Tomato *Sl3-MMP*, a member of the Matrix metalloproteinase family, is required for disease resistance against *Botrytis cinerea* and *Pseudomonas syringae* pv. *tomato* DC3000

Dayong Li, Huijuan Zhang, Qiuming Song, Lu Wang, Shixia Liu, Yongbo Hong, Lei Huang and Fengming Song*

**Abstract**

**Background:** Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases. MMPs have been characterized in detail in mammals and shown to play key roles in many physiological and pathological processes. Although MMPs in some plant species have been identified, the function of MMPs in biotic stress responses remains elusive.

**Results:** A total of five MMP genes were identified in tomato genome. qRT-PCR analysis revealed that expression of *Sl-MMP* genes was induced with distinct patterns by infection of *Botrytis cinerea* and *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 and by treatment with defense-related hormones such as salicylic acid, jasmonic acid and ethylene precursor 1-amino cyclopropane-1-carboxylic acid. Virus-induced gene silencing (VIGS)-based knockdown of individual *Sl-MMPs* and disease assays indicated that silencing of *Sl3-MMP* resulted in reduced resistance to *B. cinerea* and *Pst* DC3000, whereas silencing of other four *Sl-MMPs* did not affect the disease resistance against these two pathogens. The *Sl3-MMP*-silenced tomato plants responded with increased accumulation of reactive oxygen species and altered expression of defense genes after infection of *B. cinerea*. Transient expression of *Sl3-MMP* in leaves of *Nicotiana benthamiana* led to an enhanced resistance to *B. cinerea* and upregulated expression of defense-related genes. Biochemical assays revealed that the recombinant mature *Sl3-MMP* protein had proteolytic activities in vitro with distinct preferences for specificity of cleavage sites. The *Sl3-MMP* protein was targeted onto the plasma membrane of plant cells when transiently expressed in onion epidermal cells.

**Conclusion:** VIGS-based knockdown of *Sl3-MMP* expression in tomato and gain-of-function transient expression of *Sl3-MMP* in *N. benthamiana* demonstrate that *Sl3-MMP* functions as a positive regulator of defense response against *B. cinerea* and *Pst* DC3000.

**Keywords:** Tomato (*Solanum lycopersicum*), Matrix metalloproteinases, *Botrytis cinerea*, *Pseudomonas syringae* pv. *tomato* DC3000, Disease resistance, Proteolysis
**Background**

Proteases play key roles in the regulation of a variety of biological processes [1]. Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent proteases belonging to the metzincin clan of metalloendopeptidases, EC subclass 3.4.24, MA (M) clan according to the MEROPS database [2, 3]. The MMP family is characterized by the presence of a highly conserved catalytic domain containing an HEXXHXXGXX(H/D) zinc-binding sequence followed by a conserved methionine that forms a tight 1,4-β turn called Met-turn [4]. Members of this family have mainly been studied in mammals, but have also been found in simpler animals and plants [5].

In human, 23 MMP genes have been identified to encode proteins with similar structure, e.g., an N-terminal signal peptide for the secretory pathway, a prodomain that regulates the latency of the enzyme and a catalytic domain with the active zinc-binding site [6]. In addition, most of the human MMP proteins contain a C-terminal hemopexin (HPX)-like domain, which is believed to be important in regulating the activity and specificity of the catalytic domain [7, 8]. It has been shown that human MMPs play key roles in many physiological and pathological processes [9, 10].

Members of the MMP family have been identified in higher plants, but only few of them have been studied to date [2]. Similar to the human MMPs, the predicted primary structures of plant MMPs contain a signal peptide, a prodomain with the cysteine-switch motif and a catalytic domain containing the active zinc-binding sequence and structural zinc- and calcium-binding site followed by the conserved Met-turn [11–13]. Activation of MMPs requires physical delocalization of the prodomain from the catalytic site by proteolytic or nonproteolytic mechanisms [14]. It is believed that all plant MMPs are synthesized as inactive forms and are localized either in the plasma membrane or in the extracellular space. However, it was found that Arabidopsis At4-MMP contains a predicted non-cleavable N-terminal signal peptide and tobacco Nt1-MMP was inserted into the plasma membrane [15].

The biological function of MMP proteases in higher plants is largely unknown. Based on the expression patterns, it is proposed that the plant MMPs may be involved in remodeling of the extracellular matrix (ECM) during plant growth and development [2]. The first plant MMP was identified as an ethylenediaminetetraacetic acid (EDTA)-sensitive Azocoll-degrading enzyme in soybean [16]. In cucumber, Cs1-MMP was found to be associated with senescence and cell death in cotyledon development [17]. In Arabidopsis, 5 MMP genes were identified and were found to be differentially expressed in roots, leaves, stems and flowers [15]. The At2-MMP mutant plants exhibited altered growth in association with late flowering and early senescence, supporting a physiological and developmental role for plant MMPs [18]. In Medicago truncatula, expression of Mt1-MMP was induced in young nodules, specifically in association with Sinorhizobium meliloti infection [13]. An Mt1-MMP RNAi mutant in *M. truncatula* showed nodules with enlarged infection threads and substantial increase in the number of bacterial colonies; whereas an ectopic overexpression of Mt1-MMP in roots led to a significant decrease in nodule number [13]. On the other hand, several lines of evidence also indicate that MMPs may be involved in biotic and abiotic stress responses in plants. In soybean, Gm2-MMP was isolated as a pathogen-induced gene [19]. Expression of Gm2-MMP was induced rapidly in compatible and incompatible interactions with pathogens, but not by salicylic acid (SA) and jasmonic acid (JA), two classical pathogen response signaling molecules [19]. In the tobacco suspension line BY-2, Nt1-MMP was expressed at low level but was induced immediately after treatment with *Pseudomonas syringae* [11]. In Arabidopsis, distinct expression patterns for each MMP in response to various abiotic and biotic stresses were described in the Genevestigator analysis [20]. At3-MMP showed significant changes in transcript levels under stress conditions, while other MMPs displayed minimal transcript changes [20]. The expression of At2-MMP is tightly controlled in a tissue-responsive way during stress conditions. At2-MMP in 4-week-old plants was induced in leaves by cadmium or methyl jasmonate and in roots by sodium chloride; however, cadmium inhibited the expression of At2-MMP in inflorescence and leaves of 10-week-old plants [18].

In the present study, we characterized the MMP family in tomato and performed functional analyses for their roles in disease resistance. A total of five MMP genes were identified in tomato and their expression was induced with distinct patterns in response to pathogen infection and treatments with defense-related hormones. Silencing of *Sl3-MMP* in tomato resulted in reduced resistance to *Botrytis cinerea* and *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 whereas transient expression of *Sl3-MMP* in *Nicotiana benthamiana* led to an enhanced resistance to *B. cinerea*. Our data demonstrate that member of the MMP family may participate in the regulation of defense response in plants against pathogen infection.

**Results**

**Identification of the SI-MMP family in tomato**

To identify members of the MMP family in tomato, HMM and Blastp searches using MMP proteins previously reported from Arabidopsis and other plant species as queries against the recently published tomato genome sequences (Release Version ITAG2.40) were performed.
Five significant hits corresponding to non-redundant putative SI-MMP genes were identified (Table 1). All five SI-MMPs are intronless genes, which is consistent with structural features of genes for MMPs in Arabidopsis, soybean, cucumber and Medicago truncatula [13, 15, 17, 19, 21]. Full-length cDNAs for Sl2-MMP and Sl3-MMP were identified in the NCBI and SOL databases while no full-length cDNA was found for other three members (Table 1). We amplified and cloned all 5 SI-MMP genes using gene-specific primers and confirmed by sequencing. These sequences were submitted to GenBank for deposition and presented in Additional file 1.

The SI-MMP proteins are approximately 360 amino acids with molecular weight of ~40 kDa (Table 1). The SI-MMP proteins shared conserved structural features, e.g., a signal sequence at N terminus, a propeptide domain, a catalytic domain, a transmembrane domain at C terminus (Fig. 1a). Characteristic motifs including a PRCGxxxD motif, which is characteristic of the cysteine switch mechanism of activation [22], in the propeptide domain and a HExGHxGxxH zinc-binding region and a conserved methionine residue in the Met-turn in the catalytic domain are present in the SI-MMP proteins (Fig. 1b). In addition, each of SI-MMPs contains an invariant DLESV motif on the N-terminal side of the zinc-binding region (Fig. 1b), which is thought to be a plant-specific motif with unknown function [15]. This motif is replaced by a distinct consensus sequence of NLFLV in human and insect MMPs [4, 23] but is not present in single-celled green algae MMPs [20, 24]. In term of secondary structure feature, SI-MMPs have 2 β-strands, 4 β-sheets and 3 α-helices and they all contain three active site histidines and a catalytic glutamate residue in the zinc-binding region (Fig. 1a). Putative conserved structural ligands for binding zinc and calcium including 3 histidine (H), 2 aspartic acid (D) and 1 glutamic acid residues are present in SI-MMPs (Fig. 1a). However, like MMPs from other plants, SI-MMPs do not contain a C-terminal hemopexin domain, which is present in most human MMPs [8].

Phylogenetic tree analysis of SI-MMPs with previously identified MMPs from other plant species clearly distinguished different groups, which had distinct features linked to plant species or specific functions. Groups I and II can be further separated into two subgroups, representing MMP branches from dicots and monocots. SI2-MMP, SI3-MMP and SI4-MMP are assigned in Group I, which contain Nt1-MMP, At2-MMP and At3-MMP that are known to be pathogen-responsive [11, 18, 20]. SI1-MMP and SI5-MMP belong to Group II, whose members have been proposed to be involved in plant growth and development [20]. Group III only hosts MMPs from legumes such as Glycine max and Medicago truncatula and thus seem to be legume-specific.

**Table 1 Characterization of tomato SI-MMP genes and proteins**

| Genes | Locus in SOL | Proteins in NCBI | Size (aa) | MW (kDa) | pl | cDNAs in NCBI/SOL |
|-------|--------------|------------------|-----------|----------|----|-------------------|
| SI1-MMP | Solyc08g078550 | XP_010325488 | 356 | 40.17 | 5.20 | – |
| SI2-MMP | Solyc04g005040 | CCH68443 | 363 | 40.00 | 5.46 | AK328733/AK327627/ SGN-U573509 |
| SI3-MMP | Solyc04g005050 | NP_001266203 | 367 | 40.21 | 5.08 | AK322919/ SGN-U573510 |
| SI4-MMP | Solyc05g006360 | XP_010320628 | 357 | 40.50 | 5.60 | – |
| SI5-MMP | Solyc10g018750 | XP_004248606 | 357 | 40.80 | 7.83 | – |

Expression of SI-MMPs in response to pathogens and defense signaling-related hormones

To explore the possible involvement of SI-MMPs in defense response against pathogen infection, we first analyzed the expression changes of SI-MMPs after infection with B. cinerea. As shown in Fig. 2a, expression of the SI-MMP genes were induced upon infection of B. cinerea but showed distinct expression patterns. Generally, the expression of SI1-MMP, SI3-MMP, SI4-MMP and SI5-MMP was significantly induced with peaks at 48 h whereas the expression of SI2-MMP was induced significantly with peaks at 24 h after infection with B. cinerea, as compared with those in the mock-inoculated plants (Fig. 2a). Specifically, the expression levels of SI1-MMP, SI4-MMP and SI5-MMP was significantly induced with peaks at 48 h whereas the expression of SI2-MMP was induced significantly with peaks at 24 h after infection with B. cinerea, as compared with those in the mock-inoculated plants at 48 h after inoculation (Fig. 2a). The expressions of SI1-MMP and SI3-MMP exhibited 4-5 folds of increases over those in the mock-inoculated plants at 48 h after inoculation (Fig. 2a).

We next analyzed the expression changes of SI-MMPs after infection with Pst DC3000. As shown in Fig. 3b, only SI1-MMP, SI2-MMP and SI3-MMP were induced upon infection of Pst DC3000 and again showed different expression patterns. Generally, the expression of SI1-MMP and SI3-MMP was induced significantly with peaks at 24 h while the expression of SI2-MMP was induced significantly with peaks at 12 h after infection with Pst DC3000, as compared with those in the mock-inoculated plants (Fig. 2b). Specifically, the expression of SI1-MMP and SI3-MMP in Pst DC3000-infected plants
Fig. 1 Sequence alignment and phylogenetic tree analysis of Sl-MMP with other plant MMP proteins.  

a Predicted domains of Sl-MMP proteins. TM, transmembrane domain. b Alignment of Sl-MMPs. Numbers on the right indicate amino acid positions of the Sl-MMP proteins. The cysteine switch motif, zinc-binding sequence and DLESV sequence are boxed in red. Secondary structure features such as β-strand, β-sheet and α-helix are indicated above the aligned sequences. The active site histidine and the catalytic glutamate residues are indicated in red. Ligands of the conserved structural zinc and calcium are colored in green and yellow, respectively. The hydrophobic base forming the methionine residue of the Met-turn is highlighted in blue. c Phylogenetic tree analysis of Sl-MMPs with other plant MMPs. Phylogenetic tree was constructed by Neighbor-joining method using MEGA program. Plant MMPs used and their GenBank accessions are as follows: Arabidopsis thaliana At1-MMP (NP_193397), At2-MMP (NP_177174), At3-MMP (NP_173824), At4-MMP (NP_182030), At5-MMP (NP_176205), Glycine max Gm2-MMP (AAL27029), Gm-ACU24527 (ACU24527), Gm-Slti114 (ABW96008), Hordeum vulgare HvMMP1 (BAJ94792), HvMMP2 (BAJ95963), HvMMP3 (BAJ94176), HvMMP4 (BAJ90394), Medicago truncatula Mt1-MMP (CAA77093), Nicotiana tabacum NtMMP1 (ABF58910), Solanum lycopersicum Sl1-MMP (XP_010325468), Sl2-MMP (CCU68443), Sl3-MMP (XP_001125620), Sl4-MMP (XP_010320628), Sl5-MMP (XP_001125628), Zea mays ZmMMP1 (NP_001151749), ZmMMP2 (NP_001142095), Oryza sativa Os1-MMP (NP_001044075), Os2-MMP (NP_001057259), Os3-MMP (NP_001065361). Bootstrap values from 100 replicates are indicated at each node. Bar represents the number of amino acid differences per site.
showed >5 folds of increases over those in the mock-inoculated plants at 24 h, whereas the expression of Sl2-MMP was induced significantly with peaks at 12 and 24 h after infection with *Pst* DC3000 (Fig. 2b). Interestingly, the expression of Sl5-MMP was down-regulated at 12 h (Fig. 2b). These results indicate that the expression of Sl1-MMP, Sl2-MMP and Sl3-MMP was induced by *Pst* DC3000.

We also examined the dynamics of Sl-MMPs expressions in tomato plants after treatments with SA, methyl jasmonate (MeJA) and 1-amino cyclopropane-1-carboxylic acid (ACC) [a precursor of ethylene (ET)], three defense signaling-related hormones. As shown in Fig. 4, different expression patterns for Sl-MMPs were observed in response to these defense signaling-related hormones. In SA-treated plants, expression of Sl3-MMP and Sl4-MMP was significantly increased by 2-3 folds over that in the control plants, while expressions of Sl1-MMP, Sl2-MMP and Sl3-MMP were not affected (Fig. 3a). In JA- or ACC-treated plants, expression of Sl4-MMP was strongly induced, reaching 3-4 folds of increased at 6 h after treatment (Fig. 3b and c). Besides Sl4-MMP, the expression of Sl1-MMP, Sl2-MMP and Sl3-MMP was also induced by JA, showing an increase of 2-3 folds at 12 h (Fig. 3b). Except Sl4-MMP, expression of other four Sl-MMPs was not affected by ACC (Fig. 3c). Interestingly, the expression of Sl5-MMP was not affected by both JA and ACC during the experimental period (Fig. 3b and c). These data indicate that the tomato Sl-MMPs respond with different expression patterns to SA, JA and ET, three well-known defense signaling-related hormones.

Silencing of Sl3-MMP resulted in reduced resistance to *B. cinerea* and *Pst* DC3000

To examine the possible involvement of Sl-MMPs in disease resistance, we performed functional analyses by virus-induced gene silencing (VIGS) approach through comparing the disease phenotype between individual Sl-MMP-silenced plants and non-silenced control plants. For this purpose, specific fragment for each Sl-MMP gene (Additional file 2) was chosen to generate VIGS construct and standard VIGS procedure with a phytoene desaturase (*PDS*) construct as an indicative for VIGS efficiency of each experiment was performed on 2-week-old plants [25, 26]. Under our experiment conditions, >90 %
of the pTRV2-PDS-infiltrated plants showed bleaching phenotype (data not shown). The silencing efficiency and specificity for each Sl-MMP gene was examined by qRT-PCR analyzing the transcript level of the target Sl-MMP gene and other four Sl-MMP genes in the pTRV2-target Sl-MMP-infiltrated plants. When compared with those in the pTRV2-GUS-infiltrated plants, the transcript level of the target Sl-MMP gene was significantly reduced whereas the transcript levels of the other Sl-MMP genes were comparable in the pTRV2-Sl-silenced plants (Fig. 4). Overall, the silencing efficiency for a target Sl-MMP gene was approximately 70 % (Fig. 4). The efficiencies and specificity of silencing for each individual Sl-MMP gene were satisfied for further experiments and all the subsequent experiments were performed only on those pTRV2-Sl-MMPs-infiltrated plants with high levels of silencing efficiency (>70 %).

We first examined the possible involvement of Sl-MMPs in resistance against B. cinerea by challenging the pTRV2-Sl-MMPs-infiltrated plants with spore suspension of B. cinerea and comparing the disease severity and in planta fungal growth with those in pTRV-GUS-infiltred non-silenced plants. In our detached leaf assays, B. cinerea-caused lesions on detached leaves from the pTRV2-Sl1-MMP-, pTRV2-Sl2-MMP-, pTRV2-Sl4-MMP- and pTRV2-Sl5-MMP-infiltrated plants were similar to those on the detached leaves from pTRV2-GUS-infiltred plants (Fig. 5a), suggesting that Sl1-MMP, Sl2-MMP, Sl4-MMP and Sl5-MMP may not be involved in resistance against B. cinerea. However, B. cinerea-caused lesions on detached leaves from the pTRV2-Sl3-MMP-infiltrated plants were significantly larger and developed faster, merging into large necrotic areas, as compared with those on leaves from the

![Fig. 3 Expression patterns of Sl-MMPs in response to defense signaling hormones. Tomato plants were treated by foliar spraying of 1 mM SA (a), 100 μM MeJA (b), 100 μM ACC (c) or equal volume of solution as a control and leaf samples were collected at indicated time points. Gene expression was analyzed by qRT-PCR and relative expression levels were calculated by comparing with the corresponding values at 0 h (as a control) after treatment. Relative expression was shown as folds of the actin transcript values. Data presented are the means ± SD from three independent experiments and different letters above the columns indicate significant differences at p < 0.05 level](image-url)
pTRV2-GUS-infiltrated plants (Fig. 5a), at 3 days after inoculation (dpi), showing an approximately 60 % of increase in lesion size over those on leaves from the pTRV2-GUS-infiltrated control plants (Fig. 5c). We further analyzed and compared the disease severity and in planta fungal growth in the pTRV2-Sl3-MMP- and pTRV2-GUS-infiltrated plants after inoculation by foliar spraying with spore suspension of *B. cinerea* in whole plant inoculation experiments. As shown in Fig. 5b, the pTRV2-GUS-infiltrated control plants displayed slight disease symptoms, whereas the pTRV2-Sl3-MMP-infiltrated plants showed severe disease symptoms, showing large necrotic areas and maceration or wilting of full leaves at 5 dpi. Analysis of the transcript for the *B. cinerea* actin gene *BcActin* revealed that growth of *B. cinerea* in leaf tissues of the pTRV2-Sl3-MMP-infiltrated plants had 3 times higher than those in the pTRV2-GUS-infiltrated plants at 24 and 48 h after inoculation (Fig. 5d). These data indicate that silencing of *Sl3-MMP* resulted in reduced resistance to *B. cinerea*, demonstrating the requirement of *Sl3-MMP* for resistance to *B. cinerea*.

As mentioned above that silencing of *Sl3-MMP* resulted in a clear phenotype in change of disease resistance to *B. cinerea*, we subsequently focused our efforts on the functions in resistance to other diseases, mechanism and biochemical activity of Sl3-MMP. We examined whether *Sl3-MMP* is also involved in resistance against *Pst* DC3000, which is a hemibiotrophic bacterial pathogen that has different infection style from that of *B. cinerea*. In our experiments, necrotic lesions were observed in the inoculated leaves of the pTRV2-Sl3-MMP- and pTRV2-GUS- infiltrated plants; however, the lesions on leaves of the pTRV2-Sl3-MMP-infiltrated plants were larger and denser than those in the pTRV2-GUS-infiltrated plants (Fig. 6a). At 2 and 4 dpi, the bacterial population in the inoculated leaves of the pTRV2-Sl3-MMP-infiltrated plants showed approximately 10 and 25 folds higher over those in the pTRV2-GUS-infiltrated plants, respectively (Fig. 6b). These results indicate that silencing of *Sl3-MMP* resulted in reduced resistance to *Pst* DC3000, implying the requirement of *Sl3-MMP* for resistance against *Pst* DC3000.

**Silencing of Sl3-MMP attenuated defense response against B. cinerea**

To elucidate the possible mechanism involved in the reduced resistance in *Sl3-MMP*-silenced plants, we analyzed and compared the accumulation of reactive oxygen species (ROS), cell-death response and expression of defense genes before and after infection with *B. cinerea* between the *Sl3-MMP*-silenced plants and the control plants. No difference in accumulation of H$_2$O$_2$, as detected by 3, 3-diaminobenzidine (DAB) staining, was
observed in leaves of pTRV2-Sl3-MMP- and pTRV2-GUS-infiltrated plants without infection of B. cinerea (Fig. 7a), indicating that silencing of Sl3-MMP itself did not affect the generation and accumulation of H$_2$O$_2$ in tomato plants. After infection with B. cinerea, significant accumulation of H$_2$O$_2$ shown as brown precipitates in leaves, was detected in leaves of pTRV2-Sl3-MMP- and pTRV2-GUS-infiltrated plants (Fig. 7a). However, the leaves from pTRV2-Sl3-MMP-infiltrated plants showed consistent increase in intensity of the stained areas (Fig. 7a), showing increases of 85 % at 12 h and 24 % 24 h, when compared with those in pTRV2-GUS-infiltrated plants after infection of B. cinerea (Fig. 7b). On the other hand, levels of cell death, as detected by Trypan blue staining, and electrolyte leakage, as estimated by ion conductivity, were comparable in leaves of pTRV2-Sl3-MMP- and pTRV2-GUS-infiltrated plants without infection of B. cinerea but significantly increased after infection with B. cinerea (Fig. 7c and d). Notably, the levels of cell death and electrolyte leakage in leaves of pTRV2-Sl3-MMP-infiltrated plants were significantly higher than those in leaves of pTRV2-GUS-infiltrated plants after infection of B. cinerea (Fig. 7c), leading to 38 % increases for electrolyte leakage at 24 h after infection (Fig. 7d). These data
indicate that silencing of \textit{Sl3-MMP} resulted in increased ROS accumulation of H$_2$O$_2$ and excessive cell death in pTRV2-Sl3-MMP-infiltrated plants upon infection of \textit{B. cinerea}.

To explore the possible mechanism for the increased accumulation of H$_2$O$_2$ in the Sl3-MMP-silenced plants, we analyzed and compared the expression of genes encoding for NADPH oxidases, glutathione reductase (GR), catalases (CAT), superoxide dismutases (SOD) and ascorbate peroxidases (APX) in the pTRV2-Sl3-MMP-infiltrated plants. As shown in Fig. 8a, no significant difference in the expression levels of these selected genes was observed between the pTRV2-GUS-infiltrated and pTRV2-Sl3-MMP-infiltrated plants without infection of \textit{B. cinerea}. By contrast, the expression levels of \textit{Rboh1} and \textit{Wfi1}, two genes for NADPH oxidases, in the pTRV2-Sl3-MMP-infiltrated plants were significantly elevated upon \textit{Botrytis} infection, showing ~5-fold increases over those in the pTRV2-GUS-infiltrated plants. Similarly, the expression levels of \textit{APX} and \textit{GR} in the pTRV2-Sl3-MMP-infiltrated plants were also increased as compared with those in the pTRV2-GUS-infiltrated plants (Fig. 8a). By contrast, no significant difference

\begin{figure}
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\includegraphics[width=0.9\textwidth]{fig6}
\caption{Silencing of \textit{Sl3-MMP} resulted in reduced resistance to \textit{P. syringae} pv. tomato DC3000. Two-week-old seedlings were infiltrated with agrobacteria carrying pTRV2-Sl3-MMP or pTRV2-GUS and were inoculated by infiltration with \textit{Pst} DC3000 4 weeks after VIGS infiltration. \textbf{a} Representative disease phenotype. \textbf{b} Bacterial population. Data presented in \textbf{b} are the means ± SD from three independent experiments and different letters above the columns indicate significant differences at $p < 0.05$ level.}
\end{figure}

\begin{figure}
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\includegraphics[width=0.9\textwidth]{fig7}
\caption{Increased accumulation of H$_2$O$_2$ and cell death in Sl3-MMP-silenced plants after infection with \textit{B. cinerea}. Two-week-old seedlings were infiltrated with agrobacteria carrying pTRV2-Sl3-MMP or pTRV2-GUS and were inoculated with spore suspension of \textit{B. cinerea} or with buffer solution as a mock-inoculation control at 4 weeks after VIGS infiltration. Leaves from six individual plants were collected at 24 h after inoculation and used for analysis of cell death and H$_2$O$_2$ accumulation. \textbf{a} and \textbf{b} Accumulation of H$_2$O$_2$ by DAB staining and quantification method respectively. \textbf{c} Cell death detected by trypan blue staining. \textbf{d} Electrolyte leakage. Data presented in \textbf{b} and \textbf{d} are the means ± SD from three independent experiments and different letters above the columns indicate significant differences at $p < 0.05$ level.}
\end{figure}
was observed in the expression levels of \( CAT \) and \( SOD \) between the \( pTRV2-Sl3-MMP \) infiltrated plants and the \( pTRV2-GUS \) infiltrated plants (Fig. 8a). These results indicate that the increased ROS accumulation in the \( Sl3-MMP \)-silenced plants might be due to an increased ROS generating ability resulted from the high level of expression of the NADPH oxidases.

We next analyzed the expression changes of defense-related genes regulated by the \( JA/ET \)- and \( SA \)-mediated signaling pathways, respectively, to explore the possible molecular mechanism associated with the reduced disease resistance in \( Sl3-MMP \)-silenced plants. No significant difference in expression of \( SlPI-I \) and \( SlPI-II \), known to be regulated by the \( JA/ET \)-mediated signaling pathway [27], and \( SlLapA \), \( SlPI-I \) and \( SlPI-II \), known to be regulated by the \( JA/ET \)-mediated signaling pathway [27], was observed in mock-inoculated \( pTRV2-Sl3-MMP \)- or \( pTRV2-GUS \)-infiltrated plants (Fig. 8b), indicating that silencing of \( Sl3-MMP \) did not affect the expression of defense-related genes in tomato plants. However, the expression of these defense-related genes exhibited different patterns in \( pTRV2-Sl3-MMP \)- or \( pTRV2-GUS \)-infiltrated plants after infection of \( B. cinerea \) (Fig. 8b). The expression of \( SlPI-I \) and \( SlPI-II \) in the \( pTRV2-Sl3-MMP \)- and \( pTRV2-GUS \)-infiltrated plants was significantly upregulated after infection of \( B. cinerea \), but the levels in \( pTRV2-Sl3-MMP \)-infiltrated plants showed 7-8 folds higher than those in the \( pTRV2-GUS \)-infiltrated plants (Fig. 8b). After infection with \( B. cinerea \), the expression levels of \( SlLapA \), \( SlPI-I \) and \( SlPI-II \) in \( pTRV2-GUS \)-infiltrated plants were significantly increased, whereas the levels in \( pTRV2-Sl3-MMP \)-infiltrated plants remained unchanged, comparable to those in the mock-inoculated plants but showing 3-5 folds of decreases as compared with those in \( B. cinerea \)-infected \( pTRV2-GUS \)-infiltrated plants (Fig. 8b).

These data demonstrate that silencing of \( Sl3-MMP \) attenuated the defense response in tomato upon infection of \( B. cinerea \) through affecting the expression of defense-related genes that are regulated by the JA/ET-mediated signaling pathway.

**Transient expression of \( Sl3-MMP \) in \( N. benthamiana \) led to increased resistance against \( B. cinerea \)**

To further confirm the function of \( Sl3-MMP \) in disease resistance, we examined whether overexpression of \( Sl3-MMP \) could confer an increased resistance to \( B. cinerea \). In our qRT-PCR experiments, transcripts of putative \( N. benthamiana \) homolog(s) of \( Sl3-MMP \) was detected using \( Sl3-MMP \)-specific primers in \( GFP \)-infiltrated plants, probably due to high level of sequence similarity/identity among \( Sl3-MMP \) and the homologous \( MMP \) genes in \( N. benthamiana \). However, agroinfiltration did not significantly affect the transcript levels of endogenous \( N. benthamiana \) homologous genes in \( GFP \)-infiltrated plants (Fig. 9a). When transiently expressed in \( N. benthamiana \) leaves, high levels of \( Sl3-MMP \) expression, as estimated by the significant increases in the transcript levels of \( Sl3-MMP \) and the endogenous homologous genes in \( Sl3-MMP \)-infiltrated plants over the levels of the endogenous homologous genes in \( GFP \)-infiltrated plants, and the \( Sl3-MMP-GFP \) (a fusion of \( Sl3-MMP \) with \( GFP \) fusion protein were detected during a period of 48 h after infiltration (Fig. 9a and b). In disease assays, the lesions on leaves from \( Sl3-MMP \)-infiltrated \( N. benthamiana \) plants were significantly smaller than that in \( GFP \)-infiltrated control plants (Fig. 9c), leading to approximately 40 % of reduction in lesion size at 5 days after inoculation (Fig. 9d). To examine whether an increased defense response was linked to the enhanced resistance resulted from the transient expression of \( Sl3-MMP \), we analyzed and compared.
the expression of some selected defense-related genes in leaves of the GFP- and Sl3-MMP-infiltrated N. benthamiana plants. As shown in Fig. 9e, the expression of PR1, PR2, PR3 and PR4 in Sl3-MMP-infiltrated plants were significantly upregulated at 24 h after infiltration, showing 5-24 folds of increases over those in GFP-infiltrated plants, whereas no significant difference in the levels of these defense-related genes was observed between GFP-infiltrated plants at 0 h and 24 h and between GFP- and Sl3-MMP-infiltrated plants at 0 h after infiltration (Fig. 9e). These data demonstrate that transient expression of Sl3-MMP in N. benthamiana plants conferred an increased resistance against B. cinerea through an activated defense response resulted from the upregulated expression of defense-related genes.

Proteolytic activity and subcellular localization of Sl3-MMP
To delineate the biochemical activity of Sl3-MMP, we expressed the putative mature form of Sl3-MMP (Gly155-Ser340), in which the N-terminal propeptide domain as well as the C-terminal predicted transmembrane domains were deleted, in E. coli, and purified the recombinant mature Sl3-MMP protein (Sl3-MMPm) to homogeneity as examined on a SDS-PAGE gel (Fig. 10a). The activity of the recombinant Sl3-MMPm was examined for its ability to cleave a general protease substrate bovine myelin basic protein (MBP). As shown in Fig. 10b,
MBP was present as a single band without any degradation in absence of Sl3-MMPm and the GST tag alone did not cause degradation of MBP. However, significant degradation of MBP was detected in the presence of Sl3-MMPm, showing an additional band with small molecular weight (Fig. 10b). Meanwhile, addition of 1 mM EDTA, an inhibitor of MMP [2], in the Sl3-MMPm-MBP reaction completely abolished the degradation of MBP by Sl3-MMPm (Fig. 10b). These results demonstrate that the recombinant Sl3-MMPm had proteolytic activity on MBP. To determine the cleavage site specificity, we examined whether Sl3-MMPm was able to hydrolyze quenched fluorescent (QF) peptide substrates QF24, QF35 and QF75. As shown in Fig. 10c, the recombinant Sl3-MMPm cleaved the QF24 most efficiently, followed by QF 35 and then QF75, indicating a cleavage site specificity of Sl3-MMP towards different substrates.

Subcellular localization of Sl3-MMP was examined by transient expression of a GFP-tagged Sl3-MMP construct in onion epidermal cells. To exclude the possibility that Sl3-MMP-GFP is secreted into the apoplast and not associated with the plasma membrane, the GFP-tagged Sl3-MMP construct was introduced into onion epidermal cells by particle bombardment and GFP was observed before and after plasmolysis, which was induced by rinsing the cells with 0.8 M mannitol solution for 10 min. The GFP protein alone accumulated in the cells without specific localization; however, GFP from the GFP-tagged Sl3-MMP protein was clearly localized on the plasma membrane and cell wall (Fig. 11). These observations indicate that Sl3-MMP was targeted to plasma membrane of cells but not secreted into the apoplast.

Discussion

MMPs are a family of zinc-dependent endopeptidases widely distributed in all organisms. However, only a few of plant MMPs have been studied for their biological functions so far [2]. In the present study, we characterized the tomato MMP family, analyzed the expression patterns of Sl-MMP genes in response to pathogen infection and treatments of defense-related signaling hormones, and performed VIGS- and transient expression-based functional analyses to explore the involvement of Sl-MMPs in disease resistance. Our results demonstrate that Sl3-MMP act as a positive regulator of defense response against B. cinerea and Pst DC3000 in tomato, providing new insights into the biological function of plant MMPs.

The significance of the proteases is generally related to their substrates and the physiological consequences of these actions. No physiological substrate has been identified for higher plant MMPs. However, activity of plant MMPs was detected in vitro on general animal protease substrates [11, 12, 15, 17, 19, 28, 29]. It was also found that recombinant mature MMPs, lacking the N-terminal propeptide domains showed higher proteolytic activity than the full-length MMPs [15, 19, 28]. In the present study, we demonstrated that Sl3-MMPm, lacking the N-terminal propeptide domain, had proteolytic activity toward MBP in vitro (Fig. 10b). Similar proteolytic activity on MBP was verified for At1-MMP, At2-MMP, At5-MMP [15, 30], Gm2-MMP [19] and Pta1-MMP [12]. However, proteolytic activities of plant MMPs on other substrates were also detected, e.g., Cs1-MMP and Nt1-MMP on gelatin [17, 28] and At3-MMP and Nt1-MMP on β-casein [11, 28, 30]. Therefore, it is likely that members of plant MMP family may have different substrates in plants and such substrate specificity may determine their biological functions. On the other hand, previous...
reports have shown that both human and plant MMPs differ in their cleavage site preferences [30]. Synthetic peptides, designed for vertebrate MMPs, have been used to characterize the proteolytic activity of SMEP1/Gm1-MMP [29], At1-MMP-At5-MMP [15, 30], Cs1-MMP [17] and Nt1-MMP [28]. Among these synthetic peptides, QF24 is a substrate for all MMPs [31], QF35 is the stromelysin substrate [32] and QF75 was designed to mimic the activation cleavage site in human MMP-2 and has an amino acid sequence different from the typical MMP cleavage motif [30]. In the present study, we found that Sl3-MMPm, lacking the N-terminal propeptide domain, cleaved the QF24 with the highest efficiency, followed by QF35 and QF75 (Fig. 10c). Thus, Sl3-MMP is able to efficiently cleave general MMP substrates. This is in agreement with the differences in the cleavage site specificities observed in Arabidopsis MMPs [30]. For example, At1-MMP was found to cleave efficiently QF24 and QF35 while only At5-MMP was found to be able to cleave QF75 [15, 30].

Most human MMPs are secreted into the extracellular matrix, but six membrane-type MMPs maintain contact with the cell surface through either transmembrane domains or glycosylphosphatidylinositol (GPI) anchors [33]. Like some plant MMPs such as GmMMP2, At2-MMP, At4-MMP and At5-MMP [20], Sl3-MMP contains a signal peptide (1-21 aa) at the N-terminus and a putative GPI-anchor modification site or a transmembrane domain at the C-terminus and was predicted to be located in the extracellular space by WoLF PSORT and TargetP programs. Our experimental evidence from transient expression of the GFP-tagged Sl3-MMP in onion epidermal cells clearly demonstrated that Sl3-MMP is targeted to plasma membrane but not secreted into the apoplast (Fig. 11). This is consistent with the previous observations that both of soybean Shl114 and tobacco Nt1-MMP, which are phylogenetically closest MMPs to Sl3-MMP, localized in the plasma membrane [11, 34], but differs from the soybean SMEP1, which was reported to be tightly bound to the cell wall [21]. Animal MMPs have been recognized as a class of enzymes that plays a critical role in ECM turnover and remodeling based on their ability to hydrolyze the major protein components of the ECM [35]. However, whether Sl3-MMP exhibits its biological function through degradation of plant ECM components needs to be further investigated.

Direct evidence supporting the involvement of MMPs in plant disease resistance is lacking although members of the MMP family have been shown to be induced by pathogen infection and defense signaling hormones [2, 20]. In the present study, we observed that the expression of Sl-MMPs could be induced by both Pst DC3000 and B. cinerea (Figs. 3 and 4). This is similar to the observations that the expression of Gm2-MMP in soybean [19] and Nt1-MMP in tobacco suspension cells [11] was induced by different fungal, oomycetes or bacterial pathogens. However, pathogen-induced expression patterns varied among the Sl-MMPs, e.g., all Sl-MMPs, especially for Sl1-MMP, Sl3-MMP and Sl5-MMP, exhibited upregulated expression patterns in response to B. cinerea while only Sl1-MMP, Sl2-MMP and Sl3-MMP showed upregulated patterns in response to Pst DC3000 (Fig. 2). In addition, the expression of most of the Sl-MMPs was also induced by defense signaling hormones such as SA, JA and ACC (Fig. 3). This is consistent with At2-MMP whose expression was induced rapidly by MeJA [18] but differs from Gm2-MMP, which was not induced by SA and MeJA in soybean suspension cells [19]. Therefore, it is likely that the expression of Sl-MMPs is precisely controlled by complex mechanisms in response to infection from different pathogens and/or defense signaling hormones.

Our VIGS- and transient expression-based functional analyses led to the identification of Sl3-MMP as a positive regulator of defense response against B. cinerea. Firstly, silencing of Sl3-MMP but not other four Sl-MMPs resulted in reduced resistance against B. cinerea and Pst DC3000, as the Sl3-MMP-silenced plants exhibited severe disease symptoms and supported more in planta pathogen growth (Figs. 6 and 7). Accompanying with the reduced resistance in Sl3-MMP-silenced plants was attenuated defense responses upon infection of B. cinerea, e.g., increased ROS accumulation and cell death and downregulated expression of JA/ET-mediated signaling responsive defense-related genes (Figs. 8 and 9). B. cinerea can induce the generation of ROS in plants to benefit its infection [36–38]; however, ROS may function in different ways in the interaction between tomato and B. cinerea [39]. It is generally accepted that ROS accumulated during the late stage directly benefits the establishment of infection by B. cinerea [39] and that sustained production of ROS as a facilitator of cell death may promote susceptibility [40]. In the present study, the Sl3-MMP-silenced plants accumulated larger amount of H2O2 at 24 h than the control plants after inoculation (Fig. 7a and b). This increase in ROS accumulation in the Sl3-MMP-silenced plants was mainly due to an accelerated ROS generation rather than a reduced ROS scavenging ability because the expression levels of SlRboh1 and SlWF1, coding for NADPH oxidases that are plasma membrane-localized ROS generating enzymes [41], were significantly higher but the expression levels of SICA7 and SISOD, coding enzymes capable of scavenging ROS [42] were similar to those in the control plants after infection of B. cinerea (Fig. 8a). Increased expression levels of SlAPX and SlGR in the Sl3-MMP-silenced plants might be responses to the change in cellular redox status caused by the excessive ROS accumulated after infection of B.
cinerea. Furthermore, excessive ROS accumulation in the SI3-MMP-silenced plants thus resulted in an increased level of cell death, as revealed by Trypan blue staining and measurement of electrolyte leakage (Fig. 7c and d), which should favor the growth of B. cinerea (Fig. 5d). It is therefore likely that accelerated ROS accumulation in B. cinerea-infected SI3-MMP-silenced plants may be one of the mechanisms leading to a reduced resistance to B. cinerea. On the other hand, B. cinerea-induced expressions of SLPR1a and SLPR1b, regulated by the SA-mediated signaling pathway [27], and SLLApA, SIPI-I, and SIPI-II, regulated by the JA/ET-mediated signaling pathway [27], were significantly increased and suppressed, respectively, in SI3-MMP-silenced plants (Fig. 9b), indicating that silencing of SI3-MMP may affect the efficiency of the JA/ET-mediated signaling pathway in regulating expression of defense-related genes. Secondly, transient expression of SI3-MMP in N. benthamiana plants conferred an enhanced resistance to B. cinerea and upregulated expression of defense genes (Fig. 9), further supporting the hypothesis that SI3-MMP acts as a positive regulator of defense response against B. cinerea. This is consistent with the observations that ectopic overexpression of MtMMPL1 led to numerous abortive infections and an overall decrease in the number of nodules upon Sinorhizobium meliloti infection [13]. Additionally, silencing of SI3-MMP also led to a reduced resistance to Pst DC3000, a hemibiotrophic bacterial pathogen that has different life style from that of B. cinerea, indicating a broad involvement of SI3-MMP in regulation of disease resistance against different pathogens.

Conclusion
Tomato genome encodes five SI-MMP genes and all of them exhibited differentially expression patterns in response to pathogens and defense signaling hormones. The present study focused on the characterization of SI-MMPs in disease resistance and data from VIGS- and transient expression-based analyses demonstrated that SI3-MMP functions as a positive regulator of defense response against B. cinerea and Pst DC3000 in tomato. Further biochemical studies indicate that SI3-MMP possesses an in vitro enzymatic activity with different cleavage site specificities and is targeted on the plasma membrane. However, the biological functions of other four SI-MMPs need to be investigated. Further identification of endogenous substrates for SI3-MMPs will be helpful in elucidation of the biochemical mechanism of SI3-MMP in disease resistance.

Methods
Plant growth, treatment and disease assays
Tomato (Solanum lycopersicum L.) cv. Suhong 2003 was used for all experiments. Plants were grown in a mixture of perlite: vermiculite: plant ash (1:6:2) in a growth room under fluorescent light (200 μE m⁻² s⁻¹) at 22-24 °C with 60 % relative humidity and a 14 h light/10 h dark cycle. Pathogen inoculation, disease assays and measurement of pathogen in planta growth were performed basically according to previously described protocols [43, 44]. For analysis of gene expression in responding to pathogen infection, mock-inoculation controls were set by treating the plants with buffer solution (B. cinerea) or 10 mM MgCl₂ (Pst DC3000). For analysis of gene expression in response to defense signaling hormones, 4-week-old tomato plants were treated by foliar spraying with 100 μM MeJA, 100 μM ACC or 100 μM SA in 0.1 % ethanol and equal volume of 0.1 % ethanol solution as control. Leaf samples were collected at indicated time points after treatment or inoculation and stored at -80 °C until use.

Identification of tomato SI-MMPs and bioinformatics analyses
Arabidopsis AtMMPs were used as queries to perform multiple database searches against the proteome and genome files downloaded from the SOL Genomics Network (SGN, http://solgenomics.net) [45]. BlastP and TBlastN at NCBI (http://blast.ncbi.nlm.nih.gov) were performed with an e-value cutoff set to 1e⁻003 [46]. All protein sequences were compared with known MMP sequences using ClustalX (http://www.clustal.org/) to verify the sequences were candidate MMPs. The obtained MMP sequences were examined by domain analysis programs PFAM (http://pfam.sanger.ac.uk/) and SMART (http://smart.embl-heidelberg.de/) with the default cutoff parameters [47, 48]. The isoelectric points and molecular weights were predicted with the help of the proteomics and sequence analysis tools on the Expasy Proteomics Server (http://expasy.org/). Sequence alignment was carried out by the ClustalX program [49]. Putative signal peptides and transmembrane domains were predicted by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and TMpred (http://.ch.embnet.org/software/TMPRED_form.html), respectively. Phylogenetic trees including the tomato MMP protein sequences were constructed using the neighbor-joining (NJ) method of the MEGA6 program with the p-distance and complete deletion option parameters [50]. The reliability of the obtained trees was tested using a bootstrapping method with 1000 replicates.

Cloning of the SI-MMP genes
The coding sequence of the SI3-MMP gene was amplified using a pair of gene-specific primers (Additional file 3) from tomato cDNAs and cloned into pMD19-T vector, yielding plasmids pMD19-SI-MMPs. After confirmation by sequencing, the plasmid pMD19-SI3-MMP was used for further experiments.
Purification of recombinant SI3-MMP protein and proteolytic activity assays

The coding sequence for the mature form of SI3-MMP protein (SI3-MMPm) was amplified from pMD19-SI3-MMP with a pair of primers (Additional file 3) and cloned into pGEX-4 T-3 vector at EcoRI/XhoI sites, which was fused to glutathione-S-transferase (GST) at its N-terminal. The recombinant plasmid pGEX-SI3-MMPm and empty vector were introduced into the E. coli strain Rosetta DE3 and expression of SI3-MMP fusion protein and GST tag in E. coli cells was induced by 1 mM isopropyl-D-thiogalactoside (IPTG) at 20 °C overnight. The GST-tagged SI3-MMPm fusion protein and GST tag were purified using the Bug-Buster GST-Bind purification kit following the manufacturer’s protocols (Merck, Darmstadt, Germany). Protein concentration was determined using Bio-Rad protein assay kit (Bio-Rad, CA, USA) according to the recommended method.

For proteolytic activity assays, purified SI3-MMPm or GST (a negative control) was incubated with 5 µg of MBP (Sigma-Aldrich, St. Louis, MO, USA) in the absence (−) or presence (+) of 1 mM EDTA in 200 mM Tris–HCl, pH7.5, containing 10 mM CaCl₂, 0.1 % (w/v) Brij35 and 1× EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) at 37 °C overnight. Products were analyzed by 16 % Tricine–SDS-PAGE and stained in Colloidal Coomassie [51].

Proteolytic activity of SI3-MMP were measured using the synthetic fluorescent substrates Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (QF-24), Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH₂ (QF-35) and Mca-KESabuNLFVLKDPaR-NH₂ (QF-75) (synthesized by Invitrogen Life Technologies, Inc.). Purified recombinant SI3-MMPm was incubated to a final concentration of 1 µM in a total volume of 100 µL of 50 mM Hepes, pH7.5, 5 mM CaCl₂ and 10 µM ZnCl₂. Synthetic quenched fluorescent peptide substrates were added from a 100x stock in DMSO to a final concentration of 1 µM. The excitation and emission wavelengths were set at 320 and 405 nm, respectively and the fluorescence was measured at one minute intervals for 90 min.

Subcellular localization

The coding sequence of SI3-MMP was amplified from pMD19-SI3-MMP using a pair of primers SI3-MMP-GFP-F and SI3-MMP-GFP-R (Additional file 3) and inserted into pFGC-Egfp at BamHI/XbaI sites. The recombinant plasmid pFGC-SI3-MMP and the empty vector pFGC-Egfp were introduced into onion epidermal cells by particle bombardment method. Particle bombardment was performed with a PDS-1000 (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. GFP was detected 24 h after bombardment. Plasmolysis was achieved by treating the bombarded onion epidermal cells with 0.8 M mannitol for 10 min. Microscopic observation was performed using a Zeiss LSM 780 confocal laser scanning microscope (Carl Zeiss, Germany) and representative photographs were taken.

VIGS in tomato and transient expression in N. benthamiana

For VIGS constructs, fragments of 300-400 bp (Additional file 2) for SI-MMPs were amplified from tomato cDNAs using gene-specific primers (Additional file 3) and cloned into pTRV2 vector [26], yielding pTRV2-SI1-MMP-pTRV2-SI5-MMP. The recombinant plasmids with pTRV2-GUS (as control) were then introduced into Agrobacterium tumefaciens strain GV3101 by electroporation using GENE PULSER II Electroporation System (Bio-Rad Laboratories, Hercules, CA, USA). Agrobacteria carrying pTRV2-GUS (control) or pTRV2-SI-MMP plasmids were grown in YEP medium (50 µg/ml rifampicin, 50 µg/ml kanamycin and 25 µg/ml gentamycin) for 24 h with continuous shaking at 28 °C. Cells were centrifuged and resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES, 200 µM acetosyringone, pH5.7). Agrobacteria carrying pTRV2-GUS or pTRV2-SI-MMP were mixed with agrobacteria carrying pTRV1 in a ratio of 1:1 and adjusted to OD₆₀₀ = 1.5. The mixed agrobacteria suspension was infiltrated into the abaxial surface of 2-week-old seedlings using a 1 ml needleless syringe. Efficiency of the silencing protocol was examined using phytoene desaturase (PDS) gene as a marker of silencing in tomato plants according to the protocol described previously [26]. The VIGS-infiltrated plants were allowed to grow for three weeks under same condition as mentioned above and then used for all experiments.

For transient expression in N. benthamiana, agrobacteria carrying pFGC-SI3-MMP or pFGC-eGFP empty vector were infiltrated into leaves of 4-week-old plants using 1 ml needleless syringes. Leaf samples were collected 2 days after agroinfiltration for analyzing the expression level of SI3-MMP and were used for disease assays and physiological, biochemical and molecular analyses.

qRT-PCR analysis of gene expression

Total RNA was extracted by Trizol regent (TaKaRa, Dalian, China) according to the manufacturer’s instructions. RNA was treated with RNase-free DNase and then reverse-transcribed into cDNA using the PrimeScript RT regent kit (TaKaRa, Dalian, China). The obtained cDNAs were used for gene expression analysis with real time quantitative PCR. Each qPCR reaction contained 12.5 µL SYBR Premix Ex Taq (TaKaRa, Dalian, China), 0.1 µg cDNA and 7.5 pmol of each gene-specific primer (Additional file 3) in a final volume of 25 µL, and had three independent biological replicates. The qPCR was performed in a CFX96 real-time PCR detection system.
(BioRad, Hercules, CA, USA). Relative gene expression level was calculated using $2^{-\Delta\Delta CT}$ method as described [52].

Western blot analysis
Leaf discs were ground into 200 μl lysis buffer (50 mM Tris–HCl, pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DDT, 0.1 % (v/v) Triton X-100, and 1x protease inhibitor cocktail from Sigma plus 1 mM PMSF), followed by addition of 100 μl loading buffer. The samples were boiled for 5 min and subsequently centrifuged at 10,000× g for 10 min at 4 °C. Proteins in 20 μl of the supernatant were separated on a 12 % SDS-PAGE gel and transferred onto PVDF membrane by semi-dry transfer. Detection of GFP was performed using a polyclonal rabbit anti-GFP antibody (1:5000 dilution; GenScript, Nanjing, China) and a Horseradish peroxidase-conjugated anti-rabbit antibody (1:10,000 dilution; GenScript, Nanjing, China) according to the manufacturer’s instructions. Proteins on PVDF membranes were detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA).

Histological assays and measurement of electrolyte leakage
Detection of H$_2$O$_2$ was performed by DAB staining [53]. Leaf samples were collected from inoculated tomato plants at 24 h after inoculation or N. benthamiana plants at 48 h after infiltration for transient expression. Leaves were dipped into DAB solution (1 mg/ml, pH3.8) and incubated for 8 h in dark at room temperature. The DAB-treated leaves were removed, placed into acetic acid/glycerol/ethanol (1:1:1, vol/vol/vol), and boiled for 5 min in a water bath, followed by several changes of the solution. Subsequently, the leaves were maintained in 60 % glycerol. Accumulation of H$_2$O$_2$ was visualized using a digital camera and quantified using ImageJ software (National Institutes of Health) from DAB image. Trypan blue staining to visualize cell death and B. cinerea hypha were performed as previously described [44]. Fresh tissue was harvested, stained, and boiled for 30 s in lactophenol (10 mL of lactic acid, 10 mL of glycerol, 10 mL of liquid phenol, and 10 mL of distilled water) containing 10 mg of trypan blue. Tissue was rapidly transferred and boiled in alcoholic lactophenol (2:1 95 % ethanol:lactophenol) for 1 min, washed in 50 % ethanol at room temperature for 2 min, and stored in water. The stained leaves were examined using a digital camera.

Electrolyte leakage was measured as previously described [54]. Leaf discs (0.5 cm diameter) were washed in sterile double-distilled water for 30 min, followed by incubation for 2 h at room temperature with gentle agitation. Electrolyte leakage from the leaf samples was evaluated by measuring ion conductivity using a conductivity meter (FE30, Mettler-Toledo Group, Switzerland).

Statistical analysis
All experiments were repeated independently three times. Data obtained from three independent experiments were subjected to statistical analysis according to the Student’s t-test and the probability values of $p < 0.05$ were considered as significant difference.

Accession numbers for SI-MMPs and the defense-related genes
The SI-MMP sequences were deposited in GenBank database under the following accession numbers: SI1-MMP, KR081423; SI2-MMP, KR081424; SI3-MMP, KR081425; SI4-MMP, KR081426; SI5-MMP, KR081427. The defense-related genes used in this study and their GenBank accession numbers are as follows: SIBoh1, respiratory burst oxidase homolog 1 (NM_001247197); SIWFI1, whitefly-induced 1 (NM_001247342); SIAPX, ascorbate peroxidase (XM_006366063); SLGR, glutathione reductase (XM_010328522); SILCAT, catalase (XM_04238382); SISOD, superoxide dismutase (AF527880); PR1a, pathogenesis-related 1a (NM_001247869); PR1b, pathogenesis-related 1b (NM_001247385); SİLaP1, leucine aminopeptidase (AK319505); SIPI-I, proteinase inhibitors 1 (M13938); SIPI-II, proteinase inhibitors II (JN091682).

Availability of supporting data
The cDNA and protein sequences of SI-MMPs and the VIGS fragments for SI-MMP genes used in this study are included in Additional files 1 and 2. Phylogenetic datasets are available for download at Dryad (http://datadryad.org/; doi: 10.5061/dryad.7qh1r).

Additional files

Additional file 1: Sequences of cDNAs and proteins of SI-MMPs.
Additional file 2: Sequences of the VIGS fragments for SI-MMPs.
Additional file 3: Table S1. Primers used in this study for different purposes.

Abbreviations
ACC: 1-amino cyclopropane-1-carboxylic acid; APX: Ascorbate peroxidases; B. cinerea: Botrytis cinerea; CAT: Catalases; DAB: 3, 3-diaminobenzidine; dpi: Days post-inoculation; ECM: Extracellular matrix; EDTA: Ethylenediaminetetraacetic acid; GR: Glutathione reductase; MeJA: Methyl jasmonate; MMP: Matrix metalloproteinase; PDS: Phytoreole desaturase; Pst: Pseudomonas syringae pv. tomato; QF: Quenched fluorescent; ROS: Reactive oxygen species; qRT-PCR: Quantitative reverse transcription PCR; SA: Salicylic acid; SOD: Superoxide dismutase; VIGS: Virus-induced gene silencing.

Competing interests
The authors declare that they have no competing interests.
Author's contributions
DL and FS designed the experiments. DL, HZ, QS, LW, SL, YH and LH carried out most of the experiments. DL performed bioinformatics analysis. FS and DL wrote the paper. All authors read and approved the final manuscript.

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