Sal*I and *Cla*I. The recombinant PVX vectors were transformed into *Agrobacterium* GV3101 via the standard freeze–thaw method. *Agrobacterium* GV3101 containing chimeric PVX was grown to an OD₆₀₀ of 1.5–2.0 at 28 °C overnight. After low-speed centrifugation, the bacterial pellets were resuspended in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer containing 0.01% (v/v) acetosyringone (AS) and incubated for 2–3 h at room temperature. The suspensions were used to inoculate the back blades of *N. benthamiana* or 16c (GFP-transgenic line) using syringes without needles in the conventional agroinfiltration method.

Western blotting analysis

Leaves infected with different PVX chimeras (at 10 dpi) and healthy leaves were collected from the respective plants. Each leaf was homogenized in a 3× volume of chilled protein extraction buffer [50 mM Tris-HCl, 100 mM NaCl, 1% (v/v) Tween-20, ethylenediaminetetraacetic acid (EDTA)-free proteinase inhibitor (Roche, Basel, Swiss) and 2% (v/v) mercaptoethanol]. Debris was removed by centrifugation at 15 000 g for 20 min at 4 °C. Protein samples were added in equal volume to loading buffer, heated at 98 °C for 5 min and subjected to SDS-PAGE. After electrophoresis, gels were either used for Western blotting analysis to detect GFP or stained with Coomassie brilliant blue. For the Western blots, proteins were transferred onto poly(vinylidene difluoride) (PVDF) membranes, blocked with milk powder in 20 mM Tris-HCl, pH 7.6 (150 mM NaCl and 0.1% Tween-20) for 1 h at 37 °C, and washed three times. Next, membranes were incubated with a 1 : 3.0 × 10⁻³ dilution of the primary polyclonal antibody against GFP (GenScript, Nanjing, China) or polyclonal rabbit antibody against polypeptide B4-C1 using a 1 : 5.0 × 10⁻³ dilution. The appropriate secondary antibody conjugated with alkaline phosphatase (Sigma, Santa Clara, CA, USA) was used at a concentration of 1 : 1.0 × 10⁻⁴. Bound antibody was detected with nitroblue tetrazolium/5-bromo-4-chloroindol-3-yl phosphate (NBT/BCIP) reagents.

TEM and preparation of FITC-labelled B4

First, approximately 10 µL of protein B4 solution were loaded onto Parafilm (Polysciences Inc, Pennsylvania, USA). Next, formvar membrane-coated copper grids were placed onto the B4 suspension for 5 min, covered in 2% phosphotungstic acid (PTA) for 2 min and dried at room temperature. Samples were observed using a Hitachi H-7650 transmission electron microscope (Hitachi, Tokyo, Japan).

Protein extracts were concentrated to about 5 mg/mL using a 10-kDa molecular weight cut-off concentrator (Millipore, Massachusetts, USA) in buffer containing 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5% glycerol and 0.02% DDM. The liquid was exchanged with carbonate buffer (0.15 M, pH 9.5 containing 0.05% DDM) using ultrafiltration; 20 µL of a 10 mg/mL FITC solution were added to the B4 solution. The FITC-labelled B4 solution was dialysed against 0.1 M citrate buffer, pH 4.6 (containing 0.02% DDM), for 6 h. The FITC-labelled B4 solution was harvested and stored at –80 °C. Concentrations of FITC-labelled B4 were estimated using spectroscopy.

Investigation of the antagonistic activity of protein B4 against *F. oxysporum*

Isolated protein B4 was tested for its potential antifungal activity using *Foc*-TR4 hyphae and RDAs. *Foc*-TR4 white colonies were grown on potato

dextrose agar (PDA) until they reached approximately 4 cm in diameter. The B4 suspension was diluted and various controls [buffer alone and detergent (DDM) alone] were added to existing wells in agar containing *Foc*-TR4 lawns. Plates were incubated at 30 °C for 2 days prior to analysis.

Ten microlitres of FITC-labelled B4 were added to 50 µL of *Foc*-TR4 conidia suspension (colony-forming units about 1.0 × 10⁵ mL⁻¹) containing 0.1 M citrate buffer (pH 4.6) and incubated at 30 °C for 30 min. Following incubation, the B4-treated conidia were rinsed three times in citrate buffer. Next, conidia were loaded onto glass slides and observed using confocal microscopy (Leica, Microsystems, Wetzlar, Germany). FITC-treated conidia (in the absence of protein B4) acted as a negative control. Three synthetic peptides (B4N 1–3) were also used to investigate potential antifungal activity: ALTTERVKLFFEWFLFIGAIFIA, ITILYILLALLFEVPKYIKEI and LLALLFEVPKYIKEIVRYLVEYL. The working concentration of each peptide was approximately 100 µM.

FITC-labelled (protein)B4-treated conidia were poured onto PDA and the sporulation rates of conidia were recorded in each treatment. In parallel, the FITC- and DDM-treated conidia served as controls. Following treatment, the conidia were harvested by low-speed centrifugation (800 g for 5 min) and resuspended in 50 µL of phosphate-buffered saline (PBS), pH 7. The suspension of treated conidia was serially diluted at 1 : 5, 1 : 5², 1 : 5³, 1 : 5⁴ and 1 : 5⁵. The diluted conidia were poured onto PDA plates and incubated at 30 °C for 2 days. The relative rate of germination was calculated using $N_g/N_{total} \times 100$ (N_g , number of germinating conidia; N_{total} , total number of conidia).

Plant expression vector construction and tomato transformation

Previously amplified BBTV-encoding B4 was cloned into the pCHF3GFP binary vector (as part of a constitutive expression cassette having a 35S promoter and *Nos* terminator). The newly constructed binary vectors were inserted into *Agrobacterium tumefaciens* strain EHA105 using the freeze–thaw method before being used to transform tomato callus. The transgenic tomatoes were identified/screened using Murashige–Skoog (MS) agar medium containing 50 µg/mL kanamycin.

qPCR and reverse transcription qPCR

Tissue samples (including stems and leaves) from bananas were collected and homogenized in liquid nitrogen. Nucleic acids were extracted using standard purification kits (Omega, Bio-Tek, Norcross, GA, USA). DNA/RNA concentration and purity were estimated using a Nanodrop D-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Only samples with an OD₂₆₀/OD₂₈₀ ratio within the range 1.8–2.0 were selected for qPCR. DNA or RNA integrity was confirmed by running samples on a 1.2% agarose gel or formaldehyde-denatured gel, respectively. cDNA was synthesized from mRNA using a standard kit (TIANGEN Biotech, Beijing, China), prior to reverse transcription qPCR analyses. qPCR experiments were conducted using a Mastercycler® ep realplex (Eppendorf, Jülich, Germany) in 20-µL reaction volumes. Each reaction contained 10 µL SYBR Green qPCR mix (TOYOBO, Osaka, Japan), 0.5 µL of forward and reverse primer (20 pmol/µL) and 20–45 ng DNA. Cycling conditions were as follows: an initial denaturation step for 2 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and a combined annealing/elongation step of

60 °C for 40 s. Negative controls (absent template DNA) were included on each plate in order to check for contamination. Samples were run in triplicate. The average *Ct* values were used for quantification. *Fusarium* DNA was determined via the $2^{-\Delta\Delta C_t}$ method by normalizing the amount of target DNA/transcript to the known copy number of *actin*. All primers used are listed in Table S2 (see Supporting Information).

DGE profiling analysis

At 60 dpi with *Fol*, the leaves from WT and transgenic tomatoes were harvested, and the total RNAs were isolated using a Plant RNA extraction kit (Omega). RNA quantity and purity were screened using a Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent Technologies, Santa Clara, CA, USA) with an RNA Integrity Number (RIN) > 7.0. The polyA tails of mRNA were isolated from approximately 10 µg of total RNA using poly-dT-modified magnetic beads (Thermo-Fisher, Massachusetts, USA). Following purification, the fragmentation of mRNA was achieved using divalent cations. The cleaved RNA fragments were incorporated into the cDNA library following standard guidelines for the Illumina RNA ligation method (Illumina, San Diego, CA, USA). Briefly, the fragmented RNA was dephosphorylated at the 3'-end using phosphatase and sequentially phosphorylated at the 5'-end by polynucleotide kinase (PNK). After treatment, the RNA was isolated using the RNeasy MinElute Kit (Qiagen, Inc, Chicago IL, USA) following the manufacturer's instructions. The purified RNA was ligated with a pre-adenylated 3' adaptor, which enables the subsequent ligation of the 5' adaptor. Based on the adaptor sequence, reverse transcription followed by PCR was used to create cDNA constructs. The average insert size for the paired-end libraries was 300 bp. Single-end sequencing was performed using an Illumina HiSeq2000/2500 (LC Sciences, Houston, TX, USA).

Raw data containing adaptor sequences, tags with low-quality sequences and unknown nucleotides N were removed. Clean reads were trimmed (36 nucleotides) and assessed for quality. Outputs from genetic libraries were interrogated for correlations. All clean tags were mapped to transcript sequences by bowtie, and only 1-bp mismatch was permitted. For monitoring the mapping circumstances on both strands, both the sense and antisense sequences were included in the data collection. The number of perfect clean reads corresponding to each gene was calculated and normalized to the number of reads per kilobase of exon model per million mapped reads (RPKM). Approximately 6.5 and 5.4 million reads were acquired for WT and transgenic tomatoes, respectively (Table S3, see Supporting Information). Based on the expression levels, the significant DEGs among samples were identified ($P < 0.05$). Clustering of DEGs was performed using the common Perl and R scripts. GO analysis was conducted for the functional classification of DEGs, and pathway identifications were drafted using KEGG.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Radial diffusion assays depicting B4 inhibition of *Fusarium oxysporum* f. sp. *cubense*-Tropical Race 4 (*Foc*-TR4) hyphae. (A) Antifungal, positive control; phosphate-buffered saline (PBS), negative control; 0.05% detergent dodecyl- β -D-maltopyranoside (DDM) alone did not affect hyphal growth. (B) B4 stock solutions containing about 150 μ M B4, 0.02% DDM, Tris-buffered saline (TBS) (pH 7.5) and serial dilutions (1/3, 1/9, 1/27) were tested in duplicate.

Fig. S2 B4-GFP-transgenic tomatoes have dark-green leaves and no lesions after inoculation of *Fusarium oxysporum* f. sp. *cubense* (*Foc*). The growth of transgenic tomato line 1 at 14, 20 and 35 days post-inoculation (dpi) is shown in (A), (B) and (C), respectively. (D) Transgenic tomato fruits. (E) Transgenic tomato fruits fluorescing under UV light.

Fig. S3 Wild-type (WT) tomato is susceptible to *Fusarium oxysporum* f. sp. *cubense* (*Foc*) infection. At 14 days post-inoculation (dpi), the leaves of WT tomato showed yellowing symptoms, the severity of which gradually increased, ultimately leading to withering and falling off (denoted by a red box or arrows). The development of typical symptoms caused by *Fusarium* wilt at 14, 20 and 35 dpi is shown in (A), (B) and (C), respectively.

Fig. S4 Relative expression/abundance of genes associated with pathogen–host interaction (A) and glycolysis/photosynthesis (B) in wild-type (WT) and transgenic tomatoes responding to *Fusarium* infection. Total RNA was extracted using plant RNA and DNA isolation kits (Omega, Bio-Tek, Norcross, GA, USA). The concentrations and purity of nucleic acids were estimated using a Nanodrop D-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Only samples with an optical density ratio at 260 nm/280 nm (OD_{260}/OD_{280}) from 1.8 to 2.0 were selected for quantitative polymerase chain reaction (qPCR). (A) WRKY13, one member of the WRKY transcription factor family; RBO, respiratory burst oxidase; Sgr, senescence-inducible chloroplast stage-green protein; DNAJ, DNAJ domain. (B) Genes associated with fructose metabolism and photosynthesis. FBA, fructose-bisphosphate aldolase; CAB, chlorophyll *a/b*-binding protein.

Fig. S5 Mottling and necrosis symptoms are induced by B4. (A, B) Green islands, mosaics and necrotic patch symptoms in *Nicotiana benthamiana* induced by PVX:B4. (C) Green fluorescent protein (GFP) expression in *N. benthamiana* by PVX:B4-GFP systemic infection under UV. (D) The overexpression of B4-GFP in developing leaves results in the production of necrosis, similar to that produced by B4 at 20 days post-inoculation (dpi).

Fig. S6 N-terminal and transmembrane (TM) domains of B4 are required for suppression of RNA silencing. (A) Diagram of B4 and its mutants. (B) Reversal of the green fluorescent protein (GFP) expression in developing leaves of 16c (GFP-transgenic line) at different degrees by intact B4 or its mutants with various domain deletions. (C) GFP expressions in developing leaves of 16c reversed by B4/its mutants were confirmed by western blotting analysis, and the identical loading of total protein within each lane (verified by the Rubisco large subunit).

Table S1 Primers used for the amplification of B4 and its mutants

Table S2 Primers used for quantitative polymerase chain reaction (qPCR)

Table S3 Differential gene expression parameters