Genome-Wide Identification and Molecular Characterization of the Growth-Regulating Factors-Interacting Factor Gene Family in Tomato

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Abstract: Growth-regulating factors-interacting factor (GIF) proteins play crucial roles in the regulation of plant growth and development. However, the molecular mechanism of GIF proteins in tomato is poorly understood. Here, four SIGIF genes (named SIGRF1a, SIGIF1b, SIGIF2, and SIGIF3) were identified from the tomato genome and clustered into two major clades by phylogenetic analysis. The gene structure and motif pattern analyses showed similar exon/intron patterns and motif organizations in all the SIGIFs. We identified 33 cis-acting regulatory elements (CAREs) in the promoter regions of the SIGIFs. The expression profiling revealed the four GIFs are expressed in various tissues and stages of fruit development and induced by phytohormones (IAA and GA). The subcellular localization assays showed all four GIFs were located in nucleus. The yeast two-hybrid assay indicated various growth-regulating factors (SIGRFs) proteins interacted with the four SIGIF proteins. However, SIGRF4 was a common interactor with the SIGIF proteins. Moreover, a higher co-expression relationship was shown between three SIGIF genes and five SIGRF genes. The protein association network analysis found a chromodomain helicase DNA-binding protein (CHD) and an actin-like protein to be associated with the four SIGIF proteins. Overall, these results will improve our understanding of the potential functions of GIF genes and act as a base for further functional studies on GIFs in tomato growth and development.

Keywords: GIF; GRF; Solanum lycopersicum; transcriptional co-activator; organ size

1. Introduction

Transcription factors (TFs) are a class of proteins, which are regulators of transcription of target genes, and play essential roles in various processes of growth and development in plants [1,2]. TFs mediate expression of target genes by binding to their promoters [3–6]. Growth-regulating factors (GRFs) belong to a class of plant-specific TFs factors involved in the regulation of stem, leaf development, flower and seed formation, root development, growth processes, and response to stress [7–13]. Growth-regulating factors-interacting factors (GIFs) predominantly function as transcription co-activators of their interaction proteins, GRFs [7–9].

GIFs are a class of transcriptional activators, interacting with GRFs to form functionally transcriptional complexes [7,14–16]. The first member of the GIF family identified was AtGIF1 and used as a bait in a yeast two-hybrid assay [14]. AtGIF1 functions as a transcriptional co-activator,
involved in the control of leaf growth and morphology [14]. It also encodes a homolog of the human synovial sarcoma translocation protein (SYT), one important transcription co-activator [14]. In Arabidopsis thaliana (A. thaliana), the GIF gene family contains three proteins, GIF1, GIF2, and GIF3, and play essential roles in vegetative and reproductive organs development [17,18]. Engineered gif1 mutants involving AtGIF1 (also known as ANGUSTIFOLIA3 (AN3)) result in a decreased cell number with narrow-leaf phenotypes, and enhanced AtGIF1 expression levels leads to increased leaf areas by increasing cell numbers in leaf primordial [19]. Interestingly, an an3 mutant involving AN3 exhibits a reduced cell number, but excessively enlarged cells [20]. GIF1 is also reported to be synthesized in mesophyll cells and transported into epidermal cells to regulate the proliferation of both epidermal and mesophyll cells in leaves [21]. Additionally, AtGIF1 is involved in the establishment of cotyledon identity by suppressing ectopic root formation [22] and functions in adaxial/abaxial patterning and leaf growth [23].

In Arabidopsis, AtGIF1 interacts with AtGRF1, AtGRF2, AtGRF3, AtGRF4, AtGRF5, and AtGRF9 [14,19,24]. GIF1 affects leaf development and cell proliferation by interacting with AtGRF3 [24] and AtGRF5 [19], respectively. In rice, GIFs are involved in OsGRF4 regulation of grain size and yield [10,25]. Studies show that GIF1 also binds to a SWI/SNF chromatin remodeling complex to regulate the transcription of downstream genes [26]. Moreover, the function of the GIF1-associated SWI/SNF chromatin remodeling complex is conserved between dicots and monocots. The transcription of GRF1 and GRF10 facilitates binding with AtGIF1/AN3 in cell division and expansion which contribute to leaf growth [27]. GIF1 binds to the promoter of unranch3 (ub3), the inflorescence architecture gene, and regulates the expression of several genes involved in shoot architecture and meristem in maize [28]. GIFs function in maintaining precise expression patterns of key developmental regulators, while GIFs/AN3 complexes bind directly to the promoters of PLETHORA1 (PLT1) and SCARECROW (SCR) to fine-tune a quiescent center (QC) and root meristem during root development [29]. Recently, GIF1 was found to be the direct downstream target gene of the KIX-PPD-MYC complex in regulating seed size [30].

Tomato is an important vegetable farmed globally for essential nutrients and minerals and for industrial processing into tomato paste [31–33]. The functions of GIF genes in tomatoes remain unclear at present. In this study, we identified and characterized four tomato GIF genes, including their phylogenetic relationships, cis-acting regulatory elements (CAREs), subcellular localization, expression profiles in various tissues at varied growth stages, expression patterns in response to phytohormones (GA, IAA, and breaker (BR)), protein–protein interactions between GIFs and GRFs, co-expression relationships between GIFs and GRFs, and SIGIF genes association networks. These results provide a theoretical basis for further functional studies of SIGIF proteins in tomatoes.

2. Materials and Methods

2.1. Identification of Tomato GIF Genes

To identify GIF genes in tomatoes, the SSXT domain of the AtGIF1 protein were used as seed sequences to search the National Center for Biotechnology Information (NCBI https://www.ncbi.nlm.nih.gov/) and Sol Genomics Network (SGN https://solgenomics.net/) databases through BLASTP. To ensure all putative SIGIF genes were included, the protein sequences of the identified SIGIFs were further confirmed through the Phytozome website (http://www.phytozome.net/) and the Plaza website (http://bioinformatics.psb.ugent.be/plaza/) databases. We used the ProtParam database (https://web.expasy.org/protparam/) to assess their physio-chemical characteristics (molecular weight and isoelectric point).

We predicted the conserved motifs of the GIF proteins using the Online Conserve Domain server (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The GIF gene structure was visualized using the GeneDoc software based on the primary sequence information obtained from the SGN database.
2.2. Phylogenetic Analyses

Multiple alignment of all the GIFs proteins was performed using ClustalX [34], and phylogenetic tree was constructed by MEGA (version 6) [35] with a bootstrap of 1000 replicates using the neighbor-joining (NJ) method.

2.3. Identification of CAREs in the Promoter

Sequences from the promoter region (about 3 kb upstream of the start codon) of each gene was retrieved from the SGN database (https://solgenomics.net/organism/genome) in Generic File Format (GFF) to identify putative CAREs using the PlantCare database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The identified CAREs visualized using the Toolkit for Biologists integrating various biological data handling tools (TBtools) [36].

2.4. Plant Materials and Hormone Treatment

A Solanum lycopersicum cultivar, Alisa Craig, was used in this study. The seeds were germinated in 50-hole flats in the soil and grown in a greenhouse with a 16 h light and 8 h night photoperiod. Two-leaf-stage tomato seedlings were transplanted to 10 cm × 10 cm × 10 cm compost plastic pots and grown in a common greenhouse. Six-leaf tomato seedlings with a similar growth were chosen for the plant hormones treatment to check the expression of genes. The seedlings were sprayed with 100 µM GA, 100 µM IAA, and 100 µM BR for the hormone treatment. The seedlings treated with water were as a control. The leaves were collected after 0, 0.5, 1, 2, 4, 8, 12, and 24 h, and all the samples were frozen in liquid nitrogen and stored in -80 °C. Three biological samples in each process were obtained for the following experiments.

2.5. RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted using TRIzol reagent (Aidlab Biotechnologies, Beijing, China;). A 3 µg sample of RNA was reversely transcribed into complementary DNA (cDNA) using a HisScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China;). RT-PCR was performed to determine the transcript levels of target genes using 384-well blocks with QuantStudio (TM) 6 Flex System (ThermoFisher Scientific; Waltham, MA, USA). Three technical replicates were performed, and each replicate of 10 µL reaction containing 5 µL SYBR mix, 4.2 µL cDNA sample, and 0.4 µL of 10 µM gene-specific primes went through the following amplification process: a 3 min pre-incubation step at 95 °C, followed by 40 cycles of 95 °C for 30 s, 58 °C for 15 s, and 72 °C for 20 s. The comparative \(2^{-\Delta \Delta C_t}\) method was used to calculate the relative levels of target gene expressions [37], and the β-actin gene (Soly11g008430) was used as an internal control. The primers for RT-PCR are listed in Table S1.

2.6. Subcellular Localization

The full-length coding regions without a stop codon of each GIF genes were amplified by PCR using gene-specific primers containing homologous recombination and introduced into a yellow fluorescent protein (YFP) vector to generate a construct using the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, Jiangsu China). Four-week-old leaves of Nicotiana tabacum were used to perform a transient expression assay mediated by Agrobacterium tumefaciens strain (GV2260) carrying GIF-GFP fusion proteins and GV2260 carrying the nucleus and cytoplasm marker 35S:RFP as previously described [38]. The tobacco leaves were used for YFP and RFP fluorescence signal observation using a Leica confocal microscope (LeicaSP8). The primers for subcellular localization assays are listed in Table S1.

2.7. Yeast Two-Hybrid Assay

The full-length coding regions of the GIF genes were amplified by PCR using gene-specific primers containing homologous recombination sites and were cloned into the bait vector pGBKKT7. The full-length coding regions of the GRF genes were cloned into the prey vector pGADT7 by gene-
specific primers containing homologous recombination sites. Each pair of bait–prey vectors was co-transformed into the yeast strain AH109 following the instructions of Matchmaker Gold Two-hybrid System (Clontech, Mountain View, CA, USA). The transformed yeasts were plated on an SD medium lacking leucine and tryptophan (SD/-Trp-Leu). After the yeast cells grew at 30 °C for 3–4 days, colonies were picked and transferred to an SD medium lacking leucine, histidine, adenine, and tryptophan (SD/-Trp-Leu-His-Ade). The yeast concentrations were estimated by measuring their optical densities at 600 nm. These were maintained at the same concentration (OD_{600}:1) for protein interactions assay. The strength of the interaction depended on the yeast growth conditions [19]. The combination of SIGIFs introduced into the pGBK7 vector and the empty pGADT7 vector were used as negative controls, as well as the combination of empty pGBK7 and SIRFs introduced in the pGADT7 vector. pGBK7-53 and pGADT7-RceT were used as positive controls. The primers for the yeast two-hybrid assays are presented in Table S1.

2.8. Expression Profiles and the Correlation Coefficients Analysis

The RNA-Seq data of different tissues at various developmental stages of the fruit of the tomato cultivar, Heinz 1706, were accessed from the Tomato Expression Atlas database (TEA). Tissues including root, leaf, flower, flower bud, fruit at different sizes (1, 2, and 3 cm), mature green fruit, BR fruit, and fruit at 10 days after breaker were retrieved from the TEA database. In addition, the expression data for leaf, immature green fruit, BR fruit and fruit at 5 days after breaker were accessed from LA1589 (Solanum pimpinellifolium) [32,39]. The normalized expressions (RPKM) of SIGIFs were downloaded from the supplementary files [32].

The expression profiles of SIGIFs and SIRFs from the RNA-seq in 536 samples from 18 transcriptome assays are listed in Table S4. The RPKM of SIGIFs and SIRFs were accessed from the Tomato Functional Genomic database (http://ted.bti.cornell.edu/cgi-bin/TFGD/digital/home.cgi). The correlation coefficients between SIRFs and SIGIFs were computed using the R language (R version 3.6.3) (https://www.r-project.org/).

3. Results

3.1. Identification of GIF Genes in Tomatoes

In this study, four GIF gene members were identified in the tomato genome (Table 1). To further understand SIGIF proteins, the amino acid (aa) length, the chromosome location, the molecular weight (Mw), and the theoretical isoelectric points (pl) of the four SIGIF proteins were analyzed (Table 1). The SIGIF genes were distributed on four chromosomes (chromosomes 3, 4, 10, and 11). The lengths of SIGIF proteins varied from 199 aa residues (SIGIF2) to 222 aa residues (SIGIF1b), with the Mw ranging from 21.74 kDa (SIGIF3) to 23.59 kDa (SIGIF1b). The pl varied from 5.85 (SIGIF2) to 6.60 (SIGIF1a).

| Gene Name | Gene Loci | Chromosome Location (Strand) | aa | pIs/Mw |
|-----------|-----------|-------------------------------|----|--------|
| SIGIF1a   | Solyc04g009820.2.1 | SL2.50ch04:3139217-3143959 (+) | 208 | 6.60/22.74 KDa |
| SIGIF1b   | Solyc11g006230.1.1 | SL2.50ch11:981174-984561 (−) | 222 | 6.41/23.59 KDa |
| SIGIF2    | Solyc03g082480.2.1 | SL2.50ch03:45948144-45952630 (+) | 199 | 5.85/21.83 KDa |
| SIGIF3    | Solyc10g009280.2.1 | SL2.50ch10:3267235-3271023 (−) | 200 | 6.51/21.74 KDa |

aa refers to protein length; pl refers to the theoretical isoelectric points; Mw refers to the molecular weight.

3.2. Phylogenetic Analysis of the SIGIF Family Genes

A phylogenetic tree of the GIF genes from five species was constructed to study their evolutionary patterns in the plant kingdom. SIGIFs and their counterparts in Arabidopsis, rice, maize, and potatoes were used for the phylogenetic analysis. The unrooted phylogenetic tree was constructed after the
alignments of the full-length GIFs protein sequences from the five species containing three GIF proteins each in 
*A. thaliana*, *Oryza sativa* (*O. sativa*), and *Zea mays* (*Z. mays*) and four GIF proteins each in *Solanum tuberosum* and *Solanum lycopersicum* (Table S2). All four tomato GIFs proteins showed high similarity with AtGIF1/AN3 and were named SIGIF1a, SIGIF1b, SIGIF2, and SIGIF3 according to their sequence similarity to GIFs in Arabidopsis (Figure 1). The four SIGIF proteins were clustered into two clades (I and II) in the phylogenetic tree (Figure 1). Clade I contained SIGIF1a and SIGIF1b, while clade II contained SIGIF2 and SIGIF3.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Phylogenetic relationship of GIF proteins. The unrooted tree was constructed using MEGA6 through the neighbor-joining method at 1000 bootstrap replicates based on the alignment of GIF protein sequences in Arabidopsis, rice, maize, potatoes, and tomatoes. The different markers before protein names stand for different plants. All these GIF proteins were clustered into two clades (I and II) in the phylogenetic tree.

### 3.3. Gene Structures, Conserved Domains, and CAREs in the Promoters of SIGIF Genes in Tomatoes

To further study the potential functions of the GIF genes, the structures of the GIF gene sequences were analyzed using the PlantCare database [40]. Each of the four *SIGIF* genes contained four exons and three introns (Figure 2A). The lengths of all introns were longer than those of all the exons (Figure 2A). The N-terminal regions of the GIF proteins contained the conserved domain, SSXT (Figure 2B), which is involved in synovial sarcoma in humans [14]. The conserved domain contained the motif “LDENK*LI*I*QN*GK *EC*Q*LQ**NL*YLAAIAD*QP” (Figure 2).

The CAREs in the promoter sequences play essential roles in gene transcription. Therefore, characterizing them in the promoter of *SIGIF* genes in tomatoes may provide insights into the functions of SIGIF genes. A total of 33 CAREs with predicted functions were identified from the promoters of the four *SIGIF* genes (Table 2). Among the 33 CAREs (Figure 3), six (ABER, CAAT-box, G-box, TATA-box, TCA-element, and ARE) were common to all four SIGIF genes. TATA-box and CAAT-box were the most common CAREs. G-box was involved in light responsiveness, implying the functions of GIF proteins may be influenced by light. AERE and TCA-elements were responsive to abscisic acid (ABA) and salicylic acid (SA), indicating that GIFs may play an important role in
ABA and SA response. The remaining CAREs were divided into five groups, containing growth-, metabolism-, hormone-, stress-, and light-responsive elements (Figure 3). Phytohormone-responsive elements included auxin-responsive elements (AuxRR-core, TGA-element), MeJA-responsiveness (CGTCA-motif and TGACG-motif), and gibberellin-responsiveness (TATC-element). Interestingly, CAREs involved in circadian controls were found in SlGIF1a and SlGIF1b promoters, signifying their potential functions may be influenced by day length. This is consistent with the function of AN3 in modulating light-induced root elongation [5], as shown by the clustering of GIFs in clades I and II (Figure 3).

Table 2. Functionally described cis-elements identified in the promoters of the SlGIF genes.

| Cis-Element   | Members of GIFs                        | Functions of Cis-Element                                                                 |
|---------------|----------------------------------------|------------------------------------------------------------------------------------------|
| ABRE          | SlGIF1a, SlGIF1b, SlGIF2, and SlGIF3   | cis-acting element involved in the abscisic acid responsiveness                          |
| ACA-motif     | SlGIF3                                 | part of gapA in (gapA-CMA1) involved with light responsiveness                          |
| ACE           | SlGIF1a, SlGIF2, and SlGIF3            | cis-acting element involved in light responsiveness                                      |
| AE-box        | SlGIF3                                 | part of a module for light responsiveness                                                |
| ARE           | SlGIF1a, SlGIF1b, SlGIF2, and SlGIF3   | cis-acting regulatory element essential for the anerobic induction                       |
| AT1-motif     | SlGIF1b                                | part of a light responsive module                                                       |
| ATCT-motif    | SlGIF1b                                | part of a conserved DNA module involved in light responsiveness                         |
| AT-rich sequence | SlGIF1b and SlGIF3                    | element for maximal elicitor-mediated activation                                         |
| AuxRR-core    | SlGIF1a                                | cis-acting regulatory element involved in auxin responsiveness                           |
| Box 4         | SlGIF1a, SlGIF2, and SlGIF3            | part of a conserved DNA module involved in light responsiveness                         |
| CAAT-box      | SlGIF1a, SlGIF1b, SlGIF2, and SlGIF3   | common cis-acting element in promoter and enhancer regions                               |
| CAT-box       | SlGIF1b and SlGIF2                     | cis-acting regulatory element related to meristem expression                             |
| CGTCA-motif   | SlGIF1a and SlGIF2                     | cis-acting regulatory element involved in the MeJA-responsiveness                        |
| chs-CMA1a     | SlGIF1b and SlGIF2                     | part of a light responsive element                                                       |
| circadian     | SlGIF1a and SlGIF1b                    | cis-acting regulatory element involved in circadian control                              |
| GARE-motif    | SlGIF1a and SlGIF2                     | gibberellin-responsive element                                                          |
| G-box         | SlGIF1a, SlGIF1b, SlGIF2, and SlGIF3   | cis-acting regulatory element involved in light responsiveness                          |
| GCN4_motif    | SlGIF1b                                | cis-regulatory element involved in endosperm expression                                 |
| GT1-motif     | SlGIF1a and SlGIF2                     | Light-responsive element                                                                 |
| LAMP-element  | SlGIF1a                                | part of a light-responsive element                                                      |
| LAMP-element  | SlGIF3                                 | part of a light-responsive element                                                      |
| LTR           | SlGIF1b, SlGIF2, and SlGIF3            | cis-acting element involved in low-temperature responsiveness                           |
| MBS           | SlGIF3                                 | MYB-binding site involved in drought inducibility                                         |
| MRE           | SlGIF1a and SlGIF3                     | MYB-binding site involved in light responsiveness                                        |
| O2-site       | SlGIF1a and SlGIF1b                    | cis-acting regulatory element involved in zein metabolism regulation                    |
| Sp1           | SlGIF1b                                | Light-responsive element                                                                 |
| TATA-box      | SlGIF1a, SlGIF1b, SlGIF2, and SlGIF3   | core promoter element around –30 of transcription start                                 |
| TATC-box      | SlGIF1a                                | cis-acting element involved in gibberellin responsiveness                                |
| TCA-element   | SlGIF1a, SlGIF1b, SlGIF2, and SlGIF3   | cis-acting element involved in salicylic acid responsiveness                            |
Table 2. Cont.

| Cis-Element      | Members of GIFs | Functions of Cis-Element                      |
|------------------|-----------------|-----------------------------------------------|
| TC-rich repeats  | SlGIF1a, SlGIF1b, and SlGIF3 | *cis*-acting element involved in defense and stress responsiveness |
| TGA-box          | SlGIF2          | part of an auxin-responsive element           |
| TGACG-motif      | SlGIF1b and SlGIF3 | *cis*-acting regulatory element involved in the MeJA responsiveness |
| TGA-element      | SlGIF3          | auxin-responsive element                      |

Figure 2. Structure and conserved motif analysis of the GIF genes. (A) The exon and intron structures of the tomato GIF genes. The gene structures of the SlGIF genes were illustrated using the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) and the IBS software [41]. The red box and the green box represent the CDS and UTR, respectively. The solid line stands for the intron. (B) Motif analysis of the SlGIF proteins. The conserved motifs of all GIF proteins in this study were identified using ClustalX and GeneDoc software. (C) Detailed SSXT domains from the GIF proteins.
Based on the important functions of the GIF proteins in various growth processes in plants, we studied their expression patterns in different tomato tissues. The four GIFs were expressed in the various tissues in Heinz 1706 (Figure 4). The expression levels of SIGIF2 and SIGIF3 showed similar patterns in immature green fruit, mature green fruit, BR fruit, and red ripe fruit, but the expression of SIGIF1a exhibited higher relative expression in immature green fruit (1, 2, and 3 cm) and declined sharply in the late developmental stages of ripening (Figure 4). The expressions of SIGIF1a and SIGIF1b shared a similar expression pattern, but the expression levels of SIGIF1b were significantly higher in immature green fruit than those of SIGIF1a. In short, the expression of SIGIF1a was relatively higher in immature green fruit and decreased during ripening. The expression of SIGIF1b was low in root, leaf, bud, and flower, but it maintained higher relative expression in immature green fruit. The transcript levels of SIGIF2 recorded relatively high expression in root, 1 cm fruit, and 2 cm fruit but were lowly expressed in other tissues/stages. The expression levels of SIGIF3 were lower in leaf and 2 cm fruit stages and were relatively higher in root, bud, flower, 1 cm fruit, 3 cm fruit, BR and red ripe fruit stages. However, the expression patterns of the GIF genes in LA1589 were different from in Heinz 1706. SIGIF1a showed a relatively lower expression in immature green fruit, BR, and fruit at 5 days after breaker, and the expressions of SIGIFb were hardly be detected in IM, BR, and red ripe stages in LA1589. The expression levels of SIGIF2 and SIGIF3 showed similar patterns among the four tissues/stages in LA1589. In summary, the different expression levels of the GIF genes between Heinz 1706 and LA1589 indicated functional divergence in domesticated and wild tomatoes.
3.5. Expression Profiles of the SlGIF Genes under Phytohormone Treatments

Phytohormones play essential roles in the coordination of growth and development under various environmental conditions. BRs are a class of steroid phytohormones, known for their functions in cell division and elongation [42–45]. Similarly, GA and IAA have also been functionally implicated in cell proliferation and expansion in tomatoes [46]. Functional studies showed that GIFs play essential roles in cell proliferation and expansion in Arabidopsis [7,14,20,24]. These hormones were chosen to check their effects on GIF responses and expressions. We analyzed the expression profiles of the SlGIFs genes under phytohormone treatments with BR, GA, and IAA. Four SlGIF genes were induced by GA and IAA treatments, especially for SlGIF1a and SlGIF1b (Figure 5). They were less sensitive to BR treatments. Among the four SlGIF genes, SlGIF1b showed consistently lower relative expression levels after the BR treatment compared to GA and IAA. However, SlGIF1a sharply decreased in expression after peaking under the GA and IAA treatments, whereas the expression of SlGIF3 gradually decreased after peaking under the IAA and GA treatments. The expression levels of SlGIF1b and SlGIF2 decreased slowly after the peak under the IAA treatment. The expression levels of SlGIF1b and SlGIF2 were downregulated sharply after the peak at 4 and 1 h under the GA treatment, respectively. This suggested different roles of SlGIFs in growth signals.
The expression levels of SlGIF1b and SlGIF2 were downregulated sharply after the peak at 4 and 1 h under the GA treatment, respectively. This suggested different roles of SlGIFs in growth signals.

Figure 5. Expression profiles of SlGIF genes (GIF1a (A), GIF1b (B), GIF2 (C), and GIF3 (D)) under BR, GA, and IAA treatments. The numbers 0 H, 0.5 H, 1 H, 2 H, 4 H, 8 H, 12 H, and 24 H indicate the time after the treatment. The expressions of the treated plants were compared with those of the untreated plants after the normalization of values with an internal reference. The error bars represent the standard errors among three independent replicates, and the different letters above the bars indicate statistically significant differences at a 5% level of significance according to Tukey’s pairwise comparison tests.
3.6. Subcellular Localization of the SlGIF Proteins

To further understand the functions of SlGIFs, we confirmed the subcellular localization of SlGIFs. The SlGIF:YFP fusion proteins were constructed under the control of the CaMV 35S promoter and expressed in the tobacco leaves. The confocal observation revealed fluorescence signals for all the SlGIF:YFP proteins in the nucleus and cytoplasm (Figure 6). Thus, all the SlGIF proteins were localized in the nucleus.

![Figure 6](image-url) Subcellular localization of SlGIF– yellow fluorescent protein (YFP) fusion proteins. Tobacco leaves was infiltrated with *Agrobacterium tumefaciens* (*A. tumefaciens*) containing a recombination vector (35S: GIFs-YFP) and a nuclear marker RFP (red fluorescent protein).

3.7. Interactions between the SlGIF Proteins and the SlGRF Proteins

GIFs proteins act as the co-activators of GRFs proteins in Arabidopsis, rice, and maize [10,12,14–16,18,19,25,28]. The interaction between the GIF proteins and the GRFs in tomatoes was assessed by a yeast two-hybrid assay. Four GIFs were cloned into pGBKT7 (bait vector), and 12 GRFs [47,48] were cloned into pGADT7 (prey vector) for an interaction assay. The four GIFs showed no self-activation activity, and each GIF proteins interacted with several GRF proteins (Figure 7 and Figure S4). SlGIF1a strongly interacted with SlGRF3, SlGRF4, SlGRF12, and SlGRF13 but weakly interacted with SlGRF10. Again, SlGIF1b strongly interacted with SlGRF4 and SlGRF8 but interacted weakly with SlGRF2. SlGIF2 strongly interacted with SlGRF3, SlGRF4, SlGRF8, SlGRF10, SlGRF11, and SlGRF13. However, it weakly interacted with SlGRF1 and SlGRF6 (Figure 7). SlGIF3 strongly interacted with SlGRF4, SlGRF8, SlGRF11, and SlGRF13 and weakly interacted with SlGRF5. SlGRF4 was the only GRF protein
that interacted with the four GIF genes, indicating SlGRF4 may be involved in the functions of GIF proteins in tomatoes (Figure 7).

**Figure 7.** Interactions between GIF proteins and growth-regulating factors (GRF) proteins in a yeast two-hybrid assay. (A) GIF1a interacted with GRFs; (B) GIF1b interacted with GRFs; (C) GIF2 interacted with GRFs; (D) GIF3 interacted with GRFs; (E) the negative controls of GRFs. GIF proteins and GRF proteins were used as a bait and a prey, respectively, in different combinations. SD-/Trp-Leu was an SD medium lacking leucine and tryptophan. SD-/Trp-Leu-His was an SD medium lacking leucine,
Histidine, adenine, and tryptophan. BD was the pGBK7 vector, and AD was the pGADT7 vector. Yeast cultures with transformed yeasts adjusted to have optical densities at 600 nm (OD$_{600}$) of 1.0 and 2 µL yeast culture dilutions were spotted on SD/-Trp-Leu and SD/-Trp-Leu-His-Ade medium, respectively. The growth of the yeast strain on the SD/-Trp-Leu medium indicated that each pair of bait–prey was successfully transformed into AH109. The different growth conditions of the transformed yeasts on the SD/-Trp-Leu-His-Ade medium showed the strength of the interaction between the two proteins. Each group was performed for three repetitions.

3.8. Relative Expression between SlGIF and SlGRF Genes and SIGIF Protein

GIF1 interacts with GRF to regulate the expression of GRF in rice and Arabidopsis [7–9,15]. To further understand whether there is a regulatory relationship between the tomato GIF and GRF genes, the co-expression analyses between SIGIFs and SIGRFs were conducted. The expression profiles of SIGIFs and SIGRFs in different tissues were retrieved from the Tomato Functional Genomics Database (http://ted.bti.cornell.edu/). The expression levels of GIF and GRF genes from 536 samples (Table S3) in 18 transcriptome assays (Table S4) were employed for co-expression analysis between SIGIFs and SIGRFs. SIGRF13, SIGRF9, and SIGRF1 showed lower co-expression levels between the GIF genes (Figure 8). Among the four GIF genes, SIGIF1b recorded a lower co-expression level between GRF genes. The expression levels of SIGRF2, SIGRF3, SIGRF4 and SIGRF5 were highly correlated with SIGIF1a, SIGIF2, and SIGIF3. In summary, the expression levels of SIGIF1a, SIGIF2, and SIGIF3 had higher relationships with those of SIGRF2, SIGRF3, SIGRF4 and SIGRF5, suggesting regulatory relationships between these genes.

![Figure 8. Co-expression between GIF and GRF genes in 536 tissues and samples. The sizes and colors of the circles indicate the values of the co-expression coefficients.](image-url)
The STRING database (https://string-db.org/cgi/) was used to obtain putative protein–protein interaction among the SIGIF proteins and related proteins. Outputs from the STRING database were subsequently visualized in the standalone version of Cytoscape software [49] (Figure 9). Several proteins were predicted to associate with the SIGIF proteins, indicating diverse functions in growth and development. Among the proteins, two proteins (Solyc11g062010.1.1 and Solyc12g037980.1.1) were found to associate with all the SIGIF proteins. Based on the annotations of the proteins, Solyc11g062010.1.1 encoded a chromodomain helicase DNA-binding protein (CHD), related to chromatin remodeling [50–52], and Solyc12g037980.1.1 encoded an actin-like protein.

4. Discussion

GIF proteins have been identified in several plants, such as *A. thaliana*, *O. sativa*, and *Z. mays*. They play essential roles in various biological processes [7,14,17,21,22,25–30]. However, there is limited study about the roles of GIF genes in tomatoes. In this study, the four *SlGIF* genes were identified in the tomato genome (Table 1). All four *SlGIF* genes showed different expression profiles in Heinz 1706 and LA1589 (Figure 4) and displayed different expression patterns in response to IAA and GA.
The four SlGIF proteins were localized in the nucleus (Figure 6), interacted with various SIGRF proteins (Figure 7) and associated with the CHD protein and the actin-like protein (Figure 9). Additionally, SIGRF4 was a common protein that interacted with all four SIGIF proteins. Five SIGRF proteins and three SIGIF proteins showed higher co-expression relationships (Figure 8). Our results provide basic information of GIF proteins in tomatoes.

4.1. Phylogenetic Relationships and Structures of the SIGIF Gene Families

GIF genes were identified and distributed on four chromosomes in the tomato genome (Table 1). However, only three GIF genes were identified in Arabidopsis [14,19], rice [10], and maize [28], respectively. Gene duplications are one of significant forces driving the evolution of genomes and genetic systems in plants [53]. Our results indicated a gene duplication event occurred in the GIF gene family in tomatoes. Interestingly, two GIF genes (SIGIF1a and SIGIF1b) in tomatoes are the orthologous genes of AtGIF1/AN3, suggesting the duplication of the GIF1 in the tomato genome may have resulted in the expansion of the SIGIF genes. Remarkably, the GIF proteins in two monocots (rice and maize) and three dicots (Arabidopsis, potatoes, and tomatoes) clustered as orthologous pairs in a subgroup of each clade (OsGIF1 and ZmGIF1, OsGIF2 and ZmGIF2, and OsGIF3 and ZmGIF3) (Figure 1). The results implied the GIF gene family arose before monocots and dicots diverged. The four SIGIF genes possessed similar exon/intron structures (Figure 2A). However, AtGIF1/AN3 clustered in clade I had four exons, whereas AtGIF2 and AtGIF3 in clade II had five exons [29], indicating similar exon organization in Arabidopsis. The difference in the structures of the GIF genes in clade II between Arabidopsis and tomatoes implied varied functions of GIF genes may partly be ascribed to evolutionary divergence.

4.2. Different Expression Patterns Shown by SIGIFS

In Arabidopsis, GIFs play essential roles in the development of leaves, male and female reproductive organs, cotyledons, and roots [17,20–22,24,29]. Our study indicated that SIGIF1b and SIGIF2 had higher expressions in the early development of fruits, suggesting they play more important functions in early fruit development. Remarkably, cell division and expansion occurs in early fruit development which directly influence fruit weight and shape [54–60]. GIF genes play crucial roles in cell proliferation to determine fruit size [27,30,61]. For example, an3 mutants generated in Arabidopsis involving GIF genes caused a decrease in cell number and slender-leaf phenotypes [19]. The rest of the triple mutants (gif1, gif2, and gif3) produced abnormal carpel margin meristem [17]. The gif1 mutant in maize reduced indeterminate cells in leaf and stem, resulting in the production of narrow leaves and short internodes [28]. Enhancing the expression of OsGIF1 led to increased sizes of multiple rice organs, such as stems, leaves, and grains [10,12,61]. Generally, GIF proteins may positively regulate fruit weight and size in tomatoes.

4.3. Multifunctions in Tomatoes Played by the SIGIF Gene Family

The subcellular localization analysis indicated that SIGIF proteins were located in different organelles in a cell, including the nucleus, which is consistent with an earlier study [14]. The functional study showed that AtGIF1 proteins acted as transcriptional co-activators and interacted with AtGRF proteins in Arabidopsis [14,18,19,29], rice [10,12,16,25], and maize [28]. Thirteen (13) GRFs were identified in maize to interact with GIF1 [28]. AtGIF1 interacted with six GRF proteins in Arabidopsis [14,19,24], while OsGIF1 interacted with three GRF proteins [10,12,16]. These interactions suggested GIF genes play essential roles in complexes formed by GIF and GRF interactions. GIF genes may also mediate different pathways of plant growth and development via interacting with different GRF genes. All four SIGRFs interacted with SIGIFs in tomatoes (Figure 6), implying their multifunctions in tomatoes.

Although SIGIF1a and SIGIF1b were the orthologous genes of AtGIF1/AN3, SIGIF1a and SIGIF1b interacted with different SIGRF proteins, except for the common protein SIGRF4. This indicated
the functional divergence of SlGIF1a and SlGIF1b during evolution. Interestingly, SlGRF4 could interact with four SlGIF proteins in yeast. AtGRF5 is an SlGRF4 orthologous gene in tomatoes [48] and regulates cell proliferation in leaves [19]. These results implied the similar functions of GIF genes in cell proliferation in tomatoes by interacting with SlGRF4. This is inconsistent with the redundant functions of GIF genes in Arabidopsis [18]. Moreover, it has been reported that several GRF proteins were the downstream of the GIF genes in rice [25] and maize [27] and increasing the expression of GIF genes enhances the transcription levels of GRF genes. The different GRF and GIF functions in tomatoes may require further studies to unravel their specific functions. The co-expression analyses of SIGFs and SIGFRs in tomatoes indicated the expression levels of SIGF2, SIGF3, SIGF4, and SIGF5 had higher relationships with SIGF1α, SIGF2, and SIGF3. The higher correlation of relative expression between the SIGF and SIGFR genes showed that they may be regulated by the same TFs or the SIGFRs may function in the downstream functions of SIGF1α, SIGF2, and SIGF3. The SIGF proteins associated with the CHD protein and the actin-like protein as revealed by the protein association network analysis. This affirmed the primary function of SIGFs as co-activators [52]. This is consistent with the roles of AtGIF1/AN3 in Arabidopsis [26,27,62].

5. Conclusions

Four GIF genes were identified in the tomato genome. These genes are localized on four of the 12 tomato chromosomes. Our phylogenetic analysis classified the GIF genes into two major clades. The results from the conserved motifs, gene structure, and subcellular localization indicated that SIGF genes contain SSXT motif and are localized in the nucleus and cytosol. A significant variation was recorded in the expression profiles of these genes at different stages of tomato growth and tissues under phytohormone treatments. We identified key cis-elements in the promoter regions, assessed expression profiles, protein–protein interaction and performed gene co-expression analyses to further evaluate the functions of GIFs in tomato growth and development. The identification and characterization of GIF gene family members in tomatoes provides a foundation for further functional studies for genetic improvement of tomatoes.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/2073-4425/11/12/1435/s1. Table S1: List of primers used in this study. Table S2: Protein sequences of GIFs from Arabidopsis, rice, maize, potatoes, and tomatoes. Table S3: Expression profiles of the SlGIF and SlGRF genes in 536 samples. Table S4: List of transcriptome analysis.

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