3 Biotech (2019) 9:112
https://doi.org/10.1007/s13205-019-1650-7

REVIEW ARTICLE

RNAi-mediated protection against banana diseases and pests

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Received: 24 July 2018 / Accepted: 23 February 2019 / Published online: 28 February 2019
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Abstract
Pests and pathogens restrict the production potential of many crop plants. The losses incurred due to pests and diseases are huge threatening food security. Management strategies include use of chemical pesticides which can be detrimental to human health and environment and other physical and biological methods which have serious limitations. An alternative would be to utilize the advanced technology such as RNA interference (RNAi) to engineer disease resistance in crop plants. The phenomenon of RNAi is very well studied in organisms across genera and found to be conserved. Taking advantage of this, dsRNAs have been delivered into pests and pathogens and showed significant growth inhibition. Banana is susceptible to various groups of pathogens which results in poor yield. The proof-of-principle studies using RNAi technology have already been demonstrated in banana to develop resistance to two important groups of pathogens. Transgenic banana plants expressing small interfering RNA targeting BBTV and Fusarium pathogen have shown high level of resistance. In this review, we summarize and discuss the studies utilizing RNAi as a strategy to develop resistance to major banana diseases and encourage further research in exploiting RNAi-based resistance in other crop plants.

Keywords Banana · RNAi · *Fusarium oxysporum* f. sp. *cubense* · BBTV · PTGS · Resistance

Introduction

Pests and diseases cause significant losses to the crop plants thereby threatening food security (Strange and Scott 2005). Migration of plant pests and diseases to different regions of the world can cause serious epidemics thus menacing vulnerable farmers and food security on a global scale (Bebber et al. 2014). Banana is the staple food of many people in developing countries and ranks as the fourth most important crop (Pillay et al. 2012). Banana and plantains are sensitive to abiotic and biotic factors which reduces production drastically. Due to the shallow root system and round the year green canopy, banana plant is vulnerable to damage occurring due to water stress (Dash and Rai 2016). The plant cannot withstand temperatures lower than the ambient temperature of the tropics and sub-tropics (Turner and Lahav 1983; Sreedharan et al. 2013). Moreover, banana plants are constantly under the risk of being attacked by viruses, fungi, bacteria, nematodes and weevil which infects them and cause significant yield losses (Table 1). Banana and plantains were domesticated by humans, right from the time of the recorded history (Heslop-Harrison and Schwarzacher 2007). The popular cultivars are vegetatively propagated and cultivated as monocultures all over the world. As there is no sexual reproduction in the major edible cultivars of banana, the gene pool remained more or less dormant without any genomic variations. However, the pathogens infecting banana were constantly mutating to adapt to the host defense strategies. In the nineteenth century, when the superior clone Gros Michel was infected with the fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) race 1, the banana industry had to fetch its substitute for its sustenance (Ploetz 2006; Ghag et al. 2015a). The present day Cavendish are resistant to race 1 strain, but the recently evolved Tropical race 4 (TR4) has started infecting them. Again the banana growers have come to the same juncture where they need to identify a substitute for Cavendish. Since the gene pool of banana and plantains is limited,

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Table 1 Major diseases of banana and plantains

Diseases	Causative agent	Symptoms	References
Viral			
Banana bunchy top	*Banana bunchy top virus* (BBTV)	Stunted growth, leaf atrophy and chlorosis, young leaves appear choked or bunched appearance like rosette, leaves are upright with wavy margins, dark green dot or dash flecks along the veins	Dale and Harding (1998), Chen and Hu (2013)
Banana streak disease	*Banana streak virus* (BSV)	Yellow streaks on the banana leaves which later turns necrotic, splitting of pseudostem and rotting	Gayral et al. (2008), Manoranjitham et al. (2012)
Fungal			
Fusarium wilt	*Fusarium oxysporum* f. sp. *cubense* (Foc)	Yellowing of the leaves, splitting of the pseudostem, falling off of the older leaves around the pseudostem, discoloration of the rhizome and pseudostem, wilting of the leaves	Ploetz (2006), Ghag et al. (2015a)
Black Sigatoka	*Mycosphaerella fijiensis*	Reddish-brown flecks on the lower leaf surface which runs parallel to leaf veins, lesions turns dark brown to black and appears sunken later, heavy infestation leads to necrotic lesions and falling off leaves	Carlier et al. (2000a); Etebu and Young-Harry (2011), Kumakech et al. (2015)
Yellow Sigatoka	*Mycosphaerella musicola*	Symptoms similar to black Sigatoka except the early streaks are seen more on the upper surface of the leaves which are yellow–green, narrower and shorter	Surridge et al. (2003), Perera and Kelaniyangoda (2013)
Anthracnose	*Colletotrichum musae*	Development of dark, sunken spots or lesions on affected leaves and fruit, fruit turns black and shrivels	Khan et al. (2001); Zakaria et al. (2009)
Septoria leaf spot	*Mycosphaerella eumusae*	Light brown streaks on the lower side of the leaf, later streaks developed into oval lesions with gray centers as small necrotic, and dark brown spots	Carlier et al. (2000b); Amani and Avagyan (2014)
Bacterial			
Xanthomonas wilt	*Xanthomonas campestris* p. v. *musacearum*	Yellowing and wilting of leaves, diseased pseudostem shows yellowish bacterial ooze, immature fruits turn yellow, infected plants withers away	Biruma et al. (2007), Tripathi et al. (2009)
Moko	*Ralstonia solanacearum* (race 2)	Symptoms are similar to Fusarium wilt. Older leaves first turn chlorotic and wilt and then younger leaves are affected, internally fruit remains firm but becomes brown and later turns gray, vascular discoloration and presence of milky discharge after suspending in water	Ploetz (2003), Chaube and Pundhir (2005)
Rhizome rot	*Erwinia carotovora*/ *Erwinia chrysanthemi*	Wilting of the foliage, stunting and vascular discoloration of the pseudostem, water soaked lesions at the site of infection followed by necrotic lesions, loss of turgor in tissues, breaking of the pseudostem at the collar, rotting of rhizome and unpleasant odor	Gowen (2012), Arun et al. (2013), Nagrale et al. (2013)
Nematode			
Root-knot	*Meloidogyne incognita*/ *Meloidogyne javanica*	Swollen, galled primary and secondary roots, root tip growth ceases, yellowing of the leaves, stunted plant growth and reduced fruit yield	Jaizme-Vega et al. (1997), De Waele and Davide (1998)

Table 1 (continued)

Diseases	Causative agent	Symptoms	References
Nematode root rot	*Radopholus similis*	Root necrosis, stunted growth, delayed fruiting, toppling of the plants	Volcy (2011), Bartholomew et al. (2014)

there are very few known cultivars which show resistance to TR4, and those are diploid wild bananas unsuitable for consumption. Same is the case with other banana diseases such as banana bunchy top disease, Sigatoka disease and weevil infestation. It is essential to identify the genetic basis of resistance that can be transferred to the superior elite varieties. Banana biotechnologists all over the world are putting consorted efforts in developing varieties which can resist these pests and diseases. Elite cultivars of banana are triploid which do not set seeds and so breeding for disease resistance in banana is difficult (Bakry et al. 2009). Nonetheless, researchers in CIRAD (Guadeloupe), CARBAP (Cameroon), IITA (Nigeria), EMBRAPA (Brazil), NRCB (India) and TNAU (India) are able to breed bananas with valuable trait/s.

Several transgenic bananas have been developed in last few years which showed resistance to different groups of pests and pathogens. Genes such as defensins (Ghag et al. 2012, 2014a; Mohandas et al. 2013), chitinase (Kovács et al. 2013), *pflp* (Namukwaya et al. 2012), *hrap* (Tripathi et al. 2010), *Xa21* (Tripathi et al. 2014a) cystatins (Atkinson et al. 2004), cysteine protease inhibitors (Roderick et al. 2012; Tripathi et al. 2015), and cell death-related genes namely, *Ced9, Bcl-xL, BAG1, BI-1* and *DAD1* (Paul et al. 2011; Ghag et al. 2014b) were used to generate resistant transgenic banana lines (Table 2). Expressing defensins, chitinases and cell death-related genes in transgenic banana plants have demonstrated fungal resistance, but ectopic and constitutive expression of these proteins in banana tissues have shown to affect growth and development of banana plants. Field trials were carried out for transgenic banana plants constitutively expressing *hrap* or *pflp* genes against Xanthomonas wilt disease. Fifty-nine out of 65 plants evaluated showed no growth anomaly (Tripathi et al. 2014b). Banana plants expressing the rice *Xa21* gene were also developed and tested for Xanthomonas wilt disease resistance under glass house conditions (Tripathi et al. 2014a). Cysteine proteinase inhibitors or cystatins obstruct the activity of the major digestive enzymes, the cysteine proteinases, thereby suppressing nematode growth and reproduction. Expressing these inhibitors in transgenic banana plants offered resistance to nematodes under confined field conditions (Tripathi et al. 2015). These studies showed success under laboratory conditions barring a few, as they did not reach the farmers field due to worldwide GMO regulatory issues. Furthermore, expressing defense-related genes using strong promoters in transgenic banana plants have resulted in phenotypic abnormalities and can target beneficial organisms associated with banana plants (Paul et al. 2011; Stefani and Hamelin 2010). This state of affairs lead to the utilization of latest and robust technology like RNA interference (RNAi) for engineering disease resistance against important pests and pathogens in crop plants.

Table 2 Strategies employed to develop resistance to major diseases of banana

Diseases	Resistance strategies employed	Level of progress	References
Banana bunchy top disease	RNAi	Laboratory level	Shekhawat et al. (2012)
Fusarium wilt	Expression of defensins, magainin (MSI-99)	Laboratory level	Chakrabarti et al. (2003), Ghag et al. (2012, 2014a)
	Cell death genes	Laboratory level/field level	Paul et al. (2011), Ghag et al. (2014b)
	Resistance gene analogs	Field level	Dale et al. (2017)
	RNAi	Laboratory level	Ghag et al. (2014c)
Sigatoka	Expression of chitinase	Laboratory level	Kovács et al. (2013)
Xanthomonas wilt	Expression of *hrap* and *pflp* genes	Field level	Tripathi et al. (2014b)
Root rot	Expression of cystatins	Field level	Tripathi et al. (2015)

RNAi for disease resistance

RNAi is a sequence-specific gene silencing which involves the complex machinery called RNA-induced silencing complex (RISC) which identifies the homologous sequences and cleaves it (Hannon 2002). This mechanism is conserved across plant (called as cosuppression), animal (called as RNAi) and fungal (called as quelling) kingdom and was first elucidated in the nematode *Caenorhabditis elegans* (Fire et al. 1998). In fact, RNAi has been regarded as a natural component of antiviral defense mechanism in plants. Since the pest or pathogen populations are known to directly interact with host plants to derive nutrition, host plants can be engineered to express the RNAi construct targeting pest or pathogen transcripts. The small interfering RNA (siRNA) processed from the double-stranded RNA (dsRNA) generated in host plant finds entry into the pest or pathogen particularly pathogenic fungi and nematodes and cleave the transcripts important for its growth, development and/or pathogenicity (strategy known as host-induced gene silencing). Herein, no proteins are produced which is one of the important concerns to the GMO regulatory bodies. The dietary small RNAs are generally degraded in human gut due to its harsh environment and the ones enclosed in plant vesicles are poorly bioavailable, thereby reducing the chances of it causing any harm (Sherman et al. 2015; Chan and Snow 2017). Nonetheless, rigorous case by case investigations are warranted to establish potential effects of each of these siRNAs that finds entry into the human system. Further, since this strategy is highly sequence specific, selected genes can be targeted and, therefore, none of the other interacting or beneficial partners are affected. In addition to the detrimental effects of fungicide on environment and human health, some fungal pathogens are developing resistance to these chemicals (Hahn 2014; Hobbelen et al. 2014). Thus, RNAi technology seems to be the most sustainable solution to this problem with no significant environmental impact. This technology can be used to target single or multiple pathogens simultaneously. Moreover, this strategy can be applied using topical sprays or through transgene expression in host plant for durable resistance.

RNAi against banana diseases

The edible bananas (diploids, triploids, and tetraploids) have evolved from the natural hybridization of two originators, *Musa acuminata* (AA) and *Musa balbisiana* (BB) (Perrier et al. 2011). The triploid varieties have been cultivated throughout the world because they are more productive than the diploid counterparts. The edible bananas are propagated vegetatively and tissue culture has favored mass production of these varieties. The consumer demand for particular traits directed cultivation of certain varieties that led to monoculture cultivation over large acreage in the banana-growing regions. However, this monoculture practice increased the vulnerability to diseases causing huge economic losses. Banana plantations around the globe are devastated by the fungal diseases, Fusarium wilt and Sigatoka and viral disease, BBTD, while bacterial wilt is destroying plantations in East and central Africa (Dale 1987; Tripathi et al. 2009; Ghag et al. 2015a). Developing resistance in banana is difficult due to complex ploidy, limited gene pool and pathogen diversity. Rapid progress in developing strategies for resistance against different diseases of banana is warranted.

RNA silencing has been exploited as a dominant tool for engineering virus resistance in plants, because viral genome replicates within the host plant and is amenable for direct silencing (Wang et al. 2012). However, there are several suppressors of RNA silencing identified in plant viruses which act as counter defense (Burgyán and Havelda 2011; Pumplin and Voinnet 2013). RNAi strategy has proved effective in case of banana to develop resistance to important diseases such as Fusarium wilt and banana bunchy top disease. Banana bunchy top disease is a destructive viral disease which is transmitted by aphids causing severe stunting and distortion of leaves (Blomme et al. 2011). The infected plant do not produce bunch if infected before maturity or produces

distorted bunch if infected later. RNAi-based resistance was introduced into susceptible cultivar of banana (cv. Rasthali), wherein the master replication protein gene '*Rep'* full coding sequence or '*Rep'* partial coding sequence together with its 5′ partial upstream regulatory region of the BBTV, was used to generate RNAi vector and successfully transformed into banana (Shekhawat et al. 2012). These plants were resistant to BBTV up to 6 months post-inoculation with viruliferous aphids. Moreover, RT-PCR failed to detect any BBTV-specific transcripts in these transgenic lines. A similar study was carried out using gene fragments from the four BBTV DNA segments namely DNA 1, DNA 3, DNA 4 and DNA 5 to generate RNAi vectors. The transgenic banana plants (cv. Grand Naine) generated showed delayed viral multiplication and development of symptoms (Krishna et al. 2011). It is very important to recognize a suitable target sequence and later to identify a transgenic line which expresses correct siRNA in proper amounts which is able to resist the virus (Fig. 1).

RNAi has also been successfully applied to protect plants against fungal pathogens (Ghag 2017). Fungal pathogens are known to accumulate small RNAs from host plants during colonization (Zhang et al. 2016). The ones colonizing the host vasculature such as *Fusarium oxysporum* are more likely to take up these silencing signals from the host plants as these are its sites of transport and storage (Chitwood and Timmermans 2010). Host plant-generated RNAi has proved efficient in managing Fusarium wilt disease (Ghag et al. 2014c). Partial sequences of two crucial genes namely, the velvet protein gene and *Fusarium* transcription factor 1 gene were assembled separately in the RNAi vectors and transformed into banana plants (cv. Rasthali). The transgenic banana plants did not show external or internal symptoms of Fusarium wilt disease. These plants resisted the infection till 8 months post-inoculation with Foc after which the experiment was terminated. Moreover, small RNAs of 21 nucleotides were formed all along the length of the transgene that targeted the vital Foc transcripts. This indicates that there is no directionality or specificity to form siRNAs when it comes to transgene expression. Therefore, several crucial gene segments from single or multiple pathogens can be stitched together to target all of them simultaneously. Nevertheless, when the 'Rep' RNAi vector and velvet RNAi vector were co-expressed in the transgenic banana plants it imparted resistance to both the pathogens (BBTV and Foc) (Ghag et al. 2015b).

There are no studies to demonstrate RNAi effectiveness against banana nematodes and weevil. However, in vitro

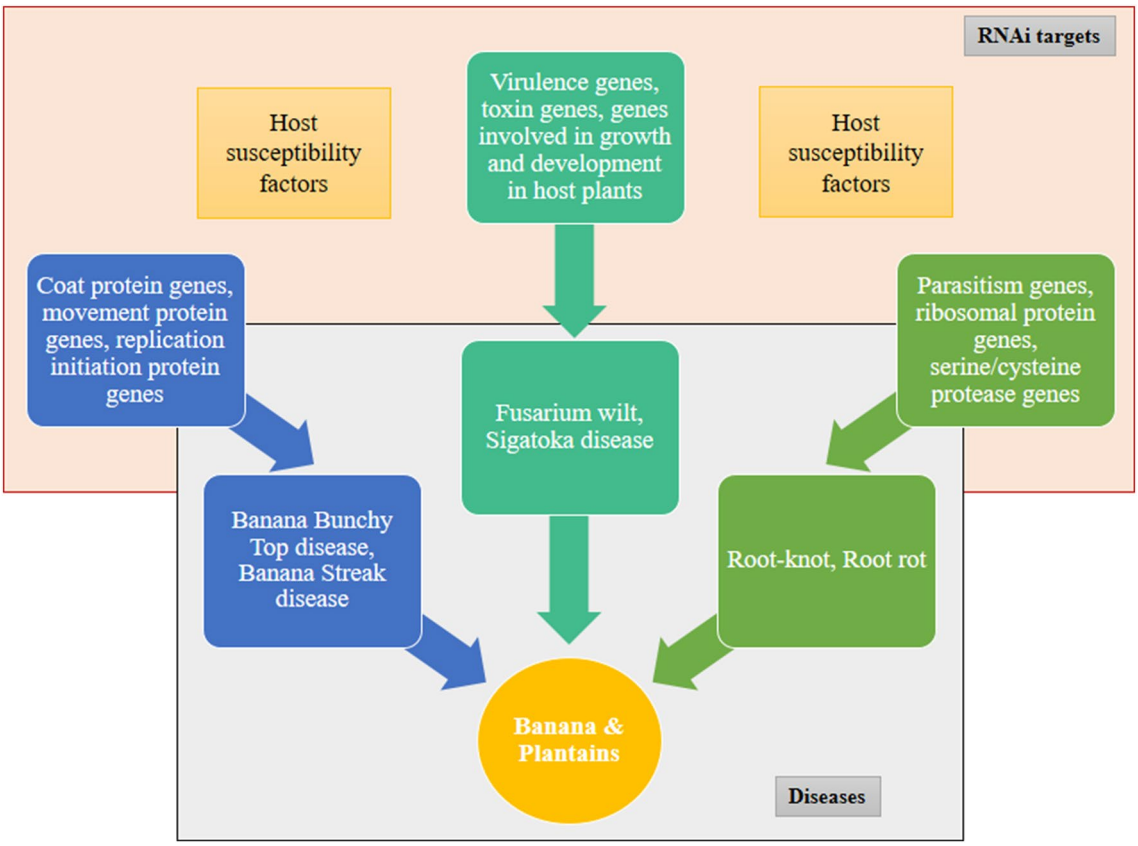

Fig. 1 RNAi targets to manage important diseases and pests of banana

RNAi-based studies were conducted on nematode *Radopholus similis* (causative agent of banana root lesion) and banana weevil *Cosmopolites sordidus*. *R. similis* Cathepsin B (*Rs-cb-1*) mRNA is expressed in the esophageal glands, intestines and gonads of the females, testes of males, juveniles and eggs which when targeted using dsRNA showed significant inhibition in development and hatching and also greatly reduced its pathogenicity (Li et al. 2015). Four pests of banana, *Radopholus similis, Pratylenchus coffeae, Meloidogyne incognita* and *Helicotylenchus multicinctus* were targeted by generating dsRNA against the conserved domains of Proteasomal alpha subunit 4 and Actin-4. In vitro Actin-4 dsRNA treatment on *R. similis* impaired motility and reduced nematode multiplication on carrot discs (Roderick et al. 2018). In vitro feeding of ubiquitin E2 gene dsRNA to banana weevil larva significantly arrested banana weevil larval growth and caused up to 100% mortality at 21 days (Ocimati et al. 2016). Transgenic banana plants expressing siRNA or hairpin RNA of the above validated genes could, therefore, potentially be used for controlling the banana nematode and weevil infestation (Fig. 1).

Future prospects

RNAi is a potential strategy to control an array of pests and pathogens without having any significant side effects; which is quite common with transgenics expressing potent defense molecules and/or using harmful chemical pesticides. Although, there are off-target effects seen in RNAi-based approaches when there is more than 95% similarity in the sequences (Xing and Zachgo 2007). Identifying suitable target sequence which is specific for a given pest or pathogen population and with no corresponding homologous sequence in the host plant or any other non-target organisms resolves this problem. With the existing technology, it is becoming easier to identify such target sequences because of the availability of large-scale sequencing data derived from the genome, transcriptome or secretome of the host and pathogen species and accessibility of numerous bioinformatic tools. Particular gene sequences can be targeted and its homologous sequences can be identified in other organism just by a simple in silico homology search tool. Nevertheless, the non-exonic regions which are poorly conserved across species can be targeted to avoid off-target and non-target effects. Moreover, particular sequence stretches from different pathogens can be stacked together in a single RNAi vector to achieve resistance to different groups of pathogens. Further, to avoid ectopic expression and causing any undesirable effect, siRNAs can be expressed particularly in specified tissues using tissue-specific promoters. For example, in banana, siRNAs targeting root pathogens such as *Fusarium* or banana nematodes can be expressed specifically in banana roots using root-specific promoters and, therefore, it will not be expressed in fruits. This approach will not only help in mitigating biosafety concerns but might also relieve some hurdles in GMO regulatory approvals. Biosafety studies are essential to determine the safety of these siRNA in food and feed. Much more research is still warranted to understand the environmental fate of these siRNAs. Once all these studies are accomplished and we have enough knowledge and understanding supported with the experimental findings, undoubtedly RNAi technology will be highly efficient in managing pests and pathogens affecting susceptible crop plants.

Acknowledgements SBG thank Department of Science and Technology, India and Indian National Science Academy for the INSPIRE Faculty award.

Compliance with ethical standards

Conflict of interest On behalf of all the authors, the corresponding author states that there is no conflict of interest.

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