Abstract
Pest and diseases are the main cause of loss in productivity of major food security crops. The objective to increase food production and feeding the increasing world population, at most times is seriously compromised by these biotic constraints. Introducing resistant traits in plants against multiple pathogens is the need of the hour. New methods such as RNA interference can be used to develop efficient resistance against several deleterious phytopathogens. RNA interference has previously been applied to control banana bunchy top virus (BBTV) and Fusarium wilt disease in banana. In this study, we introduced both these ihpRNA (intron hairpin RNA) constructs in the single transgenic banana plant by co-transformation so as to stack resistance to both the diseases. Thirty eight transgenic lines were regenerated, out of which 5 lines were found to be co-transformed. To test for resistance to BBTV and Fusarium wilt disease, the four out of these five co-transformed lines were challenged with viruliferous aphids and Fusarium mass culture. In these bioassays, two transgenic lines (VR19 and VR20) showed high level of resistance to BBTV and Fusarium wilt disease. The two resistant lines expressing both the silencing cassettes were characterized at molecular level by PCR using respective T-DNA specific primers. Further, based on these results, we propose that generation of transgenic plants transformed with multiple silencing constructs targeted towards vital pathogen genes could turn out to be most appropriate approach to develop effective resistance against a broad array of viral and fungal pathogens.

Keywords: Banana; co-transformation; intron-hairpin-RNA; banana bunchy top disease; Fusarium wilt

Introduction
Agricultural production of the important food security crops is under constant threat of damaging pests lowering the yield substantially (1). An estimated 50% of the crop productivity is lost due to destructive insects and pests (2). Banana is the fourth most important food crop after rice, wheat and maize and it is grown in over 120 countries. Banana is affected by several diseases caused by fungal, viral and bacterial pathogens that result in huge losses globally. Fungal pathogens like Fusarium oxysporum f. sp. cubense (Foc) and Mycosphaerella species and viral pathogens such as banana bunchy top virus are the most destructive banana pathogens and also especially difficult to control via conventional methods (3). Banana bunchy top disease (BBTD) is caused by the member of the Nanoviridae group, banana bunchy top virus (BBTV). Typical symptoms include ‘bunchy top’ appearance of the infected plant wherein the
plant top becomes choked with a rosette of narrow, short, erect and brittle leaves (4). BBTV is transmitted by banana aphid, *Pentalonia nigronervosa* Coquerel that can feed on all forms of vegetative planting material like suckers, corms and tissue cultured plants (5). Another disease of banana that is of great concern is the Fusarium wilt disease (also know as Panama wilt). Panama wilt caused enormous destruction of the banana plantations in prime banana growing regions of the world in the nineteenth century. Foc, a soil pathogen enters the healthy banana plant via roots and thrives in the corm tissue of the infected plant. The fungus grows further by overcoming the plant defenses and plugs the xylem vessels thereby causing wilting of the plant. The infected plant shows progressive yellowing of the leaves, discoloration of the pseudostem and finally wilts (6). There are four races identified as race1-4 that infect different banana genotypes of which the race 1 and race 4 are the most significant on a global perspective. Race 1 of Foc infects the Silk and Pome varieties, whereas race 4 (tropical race 4, TR4) infects all the varieties of banana including the race 1 resistant Cavendish varieties which dominate the world market. There are no known effective control methods available to manage this dreadful disease of banana.

Both the diseases of banana viz. Fusarium wilt and BBTD can reduce the yield from a banana plantation by 50 - 100% which can have significant impact on the economic and social conditions of small and marginal farmers in developing countries especially where this crop also serves as a cash crop (7, 8). Development of resistant cultivars is an alternative strategy to manage these diseases. Banana breeding is complicated due the triploid nature of the elite edible cultivars and unavailability of natural resistance in the available gene pool. Thus, stacking of novel genes imparting resistance to multiple pathogens is considered as the best approach to effectively control several deleterious plant diseases in a single line (9-13). In the last couple of years, RNA interference, mediated by small interfering RNA (siRNA), has been adopted as the strategy of choice for control of several plant pathogens (14). Several proof-of-principle studies have lately been published which demonstrated development of disease resistance in plants by achieving silencing of important pathogen genes (15-18, 5). This concept known as “host induced gene silencing” (HIGS) is based on the recently unearthed evidence that there is a free flow exchange of RNA between the host and the pathogen in early stages of disease development (19). We have conducted two such studies wherein host coded hairpin RNA expression cassettes were used *in planta* to generate siRNAs specifically targeted towards vital pathogen genes thereby leading to inhibition of pathogen growth in case of simulated infection (bioassay). In one of these studies, the transgenic banana plants expressing the intron hairpin RNA (ihpRNA) construct designed to silence the viral master replication initiation protein (Rep) (20) showed resistant to BBTV infection more than 6 months post-inoculation with viruliferous aphid (5). In another study, transgenic banana plants expressing ihpRNA construct targeted against the velvet protein gene of Foc imparted high level of resistance to Fusarium wilt disease of banana (18). Velvet protein (21) used in the above study was a suitable candidate for silencing because it is considered to be one of the most important general pathogenecity proteins involved in all aspects of pathogenesis and disease development (22).

In the present study, an attempt has been made to introduce T-DNAs from both these constructs (ihpRNA-Rep and ihpRNA-VEL) in a single banana plant by co-transformation to impart effective resistance against both BBTV and Fusarium wilt disease (Fig. 1). Embryogenic cell suspension cultures of banana were co-transformed with two ihpRNA constructs targeted against viral Rep gene and Foc velvet protein gene. Further, transgenic banana plants having both the T-DNAs were identified and subjected to BBTV and Foc bioassays.

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cultures were subcultured after every eight-days using fresh medium and sieved through an 85µm sieve before being used for Agrobacterium-mediated genetic transformation. ihpRNA-Rep (5) and ihpRNA-VEL (18) binary vector constructs were mobilized separately into Agrobacterium tumefaciens EHA 105 by electroporation.

**Co-transformation** : A single Agrobacterium colony of each construct (ihpRNA-Rep and ihpRNA-VEL) was inoculated in liquid medium containing 0.75% w/v yeast extract and 0.8% w/v nutrient broth supplemented with 50 mgL⁻¹ kanamycin. The bacterial cultures were incubated overnight at 27ºC with an orbital shaking of 180 rpm. Both the overnight grown Agrobacterium cultures were resuspended in the same liquid medium supplemented with kanamycin and grown for further 4-5 hours under the same conditions until an OD₆₀₀ nm of ~ 0.6-0.8 was reached. The Agrobacterium suspension cultures were centrifuged at 6,500 g for 10 minutes and resuspended in M2 medium (24) added with 100 μM acetosyringone (ACS) as described previously (25). Further, the two Agrobacterium cultures were mixed in the ratio of 1:1 and vortexed thoroughly to obtain a uniform suspension culture. The Agrobacterium suspension culture so obtained was used for cocultivation with banana ECS cultures. The cocultivated cells were aspirated onto sterile glass filter discs and were then transferred onto semi-solid M2 medium supplemented with 100 μM ACS as described previously (23). Further, the cells were transferred onto embryo induction medium supplemented with cefotaxime (400 mgL⁻¹) and hygromycin (5 mgL⁻¹). The embryos growing on the selection medium were subcultured for three rounds after every three-week. The embryos were germinated and shoots developed were multiplied to obtain clonal copies of the same transgenic line followed by rooting and hardening in green house as described previously (25).

![Schematic representation of generation of co-transformed multiple disease resistant transgenic banana plants.](image)

**Materials and methods**

**Plant material and binary vectors** : Previously established embryogenic cell suspension (ECS) cultures of banana cv. Rasthali were used for transformation experiments. This ECS culture was initiated from the shoot-tip cultures of cv. Rasthali as described before (23). The ECS
Genomic DNA PCR: The transgenic banana plants regenerated on hygromycin selection medium were sorted out based on preliminary GUS staining results and further analyzed using genomic DNA PCR using primers designed to amplify hygromycin phosphotransferase (hpt) gene to check for the presence of at least one T-DNA in the banana genome. Genomic DNA was isolated from young banana leaves using GenElute Plant Genomic DNA Miniprep Kit (Sigma, USA). Subsequently, another PCR set was carried out using primers specific for VSP (soybean vegetative storage protein) 3’ UTR and nos (nopaline synthase) 3’ UTR (Supplementary table S1) of the ihpRNA-Rep and ihpRNA-VEL constructs respectively to identify the co-transformed transgenic lines.

BBTV bioassay: The BBTV infected banana plants were maintained under contained controlled conditions. The aphid colonies obtained from the banana growing fields in India were reared in a controlled growth chamber at 25 ± 0.5°C and 12 h light/dark photoperiod. Mature aphids were fed on BBTV infected banana leaves for 24 hours and then transferred onto the transformed banana plants in vitro at 25 ± 0.5°C and 12 h light/dark photoperiod (5). After the inoculation-access periods the in vitro maintained plants were transferred to soil and subsequently all plants were sprayed with insecticide confidor (Bayer Crop Science). All the inoculated banana plantlets were grown in an insect-proof growth chamber maintained at 25 ± 0.5°C and 12 h light/dark photoperiod. The BBTD symptoms were monitored after 3 and 6 months from inoculation of viruliferous aphids.

Foc bioassay: Foc race 1 was isolated from the infected corm tissue of banana obtained from the banana growing regions of India. This Foc race 1 culture was grown in potato dextrose broth (PDB) for 5 days at 30°C with an orbital shaking of 160-180 rpm. After 5-days, the spores were separated using cheesecloth and spore count was adjusted to 8 X 10^5 ml⁻¹ using haemocytometry. The spores were inoculated in an autoclaved mixture of sand and maize bran in the ratio of 19:1 and incubated at room temperature for 4-weeks (25). Green-house hardened transgenic and untransformed control banana plants were replanted in the mixture of soil and mass culture (1:1). The plants were screened for Fusarium wilt symptoms six-week post-infection (18). For untransformed controls and each transgenic line, a minimum of four replicates were screened for Foc infection twice and representative plants were photographed.

Results

Regeneration of co-transformed banana plants: Embryogenic cell suspension cultures of banana cv. Rasthali co-cultivated with 1:1 mixture of two different Agrobacterium cultures harboring binary vectors ihpRNA-Rep and ihpRNA-VEL developed into whitish embryos on embryo induction medium supplemented with the antibiotic hygromycin at a concentration of 5 mgL⁻¹ (Fig. 2a). The untransformed cells necrosed in presence of the antibiotic. After repeated subculture on the embryo induction medium added with antibiotic, the putatively transformed embryos matured and subsequently germinated on the medium containing 0.5 mgL⁻¹ 6-benzylaminopurine (BAP). The shoots so obtained were transferred to shoot multiplication medium containing BAP (2 mgL⁻¹) to get multiple copies of the same transformation event (Fig. 2b). Each shoot was separately rooted in the 1-naphthaleneacetic acid (NAA) based medium for 4-weeks (Fig. 2c) and used for inoculation-access of BBTV followed by hardening in green house under controlled conditions or the rooted plants were first hardened in green house for 2 months (Fig. 2d) followed by challenging them with Foc mass culture. Totally, 38 independent transformed lines were regenerated from the co-transformation experiment. All the transformed plants obtained looked phenotypically similar to the untransformed control plants. The transgenic character was determined by GUS assay and PCR amplification. Out of 38 putatively transformed lines, 36 lines tested positive for GUS assay but all the 38 lines were PCR positive.
the transgenic banana plants. Among these, four co-transformed lines (VR18, VR19, VR20 and VR23) were selected for bioassay studies.

**Enhanced resistance to BBTV infection**: The selected co-transformed transgenic rooted banana plants possessing both the T-DNAs were each inoculated with 10 viruliferous aphids after 24 hours of acquisition-access to BBTV infected banana leaf. Untransformed control plants at the same growth stage were also inoculated with the viruliferous aphids in similar manner. These plants were then maintained in contained greenhouse conditions and observed for BBTD symptoms. After 3 months of BBTV assay, the untransformed control plants showed BBTV infection symptoms such as stunted growth, short narrow leaves and rosette pattern whereas none of the 4 co-transformed lines (VR18, VR19, VR20 and VR23) showed BBTD symptoms 3-months post-inoculation (Fig. 4a). The plants were maintained further under same conditions for 6 months without any visible BBTD symptoms indicating that the ihpRNA-Rep construct was effectively transcribed and processed into respective siRNAs that imparted resistance to BBTV and these did not have any deleterious effects in the transgenic plants.

**Improved resistance to Fusarium wilt disease**: Four selected co-transformed lines were subjected to Foc bioassay following the acclimatization of plants in green-house for 2-months. The untransformed control plants showed typical Fusarium wilt disease symptom development 4-weeks post-infection. There was yellowing of the older leaves, cracking and discoloration of the pseudostem. Out of the 4 co-transformed lines, VR18 behaved similar to the untransformed control wherein there was yellowing of the leaves followed by discoloration and cracking of pseudostem and eventually wilting. VR20 and VR23 showed mild to moderate symptoms but no wilting. Line VR19 was healthy and did not show any symptoms of Fusarium wilt disease (Fig. 4b). VR19 and VR20 co-transformed lines were further maintained for 6-

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Fig. 3. Genomic DNA PCR for determination of transgenic nature and co-transformation event. All the 38 lines regenerated were tested for the co-transformation event by performing genomic DNA PCR using primers specific for the T-DNA regions of each ihpRNA-Rep (a) and ihpRNA-VEL (b) constructs. The PCR products obtained using genomic DNA from the transgenic lines (VR1-38) were checked on 1% TAE-agarose gel along with the 1 kb DNA marker (M).

**Table S1.** Primers used in the present study

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<th>PCR Reaction</th>
<th>Primer Sequence (5’-3’)</th>
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| Amplification of *hygromycin phosphotransferase* gene | Fw: GTCCTGCGGGTAAATAGCTG  
                                  Rv: ATTTGTGTACGCCCGACAGT       |
| Amplification of soybean VSP 3’ UTR       | Fw: GCACATTCAAAATAGGAGCATTAGC  
                                  Rv: CTTCAAGACGTGCTCAAAATCACTAT |
| Amplification of *nos* 3’ UTR (reverse primer binds in vector backbone) | Fw: CTACCGAGCTCGAATTTCCCGATCGTT  
                                  Rv: TTCACACAGGAAACAGCTATGA     |
months to observe for any Fusarium wilt disease symptoms. These plants remained free of any Fusarium wilt symptom 6-months post-infection indicating that ihpRNA-VEL overexpressing lines effectively controlled Foc infection (Fig. 4c).

Discussion
Multigene manipulation is an important tool to incorporate two or more important agronomic traits in plants (26). Transgenic approaches for developing crops resistant to a particular phytopathogen have already proved effective in several crops against wide array of pathogens (27). Today, the emphasis is towards developing transgenic crops with multiple qualitative traits so as to enable the plant to resist several pathogens together. In the present study, transgenic banana plants were developed by introducing resistance to two important diseases of banana, BBTD and Fusarium wilt disease by co-transforming these plants with two different ihpRNA constructs to silence the expression of BBTV Rep gene and Foc velvet protein gene.

Banana is an important food security crop and it is grown as monocultures throughout the world (3). As a result, the plantations are always under the threat of a disease epidemic. Banana production is severely affected by two economically important diseases namely BBTD and Fusarium wilt disease (28). Management of these diseases is warranted as both these diseases spread easily and there are no known natural resistant edible cultivars. Genetic resistance is the most effective and sustainable method for controlling these diseases. Genetic transformation of elite edible banana varieties has gained importance due to difficult breeding strategies and involvement of huge expenditure (29). In this study, two different T-DNAs containing the silencing cassettes targeted against the BBTV Rep gene and Foc velvet protein gene were introduced in the super-virulent strain of Agrobacterium tumefaciens. The Agrobacterium cultures harboring the respective T-DNAs were mixed in the ratio of 1:1 so as to obtain co-transformed banana plants. Banana

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being a monocot, Agrobacterium-mediated genetic transformation is a difficult task even in case of single gene transformations. In our co-transformation experiments, we were able to regenerate four co-transformed lines out of the total of 38 lines. The four co-transformed lines were subjected to BBTV and Foc bioassay under greenhouse conditions. The in vitro developed plantlets were inoculated with the active aphid cultures which were previously allowed to feed on the BBTV infected banana leaf. The control plants started showing BBTV symptoms such as stunted growth, shortening and narrowing of the leaf lamina and choking of the leaves on the top 4-weeks post-inoculation. Even after the period of 3-months all the four co-transformed lines (VR18, VR19, VR20 and VR23) did not show any symptoms of BBTV infection indicating that the transgenic banana plants were able to suppress the expression of BBTV Rep gene which resulted in effective resistance.

In another set of experiments, the co-transformed plants were challenged with Foc mass culture and observed for disease development. Six-week post-infection, VR19 line was healthy and showed no signs of Foc infection. The other plants showed some level of Foc disease symptom development. The two best performing co-transformed lines (VR19 and VR20) obtained from the Foc bioassay studies were maintained under greenhouse conditions for further 6-months and they did not show any BBTV or Fusarium wilt symptoms. These two lines (VR19 and VR20) effectively resisted both the pathogens because they completely silenced the BBTV Rep gene and Foc velvet protein genes at the transcriptional level. Further, even though we used two T-DNAs having same marker and reporter cassettes, we did not observe any growth deformities in the regenerated transgenic banana plants.

Conclusions
Stacking of genes in economically important crops vulnerable to serious pathogens offers potential alternative for imparting resistance to multiple pathogens in the single crop plant. In this study, we extended gene pyramiding strategy in combination with RNA interference technology to curb the two most important diseases of banana. The plant showed resistance to both BBTV and Fusarium wilt disease when challenged under greenhouse conditions. These plants also resisted infection symptoms for 6-months after which the experiment was terminated. Thus, in this study we revalidated the potential of using genes and sequences from target pathogens for imparting effective resistance against multiple pathogens in a single transgenic line by simply co-transforming suitable binary vectors.

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Conflict of interest: The authors declare that they have no conflict of interest

Supplementary material: Supplementary table S1: Primers used in the present study

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