Tomato IncRNA23468 functions as a competing endogenous RNA to modulate NBS-LRR genes by decoying miR482b in the tomato-Phytophthora infestans interaction

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Abstract
Our previous studies indicated that tomato miR482b could negatively regulate the resistance of tomato to Phytophthora infestans and the expression of miR482b was decreased after inoculation with P. infestans. However, the mechanism by which the accumulation of miR482b is suppressed remains unclear. In this study, we wrote a program to identify 89 long noncoding RNA (IncRNA)-originated endogenous target mimics (eTMs) for 46 miRNAs from our RNA-Seq data. Three tomato IncRNAs, IncRNA23468, IncRNA01308 and IncRNA13262, contained conserved eTM sites for miR482b. When IncRNA23468 was overexpressed in tomato, miR482b expression was significantly decreased, and the expression of the target genes, NBS-LRRs, was significantly increased, resulting in enhanced resistance to P. infestans. Silencing IncRNA23468 in tomato led to the increased accumulation of miR482b and decreased accumulation of NBS-LRRs, as well as reduced resistance to P. infestans. In addition, the accumulation of both miR482b and NBS-LRRs was not significantly changed in tomato plants that overexpressed IncRNA23468 with a mutated eTM site. Based on the VIGS system, a target gene of miR482b, Solyc02g036270.2, was silenced. The disease symptoms of the VIGS-Solyco2g036270.2 tomato plants were in accordance with those of tomato plants in which IncRNA23468 was silenced after inoculation with P. infestans. More severe disease symptoms were found in the modified plants than in the control plants. Our results demonstrate that IncRNAs functioning as eTMs may modulate the effects of miRNAs in tomato and provide insight into how the IncRNA23468-miR482b-NBS-LRR module regulates tomato resistance to P. infestans.

Introduction
MicroRNAs (miRNAs) are noncoding RNAs of 20–24 nucleotides¹ that transcriptionally and post-transcriptionally regulate gene expression in various biological processes of plants². Many miRNAs act in plant responses to various biotic challenges. For example, miR482, belonging to the miR482/2118 family, functions in various plants³,⁴. After Verticillium dahliae infection, the accumulation of miR482e in potato significantly decreases⁵. Similarly, after infection with cucumber mosaic virus and V. dahliae, the expression of miR482 is suppressed in tomato and cotton, respectively⁶,⁷. To show the biological function of miRNAs, identification of their target genes is necessary and important⁸. MiR482 can silence members of the NBS-LRR gene family⁴. An NBS-LRR protein is a disease resistance (R)
protein that is involved in the effector-triggered immunity (ETI) of the plant innate immune system. An NBS-LRR protein contains a nucleotide-binding site (NBS), a leucine-rich repeat (LRR) and a toll-interleukin receptor-like (TIR) domain or a coiled coil (CC) domain. An NBS-LRR gene, GbRVd, was cloned and characterized in *Gossypium barbadense*, and its silencing enhances the susceptibility of *G. barbadense* to Verticillium wilt. The resistance of *Nicotiana benthamiana* to *Phytophthora parasitica* is effectively enhanced by the overexpression of the grapevine TIR-NB-LRR gene VaRGA. Plants infected with pathogens show an increased level of NBS-LRR transcripts and reduced level of miR482. Potato miR482 acts by suppressing NBS-LRR genes to regulate the potato resistance against *V. dahliae* infection.

Recently, long noncoding RNA (lncRNA), another type of ncRNA, has been identified and analyzed in various biological processes. LncRNAs are a set of RNA transcripts (>200 nt length) that have no protein-coding ability. LncRNAs play important roles in flowering time regulation, fruit development, photomorphogenesis, gene silencing, and biotic and abiotic stress responses. In plant-pathogen interactions, using an RNA sequencing approach, 125 putative stress-responsive lncRNAs that are induced by powdery mildew infection have been identified in wheat. A number of lncRNAs have been identified in Paulownia, which lncRNAs, as competing endogenous RNAs (ceRNAs), suppress the accumulation of miRNAs in the tomato–*P. infestans* interaction are unknown. To determine whether lncRNAs can suppress miR482b accumulation in tomato resistance to pathogen infection, RNA-Seq data were used to identify and characterize a number of lncRNAs, and bioinformatics analysis was used to predict the endogenous target mimics (eTM) of these lncRNAs. We found that lncRNA23468 modulated the accumulation of NBS-LRRs by suppressing miR482b expression in tomato plants infected with *P. infestans*. These results will improve our understanding of the regulatory mechanism of miR482b in the response of tomato to *P. infestans* infection and help future molecular-based breeding approaches of pathogen resistance.

**Materials and methods**

**Bioinformatics pipeline for identifying IncRNAs**

To identify the IncRNAs, we used two RNA-Seq datasets obtained from our previous studies. These two datasets were constructed by LC Biotech, Hangzhou, China, using the leaves of miR482b-overexpressing and Zaofen No. 2 tomatoes. The clean reads were *de novo* assembled using Cufflinks. TopHat was used to align assembled transcripts to the tomato genome iTAGv2.3 (http://phytozome.jgi.doe.gov/pz/portal.html?aln=Org_Slycopersicum). All transcripts were required to be more than 200 bp in length. The IncRNAs were identified according to the method of Cui et al. According to their genomic locations, the IncRNAs were classified into four categories. The fragments per kilobase of exon per million fragments mapped (FPKM) value was applied to represent the normalized expression value of the IncRNAs.

**Prediction of ceRNAs**

All tomato miRNAs were collected from miRBase (http://www.mirbase.org/), and the IncRNAs identified above were used as the ceRNA prediction libraries. CeRNAs for the selected tomato miRNAs were predicted using RNAhybrid software with the following rules: (i) P-value < 0.05 and minimum free energy (mfe) <-25 kcal/mol; (ii) bulges were only permitted at the ninth to 12th positions of the 5' end of a miRNA sequence; (iii) the bulge in ceRNAs should be composed of 2–4 nucleotides; (iv) G/U pairs were allowed with the ceRNA and miRNA pairing region, and perfect nucleotide pairing was required at the second to eighth positions of the 5' end of the miRNA sequence; and (v) except for the central bulge, the total mismatches within the ceRNA and miRNA pairing regions should be no more than four, with no more than two consecutive mismatches. Interaction networks among the ceRNAs and miRNAs were constructed using the software Cytoscape. WebLogo software was used to analyze the conserved residues (http://weblogo.berkeley.edu/logo.cgi).
Tomato material and *P. infestans* inoculation

Tomato Zaofen No. 2, bred by the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China, is an accession susceptible to *P. infestans*. The tomato was grown in a greenhouse under 16 h light within a temperature range of 22–28 °C. *P. infestans* strain P12103 was cultured in oat medium in the dark at 20 °C. The tomato plants (4–5-leaf stage) were inoculated with *P. infestans* spores according to the method of Jiang et al. The whole fifth leaves of each sample were collected at the indicated times (0, 1, 2, 3 and 4 dpi). All samples were quickly frozen in liquid nitrogen and stored at −80 °C until RNA isolation.

Cloning of lncRNA23468, mutation of lncRNA23468 and construction of the overexpression plasmid

According to the tomato genome and lncRNA prediction results, a pair of primers (l23468F and l23468R) were designed and used to clone lncRNA23468 from tomato plant (Table S1).

We introduced six point mutations to lncRNA23468 within sequences pairing with miR482b. LncRNA23468 mutation was generated by PCR, which involved amplification and mutagenesis using lncRNA23468 as the backbone. Two more primers were used for this: ml23468-1R and ml23468-2F (Table S1). Three rounds of PCR were performed to amplify the mutated lncRNA23468 (mlncRNA23468). First, the primers l23468F and ml23468-1R were used to amplify a fragment containing mutation points, and then ml23468-2F and l23468R were used to amplify another fragment. Finally, the PCR products of the first and second rounds were used as the template along with l23468F and l23468R. The PCR fragments of lncRNA23468 or mlncRNA23468 were subcloned into binary vector pBI121, replacing the GUS gene. In plasmids, lncRNA23468 and mlncRNA23468 were controlled by the Cauliflower mosaic virus (CaMV) 35S promoter.

Virus-induced gene silencing (VIGS) constructs

TVR-based vectors (pTRV1 and pTRV2), which were provided by Prof. Liu from Tsinghua University of China, were used for VIGS. The VIGS sequence was designed according to the SGN VIGS Tool (http://vigs.solgenomics.net/) and cloned into the pTRV2 vector by the ligation-independent cloning method.

Agrobacteria infiltration

All the plasmids were transformed into *A. tumefaciens* strain GV3101 by the freeze-thaw method. Agrobacteria infiltration was performed according to Jiang’s method.

*A. tumefaciens* containing pBI-121-lncRNA23468 or pBI-121-mlncRNA23468 plasmids was introduced into the leaves of Zaofen No. 2 tomato by infiltration. *A. tumefaciens* with an empty vector was used as a control. The leaves were harvested for the next experiments at 3 dpi.

In the tobacco (*N. tabacum*) system, we introduced *Agrobacterium* harboring pBI121-miR482b into tobacco leaf cells. After 3 days, the *Agrobacterium* harboring pBI121-lncRNA23468 and mlncRNA23468 were introduced into the tobacco leaves that expressed miR482b. The accumulation of miR482b was examined.

Agrobacterium cultures containing pTRV2 derivatives and pTRV1 were mixed at a 1:1 ratio and then infiltrated into a 2-3-leaf-stage tomato. The pTRV2 empty vector was used as the negative control. The plants were maintained for 3 weeks in a greenhouse under 16 h light within 20 °C, and leaflets were harvested from several plants for the isolation of RNA and qRT-PCR analysis to assess the degree of silencing.

*P. infestans* resistance analysis

For the tomato plants that overexpressed lncRNA23468 or mlncRNA23468, the infiltrated leaf regions were inoculated with 20 μl of *P. infestans* (10⁶ zoospores/ml) and then were placed at 20 ± 1 °C in a 100%-relative-humidity environment without light. The lesions were observed at the fifth day, and the sizes of the lesions were also calculated.

3 weeks after VIGS, detached leaves from the VIGS tomato plants were inoculated with 20 μl of the *P. infestans* zoospore suspension (1 × 10⁶ zoospores/ml) according to Jiang’s method. The whole plants were sprayed to run-off with the same zoospore suspension by the method described above. At 5 dpi, the diameters of the lesions and the abundance of *P. infestans* were calculated according to Jiang’s method.

The disease index (DI) was calculated according to disease grade (DG). The DGs were sorted from 0 to 6 on the basis of the lesion area (Table S2). The DI was calculated according to the following formula:

\[
\text{DI} = \left(\frac{\sum(DG_i \times n_i) \times 100}{n \times DG_{\text{max}}}\right)
\]

Where DGi is the value of DG, \(n_i\) is the number of plants in each DG, and \(n\) is the total number of plants. Each experiment was carried out at least three times.

RNA isolation, reverse transcription, and qRT-PCR analysis

Total RNA was extracted using RNAiso Plus (TaKaRa, Dalian, China). Reverse transcription and the qRT-PCR reactions of miRNAs were performed with TransScript Green miRNA Two-Step qRT-PCR SuperMix (Transgen Biotech, Beijing, China) according to the manufacturer’s instructions. We used PrimeScriptTM RT Master Mix (TaKaRa, Dalian, China) to synthesize the cDNAs of the mRNAs and lncRNAs. The qRT-PCR reactions were
performed by using a SYBR Premix Ex Taq™ II kit (TaKaRa, Dalian, China). The qRT-PCR reactions of the selected genes, miRNAs and lncRNAs were performed on an ABI7500. The tomato actin gene was used as an internal reference gene. All primer sequences are shown in Table S1. The gene sample Ct values were standardized, and the 2−ΔΔCt method was used to analyze the relative changes in expression. Of the nine leaves sampled in each experiment, three leaves were pooled into one biological replicate, resulting in three biological replicates.

Statistical analysis
All statistical analyses of the data were performed with SPSS19.0, and all data were expressed as the means ± SEs from three independent experiments. We used the Tukey method to estimate significance.

Results
Identification of lncRNAs
Two of our group’s RNA-Seq datasets, miR482b-overexpressing (OE482) and Zaofen No. 2 tomatoes (Slz), were used to identify the expressed lncRNAs. After assembly and mapping to the tomato genome, the non-coding transcripts were considered “putative” lncRNAs and filtered according to length, coding potentials and coverage of reads. From these analyses, approximately 9742 unique lncRNAs were obtained in two samples, and 9562 of these lncRNAs were expressed in the OE482 sample (Table S3). Among these lncRNAs, 6,785 were natural antisense transcripts (x); 2,669 were long intergenic noncoding RNAs (u); 72 were generic exonic overlaps with a reference transcript (o); and 36 were potentially novel isoforms (j) in the OE482 sample (Fig. 1a). These lncRNAs were evenly distributed across the 12 chromosomes in tomato (Fig. 1b).

Identification of ceRNAs
LncRNAs may act as ceRNAs via the eTMs of miRNAs. Therefore, a program was written to identify the lncRNAs acting as eTMs of miRNAs in tomato. In total, 89 lncRNAs were found to be bound by 46 miRNAs (Table S4). Interaction networks showed that 38 miRNA-lncRNA duplexes were formed (Fig. 1c). In these duplexes, miR482d-5p was putatively sequestered by 16 lncRNAs, and lncRNA34658 decoyed three miRNAs, including miR168a-5p, miR168b-5p, and miR477-3p. In addition, more than one-third of the regulated relationships had only two nodes, as shown in the networks in Fig. 1c.

The eTMs of miR482b and expression analysis of miR482b ceRNAs after infection with P. infestans
The predicted binding sites of miR482b among these lncRNAs (lncRNA23468, lncRNA13262 and
lncRNA01308) were well conserved (Fig. 2a). In our prediction, a two or three-nucleotide bulge on the eTMs located between the ninth and 12th positions at the 5′ end of miR482b was required for the eTMs to decoy miR482b (Fig. 2b). The minimum free energies (mfes) were \(-33.8\) kcal/mol, \(-35.8\) kcal/mol and \(-33.2\) kcal/mol in lncRNA13262-miR482b, lncRNA01308-miR482b and lncRNA23468-miR482b, respectively. The values of the mfes in lncRNA13262-miR482b and lncRNA01308-miR482b were close to that in lncRNA23468-miR482b. This suggests that the abilities of the three lncRNAs to suppress miR482b were not significantly different.

To explore whether the ceRNAs of the miR482b, lncRNA23468, lncRNA13262 and lncRNA01308 had an effect on tomato response to *P. infestans*, the relative levels of accumulation were examined in tomato leaves infected by *P. infestans*. These three lncRNAs responded to *P. infestans* infection. The changes in the expression of both lncRNA23468 and lncRNA01308 followed the same pattern. Both first increased and then decreased, reaching a peak at 2 days after *P. infestans* infection. LncRNA13262 was downregulated from 0 dpi to 1 dpi and then reached a peak at 2 dpi (Fig. 2c).

**Construction of the lncRNA23468 mutant**

To examine whether lncRNA23468 indeed functioned via pairing with miR482b, a six-point mutation was introduced into lncRNA23468 by using sequences that paired with miR482b (mlncRNA23468; Fig. 3a). The design of the mlncRNA23468 is shown in Fig. 3b. The eTM of miR482b (5′-AGAUUGGCCGGUAGGUAAAG-3′) in lncRNA23468 was replaced with the mutation sequence (5′-AGAUUCGCCCGAAUAGCUAUCA-3′) by oligonucleotide-directed mutagenesis. After three rounds of PCR, the sequence of mlncRNA23468 was amplified (Fig. 3c). As shown in Fig. 3d, the sequencing results showed that six point mutations were introduced into the eTM from lncRNA23468. In addition, except for the eTM region of miR482b, the lncRNA23468 and mlncRNA23468 sequences were conserved after sequence alignment analysis (Fig. 3e). Overall, mlncRNA23468 was successfully constructed by using lncRNA23468 as the backbone.

**LncRNA23468 as a miRNA decoy suppresses the expression of miR482b**

We investigated the possible function of lncRNA23468 using infiltration to upregulate lncRNA23468 expression...
in Zaofen No. 2 tomato. The plasmids for the overexpression of lncRNA23468 and mlncRNA23468 were carried out on the basis of the pBI121 vector (Fig. 4a). The introduction of A. tumefaciens harboring pBI121-lncRNA23468 and pBI121-mlncRNA23468 into tomato leaf cells resulted in significant upregulation of lncRNA23468 and mlncRNA23468 expression at 3 days: the expression levels of lncRNA23468 and mlncRNA23468 were approximately 6.1-fold and 6.0-fold greater than the tomato leaves that overexpressed empty vector (EV), respectively (Fig. 4b). The expression of miR482b was dramatically suppressed to approximately 40% in the tomato plants that overexpressed lncRNA23468 (OE23468), and the target genes of miR482b, NBS-LRRs, were significantly increased (Fig. 4b). The levels of the transcripts of Solyc02g036270.2, Solyc04g009070.1, Solyc05g008070.2 and Solyc12g016220.2 in the leaves of the OE23468 tomato plants were approximately 2.9-fold, 2.1-fold, 2.4-fold, and 2.1-fold, respectively, to the values in the leaves of the EV tomato plants. The overexpression of mlncRNA23468 in tomato (mOE23468) did not cause expression changes in either miR482b or its target genes (Fig. 4b).

In our previous work, miR172 and miR396 were identified to play important roles in tomato resistance to P. infestans. Five days after infection, the physical appearance of these tomato plants was assessed. Compared with the severe disease symptoms that appeared on the leaves of EV plants, the disease symptoms on the leaves of mOE23468 plants were similar, while fewer disease symptoms were exhibited on the leaves of OE23468 plants (Fig. 4c). The diameter of the lesion was significantly smaller in the leaves of P. infestans-infected OE23468 plants than in those of EV or mOE23468 plants (Fig. 4d).

As overexpression is not enough to fully understand lncRNA functions, lncRNA silencing should be performed to explore these biological functions. LncRNA23468 was silenced in Zaofen No. 2 tomato by VIGS. The empty TRV2 vector and PHYTOENE DESATURASE (PDS)-VIGS were used as the negative control and the positive control, respectively. At 21 days of VIGS treatment, the PDS-VIGS tomato showed visible bleaching, indicating that the IncRNA23468 gene was also silenced (Fig. S2).

The expression of IncRNA23468 was suppressed to approximately 40%, and the accumulation of miR482b was upregulated approximately 2-fold, while the four target genes showed decreased abundance (Fig. 5a).
The disease phenotypes for the control (TRV2) and lncRNA23468-VIGS tomato plants were assessed after infection with *P. infestans*. Compared to the leaves of TRV2 tomato plants, the detached leaves of the lncRNA23468-VIGS tomato plants showed more serious symptoms of the disease. The decreased resistance to *P. infestans* in the leaves of lncRNA23468-VIGS tomato plants was also revealed by trypan blue staining, as shown by the increased number of necrotic cells relative to those in the leaves of TRV2 tomato plants (Fig. 5c). In addition, Fig. 5d shows that the leaves of the lncRNA23468-VIGS tomato plants had larger lesions than did the leaves of the TRV2 tomato plants.

The leaves of the lncRNA23468-VIGS tomato plants also exhibited more serious symptoms of the disease after whole-plant inoculation with *P. infestans* (Fig. 5e), with a higher disease index than that observed for the TRV2 tomato plants (Fig. 5f). In addition, there were significant increases in the abundance of *P. infestans* in the lncRNA23468-VIGS tomato plants compared to the TRV2 tomato plants (Fig. 5g). These results suggest that lncRNA23468 suppressed the accumulation of miR482b, resulting in an increased expression of *NBS-LRRs* and enhanced tomato resistance to *P. infestans*.

The regulation between lncRNA23468 and miR482b was also validated using the *Nicotiana* system. We introduced *Agrobacterium* harboring pBI121-miR482b into tobacco leaf cells, which led to a significant increase in miR482b expression at 3 days compared to tobacco leaves overexpressing the empty vector (Fig. S3a). Then, the *Agrobacterium* harboring pBI121-lncRNA23468 and mlncRNA23468 were introduced into the tobacco leaves that overexpressed miR482b. The accumulation of miR482b was significantly decreased after the overexpression of lncRNA23468. The overexpression of mlncRNA23468 did not cause expression changes in miR482b (Fig. S3b). These results suggested that lncRNA23468 as a ceRNA may decoy miR482b.

**Silencing of NBS-LRR enhances tomato susceptibility to *P. infestans***

We also employed the VIGS system to test the role of the target gene of miR482b (Solyco02g036270.2) in tomato resistance to *P. infestans*. At 21 days after hand-infiltration, a further examination of Solyco02g036270.2 indicated that the abundance of transcripts was significantly suppressed in the tomato plants that silenced Solyco02g036270.2 (*NBS-LRR-VIGS*) (Fig. 5b).

More serious disease symptoms were shown in the detached leaves of the *NBS-LRR-VIGS* plants than in the TRV2 plants after infection with *P. infestans* (Fig. 5c). The darker blue marks from trypan blue staining in the detached leaves indicated that a greater number of dead cells occurred in the *NBS-LRR-VIGS* tomato plants than...
in the TRV2 tomato plants (Fig. 5c). Furthermore, these NBS-LRR-VIGS tomato leaves also had larger diameter lesions (approximately 1.91-fold increase compared to TRV2 tomato plants infected with *P. infestans*) (Fig. 5d).

In the whole-plant inoculation assay, the susceptibility of the silenced plants manifested as more serious disease symptoms (Fig. 5e), higher DI (Fig. 5f) and an increased abundance of *P. infestans* (Fig. 5g).

**Discussion**

LncRNA as ceRNA regulates the accumulation of miRNA

MicroRNAs (miRNAs), a class of approximately 22 nt endogenous small noncoding RNAs, can direct the post-transcriptional repression of target genes by base-pairing to mRNAs. The mature miRNA is separated from the miRNA/miRNA* duplex, which is processed from a pre-miRNA.

One focus of miRNA research is the regulation of miRNA accumulation. Various transcription factors can promote the expression of pre-miRNAs at the transcriptional level. For example, in *A. thaliana*, transcription factor APETALA2 (AP2) positively or negatively regulates the expression of miR156 or miR172 by binding to these miRNA genes. Another *Arabidopsis* transcription factor, AtMYB2, has been identified for its roles in the activation of miR399f expression in the context of phosphate homeostasis through sequence-specific interactions with a MYB-binding site in the promoter of the miR399f precursor. In addition, tomato ripening inhibitor (RIN) binds to a RIN-binding site in the promoter of miR172a to regulate its accumulation.

Another regulatory mechanism has been proposed to underlie the accumulation of miRNA. During plant-pathogen interactions, pathogen effectors can suppress host defense. The effectors, RNA-silencing suppressors from the pathogens, affect the resistance of plants by suppressing the accumulation of host miRNAs. *P. sojae* encoded two RNA-silencing suppressors (PsPSR1 and PsPSR2) that enhance plant susceptibility by impairing the small RNA-mediated defense of the host. PsPSR1 virulence target acts in the assembly of sRNA-processing complexes in *Arabidopsis* and soybean. PSR2 is involved...
in modifying plant gene regulation early during *Phytophthora* infection, and the overexpression of *PsPSR2* in *Arabidopsis* enhances hypersusceptibility to *P. capsici*.

Some specific endogenous lncRNAs can act as ceRNAs to interfere with miRNA pathways. Acting as a ceRNA is an effective posttranscriptional regulatory mechanism by which lncRNAs interfere with target transcripts. The disruption of the equilibrium between ceRNAs and miRNAs could be important to ceRNA activity in diseases such as cancer. For example, the lncRNA HOTAIR decoys miR-152-3p to promote malignant melanoma progression. In addition, the lncRNA CRNDE prevents miR-136-5p-mediated downregulation of Bcl-2 and Wnt2 to promote glioma malignancy. However, studies on ceRNA in plants are limited. In maize, a number of lncRNAs have been identified as miRNA decoys by using computational methods. Tobacco eTMX27 inhibits the expression of miRXX7, resulting in enhanced nicotine biosynthesis. *Arabidopsis* IPS1 contains the eTM of ath-miR399 and inhibits the expression of ath-miR399, and PDIL1 reduces the accumulation of miR399, thus regulating Pi-related pathways in *Medicago truncatula*. Wang et al. reported that the eTMs of miR160 from *Tomato* can serve as decoys for miR160 and function as the regulator in rice development.

In this study, we wrote a program that systematically identified 89 lncRNAs as 46 miRNA decoys in tomato (Fig. 1c and Table S4). Three of these lncRNAs, lncRNA23468, IncRNA01308 and IncRNA13262, contained an eTM site of miR482b (Fig. 2b). The expression of these three lncRNAs was initially increased but then decreased with *P. infestans* infection (Fig. 2c). This result was in contrast to the expression trend of miR482b upon infection by *P. infestans*, which was detected in our previous study. The expression of miR482b was decreased in the tomato plants that overexpressed lncRNA23468 in tomato infected with *P. capsici*. Similar results were reported in our previous study, showing that miR482b negatively regulates tomato resistance to *P. infestans*. To understand the biological functions of miRNAs, the identification of their target genes is an important step. In our previous study, four members of the NBS-LRR family were identified as the target genes of miR482b using the degradate data of tomato infected with *P. infestans*. NBS-LRRs are active in the resistance of other plants to pathogens, such as tobacco resistance to *P. parasitica*, wheat resistance to powdery mildew, and rice resistance to blast. In this study, NBS-LRR accumulation was suppressed by silencing lncRNA23468 and VIGS technology in tomato, resulting in the enhancement of tomato susceptibility to *P. infestans* (Figs. 4 and 5). Similarly, cotton susceptibility is also increased by silencing a CC-NBS-LRR gene.

lncRNAs are involved in many biological processes, including biotic stresses. For example, Joshi et al. found that 13 lncRNAs were predicted to be the precursors of 96 miRNAs that affect the *Brassica napus* and *Sclerotinia sclerotiorum* interaction. The silencing of GhIncNAT-ANX2 and GhIncNAT-RLP7 in cotton seedlings can induce LOX1 and LOX2 expression to increase the resistance to *V. dahliae* and *Botrytis cinerea* infection. In addition, many lncRNAs act in the response of *Arabidopsis* to *F. oxysporum* and *Pseudomonas syringae* pv. *tomato* DC3000, wheat response to powdery mildew pathogen, tomato response to TYLCV and *P. tomentosa* response to Paulownia witches’ broom. Tobacco eTMX27 inhibits the expression of LOX1 and LOX2 to stimulate lncRNA23468-induced downregulation of miR482b, resulting in enhanced nicotine biosynthesis.

In summary, lncRNA23468 increases resistance to *P. infestans* in tomato. From our results in the lncRNA23468 silenced, lncRNA23468 overexpressing and mlncRNA23468 overexpressing tomato, we propose that lncRNA23468 increases the expression levels of NBS-LRRs by the repression of miR482b. This mechanism would allow for a response to *P. infestans* stress through lncRNA23468-induced downregulation of miR482b, which increases NBS-LRRs, thus promoting tomato resistance to *P. infestans*. Our results provide insight into an effective posttranscriptional regulation mechanism of...
lnRNA and demonstrate that the lnRNA23468-miR482b-NBS-LRR network is an important component of the P. infestans network in tomato.

Acknowledgements
This work is supported by grants from the National Natural Science Foundation of China (Nos. 31471880 and 61472061). We thank Prof. Weixing Shan from Northwest A & F, University of China, for providing P. infestans strain P12103 and Yule Liu from Tsinghua University for providing the VIGS system.

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Conflict of Interest
The authors declare that they have no conflict of interest.

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Supplementary Information accompanies this paper at (https://doi.org/10.1038/s41438-018-0096-0).

Received: 8 May 2018 Revised: 7 September 2018 Accepted: 19 September 2018

Published online: 01 February 2019

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