

Reducing AsA Leads to Leaf Lesion and Defence Response in Knock-Down of the AsA Biosynthetic Enzyme GDP-D-Mannose Pyrophosphorylase Gene in Tomato Plant

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Abstract

As a vital antioxidant, L-ascorbic acid (AsA) affects diverse biological processes in higher plants. Lack of AsA in cell impairs plant development. In the present study, we manipulated a gene of GDP-mannose pyrophosphorylase which catalyzes the conversion of D-mannose-1-P to GDP-D-mannose in AsA biosynthetic pathway and found out the phenotype alteration of tomato. In the tomato genome, there are four members of *GMP* gene family and they constitutively expressed in various tissues in distinct expression patterns. As expected, over-expression of *SIGMP3* increased total AsA contents and enhanced the tolerance to oxidative stress in tomato. On the contrary, knock-down of *SIGMP3* significantly decreased AsA contents below the threshold level and altered the phenotype of tomato plants with lesions and further senescence. Further analysis indicated the causes for this symptom could result from failing to instantly deplete the reactive oxygen species (ROS) as decline of free radical scavenging activity. More ROS accumulated in the leaves and then triggered expressions of defence-related genes and mimic symptom occurred on the leaves similar to hypersensitive responses against pathogens. Consequently, the photosynthesis of leaves was dramatically fallen. These results suggested the vital roles of AsA as an antioxidant in leaf function and defence response of tomato.

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Introduction

In higher plants, reactive oxygen species (ROS) are produced as by-products in most energy-generating processes, such as photosynthesis and respiration. An electron reduction of O_2 leads to the formation of superoxide radical (O_2^-), which is then disproportionated by superoxide dismutase (SOD) to O_2 and hydrogen peroxide (H_2O_2). ROS production in plant cells is low under optimal growth conditions, but increases dramatically when plants are subjected to abiotic stresses and pathogen attack. Unfavourable environmental conditions, such as cold, heat, drought, and salt, limit the rate of carbon fixation, which results in an increase in photoinhibition and overproduction of superoxide radicals and H_2O_2 [1]. Furthermore, oxidative burst is one of the most rapid defence reactions to pathogen attack, which changes the production of superoxide and H_2O_2 at the infection site [2].

Excessive ROS can induce programmed cell death and necrosis [3]. In higher plants, the levels of ROS are strictly regulated by an efficient battery of enzymatic and non-enzymatic antioxidants [2]. Among them, ascorbic acid (AsA) acts as one of the most abundant antioxidants against oxidative stress [4]. In plants, chloroplasts are potentially the major site for the generation of ROS, in which AsA is present at a high level, at concentration of 20 mM or more.

Thus, AsA plays a central function in photoprotection, including scavenger of ROS generated by photosynthesis and respiration, cofactor for violaxanthin deepoxidase, and photosystem II electron donor [5].

Although alternative biosynthetic pathways have been proposed [6–8], Smirnoff-Wheeler's pathway [9] (Fig. 1) has been proved to be the major functional pathway by biochemical and genetic approach. GDP-D-mannose pyrophosphorylase (GMP) catalyzes the conversion of D-Mannose-1-P to GDP-D-Mannose, an initial step in the Smirnoff-Wheeler's pathway [9]. The importance of GMP in the control of AsA biosynthesis has been confirmed in some plants. The significant reduction of AsA in the *vtc1* mutant of *Arabidopsis* was caused by a point mutation in *GMP* gene [10]. Antisense inhibition of *GMP* gene in the transgenic potato (*Solanum tuberosum*) plants reduced AsA contents both in leaves and tubers [11]. In acerola (*Malpighia glabra*), the *MgGMP* gene expression displayed a strong correlation with the AsA contents in the ripening fruit [12]. In addition, over-expression of *MgGMP* gene increased AsA content by approximately two-fold in tobacco (*Nicotiana tabacum*) [13].

Tomato is a representative of edible fruit plants with an abundance of AsA. Recently, several progresses about AsA metabolism in tomato have been achieved [14–19]. Genes

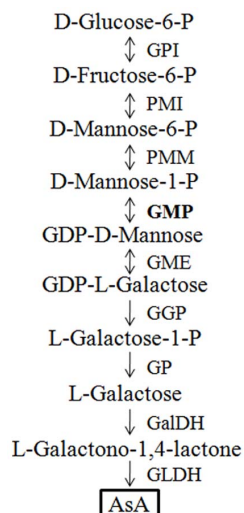


Figure 1. Major AsA biosynthetic pathway in higher plants (Smirnoff-Wheeler's pathway). GPI, glucose-6-phosphate isomerase; PMI, phosphomannose isomerase; PMM, phosphomannomutase; GMP (marked in bold), GDP-D-mannose pyrophosphorylase; GME, GDP-D-mannose-3',5'-epimerase; GGP, GDP-L-galactose-1-phosphate phosphorylase; GP, L-galactose-1-phosphate phosphatase; GalDH, L-galactose dehydrogenase; GLDH, L-galactono-1,4-lactone dehydrogenase. doi:10.1371/journal.pone.0061987.g001

involved in tomato AsA biosynthesis have been largely identified [20–24], and some of them have been functionally characterized, such as L-galactono-1,4-lactone dehydrogenase (*GLDH*) [20] and GDP-D-mannose 3', 5'-epimerase (*GME*) [21,22]. In tomato genome, GMP has four isoforms (GMP1-GMP4). Currently, some progresses have been made in understanding the biological function of tomato *GMP* genes, especially *GMP3*. Expression profiling analysis revealed that *SIGMP3* mRNA accumulated during the early stages of tomato fruit development and was down-regulated in mature green fruits subjected to ethylene and various stresses [16]. In addition, over-expression of *SIGMP3* gene in tobacco significantly increased AsA content in leaf and improved the tolerance to high and low temperature stress by enhancing antioxidation capacity [25]. However, whether *SIGMP3* affects the AsA biosynthesis and accumulation in tomato remains unclear.

To better understand the regulation of AsA biosynthesis in tomato, we generated *SIGMP3* over-expressing and knocked-down transgenic tomato plants, and analyzed the subsequent effects on AsA metabolism and plant growth. Results indicated that elevated *GMP3* expression increased total AsA levels in tomato leaves and fruits, and then enhanced photo-oxidative stress tolerance, whereas RNAi silencing of GMP in tomato could effectively reduce AsA contents, leading to ROS accumulation with leaf lesion formation and senescence, and activation of defence responses. These imply the vital role of AsA as an antioxidant in tomato.

Materials and Methods

Plant Materials

Tomato (*Solanum lycopersicum* cv. Ailsa Craig, AC) plants were grown in a naturally illuminated glasshouse. Tissues from roots, stems, leaves, flowers, and fruits at various developmental stages of AC plants were collected, immediately frozen in liquid nitrogen, and stored at -80°C until use.

Gene Cloning, Vector Construction and Tomato Transformation

The 1236 bp fragment, including the full open reading frame of *SIGMP3* (SGN-U568547), was amplified using the primer sets of GMP3F and GMP3R (Table S1), and then cloned into pMD18-T vector (Takara, Japan). Subsequently, over-expression and RNAi vectors were constructed by subcloning the full length cDNA of *SIGMP3* into pMV and pHGRV vectors, respectively, under the control of CaMV35S promoter. The constructs were introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation. Tomato seeds of Ailsa Craig were used for transformation, performed according to the methods described by Fillatti et al. [26]. Independent transgenic plants were confirmed by polymerase chain reaction (PCR) using primer combinations between CaMV35S promoter specific primers CaMV35SF or gate35SF and *SIGMP3* gene specific primer GMP3R (Table S1) and Southern blot. The transcript levels of *SIGMP3* as well as other three members of *SIGMP* gene family in the transgenic lines were analyzed via semi-quantitative reverse transcriptase (RT)-PCR and real-time RT-PCR.

Semi-quantitative and Real-time RT-PCR Analysis

Total RNA was isolated using TRIzol[®] reagent (Introvigen, USA), and DNase I was used to clean out DNA before reverse transcription. The reverse transcript reaction was performed with MMLV reverse transcriptase (Toyobo, Osaka, Japan) following the manufacturer's protocol. Reverse transcript products were used as template for semi-quantitative RT-PCR and real-time RT-PCR. All reaction was assayed in triplicates. Semi-quantitative RT-PCR was performed to preliminarily analyze the expression levels of *SIGMP3* in the transgenic lines. It was carried out using the following program: an initial denaturation of 94°C for 3 min, followed by 24–28 circles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were detected by 1% agarose gel in $1\times$ TAE with EtBr. Real-time RT-PCR was performed using LightCycler 480 SYBR Green I Master kit (Roche, Germany) according to the supplier's instruction to analyze the expression levels of the genes involved in AsA biosynthesis, photosynthesis, antioxidant system, and defence response in the transgenic tomato plants. Real-time RT-PCR amplification step consisted of a pre-incubation at 95°C for 5 min, followed by 40 circles of 95°C for 10 s, 60°C for 15 s and 72°C for 20 s. PCR products were monitored by the LightCycler 480 Real-Time PCR Detection System. Tomato β -actin gene was used as a reference gene to optimize semi-quantitative RT-PCR and as internal standard in real-time RT-PCR. The primers used are listed in Tables S1, S2, S3 and S4.

Oxidative Stress Treatment

One-month-old plantlets of *SIGMP3* over-expressing lines and wild type grown in compost plastic pots in a naturally illuminated glasshouse were sprayed with 75 μM methyl viologen (MV) solution or distilled water (control) for 2 d. The MV dissolved in 0.1% Tween-20 solution (100 mL) was applied to six plants. The fourth leaf samples were taken at 7 d post-treatment. The effect of oxidative stress on *SIGMP3* over-expressing transgenic plants was assessed by measuring the chlorophyll and malondialdehyde (MDA) contents in the leaves. The experiment was replicated three times.

Measurement of Total Ascorbate

The AsA content was determined using high-performance liquid chromatography as described by Rizzolo et al. [27]. Briefly,

samples were ground under liquid nitrogen and homogenised in 5 mL of cold 0.1% (w/v) metaphosphoric acid. The homogenate was centrifuged at 12,000 g for 10 min at 4°C. Then the supernatant was filtered through a Millipore membrane (0.22 µm). An aliquot of 300 µL was incubated with 300 µL 50 mM dithiothreitol for 15 min at room temperature. Then, the extracts were analysed by HPLC using an SB-aq column (Agilent) eluted with acetate buffer (0.2 mol/L, pH 4.5) at a flow rate of 1.0 mL/min to measure total ascorbate. Elutes were detected at 254 nm, and a standard curve from 2 to 40 µg/mL AsA was obtained.

Determination of Chlorophyll, MDA, and Net Photosynthesis Rate

Chlorophyll content was determined by grinding leaf tissues under liquid nitrogen and extracting with 80% (v/v) acetone under low light intensity using the procedure described by Wellburn's [28]. MDA was assayed for indirect evaluation of lipid peroxidation using trichloroacetic acid, as described previously by Heath and Parker [29]. Net photosynthesis rate was measured using a portable photosynthetic system (CIRAS-2, PP System, USA) according to the supplier's manual.

Histochemistry

Leaf samples taken from two-month-old tomato plants were stained with trypan blue and 3,3'-diaminobenzidine (DAB) solution to visualize dead cells and detect the presence of H₂O₂, respectively. Trypan blue staining was performed as previously described [30]. Leaves were submerged in a 70°C LPTB solution [2.5 mg/mL trypan blue, 25% (w/v) lactic acid, 23% water-saturated phenol, 25% glycerol, and H₂O], vacuum infiltrated for 5 min, and then repeated one time. Subsequently, the samples were heated over boiling water for 2 min and cooled for 1 h. The LPTB solution was then replaced with a chloral hydrate solution (25 g in 10 mL H₂O) for destaining. After multiple exchanges, the samples were equilibrated in 70% glycerol and photographed. H₂O₂ was visually detected in the leaves of tomato plants using DAB as the substrate [31]. Briefly, the leaves were cleaned and placed in 1 mg/mL DAB, pH 3.8, under light at 25°C for 8 h. The experiment was terminated by immersing the leaves in boiling 96% ethanol for 10 min. After cooling, the leaves were placed in fresh 96% ethanol for 4 h at room temperature and photographed. The deep brown polymerization product was produced via the reaction of DAB with H₂O₂.

Statistic Analysis

Data analysis was performed using SAS software, and significant differences were calculated using the Student's *t*-test at 95% confidence limit.

Results

Expression Patterns of GMP Genes in Tomato

Four unigenes corresponding to the amino acid sequences of GMP (*GMP1*, SGN-U563807; *GMP2*, SGN-U568548; *GMP3*, SGN-U568547; and *GMP4*, SGN-U584300) exist in tomato genome as reported by Massot et al. [17]. Blast results showed that *GMP1* and *GMP3* are both located on chromosome 3, and *GMP2* and *GMP4* are located on chromosome 6 and 9, respectively. The full-length cDNA of *SIGMP3* was isolated previously by our group [23]. We investigated the spatial and temporal expression patterns of four *GMP* members via real-time RT-PCR analysis. All of them were expressed constitutively in the tissues tested though distinct expression patterns (Fig. 2). For

SIGMP3, the transcript levels were high in stems, flowers, and young leaves, whereas low in roots, and fruits at breaker and red ripe stages (Fig. 2). The expressions of the other members, *SIGMP1* and *SIGMP2*, followed in a similar pattern, which were high in flowers, low in roots and breaker fruits, and slightly increased at the red ripe stage (Fig. 2). For *SIGMP4*, the expression was extremely distinct at low in most tissues, especially in vegetative tissues (Fig. 2).

Identification of Tomato Transgenic Plants

Thirty-eight *SIGMP3* over-expressing (OX) and seventeen RNAi (KD) transgenic plants were obtained and confirmed by PCR using genomic DNA as template and 35S forward and gene-specific reverse primers. The expression level of *SIGMP3* gene in young leaves of transgenic as well as wild-type plants was examined by semi-quantitative RT-PCR (Fig. 3A) and real-time RT-PCR (Fig. 3B). Two over-expressing lines (OX6 and OX19) and two RNAi lines (KD7 and KD17) with significant changes were selected for further study.

In order to make clear whether other three members of *SIGMP* gene family were affected in the *SIGMP3* transgenic plants, we investigated their expressions in young leaves (Fig. 3B). In two *SIGMP3* over-expressing lines, the expression of *SIGMP1*, *SIGMP2* and *SIGMP4* was not significantly affected. However, in *SIGMP3* RNAi lines, only *SIGMP2* was markedly down-regulated. This is due to the fact that *SIGMP2* has 86% homology to *SIGMP3* with ten ≥ 20 base pair length identity (Fig. S1), while *SIGMP1* and *SIGMP4* share low identity (only 23% and 40%, respectively) with *SIGMP3*. Due to repression of both *SIGMP2* and *SIGMP3*, *SIGMP3* RNAi lines were designated as *SIGMP2/3*-KD lines in the following.

Alteration of AsA Pool Size in Transgenic Tomato Plants

The total AsA contents of both *SIGMP3*-OX and *SIGMP2/3*-KD lines were altered compared to the non-transgenic plants. Constitutive over-expression of *SIGMP3* increased the total AsA contents in the fully expanded leaves and red ripe fruits (Fig. 4A). However, in *SIGMP2/3*-KD lines, the total AsA contents in leaves, immature green fruits, and breaker fruits decreased dramatically (Fig. 4B). With the developments of fruit, the AsA levels increased up to 46% in breaker stage compared with immature green stage in wild-type plants, whereas no major difference was observed between two stages of *SIGMP2/3*-KD lines (Fig. 4B). Moreover, a correlation between AsA content and the suppression extent of *SIGMP* genes was observed in KD7 and KD17 lines. These results indicate that over-expressing *SIGMP3* could enhance AsA accumulation, and knock-down of *SIGMP3* with *SIGMP2* significantly affects the total AsA pool size in tomato leaves and fruits.

The expression levels of other genes in the Smirnoff-Wheeler's pathway were also examined in transgenic and wild-type plants. The results showed the regulation of AsA biosynthesis altered in leaves. The transcript abundances of biosynthetic genes *GME* and GDP-L-galactose-1-phosphate phosphorylase (*GGP*) were remarkably affected. Over-expression of *SIGMP3* down-regulated *SIGME2* and *SIGGP2* (reduced nearly 50%) (Fig. 3C). On the contrary, in *SIGMP2/3*-KD transgenic line KD17, *SIGME2* and *SIGGP1* were significantly up-regulated (over one fold) compared with the wild-type plants (Fig. 3C). Most other genes of AsA biosynthetic pathway remained unchanged in transgenic plants.

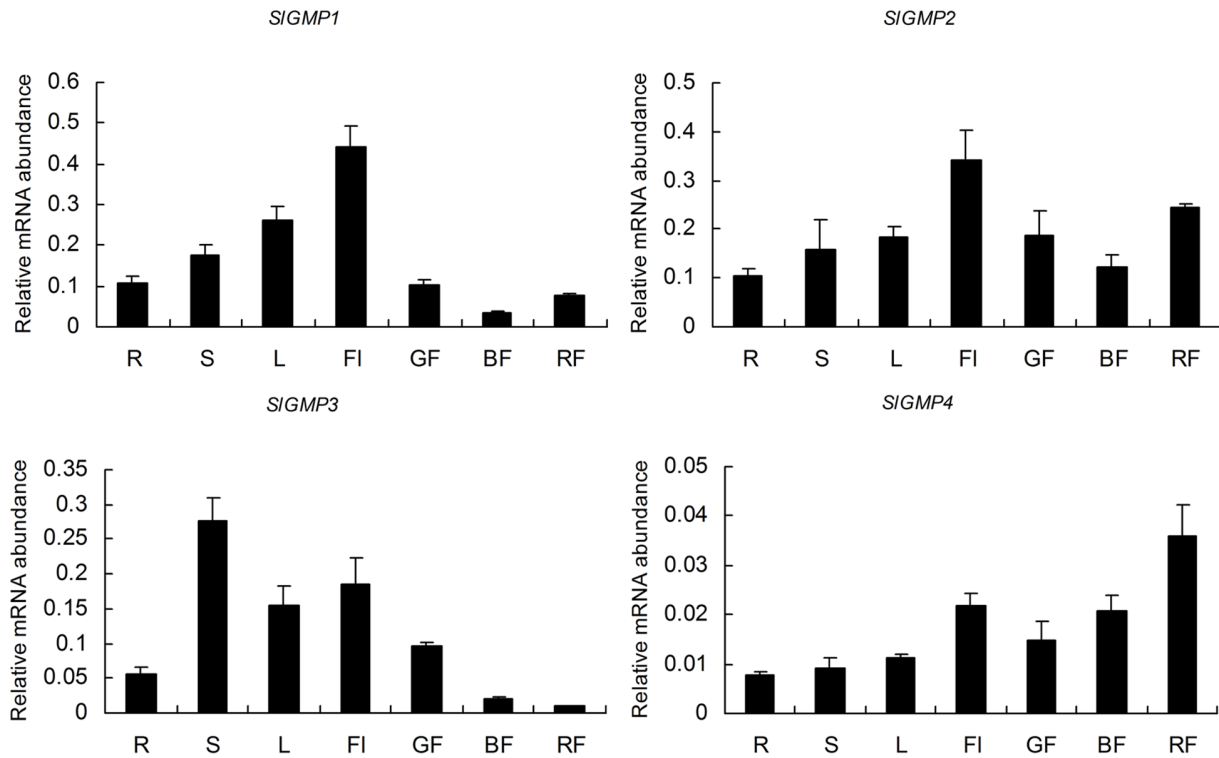


Figure 2. Relative expression analysis of four members of *SIGMP* gene family in various tissues of tomato variety Ailsa Craig. R: root, S: stem, L: leaf, FI: flower, GF: green fruit, BF: breaker fruit, and RF: red fruit. Data obtained by real-time RT-PCR were normalized against *Actin*. doi:10.1371/journal.pone.0061987.g002

Enhanced Tolerance to Oxidative Stress through Over-expressing *SIGMP3* in Tomato

To evaluate whether the over-expression of *SIGMP3* could protect transgenic plants against oxidative damage, MV was used to induce oxidative stress and sprayed on one-month-old plantlets of line OX6 and wild type. After the treatment, we found less chlorotic spots on the leaves of line OX6 than wild type. The oxidative damage caused by MV was examined through the extents of leaf chlorophyll loss and MDA (membrane-lipid peroxidation product) increase. Under normal conditions, chlorophyll content was not significantly different between wild-type and transgenic plants. However, after MV treatment, the chlorophyll content in the wild-type plants decreased by 34%, whereas no significant change in line OX6 (Fig. 5A). And the same as the MDA content in wild-type plants increased up to 70%, whereas only 21% increase was found in line OX6 (Fig. 5B). These results indicate that over-expressing *SIGMP3* improves the tolerance of the plants to oxidative stress.

Phenotypic Alteration in *SIGMP2/3*-KD Plants

The *SIGMP3*-OX plants grew normally during the whole life cycle. On the contrary, the *SIGMP2/3*-KD plants exhibited a lesion-mimic phenotype from the very early stage of seedlings, and the lesions spontaneously developed on leaves. Some lesions initially appeared on the cotyledon surfaces at three weeks post germination; the cotyledons turned yellow (Fig. 6A) and then dropped off the plants. Subsequently, lesions occurred on the lower true leaves (Fig. 6B), and spread from the bottom to the upper leaves (Fig. 6C). The necrotic lesions started from the leaflet tips of the compound leaves (Fig. 6D), and developed in a typical circular pattern and spread until the entire leaves turned chlorotic, and finally fell. The cell deaths of the lesioned leaves were

observed via trypan blue staining. The dead cells were consistent with macroscopic lesions before staining in the leaves of lines KD7 and KD17, whereas no staining was observed in wild-type leaves (Fig. 6D). The mature leaves of lines KD7 and KD17 withered faster than the wild type. With plants growing up to the reproductive stage, most leaves of line KD7 were dried out, whereas the wild-type plants remained green (Fig. 6C). We noticed the occurrence of lesions was more severe in line KD7 than line KD17. It indicates that phenotypic changes are correlated with the inhibition extent of *SIGMP* genes (Fig. 3B) in tomato.

Although the flowering time and the yield of *SIGMP2/3*-KD plants were not influenced, the fruit weight was significantly decreased in line KD7 (Fig. 6F and Table S5).

Oxidative Burst in the Leaves of *SIGMP2/3*-KD Plants

The spontaneous lesions on the leaves of *SIGMP2/3*-KD plants developed in a similar manner as a hypersensitive response after pathogen attack. Oxidative burst, wherein large quantities of ROS are generated, is an early plant response to microbial pathogen attack. Both types of leaves with and without lesions were analyzed via DAB staining to check whether the lesions observed in the leaves of *SIGMP2/3*-KD plants were caused by H_2O_2 accumulation. The lesioned leaves stained more, whereas the wild type did not (Fig. 6E), showing a close relationship between lesion and H_2O_2 levels in tomato leaves.

To examine whether the accumulation of high ROS concentrations affected the antioxidant defence system of plants, the transcript levels of several genes involved in detoxifying H_2O_2 or O_2^- were measured, including catalase (*CAT*), *SOD*, and ascorbate peroxidase (*APX*) using real-time RT-PCR. We found that *SOD* and chloroplastic *APX* genes were down-regulated in lesioned leaves of lines KD7 and KD17, whereas both their *CAT* and

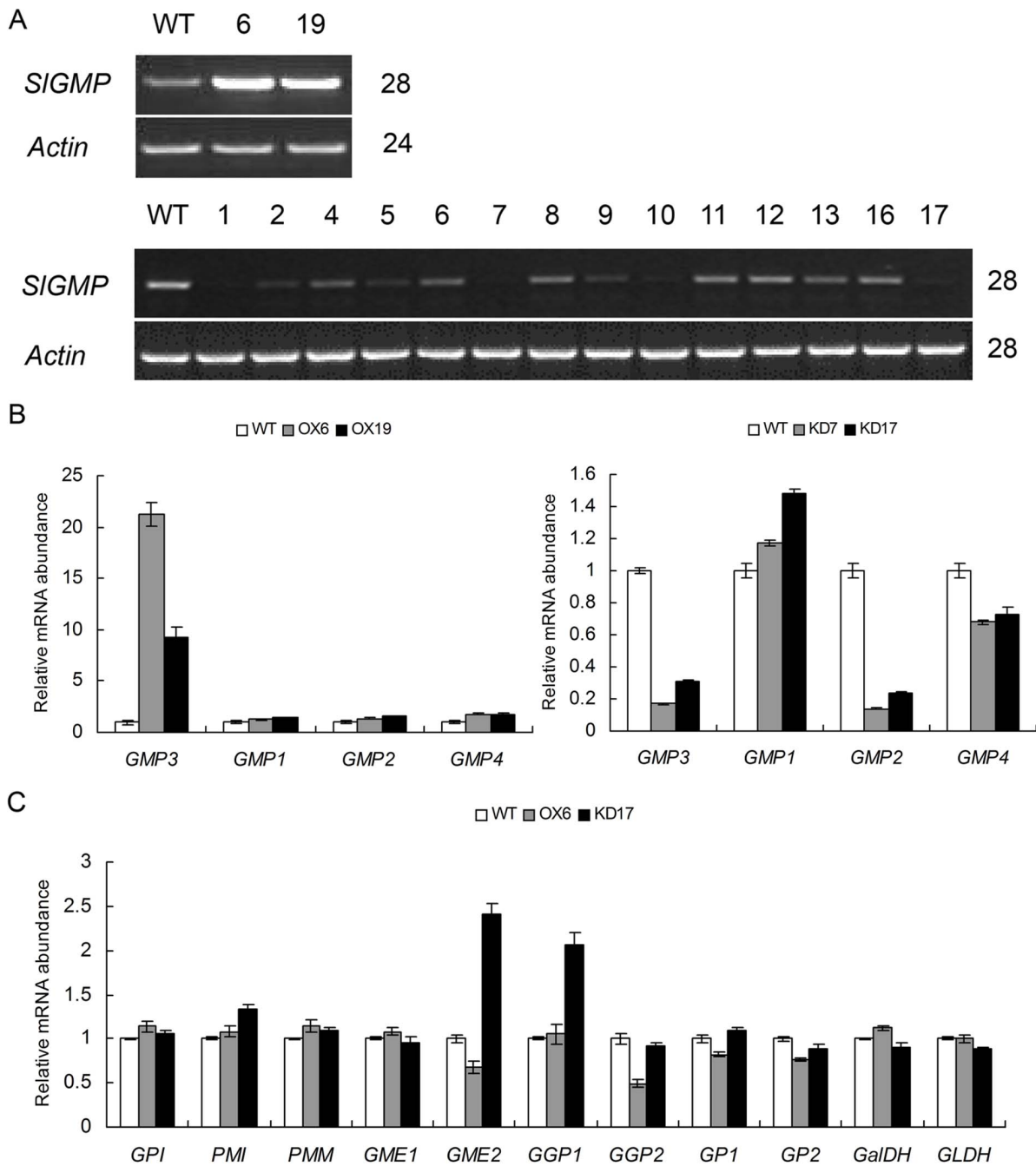


Figure 3. Expression analysis of four *SIGMPs* and other AsA biosynthesis-related genes in *SIGMP3* transgenic plants. (A) RT-PCR analysis of *SIGMP3* expression in the young leaves of two *SIGMP3*-OX lines (upper panel) and 17 *SIGMP3* RNAi lines (bottom panel). The PCR circle numbers are indicated on the right. (B) Relative expression analysis of four members of *SIGMP* gene family in the young leaves of lines OX6 and OX19 (left) and lines KD7 and KD17 (right) via real-time RT-PCR. (C) Relative expression analysis of AsA biosynthesis-related genes in the young leaves of lines OX6 and KD17 via real-time RT-PCR. Data were obtained by normalizing against *Actin* and shown as a percentage of wild-type plants. doi:10.1371/journal.pone.0061987.g003

cytosolic *APX* genes were significantly up-regulated, especially for *CAT*, which increased over two-fold compared with the wild type (Fig. 7). These results imply that the partial antioxidant defence system is triggered in response to the oxidative burst in *SIGMP2/3*-KD leaves.

Activation of Defence Response in *SIGMP2/3*-KD Plants

Many mutants with such lesions in plants have shown an enhanced systemic resistance to microbial pathogens. To reveal whether *SIGMP2/3*-KD plants activated pathogen defence response, the expressions of three pathogenesis-related (PR) genes, including *PR1b1* [32], *PR-P2* [33], and *PR-P6* [34,35], were analyzed by real-time RT-PCR. These genes are known to be strongly induced locally in tomato-pathogen interactions. The

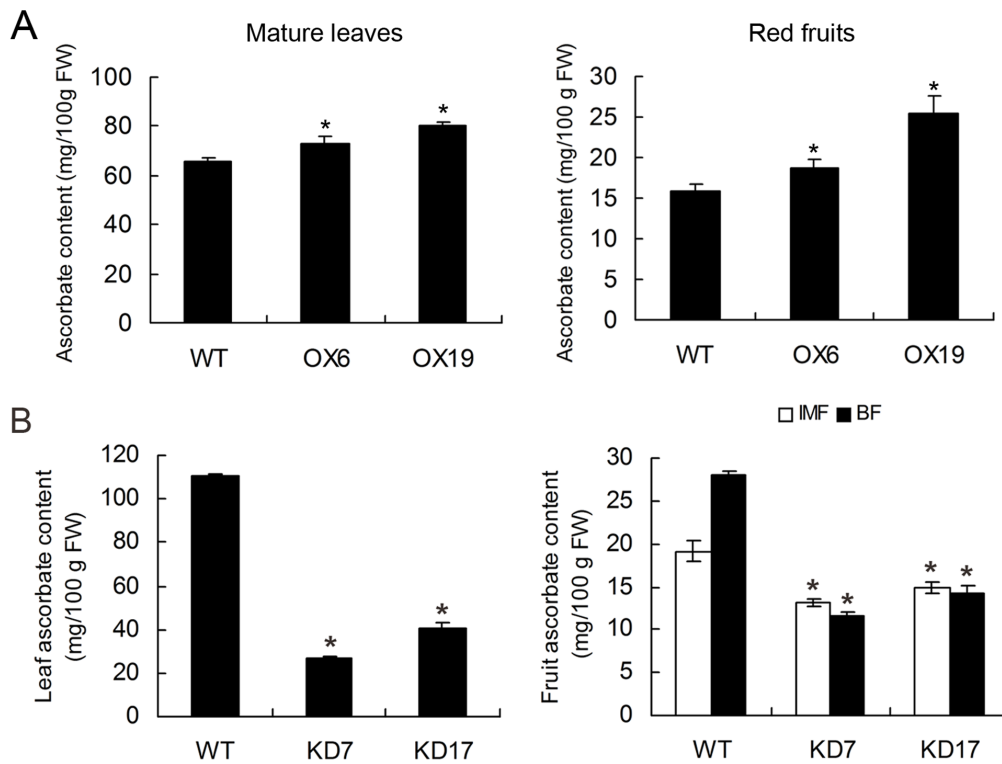


Figure 4. AsA content analysis in *SIGMP3* transgenic plants. (A) Total AsA contents in the mature leaves (left) and red fruits (right) of lines OX6 and OX19. (B) Total AsA contents in the mature leaves (left) and immature green fruits (IMF) and breaker fruits (BF) (right) of lines KD7 and KD17. As the experiments of (A) and (B) were carried at two separate times, there were some differences in AsA contents in leaves of the wild-type tomato plants. Data are presented as mean \pm SD of four independent plants per line. Asterisk indicates significant differences from the control ($P > 0.95$). doi:10.1371/journal.pone.0061987.g004

results showed that the transcripts of *PR1b1*, *PR-P2*, and *PR-P6* were more abundant in lesioned leaves of lines KD7 and KD17 than those in the wild type (Fig. 8A). Moreover, three defence genes were also activated in young leaves without any lesion (Fig. 8B) and in immature green fruits without any occurrence of lesions (Fig. 8C) though the activation extent was less than that in lesioned leaves. In addition, as shown in Fig. 8, more transcripts of *PR1b1*, *PR-P2*, and *PR-P6* were induced in leaves than immature fruits. All these results seem that systemic acquired resistance (SAR) is activated in *SIGMP2/3*-KD plants.

Impairment of the Photosynthetic System in *SIGMP2/3*-KD Plants

Primary results obtained through TOM2 Oligo chip microarray showed that some photosynthesis-related genes were up-regulated in the breaker fruits of *SIGMP3*-OX line OX19, and further confirmed via real-time RT-PCR analysis. These genes are involved in chlorophyll *a-b* binding, light harvesting processes in photosystem II, as well as ATP and phytochrome biosyntheses. Therefore, these genes were also analyzed in *SIGMP2/3*-KD plants. The nine selected photosynthesis-related genes were significantly down-regulated, and the net photosynthesis rates

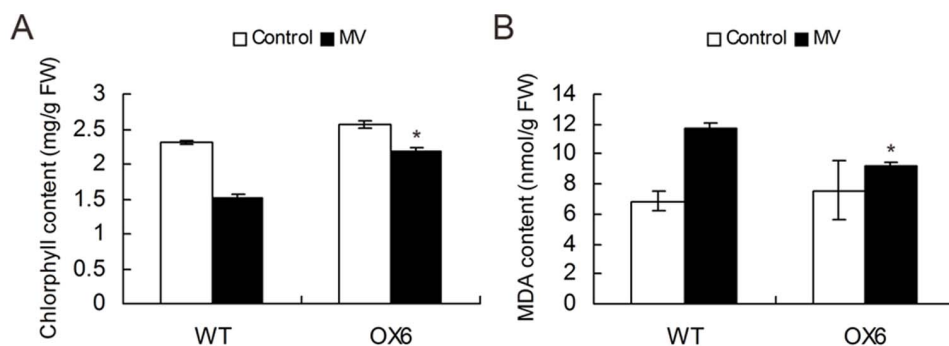


Figure 5. Improvement of the photo-oxidative stress tolerance in tomato plants via over-expressing *SIGMP3*. Chlorophyll content (A) and MDA content (B) in the fourth leaf of the MV-treated and untreated plants measured at 7 d post-treatment. Data are presented as mean \pm SD (N=6) from triplicate independent measurements. Asterisk indicates significant differences from the control ($P > 0.95$). doi:10.1371/journal.pone.0061987.g005

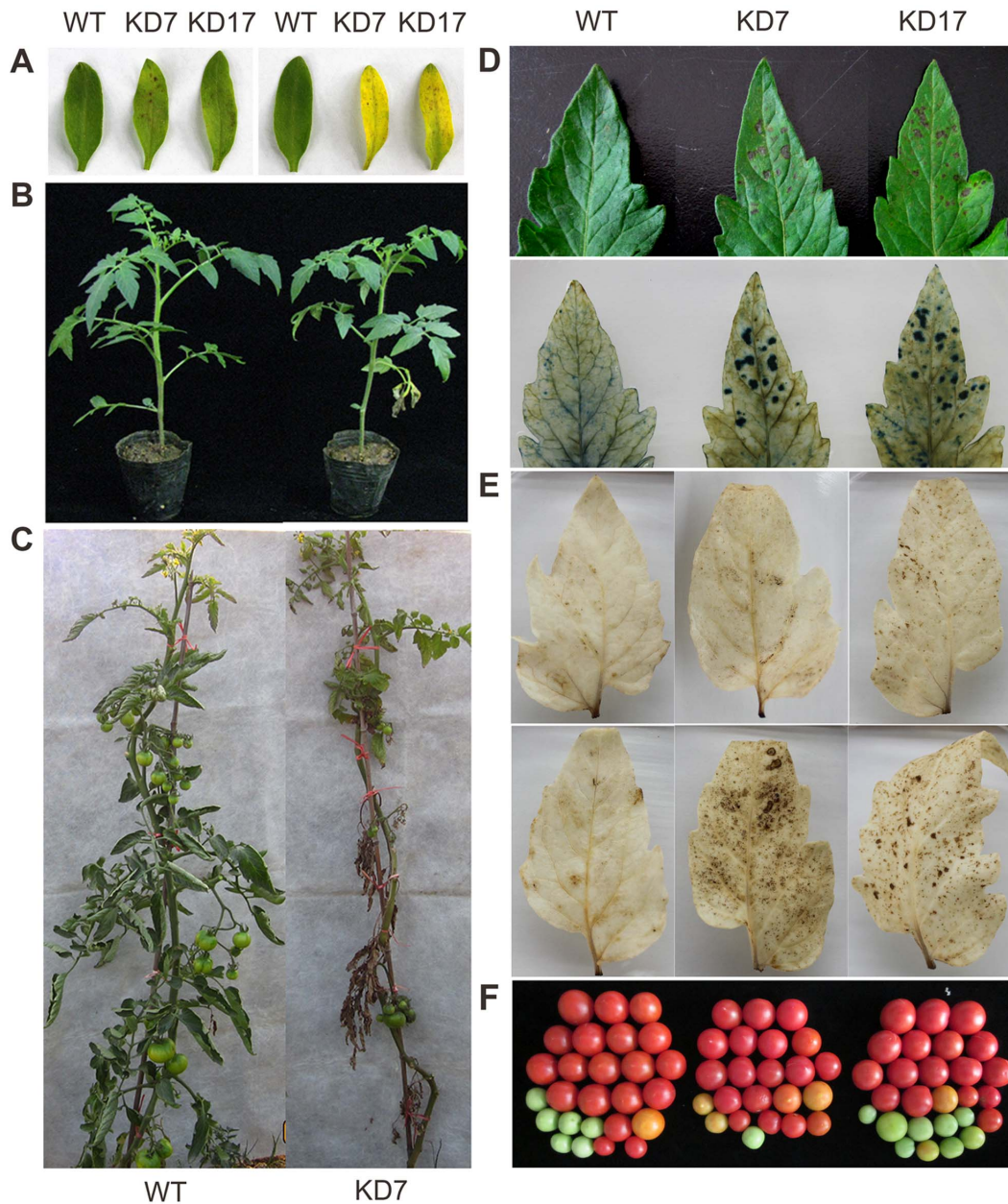


Figure 6. Phenotype comparisons of *SIGMP2/3*-KD and wild-type plants. (A) Altered cotyledon morphology of *SIGMP2/3*-KD vs wild-type plants. KD7 and KD17 cotyledons developed lesions (left) at three weeks post germination and accelerated senescence (right) compared with the wild type. (B) Seedlings of wild-type (left) and KD7 (right) plants. The bottom leaves of KD7 plant started to wilt. (C) Plant morphology of the three-month-old wild-type (left) and KD7 (right) plants. Middle and bottom leaves of KD7 plants became dry wilted. (D) Lesion formation on KD7 and KD17 leaves. The leaf lesion areas on the two-month-old plants of KD7 and KD17 (upper panel) are consistent with the areas of dead cells revealed through trypan blue staining (bottom panel). (E) H₂O₂ accumulation in the leaves of KD7 and KD17. H₂O₂ accumulation was revealed via DAB staining in leaves without (upper panel) and with (lower panel) visible lesions from the two-month-old plants of KD7 and KD17. (F) Tomato fruits harvested from four-month-old wild-type and transgenic plants.
doi:10.1371/journal.pone.0061987.g006

drastically declined in the slightly lesioned leaves of lines KD7 and KD17 (Fig. 9A, B).

Discussion

GMP has been shown to affect AsA biosynthesis in *Arabidopsis* [10], potato [11], and acerola [12,13]. Recently, expression of yeast-derived GMP gene in tomato was found to enhance AsA levels in leaves, green and red fruits [36], indicating biotechno-

logical manipulation of AsA biosynthesis in tomato can be achieved through increasing GMP activity. In recent years, four GMP isoforms are found to exist in tomato genome [17]. However, until now, whether AsA biosynthesis could be regulated by manipulation of the four *SIGMP* genes in tomato is not clear yet. In 2006, due to the limited mRNA/EST sequence information in public databases, only one *GMP* gene (*SIGMP3*) was obtained from tomato by our group. The putative amino acid sequence of *SIGMP3* has high similarity with AtGMP and StGMP

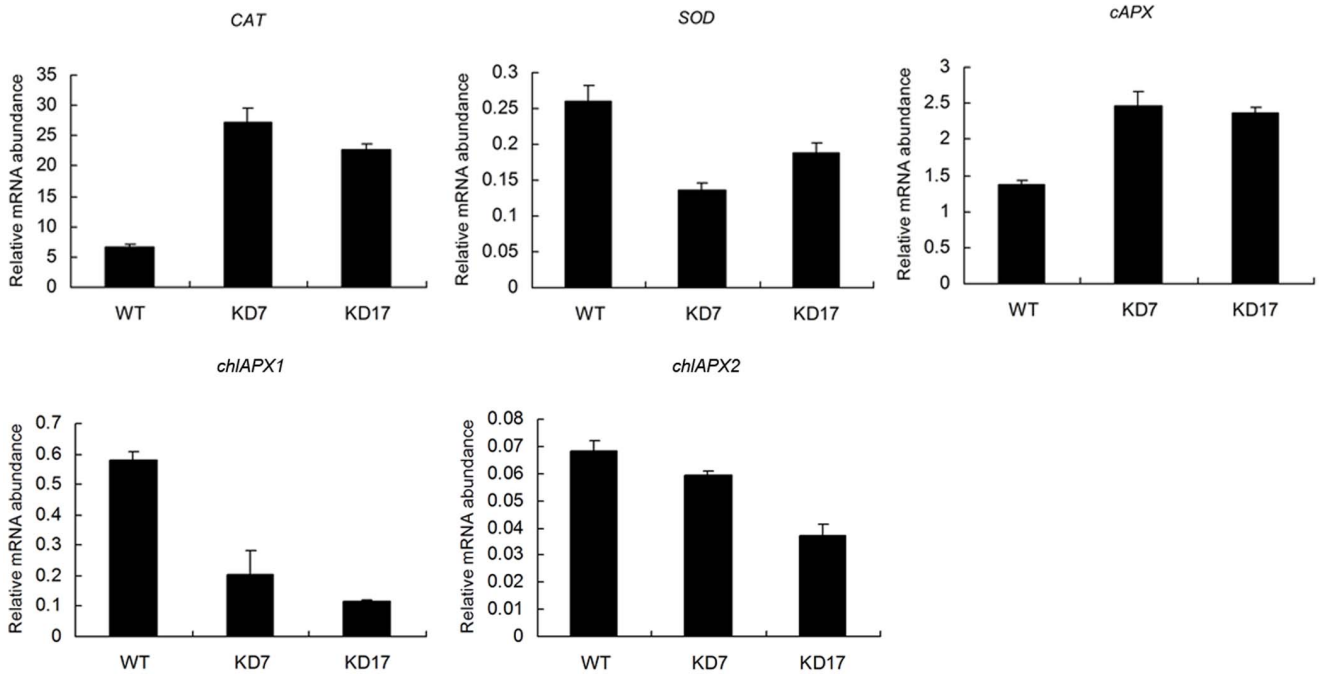


Figure 7. Relative transcript levels of the oxidative stress-related genes in the tomato lesioned leaves. The expression levels of oxidative related genes *CAT*, *SOD*, *cAPX*, and *chlAPXs* in the tomato lesioned leaves were measured by real-time RT-PCR. Data were normalized against *Actin*.
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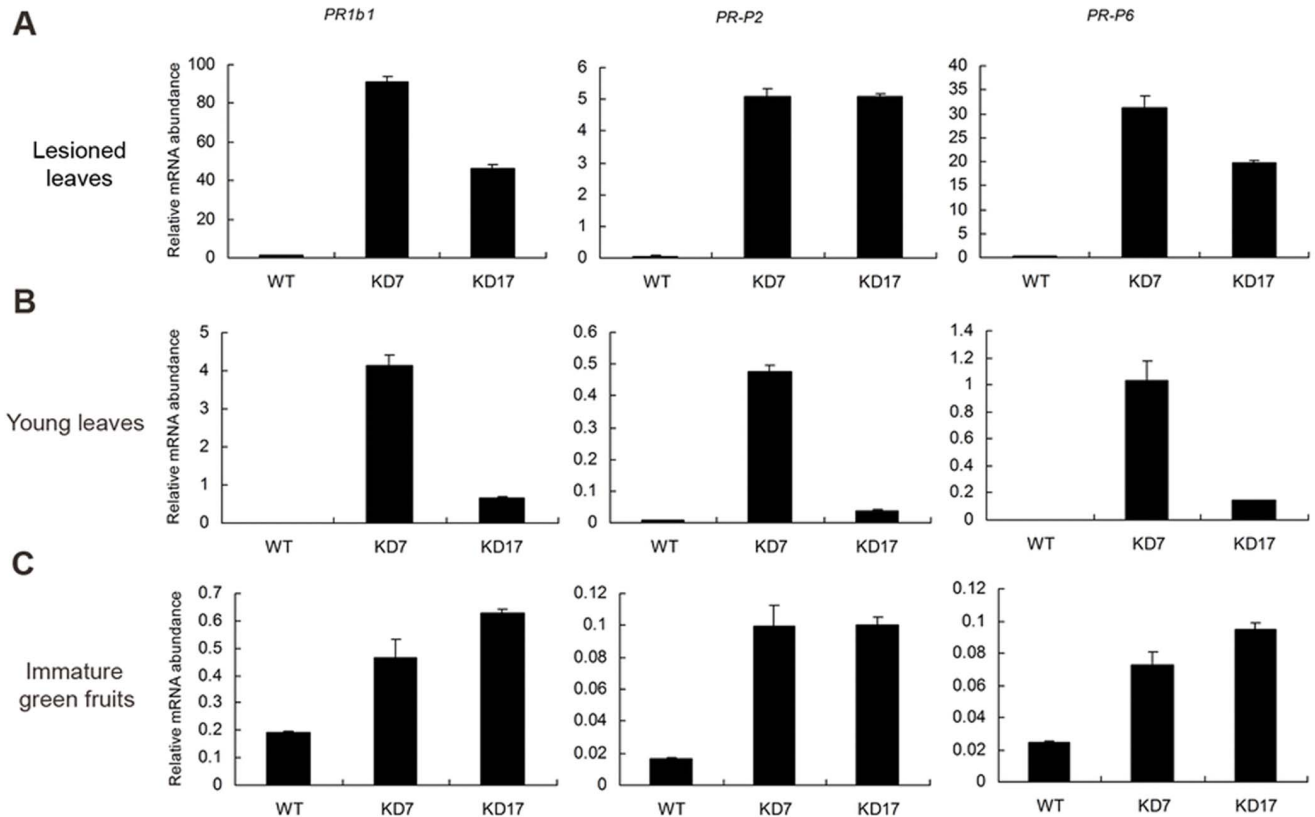


Figure 8. Relative transcript levels of the pathogenesis-related genes in the tomato lesioned and normal leaves and fruits. Expression analysis of the pathogenesis-related genes *PR1b1*, *PR-P2*, and *PR-P6* was performed in necrotic mature leaves (A), non-necrotic normal young leaves (B) and immature green fruits (C) of lines KD7 and KD17 by real-time RT-PCR. Data were normalized against *Actin*.
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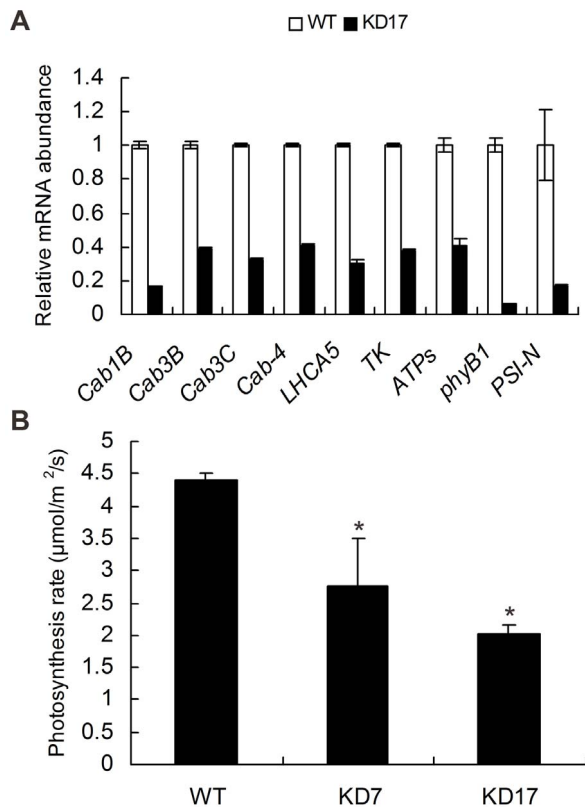


Figure 9. Photosynthetic system was impaired in leaves of *SIGMP2/3*-KD tomato plants. (A) Relative transcript levels of the photosynthesis-related genes in wild-type leaves and KD17 necrotic leaves were assayed via real-time RT-PCR. Data were normalized against *Actin* and shown as a percentage of wild-type plants. (B) Net photosynthesis rates of KD7 and KD17 necrotic leaves and wild-type leaves were measured using a portable photosynthetic system (CIRAS-2, PP System, USA). Data are presented as mean \pm SD (N=4) from triplicate independent measurements. Asterisk indicates significant differences from the control ($P>0.95$). doi:10.1371/journal.pone.0061987.g009

which have been proved to play important roles in ascorbate biosynthesis in *Arabidopsis* and potato, respectively. So we focused our work on *SIGMP3* gene to make clear its function in tomato. In this study, we generated transgenic tomato plants using constitutive over-expression and RNAi strategies for *SIGMP3* gene and studied the plant response to stress regarding the role of GMP in the Smirnoff-Wheeler's pathway and AsA as a major antioxidant of the plant cell.

Over-expression of *SIGMP3* increased the total AsA content and enhanced antioxidant capacity, which was consistent with the results in tobacco expressing *SIGMP3* [25]. Since we constructed RNAi vector using the full length cDNA of *SIGMP3*, *SIGMP2* as well as *SIGMP3* were simultaneously suppressed, leading to significant reduction in AsA contents in leaves and fruits of tomato. This confirmed the importance of the Smirnoff-Wheeler's pathway and the vital role of GMP activity in AsA biosynthesis in tomato plant. Plant AsA efflux is regulated by environmental factors and especially the light environment. Massot et al. [17] found that among the four GMP genes, *SIGMP1* and *SIGMP3* were regulated by light, whereas *SIGMP2* and *SIGMP4* were not in tomato leaves. It implies *SIGMP1* may also play an important role in controlling AsA biosynthesis in tomato. It should be mentioned that *SIGMP2* was severely suppressed in *SIGMP2/3*-KD lines as

SIGMP3, then we don't exclude the possibility that *SIGMP2* participates in controlling ascorbate content and causing phenotypic alteration of KD lines. Further investigation is needed to study on *SIGMP1* and *SIGMP2*.

Significant increase in transcript abundances of *SIGME2* and *SIGGPI* downstream of *SIGMP* was found in the *SIGMP2/3*-KD plants, possibly because of a biological process to maintain a relative, stable ascorbate output. Similarly, up-regulation of *SIGMP* genes and *SIGGPI* were observed in *SIGME* RNAi transgenic tomato plants [21]. The step controlled by GME was considered as an important control point in tomato AsA synthesis because it lies at the intersection of AsA synthesis and cell wall polysaccharides [21]. RNAi silencing of *SIGME1* and *SIGME2* in tomato effectively reduces AsA content, resulting in ROS accumulation, leaf bleaching and developmental defects [21], and over-expression of *SIGME2* could improve AsA accumulation in tomato leaves and red ripe fruits in our group [22]. For *GPP* gene, it encodes an enzyme that catalyzes the first committed step in AsA biosynthesis in plants and has been identified as rate limiting in *Arabidopsis* [37] and tobacco [38] leaves. It has been confirmed that over-expression of *GPP* gene from kiwifruit increased AsA up to six-fold in tomato fruits [39]. Recently, the vital role of *SIGGPI* in light-regulated AsA biosynthesis in tomato has been found [17]. We propose except *SIGME1*, *SIGME2* and *SIGMP3*, *SIGGPI* may be another good candidate for enhancing AsA accumulation in tomato. All these above underscore a complex regulation of the AsA pool size in tomato.

Lesions were formed on leaves throughout the whole life of *SIGMP2/3*-KD tomato plants. Cell death phenotype was previously found in the *Arabidopsis* mutant *vtc1* and GMP antisense transgenic potato plants lacking AsA [11,40]. However, the cell death pattern of *SIGMP2/3*-KD tomato plants is more similar to the GMP antisense potato plants [11] than *vtc1* [40]. Interestingly, the cell death phenotype started earlier in *SIGMP2/3*-KD tomato plants than GMP antisense potato plants. Lesions were formed on tomato leaves even from seedling stage (Fig. 6A), while GMP antisense potato plants started to develop this phenotype 10 weeks after transfer to soil, which was considered that the imbalance between ROS and antioxidants due to the reduction in AsA contents changed the phenotype in the GMP antisense transgenic potato plants [11]. In this study, DAB staining revealed that higher concentration of H_2O_2 was involved in activating cell death program, which is distinct from the *vtc1* mutant, wherein the H_2O_2 content is not changed [41]. This is consistent with the case of lesion formation on tobacco leaves due to reduced levels of the H_2O_2 -detoxifying enzyme CAT [42]. In our study, the increased AsA content improved the tolerance to photo-oxidative stress in *SIGMP3*-OX plants, which further supported the results of gene knocked-down.

CAT is the key enzyme that directly detoxifies H_2O_2 in plant antioxidant system. *CAT* mRNA abundance was significantly up-regulated in the lesioned leaves of *SIGMP2/3*-KD plants. The increased expression of *CAT* appeared to be a compensatory mechanism for the reduced levels of AsA under oxidative stress. However, the capacity of the antioxidant system is not largely changed in the *Arabidopsis* mutant *vtc1-1* [41], which might suggest that the low intracellular level of AsA is sufficient for scavenging ROS in *Arabidopsis*.

Salicylic acid (SA) is known to play an important role in the activation of defence responses in plants against microbial infection. In several identified lesion mimic mutants, the activation of PR genes was correlated with the accumulation of high SA levels [43,44]. *PR1b1* and *PR-P2*, the marker genes in tomato SA-dependent defence pathway were activated in *SIGMP2/3*-KD

plants. In previous study, H₂O₂ is considered to act upstream of SA in the signal transduction pathway [45]. We speculate that the enhanced production of H₂O₂ might induce defence response partly through SA response pathway in *SIGMP2/3*-KD plants. More studies are needed to further confirm this hypothesis.

To determine whether the activated defence response could enhance resistance to pathogens in *SIGMP2/3*-KD plants, five-week-old plantlets were challenged with the virulent bacterium *Pseudomonas syringae* pv. tomato DC3000 (*Pst*) according to the method described by Anderson [46]. However, the resistance of lines KD7 and KD17 to *Pst* DC3000 at 24/20°C day/night temperature (16 h/8 h) and 70% relative humidity was slightly improved but not statistically significant (data not shown). This result may be attributed to the low light intensity of the artificial climate chamber, in which the transgenic plants showed weaker phenotype of spontaneous cell death than that in greenhouse with a high light intensity.

Multiple functions for AsA in photosynthesis have been proposed [5]. AsA-deficient plants show zeaxanthin depletion and an inhibition of photosynthesis when exposed to high light [47]. Rubisco levels are significantly reduced in the *vtc1* mutant [48]. It is suggested that genes encoding the components of the electron transport system could be regulated by AsA [49]. In the chloroplast, AsA-GSH (reduced glutathione) cycle is the key pathway to protect the photosynthetic machinery by removing ROS. In *SIGMP2/3*-KD tomato lines, the expressions of several genes encoding photosynthetic enzymes were very significantly reduced as well as overall photosynthetic activity. This implies that the impairment of photosynthetic system may be due to the lower AsA level.

In conclusion, we have studied one of the AsA biosynthetic pathway steps using over-expression and RNAi strategies for *SIGMP3* gene in tomato plants. In doing so, we found the vital roles of AsA as an antioxidant in leaf senescence and defence response in tomato. The increased H₂O₂ level caused by low AsA levels might be responsible for the induction of the SA signal transduction pathway, which led to necrosis development and defence response activation. In addition to AsA biosynthesis, GMP is also need for synthesis of mannose, fucose and L-galactose

containing polysaccharides in the cell wall. We do not exclude the possibility that cell wall defects participate in causing some of the phenotypes seen in our experiment and this will be investigated in our further study.

Supporting Information

Figure S1 Nucleotide sequence alignment of *SIGMP2* and *SIGMP3* genes.

(TIF)

Table S1 Primers used for *SIGMP3* amplification and identification of the transformants and RT-PCR.

(DOC)

Table S2 Primers used for real-time RT-PCR of the AsA biosynthesis-related genes.

(DOC)

Table S3 Primers used for real-time RT-PCR of the photosynthesis-related genes.

(DOC)

Table S4 Primers used for real-time RT-PCR of the pathogenesis-related genes and antioxidant enzyme genes.

(DOC)

Table S5 The fruit weights and yields of *SIGMP2/3*-KD lines and wild-type plants.

(DOC)

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Author Contributions

Conceived and designed the experiments: CJZ BOY ZBY. Performed the experiments: CJZ CXY HL. Analyzed the data: YYZ HXL. Contributed reagents/materials/analysis tools: JHZ XHZ. Wrote the paper: CJZ.

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