AP2/ERF Transcription Factors Involved in Response to Tomato Yellow Leaf Curly Virus in Tomato

Ying Huang, Bao-Long Zhang, Sheng Sun, Guo-Ming Xing, Feng Wang, Meng-Yao Li, Yong-Sheng Tian, and Ai-Sheng Xiong*

Abstract
Tomato yellow leaf curl virus (TYLCV), transmitted by the whitefly (Bemisia tabaci), causes leaf curling and yellowing, plant dwarfism, and growth inhibition in tomato (Solanum lycopersicum L.). The APETALA2 (AP2) and ethylene response factor (ERF) transcription factor (TF) family, the largest plant-specific TF family, was identified to function in plant development and pathogen defense. Our study aimed to analyze the mechanism underlying the function of S. lycopersicum ERF (SlERF) TFs in response to TYLCV infection and improve useful information to increase the resistance to TYLCV in tomato. A total of 22 tomato AP2/ERF TFs in response to TYLCV were identified according to transcriptome database. Five ERF-B3 TFs were identified in cultivars Hongbei, Zheza-301, Zhefen-702 (both resistant), Jinpeng-1, and Xianke-6 (both susceptible). Interaction network indicated that SlERF TFs could interact with mitogen-activated protein kinase (MAPK). Expression profiles of five ERF-B3 genes (Soly19, Soly36, Soly66, Soly67, and Soly106) were detected by quantitative real-time–polymerase chain reaction (qRT-PCR) after TYLCV infection in five tomato cultivars. Soly106 expression was upregulated in five tomato cultivars. The expressions of three genes (Soly19, Soly67, and Soly36) were upregulated in Zheza-301 and Zhefen-702. Soly66 and Soly36 expressions were downregulated in Hongbei and Xianke-6, respectively. Yeast one-hybrid showed that the GCC-box binding ability of ERF-B3 TFs differed in resistant and susceptible tomato cultivars. Expression profiles were related to the GCC-box binding ability of SlERF TFs in resistant and susceptible tomato cultivars. The defense mechanism underlying the tomato’s response to TYLCV involved a complicated network, which provided important information for us in breeding and genetic analysis.

Core Ideas
• SIAP2/ERF factors respond to the TYLCV infection.
• GCC-box binding ability of SIERF factors was different in tomato cultivars.
• SlERFs interact with other proteins in tomato.
• The defense mechanism to TYLCV is a complicated network.

TOMATO YELLOW leaf curl virus, which belongs to the genus Begomovirus of the family Geminiviridae, is a monopartite virus containing one single-stranded DNA molecule of 2.7 to 2.8 kDa (Ge et al., 2007). Originating from the Middle East, TYLCV has been found in multiple locations worldwide such as the Mediterranean, Japan, China, and many other countries in recent years (Akad et al., 2007; Lefeuvre et al., 2010). Transmitted by the whitefly Bemisia tabaci Gennadius (Hemiptera: Aleyrodidae), TYLCV can cause serious harm to tobacco (Nicotiana tabacum L.), tomato, pumpkin (Cucurbita spp.), cassava (Manihot esculenta Crantz), cotton (Gossypium hirsutum l.).

Y. Huang, F. Wang, M.Y. Li, Y.S. Tian, and A.S. Xiong, State Key Laboratory of Crop Genetics and Germplasm Enhancement, College of Horticulture, Nanjing Agricultural University, Nanjing, China; B.L. Zhang, Provincial Key Laboratory of Agrobiology, Jiangsu Academy of Agricultural Sciences, Nanjing, China; S. Sun and G.M. Xing, College of Horticulture, Shanxi Agricultural University, Taiyuan, China. Received 8 Sept. 2015. Accepted 17 Dec. 2015. *Corresponding author (xiongaisheng@njau.edu.cn).

Abbreviations: AP2, APETALA2; AP2/ERF, APETALA2/ethylene response factor; DREB, dehydration-responsive element binding; JA, jasmonic acid; MAPK, mitogen-activated protein kinase; PCC, Pearson correlation coefficient; qRT-PCR, quantitative real-time–polymerase chain reaction; RAV, related to ABI3/VP; SA, salicylic acid; SD, synthetic dropout; SIERF, S. lycopersicum ethylene response factor; TF, transcription factor; Trp, tryptophan; TYLCV, tomato yellow leaf curl virus; Ura, uracil.
L.), and other economically important crops (Jones et al., 2008). In recent years, TYLCV has induced devastating damages in tomato production and quality because of the outbreak of its vector whitefly B. tabaci. Typical symptoms in the early onset of TYLCV include leaf yellowing and curling as well as smaller and shrunk new leaves. Infected whole plants even stop growing completely. Flowers and fruits are absised after exposure to viruliferous whiteflies for a month, leading to serious economic loss (Lapidot, 2007). Tomato breeding for resistance to TYLCV has focused on the introgression of resistance genes. At present, five important resistance genes have been identified: ty-1 (Zamir et al., 1994), ty-2 (Hanson et al., 2006), ty-3 (Ji et al., 2007), ty-4 (Ji et al., 2009), and ty-5 (Anbinder et al., 2009). Besides these five genes, numerous other genes are involved in TYLCV infection thereby forming a complex gene network.

Tomato, one of the most important vegetables worldwide, originated in western South America (Kimura and Sinha, 2008). The species belongs to the nightshade family, Solanaceae, which contains other well-known species such as pepper (Capsicum annuum L.), eggplant (Solanum melongena L.), and potato (Solanum tuberosum L.) (Mueller et al., 2005). Worldwide tomato production has continued to increase from 110 Tg in 2000 to 161 Tg in 2012 (http://faostat.fao.org/). As an economically important vegetable, tomato plays an important role in balancing people’s diets. Tomato fruits are rich in sugars, dietary fiber, minerals, essential amino acids, and vitamins (Olaiya, 2011). Tomato also contributes to the experimental sciences such as in studies on defense regulation against stresses, breeding, fruit development, and ripening (Klee and Giovannoni, 2011; Schauer et al., 2008). The number of tomato cultivation areas has been increasing on a daily basis. However, tomatoes are highly susceptible to TYLCV damage. In 2009 to 2010, a TYLCV outbreak happened in Jiangsu, Shandong, Henan, and other provinces of China, which considerably reduced tomato production and quality.

Numerous abiotic and biotic stresses influence plant growth, development, and yield (Huang et al., 2015). Numerous genes are induced to defend against stress via two modes: one mode involves engendering related functional proteins, and the other mode involves functioning as signal transduction factors in stress responses (Cao et al., 2008; Yamaguchi-Shinozaki and Shinozaki, 2006). The APETAL2/ethylene response factor (AP2/ERF) TFs family is one of the largest plant-specific TFs with a minimum of one APETAL2 (AP2) domain (Song et al., 2014). According to the number and sequence similarities of the AP2 domain, AP2/ERF TFs are divided into five subfamilies: AP2, ethylene responsive element binding factors (ERF), RAV (related to ABI3/VP), dehydration-responsive element binding (DREB), and Soloist (Licauisi et al., 2013). The AP2/ERF TFs family plays an important role in plant development processes such as flower and seed development (Tsafarissi et al., 2012; Maes et al., 2001), meristem capacity (Asahina et al., 2011), stress responses (Chen et al., 2012), and primary and secondary metabolism (Shi et al., 2011; Zhang et al., 2005).

Some research also indicated that AP2/ERF family TFs contributed to pathogen defense. TaPIEP1, a novel pathogen-induced ERF gene of wheat, enhanced resistance to fungal pathogen, Bipolaris sorokiniana (Dong et al., 2010). HvRAF, which was identified from barley (Hordeum vulgare L.), enhanced pathogen resistance and salt tolerance in Arabidopsis thaliana (L.) Heynh. (Jung et al., 2007). The ectopic expression of a tobacco stress-induced gene, Tsi1, could enhance resistance to viral, bacterial, and oomycete pathogens (Shin et al., 2012). A comparative transcriptome profiling in response to TYLCV infection between resistant tomato breeding line CLN2777A and susceptible tomato breeding line TMXA48-4-0 was conducted and found that numerous genes were induced, including several transcription factors, such as AP2/ERF, WRKY, and NAC (Chen et al., 2013). All these research works demonstrated the important link between AP2/ERF TFs and TYLCV infection, but information on the mechanisms underlying this link is lacking. In this work, we conducted in-depth research about the link between AP2/ERF TFs family and TYLCV infection in tomato.

According to a previous transcriptome database, 22 TFs from SIAP2/ERF family that were induced in response to TYLCV infection were identified in resistant and susceptible tomato cultivars. In the present work, five genes (Soly19, Soly36, Soly66, Soly67, and Soly106) belonging to ERF-B3 subfamily were selected to further analyze the functional mechanism of SIAP2/ERF TFs and TYLCV infection in five different tomato cultivars. An interaction network was constructed to analyze the interaction between SIERF TFs in response to TYLCV infection and other genes in the tomato genome. The binding ability to GCC-box of SIERF factors in response to TYLCV infection was identified by yeast one-hybrid. Quantitative real-time PCR was used to analyze the expression profiles of the five SIERF genes at 2, 4, 6, 8, 10, and 15 d after TYLCV inoculation. This work aimed to determine the mechanism underlying the function of SIERF factors in response to TYLCV infection and the improvement of TYLCV resistance in tomato.

Materials and Methods

Plant Material and Tomato Yellow Leaf Curly Virus Inoculation

To determine whether phenotypes after TYLCV infection differ between resistant and susceptible tomato cultivars, we selected five different tomato cultivars: Hongbeibei, Zhefen-702, Zheza-301, Xianke-6, and Jinpeng-1. Hongbeibei has high TYLCV resistance, Zhefen-702 and Zheza-301 have middle-level resistance, and Xianke-6 and Jinpeng-1 are susceptible to TYLCV. The five tomato cultivars were grown in a chamber programmed for 12 h photoperiod at 25 and 18°C (day vs. night) and a relative humidity of 60 to 70% with 320 μmol m⁻² s⁻¹ light intensity. Viruliferous whiteflies were fed on tomato plants in...
an insect-proof greenhouse. Two-leaf-stage tomatoes were transferred into the insect-proof greenhouse for exposure to whiteflies carrying TYLCV for 0, 2, 4, 6, 8, 10, 15, and 40 d. Leaves of five tomato cultivars were collected and frozen in liquid N\textsubscript{2} immediately after exposure.

**Sequence Database Searches**

The ERF TFs family sequences of tomato related to TYLCV were downloaded from the tomato genome view according to comparative transcriptome profiling in response to TYLCV between resistant and susceptible tomato cultivars (The Tomato Genome Consortium, 2015; Chen et al., 2013). The AP2 domain of each AP2/ERF factor was confirmed using BLASTp. The AP2/ERF TFs family was retrieved from Sol Genomics Network (ITAG2.4) (Fernandez-Pozo et al., 2015).

**Phylogenetic Analysis and Motif Recognition**

The amino acid sequences of SIAP2/ERF TFs were used to conduct phylogenetic analysis. Several AP2/ERF TFs that responded to the pathogen were identified such as PpERF (PpERF1a and PpERF1b) (Sherif et al., 2012), TaPIEP1 (Dong et al., 2010), OsERF922 (Liu et al., 2012), and Opbp1 (Guo et al., 2004). Clustal X was used to align the sequence by neighbor-joining method (Chenna et al., 2003), and MEGA5.0 was used to construct the phylogenetic tree (Tamura et al., 2011). The chemical and physical characteristics of the 22 SIAP2/ERF factors in response to TYLCV infection were determined by Expert Protein Analysis System (ExPASy) program (http://web.expasy.org/protparam/) (Gasteiger et al., 2003). Conserved motifs were detected using MEME (Version 4.9.1) (Bailey et al., 2009).

**Identification of Orthologous and Paralogous Genes**

Orthologous and paralogous genes of AP2/ERF TFs family in tomato, rice (Oryza sativa L.), and Arabidopsis were identified by OrthoMCL (Li et al., 2003). Circos software was used to display the relationship between orthologous and paralogous genes (Krzywinski et al., 2009). The databases of Arabidopsis and rice AP2/ERF TFs family were downloaded from TAIR10 and DRTF, respectively. Arabidopsis Interactions Viewer was used to construct the interaction network of Arabidopsis AP2/ERF proteins (http://bar.utoronto.ca/interactions/cgi-bin/Arabidopsis_interactions_viewer.cgi).

**RNA Extraction and Quantitative Real-Time–Polymerase Chain Reaction Analysis**

An RNA kit (RNA simple total RNA kit, Tiangen) was used to extract the total RNA of tomato. A Prime Script RT reagent kit (TaKaRa) reverse transcribed the RNA into complementary DNA. ABI7500 (Applied Biosystems7500) was used to carry out qRT-PCR with SYBR Premix Ex-Taq (TaKaRa). The PCR procedure was conducted according to the following operating instructions: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s, and melting curve analysis (61 cycles) at 65°C for 10 s.

**Table 1. Quantitative real-time–polymerase chain reaction primers of five SIERF-B3 genes and Tubulin.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’-3’</th>
<th>Reverse primer 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soly19</td>
<td>TCCATCGAAAATGAGGAGGCTCCT</td>
<td>GATCATAACATCATTGTTCAAC</td>
</tr>
<tr>
<td>Soly36</td>
<td>AGCAACAAATGAGATGAAAAC</td>
<td>GAGATAATGCTGAGAATGGTCCG</td>
</tr>
<tr>
<td>Soly106</td>
<td>TAGACATGATTGTCCTCCCTGTA</td>
<td>ATATCAGACCAAAAATCTCTCAAC</td>
</tr>
<tr>
<td>Soly67</td>
<td>TTCATCAATACAATCCTAAATTCA</td>
<td>CGAATTTCATCACTTCTTCTTTA</td>
</tr>
<tr>
<td>Soly66</td>
<td>TTCACTCAGTTAAAAGCAGGGCAT</td>
<td>GAACACTAAGCTAGG ATAATAT</td>
</tr>
<tr>
<td>Tubulin</td>
<td>TGAAGAAGT6GACAGAGGAAA</td>
<td>CTGACATTCTTTGCGAACTG</td>
</tr>
</tbody>
</table>

Three technical repeats were performed with each RNA sample of five tomato cultivars. The comparative CT method (2−ΔΔCT) method was used to measure the RNA level, which was expressed relative to the Tub gene (ΔCT = CT\textsubscript{target sample} − CT\textsubscript{Tub}) (Pfaffl, 2001). The primer sequences of each selected gene were designed using Primer Premier 5.0 software (PREMIER Biosoft, 2012). A-Tubulin (Solyco4 g077020.2) was used to regulate the expression level under the control of each primer (Chen et al., 2013). Primers used in this study are listed in Table 1.

**Gene Cloning and Yeast One-Hybrid Assay of SIERF Transcription Factors**

According to the phylogenetic tree, most of SIAP2/ERF factors in response to TYLCV were identified in the ERF group, especially in the ERF-B3 subfamily (Supplemental Table S2). According to previous studies, SIERF TFs in other plants that responded to pathogen were focused on the ERF-B3 subfamily. Five genes encoding ERF-B3 TFs (Soly19, Soly36, Soly66, Soly67, and Soly106) were selected to analyze the plant’s response to TYLCV infection. Special primers of the five genes were designed with Bam HI and Sac I enzymes at the end of each primer (Table 2). Polymerase chain reaction amplification was performed under the following conditions: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 60 s, and then a final 10-min extension at 72°C. pMD-19 simple-T vector (TaKaRa) was used to ligate the PCR product after sequencing and digestion.

The pPC87 vector, a reconstruction of pPC86 vector, which contained tryptophan (Trp) synthesis gene and a galactose-inducible protein (GAL4) activating domain, was used to ligate the coding region of each ERF-B3 gene. Recombinant plasmids were imported into the yeast EGY48, which contained the vector that could generate uracil (Ura) and carried the reporter gene LacZ generate uracil (Ura) and carried the reporter gene LacZ for the LacZ under the control of a 75-bp fragment containing a GCC target sample − CT\textsubscript{Tub} (Pfaffl, 2001). The primer sequences of each selected gene were designed using Primer Premier 5.0 software (PREMIER Biosoft, 2012). A-Tubulin (Solyco4 g077020.2) was used to regulate the expression level under the control of each primer (Chen et al., 2013). Primers used in this study are listed in Table 1.

**Gene Cloning and Yeast One-Hybrid Assay of SIERF Transcription Factors**

According to the phylogenetic tree, most of SIAP2/ERF factors in response to TYLCV were identified in the ERF group, especially in the ERF-B3 subfamily (Supplemental Table S2). According to previous studies, SIERF TFs in other plants that responded to pathogen were focused on the ERF-B3 subfamily. Five genes encoding ERF-B3 TFs (Soly19, Soly36, Soly66, Soly67, and Soly106) were selected to analyze the plant’s response to TYLCV infection. Special primers of the five genes were designed with Bam HI and Sac I enzymes at the end of each primer (Table 2). Polymerase chain reaction amplification was performed under the following conditions: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 60 s, and then a final 10-min extension at 72°C. pMD-19 simple-T vector (TaKaRa) was used to ligate the PCR product after sequencing and digestion.

The pPC87 vector, a reconstruction of pPC86 vector, which contained tryptophan (Trp) synthesis gene and a galactose-inducible protein (GAL4) activating domain, was used to ligate the coding region of each ERF-B3 gene. Recombinant plasmids were imported into the yeast EGY48, which contained the vector that could generate uracil (Ura) and carried the reporter gene LacZ under the control of a 75-bp fragment containing a GCC target sample − CT\textsubscript{Tub} (Pfaffl, 2001). The primer sequences of each selected gene were designed using Primer Premier 5.0 software (PREMIER Biosoft, 2012). A-Tubulin (Solyco4 g077020.2) was used to regulate the expression level under the control of each primer (Chen et al., 2013). Primers used in this study are listed in Table 1.
Results

Symptoms of Five Different Tomato Cultivars In Response to Tomato Yellow Leaf Curly Virus Infection

Two-leaf-stage tomato cultivars (highly tolerant cultivar, Hongbeibei; tolerant cultivars, Zheza-301 and Zhefen-702; and susceptible cultivars, Jinpeng-1 and Xianke-6) were infected with TYLCV for 0, 2, 4, 6, 8, 10, 15, and 40 d by exposure to viruliferous whiteflies. After 10 d, the symptoms of five tomato cultivars were normal. The leaves of Jinpeng-1 and Xianke-6 became curly, and those of the resistant cultivars were normal after 2 wk (Fig. 1). The symptoms of TYLCV infection in tomato cultivars became typical with increasing time of exposure, except in Hongbeibei. After 40 d, the leaf of Hongbeibei became slightly curly. Zheza-301 and Zhefen-702 became curly and slightly yellow colored. Two susceptible cultivars (Jinpeng-1 and Xianke-6) displayed typical symptoms. The cultivars showed symptoms of dwarfing with yellow and curly leaves and new leaves became smaller and shrunken (Fig. 2; Supplemental Fig. S6).

SIAP2/ERF Transcription Factors Involved in Response to Tomato Yellow Leaf Curly Virus Infection

To determine the SIAP2/ERF TFs involved in TYLCV resistance, SIAP2/ERF TFs that exhibited up- or down-regulation in tomato after TYLCV infection were identified based on transcriptome data (Chen et al., 2013). A total of 22 SIAP2/ERF TFs that responded to TYLCV infection were determined. Two genes encoding SIERF factors (Soly66 and Soly67) and one gene encoding SIAP2 (Soly165) was upregulated by four to six times in the resistant cultivar. A total of 18 downregulated SIAP2/ERF TFs and one upregulated TF (Soly147) were associated with plant defense response at different levels in the susceptible cultivar (Supplemental Fig. S7). The responses of 22 SIAP2/ERF TFs to TYLCV infection were examined by sequence alignment. These 22 TFs were conserved in an AP2 domain comprising 58 to 59 amino acids. Seven amino acid residues (Arg29, Arg31, Trp33, Glu39, Arg41, Arg49, and Trp51) that could bind the GCC-box were conserved (Supplemental Fig. S8B). Soly147, which was identified to belong to the AP2 subfamily, possessed two AP2 domains. The DREB and ERF subfamilies (Soly32 and Soly19) contained only one AP2 domain. AP2 and B3 domains were found in the transcription factor Soly165, which belonged to RAV subfamily (Supplemental Fig. S8A).

The bioinformatics resource portal ExPASy (http://web.expasy.org/translate/) was used to analyze the chemical and physical characteristics of 22 SIAP2/ERF TFs (Supplemental Table S2). The theoretical pI values

Table 2. Primers of five SIERF-B3 genes used for vector construction. Bold primer segments represent the Bam HI (GGATCC) and Sac I (GAGCTC) restriction enzyme sites.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5'-3'</th>
<th>Reverse primer 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soly19</td>
<td>CGGGATCCATGGTCCAATCTTCCAAAGTGTAT</td>
<td>GGAGCTCTTAAACATTGTTCAATAATCATCA</td>
</tr>
<tr>
<td>Soly36</td>
<td>CGGGATCCATGGTCCAATCTTCCAAAGTGTAT</td>
<td>GGAGCTCTTAAACATTGTTCAATAATCATCA</td>
</tr>
<tr>
<td>Soly106</td>
<td>CGGGATCCATGGTCCAATCTTCCAAAGTGTAT</td>
<td>GGAGCTCTTAAACATTGTTCAATAATCATCA</td>
</tr>
<tr>
<td>Soly67</td>
<td>CGGGATCCATGGTCCAATCTTCCAAAGTGTAT</td>
<td>GGAGCTCTTAAACATTGTTCAATAATCATCA</td>
</tr>
<tr>
<td>Soly66</td>
<td>CGGGATCCATGGTCCAATCTTCCAAAGTGTAT</td>
<td>GGAGCTCTTAAACATTGTTCAATAATCATCA</td>
</tr>
</tbody>
</table>
of factors (Soly83, Soly108, and Soly109) belonging to ERF-B1 were >8.0, whereas the pl values of the DREB-A1 subfamily (Soly32 and Soly87) were <6.0. The pl of ERF-B3 factors varied widely from 4.98 to 9.07. The motifs of SIAP2/ERF TFs were detected by MEME (Supplemental Fig. S9). The LOGOs of proteins are shown in Supplemental Fig. S10. The WLG element (in motif1) was conserved in all SlAP2/ERF TFs. Nearly all AP2/ERF TFs, except Soly95 and Soly165, possessed motif 2, which contained YRG amino acids. Different TFs presented different motifs that maybe related to the differences in proteins or function in response to TYLCV.

To analyze the mechanism between ERF-B3 and TYLCV infection, full lengths of five SlERF-B3 genes (Soly19, Soly36, Soly66, Soly67, and Soly106) from Zheza-301 and Xianke-6 were cloned. As shown in Supplemental Fig. S1 to S5, the genes encoded proteins with calculated molecular masses of 161, 244, 201, 365, and 240 amino acids, and one to three different amino acids were found between Zheza-301 and Xianke-6 in each SIERF factor. Sequence analysis showed that all five SIERFs possessed the AP2 domain, which consisted of a three-stranded, antiparallel β-sheet and α-helix packed approximately parallel. There were different amino acids residues among five ERF TFs though the AP2 domain was conserved (Fig. 3).

**Phylogenetic analysis of SIAP2/ERF Transcription Factors Involved in Response to Tomato Yellow Leaf Curly Virus Infection**

A phylogenetic tree was constructed to analyze phylogenetic development. A total of 172 AP2/ERFs (167 tomato SIAP2/ERFs and five ERF factors responding to the pathogen) were used. Among the 22 SIAP2/ERFs involved in the plant’s response to TYLCV infection, four factors (Soly87, Soly95, Soly97, and Soly32) were classified as DREB TFs, whereas 16 factors belonged to the ERF subfamily. Only Soly165 and Soly147 TFs were classified into the RAV and AP2 subfamilies, respectively (Fig. 4A). A total of 16 factors (72.73%) were identified in the ERF group. Eight factors were categorized into the ERF-B3 group, whereas three factors belonged to the ERF-B1 group. The ERF-B5 subfamily contained only one factor (Supplemental Table S2). The different proportions of the 22 SIAP2/ERF TFs indicated that the ERF group may play a key role in the response to TYLCV.

Eight TFs of 22 AP2/ERFs belonged to ERF-B3 subfamily (Fig. 4A; Supplemental Table S2), which showed there was close relationship between ERF-B3 and TYLCV infection. By protein alignment, five SIERF factors (Soly19, Soly36, Soly66, Soly67, and Soly106) belonging to ERF-B3 showed different degrees of similarity to other plant ERF proteins that were identified to be involved in defense responses such as PpERFs, OPBP1, Pti4, and AtERF2 (Fig. 4B).

**Evolution of AP2/ERF Family Transcription Factor in Plant Species**

To analyze the evolution of AP2/ERF family TFs, comparisons were constructed among different plant species.
Fig. 4. Phylogenetic tree of APETALA2 (AP2)/ethylene responsive element binding factors (ERF) transcription factors in tomato. (A) Phylogenetic tree of 167 SlAP2/ERF factors and other ERF factors; (B) Phylogenetic tree of ERF transcription factors from different plant species. GenBank accession numbers of ERF proteins are listed as follows: tobacco, Opbp1 (U81157); peach, PpERF-1.a (HQ825094), PpERF-1.b (HQ825095), PpERF-2.a (HQ825096), PpERF-2.b (HQ825097) and PpERF-2.c (HQ825098); rice, OsERF922 (BAD87106) and OsBiERF3 (AAV98702); wheat, TaPIEP (ABU62817.1) and TaERF2 (AAP32468); tomato, Pti4 (AAC50047) and JERF3 (AAQ91334); pepper, CaPF1 (AAP72289); Arabidopsis, AtERF2 (AAM64544).
A total of 3332 AP2/ERF factors were identified (Fig. 5). All five subfamilies, namely DREB, ERF, AP2, RAV, and Soloist, were identified in land plants except in rice and Arabidopsis lyrata (L.) O’Kane & Al-Shehbaz, which did not contain a factor from the Soloist subfamily. Among 20 plant species, Chinese cabbage [Brassica rapa L. subsp. chinensis (L.) Hanelt] contained the most number of AP2/ERF family TFs (289 AP2/ERF factors). In dicots, the number of AP2/ERF factors in tomato (167) exceeded those of A. thaliana (147), watermelon [Citrullus lanatus (Thunb.) Matsum. & Nakai] (145), and grapevine (Vitis vinifera L.) (132). However, more AP2/ERF TFs were found in turnip (B. rapa L.) (289), apple (Malus domestica Borkh.) (259), and potato (231). The AP2/ERF protein density in tomato (0.186) was the lowest among dicots. The number of AP2/ERF TFs in land plants was larger than in algae. Only 24 and nine factors were found in Chlamydomonas reinhardtii and Ostreococcus lucimarinus, respectively.

As shown in Fig. 5, the TF numbers in the ERF subfamily were the largest among the five subfamilies except in A. lyrata, watermelon, and Brachypodium distachyon (L.) Beauv. Numbers in the ERF subfamily were twice that in the DREB subfamily in certain species, such as potato, maize (Zea mays L.), and barley. In tomato, the number of ERF factors (88) was considerably larger than those from other subfamilies (DERB, 49; AP2, 26; RAV, 3; and Soloist, 1). At the same time, 16 factors that were induced in response to TYLCV infection were classified into the ERF subfamily, and only four, one, and one factor belonged to DERB, AP2, and RAV subfamilies, respectively.

As model plants, Arabidopsis and rice were selected to construct a comparative analysis with tomato to analyze the paralogs and orthologs (Fig. 6). Fifty-seven and 32 pairs of orthologous AP2/ERF factors were identified between tomato and Arabidopsis and between tomato and rice, respectively. Moreover, 83, 43, and 76 paralogs were located in Arabidopsis, rice, and tomato, respectively. Among 22 SIAP2/ERF TFs that were induced in response to TYLCV infection, seven and one orthologs were found between tomato and Arabidopsis and between tomato and rice, respectively (Supplemental Table S3). Twelve paralogs were identified in tomato (Supplemental Table S4).

**Distribution of SIAP2/ERF Transcription Factors Involved in Response to Tomato Yellow Leaf Curly Virus in Tomato Chromosome**

A total of 167 AP2/ERF TFs were distributed on 12 tomato chromosomes (Fig. 7). The AP2/ERF TFs of tomato were mainly distributed on chromosome 12 (147), watermelon [Citrullus lanatus (Thunb.) Matsum. & Nakai] (145), and grapevine (Vitis vinifera L.) (132). However, more AP2/ERF TFs were found in turnip (B. rapa L.) (289), apple (Malus domestica Borkh.) (259), and potato (231). The AP2/ERF protein density in tomato (0.186) was the lowest among dicots. The number of AP2/ERF TFs in land plants was larger than in algae. Only 24 and nine factors were found in Chlamydomonas reinhardtii and Ostreococcus lucimarinus, respectively.

As shown in Fig. 5, the TF numbers in the ERF subfamily were the largest among the five subfamilies except in A. lyrata, watermelon, and Brachypodium distachyon (L.) Beauv. Numbers in the ERF subfamily were twice that in the DREB subfamily in certain species, such as potato, maize (Zea mays L.), and barley. In tomato, the number of ERF factors (88) was considerably larger than those from other subfamilies (DERB, 49; AP2, 26; RAV, 3; and Soloist, 1). At the same time, 16 factors that were induced in response to TYLCV infection were classified into the ERF subfamily, and only four, one, and one factor belonged to DERB, AP2, and RAV subfamilies, respectively.

As model plants, Arabidopsis and rice were selected to construct a comparative analysis with tomato to analyze the paralogs and orthologs (Fig. 6). Fifty-seven and 32 pairs of orthologous AP2/ERF factors were identified between tomato and Arabidopsis and between tomato and rice, respectively. Moreover, 83, 43, and 76 paralogs were located in Arabidopsis, rice, and tomato, respectively. Among 22 SIAP2/ERF TFs that were induced in response to TYLCV infection, seven and one orthologs were found between tomato and Arabidopsis and between tomato and rice, respectively (Supplemental Table S3). Twelve paralogs were identified in tomato (Supplemental Table S4).

**Interaction Network of AP2/ERF Factors Involved in Tomato Yellow Leaf Curly Virus Infection**

An interaction network of AP2/ERF factors in tomato was developed using the orthologous TFs in Arabidopsis to further analyze the correlations among tomato AP2/ERF factors (Fig. 8). Red, blue, and green lines represented the correlation of AP2/ERF factors under different values of Pearson correlation coefficient (PCC). A total of 115 pairs (PCC > 0) were
identified, and 43 pairs showed negative correlation (PCC < 0). A total of 21 pairs could not be calculated.

As shown in Fig. 8, numerous AP2/ERF proteins were identified to interact with other proteins in the tomato genome. Three AP2/ERF proteins (Solyc01 g108240.2.1, Solyc08 g078190.1.1, and Solyc09 g089930.1.1) that responded to TYLCV interacted with MAPK 2 (Solyc08 g014420.2.1). Solyc01 g108240.2.1 and Solyc08 g078190.1.1 showed positive correlation with MAPK, whereas Solyc09 g089930.1.1 was potentially negatively correlated with MAPK. As universal signal transduction pathways, MAPK cascades could activate plant defense against fungal and viral attacks (Desikan et al., 2001; Nuhse et al., 2000). Previous study had shown that after inoculation with TYLCV, the abundance of MAPK showed a less pronounced decline (Gorovits et al., 2007).

Transcription factor proteins Solyc11 g072600.1.1, Solyc06 g075510.2.1, and Solyc02 g077840.1.1 showed potential contact with numerous proteins, such as TCP.
and thioredoxin, which indicated that ERF TFs played important roles in transcriptional level regulation (Fig. 8). The interaction network of SIAP2/ERF factors in tomato showed that the response mechanisms to TYLCV in tomato were complicated by interacting with other proteins in tomato genome.

**SIERF Proteins Interact Specifically with GCC-Box**

The AP2/ERF TFs could bind to the GCC-box AGCC-GCC (Gutterson and Reuber, 2004). To analyze whether the tomato SIERF TFs involved in TYLCV infection possessed the ability to bind to the GCC-box element, yeast one-hybrid method was used; this method was identified as effective for this purpose (Ou et al., 2011; Ye et al., 2004). The GCC-box binding activities of five selected genes belonging to ERF-B3 subfamily were identified in two tomato cultivars, namely Zheza-301 (resistant cultivar) and Xianke-6 (susceptible cultivar) (Fig. 9). Beta-galactosidase activity indicated that the SIERF factors could bind to GCC-box (Fig. 9B). Soly106 showed high binding ability in both Zheza-301 and Xianke-6. The binding ability of Soly19 to the GCC element in Zheza-301 was higher than that in Xianke-6. The binding ability of Soly36 and Soly66 in Zheza-301 was weaker than in Xianke-6. However, Soly67 did not bind or presented weak binding ability to GCC-box in Xianke-6.

**Expression Profiles of Selected SIERF Genes in Response to Tomato Yellow Leaf Curly Virus Infection in Tomato**

The expression profiles of five genes (Soly19, Soly36, Soly106, Soly67, and Soly66) were analyzed to confirm whether SIERFs were involved in the defense responses of tomato to TYLCV. The five SIERF genes, classified into ERF-B3 subfamily, had close relationship with genes PpERFs, OsERF922, TaPIEP1, and Opbp1. As shown in Fig. 10, the expression levels varied not only among genes but also among tomato cultivars. In the overall trend, the expression levels of five genes generally increased after inoculation with TYLCV, except for Soly36 and Soly66, which were downregulated in Xianke-6 and Hongbeibeici, respectively. In the highly resistant cultivar Hongbeibeici,
Discussion

As a major plant crop and a model system for scientific research, tomato production worldwide was threatened by the whitefly-transmitted geminiviruses, especially TYLCV (Jones et al., 2008; Liu et al., 2012). The resistant ability to TYLCV infection varies following the different tomato cultivars. After TYLCV infection for 2 mo, Hongbeibei has high TYLCV resistance with only 15% disease incidence, Zhefen-702 and Zheza-301 have middle level resistance with 50 and 45% disease incidence, respectively, the expression patterns of Soly19 and Soly106 increased by 4.5 (after 15 d) and 7.93 times (after 8 d), respectively. Four genes (Soly19, Soly36, Soly67, and Soly106) were upregulated in resistant cultivars Zheza-301 and Zhefen-702. In susceptible cultivars, the expression levels of Soly36 were insensitive in Jinpeng-1 and downregulated in Xianke-6. Soly106 showed upregulated expression patterns in the five tomato cultivars.

Fig. 8. The interaction network of AP2/ERF factors in tomato according to orthologs in Arabidopsis.

Fig. 9. Yeast one-hybrid and β-galactosidase activity assays. (A) GCC-box-binding of five ERF-B3 transcription factors. The pictures were captured after 12 h at 30°C with 20 μg mL−1 X-gal incubation on SD/-Ura/-Trp medium. (B) Relative β-galactosidase activity of ERFs that bind to the GCC-box element. The data was calculated with independent clones for three replicates.
Genes in Response to Tomato Yellow Leaf Curly Virus Infection

When faced with pathogen infection, a defense system that combines physical and chemical barriers to protect the plant from damage is induced (Sade et al., 2015). Plant diseases can cause considerable losses to agriculture worldwide (Anderson et al., 2004). As the model plant of the Solanaceae family, tomato production decreases because of damage from TYLCV, which is transmitted by the whitefly *B. tabaci*. At present, the genetic structure and population variability of TYLCV is extensively studied (Ge et al., 2007). Identifying five important resistance genes, namely Ty-1, Ty-2, Ty-3, Ty-4, and Ty-5, was crucial. Aside from the five major genes, numerous other genes play important roles in resistance against TYLCV such as *GroEL* (Morin et al., 1999) and *CLNLR* (Li et al., 2014). The TYLCV defense mechanism in tomato involves a complex signal transduction
AP2/ERF Factors Involved in Response to Tomato Yellow Leaf Curly Virus Infection in Higher Plants

As a large transcription factor family, AP2/ERF factors play important roles not only in plant development but also in pathogen defense. Numerous research works demonstrated that overexpression of the ERF factor could increase the resistance to fungal, bacterial, and viral pathogens. In our study, 167 SlAP2/ERF TFs were identified in tomato, and according to the transcriptome data, 22 AP2/ERF TFs were identified at different expression levels in response to TYLCV infection in resistant vs. susceptible tomato cultivars (Chen et al., 2013). Sequence alignment showed that the AP2 domain consisting of ~58 to 59 amino acids was identified in all 22 SlAP2/ERF factors. Seven conserved amino acids existed in three-stranded, antiparallel β sheets of the AP2 domain. Among the 22 SlAP2/ERF factors, a total of 16 factors belonged to the ERF subfamily, whereas only four, one, and one factors were identified in the DERB, AP2, and RAV subfamilies, respectively.

The GCC-box Binding Ability of SlERF Factors in Response to Tomato Yellow Leaf Curly Virus Infection in Different Tomato Cultivars

Ethylene response factors can bind a short cis-acting element GCC-box (AGCCGCC) by seven amino acid residues, namely arginine29 (Arg29), arginine31 (Arg31), tryptophan33 (Trp33), glutamic acid39 (Glu39), arginine41 (Arg41), arginine49 (Arg49), and tryptophan51 (Trp5) (Allen et al., 1998; Ohme-Takagi and Shinshi, 1995). Consisting of seven amino acids residues, the β sheet in the AP2 domain may play an important role in the formation of the domain GCC-box complex (Allen et al., 1998). Yeast one-hybrid was constructed to analyze the GCC-box binding ability of five factors in Zheza-301 (resistant cultivar) and Xianke-6 (susceptible cultivar) (Fig. 9). According to β-galactosidase activity, each factor could bind the GCC-box except for Soly67, which exhibited weak or no binding ability in Xianke-6. Soly19 factor showed the strongest binding ability with a 19-fold increase. As shown in Fig. 3, the AP2 domain was identified in five ERF factors with seven conserved residues and other nonconserved residues. The binding specificity of the seven amino acid residues to the GCC-box varied (Wang et al., 2009). Arg29, Glu39, and Arg41 showed the primary DNA binding capability of AtERFs, whereas Arg31, Arg49, and Trp51 exhibited different binding preferences. Differences may also exist among five SlERF factors, thereby resulting in different binding abilities to the GCC-box. Previous studies showed that the change of Glu156 to Arg and of phenylalanine62 (Phe62) to serine (Ser) increased the GCC-box binding ability of BnaERF-B3-hy15 (Jin et al., 2010, Xiong et al., 2013). The alanine14 (Ala14) and aspartic acid19 (Asp19) played important roles in recognizing the DNA-binding sequence (Liu et al., 2006). The diverse binding ability among five SlERF factors may be related to the binding specificity of the seven conserved amino acids. Other key residues that may affect the binding ability also need to be analyzed. Results indicated that the binding ability between ERF factors and GCC-box was necessary but was complicated.

Interaction network indicated AP2/ERF TFs could interact with other proteins such as MAPK, which could regulate the expression of MAPKs. As we know, MAPK cascades took part in regulation of response to hormones, biological and environment stresses, and showed decreased expression levels after TYLCV infection (Gorovits et al., 2007). Previous study demonstrated that OsERF3 positively regulated transcriptional of at least two MAPKs (Lu et al., 2011). As upstream regulators, MAPKs also mediated some WRKY TFs, which could regulated the biosynthesis of jasmonic acid (JA), salicylic acid (SA), ethylene, and H2O2 (Pandey and Somssich, 2009). OsERBF1 could be phosphorylated by BWMW1 in vitro, a rice MAPK protein, and enhanced the ability of OsERFPF1 to bind the GCC-box DNA motif, which was suggested to be an advantageous function in multiple stresses in plants (Cheong et al., 2003). Those studies suggest that when faced with TYLCV infection, ERF TFs in tomato may regulate the signal pathway of JA, SA, ethylene, and H2O2 by mediating MAPKs and WRKY to resist the stress. Apart from MAPK, ERF TFs could interact with TCP and thioredoxin in tomato as shown in Fig. 8. All the results showed a complex defense mechanism of AP2/ERF TFs in response to TYLCV infection.

Expression Profiles of SlERF Genes in Response to Tomato Yellow Leaf Curly Virus Infection in Different Tomato Cultivars

To investigate whether SlERF factors are involved in responses to TYLCV, the expression patterns of five genes encoding SlERFs were analyzed in resistant and susceptible tomato cultivars. As shown in Fig. 10, the expression levels of five SlERF genes varied. Three SlERF genes (Soly19, Soly67, and Soly106) showed more rapid and vigorous expression levels in resistant and susceptible tomato cultivars, whereas the expression levels of Soly66 in the resistant cultivar were much smaller than in the susceptible cultivar. The expression level of this gene increased by 6.3-fold in Xianke-6 after 4 d. Soly36 also showed different expression profiles in resistant and susceptible cultivars. The expression levels were considerably higher in resistant cultivars, reaching 16 times in Zheza-301 after 8 d. There was research found that the expression patterns of TaERF3 in resistant and susceptible cultivars varied after inoculation with various pathogens (Zhang et al., 2007). Five ERF genes (Pp-ERFI.a,
Resistance against TYLCV varied among ERFs in different tomato cultivars. The results indicated that the ability to induce the GCC-box binding ability in Xianke-6 than in Zheza-301. The expression levels of Soly19 and Soly67 in Zheza-301 (resistant cultivar) were considerably higher than in Xianke-6 (susceptible cultivar), thereby indicating that the binding ability to GCC-box in Zheza-301 was stronger than in Xianke-6. Soly106, which exhibited GCC-box binding ability that is similar to Soly19, showed the same expression patterns in the two tomato cultivars. By contrast, Soly66 showed a higher expression level that corresponded to GCC-box binding ability in Xianke-6 than in Zheza-301. The results indicated that the ability to induce resistance against TYLCV varied among ERFs in different tomato cultivars, and such difference may be related to the binding ability of the transcription factors to the GCC-box. Several amino acids differ in each SIERF between Zheza-301 and Xianke-6, which may have affected the expression response to TYLCV infection.

Numerous studies have demonstrated that AP2/ERF TFs play important roles during various stress response such as abiotic and biotic stresses (Dong et al., 2010; Guo et al., 2004; Licausi et al., 2013). Less information is available about their function in defense against TYLCV infection. The expression levels of AP2/ERF TFs in resistant and susceptible tomato cultivars showed different function patterns in anti-TYLCV infection, which, given the difference, choose for tomato breeders. Based on our research, a new opportunity is provided for further understanding the new function of AP2/ERF TFs family in the process of mediating TYLCV infection. The study also paves the way to investigate the unknown defense mechanism against TYLCV infection in tomato.

Conclusions
When encountered with TYLCV infection, tomato plants may be considered to inhibit the replication and movement of virus by interconnecting gene networks and signaling pathways. Our study is the first to analyze the relationship between ERFs and TYLCV infection in tomato. Previous research indicated that AP2/ERF factors interacted with numerous other tomato genes (such as MAPK), which played important roles in activating plant defense against fungal and viral attacks. The AP2/ERF factors could respond to TYLCV infection with positive or negative expression patterns and by interacting with other genes and regulating the signal pathway of JA, SA, ethylene, and H₂O₂. The TYLCV defense mechanisms in tomato are a complicated network. At present, the transgenic system and proteomics are being developed by our group, and we are preparing to transfer the five SIERF genes to different tomato cultivars to further analyze the functional mechanisms of ERFs and TYLCV. Moreover, as functional molecules, proteins play vital roles in the living cell, and we are preparing to use the proteomics approach to identify the protein that respond to TYLCV infection to improve tomato TYLCV resistance.

Competing Interests
The authors declare that they have no competing interests.

Author Contributions
Conceived and designed the experiments: ASX YH. Performed the experiments: YH, MYL, FW, SS, GMX, YST, BLZ, ASX. Analyzed the data: YH, MYL, ASX. Contributed reagents/materials/analysis tools: ASX. Wrote the paper: YH. Revised the paper: ASX YH. All authors read and approved the final manuscript.

Acknowledgments
The research was supported by Jiangsu Natural Science Foundation (BK20130027), Shanxi Province Coal Based Key Scientific and Technological Project (FT201402-07), New Century Excellent Talents in University (NCET-11-0670), and Priority Academic Program Development of Jiangsu Higher Education Institutions Project.

References


Krzywinski, M. 2016. THE PLANT GENOME ■ JULY 2016 ■ VOL. 9, NO. 2


Sade, D., O. Shriki, A. Cuadros-Inostroza, T. Tohge, Y. Semel, Y. Havivet, et al. 2015. Comparative metabolomics and transcriptomics of plant response to Tomato yellow leaf curl virus infection in resistant and...
susceptible tomato cultivars. Metabolomics 11:81–97. doi:10.1007/s11306-014-0670-x


