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RESEARCH ARTICLE

RNAi Mediated Silencing of *ftf1* Gene Enhances Resistance in Banana to *Fusarium* Wilt

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ABSTRACT

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*) is a devastating disease of banana. Race 1 of *Fusarium* wilt is a serious threat to *Musa* spp. cv. Rasthali (AAB, Silk subgroup), which is a choice variety traditionally grown in most of the South East Asian countries. The investigation was an attempt to study whether RNAi-mediated expression of small interfering RNAs (siRNAs) targeted against *ftf1* (*fusarium transcription factor1*) gene in transgenic banana could impart resistance against *Foc* race 1. Partial sequences comprising sense and antisense sequences of *ftf1* gene were assembled as siRNA construct in a binary vector background and transformed into embryogenic cell suspensions of banana cv. Rasthali by *Agrobacterium* mediated genetic transformation. Presence of RNAi construct in transgenic banana plants were confirmed by polymerase chain reaction assay. Forty- six transformed lines derived from RNAi-FTF1 were found to be free of external symptoms of *Foc* after 3 months of challenge inoculation. Seventeen transgenic lines continued to resist the *Foc* race 1 even after six months of post inoculation. The present study confirmed that the host induced RNAi-mediated gene silencing of a fungal gene could confer resistance against *Foc* in banana.

Keywords: *Banana Fusarium wilt; Fusarium oxysporum f. sp. cubense; RNA interference; fusarium transcription factor 1.*

INTRODUCTION

Banana (*Musa* spp.) is one of the most important fruit crops as well as staple food crop for millions of people in developing countries. Most of the edible bananas are triploid, which are formed by inter-specific hybridization between two diploid progenitors, namely *Musa acuminata* (AA genome) and *Musa balbisiana* (BB genome). A major threat to banana production is Panama disease or *Fusarium* wilt caused by a soil borne pathogen, *Fusarium oxysporum* f.sp. *cubense* (*Foc*). *Foc* invades banana root system, colonizes the corm tissue and clogs the water conducting vessels, thereby causing wilting of the aerial parts of infected banana plant. The infected plant shows typical discoloration of the corm and pseudostem and yellowing of the foliage followed by complete wilting (Dale *et al*., 2017).

The *Fusarium* wilt was first discovered in banana var. Sugar (Silk AAB) in Brisbane, Queensland, Australia during 1876 (Bancroft, 1876). The pathogen has been classified into four races, namely races 1, 2, 3 and 4. Race 1 which infected Gros Michel (AAA) earlier, now infects Silk and Pome (AAB) and Pisang Awak (ABB); race 2 infects cooking bananas namely Bluggoe (ABB) subgroup; race 3 infects ornamental namely *Heliconia* sp., (Tropical American banana relatives) and race 4 affects all cultivars in the Cavendish (AAA) subgroup in addition to the varieties susceptible to races 1 and 2 (Buddenhagen, 2009; Chittarath *et al.,* 2018; Zheng *et al.*, 2018; Thangavelu *et al.,* 2021). Rasthali cultivar of banana (AAB) is exceptionally valued for its unique fragrance, taste and keeping quality. Moreover, it is a premium variety cultivated across the globe, more extensively in the South East Asian countries. Unfortunately, Rasthali is highly susceptible to *Fusarium* wilt (race 1) resulting in heavy yield loss to the farmers. In India, besides Rasthali, the major varieties affected are Amrithapani, Karpuravalli, Monthan, Ney Poovan, and Virupakshi (Mustafa and Thangavelu, 2010).

Various agronomic practices like crop rotations, addition of organic amendments, flooding the field etc., have proved to be ineffective in controlling this disease (Bakry *et al.,* 2009). Once the fungal spores get established in field, they persist in soil for almost three decades. Moreover, the disease is difficult to manage using fungicides (Thangavelu and Mustafa, 2010). One of the sustainable solutions to manage this disease is to develop resistant cultivars by either conventional breeding or genetic engineering. Conventional breeding in banana faces challenges due to various sexual reproduction barriers such as high sterility, complex genetic background, polypoid and parthenogenesis (Ghag *et al.,* 2014), making it extremely difficult to develop new disease resistant bananas (Czislowski *et al.,* 2018). Therefore, host-induced gene silencing (HIGS) has increasingly become one of the most practical approaches for enhancing new disease resistant crop varieties (Qi *et al*., 2019; Zhang *et al*., 2016).

RNA interference (RNAi), also known as Host-Delivered RNAi (HD-RNAi) or Host Induced Gene Silencing (HIGS) or pathogen derived resistance, uses the host plant as a delivery system to cause gene silencing in the pathogen (Nunes and Dean, 2012; Ghag *et al.*, 2014; Mat Jalaluddin *et al.*, 2019). Plant siRNAs are 21 to 24 nucleotide long double-stranded RNAs with 3′ 2-nucleotide overhangs and generally carry a 2′-O methyl group on the 3′-terminal ribose (Yu *et al*., 2010). Double stranded RNAs, the progenitors of siRNAs are processed by dicer proteins in the nucleus and then the siRNAs are incorporated into the RNA-induced silencing complex (RISC) in the cytoplasm and thus results in sequence specific mRNA degradation (Bernstein *et al.,* 2001; Baulcombe, 2004; Ghildiyal and Zamore, 2009). Hemi biotrophic fungus like *Foc* have a short biotrophic phase followed by all out necrotrophy of the targeted plant. During the short biotrophic phase, the fungal haustoria penetrate the cell wall to derive nutrition, activating plant necrosis through a cascade of plant signaling system (Thaler *et al*., 2004). It is probably during this short biotrophic phase, exchange of cellular components occurs between host and the pathogen. The host derived siRNAs (targeted against vital fungal genes) finds entry into the fungal cytosol leading to host-induced post-transcriptional gene silencing of the target fungal genes.

Genetic engineering employing RNA interference technology has been successfully used to incorporate resistance against viruses (Shekhawat *et al*., 2012), nematodes (Huang *et al*., 2006), insect

pests (Mao *et al*., 2007) and fungal pathogens (Nowara *et al*., 2010; Panwar *et al*., 2013; Ghag *et al*., 2014 and Sunisha *et al*., 2020). The *ftf1* gene encoding a Zn (II) 2-Cys6 binuclear DNA-binding protein is up-regulated several folds in *Fusarium oxysporum* f. sp. *phaseoli* during its colonization in common bean (De Vega-Bartol *et al.,* 2011). This indicated that the down regulation of *ftf1* gene could potentially impart resistance to *Fusarium*. Besides *ftf1* gene, *velvet gene* was reported to play a key role in virulence of the pathogen. Down regulation of *ftf1* or *velvet* gene could confer resistance to *Fusarium* in banana (Ghag *et al*., 2014). Considering these leads, an attempt was made to develop *Foc* race 1 resistant transgenic banana in Rasthali by silencing *ftf1* (*fusarium transcription factor 1*) gene.

MATERIAL AND METHODS

Construction of RNAi vector

Partial sequence of *ftf1* gene (323 bp) was amplified from genomic DNA of *Foc* race 1 as described by Ghag *et al.* (2014) using gene specific primers designed to create a restriction site on either side of the amplified product (Table 1). The amplified products were cloned between *Xho*I and *Xba*I sites of pJET vector. The cloned sequences were confirmed by sequencing. The partial *ftf1* sequence (antisense sequence) was cloned between *Kpn*I and *Spe*I (New England Biolabs, UK) sites of an intermediate vector, pSTARLINGA followed by cloning of sense sequence between *BamH*I & *Asc*I sites. Finally, the RNAi cassette (*ftf1*sense-intron-*ftf1*antisense) (Figure. 1) was released from recombinant pSTARLINGA using *Not*I and subcloned in the *Not*I site of a binary vector (pART27). The vector was then mobilized into *Agrobacterium tumefaciens* strain, AGL1 by triparental mating. *Agrobacterium* strain, AGL1 harboring recombinant pART27 was used for genetic transformation of Rasthali.

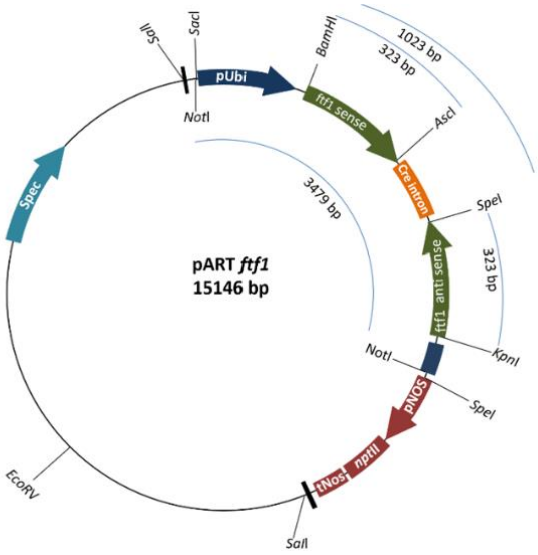


Figure 1. Physical map of pART27 harboring *ftf1* RNAi cassette

Generation and molecular analysis of transgenic banana plants

A single colony of AGL1 strain harboring *ftf1* gene was inoculated in YEMA (Yeast Extract Maltose Agar) liquid medium added with 100 mg L^{-1} spectinomycin and grown in an orbital shaker for 12 h at 28 °C. An overnight grown *Agrobacterium* culture (1 mL) was transferred to 50 mL of AB minimal medium augmented with 100 mg L^{-1} spectinomycin and incubated under the same conditions. The *Agrobacterium* culture was pelleted (3500 rpm for 10 min), suspended in AB induction medium with 100 mM acetosyringone (ACS), and allowed to grow at 28 °C (90 rpm for 3-4 h). Following induction, the bacterial

culture was centrifuged (3500 rpm for 10 min) and resuspended in the same medium with the addition of pluronic acid F68 (0.02 %) to a final OD_{600} nm of 0.5.

Name of gene	Forward (F) and reverse (R) primers (5' to3')	Amplicon size (bp)	PCR conditions

The transformation was performed using the protocol as suggested in technical guideline bulletin 8 of INIBAP (Stross *et al.,* 2003) with few modifications. About 0.5 ml of settled cell volume (SCV) of banana embryogenic cells were subjected for heat shock at 45 °C for 5 min followed by co-cultivation with *Agrobacterium* by centrifugal method. The co-cultivated cells were inoculated on co-cultivation medium (CCM) with 100 µM ACS and incubated for 3 days at 28 °C. The cells were washed in MA2 liquid medium and plated on semi-solid MA2 medium supplemented with cefotaxime (250 mg L^{-1}) for 2 weeks. Cells were transformed on MA3 medium (250 mg L^{-1} of cefotaxime and 100 mg L^{-1} of kanamycin) for maturation of somatic embryos. Matured embryos were then plated on MA4 medium supplemented with BAP 0.5 mg L^{-1} for shoot regeneration. The rooting was achieved on MA5 medium supplemented with 1 mg L^{-1} BAP and 2 mg L^{-1} IBA. For plantlet acclimatization, the rooted plants were hardened in greenhouse and maintained further for molecular analysis.

All the transgenic lines were further confirmed using primers specific for the *neomycin phosphotransferase II* gene and *ftf1* gene sequences (Table 1). Genomic DNA was isolated from leaves of transgenic and non-transformed banana plants using CTAB method (Dellaporta, 1983). The conditions for PCR were as follows: initial denaturation at 94 °C for 4 min; denaturation at 94 °C for 1 min; annealing at 57 °C for 45 s; extension at 72 °C for 1 min and 35 cycles were performed before the final extension at 72 °C for 10 min.

Table 1. List of primers used to amplify target genes

Partial *ftf1* sense and antisense	Antisense *ftf*1 F* **CTTC**GGTACCCTTGAAGCTAGTCTGCCCTTG Antisense *ftf*1 R* **GAAG**ACTAGTGAGGAAAAGGCGCAGTATCCA Sense *ftf*1 F** **TGTT**GGATCCCTTGAAGCTAGTCTGCCCTTG Sense *ftf*1 R** **AACA**GGCGCGCCGAGGAAAAGGCGCAGTATCCA	323	94°C for 5 min:1 cycle 94°C for 1 min 54 °C for 30sec 72°C for 30 sec (35 cycles) 72°C for 10 min: 1 cycle
ftf1	*ftf1* F AGTCTGCCCTTGTCACTCAA *ftf1* R AGGAAAGGCGCAGTATCCAG	287	94°C for 5 min:1 cycle 94°C for 1 min 54°C for 30sec 72°C for 30 sec (35 cycles) 72°C for 10 min: 1 cycle
nptII	*nptII* F CTGATGCTCTTCGTCCAGAT *nptII* R AGAGGCTATTCGGCTATGACT	440	94°C for 5 min:1 cycle 94°C for 1 min 63°C for 40sec 72°C for 45 sec (30 cycles) 72°C for 10 min: 1 cycle
SIX6B	SIX6B F ACGCTTCCCAATACCGTCTGT SIX6B R AAGTTGGTGAGTATCAATGC	220	94°C for 5 min:1 cycle 94°C for 1 min 63°C for 40sec 72°C for 45 sec (30 cycles) 72°C for 10 min: 1 cycle

*Restriction sites of *Kpn*I and *Spe*I; **Restriction sites of *BamH*I and *Asc*I

Screening of transformants for resistance to *Foc* race 1

A highly virulent fungal strain (*Foc* race 1), KP used in this study was maintained in PDA plates. For inoculum production, 500 mL Erlenmeyer flasks containing 250 mL of potato dextrose liquid medium were inoculated with 9 mm dia mycelial plugs of *Foc* race 1 excised from 5-day-old colonies grown on Potato Dextrose Agar (PDA) medium and incubated for 6 days (28±2 °C, 200 rpm). In addition, *Foc* mycelial discs were grown in 250 mL flasks containing 50 g of sterilized bajra kernels (28±2 °C, 10 days) as described by Dita *et al*. (2010).

Screening for resistance against *Fusarium* wilt of banana was conducted in transgenic events under contained conditions, using non-transgenic Rasthali control as susceptible and Grand naine as resistant cultivar. To evaluate fungal resistance, transgenic and control plants were inoculated with *Foc* race 1 strain, KP at five-leaf stage. Prior to inoculation, plants were removed from pots and the roots were trimmed to a length of ~40 cm. Inoculation was performed by root dipping (30 min, 10^6 conidia mL^{-1}) and inoculated plants were transferred to pots, watered with tap water daily and supplemented with 50 ml of Hoagland's solution at weekly interval. The disease severity of each plant was scored at 45 days and 3 months post inoculation. For resistance evaluation, the standard evaluation method described by Perez-Vicente *et al.* (2014) was adopted. The following scale was used to phenotype the plants based on the percentage of yellowing of leaves: 0 = no symptoms; 1 = initial yellowing, mainly in the lower leaves; 2 = yellowing of all the lower leaves with some discoloration of younger leaves; 3 = all leaves with intense yellowing or the plant died. Both transformed and non-transformed plants were scored based on their symptoms.

Detection of *Foc* in transgenic plants by PCR

Total genomic DNA was isolated from infected plants using the CTAB method as stated above. PCR analysis was done using *Foc* race 1 specific *SIX*6B gene primers to detect the presence of *Foc* (Table 1).

RESULTS AND DISCUSSION

The production and trade of banana is threatened by major fungal disease such as *Fusarium* wilt which can damage huge plantations in a short span of time. Banana cv. Rasthali (AAB) is considered to be highly susceptible to this disease and is threatened with extinction (Thangavelu *et al.,* 2001). In banana, a vegetatively propagated crop with triploid genome, recombination breeding has not been successful in

introducing resistance from tolerant genomes. RNAi phenomenon is being exploited in plants for resistance against biotic stresses that affects crop productivity significantly and this technology has been recognized as key biotechnology strategy in plant stress management. The expression of dsRNA, targeting essential genes of pathogens to enhance resistance against a number of pathogens have been reported in several crop plants including wheat, barley, banana and tomato (Nowara *et al.,* 2010; Ghag *et al*., 2012, 2014; Singh *et al.,* 2020). The regulatory approved Brazilian transgenic bean employing RNAi technology to combat bean golden mosaic virus is an example. Realizing the potential of the RNAi technology, this approach was used to engineer expression of dsRNA specific to *Fusarium transcription factor* 1 gene in banana plants with a view to inhibit the gene expression. The production of fungal gene-specific siRNAs in banana plants was expected to inhibit the invasion of host cells by the fungus and thereby reducing disease severity. An RNAi vector in the backbone of binary plasmid, pART27 was constructed and mobilized into *Agrobacterium tumefaciens* and used for genetic transformation of embryogenic cell suspension of Rasthali.

Generation and molecular analysis of transgenic banana plants expressing RNAi constructs

Co-cultivation of embryogenic cell suspension cultures of banana cv. Rasthali using *Agrobacterium tumefaciens* AGL1 strain harboring pART27-RNAi-FTF1 resulted in 444 plantlets (Figure 2a-e). Genomic DNA of all the putatively transformed plants and plants derived from non-transformed ECS were used as template in PCR using primers specific to the *neomycin phosphotransferase II* and *ftf1* gene. Amplification of internal sequences of 440 bp of *nptII* and 287 bp of *ftf1* gene was observed in 441 plants derived from RNAi-FTF1 construct, whereas the same was absent in non-transformed controls (Figure 3 a & b).

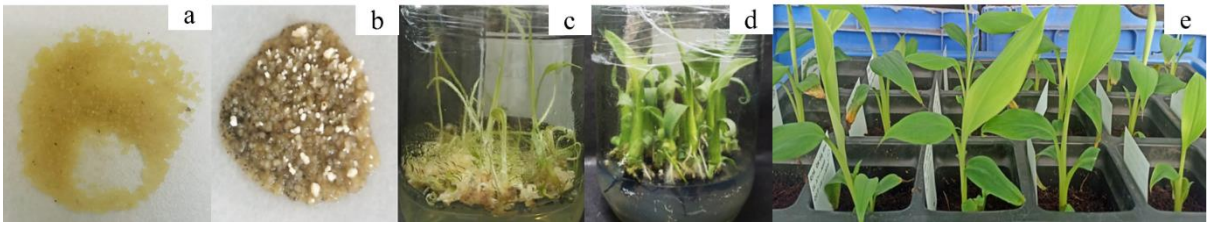
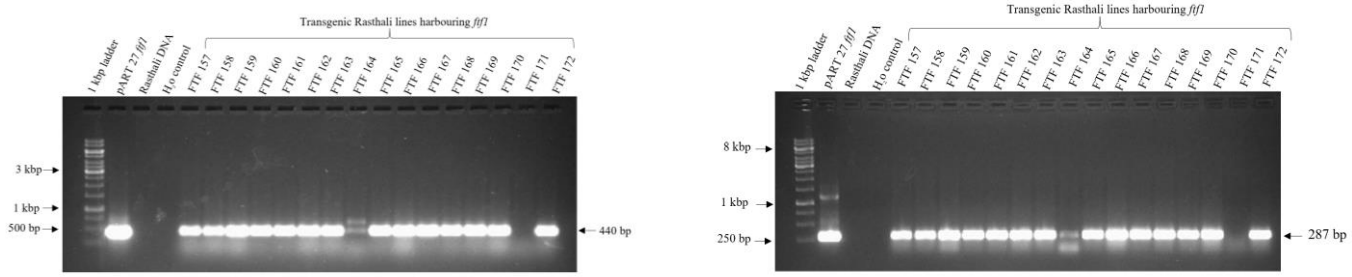


Figure 2. Transformation of banana cv. Rasthali. Embryogenic cells were co-cultivated and plated on co-cultivation medium, b) developed somatic embryo cells on MA3 medium, c) germination of plantlets from the somatic embryos on MA4 medium, d) rooted plants and e) putative transformants acclimatized in protray



a) PCR analysis for *nptII* b) PCR analysis for *ftf1*

Figure 3. PCR analysis of putative transgenic banana cv. Rasthali. Lane 1- 1 kbp marker, lane 2-positive control (pART27 *ftf*1), lane 3-negative control (Rasthali plant DNA), lane 4-water control, lanes 5 to 20-banana transformants

Bioassay of transgenic plants against *Foc* race 1

Transgenic banana plants (434 plants) harboring RNAi-*ftf1* construct were subjected to fungal bioassay following acclimatization for 2 months in greenhouse. Transgenic plants were found to be apparently healthy and exhibited normal phenotype except a few plants which grew slower than the controls. This may be due to the fact that *Fusarium ftf1* gene is a zinc finger transcription factor. The zinc

finger proteins are present throughout the higher organisms; the siRNAs derived from the partial *ftf1* sequence have influenced the expression of some of the zinc finger genes in banana plant. Moreover, zinc finger proteins are involved in a multitude of physiological processes in plants; it is extremely difficult to pinpoint the cause of this deviation (Ghag *et al.*, 2014).

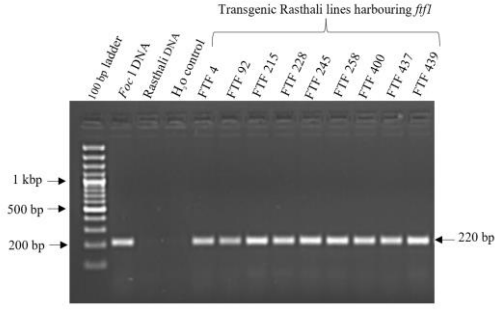
For pathogenicity studies, both transformed and untransformed plants were inoculated with *Foc* race 1 at a spore concentration of 10^6 mL^{-1}. To evaluate the performance of transgenic Rasthali plants under *Foc* root challenge bioassay, the PCR positive transgenic and non-transformed control plants were transplanted in polybags (30x15 cm) for long term assessment (Sunisha *et al.,* 2020). The plants were monitored for external symptoms and scored periodically. Control plants exhibited yellowing of the older leaves, wilting of lower leaves and pseudostem splitting at 45 days of post inoculation, whereas, no such symptom was observed in transgenic plants (Figure 4a). Expression of dsRNAs targeted against important fungal genes in transgenic plants has been tested successfully in barley, wheat, banana and lettuce (Nowara *et al.,* 2010, Panwar *et al.,* 2013, Ghag *et al*., 2014 and Govindarajulu *et al.,* 2015). The accumulation of double-stranded or antisense RNA targeting vital fungal transcripts (effector gene *Avra10*) in transgenic barley and wheat has been demonstrated to inhibit the development of the powdery mildew fungus *Blumeria graminis* (Nowara *et al.,* 2010). HIGS for wheat leaf rust fungus caused by *Puccinia triticina* was achieved by targeting three pathogenicity genes of this fungus *viz.,* MAPK, cyclophilin and calcineurin regulatory subunit (Panwar *et al.,* 2013). Similarly, HIGS of HAM34 (Highly Abundant Message #34), CES1 (Cellulose Synthase 1) genes against downy mildew in lettuce was reported by Govindarajulu *et al.* (2015)

Subsequently, after three months of *Foc* inoculation, 304 transgenic plants exhibited complete wilting (disease severity scale between 2-3) followed by initial yellowing of lower leaves in 84 plants. However, no external *Foc* symptoms were observed in 46 plants (Figure 4b; Table 2). Both the susceptible and resistant transgenic plants were further subjected to PCR assay for the presence of *Foc* using specific primer (to amplify *SIX* gene) and it was found that all the plants had fungal infection (Figure 4 c & d). This indicated that the transgenic plants did not exhibit symptoms despite the presence of fungus in the plants. The RNAi mediated silencing of vital fungal genes (*ftf1, velvet, ERG6/11*) against *Fusarium* wilt in banana (Ghag *et al*., 2014; Dou *et al*., 2020) supports the findings of this study. The results of this study showed that HIGS can potentially play a major role in controlling *Fusarium* wilt disease (race 1) in banana, where there is neither a natural resistance gene pool known nor the conventional breeding methods available (Ghag *et al*., 2012). Moreover, the pathogen control conferred by HIGS is likely to be more durable than resistance mediated by major genes for resistance (Govindarajulu *et al.,* 2015). These 46 *ftf1* transgenic plants were further observed up to 6 months post-inoculation for any delayed *Foc* infection. Nine plants exhibited complete wilting, followed by marginal yellowing of older leaves in 20 plants. Seventeen transgenic lines were free of *Foc*-related symptoms even.

Table 2. Disease severity scale of transgenic plants against *Foc* race 1 at 3 months post inoculation

Treatment	No. of plants showing *			
	Grade 0	Grade 1	Grade 2	Grade 3
Rasthali control (Un-inoculated)	30	-	-	-
Rasthali control (Inoculated with *Foc*)	-	-	1	124
Grandnaine control (Un-inoculated)	12	-	-	-
Grandnaine control (Inoculated with *Foc*)	40	-	-	-
Rasthali *ftf1* (Un-inoculated)	7	-	-	-
Rasthali *ftf1* (Inoculated with *Foc*)	46	84	184	120

a) b)



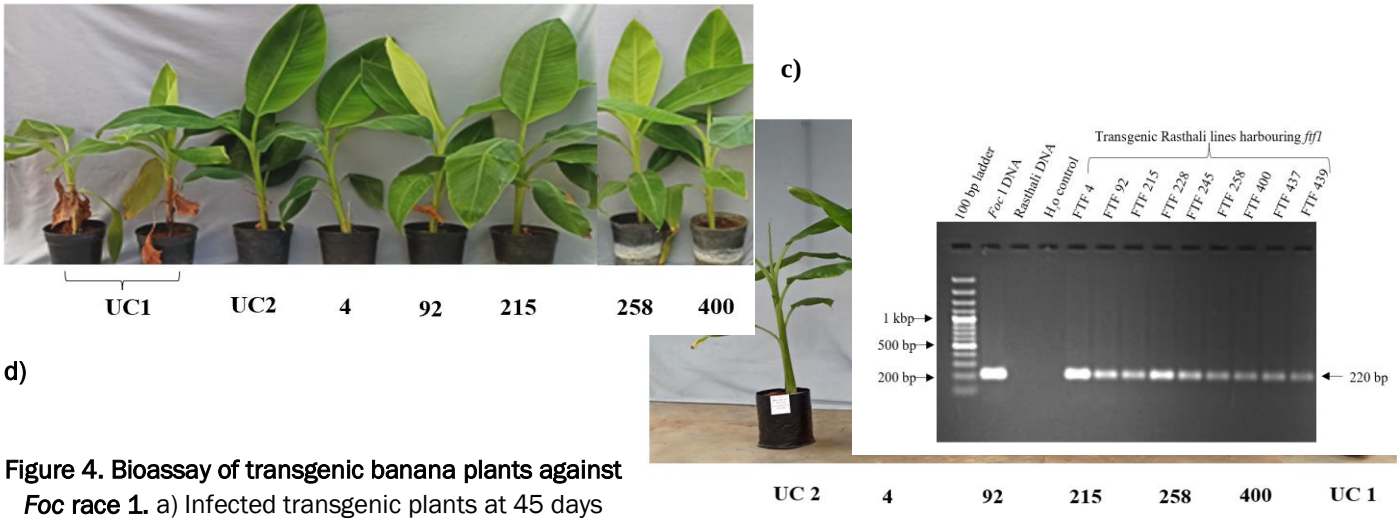


Figure 4. Bioassay of transgenic banana plants against *Foc* race 1. a) Infected transgenic plants at 45 days post inoculation, UC 1- wild type Rasthali, UC 2- wild type Grand naine; b) PCR analysis of infected transgenic plants 45 days post inoculation; c) Infected transgenic plants 3 months post inoculation, d) PCR analysis of infected transgenic plants 3 months post inoculation (UC - Un-transformed control).

CONCLUSION

The results of this study have demonstrated the effectiveness of RNAi technology in imparting resistance to *Foc* race 1 in banana cv. Rasthali. Further, the durability of the resistance needs to be studied in these disease free plants. This strategy could potentially be employed in other banana cultivars that are susceptible to *Foc* race 1.

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Disease severity scale

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