Brief Communication

**SlymiR482e-3p mediates tomato wilt disease by modulating ethylene response pathway**

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Fusarium oxysporum f. sp. Lycopersici, a necrotrophic pathogen, is a causal agent of tomato wilt disease. Plants have two major sophisticated innate immune systems, Pathogen-Associated Molecular Pattern (PAMP)-triggered immunity (PTI) and Effector-Triggered Immunity (ETI), to perceive and resist pathogen infections (Jones and Dangl, 2006). MicroRNAs (miRNAs) contribute to PTI and ETI by fine-tuning plant hormones and/or silencing the genes involved in pathogen virulence by regulating the expression of target genes, thereby acting as crucial regulators of the plant immune system (Fei et al., 2016). Many plants produce microRNAs belonging to the miRNA482/2118 superfamily. These miRNAs target R-genes of the class NBS-LRR (nucleotide-binding site-leucine rich repeat) through recognizing the P-loop motif in the NBS-LRR mRNA. Our previous studies showed that SlymiR482e-3p, a member of the miR482/2118 superfamily in tomato, negatively regulated the resistance to *Fusarium oxysporum* f. sp. *lycopersici* (race 2) (Fol) by targeting several NBS-LRR genes (Ouyang et al., 2014). However, the exact mechanism underlying the basic function of SlymiR482e-3p during the response to Fol attack needs further exploration. In this study, two near-isogenic tomato cultivars, Moneymaker (susceptible) and Motelle (resistant) (Figure 1b), were greatly decreased in the presence of Fol infection, were recruited (Ouyang et al., 2014).

To characterize the functions of SlymiR482e-3p in response to tomato wilt disease, we generated a CRISPR/Cas9-related knock-out mutant lacking the SlymiR482e-3p gene in the susceptible cultivar Moneymaker (Deng et al., 2018). Three regenerated plants, termed as SlymiR482e-3p-KO-Line 3, 7 and 11, carried 2-, 9- and 6-nucleotide deletion in front of the mature miRNA region respectively, were identified (Figure 1a). Compared with the control, the expression levels of SlymiR482e-3p was dramatically reduced by more than 90% in individual transgenic plants (Figure 1b). SlymiR482e-3p has been proved as a negative regulator for several targeted NBS-LRR genes, including Soly08g075630 and Soly08g076000 in tomato (Ouyang et al., 2014). As expected, basal expression levels of both Soly08g075630 and Soly08g076000 were increased in all transgenic Moneymaker plants (Figure 1b). Furthermore, no visible difference in major agronomic traits, including leaves, flowers and fruits, were observed in transgenic plants compared with the control (Figure 1c).

To further evaluate the function of SlymiR482e-3p in tomato wilt disease susceptibility, we inoculated the SlymiR482e-3p-KO transgenic plants as well as resistant Motelle and susceptible Moneymaker controls with Fol. As gauged, SlymiR482e-3p-KO plants exhibited enhanced resistance to Fol relative to the Moneymaker control while displayed an appearance similar to the treated Motelle plants (Figure 1d). This result further confirms that SlymiR482e-3p functions as a negative regulator of resistance to tomato wilt disease.

We utilized the psRNATarget algorithm (Dai et al., 2018) to predict potential targets of SlymiR482e-3p. Intriguingly, Soly-c11g010660, a homolog of SGT1 (suppressor of the G2 allele of *skp1*), was predicted as a target of SlymiR482e-3p and termed as SISGT1. SGT1 was first reported as a component of the SCF E3 ubiquitin ligase complex in yeast (Kitagawa et al., 1999) and interacted with RAR1 to trigger disease resistance in plants (Azevedo et al., 2002). It has been documented that SGT1 homologs in plants are triggered by various plant defence response pathways, including ethylene-mediated cross-talk between calcium-dependent protein kinases (CDPK) and mitogen-activated protein kinase (MAPK) signalling (Ludwig et al., 2005; Peart et al., 2002). To determine whether SlymiR482e-3p regulate the SISGT1 expression, we conducted an *Agrobacterium*-mediated transient co-expression experiment in *N. benthamiana*, as previously implemented in our laboratory (Ouyang et al., 2014). qRT-PCR data showed that the SISGT1 transcripts were greatly decreased in the presence of SlymiR482e-3p (Figure 1e). Consistently, GFP fluorescence and Western blot assays using an anti-GFP antibody further demonstrated that SISGT1 protein levels were significantly down-regulated in the presence of SlymiR482e-3p (Figure 1f). To identify the cleavage site in the SISGT1 mRNA targeted by SlymiR482e-3p, we performed a 5'-RNA ligase-mediated rapid amplification of cDNA ends (5' RLM- RACE) analysis. The result showed the cleavage site occurred at the 999th nt of the SISGT1 mRNA in 13 out of 14 clones (Figure 1g). AdSGT1 transcripts were strong up-regulated by ethepohon resulting in enhanced disease resistance in tobacco and peanut (Kumar and Kirti, 2015). In this study, SISGT1 was dramatically induced during Fol infection in the susceptible cultivar Moneymaker, meanwhile, the basal level of SISGT1 was elevated in the SlymiR482e-3p-KO mutant possibly resulting in

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the resistance to *Fol* (Figure 1h). Considering all of the above results, we concluded that *Sly*miR482e-3p regulates *SlSGT1* expression by chopping the intact mRNA.

To further understand the role of *Sly*miR482e-3p in mediating resistance to *Fol* in tomato, we constructed and sequenced six RNA-seq libraries, including Moneymaker treated with water (*MM H₂O*) or *Fol* (*MM_Fol*), as well as *Sly*miR482e-3p-KO lines 3 and 7 treated with water or *Fol* (*KO-Line3_H₂O*, *KO-Line3_Fol*, *KO-Line7_H₂O*, and *KO-Line7_Fol*) (The raw sequence data are available in the Genome Sequence Archive in BIG Data Centre.

Figure 1 *Sly*miR482e-3p mediates tomato wilt disease by modulating ethylene response pathway. (a) Clustal nucleic acid sequence alignments of *Sly*miR482e-3p-KO plants. The sequence of mature miRNA is highlighted with grey. (b) Expression of known targets of *Sly*miR482e-3p in KO plants. (c) Agricultural phenotypic traits of *Sly*miR482e-3p-KO plants. (d) Knock-out of *Sly*miR482e-3p enhances the resistance to *Fol* in Moneymaker. Two-week-old seedlings of the indicated control or transgenic plants were treated with water or *Fol* and photographed 2 weeks later. Red arrows indicating vascular tissue. (e) Level of target mRNAs. qRT-PCR was used to determine relative levels of *SISGT1* in *Nicotiana benthamiana* leaves expressing target mRNA and empty vector, target mRNA and the appropriate miRNA, target mRNA and a control miRNA (*Sly*miR166). Values were normalized to *N. benthamiana* actin. Asterisks indicated significant differences (*P* < 0.05, **P** < 0.01). (f) *SISGT1* protein level was detected by Western blot using anti-GFP antibody. (g) The cleavage site in the *SISGT1* mRNA was determined using 5′ RLM-RACE. The arrow indicates the 5′ terminus of miRNA-guided cleavage products and the frequency of clones (13/14) was shown. (h) Accumulation of *SISGT1* during a time course in different cultivars and transgenic tomato plants infected with *Fol*. (i) *Sly*miR482e-3p regulates ethylene signalling by suppressing the expression of *SIERFs*. Total RNA was isolated from tomato seedling root at 24 hpi and subjected to qRT-PCR to evaluate expression with gene-specific primers. (j) Effect of exogenous ethylene on *Fol* defence in wild and *Sly*miR482e-3p-KO plants. Two-week-old tomato seedlings were inoculated with *Fol* for 30 min followed by the first spraying with 1-Aminocyclopropane-1-carboxylic acid (*ACC, 100 μM*). All plants were treated three times with an interval of 24 h (Left). Accumulation of *Fol* in tomato stems as visualized by staining with lactic acid phenol Medan dye (Middle). Red arrows indicate vascular tissue boundary of stems with extensive staining. Recovery of *Fol* from tomato stem slices incubated on PDA medium (Right). (k) Model for *Sly*miR482e-3p-mediated ethylene signalling in promoting resistance to *Fol*. During fungal pathogen *Fol* invasion, down-regulated endogenous *Sly*miR482e-3p releases *SISGT1* accumulation, triggering CDPK-depending programmed cell death (PCD). Consequently, *SIERFs*, components of the ethylene signalling transduction pathway, are repressed to enhance the resistance to tomato wilt disease caused by *Fol*. © 2020 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 19, 17–19.
under accession numbers CRA002427). Intriguingly, we determined that genes in several phytohormone signalling pathways, particularly the ethylene (ET) signal transduction pathway, may participate in the response to Fol infection in tomato. To evaluate further a possible role for SlymiR482e-3p in regulating ethylene signalling, we monitored expression of key genes in the pathway in tomato plants after inoculation with Fol spores or water over a 24 h period. The basal expression levels (water control) of SIERF1, SIERF3, SIERF4, SIERFS, SIERF9, and SIERF11 were depressed in all SlymiR482e-3p-KO plants relative to Moneymaker. However, all these genes except SIERF3 were induced after Fol infection in both Moneymaker and SlymiR482e-3p-KO plants (Figure 1i). These results prompted us to speculate that the ethylene signal transduction pathway might be important during the response to Fol infection. We next asked whether application of a precursor of ET biosynthesis, 1-Aminocyclopropane-1-carboxylic acid (ACC), would exacerbate wilt disease symptoms. For these experiments, WT and transgenic plants were treated with Fol (ACC), would exacerbate wilt disease symptoms. For these of ET biosynthesis, 1-Aminocyclopropane-1-carboxylic acid in tomato plants after inoculation with Fol spores or water over a 24 h period. The basal expression levels (water control) of SIERF1, SIERF3, SIERF4, SIERFS, SIERF9, and SIERF11 were depressed in all SlymiR482e-3p-KO plants relative to Moneymaker. However, all these genes except SIERF3 were induced after Fol infection in both Moneymaker and SlymiR482e-3p-KO plants (Figure 1i). These results prompted us to speculate that the ethylene signal transduction pathway might be important during the response to Fol infection. We next asked whether application of a precursor of ET biosynthesis, 1-Aminocyclopropane-1-carboxylic acid (ACC), would exacerbate wilt disease symptoms. For these experiments, WT and transgenic plants were treated with Fol (optimal concentration was determined through our preliminary experiments). After ACC treatment, all tomato plants displayed aggravated wilt disease symptoms and faster disease progression compared to treatment with Fol alone (Figure 1j). Particularly, ACC overrode the resistance to Fol infection in Motelle against Fol (Figure 1j).

In summary, we present evidence that supports a key role of SlymiR482e-3p-mediated ethylene signalling in promoting resistance to a fungal necrotroph Fol. We propose that during fungal pathogen Fol invasion, endogenous SlymiR482e-3p promotes SGT1 accumulation, thereby triggering CDPK-depending PCD in tomato. Consequently, SIERFs, components of the ethylene signalling pathway are regulated to enhance resistance to tomato wilt disease (Figure 1k). Our research provides a basis to elucidate the complex SlymiR482e-3p-mediated resistance to Fol in tomato, which will be beneficial for the design of strategies to improve tomato wilt disease resistance.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

SQO designed the experiments. SQO contributed to data analysis and interpretation and wrote the paper. KAB contributed to design this project and revised this manuscript. YG and SJL performed the experiments in cooperation with SWZ, TF, ZYZ, SJL and HYM. All authors read and approved the final manuscript.

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