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In *Nicotiana* species, an artificial microRNA corresponding to the virulence modulating region of *Potato spindle tuber viroid* directs RNA silencing of a *soluble inorganic pyrophosphatase* gene and the development of abnormal phenotypes

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ABSTRACT

Potato spindle tuber viroid (PSTVd) is a small non-protein-coding RNA pathogen that can induce disease symptoms in a variety of plant species. How PSTVd induces disease symptoms is a long standing question. It has been suggested that PSTVd-derived small RNAs (sRNAs) could direct RNA silencing of a targeted host gene(s) resulting in symptom development. To test this, we expressed PSTVd sequences as artificial microRNAs (amiRNAs) in *Nicotiana tabacum* and *Nicotiana benthamiana*. One amiRNA, amiR46 that corresponds to sequences within the PSTVd virulence modulating region (VMR), induced abnormal phenotypes in both *Nicotiana* species that closely resemble those displayed by PSTVd infected plants. In *N. tabacum* amiR46 plants, phenotype severity correlated with amiR46 accumulation and expression down-regulation of the bioinformatically-identified target gene, a *Nicotiana soluble inorganic pyrophosphatase* (*siPPase*). Taken together, our phenotypic and molecular analyses suggest that disease symptom development in *Nicotiana* species following PSTVd infection results from sRNA-directed RNA silencing of the host gene, *siPPase*.

Introduction

Viroids are the smallest plant RNA pathogen with genomes ranging in size from 246 to 401 nucleotides (nt) (Rocheleau and Pelchat, 2006). Viroids are non-protein-coding RNAs that require a host encoded RNA polymerase for their replication. Most viroids replicate in the nucleus of the infected plant cell, however members of the Avsunviroidae family replicate exclusively in chloroplasts (Flores et al., 2005). Viroids infect and induce disease symptoms in a variety of plants, including some important horticultural crop species that are normally propagated clonally (Daròs et al., 2006). Each viroid species is usually comprised of multiple sequence variants within its natural population and such variants often induce different levels of disease symptom severity upon infection (Góra-Sochacka et al., 1997; 2001; Hammond, 1992; Nie, 2012; Wassenegger et al., 1996). However, the mechanism by which viroids induce disease symptom development upon infection remains poorly understood.

RNA silencing is a sequence-specific RNA degradation process directed by 21–24 nt small RNAs (sRNAs) processed by Dicer-like (DCL) endonucleases from double-stranded RNA (dsRNA) or self-complementary hairpin RNA (hpRNA) structures (Baumberger and Baulcombe, 2005; Brodersen and Vionnet, 2006; Gregory et al., 2005). The diced sRNAs are loaded onto an Argonaute (AGO) protein that forms the catalytic core of RNA-induced silencing complex (RISC). AGO-loaded sRNAs are used as guides by RISC to bind to and subsequently degrade cognate single-stranded RNAs, and in plants this is predominantly via an AGO-mediated RNA cleavage-based mechanism of RNA silencing (Baumberger and Baulcombe, 2005; Liu et al., 2007; Rand et al., 2005).

Like all plant viruses and subviral agents, viroid replication is associated with the accumulation of abundant small-interfering RNAs (siRNAs), derived from the whole viroid genome (Di Serio et al., 2009; Diermann et al., 2010; Itaya et al., 2007; Li et al., 2012; Navarro et al., 2009). We have previously shown that expression of a hpRNA transgene, encoding a near full length sequence of the severe *Potato spindle tuber viroid* (PSTVd) strain RG1 (PSTVd-RG1),

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induced PSTVd disease symptom-like phenotypes in tomato (Wang et al., 2004). This study led us to propose that siRNAs derived from PSTVd may have sequence complementarity to a host gene(s), thereby directing RNA silencing of the targeted host gene(s), leading to disease symptom development. Such a host gene RNA silencing-directed disease model has recently been demonstrated for the albinism phenotype displayed by peach (*Prunus persica*) plants infected with *Peach latent mosaic viroid* (PLMVd) (Navarro et al., 2012), and the yellowing symptoms in *Nicotiana* species induced by another subviral RNA, the *Cucumber mosaic virus* Y-Satellite RNA (Shimura et al., 2011; Smith et al., 2011). A 30 nt sequence of the 359 nt PSTVd genome, termed the pathogenicity domain or virulence modulating region (VMR), is essential for disease symptom development in PSTVd infected plants (Keese and Symons, 1985). The VMR is located in the upstream half of the rod-like structured PSTVd RNA, and sequence variation within this region among PSTVd variants is often associated with dramatic differences in PSTVd disease symptom severity (Góra-Sochacka et al., 1997; 2001; Hammond, 1992; Nie, 2012; Wassenegger et al., 1996). This observation suggests that VMR-derived siRNAs are potentially directing RNA silencing of a host target gene(s). However, to date, no causative sRNA or complementary host target gene have been identified to support the sRNA-directed PSTVd disease model.

We developed a series of artificial microRNA (amiRNA) plant expression vectors to assess whether sRNAs derived from either the VMR or its flanking genomic sequences could induce PSTVd disease symptom-like phenotypes in two *Nicotiana* species of the Solanaceae family, *Nicotiana tabacum* and *Nicotiana benthamiana*. Here we report that one amiRNA vector, the amiR46 vector, from which a 21 nt amiRNA sRNA is generated corresponding to nts 46–66 of the PSTVd genome, lying within the VMR, induced expression of phenotypes in transformed *Nicotiana* plant lines that closely resemble those displayed by PSTVd infected plants. Transformed *Nicotiana* plants expressing either the amiR24 or amiR71 vectors, that generate amiRNAs corresponding to PSTVd-RG1 genomic sequences immediately flanking the VMR, failed to develop any phenotypes. Bioinformatic interrogation of publically available *N. tabacum* and *N. benthamiana* transcriptome datasets identified a *Nicotiana soluble inorganic pyrophosphatase* (*siPPase*) transcript harbouring a partially complementary amiR46 target sequence. Molecular analyses revealed that the severity of the expressed phenotype strongly correlated with amiR46 accumulation and RNA silencing of the putative *N. tabacum* target gene *Nt. siPPase*. Taken together, the molecular and phenotypic data presented here suggests that the phenotypes expressed by *N. tabacum* amiR46 plants, phenotypes that closely resemble those displayed by PSTVd infected plants, result from sRNA-directed RNA silencing of the host gene *Nt. siPPase*. These findings also add weight to the sRNA-directed host gene RNA silencing model for disease symptom development in other PSTVd infected plant species.

Results

Expression of PSTVd VMR-specific artificial miRNAs induce phenotypes in N. tabacum plants

Six PSTVd-specific amiRNA plant expression vectors were generated using the pBlueGreen amiRNA cassette that is based on the *A. thaliana MIR159B* precursor transcript (Fig. 1A) (Eamens et al., 2009; Eamens et al., 2011; Eamens and Waterhouse, 2011). Four of the six amiRNA vectors were designed to generate a mature 21 nt amiRNA sRNA that largely (amiR45) or fully (amiR46, amiR47, amiR50) corresponded to VMR sequences (Fig. 1B), starting at nt positions 45, 46, 47 and 50 of the PSTVd-RG1 genome respectively (Fig. 1C). The amiR24 and amiR71 vectors were designed to generate mature amiRNAs corresponding to upstream and downstream genomic sequences respectively that immediately flank the PSTVd-RG1 VMR (Fig. 1C). These two amiRNA vectors were included in this study as negative controls for the proposed PSTVd VMR sRNA-directed disease model. All six amiRNA plant expression vectors were used to transform *N. tabacum* and *N. benthamiana* plants via *A. tumefaciens* (*Agrobacterium*)-mediated transformation (Ellis et al., 1987). Table 1 lists the number of primary (T0) *N. tabacum* and *N. benthamiana* transformants randomly selected for further phenotypic analysis of the six PSTVd amiRNA plant expression vectors.

In *N. tabacum* transformants, amiR24, amiR45, amiR47 and amiR71 expressing plants all displayed wild-type (WT)-like phenotypes. Six of the 14 amiR50 *N. tabacum* plants displayed elongated leaves with mild discolouration (yellowing) during early vegetative development (Figure S1A), suggesting that the amiR50 sRNA was potentially targeting a tobacco gene(s) for amiRNA-directed RNA silencing. However, this phenotypic abnormality disappeared as plants matured, displaying WT-like phenotypes during the later reproductive stage of development (Figure S1B). Fig. 2 clearly shows that in contrast to *N. tabacum* plants transformed with amiR24, amiR45, amiR47, amiR50 and amiR71 vectors, all 23 plants expressing the amiR46 vector displayed a distinct phenotype. During the rooting stage of *Agrobacterium*-mediated transformation, amiR46 *N. tabacum* plants developed significantly fewer roots than plants expressing either control vector or the three other VMR-specific amiRNAs (Fig. 2A). During vegetative growth, 20 of the 23 primary *N. tabacum* amiR46 transformants had a short stature with multiple shoots (Fig. 2B). The majority of *N. tabacum* amiR46 phenotype-expressing plants also developed elongated leaves that were discoloured (Figs. 2B and 4A). The vegetative phenotypic abnormalities of *N. tabacum* amiR46 transformants resemble the stunting, bushiness and leaf discolouration symptoms previously associated with PSTVd infection of tomato (Schnölzer et al., 1985), or for tomato plants transformed with a PSTVd-RG1 hpRNA transgene (Wang et al., 2004). At the reproductive stage, all amiR46 plants had low fertility, primarily due to the development of anthers with shortened filaments, but also due to greatly reduced amounts of pollen (Fig. 2C and data not shown). As a consequence, most seed pods were reduced in size and contained very few seed (Fig. 2D and data not shown). Manual pollination with anthers from the same flower, or from WT flowers recovered fertility, but the resulting seed pods remained reduced in size compared to *N. tabacum* plants expressing the other PSTVd amiRNA vectors (Fig. 2D).

Expression of PSTVd VMR-specific artificial miRNAs induce phenotypes in N. benthamiana plants

As described for *N. tabacum* plants expressing the PSTVd amiRNA vector series, *N. benthamiana* primary transformants expressing the amiR24, amiR47 and amiR71 vectors were WT in appearance (Fig. 3 and data not shown). In addition, all amiR50 expressing *N. benthamiana* plants displayed a WT-like phenotype (data not shown). However, three of nine amiR45 *N. benthamiana* plants were reduced in overall size and developed rough-surfaced, mottled leaves (Figure S2A, S2B). At the reproductive stage, these three *N. benthamiana* amiR45 plants developed sterility defects similar to those reported for *N. tabacum* amiR46 lines (data not shown). Consistent with our observations of *N. tabacum* amiR46 transformants, all 15 *N. benthamiana* amiR46 plants displayed a readily observable phenotype (Table 1), especially at the reproductive stage of plant development. During vegetative growth, *N. benthamiana* amiR46 plants expressed milder phenotypes than those described for *N. tabacum* amiR46 transformants, characterised by stunting, bushiness and smaller sized leaves that were mottled (Fig. 3). The most striking phenotype expressed by *N. benthamiana* amiR46 plants was during the later stages of plant development. All 15 amiR46 lines had a shorter stature