Biochemical and Biophysical Research Communications xxx (2016) 1–5

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Transcriptional and posttranscriptional regulation of the tomato leaf mould disease resistance gene *Cf-9*

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ARTICLE INFO

Article history:
Received 2 January 2016
Accepted 4 January 2016
Available online xxx

Keywords:
Cf-9
Avr9
Transcriptional regulation
Temperature sensitivity
Posttranscriptional regulation
miR6022

ABSTRACT

Plant disease resistance (*R*) genes confer effector-triggered immunity (ETI) to pathogens carrying complementary effector/avirulence (*Avr*) genes. They are traditionally recognized to function at translational and/or posttranslational levels. In this study, however, transcriptional and posttranscriptional regulation of *Cf-9*, a tomato *R* gene conferring resistance to leaf mould fungal pathogen carrying *Avr9*, was demonstrated. Expression of the *Cf-9* gene was 10.8–54.7 folds higher in the *Cf-9/Avr9* tomato lines than in the *Cf-9* lines depending on the seedling age, indicating that the *Cf-9* gene expression was strongly induced by Avr9. Moreover, expression of the *Cf-9* gene in the 5-day-old *Cf-9/Avr9* seedlings at 33 °C was approximately 80 folds lower than that at 25 °C, and was enhanced by 23.4 folds at only 4 h post temperature shift from 33 °C to 25 °C, demonstrating that the Avr9-mediated induction of the *Cf-9* gene expression is reversibly repressed by high temperature. Expression of the *Cf-9* gene in the *Cf-9* seedlings was similarly affected by temperature as in the *Cf-9/Avr9* seedlings, implying that the genetic control of temperature sensitivity of the *Cf-9* gene expression is epistasis to its Avr9-mediated induction. Additionally, a miRNA sly-miR6022, TGGAAGGGAGAATATCCAGGA, targeting the leucine-rich repeat (LRR) domain spanning LRR13-LRR14 of the *Cf-9* gene transcript was predicted. Over-expression of this miRNA resulted in over 88% reduction of the *Cf-9* gene transcripts in both *Nicotiana benthamiana* and tomato, and thus verifying the function of sly-miR6022 in degrading the *Cf-9* gene transcripts. Collectively, our results reveal that the tomato *R* gene *Cf-9* is strongly regulated at transcriptional level by pathogen Avr9 in a temperature-sensitive manner and is also regulated at posttranscriptional level by a miRNA sly-miR6022.

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1. Introduction

Plant disease resistance (*R*) genes confer gene-for-gene resistance or later designated effector-triggered immunity (ETI) to pathogens carrying complementary avirulence (*Avr*) or more broadly effector genes [1–3]. Typical R proteins possess a nucleotide binding site (NBS) domain and a leucine rich repeat (LRR) domain or mainly an extracellular LRR domain. Traditionally, R proteins serve as guards to monitor plants against pathogen infection. They can recognize pathogen attacks by Avrs and subsequently activate defense responses. Therefore, at molecular level, *R* genes are classically recognized to function at protein (translational and/or posttranslational) level [1–4]. Transcriptional regulation of *R* genes by complementary Avrs is rarely reported. Nevertheless, two non-typical *R* genes, rice *Xa27* and pepper *Bs3*, whose products contain neither NBS nor LRR, are transcriptionally regulated by their complementary Avrs, AvrXa27 of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and AvrBs3 of *X. campestris* pv. *vesicatoria* (*Xcv*), respectively [5–7]. *Xa27* encodes an unknown protein [5] while Bs3 is a flavin monooxygenase [6]. Their complementary Avrs, AvrXa27 and AvrBs3 act as TALEs (Transcription activator like effectors), directly bind to the UPT (Upregulated by TALE) box of the *Xa27* and *Bs3* gene promoters, thereby promote their expression and consequently activate defense responses [5–7]. However, transcriptional regulation of typical *R* genes by complementary Avrs remains largely unclear.

In addition, *R* gene regulation at posttranscriptional level has recently been discovered. Several families of miRNAs target diverse plant *R* genes have been identified. Among them are two tobacco

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http://dx.doi.org/10.1016/j.bbrc.2016.01.015
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Please cite this article in press as: W. Li, et al., Transcriptional and posttranscriptional regulation of the tomato leaf mould disease resistance gene *Cf-9*, Biochemical and Biophysical Research Communications (2016), http://dx.doi.org/10.1016/j.bbrc.2016.01.015

miRNAs (nta-miR6019 and nta-miR6020) targeting TMV resistance gene *N* [8], miR482 superfamily targeting various NBS-LRR type *R* genes [9] and others [10–12]. Additionally, a number of miRNAs targeting diverse *R* gene homologs were predicted. For example, miR6022 and miR6023 families were predicted to target *Hcr9-0* genes (Homologue of *Cladosporium fulvum* resistance gene 9) [8]. However, function of these predicted miRNAs awaits experimental confirmation.

Tomato and leaf mould disease pathogen *Cladosporium fulvum* is one of the model pathosystems to study ETI [1,13]. Tomato *Cf* genes confer ETI to *C. fulvum* carrying the complementary *Avrs*. As the first cloned typical fungal resistant *R* gene, *Cf-9* is one of the most studied *Cf* genes. *Cf-9* confers ETI to *C. fulvum* carrying *Avr9*. Recognition of Avr9 by Cf-9 results in strong hypersensitive response (HR), a hallmark of ETI, and this HR is repressed by high temperature and high humidity [14–16]. Nevertheless, the molecular mechanism underlying the interaction between *Cf-9* and *Avr9* remains largely unknown. In this study, we probed the transcriptional and posttranscriptional regulation of *Cf-9*. We demonstrated that the tomato *R* gene *Cf-9* is strongly regulated at transcriptional level by pathogen Avr9 in a temperature-sensitive manner and is also regulated at posttranscriptional level by sly-miR6022. Our findings provide insights into molecular mechanisms underlying the interactions between plant *R* genes and pathogen *Avr* genes.

2. Materials and methods

2.1. Plant materials and sample collection

Tomato cultivar Moneymaker carrying the *Cf-9* gene (hereafter abbreviated as *Cf-9* line) and the cross lines containing both *Cf-9* and *Avr9* genes (hereafter abbreviated as *Cf-9*/*Avr9* line) were used in this study. The *Cf-9*/*Avr9* lines were the F_1 obtained from crossing between *Avr9* transgenic Moneymaker (without carrying any *Cf* gene, and hereafter abbreviated as *Avr9* line) and the *Cf-9* line [14]. Seeds were sown and grown at 25 °C as described [16]. For the temperature shift experiments, seeds were sown and grown at 33 °C for 5 days, and then shifted to 25 °C. Cotyledons of the seedlings were sampled at the time-points stated in the results section and were subjected to *Cf-9* gene expression analyses.

2.2. Gene expression analyses with real time PCR

Gene expression was detected by real time quantitative PCR (RT-qPCR) as described [17]. Briefly, RNA was extracted using RNAiso Plus kit and was used for the first strand of cDNA synthesis with PrimeScript II. RT-qPCR was performed in StepOne Real-Time PCR System (Applied Biosystems, USA) using SYBER Premix Ex Taq reagents (TaKaRa Biotechnology, China) following the program: 95 °C for 30 s, 95 °C for 5 s and 60 °C for 45 s for 40 cycles. To normalize the sample variance, 18S rDNA gene was served as the internal control. Relative gene expression values were calculated using the $2^{-\triangle\triangle Ct}$ method. The gene-specific primers used for gene expression analysis are listed at Table 1.

The experiments were conducted three times, each containing three replicates for all genes. For the statistical analysis of the gene expression data, ANOVA (analysis of variance) analysis was performed with SPSS software (Version 19.0, IBM, USA). Significance of the differences between mean values was determined by Student's *t*-test.

2.3. Prediction of miRNAs targeting Cf-9

MicroRNAs targeting the *Cf-9* gene was predicted using the SoMART database (http://somart.ist.berkeley.edu) as described [18]. Briefly, the *Cf-9* mRNA sequence was input and potential miRNAs targeting this gene were predicted using Slicer Detector program and SLY1 as tomato sequence database. The targets of the predicted miRNAs were confirmed using dRNA Mapper program with degradome database. The pre-miRNA sequences of the predicted miRNAs were obtained using PreMIR Detector program.

2.4. Verification of the miRNA targeting Cf-9

Tomato genomic DNA was extracted using Plant DNAzol reagents (Life Science, China) following the procedure recommended by the manufactory. Pri-miRNA sequence of the predicted miRNA was amplified from tomato genomic DNA while cDNA sequence of its potential target gene *Cf-9* was amplified from tomato cDNA with the primers listed at Table 1, which were then cloned into binary vector pCHF3 with *Kpn* I/*Sal* I and *Bam*H I/*Sal* I, respectively, to release the recombinant constructs pCHF3::pri-miRNA and pCHF3::*Cf-9*. These recombinant plasmids and the empty plasmid as a control were transformed into *Agrobacterium tumefaciens* strain GV3101. Mixtures at 1:1 ratio of *Agrobacterium* suspensions carrying pCHF3::*Cf-9* and those containing pCHF3::pri-miRNA or pCHF3 empty vector with a final OD_{600} of 2.0 for each type of bacteria were prepared as described [19,20], and were infiltrated into *Nicotiana benthamiana* leaves using needleless syringes. For assays in *Cf-9* tomato plants, solely *Agrobacterium* suspensions carrying pCHF3::pri-miRNA or pCHF3 empty vector as a control were infiltrated into tomato leaves. After infiltration, the plants were grown at 25 °C. Expression of the *Cf-9* gene in the agro-infiltrated areas was detected by RT-qPCR at 3 d post infiltration.

3. Results

3.1. Expression of the tomato resistance gene Cf-9 is strongly induced by the leaf mould pathogen effector Avr9

RT-qPCR detection assays using *Cf-9* gene specific primers showed that expression of the *Cf-9* gene in the tomato seedlings (cv. Moneymaker) was mildly enhanced along with the increase of the seedling age. It was 2.4–2.6 folds as high in seedlings at 3–7 days

Table 1
Primers used in this study.

Primer name	Sequence (5′→3′)	Enzyme included	Size of product (bp)
Cf-9-F	AGTGATTGCGCTTGACCT		89
Cf-9-R	GCCTTTTGAGATTGGAGAG		
Sl 18S rDNA-F	GCCGGCGACGCATCATTCAAA		154
Sl 18S rDNA-R	CGCGCCTGCTGCCTTCCTT		
pri-miR6022-F	ggtaccGCCAGAGCTGCAAGGTTTAG	*Kpn* I	1060
pri-miR6022-R	gtcgacAGCATAGTCACTCAGAATCAT	*Sal* I	
miR6022 target-F	ggatccGAAAAGCTCAAGAGGTTATCACT	*Bam* HI	365
miR6022 target-R	gtcgacAATTGTAGGTTCTTCTGGTTTA	*Sal* I	

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