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vsiRNAs derived from the miRNA-generating sites of pri-tae-miR159a based on the BSMV system play positive roles in the wheat response to *Puccinia striiformis* f. sp. *tritici* through the regulation of *taMyb3* expression

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ABSTRACT

Plants live in a complex environment, exposed to stresses, such as unsuitable climates, pests and pathogenic microorganisms. Pathogens are one of the most serious factors that threaten plant growth. Wheat stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), is one of the most destructive diseases worldwide. Virus-induced gene silencing (VIGS) is a popular tool for the functional analysis of wheat genes, generating abundant small RNAs (sRNAs). sRNAs are key components in gene regulatory networks, silencing corresponding genes at the post-transcriptional level. In this study, we transduced pri-tae-miR159a into plant tissues using the barley stripe mosaic virus (BSMV) system, and demonstrated that vsiRNAs were generated from the same miRNAs generating sites of pri-tae-miR159a, with the function of Dicer RNase III-like classes of endonucleases (DCL4). In addition, the accumulation of vsiRNAs in wheat leaves challenged with *Pst* Chinese yellow rust 23 (CYR23), resulted in a resistant phenotype, and in the compatible interaction, the sporation of *Pst* was limited. Whereas, infection with a control construct had no effect on the resistance or susceptibility. The results of the histological observation also supported these phenotype changes. Interestingly, vsiRNAs were also involved in the interactions between wheat and *Pst* through the tae-miR159-mediated regulation of *taMyb3* expression. Moreover, these results also supported the speculation that vsiRNAs were generated from the same sites of pri-tae-miR159a. These studies indicated that vsiRNAs from miRNAs generating sites of pri-tae-miR159a based on the BSMV system play positive roles in the wheat response to *Pst* through the regulation of *taMyb3* expression.

1. Introduction

Plants live in a complex environment, exposed to stresses, such as unsuitable climate, pests and pathogenic microorganisms. Pathogens are one of the most serious factors that threaten plant growth. Wheat stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), is one of the most destructive plant diseases worldwide. Stripe rust greatly reduces or even completely destroys wheat yield, depending on the extent of the disease. To reduce the damage, plants have evolved many adaptive response mechanisms to improve tolerance and resistance at the transcriptional, post-transcriptional and post-translational levels [1]. Post-transcriptional gene silencing (PTGS) was first implicated in plant defense toward pathogen aggression [2]. In recent studies, small RNAs (sRNAs) have been implicated as key components in gene regulatory networks through silencing corresponding genes at the post-transcriptional level in most eukaryotic genomes. Based upon their origins, structures, associated effector proteins, and biological functions, sRNAs are classified into three major categories: microRNAs (miRNAs), small interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs) [3].

miRNA are a class of 19–24 nucleotide (nt) non-coding small RNAs. In plants, mature miRNAs are processed from RNA transcripts

Abbreviations: BSMV, barley stripe mosaic virus; CYR, Chinese yellow rust; DCL, Dicer RNase III-like class of endonucleases; dsRNA, double-stranded RNA; hpi, hours post inoculation; miRNAs, microRNAs; piRNAs, piwi-interacting RNAs; *Pst*, *Puccinia striiformis* f. sp. *tritici*; PTGS, post-transcriptional gene silencing; RISC, RNA induced silencing complex; siRNAs, small interfering RNAs; sRNAs, small RNAs; tasiRNAs, transacting siRNAs; VIGS, virus induced gene silencing; vsiRNAs, virus small interfering RNAs.

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folded into a double-strand RNAs (dsRNAs) with a stem-loop structures. The dsRNA stem-loop structures are recognized and sequentially processed by the Dicer RNase III-like class of endonucleases 1 (DCL1) in the nucleus [4]. Once released from the precursor transcript, the miRNA/miRNA* duplex is exported to the cytoplasm. The mature miRNA guide strand is used as an sRNA silencing signal by the RNA-induced silencing complex, inducing local transcript cleavage and subsequent degradation [5]. In most cases, endogenous siRNAs are derived from long double-stranded RNA (dsRNA) with the cleavage of Dicer RNase III-like class of endonucleases 4 (DCL4) in the cytoplasm. siRNAs typically perform auto silencing by targeting the transcript loci from which they derive [3]. Exceptions include the 21 nt transacting siRNAs (tasiRNAs), which are generated from primary RNA transcripts from non-coding TAS genes. The known TAS transcripts are initially targeted and cleaved, post-transcriptionally through a miRNA-programmed silencing complex [6,7].

Large amounts of siRNAs, derived from the dsRNA accumulated during virus replication, trigger virus-induced gene silencing (VIGS), and the subsequent antiviral responses ultimately result in specific RNA degradation [8,9]. In recent years, the VIGS system has been widely used in dicotyledonous plants, such as *Nicotiana benthemiana*, tomato and potato [8–10]. For monocotyledonous plants, barley stripe mosaic virus (BSMV) has recently been adapted as a VIGS vector [11,12] and used extensively in the functional analysis of disease resistance genes in barley [13–17]. No active wheat transposons have been discovered, and the transgenic systems for wheat exhibit defects due to the large genome of this plant. Taken together, these characteristics make VIGS a useful tool for the analysis of gene function, which does not require the generation of transgenic plants [18,19].

In plant viruses, there are several types of RNAs that might account for dsRNA production. Typically, abundant copy replication intermediates of virus RNA is observed. These replication intermediates might form perfect long dsRNAs molecules that constitute an obvious substrate for DCLs [20]. Indeed, virus small interfering RNAs (vsiRNAs) from *Grapevine fleck virus* are primarily derived from a viral strand and not replication intermediates, suggesting that dsRNA-like secondary structures within single-stranded viral RNA are more likely to constitute the primary source of vsiRNAs in infected tissues than dsRNA replication intermediates [21].

miR159 is a highly abundant miRNA that regulates MYB transcription factors involved in plant development [22]. In addition, the biogenesis of miR159 is processed through a non-canonical pathway. The biogenesis of miR159 begins with the cleavage of a loop, instead of the usual cut at the base of the stem-loop structure [23]. The tae-miR159 of wheat also showed a consistent feature [24]. In 2010, a study showed that the miR159a precursor of *Phaseolus vulgaris* encoded two differentially expressed miRNAs [25]. Importantly, the expression of miR159, which is involved in regulating plant gene expression, could be sequestered through a virus-encoded post-transcriptional gene silencing suppressor that manipulates the cell system and promotes the development of diseases in plants [26].

Here, we identified a novel class of vsiRNAs generated from the same miRNAs generating sites of pri-tae-miR159 based on the BSMV system, and demonstrated these vsiRNAs play positive roles in the wheat response to *Pst* through the regulation of *taMyb3* expression.

2. Results

2.1. vsiRNAs were generated from the same miRNAs generating sites of pri-tae-miR159 effectively

We sampled wheat leaves inoculated with BSMV-pri-tae-miR159a and BSMV (control). The DNA probe, designed according to the sequence of the mature tae-miR159, was used for northern blotting. In the control, a signal was also detected at the 21 nt position as the expression of ta-miR159. While, the signal at the 21–24 nt position in BSMV-pri-tae-miR159a line was stronger than that in the control, suggesting that the sRNAs in viral-infected tissues were not miRNAs derived from the pri-tae-miR159a. The control gene U6 exhibited stable expression (Fig. 1).

As plant miRNAs are processed through DCL1 in the nucleus, the RNA virus is replicated in the cytoplasm. Therefore, we detected the relative expression of DCL1 and DCL4, which plays an important role in the generating of miRNAs and siRNAs. As shown in Fig. 2, the relative expression of DCL4 was approximately 5 times greater in the BSMV and BSMV-pri-tae-miR159a lines than that in the leaves without virus inoculation (CK). The accumulation of DCL1 showed no significant change among the CK, BSMV and BSMV-pri-tae-miR159a lines.

Hence, we concluded that the vsiRNAs were derived from the same region near the cleavage sties of the pri-tae-miR159 as tae-miR159 with the function of DCL4.

2.2. vsiRNAs play positive roles in the wheat response to P. striiformis f. sp. tritici through the regulation of taMyb3 expression

Wheat stripe rust is a serious disease worldwide. The interactions between wheat and *Pst* are complex. Whether the accumulation of these vsiRNAs affects the response of wheat to *Pst* remains unknown.

As shown in Fig. 3, the BSMV-inoculated plants displayed mild chlorotic mosaic symptoms at 10 dpi, but had no obvious defects on further leaf growth. Conspicuous HR was elicited through *Pst* race CYR23 on leaves of plants pre-infected with CK and BSMV at 14 dpi, so the virus did not affect the process of *Pst* infection. At 14 days post-inoculation with *Pst* race CYR23, smaller necrotic spots were observed on the leaves of wheat pre-inoculated with BSMV-pri-tae-miR159a. In the compatible interaction, fungal sporulation was limited on the leaves of seedlings pre-inoculated with BSMV-pri-tae-miR159a. Thus, the accumulation of vsiRNAs results in enhanced wheat resistance to *Pst*.

To further observe the detailed histological changes associated with enhanced resistance to *Pst* in vsiRNAs-generated plants, the leaf segments from plants inoculated with CYR23 and CYR31 were sampled for histological observation. At 120 hpi, the proportion of the necrotic areas in wheat leaves pre-inoculated with BSMV-pri-tae-159a was significantly smaller than that in leaves pre-inoculated with BSMV ($P < 0.05$). The hyphal lengths of *Pst* in leaves pre-inoculated with BSMV-pri-tae-159a were significantly shorter than those of the control at 120 hpi ($P < 0.05$) (Table 1 and Fig. 4). In the compatible interaction, the proportion of the necrotic areas in wheat leaves pre-inoculated with BSMV-pri-tae-159a was significantly higher than that of the control at 120 hpi ($P < 0.05$). The hyphal branches of *Pst* in leaves pre-inoculated with BSMV-pri-tae-159a were significantly greater than those of the control at 48 hpi ($P < 0.05$). The hyphal lengths of *Pst* in leaves pre-inoculated

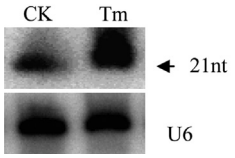

Fig. 1. Validation of vsiRNA in wheat leaves inoculated with BSMV-pri-tae-miR159a. CK, leaves injected with BSMV; Tm, leaves injected with BSMV-pri-tae-miR159a. U6 was selected as a control gene.