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A Tomato Putative Metalloprotease SlEGY2 Plays a Positive Role in Thermotolerance

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Abstract: Intramembrane proteases play very important roles in plants, such as chloroplast development, flower morphology, and response to abiotic stress. In this study, a putative metalloprotease gene, homologous to Ethylene-dependent Gravitropism deficient and Yellow-green2 (EGY2) of Arabidopsis, was isolated from tomato (*Solanum lycopersicum*) plants and named SlEGY2. We found that SlEGY2 was a member of the metalloprotease family M50 which contained conserved motifs HEXXH and NPDG and was localized in the chloroplast. SlEGY2 antisense transgenic tomato plants (AS) have similar hypocotyls phenotype to the Arabidopsis egy2 mutant. Heat (42 °C), PEG, ABA and MeJA treatments can upregulate the expression of SlEGY2. Under heat stress, SlEGY2 AS lines are more sensitive, with more water loss (lower fresh weight), seriously damaged membrane, and ROS accumulation, but lower activities of APX and CAT. In addition, suppression of SlEGY2 decreases the content of chlorophyll and photosynthetic activities, especially photosystem II. These results suggest that SlEGY2 can regulate the thermotolerance of tomatoes by affecting ROS accumulation and photosynthetic activities.

Keywords: tomato; chloroplast; metalloprotease; SlEGY2; heat stress

Citation: Zhang, S.; Chen, C.; Dai, S.; Yang, M.; Meng, Q.; Lv, W.; Ma, N. A Tomato Putative Metalloprotease SlEGY2 Plays a Positive Role in Thermotolerance. *Agriculture* **2022**, *12*, 940. https://doi.org/10.3390/agriculture12070940

Academic Editor: Andres Belver Cano

Received: 20 May 2022
Accepted: 26 June 2022
Published: 29 June 2022

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1. Introduction

With global warming, the restriction of high temperature stress on crop growth and yield is becoming more and more significant. Therefore, improving crop thermotolerance is an important measure to develop sustainable agriculture.

Photosynthesis is the primary source of raw materials for all forms of agricultural products, such as carbohydrates, amino acids, and fatty acids. In nature, plants are exposed to daily and seasonal temperature fluctuations, resulting in a frequent influence on photosynthesis. Previous studies have shown that photosynthesis is extremely sensitive to high temperatures, especially thylakoid membranes and the photosynthetic apparatus, so they are also considered to be the primary targets of heat stress on plants [1,2]. High temperature stress negatively affects essentially all aspects of photosynthetic activities, including decreasing enzyme activities and electron transfer rate (ETR), altering membrane fluidity, and destroying the photosystem protein complex, especially photosystem II (PSII) [2–4]. Therefore, understanding the mechanisms by which plant photosynthetic systems respond and adapt to temperature upshifts in their growing environment is critical for maintaining efficient photosynthesis under heat stress.

Chloroplasts are the organelle for photosynthesis. Proteolysis in chloroplasts is necessary for plant growth and development, and it is considered to be a common regulatory mechanism for plants to respond to environmental changes. Chloroplast proteases play

an important role in this process, which maintain proteostasis in the chloroplast to ensure the orderly life activities of organisms [5]. Mutation of Arabidopsis FtsH11 (filamentation temperature-sensitive H, FtsH) resulted in reduced thermotolerance of plants, with a significant decrease in ETR of two photosystems (PS) and photosynthetic capacity, as well as an increase in non-photochemical quenching (NPQ) at 30 °C, indicating that AtFtsH11 is critical for the adaption of photosynthesis to elevated temperature [6,7]. In addition, major chloroplast-located FtsH proteases of Arabidopsis (FtsH1/5, FtsH2/8, FtsH6 and FtsH7/9) have been reported to participate in alleviating and repairing high light-induced photodamages to PS, especially in the turnover of photodamaged D1 protein in PSII [8–13]. In 2016, Sedaghatmehr et al. found that AtFtsH6 regulates thermo-memory in Arabidopsis by controlling the abundance of Hsp21 [14]. Deg (degradation of periplasmic), another kind of chloroplast protease, is also vital for the PSII repair cycle and participates in the preliminary cutting of photodamaged D1, D2, CP47, and CP43 [15–17]. These studies suggest that chloroplast proteases play important roles in maintaining photosynthetic activities under abiotic stresses.

Regulated intramembrane proteolysis (RIP) is a mechanism that regulates gene expression at the transcriptional level. This process requires intramembrane cleaving proteases (I-CLiPs) to activate the membrane-anchored regulators in the cell via proteolytic cleavage, which causes them to be released from the membrane. Site-2 protease (S2P), zinc-containing metalloprotease, is a member of the I-CLiPs family and can perform proteolytic cleavage within the membrane [18]. S2P was first found in human and it activates cholesterol and fatty acid biosynthesis by cleaving transcription factor SREBP (Sterol Regulatory Element Binding Protein) after site-1 protease (S1P) cleavage [19,20]. In higher photosynthetic organisms, S2P members have been characterized, such as EGY1 and EGY2 in Arabidopsis, and SlL2 in tomato [21–23]. EGY1, a chloroplast membrane-bound and ATP-independent metalloprotease, is required for thylakoid grana development and accumulation of chlorophyll and chlorophyll a/b binding proteins [22]. Moreover, EGY1 also regulates endodermal plastid size and number, as well as the stimulatory effect of ethylene on hypocotyl gravitropism [24], participates in ammonium [25] and phosphate [26] stresses via the abscisic acid signaling pathway, and delays leaf senescence [27]. EGY2, located in the chloroplast, plays a role in hypocotyl elongation and fatty acid biosynthesis [23] and affects the expression level of genes encoding crucial subunits of PSII [28]. SlL2, encoding the AtEGY1 homologs, is involved in chlorophyll metabolism and fruit ripening [21]. These studies illuminate that S2P members can regulate chloroplast development and respond to nutrient stress. However, whether S2P members function in plant response to temperature stress has not been reported.

In this study, a homolog of Arabidopsis EGY2 was identified from tomato and named SlEGY2. We investigated its subcellular localization, explored its function in tomato response to heat stress, and found that it positively regulated tomato thermotolerance.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The wild-type (WT) plant of tomato (*Solanum lycopersicum* cv. L-402) and three antisense (A1, A3, A33) lines of the T2 generation were used in this study.

The sterilized seeds were sown on MS (Murashige and Skoog) medium, then put into a 25 °C incubator and grown for 10 days under a photoperiod of 16 h/8 h (light/dark). The young seedlings were planted in pots and grown in a greenhouse with a photon flux density (PFD) of 200 μmol m^{-2} s^{-1}, a temperature cycle of 25 °C/20 °C (day/night), and a photoperiod of 16 h/8 h (light/dark). To ensure their regular growth, these plants were irrigated with Hoagland nutrients every two days.

2.2. Isolation and Analysis of SlEGY2

Total RNAs of WT plants were extracted from leaves using RNA Simple Total RNA Kit (TIANGEN, Beijing, China). Then, the first-strand of cDNA was obtained by the Fast Quant

RT Kit containing gDNase (TIANGEN). A pair of specific primers were designed to amplify the gene according to the sequence in Solanaceae Genomics Network (Solyc06g019200.4.1). Next, the amplification products were inserted into the pMD19-T simple vector (TaKaRa, Beijing, China) and then sequenced. All the primers were synthesized by Beijing Genomics Institute Sequencing (BGI, Beijing, China). DNAMAN and MEGA 4.1 software were used for multiple sequence alignments and phylogenetic tree construction, respectively.

2.3. Subcellular Localization of SlEGY2

The coding region of SlEGY2 (termination codon excluded) was cloned and inserted into the binary vector pZP211 driven by the cauliflower mosaic virus 35S (CaMV 35S) promoter to make it in front of the GFP protein. The recombinant plasmid of p35S-SlEGY2-GFP and p35S-GFP (control vector) were injected into tobacco leaves via the Agrobacterium-mediated method, respectively. After two days of culture, the fluorescence emission of SlEGY2-GFP was observed using a two-photon laser confocal microscope (LSM880NLO, ZEISS, Jena, Germany).

2.4. Stress Treatments

Six-week-old WT tomato plants were used for abiotic stress treatment. For heat stress, the plants were put into an incubation chamber at 42 °C with a 16 h/8 h (light/dark) photoperiod. For osmotic stress, the roots of tomato plants were completely soaked in 20% PEG solution. For ABA and MeJA treatments, the plant leaves were sprayed with 100 μM ABA and 100 μM MeJA solution, respectively. The leaves of WT plants under the above four stress treatments were sampled at 0, 3, 6, 9, 12, and 24 h, frozen in liquid nitrogen, and stored at −80 °C until they were used for RNA extraction. For phenotypic observation and determination of physiological indexes, 6-week-old wild-type and antisense lines were treated at 42 °C in an incubator with 200 μmol m^{-2} s^{-1} PFD until significant phenotypic differences appeared (about 30 h).

2.5. Plant Transformation and Transgenic Tomato Identification

The full length of SlEGY2 was cloned and inserted in antisense orientation into the expression vector pBI121 using the restriction sites SalI and Xbal. Then, the recombinant plasmid was introduced into Agrobacterium tumefaciens strain LBA4404. Genetic transformation of tomato plants was performed via the leaf disc method as previously described [29]. When T0 generation transgenic plants were obtained, the DNAs were extracted and identified by PCR assay using 35S forward primer (35S) and SlEGY2 forward primer (SlEGY2-AF) listed in Supplementary Table S1.

2.6. Quantitative Real-Time PCR (qRT-PCR) Analysis

The qRT-PCR was performed as described by Zhuang et al. [30] with slight modification on a QuanStudio 6 real-time PCR system (Thermo Fisher, Waltham, MA, USA) using UltraSYBR Mixture (CWBIO, Beijing, China) and EF-1α (elongation factor 1α, GenBank accession no. X144491) was used as the internal control. The reaction was operated as follows: 95 °C for 30 s (pre-denaturation), 40 cycles of 95 °C for 15 s (denaturation), 56 °C for 15 s (annealing), 72 °C for 15 s (extension), and the final stage (55–95 °C). Three biological replicates were carried out for each qPCR. The primers used in this study are listed in Supplementary Table S1.

2.7. Measurement of Chlorophyll Content, MDA, REC, Pn, and Fv/Fm

The measurement of chlorophyll content was carried out using the method described by Liu et al. [31] with slight modification. One gram of leaves was immersed in 20 mL 80% acetone for 48 h and then centrifuged at 6000 rpm for 5 min. The absorbance of the supernatant was measured at OD_{663}, OD_{646} and OD_{470}. MDA (malondialdehyde), REC (relative electrical conductivity) were measured as described by Kong et al. [32] with slight modification. MDA was isolated from 1 g of leaves by using 10 mL 10% TCA (trichloroacetic

acid). Then, 2 mL 0.6% thiobarbituric acid reagent was added and reacted with MDA for 15 min at 100 °C. The absorbance of the supernatant was measured at OD_{450}, OD_{532} and OD_{600}. For REC, ten leaf discs were immersed in 20 mL distilled water and vacuumed for 30 min. After shaking for 3 h, the solution was measured as initial electric conductivity (S1). Then the solution was incubated at 100 °C for 30 min and final electric conductivity (S2) was measured after cooling at room temperature. REC was calculated via the formula: REC = ((S1 − S0)/(S2 − S0)) × 100.

Pn (net photosynthetic rate) and Fv/Fm (the maximum photochemical efficiency of PSII) were determined according to the method described by Kong et al. [32]. Pn was measured under ambient CO_2 conditions with a PFD of 800 μmol m^{-2} s^{-1} and relative humidity of 80% using CIRAS-2 (PP Systems, Haverhill, MA, USA). Before the measurement, the leaves were adapted in darkness for at least half an hour. Then, Fv/Fm was measured with the Handy Plant Efficiency Analyzer (Hansatech Instruments, Hongkong, China).

2.8. Histochemical Staining and Detection of ROS

The staining and quantitative determination of H_2O_2 and $O_2^{\bullet-}$ were performed using the methods described in Ma et al. [33]. Eight detached leaves from each line under both normal and heat stress conditions were infiltrated in 10 mL DAB (3,3′-diaminobenzidine, 1 mg mL^{-1}, pH 3.8) and NBT (nitroblue tetrazolium, 0.5 mg mL^{-1} dissolved in 25 mM phosphate buffer saline (PBS), pH 7.8), respectively, and placed in the dark overnight. Then, the leaves were decolorized in solution (acetic acid: glycerol: ethanol = 1:1:4).

An amount of 0.5 g leaves are ground into powder with liquid nitrogen. PBS (3 mL, 50 mM, pH = 6.8) was added and then centrifuged. A volume of 3 mL of supernatant and 1 mL of 0.1% titanium sulfate were mixed and centrifuged. Then the absorbance was measured at 410 nm. The levels of H_2O_2 were calculated according to the standard curve. For the $O_2^{\bullet-}$ assay, 0.5 g fine powder of leaves was added to 5 mL of PBS (50 mM, pH = 7.8) and centrifuged. The supernatant was mixed with PBS and hydroxylammonium chloride (1 M) and heated (25 °C, 20 min). Then, p-aminobenzene sulfonic acid (17 mM) and α-naphthylamine (7 mM) were added and the mixture was heated again (25 °C, 20 min). Then the absorbance was measured at 410 nm. The levels of $O_2^{\bullet-}$ were calculated according to the standard curve. Trypan blue staining was performed using the method in Kong et al. [32]. A volume of 10 mL lactic acid, 10 mL glycerol, 10 g phenol, and 10 mg trypan blue were dissolved in 10 mL distilled water to configure the lactophenol-trypan blue solution. Eight whole detached leaves from each line under both normal and heat stress conditions were boiled for approximately 1 min in the above staining solution and then decolorized in chloral hydrate (2.5 g chloral hydrate dissolved in 1 mL distilled water). The decolorized leaves were embedded in 60% glycerol and representative phenotypes were photographed.

2.9. Enzyme Activity Analysis of APX and CAT

The enzyme activity analysis of APX and CAT was performed following the method described by Zhang et al. [34] and Ma et al. [33], respectively. 0.5 g fine powder of leaves was suspended using homogenization buffer (pH = 7.0) and centrifuged at 4 °C. Then the supernatants were used to analyze the enzyme activity at 290 nm. The extraction of CAT was referred to as the $O_2^{\bullet-}$ assay. H_2O_2 (10 mM) was added to the supernatant and the OD was measured at 240 nm.

2.10. Statistical Analysis

The data in this study were presented as the average of three replications. Statistical analysis was tested by using SigmaPlot 12.5. * ($p < 0.05$) and ** ($p < 0.01$) indicate significant differences between the WT and transgenic tomato plant.

3. Results

3.1. Sequence Analysis and Subcellular Localization of SlEGY2

A novel tomato metalloprotease gene (Solyc06g019200.4.1) was cloned from tomato plants using a pair of specific primers. The full length of this gene is 1524 bp, containing a coding region of 1335 bp, which encodes a polypeptide of 444 amino acids. The molecular weight of the SlEGY2 protein is about 48.56 kDa and its isoelectric point is 6.19. In addition, there are five trans-membrane domains (A-E), which were predicted via a TMHMM program. Based on the results of amino acid sequence alignment, our metalloprotease contains the conserved zinc-binding motif HEXXH, and signature motif NPDG and GNLR which are unique to EGY and EGY-like proteins (Figure 1a). Furthermore, a phylogenetic tree was constructed on the basis of different EGY proteins from various plants (Figure 1b), which revealed that our protein has high homology with EGY metalloproteases. Thus, this metalloprotease was named SlEGY2.

According to TargetP (http://www.cbs.dtu.dk/services/TargetP/, accessed on 1 November 2018), the putative metalloprotease SlEGY2 was predicted to be located in the chloroplast. To determine its subcellular localization, a SlEGY2-GFP fusion protein was constructed, which was driven by the CaMV 35S promoter (Figure 2a). Then the fusion protein p35S-SlEGY2-GFP and p35S-GFP (control vector) were transiently expressed in Nicotiana benthamiana. As shown in Figure 2b, SlEGY2-GFP fluorescence co-localized with the auto-fluorescence of chloroplasts, while GFP green fluorescence was only found in both the cytoplasm and nucleus. Thus, it was demonstrated that SlEGY2 locates in the chloroplast.

3.2. Expression Profile Analysis

To analyze the expression pattern of SlEGY2 under different abiotic stress treatments, quantitative RT-PCR was performed. The SlEGY2 gene showed various expression pattern in different conditions (Figure 3). Under 42 °C and PEG treatment, SlEGY2 was induced and the transcripts accumulated to approximately four-fold than that of the control (Figure 3a,b). The SlEGY2 expression was also obviously increased under ABA and MeJA treatments. The mRNA accumulation of SlEGY2 reached five times at 24 h with ABA and six times at 12 h with MeJA treatment (Figure 3c,d), respectively. These results could speculate that SlEGY2 may participate in the abiotic stress response, especially in heat stress.

In addition, the expression patterns of SlEGY2 in various organs were detected. As shown in Supplementary Figure S1, different organs contained different mRNA levels of SlEGY2. It could be found that, the highest transcript level of SlEGY2 was in the leaf, while it was lower in the young leaf. This result was in accord with SlEGY2's chloroplast-localization.

3.3. Suppression of SlEGY2 Reduced Heat Stress Resistance

To further analyze the function of SlEGY2 in vivo, five individual lines of antisense transgenic tomato (AS) were obtained through tissue culture and screened by PCR (Figure 4a). Then, the gene expression levels of these five antisense transgenic lines were evaluated by qRT-PCR. The results showed that the relative mRNA levels of SlEGY2 in antisense transgenic lines decreased significantly than those in WT plants, especially in A1, A3, and A33 (Figure 4b). Therefore, A1, A3, and A33 lines were selected for further experiments.

Based on the expression pattern of SlEGY2, we performed heat treatment on six-week-old WT and three AS lines (A1, A3, and A33). Under normal conditions, both AS lines and WT plants grew well and showed no obvious difference in phenotype and fresh weight. However, after being treated at 42 °C for 30 h, the leaves of all lines exhibited a different degree of wilting. Compared with WT, leaf wilting was much more serious in AS plants (Figure 4c). Similarly, the fresh weight of AS lines was much lower than that of WT plants after heat treatment (Figure 4d). The phenotypic analysis indicates that suppression of SlEGY2 reduced the heat-resistance of tomato plants.

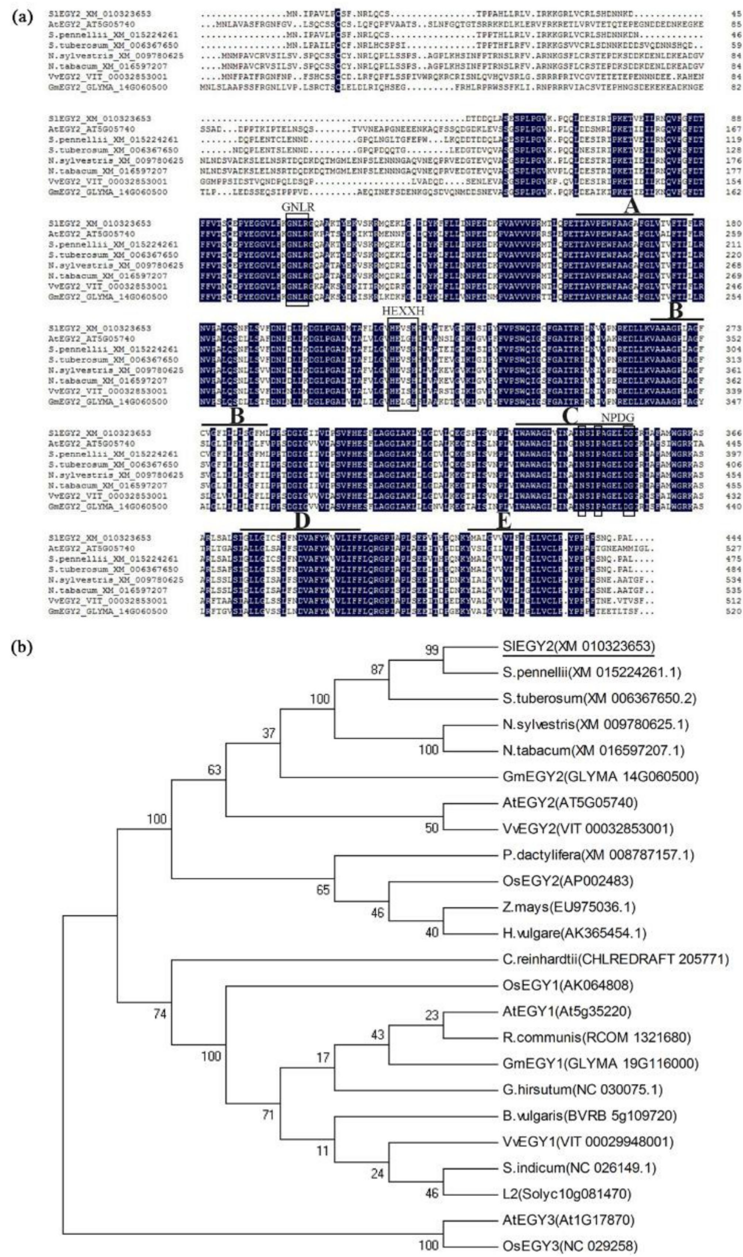

Figure 1. The homologous amino acids sequence alignment and phylogenetic tree analysis of SlEGY2. (**a**) Multiple sequences alignment of SlEGY2 with other homologous proteins in other species. Three conserved motifs (HEXXH, GNLR, and NPDG) in metalloproteases are remarked in black framers. Six predicted transmembrane topologies (A–E) are marked in black lines. Transmembrane topology is predicted by ExPASy (http://web.expasy.org/protscale/, accessed on 6 October 2018). (**b**) Phylogeny analysis of SlEGY2. Phylogeny was generated by using the Neighbor Joining (NJ) method of MEGA. Bootstrap analyses were obtained with 1000 replicates and the percentage values over 50 were brought out. The accession numbers of proteins are shown in parentheses.

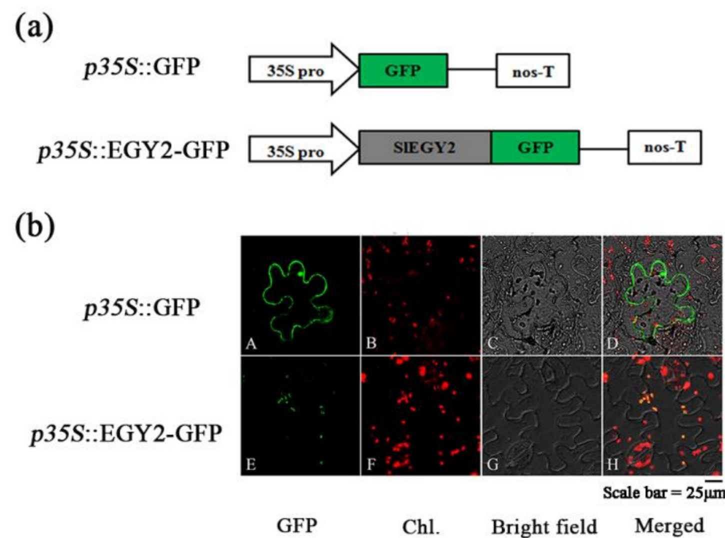

Figure 2. Subcellular localization of SlEGY2 in epidermal cells from Nicotiana benthamiana. (**a**) Schematic of the GFP recombinant plasmid. (**b**) The SlEGY2-GFP fusion protein was a target to chloroplasts. (A, E) Green fluorescence of GFP (A) and the EGY2-GFP fusion protein (E); (B, F) Red autofluorescence of chlorophyll; (C, G) Bright-field images of tobacco epidermal cells. (D) Merged images of A, B, and C. (H) Merged images of E, F, and G.

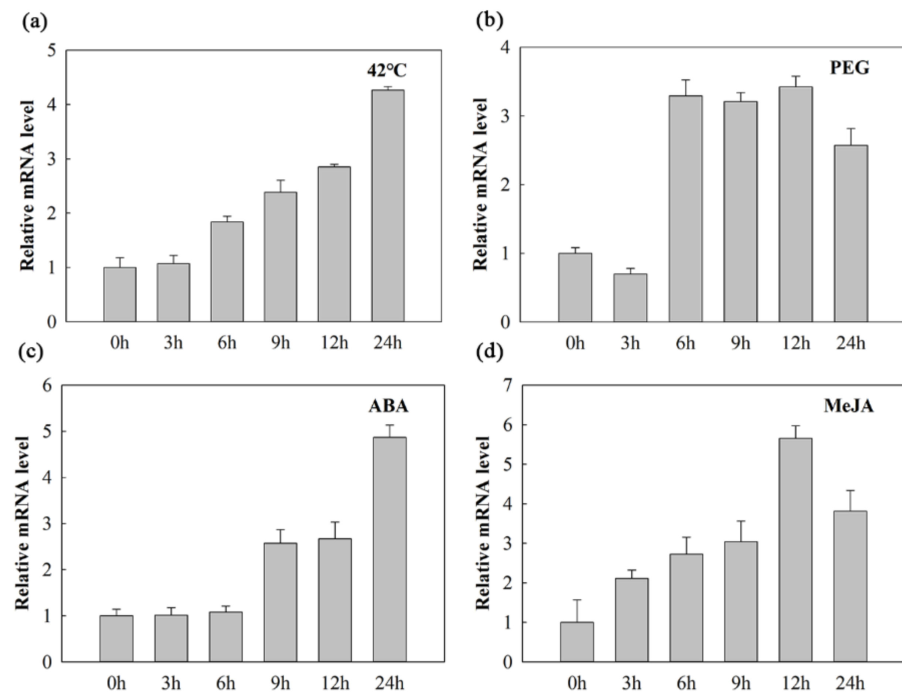

Figure 3. Expression profile analysis of SlEGY2 under different abiotic stress treatments. (**a**) 42 °C. (**b**) 20% PEG6000 (w/v). (**c**) 100 μM ABA. (**d**) 100 mM MeJA.

3.4. Inhibition of the Expression of SlEGY2 Increased Membrane Damage and Photoinhibition of PSII

The integrity of the biological membrane is not only the premise for maintaining metabolic homeostasis, but also the primary target of environmental stress. To investigate the degree of damage of the biological membrane, trypan blue staining was first performed. As shown in Figure 5a, heat stress caused more serious damage to the biological membrane of AS lines. The accumulation of MDA and REC were used as indicators of membrane damage. Under normal conditions, there was no significant difference in the accumulation

of MDA and REC between WT and AS lines. After heat treatment, the accumulation of MDA and REC were both increased, but the increasing degree in AS lines was much greater than in WT plants (Figure 5b,c). These results suggest that inhibition of SlEGY2 increases membrane damage under heat stress.

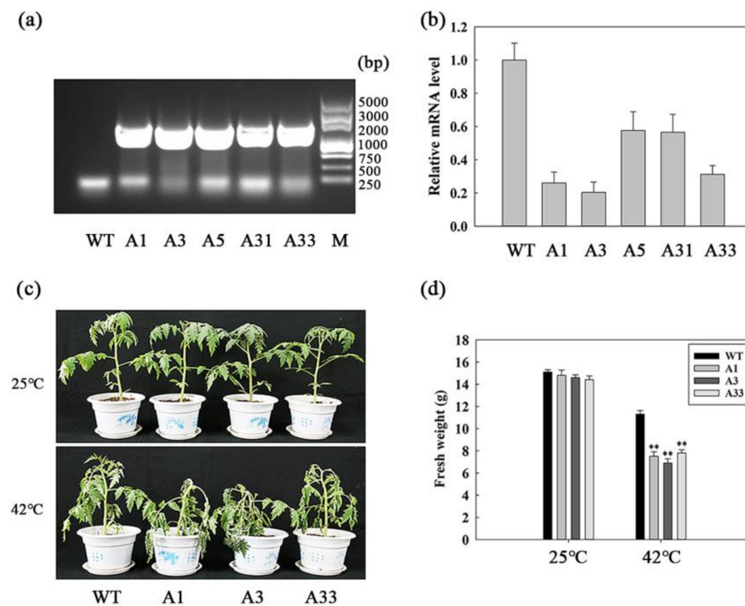

Figure 4. Identification of the AS transgenic tomato and the phenotype analysis of WT and AS lines under heat stress. (**a**) Identification of SlEGY2 AS lines using PCR. (**b**) The relative mRNA level of SlEGY2 in WT and AS lines. (**c**) The phenotype of WT and AS lines. (**d**) Fresh weight of WT and AS lines. ** ($p < 0.01$) indicate significant differences relative to the WT plants.

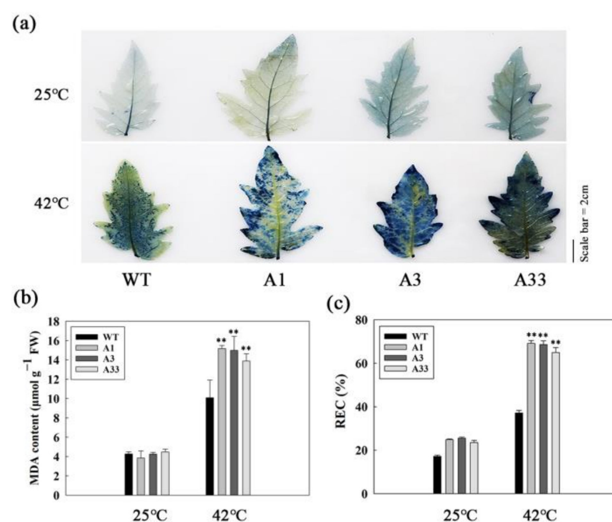

Figure 5. The integrity of biological membrane of WT and AS lines. (**a**) Trypan blue staining. (**b**) Content of MDA. (**c**) Ion leakage. ** ($p < 0.01$) indicate significant differences relative to the WT plants.

Photosystems, particularly PSII, are considered to be one of the heat stress-sensitive sites in plants [35]. In order to reveal the effect of SlEGY2 on photosynthesis, we measured the content of chlorophyll, Pn, and Fv/Fm. Under normal conditions, all the three indexes had no significant difference between WT and AS lines. Nevertheless, after heat treatment, compared with WT, the three indexes of AS lines all decreased to a greater extent (Figure 6a–c). This means that inhibiting the expression of SlEGY2 enhances the photoinhibition of PSII.

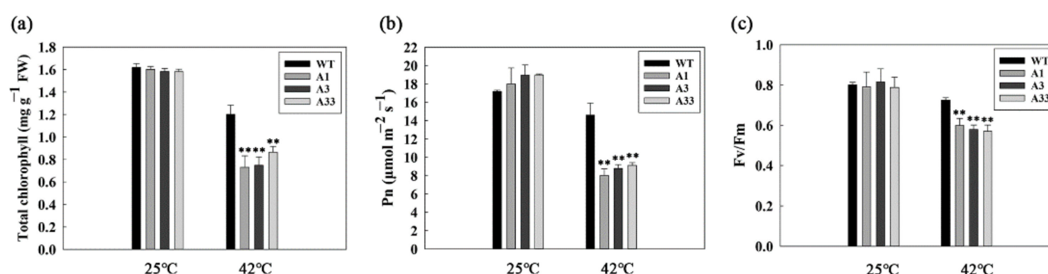

Figure 6. Content of chlorophyll (**a**), Pn (**b**), and Fv/Fm (**c**) in WT and AS lines. ** ($p < 0.01$) indicate significant differences relative to the WT plants.

3.5. SlEGY2 Regulated Accumulation of ROS by Affecting Antioxidative Enzyme Activity

One of the earliest responses of plants to stress is a rapid burst of reactive oxygen species (ROS). H_2O_2 and $O_2^{\bullet-}$ are two main kinds of ROS produced in the chloroplast. They were assessed by DAB and NBT staining, respectively. Under normal conditions, the accumulation of H_2O_2 and $O_2^{\bullet-}$ in intracellular leaves had no significant difference between AS and WT plants. However, AS lines were stained more deeply than WT plants after heat treatment (Figure 7a,b). In addition, similar results were observed by using quantitative analysis. Although the contents of H_2O_2 and $O_2^{\bullet-}$ increased in all plants subjected to heat stress, the contents of H_2O_2 and $O_2^{\bullet-}$ in AS lines were much higher than those in WT plants (Figure 7c,d).

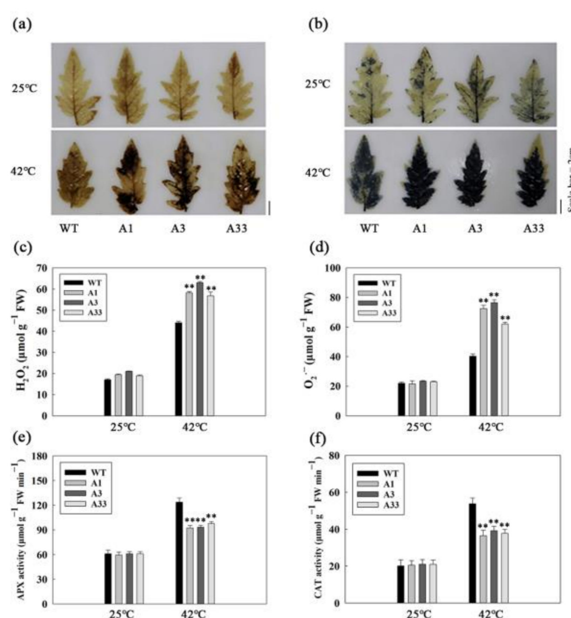

Figure 7. DAB (**a**) staining, NBT staining (**b**), H_2O_2 content (**c**), $O_2^{\bullet-}$ content (**d**) and activities of APX (**e**) and CAT (**f**) in WT and AS lines. ** ($p < 0.01$) indicate significant differences relative to the WT plants.

Then, we tested the activities of ascorbate peroxidase (APX) and catalase (CAT) in both AS and WT lines. The activities APX and CAT were higher in WT than those in AS plants after heat stress (Figure 7e,f). Thus, the lower activities of APX and CAT led to a higher accumulation of H_2O_2 and $O_2^{\bullet-}$ in AS lines. These results indicate that SlEGY2 plays a positive role in tomato response to oxidative stress caused by heat stress.

4. Discussion

EGY2, a novel nuclear-encoded plastid-localized metalloprotease, has been reported to play a role in hypocotyl elongation according to data from molecular, cellular, genetic

and physiological experiments [23]. However, the physiological function of this protease has been poorly investigated. In the current study, SlEGY2 was cloned and identified from tomatoes. Through amino acid alignment with other zinc metalloproteases, it was found that SlEGY2 belongs to the M50 metalloprotease family, containing the conserved motifs HEXXH and NPDG (Figure 1a). Phylogenetic analysis revealed that SlEGY2 was closely related to AtEGY2 (Figure 1b), and SlEGY2 was also localized in the chloroplast (Figure 2b). This implied that SlEGY2 and AtEGY2 may have a similar function. Indeed, in seedlings of SlEGY2 AS lines, we did observe the shorter hypocotyl phenotype (Supplementary Figure S2a,b) similar to the Arabidopsis egy2 mutant [23]. Interestingly, this difference exists only in young seedlings and disappears in adult plants (see Figure 4c). Moreover, we found that all seedlings appeared with a yellowing phenomenon and apical curved hook (typical ethylene triple-response phenotype) after being grown at 25 °C in darkness for 10 days, but the curvature of AS tomato seedlings was more severe than WT (data not shown). These phenotypic data suggest that SlEGY2 functions similarly to AtEGY2 in regulation hypocotyl elongation. Nevertheless, the regulation mechanism may be different. AtEGY2 regulated hypocotyl elongation possibly by affecting fatty acid content, while SlEGY2 may regulate hypocotyl elongation through the ethylene pathway.

High temperature often impairs plant growth, survival, and productivity. Heat stress affects the integrity of the proteome by causing misfolding and/or denaturation of proteins, thereby negatively affecting cell viability [14]. Proteases play an important regulatory role in maintaining proteome homeostasis in most subcellular compartments. S2P proteases are involved in regulated intramembrane proteolysis which can regulate gene expression at the transcription level [28]. However, very little is known about the physiological functions of S2P proteases in plants. Here, we found that suppressing the expression of S2P protease SlEGY2 reduced the thermotolerance of transgenic plants (Figure 4). One of the earliest responses of plants to stress is a rapid burst of ROS in the chloroplast [36]. It is well known that ROS, such as H_2O_2 and $O_2^{\bullet-}$, lead to oxidative stress [37]. After heat stress, the accumulation of H_2O_2 and $O_2^{\bullet-}$ was much more in AS lines than those in WT lines (Figure 7). Though plants can detoxify harmful ROS through the enzymatic system, the activities of APX and CAT were much lower in AS lines, compared with WT (Figure 7), thus leading to excess accumulation of ROS, which resulted in more MDA and ion leakage to damage the biological membrane system (Figure 5). Nevertheless, the mechanism by which SlEGY2 affects the activities of antioxidant enzymes under heat stress needs to be further explored.

In addition to attacking the membrane system, the photosynthetic apparatus is also the main target of ROS. Once damaged by ROS, the activity of the photosynthetic apparatus will decrease and lead to photoinhibition, even photodamage [38]. The damaged proteins must be degraded rapidly to ensure proteostasis in cells. AtEGY1, a member of S2P, has been reported to be required for proper chloroplast development [22]. Although the mRNA level of SlEGY2 was the highest in leaves (Supplementary Figure S1), SlEGY2 does not seem to regulate chloroplast development under non-stress conditions, which was consistent with AtEGY2 [23]. However, the lack of AtEGY2 caused changes in non-photochemical quenching (NPQ) and minimum fluorescence yield (F0) as well as a higher sensitivity of PSII to photoinhibition. Moreover, mutation of this gene led to a significant increase in the level of PsbA (D1) with a simultaneous decrease in the accumulation levels of PsbC (CP43) and PsbD (D2) [28]. Our results showed that inhibiting the expression of SlEGY2 aggravated the photoinhibition of PSII (Figure 6). This is contrary to the findings in Arabidopsis. Because SlEGY2 and AtEGY2 have only 54.5% sequence similarity (Supplementary Figure S3), there is a great possibility that they function differently. Furthermore, Arabidopsis is a chilling-resistant plant, while tomato is a chilling-sensitive plant. There are some particular regulatory mechanisms in chilling-sensitive plants, which are not found in Arabidopsis and other crops. For example, SlWHY1 can directly bind to the GTTACCCT element in the promoter of SlPsbA to increase the D1 level under chilling stress, but this element does not exist in Arabidopsis, maize, and rice while it is found in

pepper (another chilling-sensitive plant) [29]. Thus, tomato also has some specific mechanisms in response to heat stress, although it is not clear now. In addition, the expression patterns of AtEGY2 and SlEGY2 are different under heat stress. Through the eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi, accessed on 15 May 2022), we found that the expression of AtEGY2 was down-regulated under heat stress, while SlEGY2 was up-regulated (Figure 3). It also indicates that the two genes respond to heat stress via different mechanisms.

5. Conclusions

SlEGY2 is targeted to the chloroplast, and belongs to the metalloprotease family M50. Suppression of SlEGY2 reduced thermotolerance of transgenic plants via decreasing the scavenging ability of ROS, thus enhancing the photoinhibition of PSII. Although we demonstrated the roles of SlEGY2 in tomato response to high temperature, the detailed molecular mechanism needs to be explored in the future.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agriculture12070940/s1, Figure S1: The expression level of SlEGY2 in different tissues; Figure S2: The phenotype of WT and AS young seedlings. The phenotype of hypocotyl (a) and the statistical analysis of the length of hypocotyl (b) in WT and AS lines. ** ($p < 0.01$) and * ($p < 0.05$) indicate significant differences relative to the WT plants; Figure S3: Amino acid sequence alignment of AtEGY2 and SlEGY2; Table S1: Primers used in this study.

Author Contributions: N.M. and W.L. designed the research; S.Z., C.C., S.D. and M.Y. performed the research and analyzed the data; N.M. and S.Z. wrote the paper; W.L. and Q.M. provided advice and revised the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Natural Science Foundation of China: 31900208, 31870277, 31870239 and Natural Science Foundation of Shandong Province: ZR2019BC069.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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