Title: Eugenol confers resistance to *Tomato yellow leaf curl virus* (TYLCV) by regulating the expression of *SlPer1* in tomato plants

Author: Wei-Jie Su Wen-Jing Lv Li-Na Li Gan Yin Xiaofang Hang Yanfeng Xue Jian Chen Zhiqi Shi

PII: S1871-6784(16)00004-2
DOI: http://dx.doi.org/doi:10.1016/j.nbt.2016.01.001
Reference: NBT 848

To appear in:

Received date: 6-8-2014
Revised date: 14-12-2015
Accepted date: 7-1-2016

Please cite this article as: Su, W.-J., Lv, W.-J., Li, L.-N., Yin, G., Hang, X., Xue, Y., Chen, J., Shi, Z.,Eugenol confers resistance to *Tomato yellow leaf curl virus* (TYLCV) by regulating the expression of *SlPer1* in tomato plants, *New Biotechnology* (2016), http://dx.doi.org/10.1016/j.nbt.2016.01.001

Highlights

- Eugenol showed effective anti-TYLCV activity.
- Eugenol could stimulate plant immune signals.
- Eugenol strongly induced the expression of a host specific *R* gene *SlPer1*.
- *SlPer1* could be differentially regulated by multiple defensive signaling.

Title: Eugenol confers resistance to *Tomato yellow leaf curl virus* (TYLCV) by regulating the expression of *SlPer1* in tomato plants

Authors:

Wei-Jie Su[1,†], Wen-Jing Lv[2,†], Li-Na Li[2,†], Gan Yin[3], Xiaofang Hang[3], Yanfeng Xue[3, 4], Jian Chen[3, 4*] and Zhiqi Shi[1, 3, 4*]

Affiliations:

[1] College of Plant Protection, Nanjing Agricultural University, Weigang 1, Nanjing 210095, China

[2] College of Horticulture, Nanjing Agricultural University, Weigang 1, Nanjing 210095, China

[3] Institute of Food Quality and Safety, Jiangsu Academy of Agricultural Sciences, 50 Zhongling Street, Nanjing 210014, China

[4] Key Laboratory of Control Technology and Standard for Agro-product Safety and Quality, Ministry of Agriculture, China. 50 Zhongling Street, Nanjing 210014, China

[†] These authors contributed equally to this work.

* Corresponding author: Zhiqi Shi or Jian Chen

E-mail: jaasinput@gmail.com; jacksonchen206@gmail.com

Tel: +86-25-84391863; Fax: +86-25-84390422

Mailing address: College of Plant Protection, Nanjing Agricultural University, Weigang 1, Nanjing 210095, China

Abstract

Tomato yellow leaf curl virus (TYLCV) is one of the most devastating plant diseases, and poses a significant agricultural concern because of the lack of an efficient control method. Eugenol is a plant-derived natural compound that has been widely used as a food additive and in medicine. In the present study, we demonstrated the potential of eugenol to enhance the resistance of tomato plants to TYLCV. The anti-TYLCV efficiency of eugenol was significantly higher than that of moroxydine hydrochloride (MH), a widely used commercial antiviral agent. Eugenol application stimulated the production of endogenous nitric oxide (NO) and salicylic acid (SA) in tomato plants. The full-length cDNA of *SlPer1*, which has been suggested to be a host *R* gene specific to TYLCV, was isolated from tomato plants. A sequence analysis suggested that SlPer1 might be a nucleobase-ascorbate transporter (NAT) belonging to the permease family. The transcript levels of *SlPer1* increased markedly in response to treatment with eugenol or TYLCV inoculation. The results of this study also showed that *SlPer1* expression was strongly induced by SA, MeJA (jasmonic acid methyl ester), and NO. Thus, we propose that the increased transcription of *SlPer1* contributed to the high anti-TYLCV efficiency of eugenol, which might involve in the generation of endogenous SA and NO. Such findings provide the basis for the development of eugenol as an environmental-friendly agricultural antiviral agent.

Keywords: Eugenol; Nitric oxide; Permease; Salicylic acid; TYLCV

Introduction

Tomato yellow leaf curl virus (TYLCV) is emerging as one of the major threats to crop production worldwide because it can infect many important agricultural dicotyledonous crop plants, such as tomato, common bean, cowpea, pepper, eggplant, squash, and *Acalypha australis* [1-6]. TYLCV, which belongs to the genus *Begomovirus*, was first found in Middle East, but has spread rapidly around the world [7]. In recent years, TYLCV disease outbreaks have occurred in a large area of China, and have severely damaged tomato production [8]. Given that several TYLCV resistance loci have been identified from wild tomato species [9-12], breeding of TYLCV-resistant cultivars provides a promising approach to control this disease. However, this breeding strategy is time-consuming. In addition, some domestic cultivars with a high and stable resistance to TYLCV have been obtained through introgressing resistance traits. However, these cultivars have shown unsatisfactory horticultural quality, which limits their cultivation under many production and climatic conditions, and limits their market appeal [13,14]. Therefore, there is a need for an integrated strategy for the management of TYLCV. Unlike fungi and bacteria, viruses are difficult to kill using direct application methods. The best approach for controlling a virus disease is to enhance the intrinsic antiviral immunity in host plants [15]. Various chemicals have been reported to induce plant immunity [16,17]. Therefore, mining for specific exogenous inducers that are able to trigger plant immunity against TYLCV represents an efficient alternative for controlling TYLCV disease.

The identification of TYLCV-resistant mechanisms provides an important basis for research on chemical-induced resistance to this pathogen. Among the five major resistant loci (*Ty-1*, *Ty-2*, *Ty-3*, *Ty-4*, and Ty-5) [9,18,19], only *Ty-1* and *Ty-3* had been identified as alleles encoding DFDGD-class RNA-dependent RNA polymerases [10]. A reverse genetics approach that compared cDNA libraries of TYLCV-resistant and -susceptible tomato cultivars revealed a series of tomato genes that were preferentially expressed in resistant cultivars upon TYLCV infection [20]. Three of those genes, *permease I-like protein*, *hexose transporter* (*LeHT1*), and *lipocalin-like protein* (*SlVRSLip*) were subsequently identified as TYLCV-resistant genes, because plants in which these genes were silenced lost their resistance to TYLCV [20-23]. These three genes seem to confer TYLCV resistance independently [22]. Plant immune responses against viruses involve basic resistance mediated by several important plant hormones, such as salicylic acid (SA), jasmonic acid (JA), and nitric oxide (NO) [15]. Transcriptome analyses revealed that TYLCV-resistant cultivars had activated SA biosynthesis and repressed JA-dependent signaling pathways [11,24].

Eugenol (4-allyl-2-methoxyphenol) is a natural plant-derived compound that has been shown to have anti-fungal, anti-bacterial, and anti-insect activities in pharmacological and food-protection studies [25,26]. A recent study showed that eugenol also inhibits the growth of plant pathogenic fungi [27]. In addition, eugenol exhibits antidiarrheal activity, which is attributed to its ability to inhibit the Ca^{2+}-activated Cl^- channel, and anti-colon cancer activity, which is attributed to its apoptosis-inducing activity in mammalian cells [28,29]. However, the mechanisms by

which eugenol modulates plant immunity and functions as an anti-viral agent remain unclear. In the present study, we investigated the anti-viral activity of eugenol against TYLCV. We explored the possible anti-viral mechanism of eugenol by evaluating its ability to regulate hormones and induce expression of *permease I-like protein* (also known as *SlPer1*).

Materials and methods

Plant cultivation

Tomato (*Solanum lycopersicum*, Suhong2003 wild type) seeds were surface-sterilized with 1% NaClO for 10 min and then washed with distilled water. Seeds were germinated in dark for 12 h in Petri dishes on filter papers moistened with distilled water. Then, the germinated seeds were transferred into potting mix in pots and grown in a greenhouse under the following conditions: 12-h light/12-h dark photoperiod with photosynthetic active radiation of 200 mmol m^{-2} s^{-1}, at 25±1 °C.

Agro-inoculation of plants with infectious TYLCV clone

The *Agrobacterium tumefaciens* strain EHA105 with an infectious clone of TYLCV (PTYj01) was provided by the Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences, China. *Agrobacteria* cells were cultured in YEP medium containing 50 mg L^{-1} of kanamycin and 50 mg L^{-1} of rifampicin at 28 ºC with shaking at 150 rpm. Tomato plants with 4–5 leaves were selected for TYLCV inoculation. *Agrobacterium* cultures with OD_{600} 0.6–0.8 were injected into the stems of tomato plants [30].

Chemicals and treatments

Eugenol at different concentrations (50, 100, and 200 μg mL^{-1}) was sprayed evenly onto both sides of tomato leaves 24 h before TYLCV inoculation. Control plants were sprayed with distilled water. The commercial antiviral agent moroxydine hydrochloride (MH) was sprayed at 450 μg mL^{-1} as a positive control.

For chemical response analyses, tomato shoots were sprayed with 50 μM gibberellin, 200 μM SA, 100 μM ethephon (an ethylene-releasing compound), 100 μM MeJA (jasmonic acid methyl ester), 200 μM SNP (sodium nitroprusside, an NO donor), or 200 μM NaHS (sodium hydrogen sulfide, a hydrogen sulfide (H_2S) donor). After various treatments, plant samples were harvested at indicated times for analyses.

Identification of TYLCV infection in tomato plants

Symptoms were evaluated according to the visual symptom-severity scale described by García-Cano et al. [31] in which 0, 1, 2, 3, 4, and 5 represent no visible symptoms, 20%, 40%, 60%, 80%, and 100% of plants showing symptoms, respectively. The disease index (DI) was calculated as follows:

$$DI\ (\%) = \Sigma\ (\text{scale no.} \times \text{no. of plants at corresponding disease scale}) \times 100 / (\text{highest scale} \times \text{no. of total tested plants})$$

The control efficiency (%) of eugenol was calculated as follows:

$$\text{Control efficiency}\ (\%) = (C_{di}\text{-}T_{di}) \times 100/C_{di}$$

where C_{di} is the mean value of the DI of TYLCV-infected plants sprayed with distilled water, and T_{di} is the mean DI of TYLCV-infected plants sprayed with eugenol.

Semi-quantitative RT-PCR was used to determine the transcript levels of three viral genes, *capsid protein* (*CP*), *replication initiator protein* (*Rep*), and *replication enhancer protein* (*Ren*). Total RNA was extracted from shoot tissues using Trizol

(Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed at 42°C in a 25-μL reaction mixture containing 3 μg RNA, 0.5 μg oligo(dT) primers, 12.5 nmol dNTPs, 20 units RNase inhibitor, and 200 units M-MLV. The first cDNA was used as a template for PCR and to analyze the transcript levels of genes. The following primers were used for amplifications: *CP*, sense 5′-CTTATGAGCAACGGGATG-3′ and antisense 5′-CCAAGAAGAACACGACCT-3′; *Rep*, sense 5′-TCTCGGCGACCCACTCTT-3′ and antisense 5′-GTCAGCAATCTGCCAACG-3′; *Ren*, sense 5′-CCAAGAAGAACACGACCT-3′ and antisense 5′-GCTGTAATGTCGTCCAAA-3′; *Actin*, sense 5′-AGAGCTATGAGCTCCCAGATGG-3′ and antisense 5′-TTAATCTTCATGCTGCTAGGAGC-3′.

To standardize the transcript levels of tested genes, the relative abundance of *Actin* was used as an internal standard. First, the PCR product of *Actin* for each sample was loaded onto a 1% (w/v) agarose gel containing ethidium bromide and subjected to electrophoresis. Then, the bands were visualized under an ultraviolet transilluminator and photographed with a CCD camera. Densitometric scanning was used to quantify the signal intensity of each band. The loading cDNA template for each sample was quantitatively adjusted according to the signal intensity of the *Actin* band. Finally, the band intensity for the PCR product of the target gene represented the relative mRNA abundance of the gene.

Cloning of full-length SlPer1 cDNA from tomato plants

The putative tomato *SlPer1* gene sequence (SGN-U564503) was retrieved from the tomato genome database (Sol Genomics Network, http://solgenomics.net/organism/Solanum_lycopersicum/genome). This sequence was

used to design a primer pair for PCR (sense 5′-GGGTTTGCAGTTTGCACCGC-3′ and antisense 5′-TGCGCACCAATACACAGCTC-3′), which would amplify the entire coding region of tomato *SlPer1* cDNA.

Total RNA extraction and reverse transcription were performed as described above. The PCR conditions for amplifying *SlPer1* were as follows: 94°C for 5 min; followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1.5 min, with final extension at 72°C for 5 min. The PCR product was gel-purified with an AxyPrep Gel DNA Extraction Kit (Axygen, Hangzhou, China) according to the manufacturer's protocol. Then, the purified PCR product was cloned into the pMD 19-T vector (TaKaRa, Otsu, Japan) for sequencing (Invitrogen, Shanghai, China).

We conducted RT-PCR to analyze the transcript levels of *SlPer1* in tomato plants under various treatment conditions. Total RNA extraction and reverse transcription were performed as described above. A 202-bp fragment within the coding region of *SlPer1* was amplified using the following primers: sense 5′-TTAACGTGCCATTCTCATCG-3′ and antisense 5′-CACACGGATGGGAAATACTT-3′. To standardize the transcript levels of *SlPer1*, the relative abundance of *Actin* was used as an internal standard.

Bioinformatics analysis

The open reading frame (ORF) was analyzed using the online tool ORF Finder at http://www.ncbi.nlm.nih.gov/gorf/gorf.html. Then, DNA sequences 2-kb in length from the region upstream of *SlPer1* were retrieved to analyze *cis*-elements using the online tool PLACE at http://www.dna.affrc.go.jp/PLACE/signalscan.html [32]. Analyses of the chemical and physical properties of the protein were performed by

ProtParam at http://web.expasy.org/protparam/ [33]. Protein structure prediction was performed by SMART at http://smart.embl-heidelberg.de/ [34]. The protein subcellular location was predicted by WoLF PSORT at http://wolfpsort.org/ [35]. The analysis of transmembrane regions of the protein was performed using the TMHMM tool at http://www.cbs.dtu.dk/services/TMHMM-2.0 [36]. Multiple alignment of amino acid sequences of the tested proteins was conducted by using CLUSTALX 2.0 software [37]. The phylogenetic trees were constructed using the maximum likelihood method in MEGA6 [38].

All the sequences analyzed in the present study were obtained from BRAD (Brassica database; http://brassicadb.org/brad/index.php) or NCBI (National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov/). The following sequences were obtained from BRAD: Bra013170 for *Brassica rapa* nucleobase-ascorbate transporter 1 (BrNAT1), Bra005445 for *B. rapa* NAT2 (BrNAT2), Bra000543 for *B. rapa* NAT3 (BrNAT3), Bra014228 for *B. rapa* NAT4 (BrNAT4), Bra037944 for *B. rapa* NAT5 (BrNAT5), Bra010129 for *B. rapa* NAT6A (BrNAT6A), Bra035875 for *B. rapa* NAT6B (BrNAT6B), Bra029239 for *B. rapa* NAT6C (BrNAT6C), Bra035409 for *B. rapa* NAT7 (BrNAT7), Bra036544 for *B. rapa* NAT10A (BrNAT10A), Bra009839 for *B. rapa* NAT10B (BrNAT10B), Bra011804 for *B. rapa* NAT11 (BrNAT11), Bra034371 for *B. rapa* NAT12A (BrNAT12A), Bra011995 for *B. rapa* NAT12B (BrNAT12B). The following sequences were obtained from NCBI: NP_178636.1 for *Arabidopsis thaliana* NAT1 (AtNAT1), NP_180966.1 for *A. thaliana* NAT2 (AtNAT2), NP_180219.1 for *A. thaliana* NAT3

(AtNAT3), NP_175418.1 for *A. thaliana* NAT4 (AtNAT4), NP_199810.2 for *A. thaliana* NAT5 (AtNAT5), NP_201094.1 for *A. thaliana* NAT6 (AtNAT6), NP_176211.2 for *A. thaliana* NAT7 (AtNAT7), NP_172524.1 for *A. thaliana* NAT8 (AtNAT8), NP_197924.1 for *A. thaliana* NAT9 (AtNAT9), NP_176733.2 for *A. thaliana* NAT10 (AtNAT10), NP_195518.2 for *A. thaliana* NAT11 (AtNAT11), NP_850108.1 for *A. thaliana* NAT12 (AtNAT12), and NP_001061818.1 for *Oryza sativa* NAT6 (OsNAT6).

Histochemical detection of endogenous NO in tomato plants

Intracellular NO was visualized using the fluorescent probe DAF-FM DA (3-amino, 4-aminomethyl-2',7'- difluorescein diacetate) as described by Guo et al [39]. Root and stem samples from eugenol-treated plants were incubated in 20 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-NaOH buffer solution (pH 7.5) containing 15 μM DAF-FM DA at 25°C for 15 min in the dark. The roots were rinsed with distilled water three times and then visualized under a fluorescence microscope (ECLIPSE, TE2000-S, Nikon, Tokyo, Japan) (excitation 490 nm and emission 525 nm). The relative fluorescence density of the images was analyzed using Image-Pro Plus 6.0 (Media Cybernetics Inc., Silver Spring, MD, USA).

Determination of endogenous SA in tomato plants

The concentration of SA in plant leaves was determined by using a Plant SA ELISA Kit (Shanghai Jianglai Bioengineering Institute Co., Ltd., Shanghai, China) according to the manufacturer's instructions. Briefly, 1 g leaf tissue was homogenized in 10 mL

ice-cold phosphate buffer (50 mM, pH 7.4). The homogenate was centrifuged at 3,000 g for 20 min at 4°C. The supernatant was used for determination of SA. A 10-μL aliquot of the sample was mixed with 40 μL sample dilution and then added to a well in a plate embedded with HRP (horse radish peroxidase)-labeled SA antibody. Then, the plate was incubated at 37°C for 30 min followed by washing and drying. The substrate TMB (3,3',5,5'-tetramethylbenzidine), which reacts with HRP to form a blue product, was added to each well, and the plate was incubated in the dark at 37°C for 15 min. The reaction was terminated by adding sulphuric acid. The color change was measured spectrophotometrically at 450 nm with a Mithras LB 940 Multimode Reader (Berthold, Germany). The concentration of SA in the samples was determined by comparing the OD_{450nm} of the samples to an SA standard curve.

Quantification of virus

The virus was quantified as described elsewhere [40,41], with minor changes. Genomic DNA was extracted from TYLCV-infected plant leaves using an EaxyPure Plant Genomic DNA Kit (Transgene Biotech, Beijing, China) according to the manufacturer's instructions. The concentration of DNA was determined by spectrophotometric analysis (NanoVue^{Tm} Plus, GE Healthcare). The integrity of DNA samples was assessed by agarose gel electrophoresis.

Quantitative real-time PCR (qPCR) was performed using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The amplification was performed using an SYBR® Premix Ex Taq™ (TIi RNaseH Plus)

qPCR Kit (Takara Bio, Dalian, China) according to the manufacturer's instructions. The primers used to amplify *CP* genes, as described above, were used to perform qPCR. The qPCR procedure was as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s. Data were collected and analyzed by using ABI 7500 software (v. 2.0.6, Applied Biosystems). The threshold cycle (C_t) was determined as the cycle number with the detection of a significant increase in reporter fluorescence. To develop a standard curve for quantifying the number of TYLCV copies in infected tomato leaves, a plasmid (pGMT) containing the pure full-length TYLCV genome was prepared for qRT-PCR and calculations, based on the methods described by Mason et al. [40].

Statistical analysis

Data shown are mean ± standard deviation (SD) of at least three replicated measurements. Significant differences between treatments were statistically evaluated by SD and one-way analysis of variance (ANOVA) using SPSS 2.0. Two specific different treatments were compared statistically by ANOVA, followed by F-test if the ANOVA result was significant at $P<0.05$. For multiple comparison analyses, least significant difference test (LSD) was performed on all data after ANOVA when significant differences ($P<0.05$) were detected among different treatments.

Results

Eugenol significantly protected tomato plants against TYLCV infection

In China, MH is a widely used anti-viral agent for controlling viral diseases of

vegetables [42]. In the present study, the control efficiency of MH against TYLCV was 53.8% (Fig. 1A), which was lower than that of eugenol (controlling efficiency of eugenol at 200 μg mL^{-1} was 77.4%). Eugenol remarkably attenuated the TYLCV-induced disease symptoms, such as leaf yellowing, leaf curling, and growth stunting (Fig. 1A, B). The TYLCV infection was verified by the detection of transcripts of three viral genes (*CP*, *Rep*, and *Ren*) in leaves (Fig. 1C). Treatment with eugenol at 200 μg mL^{-1} effectively decreased the transcript levels of these virus genes (Fig. 1C), suggesting that viral replication was inhibited in eugenol-treated plants. In addition, eugenol treatment led to marked decreases in virus titer in the young leaves of TYLCV-infected plants (Fig. 1D).

Cloning and sequence analysis of cDNA encoding SlPer1

The full-length cDNA sequence of *SlPer1* was isolated from tomato leaves using an RT-PCR-based strategy. The entire ORF of *SlPer1* cDNA was 1725-bp long, and encoded a protein of 574 amino acid (aa) residues with a predicted molecular mass of 62.4 KDa and a theoretical isoelectric point of 9.38. The results of online BLAST analyses indicated that SlPer1 showed high similarities with plant NATs (nucleobase-ascorbate transporters). The polygenetic analysis indicated that SlPer1 was in a subgroup with NAT5 and NAT6 from *A. thaliana* and *B. rapa,* respectively (Fig. 2).

SlPer1 was predicted to have 11 transmembrane domains, and it contained a Xan_ur_permease conserved domain (from 31–437 aa), which is a characteristic of the permease family (Fig. 3). This family includes NATs, which function in the transport of diverse substrates such as xanthine, uracil, nucleobase, and ascorbate. It

has been suggested that NAT family members can be recognized by the NAT signature motif, (Q/E/P)-N-X-G-X-X-X-X-T-(R/K/G) [43]. A typical NAT motif was detected in the peptide sequence of SlPer1: ENVGLLALTR located from 344 − 353 aa (Fig. 3B).

The analysis of *cis*-elements suggested that there were several kinds of disease-responsive elements in the promoter region of *SlPer1*, including two elicitor-responsive elements, five SA-responsive elements, and one JA-responsive element (Table 1).

Transcription of SlPer1 was stimulated by TYLCV and eugenol

The transcriptional pattern of *SlPer1* was monitored in tomato leaves after inoculation with TYLCV. Compared with mock-inoculated plants, those infected with TYLCV showed 58%, 70%, and 91% increases in *SlPer1* transcript levels in their infected leaves at 7, 14, and 21 dpi, respectively (Fig. 4A, B). Next, we investigated the effect of eugenol on the transcription of *SlPer1*. Compared with the control, the plants treated with eugenol at 100 and 200 μg mL^{-1} showed 408% and 289% increases, respectively, in *SlPer1* transcript levels in the leaves (Fig. 4C, D).

Transcriptional patterns of SlPer1in response to plant hormones

The transcript levels of *SlPer1* in tomato leaves were analyzed in plants treated with well-known plant hormones. The results of time-course experiments showed that the transcription of *SlPer1* was regulated differentially by plant hormones (Fig. 5). Both SA and MeJA induced *SlPer1* transcription. The maximal induction of *SlPer1* by SA

and MeJA was at 12 h and 1 h after treatments, respectively (Fig. 5A–C). However, both gibberellin and ethephon inhibited *SlPer1* transcription in tomato leaves (Fig. 5A, D, E).

Next, we investigated the response of *SlPer1* transcription to SNP (an NO donor) and NaHS (an H_2S donor) (Fig. 6). The transcription of *SlPer1* was induced by both of these chemicals. The maximal induction of *SlPer1* by SNP and NaHS was at 24 h after treatment (Fig. 6).

Eugenol induced accumulation of endogenous NO and SA in tomato plants

The NO-specific fluorescent probe DCF-FM DA was used to analyze the effect of eugenol on endogenous NO accumulation in tomato plants. Compared with the control, plants treated with eugenol at 200 μg mL^{-1} showed a significant increase in endogenous NO accumulation in both roots and stems (Fig. 7). Additionally, eugenol treatments resulted in significantly increased SA concentrations in tomato leaves in a time-dependent manner (Fig. 8). Compared with the control, the plants treated with eugenol for 96 h showed a 128% increase in the SA concentration in leaves (Fig. 8).

Discussion

TYLCV disease has become one of the biggest threats to agricultural production because of the inefficiency of the current control strategies. Here, we demonstrated that a plant-derived compound, eugenol, exhibits great potential to trigger plant immune responses. Thus, eugenol may be used as an environmental friendly anti-viral

agent to control TYLCV disease. The anti-TYLCV activity of eugenol may result from the induction of the expression of a specific *R* gene, *SlPer1,* in tomato plants. Additionally, NO and SA may mediate the eugenol-induced expression of *SlPer1*.

Plant activators are agrochemicals that confer disease resistance upon crops by activating plant immunity. Consequently, these compounds have attracted much attention worldwide [44,45]. Traditional plant activators include peptides (also called elicitors) and chitosan, which act as primers of plant immunity [46,47]. However, agrochemical-derived plant activators with relatively simple structures have proven to be more durable in the field, compared with peptides or chitosan, because they are not pathogen-specific [16]. Several recent studies have suggested that the main function of plant immune-activating agrochemicals is to activate the free SA pool in host plants [16,48]. In the present study, two lines of evidence suggested that eugenol might be a novel plant immune-priming agrochemical. First, eugenol induced the accumulation of SA in host tomato plants. Second, treatment with eugenol before virus inoculation reduced the severity of TYLCV disease symptoms in tomato plants. These results indicated that eugenol-primed immunity conferred resistance to subsequent viral attack. Given that most plant activators are synthetic molecules [16,48-51], their toxicities are unknown. Eugenol is a naturally plant derived compound that has been widely used as a food additive and medicine [25,26]. According to the official report from the United States Environmental Protection Agency (EPA), eugenol has minimal potential toxicity and poses minimal risks to humans and the environment. Eugenol is included on the list of pesticides that are exempt from all provisions of the FIFRA

(Federal Insecticide, Fungicide and Rodenticide Act) when intended for continued use [52,53]. Therefore, eugenol has great potential to be developed as an environmentally friendly pesticide to control virus diseases.

The role of *SlPer1* in the response to TYLCV infection was first reported by Eybishtz et al., who found that TYLCV resistance could be abolished by VIGS-mediated silencing of *SlPer1* in a resistant cultivar [20]. *SlPer1* encodes a permease in the NAT family, whose members transport various macromolecules including xanthine, uric acid, purines, and ascorbate. Ascorbate synthesized on the inner mitochondrial membrane can be transported by NATs to different cellular compartments, including the apoplast [54]. Ascorbate in the apoplast is essential for the deposition of callose in plasmodesmata [55], which is important for host plants to limit the cell-to-cell movement of the virus [56]. This is consistent with the view that *SlPer1* may be involved in transporting macromolecules or small signaling metabolites underlying virus resistance [20].

Eybishtz et al. [20] reported that *SlPer1* was preferentially expressed in a TYLCV-resistant cultivar (line 902) as compared to a TYLCV-susceptible cultivar (line 906-4). These authors found that the transcription of *SlPer1* was barely detected in line 906-4, even after 35 PCR cycles [20]. In the present study, we detected *SlPer1* PCR products in untreated tomato plant samples after 30 PCR cycles (Fig. 4). Both line 906-4 and the cultivar used in the present study were susceptible to TYLCV infection, but the discrepancy in the basic expression level of *SlPer1* may result from the different genetic backgrounds of the two cultivars. A comparison of the sequences

383 of *SlPer1* and its promoter region between susceptible and resistant genotypes may
384 provide alternative explanations for differences in *SlPer1* transcription in different
385 genetic backgrounds [20]. It has been suggested that *SlPer1* functions at an early stage
386 of events leading to TYLCV resistance, probably by restricting virus entry and
387 replication in resistant cultivars. In the present study, inoculation with TYLCV for
388 7–21 days resulted in 58% − 91% increases in the transcript levels of *SlPer1* (Fig. 4B).
389 However, treatment with eugenol for only 1 day led to a four-fold increase in the
390 relative expression level of *SlPer1* (Fig. 4D), similar to the rapid increase (six-fold
391 change at 3 dpi) in *SlPer1* expression reported for a resistant cultivar (line 902) upon
392 TYLCV infection [20]. A pretreatment with eugenol before TYLCV inoculation
393 alleviated the disease symptoms (Fig. 1). Overall, these data suggested that
394 eugenol-induced resistance to TYLCV is related to the rapidly enhanced transcription
395 of *SlPer1*.

396 In plants, SA is a multifaceted hormone involved in combating diseases caused by
397 multiple pathogens, including viruses [57]. The results of metabolomic and
398 transcriptomic studies have highlighted the differentially regulated pathways,
399 including the SA biosynthetic pathway, between resistant and susceptible plants upon
400 TYLCV infection [24]. Activated SA accumulation and the transcription of genes
401 related to SA biosynthesis have been observed in resistant plants in response to
402 TYLCV inoculation [24]. The presence of SA-responsive elements in the promoter
403 region of *SlPer1* explains the induction of *SlPer1* expression by SA (Fig. 5B; Table 1).
404 This may also explain the coordinated activation of SA and *SlPer1* for resistance to

TYLCV (Fig. 4D and 8). Eugenol stimulated generation of endogenous SA; therefore, SA might mediate eugenol-induced expression of *SlPer1*. Crosstalk between NO and SA has been demonstrated to be vital for host plants to combat disease [57]. Based on our current results, NO may also mediate eugenol-induced up-regulation of *SlPer1*. However, further research is required to determine how eugenol induces the expression of *SlPer1* by regulating the interplay between SA and NO.

In plants, JA and its derivative MeJA play important roles in regulating signaling responses to various biotic stresses [58]. Antagonism between SA and JA defensive signaling frequently occurs upon infection by pathogens, including viruses [58,59]. For instance, SA strongly inhibits JA-dependent defense pathways in *Nicotiana tabacum* in response to tobacco mosaic virus (TMV), an RNA virus [60]. A key component of the SA signaling pathway, NPR1 (nonexpressor of pathogenesis-related genes 1), suppresses the JA signaling pathway [59]. Our previous study indicated that eugenol enhanced TMV resistance by inducing SA accumulation and up-regulating*NPR1* expression in *N. tabacum*, suggesting that eugenol may suppress the JA-dependent signaling pathway [61]. Metabolomics analyses showed that TYLCV-resistant plants accumulated high levels of hydroxyjasmonic acid, a commonly occurring metabolite of JA that negatively regulates JA signaling [24, 62]. Considering that both TYLCV and eugenol activated SA biosynthesis, it is possible that eugenol may confer TYLCV resistance by differentially regulating SA- and JA-dependent defensive signaling pathways. In addition, we identified JA-responsive elements in the promoter region of *SlPer1*, which may explain why exogenous MeJA

427 is able to induce *SlPer1* expression. A recent study suggested that SA and JA act
428 synergistically to trigger systemic resistance against TMV [63]. Therefore, we
429 speculated that eugenol may induce *SlPer1* expression by coordinating synergistic
430 cross-talk between SA and JA. This should be verified by conducting further research
431 on the anti-viral mechanism of eugenol against DNA and RNA viruses.
432 Recent studies have suggested that H_2S may be a novel gaseous messenger
433 modulating defense responses in plants [64]. Research on the biological role of H_2S in
434 plants is just beginning, but cross-talk between H_2S and NO has been shown to
435 regulate various physiological processes in tomato [65]. In the present study, the
436 transcription of the tomato *R* gene *SlPer1* was strongly induced by the H_2S donor
437 NaHS. Therefore, the role of H_2S in eugenol-induced expression of *SlPer1* in tomato
438 plants to provide resistance against TYLCV would be an interesting topic for further
439 research.

440

441 ## Conclusions

442 The results of this study indicated that eugenol shows great potential to stimulate
443 plant immune responses against TYLCV attack. In addition to the general indicators
444 of defensive responses, such as the increases in SA and NO, eugenol stimulated the
445 expression of a specific host *R* gene, *SlPer1,* against TYLCV. The mechanisms by
446 which eugenol induces *SlPer1* expression are still unknown, but our data indicated
447 that SA and NO may play important roles in this process. Further work is required to

identify and characterize the eugenol-induced resistance network against TYLCV. Such analyses would provide a valuable basis for developing eugenol as a novel environmentally friendly plant activator.

Acknowledgements

This work was supported by the Natural Science Foundation of Jiangsu Province, China (BK20130703), National Natural Science Foundation of China (21207054), and the Hi-Tech Research and Development Program of China (2011AA10A202). We thank Dr. Yinghua Ji (Institute for Plant Protection, Jiangsu Academy of Agricultural Sciences) for providing *A. tumefaciens* strain EHA105. We also thank Dr. Neng Yi (Institute of Agricultural Resources and Environment, Jiangsu Academy of Agricultural Sciences) for helping with virus quantification. We thank Dr. Fengxiang X. Han (Jackson State University) for language assistance.

References

[1] Dai FM, Zeng R, Chen WJ, Lu JP. First report of tomato yellow leaf curl virus infecting cowpea in China. Plant Dis 2010;95:362.

[2] Ji YH, Zhang H, Zhang K, Li G, Lian S, Cheng ZB, et al. First report of tomato yellow leaf curl virus in *Acalypha australis* in China. Plant Dis 2013;97:430.

[3] Shahid MS, Natsuaki KT. Identification of tomato yellow leaf curl virus naturally

infecting Common bean in Japan. Plant Dis 2014.

[4] Zubiaur YM, Fonseca D, Quiñones M, Palenzuela I. Presence of tomato yellow leaf curl virus infecting squash (*Curcubita pepo*) in Cuba. Plant Dis 2004;88:572.

[5] Salati R, Shorey M, Briggs A, Calderon J, Rojas MR, Chen LF, et al. First report of tomato yellow leaf curl virus infecting tomato, tomatillo, and peppers in Guatemala. Plant Dis 2010;94:482.

[6] Tang YF, He ZF, Du ZG, Lu LH. First report of tomato yellow leaf curl Kanchanaburi virus infecting eggplant in Laos. Plant Dis 2013;98:428.

[7] Lefeuvre P, Martin DP, Harkins G, Lemey P, Gray AJA, Meredith S, et al. The spread of tomato yellow leaf curl virus from the Middle East to the world. PLoS Pathog 2010;6:e1001164.

[8] Zhang H, Gong H, Zhou X. Molecular characterization and pathogenicity of tomato yellow leaf curl virus in China. Virus Genes 2009;39:249-55.

[9] Ji Y, Scott JW, Schuster DJ, Maxwell DP. Molecular Mapping of *Ty-4*, a new tomato yellow leaf curl virus resistance locus on chromosome 3 of tomato. J Am Soc Hortic Sci 2009;134:281-8.

[10] Verlaan MG, Hutton SF, Ibrahem RM, Kormelink R, Visser RGF, Scott JW, et al. The tomato yellow leaf curl virus resistance fenes *Ty-1* and *Ty-3* are allelic and code for DFDGD-class RNA–dependent RNA polymerases. PLoS Genet 2013;9:e1003399.

[11] Chen T, Lv Y, Zhao T, Li N, Yang Y, Yu W, et al. Comparative transcriptome profiling of a resistant vs. susceptible tomato (*Solanum lycopersicum*) cultivar in response to infection by tomato yellow leaf curl virus. PLoS ONE 2013;8:e80816.

[12] Ji Y, Scott JW, Schuster DJ. Toward fine mapping of the *tomato yellow leaf curl virus* resistance gene *Ty-2* on chromosome 11 of tomato. Hortscience 2009;44:614-8.

[13] Polston J, Lapidot M. Management of Tomato yellow leaf curl virus: US and Israel Perspectives. In: Czosnek H, editors. Tomato Yellow Leaf Curl Virus Disease, Springer Netherlands; 2007, p. 251-62.

[14] Antignus Y. The management of Tomato yellow Leaf Curl Virus in Greenhouses and the Open Field, a Strategy of Manipulation. In: Czosnek H, editors. Tomato Yellow Leaf Curl Virus Disease, Springer Netherlands; 2007, p. 263-78.

[15] Mandadi KK, Scholthof K-BG. Plant immune responses against viruses: How does a virus cause disease? Plant Cell 2013;25:1489-505.

[16] Noutoshi Y, Okazaki M, Kida T, Nishina Y, Morishita Y, Ogawa T, et al. Novel plant immune-priming compounds identified via high-throughput chemical screening target salicylic acid glucosyltransferases in Arabidopsis. Plant Cell 2012;24:3795-804.

[17] Liu T, Liu Z, Song C, Hu Y, Han Z, She J, et al. Chitin-induced dimerization activates a plant immune receptor. Science 2012;336:1160-4.

[18] Ji Y, Scott J, Hanson P, Graham E, Maxwell D. Sources of Resistance, Inheritance, and Location of Genetic Loci Conferring Resistance to Members of the Tomato-Infecting Begomoviruses. In: Czosnek H, editors. Tomato Yellow Leaf Curl Virus Disease, Springer Netherlands; 2007, p. 343-62.

[19] Anbinder I, Reuveni M, Azari R, Paran I, Nahon S, Shlomo H, et al. Molecular dissection of *Tomato leaf curl virus* resistance in tomato line TY172 derived from *Solanum peruvianum*. Theor Appl Genet 2009;119:519-30.

512 [20] Eybishtz A, Peretz Y, Sade D, Akad F, Czosnek H. Silencing of a single gene in
513 tomato plants resistant to *Tomato yellow leaf curl virus* renders them susceptible to the
514 virus. Plant Mol Biol 2009;71:157-71.
515 [21] Eybishtz A, Peretz Y, Sade D, Gorovits R, Czosnek H. *Tomato yellow leaf curl*
516 *virus* infection of a resistant tomato line with a silenced sucrose transporter gene
517 *LeHT1* results in inhibition of growth, enhanced virus spread, and necrosis. Planta
518 2010;231:537-48.
519 [22] Sade D, Eybishtz A, Gorovits R, Sobol I, Czosnek H. A developmentally
520 regulated lipocalin-like gene is overexpressed in *Tomato yellow leaf curl*
521 *virus*-resistant tomato plants upon virus inoculation, and its silencing abolishes
522 resistance. Plant Mol Biol 2012;80:273-87.
523 [23] Sade D, Brotman Y, Eybishtz A, Cuadros-Inostroza Á, Fernie AR, Willmitzer L,
524 et al. Involvement of the hexose transporter gene *LeHT1* and of sugars in resistance of
525 tomato to *Tomato yellow leaf curl virus*. Molecular Plant 2013;6:1707-10.
526 [24] Sade D, Shriki O, Cuadros-Inostroza A, Tohge T, Semel Y, Haviv Y, et al.
527 Comparative metabolomics and transcriptomics of plant response to *Tomato yellow*
528 *leaf curl virus* infection in resistant and susceptible tomato cultivars. Metabolomics
529 2015;11:81-97.
530 [25] Kamatou GP, Vermaak I, Viljoen AM. Eugenol-from the remote Maluku Islands
531 to the international market place: a review of a remarkable and versatile molecule.
532 Molecules 2012;17:6953-81.
533 [26] Kong X, Liu X, Li J, Yang Y. Advances in pharmacological research of eugenol.

534 Curr Opin Complement Alternat Med 2014;1:8-11.

535 [27] Morcia C, Malnati M, Terzi V. *In vitro* antifungal activity of terpinen-4-ol,

536 eugenol, carvone, 1,8-cineole (eucalyptol) and thymol against mycotoxigenic plant

537 pathogens. Food Addit Contam Part A 2011;29:415-22.

538 [28] Yao Z, Namkung W, Ko EA, Park J, Tradtrantip L, Verkman AS. Fractionation of

539 a herbal antidiarrheal medicine reveals eugenol as an Inhibitor of Ca^{2+}-activated Cl^{-}

540 channel TMEM16A. PLoS ONE 2012;7:e38030.

541 [29] Jaganathan SK, Mazumdar A, Mondhe D, Mandal M. Apoptotic effect of eugenol

542 in human colon cancer cell lines. Cell Biol Int 2011;35:607-15.

543 [30] Ji YH, Sun HX, Zhou XW, Zhao TM, Yun WG, Zhou YJ, 2011. The identification

544 of *Tomato yellow leaf curl virus* in Jiangsu Province, China, In The 2011 Congrass of

545 Chinese Society of Plant Pathology, The 2011 Congrass of Chinese Society of Plant

546 Pathology.

547 [31] Garcia-Cano E, Navas-Castillo J, Moriones E, Fernandez-Munoz R. Resistance to

548 *Tomato chlorosis virus* in wild tomato species that impair virus accumulation and

549 disease symptom expression. Phytopathology 2010;100:582-92.

550 [32] Higo K, Ugawa Y, Iwamoto M, Korenage T. Plant cis-acting regulatory DNA

551 elements (PLACE) database. Nucleic Acids Res 1999;27:297-300.

552 [33] Wilkins MR, Gasteiger E, Bairoch A, Sanchez JC, Williams KL, Appel RD, et al.

553 Protein identification and analysis tools in the ExPASy server. Methods Mol Biol

554 1999;112:531-52.

555 [34] Letunic I, Doerks T, Bork P. SMART 7: recent updates to the protein domain

556 annotation resource. Nucleic Acids Res 2012;40:D302-5.

557 [35] Horton P, Park KJ, Obayashi T, Fujita N, Harada H, Adams-Collier CJ, et al.

558 WoLF PSORT: protein localization predictor. Nucleic Acids Res 2007;35:W585-7.

559 [36] Krogh A, Larsson B, von Heijne G, Sonnhammer E. Predicting transmembrane

560 protein topology with a hidden markov model: application to complete genomes. J

561 Mol Biol 2001;305:567-80.

562 [37] Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H,

563 et al. Clustal W and Clustal X version 2.0. Bioinformatics 2007;23:2947-8.

564 [38] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular

565 Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 2013;30:2725-9.

566 [39] Guo K, Xia K, Yang ZM. Regulation of tomato lateral root development by

567 carbon monoxide and involvement in auxin and nitric oxide. J Exp Bot

568 2008;59:3443-52.

569 [40] Mason G, Caciagli P, Accotto GP, Noris E. Real-time PCR for the quantitation of

570 *Tomato yellow leaf curl Sardinia virus* in tomato plants and in *Bemisia tabaci*. J Virol

571 Methods 2008;147:282-9.

572 [41] Zhou T, Du L, Lan Y, Sun F, Fan Y, Zhou Y. Development of SYBR Green

573 I-Based One-Step Real Time RT-PCR Assay for Quantifying *Southern rice*

574 *black-streaked dwarf virus* in Rice. Journal of phytopathology 2014;162:26-32.

575 [42] Wen J, Zhang Y, Zeng L. Effect of moroxydine hydrochloride to control tomato

576 virus. J Changjiang Vege 2008;11b:68-9.

577 [43] Maurino VG, Grube E, Zielinski J, Schild A, Fischer K, Flügge U-I. Identification

578 and expression analysis of twelve members of the nucleobase-ascorbate transporter
579 (NAT) gene family in *Arabidopsis thaliana*. Plant Cell Physiol 2006;47:1381-93.
580 [44] Knoth C, Salus MS, Girke T, Eulgem T. The synthetic elicitor
581 3,5-dichloroanthranilic acid induces NPR1-dependent and NPR1-independent
582 mechanisms of disease resistance in Arabidopsis. Plant Physiol 2009;150:333-47.
583 [45] Serrano M, Robatzek S, Torres M, Kombrink E, Somssich IE, Robinson M, et al.
584 Chemical interference of pathogen-associated molecular pattern-triggered immune
585 responses in Arabidopsis reveals a potential role for fatty-acid synthase type II
586 complex-derived lipid signals. J Biol Chem 2007;282:6803-11.
587 [46] Thakur M, Sohal SB. Role of elicitors in inducing resistance in plants against
588 pathogen infection: a review. ISRN Biochemistry 2013;2013:762412.
589 [47] Bueter CL, Specht CA, Levitz SM. Innate sensing of chitin and chitosan. PLoS
590 Pathog 2013;9:e1003080.
591 [48] Noutoshi Y, Ikeda M, Saito T, Osada H, Shirasu K. Sulfonamides identified as
592 plant immune-priming compounds in high-throughput chemical screening increase
593 disease resistance in *Arabidopsis thaliana*. Front Plant Sci 2012;3:245.
594 [49] Noutoshi Y, Jikumaru Y, Kamiya Y, Shirasu K. ImprimatinC1, a novel plant
595 immune-priming compound, functions as a partial agonist of salicylic acid. Sci. Rep.
596 2012;2.
597 [50] Noutoshi Y, Okazaki M, Shirasu K. Isolation and characterization of the plant
598 immune-priming compounds Imprimatin B3 and -B4, potentiators of disease
599 resistance in Arabidopsis thaliana. Plant Signal Behav 2012;7:1526-8.

600 [51] Xuan W, Murphy E, Beeckman T, Audenaert D, De Smet I. Synthetic molecules:
601 helping to unravel plant signal transduction. J Chem Biol 2013;6:43-50.
602 [52] Agency USEP, 1988, EPA 738-R-98-002. Prevention, Pesticides and Toxic
603 Substances; Status of Pesticides in Registration, Reregistration, and Special Review, p.
604 p. 311.
605 [53] Agency USEP, June 21, 2012. U.S. National Archives and Records
606 Administration's Electronic Code of Federal Regulations, p. 40 CFR 152.25 (USEPA).
607 [54] Venkatesh J, Park SW. Role of L-ascorbate in alleviating abiotic stresses in crop
608 plants. Bot Stud 2014;55:38.
609 [55] Luna E, Pastor V, Robert J, Flors V, Mauch-Mani B, Ton J. Callose deposition: a
610 multifaceted plant defense response. Mol Plant Microbe Interact 2011;24:183-93.
611 [56] Zavaliev R, Ueki S, Epel B, Citovsky V. Biology of callose (β-1,3-glucan)
612 turnover at plasmodesmata. Protoplasma 2011;248:117-30.
613 [57] Vlot AC, Dempsey DMA, Klessig DF. Salicylic acid, a multifaceted hormone to
614 combat disease. Annu Rev Phytopathol 2009;47:177-206.
615 [58] Avanci NC, Luche DD, Goldman GH, Goldman MHS. Jasmonates are
616 phytohormones with multiple functions, including plant defense and reproduction.
617 Genet Mol Res 2010;9:484-505.
618 [59] Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC. Networking by
619 small-molecule hormones in plant immunity. Nat Chem Biol 2009;5:308-16.
620 [60] Preston CA, Lewandowski C, Enyedi AJ, Baldwin IT. Tobacco mosaic virus
621 inoculation inhibits wound-induced jasmonic acid-mediated responses within but not

between plants. Planta 1999;209:87-95.

[61] Huang XF, Su H, Wang CM, Chen H, Shi ZQ. Mechanism of plant-derived antiviral agent eugenol against tobacco mosaic virus disease in tobacco (Chinese version with English abstract). Jiangsu J Agr Sci 2013;29:749-54.

[62] Miersch O, Neumerkel J, Dippe M, Stenzel I, Wasternack C. Hydroxylated jasmonates are commonly occurring metabolites of jasmonic acid and contribute to a partial switch-off in jasmonate signaling. New Phytol 2008;177:114-27.

[63] Zhu F, Xi D-H, Yuan S, Xu F, Zhang D-W, Lin H-H. Salicylic acid and jasmonic acid are essential for systemic resistance against *Tobacco mosaic virus* in *Nicotiana benthamiana*. Mol Plant-Microbe Interact 2014;27:567-77.

[64] Lisjak M, Teklic T, Wilson ID, Whiteman M, Hancock JT. Hydrogen sulfide: environmental factor or signalling molecule? Plant Cell Environ 2013;36:1607-16.

[65] Li YJ, Chen J, Xian M, Zhou LG, Han FX, Gan LJ, et al. *In site* bioimaging of hydrogen sulfide uncovers its pivotal role in regulating nitric oxide-induced lateral root formation. PLoS ONE 2014;9:e90340.

Figure legends

644 **FIGURE 1** Anti-viral effect of eugenol against TYLCV. (A) Hydrochloride (MH, 450
645 μg mL^{-1}) and eugenol (50, 100, and 200 μg mL^{-1}) were sprayed on the tomato leaves
646 24 h before TYLCV inoculation. Disease index (DI) was measured to determine
647 control efficiency at 28 days after inoculation with TYLCV. Values shown are means.
648 Different letters indicate significant differences among treatments (P<0.05, ANOVA,
649 LSD). (B) Eugenol (200 μg mL^{-1}) was sprayed onto tomato leaves 24 h before
650 TYLCV inoculation. Control plants were similarly sprayed with distilled water. Plants
651 were photographed at 28 days after inoculation with TYLCV. (C) MH (450 μg mL^{-1})
652 and eugenol (200 μg mL^{-1}) were sprayed onto tomato leaves 24 h before TYLCV
653 inoculation. Total RNA was extracted from uppermost leaves for RT-PCR analysis
654 of transcript levels of viral genes (*CP*, *Rep*, and *Ren*). *Actin* was used for cDNA
655 normalization. (D) Eugenol (200 μg mL^{-1}) was sprayed onto tomato leaves 24 h
656 before TYLCV inoculation. Control plants were similarly sprayed with distilled water.
657 Total DNA was extracted from uppermost leaves for quantification of virus.
658
659 **FIGURE 2** Phylogenetic relationship of SlPer1 and related NAT members in
660 *Brassica rapa* and *Arabidopsis thaliana*. Database accession numbers of proteins are
661 provided in “Materials and Methods”.
662
663 **FIGURE 3** Sequence structure of SlPer1 and multiple alignment of predicted amino
664 acid sequence of SlPer1 with NAT6 members from *Arabidopsis thaliana*, *Brassica*
665 *rapa*, and *Oryza sativa*. (A) Orange box indicates conserved Xan_ur_permease

domain in SlPer1. Bar indicates 50 amino acids. (B) Dark shading with white letters and gray shading with black letters indicate 100% and 75% sequence similarity, respectively. Database accession numbers for proteins are provided in "Materials and Methods". Black lines show transmembrane domains (TMD); red box indicates NAT signature motif *NXGXXXXT#, where * and # indicate (Q/E/P) and (R/K/G), respectively.

FIGURE 4 Effects of TYLCV inoculation or eugenol treatment on transcription of *SlPer1*. (A) Relative transcript levels of *SlPer1* in leaves were analyzed at 0, 7, 14, and 21 days after inoculation with TYLCV. (C) Eugenol (0, 100, and 200 μg mL^{-1}) was sprayed onto tomato leaves, and then leaves were harvested 24 h later for analyses of *SlPer1* transcript levels. Numbers below band indicate relative abundance of genes with respect to loading control *Actin* (as determined by densitometric analysis). (B) and (D) Quantitative analysis of gene transcript levels from (A) and (C), respectively. Values shown are mean of three replicates. Asterisk indicates significant difference between treatment and control ($P<0.05$, ANOVA).

FIGURE 5 Effects of plant hormones on *SlPer1* transcription. (A) Tomato leaves were sprayed with salicylic acid (SA, 200 μM), methyl jasmonate (MeJA, 100 μM), gibberellin (50 μM), and ethephon (100 μM), and then leaves were harvested at indicated times for analyses of *SlPer1* transcript levels. Numbers below band indicate relative abundance of genes with respect to loading control *Actin* (as determined by

688 densitometric analysis). (B–E) Quantitative analysis of *SlPer1* transcript levels in
689 response to SA, MeJA, gibberellin, and ethephon treatments. Values shown are mean
690 of three replicates. Asterisk indicates significant difference between treatment and
691 control (P<0.05, ANOVA).
692
693 **FIGURE 6** Effects of sodium nitroprusside (SNP) and sodium hydrogen sulfide
694 (NaHS) on *SlPer1* transcription. (A) Tomato leaves were sprayed with SNP (200 μM)
695 or NaHS (200 μM), and then leaves were harvested at indicated times to analyze
696 *SlPer1* transcript levels. Numbers below band indicate the relative abundance of
697 genes with respect to loading control *Actin* (as determined by densitometric analysis).
698 (B) and (C) Quantitative analysis of *SlPer1* transcript levels in response to SNP and
699 NaHS treatments, respectively. Values shown are mean of three replicates. Asterisk
700 indicates significant difference between treatment and control (P<0.05, ANOVA).
701
702 **FIGURE 7** Effect of eugenol on generation of endogenous NO in tomato plants.
703 Roots and stems of seedlings were exposed to 100 μg mL^{-1} eugenol for 24 h. Then,
704 the plant samples were loaded with DAF-FM DA for 15 min and immediately
705 photographed (A − B). Relative DAF-FM fluorescent density in roots and stems (C
706 − D). Values shown are mean of three replicates. Asterisk indicates significant
707 difference between treatment and control (P<0.05, ANOVA).
708
709 **FIGURE 8** Effect of eugenol on generation of endogenous salicylic acid (SA) in

710 tomato leaves. Leaves of seedlings were treated with 200 μg mL^{-1} eugenol and then
711 harvested after 6, 12, 18, 24, 48, and 96 h for analysis of SA contents. Asterisk
712 indicates significant difference between treatment and control (P<0.05, ANOVA).
713
714 **TABLE 1** Disease-responsive elements in promoter region of *SlPer1*. *cis*-Elements
715 were predicted using PLACE as described in "Materials and Methods".
716

716

Disease-responsive elements in the promoter region of *SlPer1*. The *cis*-elements were predicted by using online PLACE program.

Factor or site name	Site (strand) sequence	Element
WBOXNTCHN48	401 (+) CTGACY 1892 (+) CTGACY	Elicitor-responsiveness
ELRECOREPCRP1	1734 (+) TTGACC	Elicitor- and/or SA-responsiveness
WBOXATNPR1	692 (+) TTGAC 1734 (+) TTGAC 698 (-) TTGAC 1709 (-) TTGAC	SA-responsiveness
T/GBOXATPIN2	331 (+) TAAAG	JA-responsiveness

717

718 **TABLE 1**

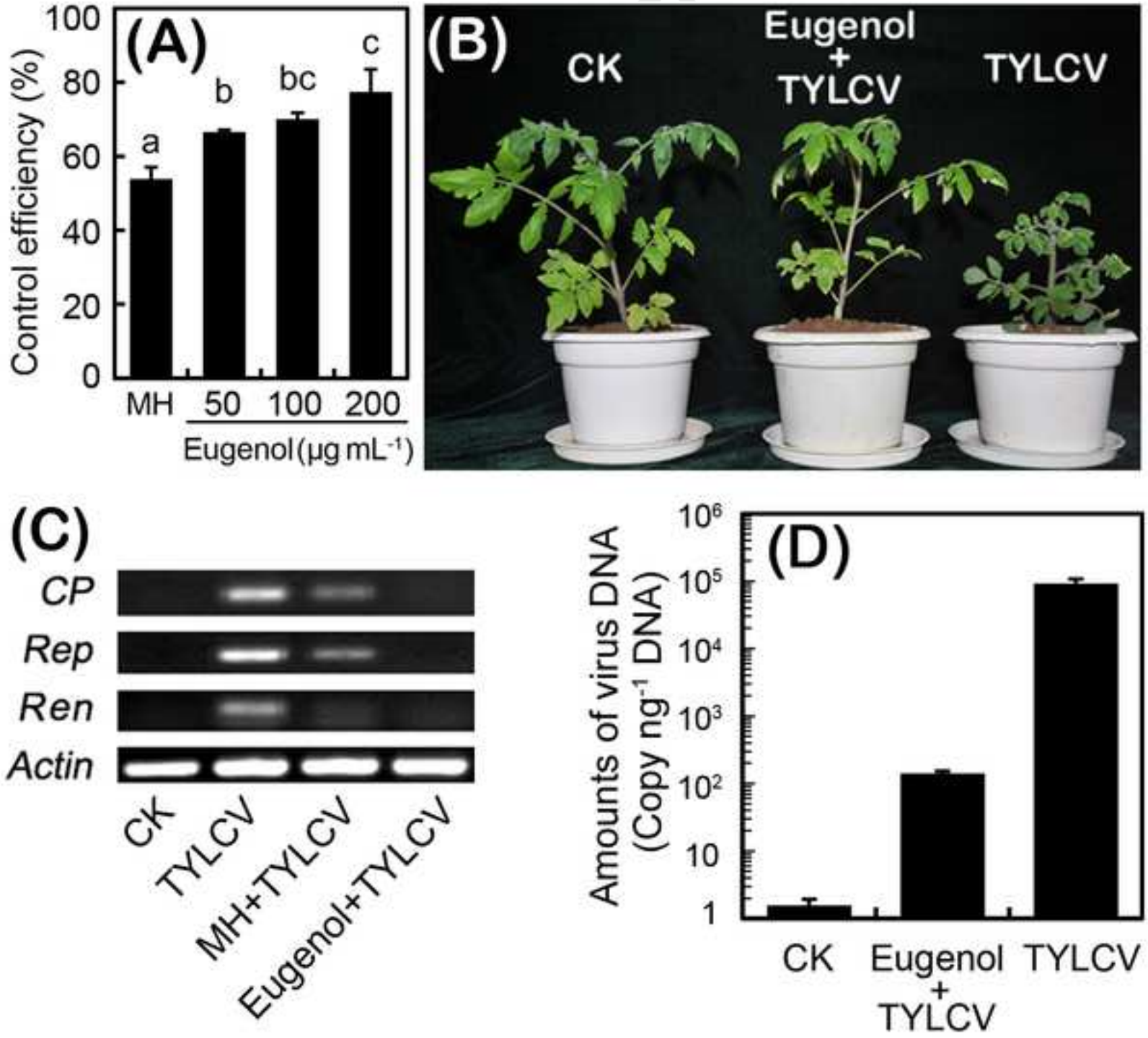
719

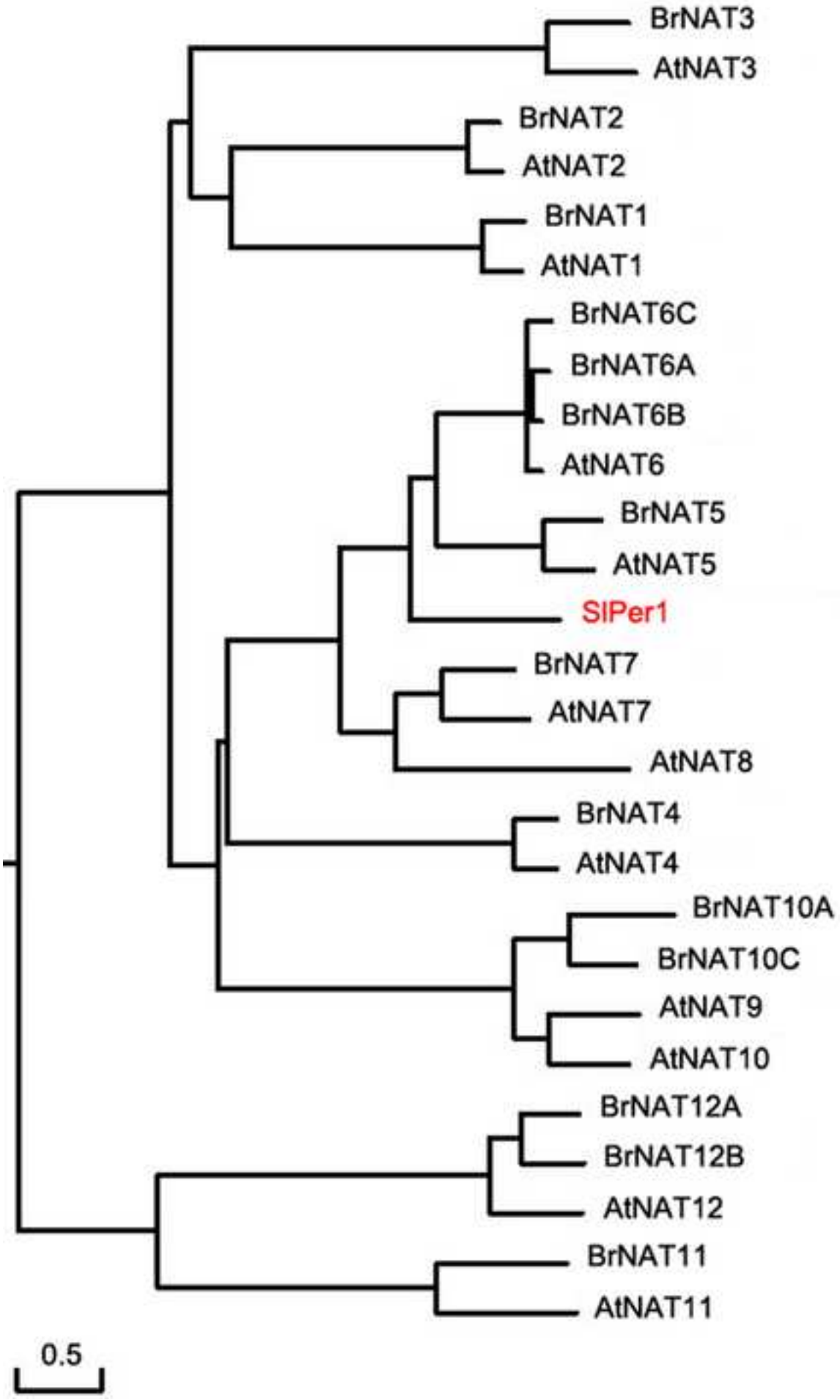
720

721

722

Figure 1


(A)
Control efficiency (%)
100
80
60
40
20
0
a
b
bc
c
MH
50
100
200
Eugenol (μg mL-1)
(B)
CK
Eugenol + TYLCV
TYLCV
(C)
CP
Rep
Ren
Actin
CK
TYLCV
MH+TYLCV
Eugenol+TYLCV
(D)
Amounts of virus DNA (Copy ng-1 DNA)
10^6
10^5
10^4
10^3
10^2
10
1
CK
Eugenol + TYLCV
TYLCV



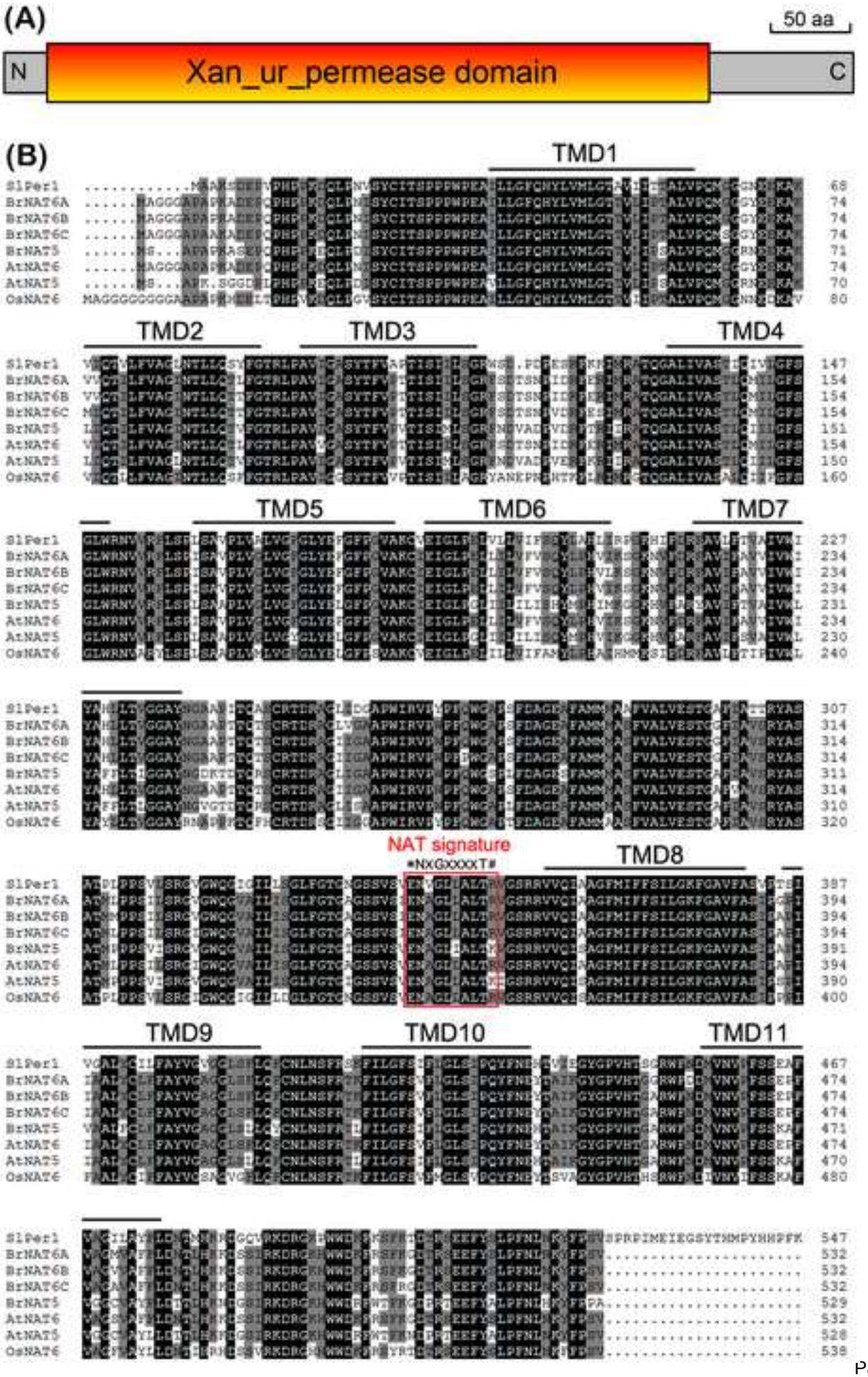
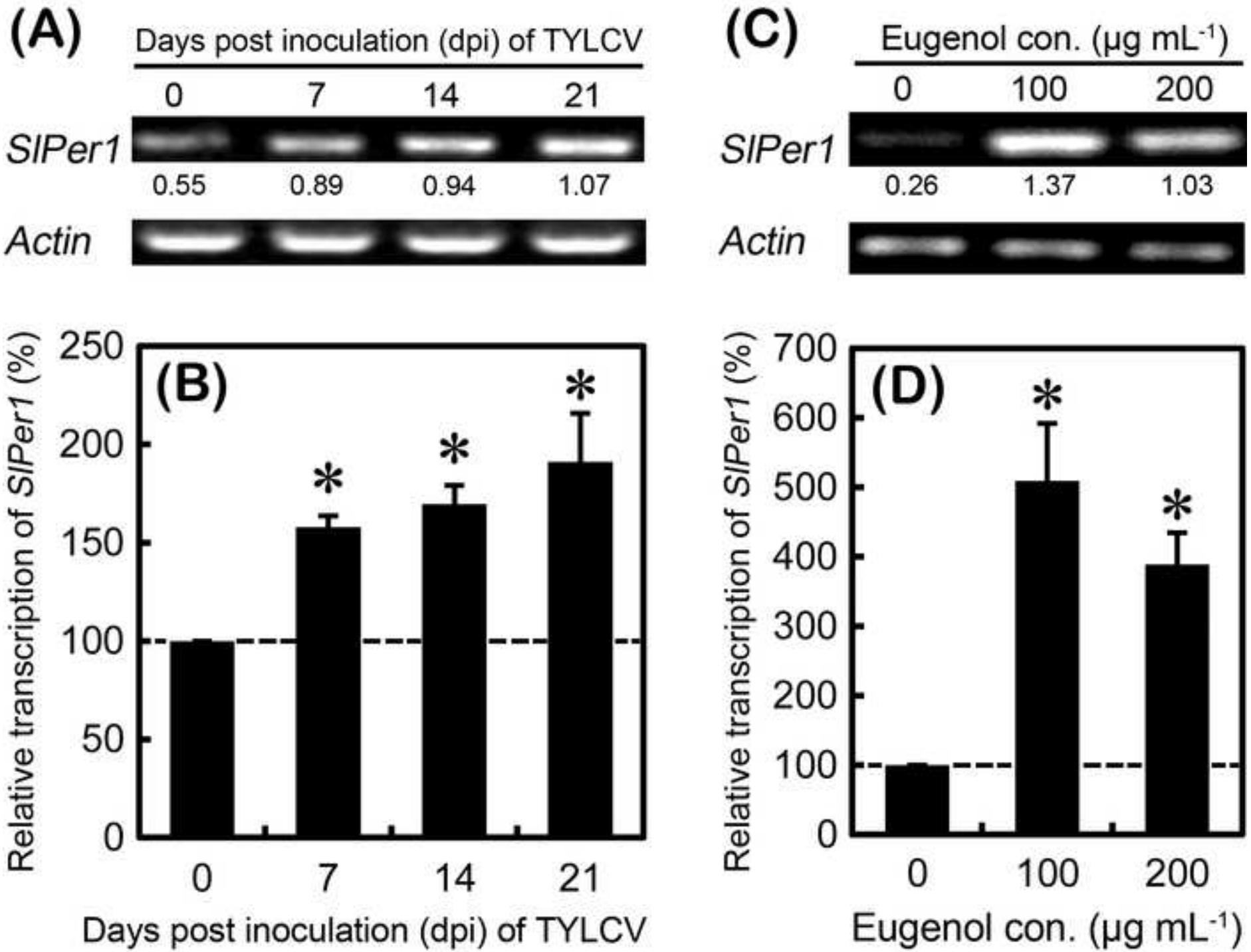
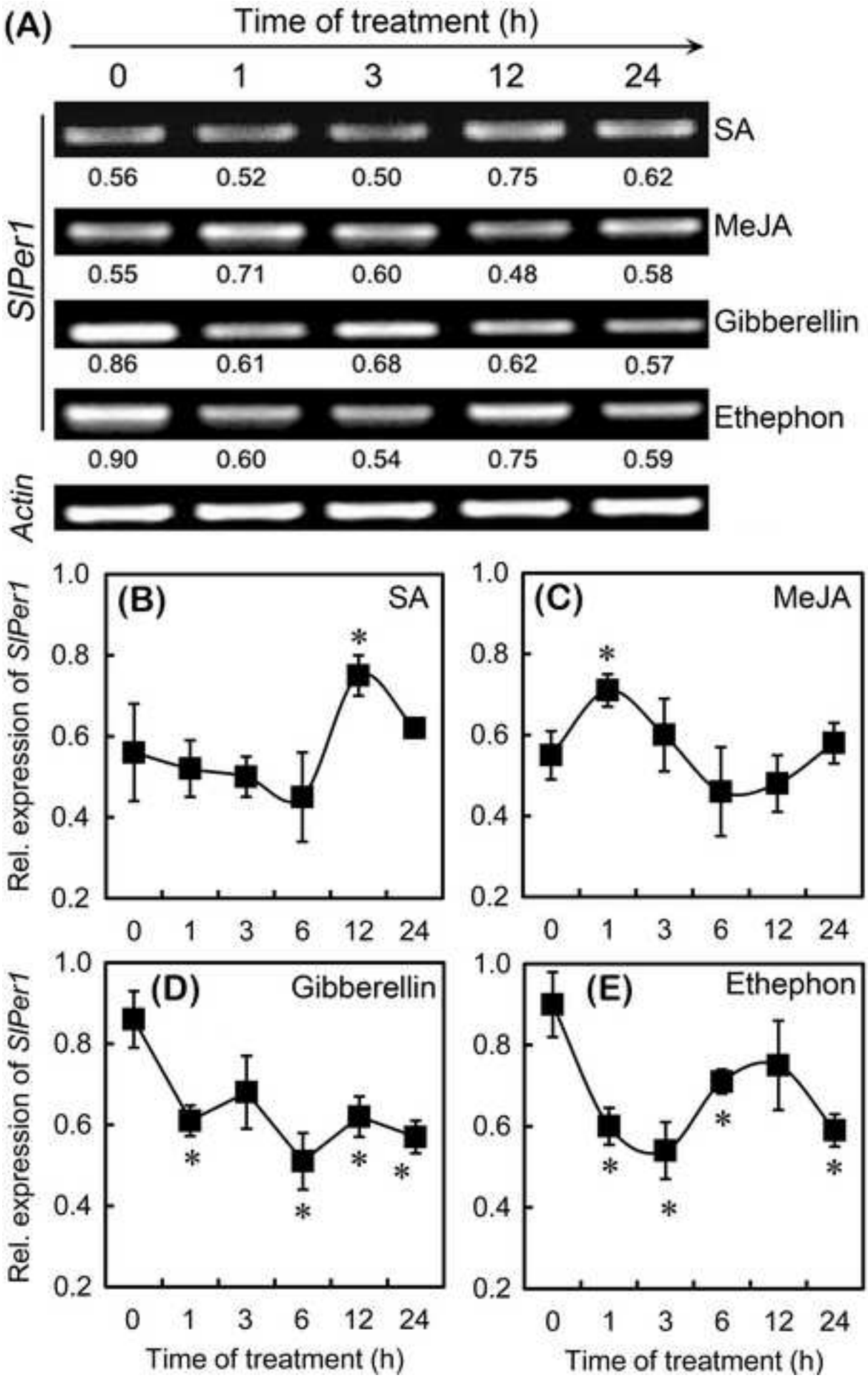
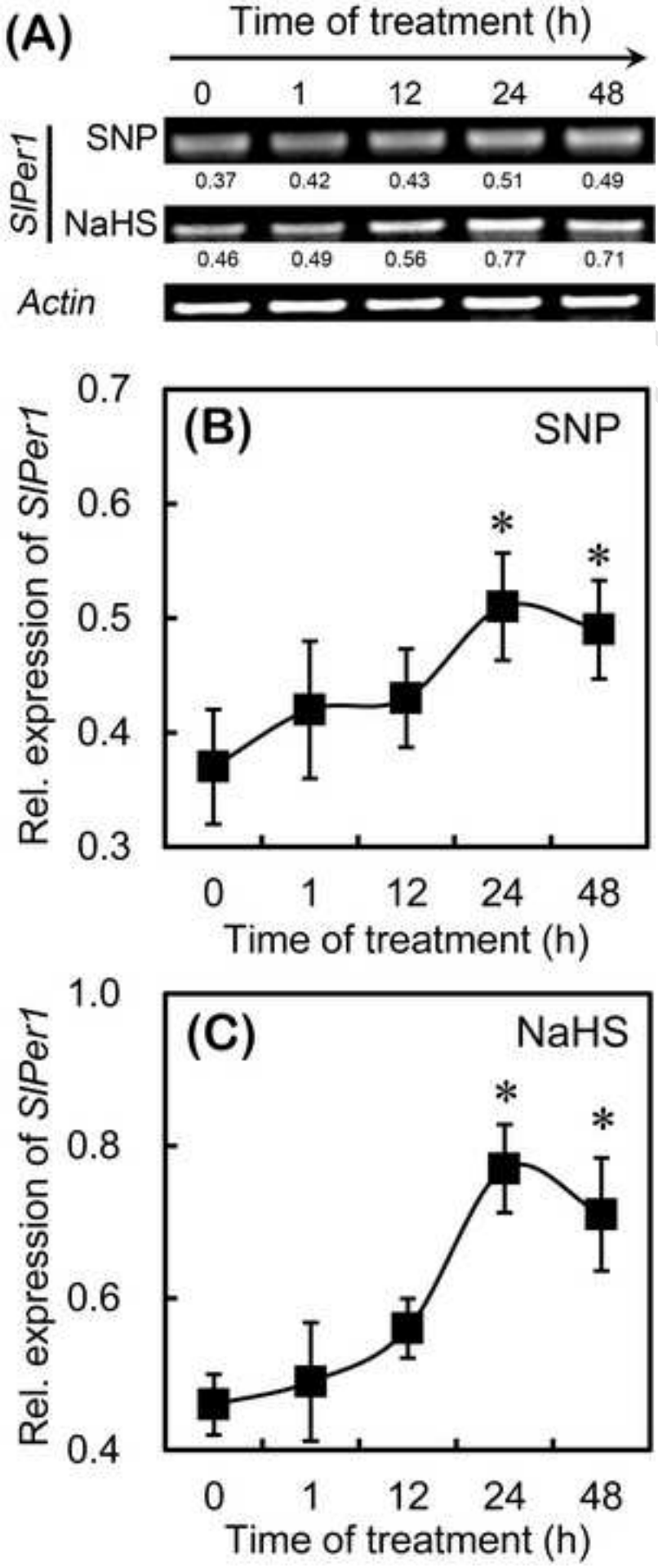


Figure 4


(A)
Days post inoculation (dpi) of TYLCV
0
7
14
21
SlPer1
0.55
0.89
0.94
1.07
Actin
(B)
Relative transcription of SlPer1 (%)
250
200
150
100
50
0
*
*
*
0
7
14
21
Days post inoculation (dpi) of TYLCV
(C)
Eugenol con. (μg mL-1)
0
100
200
SlPer1
0.26
1.37
1.03
Actin
(D)
Relative transcription of SlPer1 (%)
700
600
500
400
300
200
100
0
*
*
0
100
200
Eugenol con. (μg mL-1)





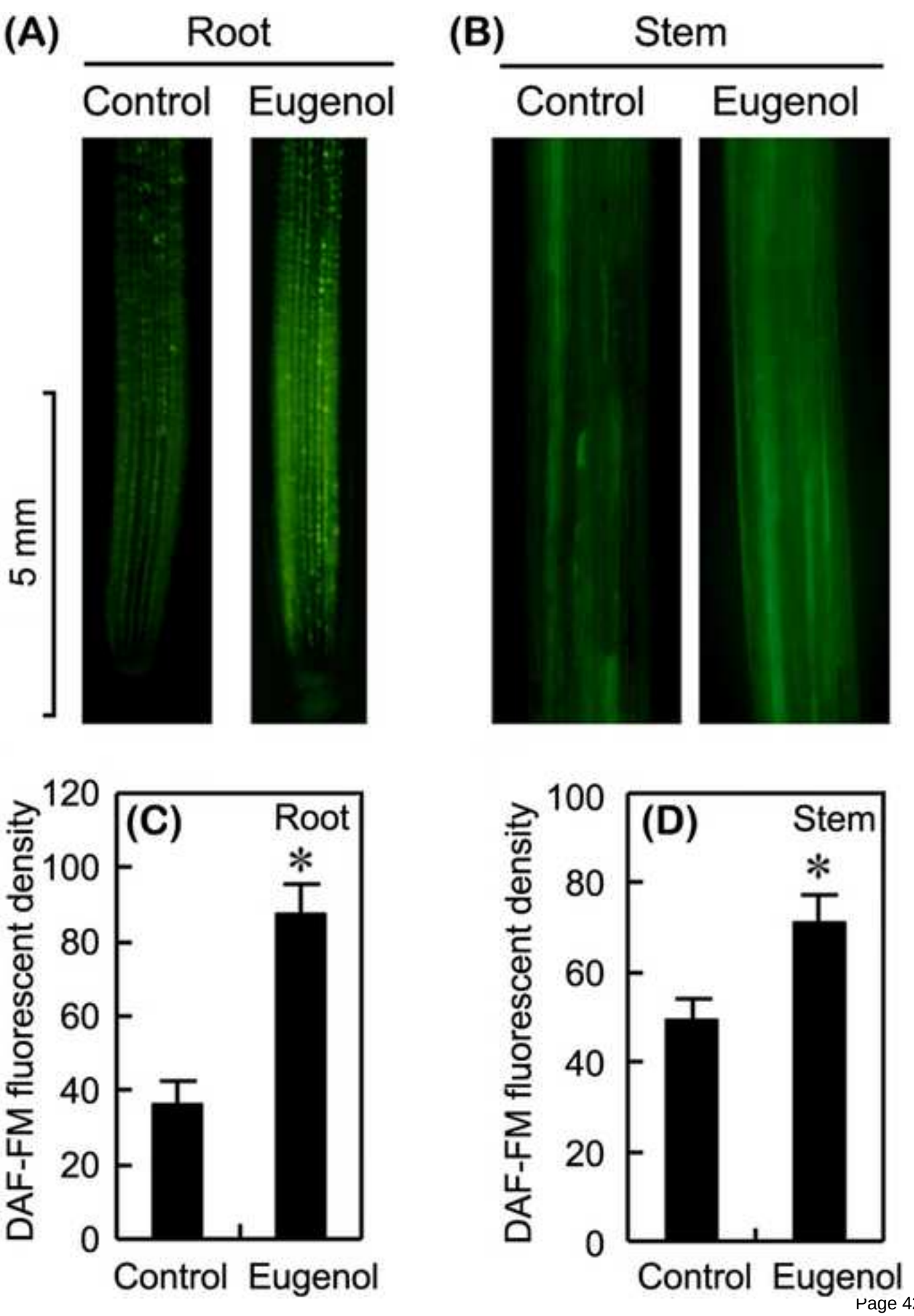
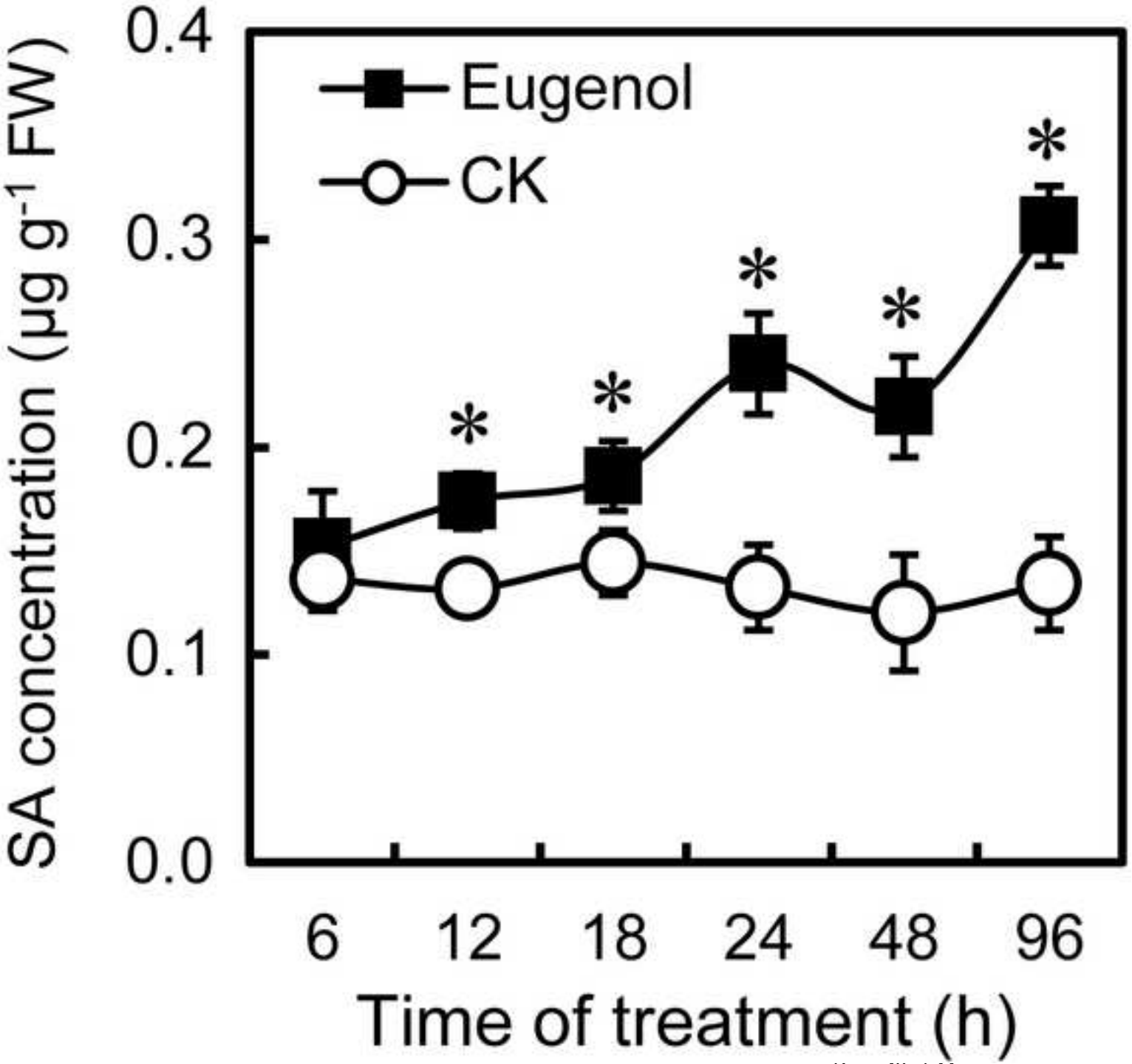


Figure 8


Eugenol
CK
SA concentration (μg g-1 FW)
0.0
0.1
0.2
0.3
0.4
6
12
18
24
48
96
Time of treatment (h)
*
*
*
*
*

Graphical Abstract