Apoptosis-related genes confer resistance to Fusarium wilt in transgenic 'Lady Finger' bananas

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Summary

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cubense* (Foc), is one of the most devastating diseases of banana (*Musa* spp.). Apart from resistant cultivars, there are no effective control measures for the disease. We investigated whether the transgenic expression of apoptosis-inhibition related genes in banana could be used to confer disease resistance. Embryogenic cell suspensions of the banana cultivar, 'Lady Finger', were stably transformed with animal genes that negatively regulate apoptosis, namely *Bcl-xL*, *Ced-9* and *Bcl-2* 3' UTR, and independently transformed plant lines were regenerated for testing. Following a 12 week exposure to Foc race 1 in small-plant glasshouse bioassays, seven transgenic lines (2 x *Bcl-xL*, 3 x *Ced-9* and 2 x *Bcl-2* 3' UTR) showed significantly less internal and external disease symptoms than the wild-type susceptible 'Lady Finger' banana plants used as positive controls. Of these, one *Bcl-2* 3' UTR line showed resistance that was equivalent to that of wild-type Cavendish bananas that were included as resistant negative controls. Further, the resistance of this line continued for 23 weeks post-inoculation at which time the experiment was terminated. Using TUNEL assays, Foc race 1 was shown to induce apoptosis-like features in the roots of wild-type 'Lady Finger' plants consistent with a necrotrophic phase in the lifecycle of this pathogen. This was further supported by the observed reduction of these effects in the roots of the resistant *Bcl-2* 3' UTR transgenic line. This is the first report on the generation of transgenic banana plants with resistance to Fusarium wilt.

Introduction

Fusarium wilt, also known as Panama disease, has been and continues to be a major constraint and serious threat to banana (*Musa* spp.) production worldwide (Ploetz and Pegg, 2000). The disease is caused by the soil-borne fungal pathogen *Fusarium oxysporum* forma specialis (f. sp.) *cubense* (Foc) of which four physiologically distinct "races" (referred to as races 1-4) have been identified based on their variation in virulence to specific host cultivars. Races 2 and 3 are not considered economically important, as they do not infect commercially relevant banana cultivars. Foc race 1, however, infects commercially important cultivars such as Gros Michel (*Musa* spp. AAA group) and 'Lady Finger' (AAB). In the mid 1950's, Foc race 1 decimated the major export cultivar (Gros Michel) in South and Central America, essentially eliminating its use and leading to the adoption of the race 1-resistant Cavendish subgroup of cultivars (AAA) as the dominant export commodity. Foc race 4 infects all race 1-susceptible cultivars as well as the Cavendish cultivars and, until relatively recently, only affected bananas in subtropical climates (and was therefore designated subtropical race 4 (SR4)). More recently, a newly discovered Foc variant called tropical race 4 (TR4) has been identified which affects Cavendish cultivars, and other locally important types such as the plantains, growing in tropical regions. This variant is apparently spreading and has been responsible for significant plantation losses is Southeast Asia, particularly Malaysia, China, Philippines and Indonesia as well as northern Australia (Ploetz and Pegg, 2000; Ploetz, 2006).

There are currently no effective methods available for controlling Foc. Chemical control is ineffective, and Fusarium chlamydospores remain viable in the soil for several decades rendering infested ground unsuitable for growing susceptible banana cultivars. It is generally accepted that the only option for controlling the disease is through the use of genetically resistant cultivars generated by conventional breeding or genetic modification. Although sources of resistance to Foc have been identified in wild bananas (Ploetz, 2006), the exploitation of these "resistance genes" by conventional breeding has been hampered by the extremely low fertility of commercial banana cultivars, which essentially do no set seeds, thus precluding traditional breeding strategies. As such, genetic modification is generally regarded as the most viable strategy for developing bananas with enhanced agronomic traits, particularly with the recent availability of efficient and reliable banana transformation protocols (Becker *et al.*, 2000; Khanna *et al.*, 2004).

Different forms of programmed cell death (PCD), including apoptosis and autophagy, occur in plants during normal growth and development as well as in response to environmental stresses and pathogen attack (Greenberg, 1996; Lenz *et al.*, 2011). We have previously shown that the extensive cell death observed in banana cell suspensions exposed to *Agrobacterium tumefaciens* was accompanied by several features characteristic of apoptosis, including DNA laddering and fragmentation and the formation of apoptotic-like bodies (Khanna *et al*. 2007). Further, these cellular responses were shown to be inhibited in cells expressing the anti-apoptosis genes *Bcl-xL*, *Bcl-2* 3' UTR and *Ced-9*. The transgenic modification of pathways controlling PCD in plants has indicated that engineering resistance to biotic stresses in plants is a valid

strategy (Dickman *et al.*, 2001). Dickman *et al.* (2001) showed that the constitutive expression of various anti-apoptotic *Bcl-2* gene family members (including *Bcl-xL*, *Ced-9* and *Bcl-2* 3' UTR) in transgenic tobacco plants resulted in high levels of resistance to a broad range of necrotrophic fungi. Since *Fusarium oxysporum* is a necrotrophic fungus (Vajna, 1985; Trusov *et al.*, 2006) that kills host cells prior to infection through the predicted deployment of toxins and enzymes that induce cell death, we hypothesized that transgenic expression of anti-apoptosis genes would confer resistance to Foc. In this paper, we provide proof-of-principle that anti-apoptosis transgenes do confer resistance against Foc race 1 in banana.

Results

Generation and characterization of transgenic plants

Binary vectors were generated containing the anti-apoptosis genes *Bcl-xL*, *Ced-9* and *Bcl-2 3' UTR*, all under the control of the maize polyubiquitin (Ubi-1) constitutive promoter. Embryogenic cell suspensions (ECS) of the banana cultivar 'Lady Finger' were transformed with each of the constructs using an *Agrobacterium*-mediated transformation protocol from Khanna *et al.* (2004) and transgenic lines were regenerated. Thirty-one independently transformed lines (7 x *Bcl-xL*, 11 x *Ced-9*, 13 x *Bcl-2* 3' UTR) were identified based on PCR and Southern analysis and these were selected for further study.

To determine whether transgene expression resulted in any deleterious phenotypic effects, each of the transgenic lines was multiplied and 10 plants from each line were acclimatised and grown in the glasshouse for eight weeks. Plants from three

123 *Bcl-xL* lines, two *Ced-9* lines and five *Bcl-2* 3' UTR lines displayed a range of mild
124 phenotypic abnormalities which included stunting as well as altered leaf morphology
125 and phyllotaxy. In most cases, the abnormal phenotype was present in every replicate
126 of a given line. None of the cell control lines displayed abnormalities. Phenotypic
127 abnormalities have previously been reported in plants expressing high levels of anti-
128 apoptosis genes (Dickman *et al*. 2001); however, abnormal phenotypes, or 'off-types',
129 also occur naturally in non-transgenic tissue-cultured banana due to somaclonal
130 variations (Côte *et al.*, 1993; Israeli *et al.*, 1996; Reuveni *et al.*, 1996). It is not known
131 whether the abnormalities observed in some transgenic plants generated in this study
132 were the result of naturally occurring somaclonal variations or transgene expression or
133 both. However, the observation that (i) all but one of these abnormalities were off-
134 types commonly observed associated with somaclonal variation in tissue-cultured,
135 non-transgenic banana plants (Israeli *et al.*, 1991) and (ii) none of the abnormalities
136 were typical of those previously associated with anti-apoptosis transgene expression
137 (Dickman *et al*. 2001), suggested that they were unlikely to be transgene-related.

138 **Glasshouse trials**

139 Two independent "small-plant" bioassay glasshouse trials were conducted to assess
140 the transgenic banana lines for resistance to Foc race 1. The first trial (T1) included 15
141 independently transformed banana lines (6 x *Bcl-xL*, 5 x *Ced-9* and 4 x *Bcl-2* 3' UTR)
142 while the second trial (T2) included the most promising line/s from trial 1 plus an
143 additional 16 independently transformed banana lines (1 x *Bcl-xL*, 6 x *Ced-9* and 9 x
144 *Bcl-2* 3' UTR). For each of the trials, 10 plants of each transgenic line were inoculated

while 10 plants of both wild-type susceptible ‘Lady Finger’ and resistant Cavendish cv ''Grand Naine'' plants were included as controls. Two plants from each transgenic and wild-type line were also included as non-inoculated controls. Twelve weeks post-inoculation, the external (yellowing, wilting, stem-splitting) and internal symptoms (corm discoloration) of Fusarium wilt infection were assessed. In the second trial, a subset of plants was evaluated at 23 weeks post-inoculation to assess the potential for long term resistance to Fusarium wilt in the glasshouse.

In both trials, typical external disease symptoms first appeared on the susceptible ‘Lady Finger’ wild-type positive control plants within 3 to 5 weeks. At 12 weeks post-inoculation (Figure 1a), these plants showed mean yellowing and wilting scores of 2 and 2.2, respectively (on a scale of 1-5), and mean stem-splitting scores of 2.8 (on a scale of 1-3) in T1 and scores of 2.6, 2.9 and 2.6, respectively, in T2 (Table 1). When the internal symptoms were assessed, an average of 44.9% and 50.6% corm discoloration was observed in trials 1 and 2, respectively (Table 1 and Figure 2a). As expected, plants from the resistant ‘Grand Naine’ wild-type line treated with Foc race 1 displayed no symptoms of wilting and stem-splitting, with corm discoloration at 2.8% and 0.1% in trials 1 and 2, respectively (Table 1 and Figure 2a). For the ‘Grand Naine’ controls, the average wilting and stem-splitting ratings were significantly less than the ‘Lady Finger’ controls; however, the average yellowing ratings were not, suggesting this symptom was not a reliable disease indicator in small plant assays. None of the non-inoculated wild-type plants of either cultivar showed external or internal signs of infection (Figures 1 and 2b).

167 Seven transgenic lines (2 x *Bcl-xL* (15 and 30), 3 x *Ced-9* (1, 4 and 6) and 2 x *Bcl-*
168 *2* 3' UTR (6 and 17)) identified from both trials had significantly lower disease ratings
169 than the wild-type susceptible 'Lady Finger' control banana plants. For line *Bcl-xL*-15
170 and line *Ced-9*-6, only the stem-splitting rating and percentage corm discoloration
171 were significantly lower than the susceptible controls while for line *Bcl-xL*-30, the
172 wilting and stem-splitting ratings and percentage corm discoloration were significantly
173 lower (Table 1 and Figure 2). For two *Ced-9* lines (*Ced-9*-1 and *Ced-9*-4) and two *Bcl-2*
174 3' UTR lines (*Bcl-2* 3' UTR-6 and *Bcl-2* 3' UTR-17), all external symptom ratings and the
175 amount of vascular discoloration were significantly lower than the susceptible controls
176 (Table 1, Figures 1b,c and 2).
177 Since all of the external symptom ratings for *Ced-9*-4, *Bcl-2* 3' UTR-6 and 17 in
178 trial 2 were significantly lower than the susceptible controls, one replicate for each line
179 was not sacrificed for evaluation of internal symptoms at week 12, but was instead
180 kept in the glasshouse for an additional 11 weeks. Non-inoculated wild-type 'Lady
181 Finger' plants were also kept as controls. At 23 weeks post-inoculation, the external
182 and internal symptoms were assessed. The *Bcl-2* 3' UTR-17, *Ced-9*-4 and *Bcl-2* 3' UTR-
183 17 plants had increased external symptom ratings over that additional 11 weeks
184 period along with an increase in the amount of corm discoloration. In contrast, none of
185 the external symptom ratings of the *Bcl-2* 3' UTR-6 plant increased over that period
186 nor did the amount of vascular discoloration.

187 **Further characterisation of the most resistant plant lines**

188 To determine whether there was any correlation between the expression of the anti-
189 apoptosis transgenes and the level of disease resistance, the seven most promising
190 transgenic lines were analyzed by semi-quantitative RT-PCR and/or Western analysis.
191 Due to a lack of suitable antibodies against Ced-9, Western analysis could only be done
192 on the 2 x *Bcl-xL* (15 and 30) lines. For the 3 x *Ced-9* (1, 4 and 6) and 2 x *Bcl-2* 3' UTR (6
193 and 17) lines, RT-PCR was used to assess transcript levels. Both RT-PCR and Western
194 analyses revealed that higher transcript/protein levels were correlated with the
195 resistance phenotype. For example, the amounts of *Ced-9* and *Bcl-2* 3' UTR-specific
196 transcripts were found to be highest in the most resistant lines, *Ced-9*-1 and *Bcl-2*
197 3'UTR-6 (Figure 3a), respectively. Similarly, Western analysis revealed that higher
198 amounts of Bcl-xL were present in line *Bcl-xL*-30 than in line *Bcl-xL*-15 (Figure 3b), with
199 the former line also displaying a higher level of disease resistance. An unexpected
200 observation was the cross-reactivity of the anti-Bcl-xL antibodies with a lower
201 molecular weight protein present in all wild-type and transgenic lines tested.

202 **Apoptotic response of banana roots to Foc**

203 Foc is considered to be a necrotrophic pathogen. Since necrotrophic pathogen appear
204 to induce PCD during the course of infection (Dickman *et al.*, 2001), we examined
205 whether Foc could induce PCD-like features in banana roots and, if so, whether these
206 effects could be prevented/reduced in the resistant transgenic banana line *Bcl-2* 3'
207 UTR-6. The terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling
208 (TUNEL) assay is commonly used to detect two characteristic features of PCD
209 associated with apoptosis, namely DNA fragmentation and the formation of apoptotic

210 bodies, which occur from coalescence of specifically cleaved DNA. The assay relies on
211 the presence of specific nicks in the DNA which can be identified by terminal
212 deoxynucleotidyl transferase, an enzyme that catalyzes the addition of dUTPs that are
213 secondarily labelled with a marker (fluorescein).

214 Roots from three wild-type susceptible 'Lady Finger' plants and three wild-type
215 resistant 'Grand Naine' plants, as well as three clones of the transgenic banana line
216 *Bcl-2* 3' UTR-6, were initially incubated separately in a solution of sugar-free liquid
217 rooting medium without Foc as controls. Root tissue samples taken at 48 h were
218 analysed by TUNEL assays and all samples contained largely intact nuclei with very few,
219 if any, green (TUNEL-positive) apoptotic cells indicating that experimental conditions
220 were not causing any background apoptotic effects (Figure 4a(i), b(i), c(i)). In contrast,
221 DNase-treated roots included as positive controls displayed a relatively large number
222 of green, apoptotic cells (Figure 4a(iii), b(iii), c(iii)).

223 When roots from wild-type 'Lady Finger' banana plants were incubated with an
224 Foc spore suspension, TUNEL-positive nuclei were observed as early as 6 h post-
225 inoculation (Figure 4a(v)) with the incidence of TUNEL-positive cells increasing to a
226 maximum at 48 h (Figure 4a(vii), (ix), (xi))**.** At 48 h post-inoculation, a slight
227 necrosis/browning type cell death was observed in the root tissue, at which time
228 distorted nuclei and apoptotic-like bodies containing fragmented DNA were also
229 observed. In comparison, roots from the *Bcl-2* 3' UTR-transgenic plants exposed to Foc
230 contained very few TUNEL-positive nuclei, like the non-exposed controls, and these
231 were only detected after 12 and 24 h exposure to Foc (Figure 4b(v), (vii)). In addition,

232 the tissue browning phenotype previously noted in Foc-exposed wild-type plants was
233 not observed.

234 As a further control, the roots from the wild-type 'Grand Naine' plants, which
235 are known to show field resistance to Foc race 1, were exposed to an Foc race 1 spore
236 suspension. TUNEL assays (Figure 4c(vii)) revealed that only a small number of positive
237 cells, similar to non-exposed controls, were present in roots of the 'Grand Naine'
238 cultivar primarily after 24 h exposure. These results indicate that (i) DNA
239 fragmentation and cell browning observed in the wild-type 'Lady Finger' banana roots
240 following exposure to Foc is a consequence of PCD which, in the case of the *Bcl-2* 3'
241 UTR-transgenic plants, could be reduced via expression of anti-apoptosis genes and (ii)
242 the lack of PCD in banana root cells following exposure to Foc is correlated with a
243 resistance phenotype.

244 **Discussion**

245 The transgenic modification of pathways regulating PCD in plants is emerging as a
246 promising strategy for engineering broad-spectrum resistance to both biotic and
247 abiotic stresses in plants (Dickman *et al.*, 2001; Lincoln *et al.*, 2002; Li and Dickman,
248 2004). Such a strategy is particularly suited for generating disease resistant bananas as
249 there are limited options for genetic improvement of this crop. In this study, we have
250 provided proof-of-principle that anti-apoptosis genes can be used in banana to confer
251 resistance against Foc race 1. Of the 31 transgenic 'Lady Finger' banana lines
252 challenged with Foc race 1 in small-plant glasshouse bioassays, two *Bcl-xL*, three *Ced-9*
253 and two *Bcl-2* 3' UTR-transgenic lines exhibited significantly less external and internal

disease symptoms than wild-type susceptible 'Lady Finger' control plants after a 12 week exposure to the pathogen. Of these lines, the transgenic line *Bcl-2* 3' UTR-6 showed a level of resistance similar to the 'Grand Naine' cultivar. Importantly, further monitoring of the line *Bcl-2* 3' UTR-6 transgenic showed that the resistance continued for at least 5 months after inoculation.

Despite limited published evidence, Foc is considered to be a necrotrophic pathogen. Such pathogens, by definition, require dead cells for nutrition, growth and development. A characteristic feature associated with many necrotrophs is the appearance of host PCD during the course of infection. Dickman *et al*. (2001) reported apoptotic responses in plants infected with several different necrotrophic fungi, including *Sclerotinia sclerotiorum, Botrytis cinerea and Cercospora nicotianae*, and concluded that apoptosis-like PCD occurs in compatible plant-necrotrophic pathogen interactions. In this study, we used TUNEL assays to demonstrate that an apoptotic-like cell death is triggered in susceptible wild-type banana root cells as early as 6 hours post-exposure to a suspension of Foc spores. Further evidence suggesting that the observed DNA fragmentation was apoptotic in nature was provided from TUNEL assays on roots from a Foc race 1-resistant *Bcl-2* 3' UTR-transgenic line in which these effects were significantly attenuated or absent. These results provide further evidence that Foc race 1 is a necrotrophic pathogen or at least has a necrotrophic stage in its life cycle.

Little is known regarding the molecular mechanisms underlying Foc infection and PCD induction in banana. Toxins produced by many necrotrophic fungi, including oxalic acid from *Sclerotinia sclerotiorum*, AAL from *Alternaria alternata*, Fumonisin B1

277 from *F. verticillioides* and victorin from *Cochliobolus victoriae*, induce characteristic
278 hallmarks of apoptosis in plants (Abbas *et al.*, 1995; Navarre and Wolpert, 1999; Kim *et*
279 *al.*, 2008). Further, in *Fusarium* species such as *F. verticillioides* and *F. moniliforme,*
280 several secondary metabolites, including fumonisins, are produced during infections
281 and act as powerful elicitors of PCD (Wang *et al.*, 1996; Stone *et al.*, 2000). Based on
282 what is known about other *F. oxysporum* f. sp, Foc is predicted to produce mycotoxins
283 which may be involved in stimulating cell death in the host and facilitating fungal
284 growth. However, whether this and/or other mycotoxins are responsible for the PCD
285 observed in Foc-infected 'Lady Finger' banana cells is unknown.

286 The mechanism(s) by which anti-apoptosis genes confer protection against
287 necrotrophic fungi is still unclear. Cytological studies on tobacco transformed with *Bcl-*
288 *xL* and *Ced-9* revealed that these proteins localized to mitochondria and other
289 organelles including the vacuole and chloroplast (Chen and Dickman, 2004). Based on
290 these observations, it was suggested that the proteins might improve the overall
291 function of organelles by assisting in the generation of ATP in the mitochondrion or
292 photorespiration to prevent ROS production in the chloroplast under stress conditions
293 (Chen *et al.*, 2003; Chen and Dickman, 2004; Li and Dickman, 2004). By analogy, Bcl-xL
294 and/or Ced-9 may prevent cell death and enhance plant resistance characteristics by
295 contributing to the maintenance of organelle homeostasis (Qiao *et al.*, 2002). The
296 mechanism/s by which *Bcl-2* 3' UTR confers resistance to plant cells is also unknown.
297 However, the *Bcl-2* 3' UTR transcript is thought to interact with pro-apoptotic proteins
298 at the RNA level (Dickman *et al.*, 2001) to mediate their degradation via the
299 ubiquitin/proteasome pathway (Martin Dickman, unpublished).

The generation of 'Lady Finger' banana plants with resistance to Foc race 1 is significant considering the serious economic and social impact caused by Fusarium wilt and the fact that no sustainable control strategy currently exists for this disease. The glasshouse tested resistant lines described here are very promising, however, testing of the transgenic plants under field conditions will ultimately be required, preferably through at least two crop cycles as symptoms of Foc infection can sometimes take up to 12 months to appear. Although the concentration of Foc in naturally infested soils is typically unknown, it is likely that the Foc inoculum pressure in the field sites would be dramatically lower than that of the very high, confined inoculum load used in our small-plant glasshouse assays. As such, the number of transgenic plants resisting infection to Foc race 1 in the field might be considerably higher than that observed in glasshouse trials.

A further important next step will be to test the transgenic banana lines generated in this study for resistance to Foc race 4, and particularly Foc TR4 which is emerging as a major threat to the global banana industry (Ploetz, 2006; Buddenhagen, 2009; Dita *et al.*, 2010). Since the apoptosis-related genes used in this study have been previously shown to generate broad resistance to a diversity of necrotrophic pathogens in other plants, it is possible that the transgenic banana generated in this study will also show resistance to the necrotrophic fungal disease black Sigatoka, caused by *Mycosphaerella fijiensis*, which also threatens the international viability of the crop. Assessment of plants for developmental and agronomic qualities under field conditions is also required, as well as evaluation of any increased susceptibility to biotrophic banana pathogens such as viruses.

Experimental procedures

Transformation constructs

Binary vectors pPTN254, pPTN261, pPTN396 and pPTN290 containing the maize polyubiquitin-1 (Ubi-1) promoter controlling the expression of anti-apoptosis genes *Bcl-xL*, *Ced-9*, *Bcl-2* 3' UTR and the *UidA* reporter gene encoding β-glucuronidase (GUS), respectively, were as described previously (Khanna *et al.*, 2007). All genes were fully sequenced in their original vector prior to transformation to verify the presence and integrity of the coding sequences and the promoter/gene and gene/terminator borders.

Transformation of banana and molecular characterization of transgenic plants

Embryogenic cell suspensions (ECSs) of the banana cultivar 'Lady Finger' (*Musa* spp. AAB group) were initiated, maintained and transformed as described previously (Khanna *et al.*, 2004). The molecular analysis of transgenic plants was essentially done as previously described by Khanna *et al.* (2007). RNA was extracted from 50 mg of fresh leaf tissue using an RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions, and was treated with RNase-free DNase (Promega, Madison, WI, USA). RT-PCR was done using a Titan One Tube RT-PCR Kit (Roche Diagnostics Australia Pty Ltd, Castle Hill, NSW, Australia), with each reaction containing 10 ng DNase-treated total RNA and the appropriate gene-specific primers. For Western analysis, crude plant protein extract (20 μg) from 100 mg of leaf tissue was separated on a 10% denaturing SDS polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was incubated with rabbit monoclonal anti-Bcl-xL (BL804)

345 (Bethyl Laboratories, Inc., Montgomery, TX, USA) primary antibody (diluted 1/1000 in
346 blocking solution) for 16 h at 4°C. Following a subsequent incubation in HRP-
347 conjugated secondary antibody in blocking solution for 1 h at 4°C, the SuperSignal
348 West Femto (Pierce Biotechnology, Inc., Rockford, IL, USA) substrate was used for
349 signal detection at 4°C until the desired intensity was achieved.

350 **Fungal cultures and inoculum preparation**

351 *Fusarium oxysporum* f. sp. *cubense* race 1 (VCG 0124/5) isolate was obtained from the
352 Department of Employment, Economic Development and Innovation (DEEDI)
353 herbarium, Indooroopilly Research Centre, Australia. The fungus was grown on 1/4
354 strength potato dextrose agar (PDA) supplemented with 50 mg/L streptomycin and
355 incubated for 3 to 5 days at 25°C. The inoculum used for small-plant bioassays was in
356 the form of Foc-colonized Japanese millet (*Echinochloa esculenta*) grain (Smith *et al.*,
357 2008). For *in vitro* root assays, spore suspensions were used as inoculum. These were
358 prepared by inoculating 100 mL of *Fusarium* liquid growth medium-A (without yeast
359 extract and supplemented with 20.6 g/L tri-sodium citrate) (Ahamed and Vermette,
360 2009) with two 7 mm diameter plugs of PDA-grown Foc. After shaking at 120 rpm for
361 three days at 25°C, the medium was filtered through three layers of cheesecloth,
362 centrifuged at 3850 g for 5 min and the pelleted conidia (a mixture of macroconidia
363 and microconidia) were resuspended in 5 mL distilled water. Spore concentration was
364 calculated using a haemocytometer before dilution to 10^5 spores/mL in sugar-free
365 liquid rooting medium M5 (Côte *et al.*, 1996).

366 **Small-plant bioassays**

Tissue-cultured transgenic plants of the 'Lady Finger' cultivar and wild-type banana plants of 'Lady Finger' and Cavendish cv 'Grand Naine' were acclimatised and transplanted into 100 mm diameter pots in soil (Searles Premium Potting Mix, Kilcoy, Australia) for 8 weeks in a glasshouse at 27°C under natural light conditions.

Selected transgenic banana lines and wild-type control plants were inoculated with Foc race 1 in two independent glasshouse trials. For logistical reasons, each independent transgenic line was assayed once in trial 1 or trial 2 with the exception for *Bcl-2* 3' UTR line 6 which was assayed in both trials. For each line tested in both trials, 10 replicate plants were inoculated and two plants were non-inoculated. Plants were inoculated with Foc race 1 using a modified version of the Foc small-plant bioassay protocol described by Smith *et al.* (2008). Briefly, 200 mm pots were half-filled with potting mix (7 mm gravel, sand, perlite, and vermiculite in a ratio of 2:2:1:1) and 20 mL of Foc-colonized millet grain (corresponding to an average of 2 x 10^8 Foc spores) was placed on the surface. Acclimatized banana plants were placed directly on the millet grain and the pots were filled with potting mixture. A tablespoon of Osmocote Plus slow-release fertilizer (Scotts Australia Pty Ltd, Baulkham Hills, NSW, Australia) was added to each pot. Plants were maintained in a greenhouse with an average temperature between 23-25°C during the summer and autumn months.

Assessment of disease symptoms

Twelve weeks after inoculation, external and internal symptoms of Fusarium wilt infection were assessed using a modified version of the method described by Smith *et al.* (2008). External symptoms were assessed by scoring each plant for the intensity of

389 the three main disease symptoms. Yellowing and wilting were assessed using a 5 point
390 scoring scale where 1 = healthy, no sign of symptoms (except natural degradation of
391 lower leaves), 2 = slight symptoms, mainly on lower leaves, 3 = advanced symptoms
392 (~50%), 4 = extensive symptoms (~90%) and 5 = entire plant affected (dead plant).
393 Splitting of the stem was assessed using a 3 point scoring scale where 1 = no sign of
394 splitting, 2 = slight splitting at the base of the plant and 3 = extensive splitting.
395 Following assessment of external symptoms, plants were removed from their pots, the
396 stem was cut longitudinally and digital images of the rhizome were taken using a
397 Canon Ixus 75 digital camera. The percentage of discoloration (browning) of the stellar
398 region of the corm was subsequently assessed from the digital images using a
399 MATLAB®-based program to ensure accuracy and reproducibility.

400 ***In vitro* root assays**

401 Tissue-cultured wild-type and transgenic 'Lady Finger' banana plants (5-7 cm tall)
402 growing on M5 medium were transferred into liquid M5 medium in a 50 mL tube and
403 roots were allowed to develop for two weeks. The plants were then transferred into
404 separate fresh 50 mL tubes containing 7 mL Foc spore suspension (10^5 spores/mL) and
405 incubated at 25°C with shaking (120 rpm) for 48 h. Negative control samples were
406 incubated in sugar-free M5 medium only. Root tip fragments (10 mm) for terminal
407 deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assays were
408 taken from each plant at 6, 12, 24 and 48 h post-inoculation, washed in 10 mM
409 phosphate-buffered saline (PBS), fixed for 24 h in 4% (v/v) paraformaldehyde in 10 mM
410 PBS (pH 7.4) at 4°C and stored at 4°C in 70% ethanol.

411 **TUNEL assays**

412 Root tip fragments were washed three times in fresh 10 mM PBS (pH 7.4) before being
413 assayed by TUNEL essentially as described by Khanna *et al.* (2007). Positive control
414 samples were made by subjecting root tissue to 1 unit of RNase-free DNase (Promega)
415 for 10 min at room temperature. Stained root fragments were squash-mounted onto
416 slides and examined under a BX41 microscope (Olympus Imaging Australia Pty Ltd,
417 Macquarie Park, NSW, Australia) equipped with U-MWIBA3 and U-MWIY2 filters
418 (Olympus), a DP71 microscope digital camera (Olympus) and the DP Manager software
419 (Olympus).

420 **Statistical analysis**

421 Correlations between data were established using a correlation matrix of all external
422 and internal symptoms. Due to the ordinal scale of measurements, a Spearman's Rho
423 correlation coefficient was calculated to determine the most appropriate choice for
424 further statistical analysis. Based on a high correlation coefficient, a Multivariate
425 Analysis of Variant (MANOVA) model was fitted using a general linear model (GLM)
426 approach. This model included all four symptoms (yellowing, wilting, stem-splitting
427 and vascular discoloration) concurrently. The LSD Post Hoc test was used to compare
428 each plant line against the wild-type 'Lady Finger' control line with statistical
429 significance reported at a level of $P < 0.05$, using a two-tailed test. Statistical analysis
430 was done using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

431 ## Acknowledgements

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Tables

Table 1 Assessment of external symptoms of Foc race 1 infection on *Bcl-xL*, *Ced-9* and *Bcl-2* 3' UTR transgenic lines

Line	External symptom scores			Internal symptoms
	Yellowing$^{\phi}$	Wilting$^{\phi}$	Stem-splitting$^{\lambda}$	% discoloration
GN (T1)	2.0	1.0*	1.0*	2.8*
LF (T1)	2.0	2.2	2.8	44.9
GN (T2)	1.9	1.0*	1.0*	0.1*
LF (T2)	2.6	2.9	2.6	50.6
Bcl-xL-15 (T1)	1.8	1.8	1.9*	26.8*
Bcl-xL-30 (T1)	2.0	1.3*	1.9*	12.6*
Ced-9-1 (T2)	1.2*	1.0*	1.0*	10.4*
Ced-9-4 (T2)	1.0*	1.0*	1.0*	23.6*
Ced-9-6 (T1)	2.0	2.0	2.0*	19.7*
Bcl-2 3' UTR-6 (T1)	1.0*	1.1*	1.4*	6.9*
Bcl-2 3' UTR-6 (T2)	1.0*	1.0*	1.0*	0.6*
Bcl-2 3' UTR-17 (T2)	1.2*	1.0*	1.5*	33.8*

Results are presented as score means based on 10 replicates.

$^{\phi}$ Based on 1-5 scale

$^{\lambda}$ Based on 1-3 scale

GN Untransformed wild-type 'Grand Naine' cultivar

LF Untransformed wild-type 'Lady Finger' cultivar

***** Significantly different from their respective susceptible LF control lines in either trial 1 (T1) or trial 2 (T2) with $P < 0.05$ based on LSD Post Hoc test.

Figure Legends

Figure 1 Representative photographs of the external symptoms of Fusarium wilt infection on susceptible, wild-type 'Lady Finger' (a) and resistant, transgenic 'Lady Finger' *Ced-9*-1 (b) and *Bcl-2* 3' UTR-6 (c) banana plants at 12 weeks post-inoculation. Inoculated (I) and non-inoculated (NI) plants are shown as indicated.

Figure 2 Assessment of the internal symptoms of Fusarium wilt in small-plant bioassays. (a) Quantitative assessment of the internal symptoms of Foc race 1 infection on resistant wild-type 'Grand Naine' (GN), susceptible wild-type 'Lady Finger' (LF) plants and selected transgenic lines harbouring transgenes *Bcl-xL*, *Ced-9* and *Bcl-2 3' UTR*. (b) Representative photographs of the internal symptoms of Fusarium wilt infection at 12 weeks post-inoculation. Susceptible wild-type 'Lady Finger' plants (1 and 2), resistant wild-type 'Grand Naine' plants (3 and 4), transgenic 'Lady Finger' *Ced-9*-6 (5 and 6) and *Bcl-2* 3' UTR-6 (7 and 8) are shown. Transgenic banana lines were inoculated with Foc race 1 and grown in the glasshouse. After 12 weeks, the plants were scored for the characteristic external and internal symptoms. Results are presented as score means ± standard errors based on 10 replicates. The significance of differences between mean values was evaluated by the LSD Post Hoc test. Differences were considered significant at $P < 0.05$. *Denotes the results are statistically significant from their respective susceptible LF line in either trial 1 (T1) or trial 2 (T2).

Figure 3 Transgene expression analysis in selected 'Lady Finger' transgenic banana lines. (a) RT-PCR transcript analysis of *Ced-9* lines 1, 4 and 6 and *Bcl-2* 3' UTR lines 6 and 17. (b) Western analysis of *Bcl*-xL transgenic lines 15 and 30 (Bcl-xL expected size is 28 kDa). P = Bcl-xL positive control consisting of 6His-Bcl-xL-6His protein expressed in *E. coli* (expected size is 31 kDa). WT = wild-type negative control.

Figure 4 Response of banana root cells to Foc race 1. Root tips from tissue-cultured susceptible wild-type 'Lady Finger' (a), resistant wild-type 'Grand Naine' (b) and transgenic 'Lady Finger' *Bcl-2* 3' UTR-6 were exposed to either liquid rooting media alone [Negative control (N) for 48 h] or rooting media containing 10^5 Foc spores/mL, and subjected to TUNEL assays and propidium iodide counter-staining at 6, 12, 24 and 48 h post-inoculation. Nucleic acid in TUNEL positive cells is selectively stained and fluoresces green, indicating the presence of apoptotic-like bodies, whereas all nucleic acid is counter-stained with propidium iodide and fluoresces red. Roots treated with DNase were used as a positive control (P). Magnification as indicated.

Figures

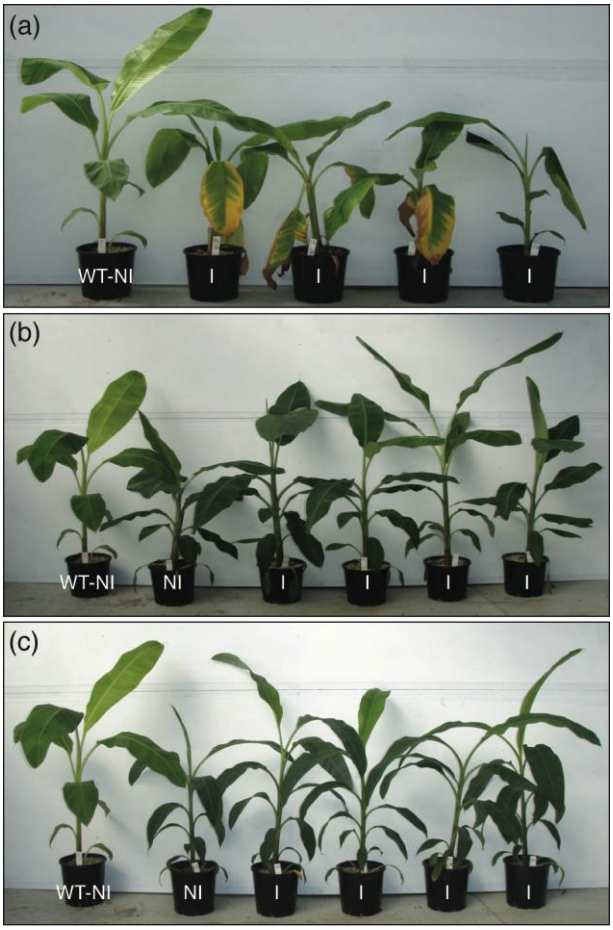


Figure 1

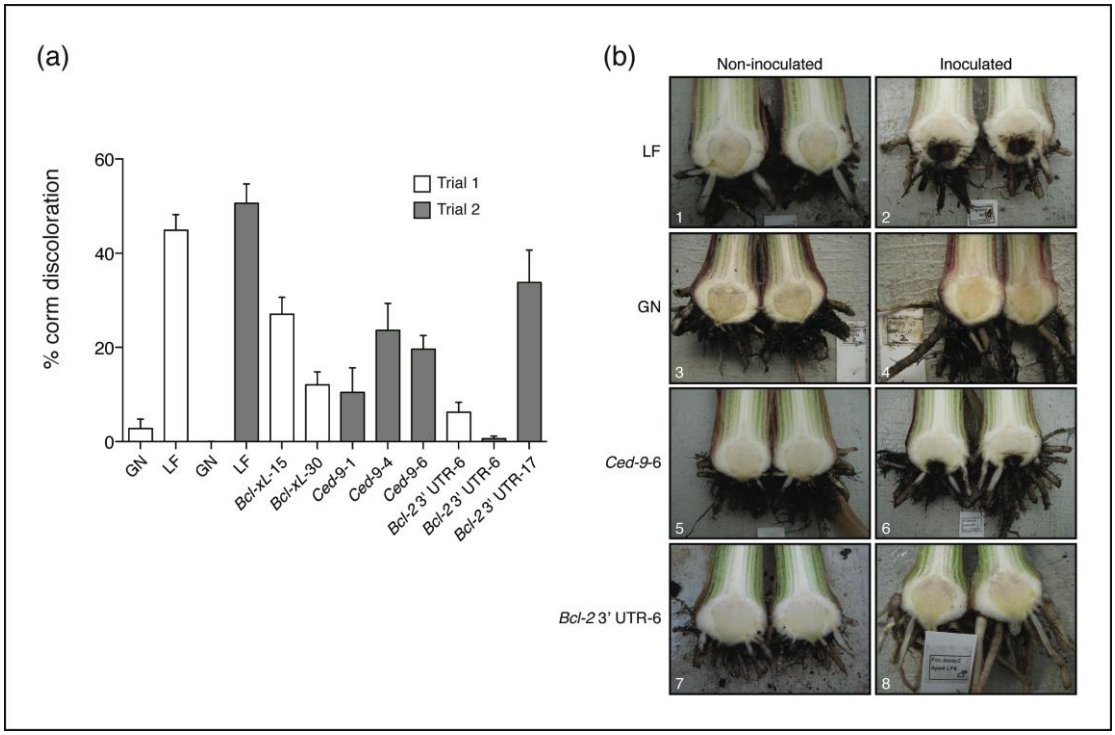

(a)
% corm discoloration
60
40
20
0
Trial 1
Trial 2
GN
LF
GN
LF
Bcl-xL-15
Bcl-xL-30
Ced-9-1
Ced-9-4
Ced-9-6
Bcl-2 3' UTR-6
Bcl-2 3' UTR-6
Bcl-2 3' UTR-17
(b)
Non-inoculated
Inoculated
LF
GN
Ced-9-6
Bcl-2 3' UTR-6
1
2
3
4
5
6
7
8

Figure 2

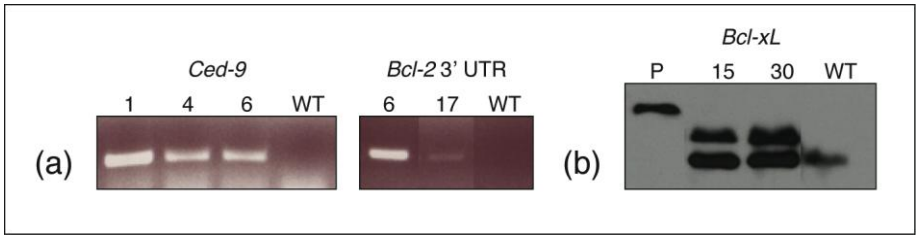
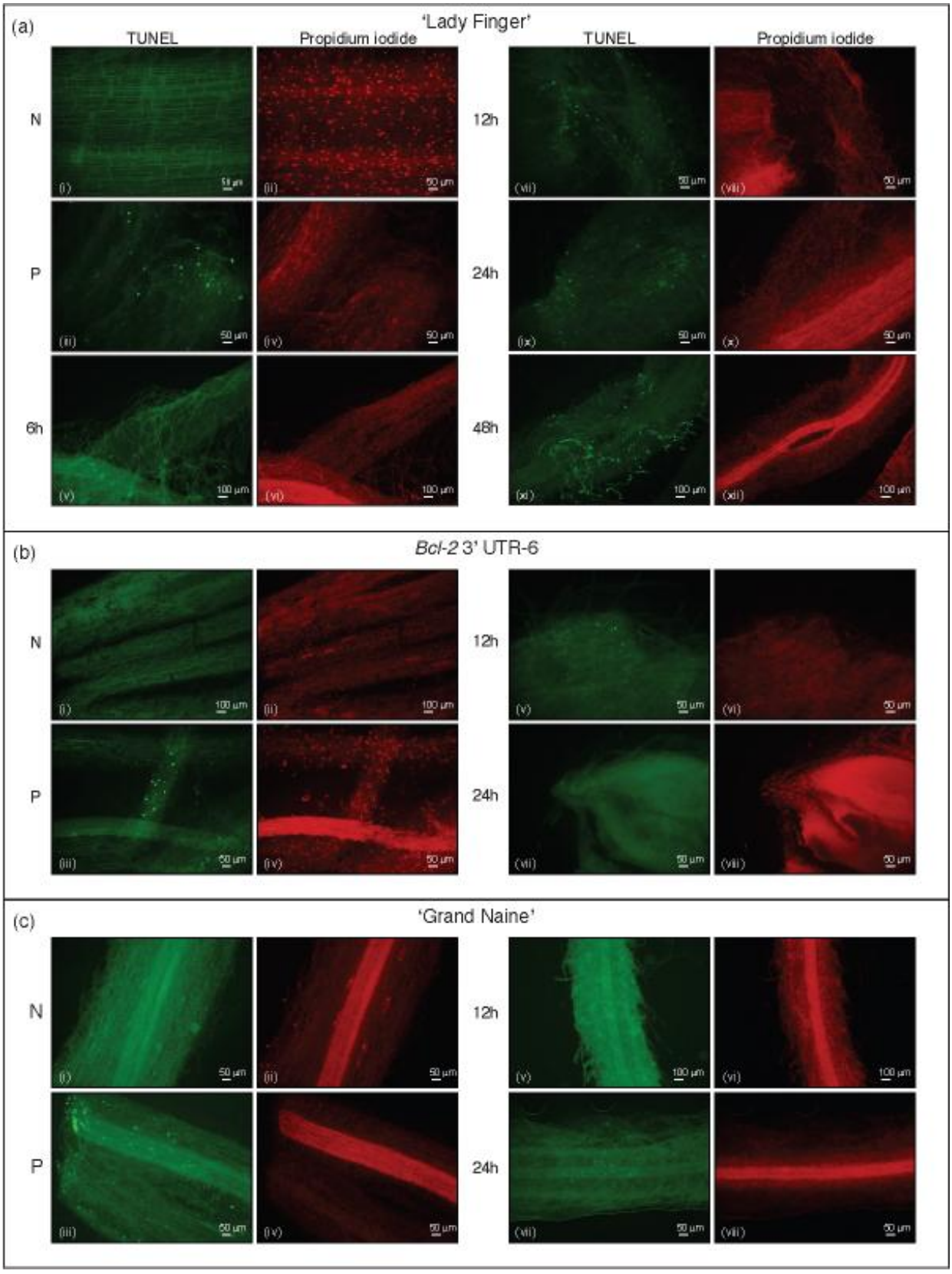


Figure 3



665

666 **Figure 4**