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Evaluation and identification of candidate genes for artificial microRNA-mediated resistance to tomato spotted wilt virus

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ABSTRACT

Tomato spotted wilt virus (TSWV) is an economically important viral pathogen of a wide range of field and horticultural crops. We developed an artificial microRNA (amiRNA) strategy against TSWV, targeting the nucleoprotein (N) and silencing suppressor (NSs) genes. The amiRNA constructs replaced the natural miRNA in a shortened *Arabidopsis* 173-nucleotide (nt) miR159a precursor backbone (athmiR159a) without the stem base extending beyond the miR/miR* duplex. Further, each amiRNA was modified to contain a mismatch (wobble) sequence at nucleotide position 12 and 13 on the complementary strand amiRNA*, mimicking the endogenous miR159a sequence structure. Transient expression in *Nicotiana benthamiana* demonstrated that the introduction of a wobble sequence did not alter amiRNA expression levels. Following challenge inoculation with TSWV, plants expressing N-specific amiRNAs with or without the wobble remained asymptomatic and were negative for TSWV by ELISA. In contrast, plants expressing the NSs-specific amiRNAs were symptomatic and accumulated high levels of TSWV. Similar findings were obtained in stably transformed *Nicotiana tabacum* plants. Our results show that a shortened 173-nt athmiR159a backbone is sufficient to express amiRNAs and that the presence of mismatch at position 12–13 does not influence amiRNA expression or conferring of resistance. We also show that selection of target gene and positional effect are critical in amiRNA-based approach for introducing resistance. These findings open the possibility of employing the amiRNA approach for broad-spectrum resistance to tospoviruses as well as other viruses.

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1. Introduction

Plants have evolved elaborate defense mechanisms to protect themselves from invading pathogens. RNA silencing is a conserved eukaryotic mechanism that serves to modulate the expression of host endogenous genes as well as suppression of foreign or aberrant DNA elements and acts as a natural plant defense system against viral infection (Bartell, 2004; Baulcombe, 2004; Voinnet, 2005; Agius et al., 2012). RNA silencing is triggered by processing of double stranded RNA (dsRNA) into small interfering RNAs (siRNAs) of 21–25 nucleotides, followed by incorporation into the RNA-induced silencing complex (RISC). The RISC complex can then find and destroy the targeted mRNA in a homology-dependent manner (Khvorova et al., 2003; Schwarz et al., 2003; Bartel, 2004; Filipowicz et al., 2005; Voinnet, 2009). In plants, RNA silencing is classified into three major pathways (Baulcombe, 2004). The first is the siRNA pathway that is involved in antiviral defense by degradation of viral transcripts. The second is the microRNA (miRNA) pathway, which modulates the plant gene expression by either suppression of protein translation or RNA cleavage. The last is the chromatin-silencing pathway that is implicated in heterochromatin formation.

The siRNA-mediated antiviral resistance approach triggers the siRNA pathway by deliberate production of sequence-specific dsRNA after the introduction into plants of sense, antisense or hairpin transgenes derived from the pathogen or pest genome (Dietzgen and Mitter, 2006; Duan et al., 2012). Artificial miRNA-based antiviral resistance approach imitates the natural miRNA pathway to degrade the viral target by expressing virus specific miRNAs using a host precursor miRNA (pre-miRNA) backbone. (Niu et al., 2006; Qu et al., 2007; Duan et al., 2008; Wu et al., 2010; Ai

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et al., 2011; Kung et al., 2012). The strategy of expressing amiRNAs was first adopted to knock out/down endogenous genes for functional analysis (Schwab et al., 2005). It was shown that biogenesis of mature miRNA is not affected even if the 21-nt region of a mature miRNA is replaced with a specific sequence complementary to the target in the pre-miRNA.

Currently, amiRNA technology for virus resistance utilizes pre-miRNAs such as miR159, miR167b, miR171, miR172 and miR319. Niu et al. (2006) first showed that the amiRNA-based approach could be used to confer resistance to turnip yellow mosaic virus (TYMV) and turnip mosaic virus (TuMV) by targeting silencing suppressor P69 and HC-Pro, respectively, using *Arabidopsis thaliana* mir159a (273 nt) sequence as the backbone. In another report, amiRNA-based resistance against cucumber mosaic virus (CMV) by targeting the 2b gene showed that the level of resistance is closely correlated to the level of miRNA expression (Qu et al., 2007). In addition, the comparison of CMV resistance between amiRNA-induced and siRNA-induced resistance led them to conclude that amiRNA approach is more efficient (Qu et al., 2007). Similarly, Duan et al. (2008) reported amiRNA-based resistance to CMV by targeting the 3′ untranslated region (UTR). Other research in this field has produced resistance against potato virus Y (PVY) and potato virus X (PVX) by targeting their silencing suppressor genes, HC-Pro, P25, Coat Protein (CP), Nuclear Inclusion Proteins a and b (NIa and NIb) and Central Inculsion Protein (CI), respectively (Ai et al., 2011; Song et al., 2014). Recently, Guo et al. (2014) have shown that the transgenic plants expressing amiRNAs targeting two different sites in the acetylcholinesterase 2 coding gene (*MpAChE2*) gene exhibited better aphid resistance than the plants expressing *MpAChE2*-specific dsRNA. Together, these studies provide the proof of the feasibility of this emerging technology in engineering antiviral resistance in plants.

Tomato spotted wilt virus (TSWV) is one of the most economically important viral pathogen, considered to be one of the top ten economically important viruses in the world (Scholthof et al., 2011). It infects a wide range of crops (more than 1000 plant species) that includes a broad range of staple and horticultural crops, impacting agriculture in tropical and subtropical areas globally. The global yield losses account for up to US$1 billion per year due to TSWV (Pappu et al., 2009). TSWV is the type species of the genus *Tospovirus* in the family Bunyaviridae and is exclusively transmitted by thrips. Its tripartite genome contains three linear single-stranded RNAs, denoted L RNA (8897 nt), M RNA (4821 nt) and S RNA (2961 nt) (Turina et al., 2012); both M and S RNAs have two genes in an ambisense arrangement. S RNA codes for the nucleoprotein (N) and a silencing suppressor protein (NSs), M RNA codes for the precursor to the membrane glycoproteins G_N and G_C and the viral movement protein NSm, and L RNA encodes an RNA-dependent RNA polymerase (RdRp) (Takeda et al., 2002; Lewandowski and Adkins, 2005; Whitfield et al., 2008; Li et al., 2009; Turina et al., 2012).

Previous work on engineering TSVW resistance in transgenic plants by siRNA- mediated silencing strategy has shown that of several genes of TSWV, only the N and NSm gene constructs resulted in resistance (MacKenzie and Ellis, 1992; Prins et al., 1996; Whitfield et al., 2005). Subsequent work showed that sequences as short as 110-nts from the TSWV N gene were sufficient to efficiently induce RNA silencing, and hence virus resistance, but only when fused to a carrier mRNA such as green fluorescent protein (Jan et al., 2000). Bucher et al. (2006) further extended this approach to deliver broad-spectrum resistance against multiple tospoviruses by targeting the N gene. Recently, Peng et al. (2014) have shown that targeting the conserved motifs of tospoviral L gene as a transgene for expressing dsRNA generates broad-spectrum resistance against tospoviruses at the genus level.

In spite of the effectiveness of siRNA mediated virus resistance, the approach exhibits weaknesses such as problems of biosafety, loss of potency in short-term and off-target effects. The amiRNA approach utilizes only 21-nts from the viral genome compared to siRNAs from longer hairpin sequences (>300 nt) thereby reducing the extent of off-target effects and increasing the biosafety of transgenic crops. The chance of off-target effects have been reported to increase with greater length of initial dsRNA sequence used (Qiu et al., 2005; Filichkin et al., 2007; Stephan et al., 2008; Warthmann et al., 2008). The relaxed demand on sequence length makes amiRNAs especially useful and facilitates targeting of conserved regions. Moreover, amiRNA-based resistance has been shown to be stable at wider temperatures (Niu et al., 2006).

Table 1
Description of amiRNA constructs targeting the TSWV N and NSs genes.

amiRNA construct	TSWV target gene	Sequence position (nt)	Primer sequence 5′–3′
amiTSWV-N1	N gene	486 ⟵ 506	TATATAGCCAAGACAACACTG*
amiTSWV-N1w			TATATAGCACAGACAACACTG
amiTSWV-N2	N gene	728 ⟵ 748	TTTTAACCCCGAACATTTCAT*
amiTSWV-N2w			TTTTAACCAAGAACATTTCAT
amiTSWV-NSs7	NSs gene	511 → 531	ATTATAGCACATCTCGATCTT*
amiTSWV-NSs7w			ATTATAGCCAATCTCGATCTT
amiTSWV-NSs8	NSs gene	520 → 540	TAAAGCCTGATTATAGCACAT*
amiTSWV-NSs8w			TAAAGCCTTCTTATAGCACAT

Artificial microRNA TSWV constructs are named based on their target positions in TSWV genome. Each sequence was modified to contain a wobble sequence at position 12 and 13 (indicated by w) on the complementary strand and is underlined in the primer sequence. (*) Complementary sequence used as an oligonucleotide probe for detection of miR and the primer sequence itself used as an oligonucleotide probe for detection of miR*. Probe sequence for miR159a 5′-TAGAGCTCCCTTCAATCCAAA-3′ and U6 loading control 5′-CACGAATTTGCGTGTCATCCTT-3′.

In the present study, amiRNAs designed to target the viral transcripts encoding the TSWV N and NS proteins were expressed using only 173-nts of pre-miR159a without the stem base extending beyond the mature microRNA. We also investigated if the presence of a wobble sequence at nucleotide position 12 and 13 on the complementary strand amiRNA*, mimicking the endogenous miR159a sequence structure will have any effect on amiRNA expression and resistance to the virus. Transient expression of amiRNAs in *Nicotiana benthamiana* (*N. benthamiana*) by agroinfiltration and transgenic tobacco plants confirmed expression of virus-specific amiRNAs using the shortened backbone of 173-nts with and without mismatch. Interestingly resistance to TSWV was conferred by amiRNAs targeting N gene and not by amiRNAs targeting NSs gene of the virus in both transient as well as stable expression systems.

2. Materials and methods

2.1. Construction of artificial pre-amiRNAs

Using Web MicroRNA designer program WMD3-http://wmd3.weigelworld.org/cgi-bin/webapp.cgi, two 21-nt amiRNA sequences were selected for TSWV N and NSs genes (Accession number AB088385.1). Table 1 shows the sequence positions of the TSWV N and NSs genes targeted. Next, we chose an *A. thaliana* 173-nt pre-miR159a hairpin structure as a backbone for the construction and expression of amiRNAs, so the original mature miR159a was substituted by amiRNA sequences listed.

A wobble sequence was introduced for each TSWV amiRNA construct that had a mismatch at nucleotide position 12 and 13 on the complementary strand amiRNA*, mimicking the endogenous miR159a sequence structure (Table 1). The constructs were synthesized in pUC57 (Gen Script, USA) and cloned into the *35S:mcs:ocs* cassette of the pART7 plasmid vector (Gleave, 1992). The expression cassette was subsequently inserted within the left and right borders of the T-DNA, in the pART27 binary vector and transformed into *Agrobacterium tumefaciens GV3101*. These con-

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