Silencing a putative cytosolic NADP-malic enzyme gene compromised tomato resistance to *Oidium neolycopersici*

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Abstract: Tomato powdery mildew caused by *Oidium neolycopersici* is a worldwide plant fungal disease distributed in Europe, African, South and North America and Asian, which is responsible for a remarkable reduction in quality and yield of tomato. The most effective way to control this disease is resistant breeding, which depends on the resistant materials and resistance genes. In our previous study, a putative enzyme gene (named *ShME1*) was found to be up-regulated in the *Ol-1* mediated tomato resistance response to *Oidium neolycopersici*. In this study, in order to know whether this gene is a key gene of the resistance response, we further analyzed the function of the gene using virus induced gene silencing (VIGS) in resistant tomato plants *Solanum habrochiates* G1.1560 carrying *Ol-1* gene. It was shown that the resistant *S. habrochiates* G1.1560 became susceptible to *O. neolycopersici* after *ShME1* was silenced in it. Microscopic observation demonstrated that *O. neolycopersici* was able to complete its life cycle on silenced resistant plants, it indicated that *ShME1* was required for *Ol-1* mediated tomato resistance against *O. neolycopersici.* It also suggested that *ShME1* could be involved in hypersensitive response (HR) and H_2O_2 accumulation, which was thought to be tightly linked to the establishment of tomato resistance to *O. neolycopersici.*
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1. Introduction

NADP-malic enzyme (NADP-ME, EC1.1.1.40), which is widely distributed in nature, catalyses the oxidative decarboxylation of L-malate to form pyruvate and CO_2 with concomitant reduction of NADP to NADPH (Chang and Tong, 2003). In plants, NADP-ME functions in different metabolic pathways, the best studied isoform of NADP-ME is the one involved in C_4 photosynthesis, which releases CO_2 to be used in carbon fixation by Ribulose-1,5-bisphosphate Carboxylase Oxygenase (RuBisCO). Apart from this specialized role, in C3 plants, the expression of NADP-ME gene were activated by UV-B, wounding, fungal effectors, NaCl, carbonates, environment pH changes etc (Walter etal., 1994; Schaaf etal., 1995; Pinto etal., 1999; Casati etal., 1999; Cheng and Long, 2007; Liu etal., 2007). It showed that NADP-ME was involved in plant defense responses and stress responses.

Powdery mildew caused by the biotrophic fungus, *O. neolycopersici*, has recently become a very important disease of tomato (*Solanum lycopersicum*) worldwide (Jones etal., 2001). Meanwhile, insecticide spraying to control the disease results in serious environmental contamination. In March of 2008, powdery mildew appeared as circular, white colonies on leaves, petioles, and stems of tomato plants grown in greenhouses in Shangqiu, Henan Province, China. It is first reported and identified as *O. neolycopersici* in China (Li etal., 2008).

Although the cultivated tomato is susceptible to the fungus, resistance occurs in many wild species of tomato. Researchers have found six resistance genes (termed *Ol-X*) and three resistance QTLs in tomato wild types, which mediate different resistance responses to *O. neolycopersici* (Lindhout et al., 1994 a&b; Ciccarese et al., 2000; Bai et al., 2003 & 2005). In our previous study, gene expression profiles were investigated of susceptible, monogenic and polygenic resistant tomato genotypes in response to *O. neolycopersici* infection using cDNA-AFLP method (Li etal., 2006 & 2007). Among the identified TDFs (Transcript Derived Fragments), eight hundreds and eighty seven TDFs were differentially expressed TDFs (DE-TDFs) upon *O. neolycopersici* challenge. By annotating the sequenced DE-TDFs, the corresponding transcripts were classified to be involved in plant defense, signal transduction,

regulation, protein synthesis and degradation, energy metabolism, etc (Li etal., 2006).

For further functional characterization of important DE-TDFs, a technology VIGS is exploited. VIGS is a widely used, powerful technique for reverse genetics with methodological simplicity, robustness, and speedy results. Many silencing vectors have been derived from viruses recently. Vectors derived from the *Tobacco rattle virus* (TRV) are among the most popular viruses for VIGS, because it is able to spread more vigorously throughout the entire plant, including meristem tissue, causing mild symptoms of infection compared with other viruses. The improved TRV VIGS vector allows the insertion of gene silencing fragments by ligation-independent cloning (LIC). The vector has several advantages over previous vectors, particularly for large-scale gene function analysis, since TRV-LIC vectors containing the desired insert are obtained with 100% efficiency (Dong etal., 2007).

We generated a collection of silencing vectors in this TRV-LIC background based on gene expression pattern and annotation information of the tomato DE-TDFs. Silencing a DE-TDF among them, which is annotated to putative NADP-malic enzyme, led to the compromise of *Ol-1* mediated resistance to *O. neolycopersici* in *S. habrochaites* G1.1560. Microscopic observation demonstrated the influence of silencing the putative NADP-malic gene on hypersensitive response (HR) and H_2O_2 accumulation in tomato against *O. neolycopersici*.

2. Material and Methods

2.1 Plant materials

Different tomato cultivars and species including *S. lycopersicum* Mill [cvs. Moneymaker (MM), Micro-Tom, Zaofen, Fenguo and Zhongza series], and wide type *S. habrochaites* G1.1560 were used in this study.

2.2 Tomato powdery mildew fungi

The Shangqiu isolate of powdery mildew fungus from tomato plants in Henan Province of China, which was identified as *O. neolycopersici* based on morphological, histological and molecular characteristics, was used as the pathogen.

2.3 Pathogen preservation and inoculation

The *O. neolycopersici* was successfully preserved on tomato plants in climate cell under the condition (20±3℃with 70±5% humidity and a 16h photoperiod). The fresh conidia were collected from infected plant leaves with sterile water. Four-week plants were used for inoculation by spraying a spore suspension of 5×10^4 conidia/ml on the whole plants for the histological study.

2.4 RNA extraction and cloning of target tomato DE-TDF

Total RNA was extracted from leaves of wide type G1.1560 using RNAiso Reagent (TaKaRa). First-strand cDNA was synthesized using 1 ug of total RNA and M-MLV RTase cDNA Synthesis kit (TaKaRa) according to the manufacturer's protocol. The target DE-TDF was amplified with primers: 5'-CGACGACAAGACCCT-gaggcgtgtcaaaaat-3' and 5'-GAGGAGAAGAGCCCT-gctgtcccctgtatatc-3' by RT-PCR（The capital letters stand for the adapter sequence of ligation-independent cloning TRV vector, the lowercase letters stand for the *ShME1* gene specific sequence）.

2.5 Vector construction

RT-PCR products were cloned into TRV2-LIC vector (pYY13). The recombinant TRV2-LIC-*ShME1* vector carrying target sequence was constructed by the protocol as describe by (Dong etal., 2007).

2.6 VIGS agroinfiltration

After successful construction, VIGS TRV1 and TRV2-LIC-*ShME1* were introduced into *Agrobacterium tumefaciens* strain GV3101 by heat shock. 5mL overnight cultures were grown at 28℃ in the appropriate antibiotic selection medium in 15mL glass tube for one day, then cultures were spun down and cells were re-suspended in infiltration medium (10 mM MES, 10 mM MgCl2, 200μM acetosyringone), adjusted to OD600 of 1, and incubated at room temperature for 3 h. Agroinfiltration was performed as previously reported (Liu etal., 2002). We infiltrated the first and second leaves of four-leaf stage plants with a 1:1 mixture of TRV1 and TRV2-LIC-*ShME1* fragment for the clone. The empty vector TRV2-LIC was as control. After 10 days post inoculation (dpi), the plants were inoculated with *O. neolycopersici*. Ten plants were inoculated per trial and three trials were done.

2.7 Semi-quantitative RT-PCR analysis

The degree of silencing of the target gene monitored by semi-quantitative RT-PCR using gene specific primers 5'-CTATTGTCTACACTCCAAC-TGTCG-3' and 5'-ATGCAATAAGCCCTGC-AAGT-3'. Tomato *Actin* gene (GenBank U60480) was as internal control, which was amplified with primers 5'-CCCAAAGGCTAATCGTGAAA-3' and 5' -GCAGCTTCCATTCCAATCAT-3'.

2.8 H_2O_2 accumulation and Microscopic analysis

For detecting H_2O_2 accumulation, the infected leaflets were immersed in the DAB solution (1mg/mL pH3.8) for 8-12 hours till DAB stain can be seen at the vein of leaflet top. Then DAB stained leaflets were fixed and stained with trypan blue as described by Huang and associates (1998). Samples were observed under differential-interference contrast

microscope (Carl Zeiss, Germany), while pictures were taken by using a Color Video Camera with image analyzing software (ImageProPlus 4.1, Media Cybernetics, L.P.).

3. Results

3.1 Identification of tomato cultivars resistance to *O. neolycopersici* Shangqiu isolate

Shangqiu isolate of *O. neolycopersici* from tomato plants in Henan Province of China was identified for the first time (Li etal., 2008). Different tomato cultivars and species including *S. lycopersicum* Mill (cvs. MM, Micro-Tom, Zaofen, Fenguo, and Zhongza series), *S. habrochaites* G1.1560 were inoculated with a conidial suspension of the Shangqiu isolate of *O. neolycopersici* with a concentration of 5×10^4 conidia/ml for disease test. Susceptible symptoms developed on all *S. lycopersicum* cultivars which developed symptoms of powdery mildew as early as 4 days after inoculation, while *S. habrochaites* G1.1560 (carrying *Ol-1*) displayed high resistance to the Shangqiu isolate of *O. neolycopersici* (Figure 1), thirty plants were tested and all of them were free of fungal colonies 14 dpi. In this paper, *S. habrochaites* G1.1560 and MM were chosen as the resistant and susceptible tomato plants for the following experiments, respectively.

Figure 1. Phenotypes of resistant plant G1.1560 and susceptible plant MM inoculated with *O. neolycopersici* Shangqiu isolate

3.2 *In silico* cloning and annotation of a candidate DE-TDF

A DE-TDF was sequenced and annotated in SGN (Solanaceae Genomics Network), obtained full-length sequence of the gene (named *ShME1*) showed 99% similarity to a unigene (SGN-U577798), which was annotated to a putative cytosolic NADP-malic enzyme. Based on the location information of the unigene on tomato genome through *in silico* analysis in SGN, the putative NADP-malic enzyme gene was located on chromosome 5.

3.3 At macroscopic observation, silencing *ShME1* can compromise *Ol-1* mediated resistance against *O. neolycopersici*

ShME1 was silenced in *S. habrochaites* G1.1560 plants by using VIGS, which are wild type tomato carrying *Ol-1* gene. Ten days after the VIGS infiltration, the silenced resistant plants were inoculated with *O. neolycopersici*. Obvious fungal colonies were observed on leaflets of all silenced resistant plants 7 dpi, while no fungal colonies were found on the control empty vector-treated resistant plants (Figure 2). The results showed that silenced G1.1560 plants showed susceptibility to *O. neolycopersici* compared to the high resistance in non-silenced G1.1560 plants. It indicated that silencing NADP-malic enzyme gene can macroscopically compromise *Ol-1* mediated resistance response against *O. neolycopersici*.

Figure 2. The susceptible phenotype of *ShME1* silenced resistant plant leaflet to *O. neolycopersici*

The *ShME1* silenced resistant plant through VIGS method (right); control resistant plant (left).

3.4 At microscopic observation, silencing *ShME1* resulted in slow HR induced by *O. neolycopersici*

Further microscopic analysis was conducted to check how silenced *ShME1* in resistant plants affected tomato responses to *O. neolycopersici* and finally influenced the fungal growth. The hypha grew long enough to form plenty of conidiophores with conidia on the *ShME1* silenced resistant plants, resulting in successful fungal sporulation (Figure 3A). The haustoria in the live cells were in good shape suggesting with normal function, which promised the fungal growth and conidiophore formation. Microscopic analysis also showed that non-silenced G1.1560 displayed a fast HR, in which the tomato cell intruded by primary haustoria of *O. neolycopersici* became dead quickly, accompanying with H_2O_2 accumulation in response to *O. neolycopersici* (Figure 3B), while *ShME1* silenced resistant plants showed a slow HR, tomato cell intruded by fungal primary haustoria kept alive and cell death happened in tomato cells intruded by secondary haustoria (Figure 3C).

The micrograph of conidiophore of *O. neolycopersici* in silenced plant G1.1560 (Figure 3A), epidermal cells in which primary haustoria formed became necrotic in non-silenced resistant plant (Figure 3B), epidermal cell in which secondary haustoria formed became necrotic, indicating HR and H_2O_2 accumulation in *ShME1* silenced resistant plant (Figure 3C). The bars equal 50 μm.

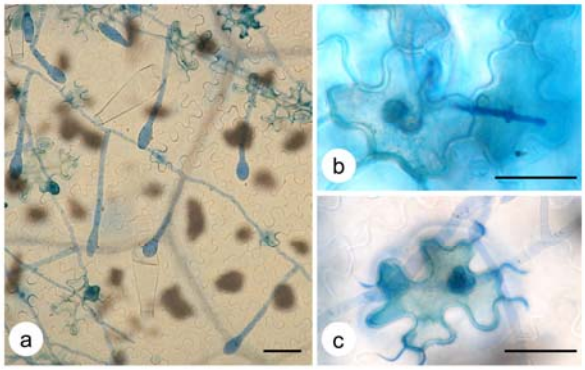

Figure 3. Microscopic observation of *O. neolycopersici* in resistant plant G1.1560.

3.5 RT-PCR confirmation of VIGS effect

Semi-quantitative RT-PCR was conducted to determine whether the target gene was silenced, *Actin* was used as an internal standard (Figure 4). It was estimated that the *ShME1* transcript level was more than 60% reduction in the silenced plants compared to the control plants, suggesting that VIGS of the *ShME1* was successful.

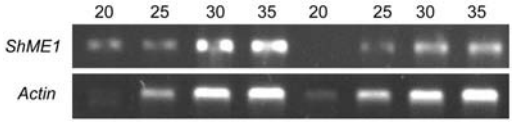

Figure 4. semi-quantitative RT-PCR analysis of *ShME1* VIGS effect

Resistant plant Control plants (left); *ShME1* silenced plants (right); 20, 25, 30 and 35 stand for cycles of PCR； levels of *Actin* transcript were determined as internal controls.

4. Discussion

NADP-ME has multiple functions in plants through reducing NADP to NADPH. NADPH-consuming steps were reported to be involved in plant defense reactions, for example, in phytoalexin biosynthesis (Beggs and Wellman, 1994), in the reductase activity of a disease resistance gene (Johal and Briggs, 1992), and in defense-related deposition of lignin (Whetten and Sederoff, 1995), in which processes the reductive power could be supplied through NADP-ME. In this paper, we demonstrated that silencing of the NADP-ME gene *ShME1* in resistant tomato plants carrying *Ol-1* gene resulted in the loss of resistance. It indicated that NADP-ME is a key gene in the *Ol-1* mediated disease resistance response of tomato against *O. neolycopersici*. It implied that NADPH production catalyzed by NADP-ME may play an important role in tomato resistance to *O. neolycopersici*.

During pathogen attacks, reactive oxygen species (ROS) including H_2O_2 are often produced to defend pathogens (Sutherland, 1991), the electron donor for activation of oxygen to form ROS is NADPH, NADP-ME is involved in mechanisms producing NADPH, therefore NADP-ME could contribute to provide the reducing power for synthesis of ROS (Minard and McAlister-Henn 2001; Møller, 2001). Previous studies showed that *Ol-1* mediated resistance to *O. neolycopersici* was associated with HR, in which a high frequency of necrosis of epidermal cells accompanying with H_2O_2 accumulation induced by the fungal haustoria was microscopically observed (Bai etal., 2005). The cell necrosis together with H_2O_2 accumulation is the main mechanism to prevent the fungal sporulation in resistant G1.1560 plants. In this paper, compared to non-silenced resistant G1.1560 plants displaying fast HR, the *ShME1* silenced GI.1560 showed slow HR resulted in the loss of *O. neolycopersici* resistance. The point further supports our previous findings that transcriptomic differences between compatible and incompatible interactions of tomato and *O. neolycopersici* are mainly in timing (Li etal., 2006). It also indicated that the expression level of *ShME1* gene affected the level of *O. neolycopersici* induced cell necrosis and H_2O_2 accumulation in tomato, implying that NADP-ME could play a role in tomato and *O. neolycopersici* interaction through adjusting the level of cell necrosis and H_2O_2 accumulation.

In conclusion, our findings suggest that the putative tomato NADP-malic enzyme gene from G1.1560 tomato could be a key gene of *Ol-1* mediated tomato resistance to *O. neolycopersici* being involved in HR forming and H_2O_2 accumulation. However, more evidence is needed to clarify the mechanism.

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