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Genome-wide identification and transcriptional profiling analysis of auxin response-related gene families in cucumber

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Abstract

Background: Auxin signaling has a vital function in the regulation of plant growth and development, both which are known to be mediated by auxin-responsive genes. So far, significant progress has been made toward the identification and characterization of auxin-response genes in several model plants, while no systematic analysis for these families was reported in cucumber (*Cucumis sativus* L.), a reference species for Cucurbitaceae crops. The comprehensive analyses will help design experiments for functional validation of their precise roles in plant development and stress responses.

Results: A genome-wide search for auxin-response gene homologues identified 16 auxin-response factors (ARFs), 27 auxin/indole acetic acids (Aux/IAAs), 10 Gretchen Hagen 3 (GH3s), 61 small auxin-up mRNAs (SAURs), and 39 lateral organ boundaries (LBDs) in cucumber. Sequence analysis together with the organization of putative motifs indicated the potential diverse functions of these five auxin-related family members. The distribution and density of auxin response-related genes on chromosomes were not uniform. Evolutionary analysis showed that the chromosomal segment duplications mainly contributed to the expansion of the CsARF, CsIAA, CsGH3, and CsLBD gene families. Quantitative real-time RT-PCR analysis demonstrated that many ARFs, AUX/IAAs, GH3s, SAURs, and LBD genes were expressed in diverse patterns within different organs/tissues and during different development stages. They were also implicated in IAA, methyl jasmonic acid, or salicylic acid response, which is consistent with the finding that a great number of diverse cis-elements are present in their promoter regions involving a variety of signaling transduction pathways.

Conclusion: Genome-wide comparative analysis of auxin response-related family genes and their expression analysis provide new evidence for the potential role of auxin in development and hormone response of plants. Our data imply that the auxin response genes may be involved in various vegetative and reproductive developmental processes. Furthermore, they will be involved in different signal pathways and may mediate the crosstalk between various hormone responses.

Background

Auxin, which is widely distributed in higher plants, has long been recognized as an essential plant hormone involved in diverse processes of plant growth and development, including plant root formation, apical dominance, senescence, fruit development, and abiotic and biotic stress responses. Auxin often rapidly induces the expression alteration of many auxin response-related genes, referred to as primary or early auxin response genes, including auxin/indoleacetic acid (Aux/IAA), Gretchen Hagen 3 (GH3), small auxin up mRNA (SAUR), and lateral organ boundaries (LBD) [1-4]. Molecular genetic and biochemical findings have suggested that the interaction of Aux/IAAs and auxin response factor (ARF) has a central function in the auxin signaling transduction pathway. Under low auxin concentration, ARF protein activities are inhibited by dimerization with Aux/IAAs [1,5,6]. Elevated auxin concentration causes ARFs to be released from a repressor heterodimer by promoting the degradation of Aux/IAA proteins through the ubiquitin-proteasome protein (TIR1) pathway [7-9]. The released

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ARFs can activate or repress the expression of other primary/early auxin response genes by binding to auxin response elements (AuxREs) on the promoters of these genes [7].

A typical ARF protein contains a conserved N-terminal B3-like DNA-binding domain (DBD) that regulates the expression of auxin response genes, a conserved C-terminal dimerization domain (CTD) that resembles domains III and IV in Aux/IAA proteins, and a variable middle region (MR) [10,11]. Aux/IAA proteins generally have four characteristic domains: I, II, III, and IV [11,12]. Except for domain II that is known to be involved in protein stability, domains I, III, and IV are repression domains (RDs) with additional functions in different processes. Domain I is an N-terminal RD represented by an LxLxL motif [13]. This domain can interact with the TOPLESS co-repressor [14]. Domains III and IV, the C-terminal domains, can repress the function of ARFs and subsequently repress auxin signaling transduction through the dimerization of ARFs with the CTD [6,15-17]. However, no conserved motif or domain has ever been found in the GH3 and SAUR proteins [1,18,19]. Although SAUR proteins are not highly homologous to any other published domains, the central regions of these proteins are quite conservative [19]. The N-terminal lateral organ boundaries (LOB) domain is approximately 100 amino acids in length [20] and typically contains three highly conservative regions, including C-domain, Gly-Ala-Ser (GAS) block, and predicted coiled-coil motif [20,21]. The C-domain contains four highly conserved cysteine (C) residues arranged in a $CX_2CX_6CX_3C$ motif, which is required for DNA binding. Similarly, the predicted coiled-coil motif contains four perfectly conserved leucine residues in a $LX_6LX_3LX_6L$ spacing that is reminiscent of a leucine zipper and may provide protein interaction [22].

Genome-wide analysis indicated that ARFs and AUX/IAAs are encoded by relatively large gene families in *Arabidopsis*, rice, maize, sorghum, *Populus trichocarpa*, tomato, soybean etc. [1,10,18,19,23-27]. Functional identification revealed that these genes have important functions in many aspects of plant development. In *Arabidopsis*, AtARF1 and AtARF2 can regulate leaf senescence and floral organ abscission in a partially redundant manner [28]. AtARF2 also functions as a transcriptional repressor involved in the auxin-mediated control of *Arabidopsis* leaf longevity [29]. IAA28 can promote lateral root initiation in response to auxin signals as a transcription repressor [30]. In rice, OsARF12 is implicated in regulating root elongation as a transcription activator [31]. In tomato, three ARF genes (SlARF4, SlARF7, and SlARF10) and one Aux/IAA gene (SlIAA9) exhibit different functions during fruit development [32-35]. Although many GH3 and SAUR genes from different species have been published, the precise functions of these genes remain unclear. Some GH3

genes in *Arabidopsis* are involved in maintaining auxin homeostasis by conjugating excess IAA to amino acids [36]. JAR1 (GH3.11) can conjugate jasmonic acid (JA) to amino acids [37]. SAUR genes may have important functions in the regulation of cell elongation in soybean and maize [38-40] and cell expansion in *Arabidopsis* [41]. In rice, OsAUR39 acts as a repressor of auxin synthesis and transport [42]. A few reports on the biological roles of LBD genes are available. AtAS2 (AtLBD6) has an important role in flat leaf formation and flower development [43]. LBD16 and LBD18 can influence lateral root formation in *Arabidopsis* [44]. LBD18 is also involved in regulating tracheary element differentiation [45]. The ortholog of AtLBD16 in rice can regulate leaf formation [46].

Cucumber (*Cucumis sativus* L.), an economically important crop of the botanical family Cucurbitaceae, is considered as one of the model dicot plants for molecular and genetic studies. As a fresh-fruit plant, cucumber has a few traits that may have been consequences of various auxin gene networks. However, only five CsARFs and three CsIAAs have previously been identified in cucumber [47,48]. To the best of our knowledge, no systematic investigations on auxin response gene families have been reported in cucumber. Taking advantage of the available cucumber genome database [49], a genome-wide search was carried out in the present study to find the homologues of auxin response gene families in cucumber. A total of 16 ARFs, 27 IAAs, 10 GH3s, 61 SAURs, and 39 LBDs were identified from the cucumber genome. Detailed information on the genomic structures, chromosomal locations, and sequence homology of these genes was presented. In addition, the phylogenetic relationship between the auxin response genes in cucumber and those in *Arabidopsis*, rice, and maize were also compared. Subsequently, the different temporal and spatial expression patterns of this family of genes during fruiting and under IAA, JA and SA treatment in cucumber plants were also compared through quantitative real-time PCR (qRT-PCR).

Results and discussion
Identification of auxin response genes in cucumber
To identify all auxin response genes in cucumber, BLAST searches on the cucumber genome database were performed using the published peptide sequences of ARF, AUX/IAA, GH3, SAUR, and LBD genes from *Arabidopsis*, rice, tomato, and maize as query sequences. A total of 181 candidates for auxin-related genes were predicted from the cucumber genome database using the TBLASTN program. These predicted peptide sequences of the candidates were analyzed through BLASTP of NCBI to check their corresponding conserved domains. The ones without anticipant domains were removed. After these analyses, the cucumber genomes appeared to have 16 ARF, 27 AUX/IAA, 10 GH3, 61 SAUR, and 39

LBD genes, referred to as *CsARF, CsIAA, CsGH3, CsSAUR,* and *CsLBD,* respectively (Additional file 1: Table S1). The number of CsARF, CsIAA, CsGH3, CsSAUR, and CsLBD members of cucumber is comparable with that of *Arabidopsis* (23 AtARFs, 29 AtIAAs, 20 AtGH3s, 72 AtSAURs, and 42 AtLBDs) and rice (25 OsARFs, 31 OsIAAs, 13 OsGH3s, 58 OsSAURs, and 35 OsLBDs). However, cucumber, *Arabidopsis,* and rice have different genome sizes (cucumber, ~243.5 Mb; *Arabidopsis,* ~125 Mb; and rice, ~420 Mb) [49]. The observed similarity partially accounts for the conservation of auxin response genes in these three species.

It is worth mentioning the nomenclature system for auxin response genes used in the present study. Distinctive names for the CsARF, CsIAA, and CsGH3 families were given according to their homologous genes in *Arabidopsis.* Some ARF, IAA, and GH3 family genes identified in cucumber without apparent homologous genes in *Arabidopsis* were named according to the order of identification. Conversely, given that sequence analysis indicated that the similarity in SAUR and LBD amino acid sequences between *Arabidopsis* and cucumber was low, distinctive names for each of the SAUR and LBD family members identified in this study were given according to their position from the top to the bottom on the cucumber chromosomes 1 to 7.

Classification and gene structure analysis

Phylogenetic analysis showed that 16 CsARF proteins can be divided into three major groups (groups I to III), wherein groups I and II can be further divided two subgroups (Figure 1a). A similar scenario was reported in *Arabidopsis* and tomato [26]. The CsARF genes in the same group display similar exon and intron structures, especially those in group III, with much fewer intron numbers (Figure 1a; Additional file 1: Table S1-1). Interestingly, all the Q-rich SlARFs clustered in group II. Moreover, they formed one triplet and one sister pair (*CsARF6/CsARF14/CsARF8* and *CsARF7/CsARF19*) with very strong bootstrap support (>99%).

Similar to tomato and *Arabidopsis,* 27 *CsIAA* genes formed two groups (groups I and II) (Figure 1b). However, the gene structure was different within the same group, although all *CsIAA* genes, except for *CsIAA25,* were interrupted by introns. The number of introns ranged from one (*CsIAA12, CsIAA18, CsIAA26,* and *CsIAA33*) to seven (*CsIAA10*) (Figure 1b; Additional file 1: Table S1-2). Similarly, all 61 *CsSAURs* can be divided into two distinct groups with 9 and 52 members (Additional file 2: Figure S1-4). However, no intron was identified from most *CsSAUR* genes, and only seven *CsSAUR* genes, including *CsSAUR14, CsSAUR15, CsSAUR19, CsSAUR32, CsSAUR42, CsSAUR50,* and *CsSAUR56,* contained one intron (Additional file 1: Table S1-5).

The GH3 gene family is highly conserved in both dicots and monocots [18]. Alignment of deduced amino acid sequences of cucumber *CsGH3* revealed that 10 *CsGH3* genes can be divided into two groups with two and eight members, respectively. The GH3 domains among these proteins were highly conserved. The multiple sequence alignment of the full-length OsGH3 proteins using ClustalX revealed high similarity, ranging from 94% to 100%. Furthermore, a comparison of the full-length cDNA sequences with corresponding genomic DNA sequences showed that the gene structure was also similar among the *CsGH3* genes. The coding sequences of all 10 *CsGH3s* were disrupted by two or three introns (Figure 1c; Additional file 1: Table S1-3). This finding is consistent with a previous report in rice [18].

According to the sequence homologs, all 39 *CsLBD* proteins were divided into two groups (groups I and II) (Figure 1d). A similar classification was reported in *Arabidopsis,* rice, and sorghum [26]. Group I consisted of 35 members, whereas group II contained the remaining four members. Most *CsLBDs* contain one or two introns (Figure 1d; Additional file 1: Table S1-4).

Protein sequences analysis in auxin-related genes

All cucumber CsARF protein sequences were found to contain DBDs, MRs, and CTDs (domains III and IV), except for *CsARF17* that lacked the CTD domain (Figure 2; Additional file 1: Table S1-1; Additional file 3: Figure S2-1). A previous study proved that the ARF MRs function either as activation domains (ADs) or RDs [50]. In *Arabidopsis,* protoplast transfection assays indicated that *AtARF1, AtARF2, AtARF4,* and *AtARF9* containing MRs rich in proline (P), serine (S), and threonine (T) act as repressors and that *AtARF5, AtARF6, AtARF7,* and *AtARF8* containing MRs rich in glutamine (Q) act as transcriptional activators [7,51]. In the present study, *CsARF1-5, CsARF9-13,* and *CsARF17* in cucumber possessed MRs rich in proline (P), serine (S), or threonine (T), indicating their role as repressors. By contrast, *CsARF6-8, CsARF14,* and *CsARF19* had MRs rich in glutamine (Q), implying their role as transcriptional activators (Figure 2). Only *CsARF17* lacked the CTD, indicating that it may regulate the expression of other auxin response genes in an auxin-independent manner [52].

Eighteen CsIAA proteins, including CsIAA1-CsIAA10, CsIAA12-CsIAA17, CsIAA19, and CsIAA25, contained all four highly conserved domains (domains I, II, III, and IV). However, some CsIAA genes lacked domain I, II, or IV, whereas others contained only one or two of these conserved domains (Figure 2; Additional file 1: Table S1-2; Additional file 3: Figure S2-2). *CsIAA24, CsIAA26, and CsIAA33* did not contain domain I, an active repression domain that was transferable and dominant over activation domains. Five CsIAA proteins, including CsIAA11, CsIAA12, CsIAA20, CsIAA21, and CsIAA33 lacked
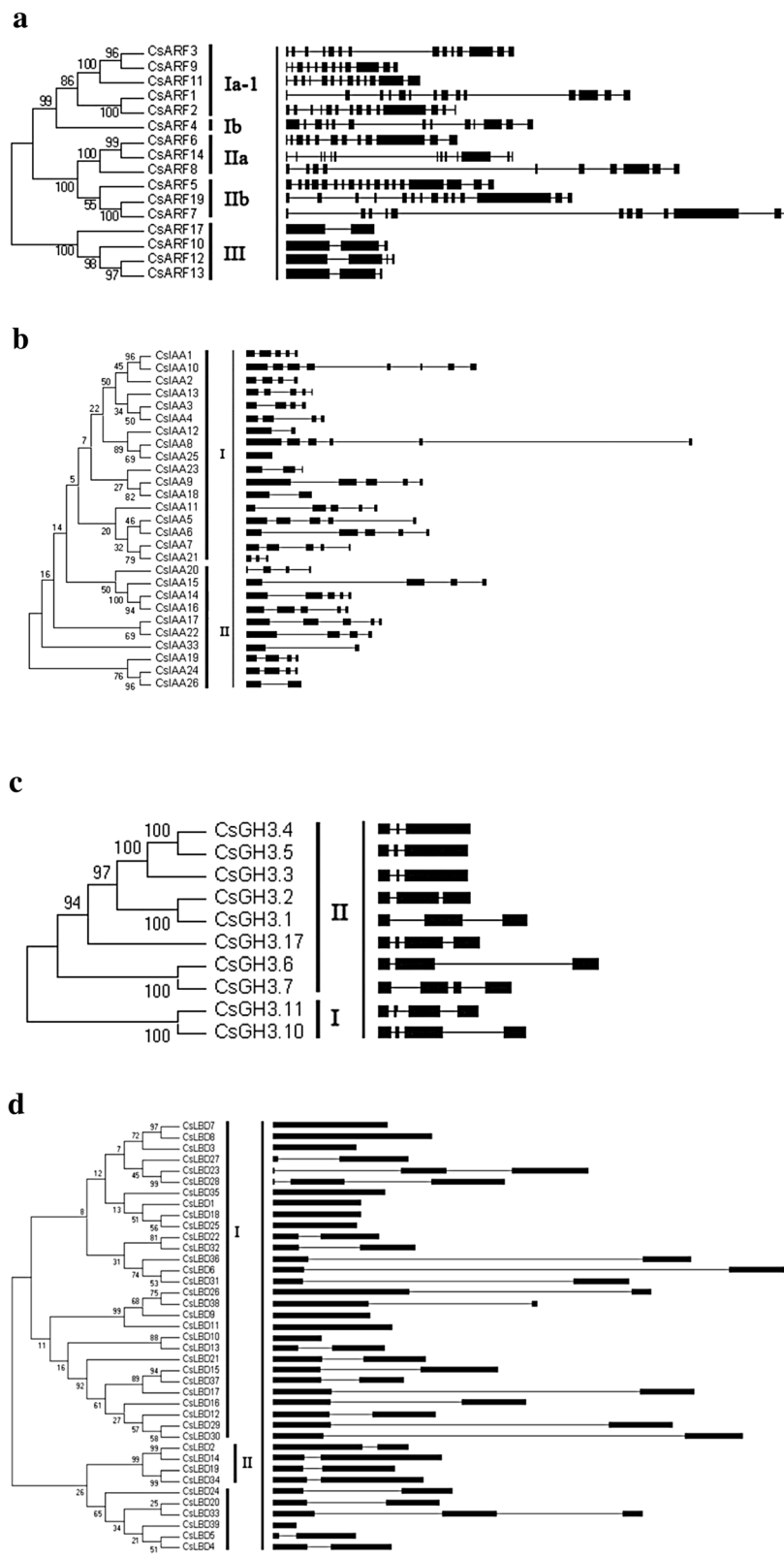
Figure 1 (See legend on next page.)

(See figure on previous page.)
Figure 1 The phylogenetic relationships and gene structure of cucumber auxin response-related genes. The left part in different panel illustrates the relationships among the cucumber ARF **(a)**, AUX/IAA **(b)** GH3 **(c)**, LBD **(d)** proteins. The unrooted phylogenetic tree was generated using MEGA4.1 through the neighbor joining method. Bootstrap values (above 50%) from 1,000 replicates are indicated at each branch. The right part illustrates the exon–intron organization of ARF **(a)**, AUX/IAA **(b)** GH3 **(c)**, LBD **(d)** family genes. The exons and introns are represented by black boxes and lines, respectively.

domain II, which plays important functions in protein stability [8,17-20]. Furthermore, domain II is responsible for the degradation of AUX/IAA proteins by physically interacting with TIR1 under a high level of auxin [53]. Previous studies demonstrated that the half-lives of proteins without domain II were much longer than those of canonical Aux/IAA proteins [54]. Similarly, two and six non-canonical AUX/IAA genes were found in tomato and *Arabidopsis*, respectively [55]. The expression levels of these non-canonical Aux/IAA genes reported so far were low in *Arabidopsis* and tomato. These genes might be relatively insensitive to IAA treatment, indicating that they might have a specific function in mediating auxin signaling during well-defined plant developmental events [53-56]. However, no consistent roles can be assigned to these Aux/IAA proteins that lack domain II until now [54]. The deduced CsIAA20 and CsIAA21 might be pseudogenes because they only contain domain IV. No information about their expression is available.

No previously-known conserved motifs or domains were found in cucumber GH3 and SAUR proteins (Figure 2; Additional file 1: Table S1-3, 4; Additional file 3: Figure S2, 3, 4). A similar finding was reported in previous studies [18]. However, the central regions of GH3 and SAUR proteins in rice and *Arabidopsis* are highly conserved [18,54]. Our data also showed that CsGH3 proteins contained the conserved central regions in cucumber (Additional file 3: Figure S2-3), implying that they might be essential and might be performing similar functions. Similarly, five putative motifs were identified from 61 CsSAURs. Interestingly, all deduced SAUR proteins contained motifs 1 and 2 (Additional file 3: Figure S2-4), indicating that these two motifs were extremely conserved during the evolutionary history of different species and that they are essential for SAUR functions. Motifs 3 to 5 can only be found in about half of the members, indicating their distinct origin and function (Additional file 3: Figure S2-4).
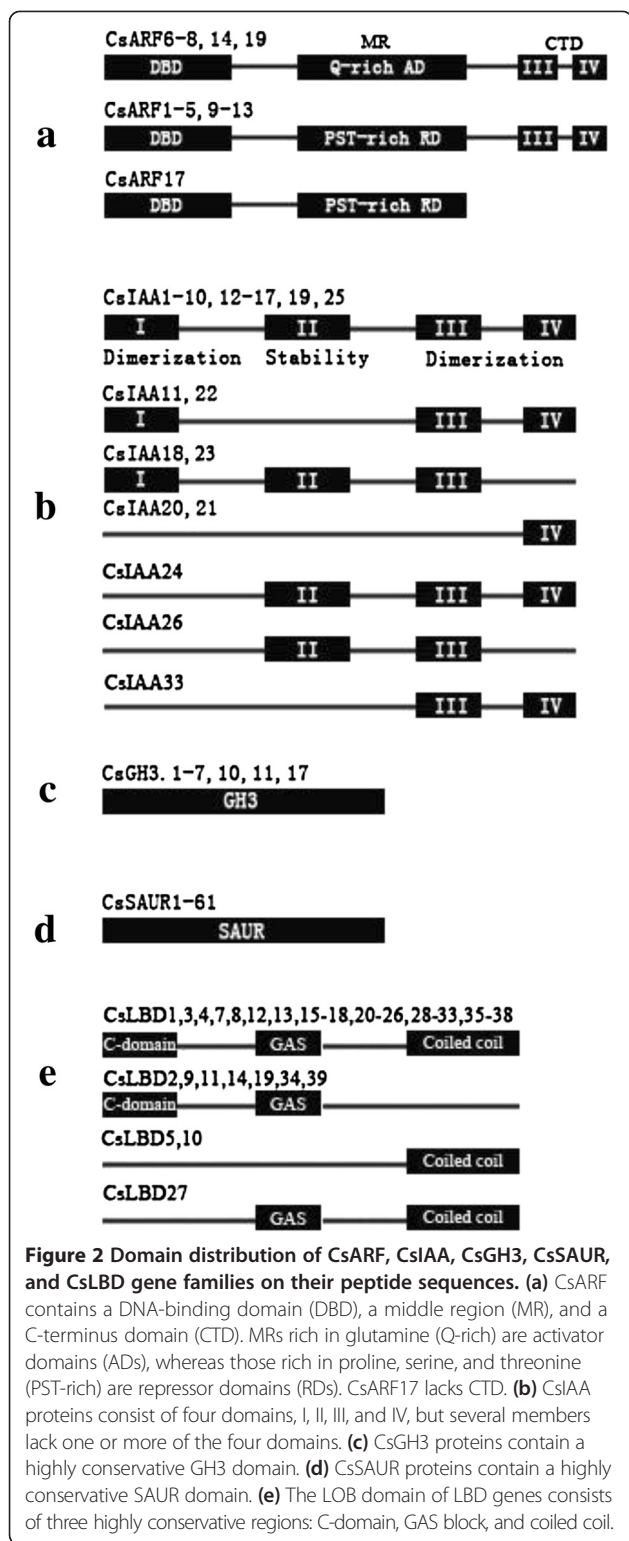
The LBD gene family encodes proteins harboring a conserved plant-specific LOB domain [20]. In cucumber, 29 CsLBD proteins all contained highly conserved regions within the LOB domain, including a conserved four-Cys motif (C-domain), a GAS block, and a leucine zipper-like coiled-coil motif. Previous studies showed that the LBD genes classified to class II all lack the coiled-coil motif in their LOB domain [20-22]. In the present study, four CsLBD genes in class II were found to lack the coiled-coil region:

CsLBD2, CsLBD14, CsLBD19, and CsLBD34 (Figure 2; Additional file 3: Figure 2-5). Unexpectively, CsLBD9, CsLBD11, and CsLBD39 belonging to class I also lacked the coiled-coil region (Figure 2; Additional file 3: Figure S2-5). Unfortunately, limited functional information is available for these non-canonical genes in the LBD family.

Chromosomal distribution and tandem duplication

The chromosomal locations of all auxin-related genes were determined and demonstrated using BLASTN analysis on the cucumber genome database. The distribution and density of the auxin-responsive genes on chromosomes were not uniform. Cucumber *ARF*, *SAUR*, and *LBD* genes were present on all seven chromosomes (Figure 3; Additional file 1: Tables S1-1, 4, and 5), and the *CsIAA* genes were present on all chromosomes, except on chromosomes 2 and 4. Conversely, the *CsGH3* genes were only localized on chromosomes 2, 3, 4, and 6. A total of 19 *CsSAUR* genes were localized on chromosome 2, 18 of which clustered on the same region. Similarly, 16 out of the 20 *CsSAUR* genes localized on chromosome 7 formed a cluster (Figure 3). Moreover, all members of these two clusters were also gathered into three clades on the phylogenetic tree (Additional file 2; Figure S1-4, colored in blue). Hence, tandem duplications might have had a crucial function in the evolution of the CsSAUR gene family. In tomato, our previous study found that eight gene clusters were located physically near each other in four chromosomes because many tandem-duplications were present in the tomato genome.

Large-scale or whole-genome duplication and tandem duplications of the cucumber genome have been reported [49]. Phylogenetic analysis revealed one triplet (CsARF6/CsARF8/CsARF14) and two sister pairs (CsARF1/CsARF2 and CsARF7/CsARF19) of CsARFs, three sister pairs (CsGH3.1/CsGH3.2, CsGH3.6/CsGH3.7, and CsGH3.10/CsGH3.11) and one triplet (CsGH3.3/CsGH3.4/CsGH3.5) of CsGH3s, and three CsLBD sister pairs (CsLBD23/CsLBD28, CsLBD2/CsLBD14, and CsLBD19/CsLBD34) in the phylogenetic tree. However, when all sister pairs and triplets were compared with their corresponding chromosomal locations, none of these sister pairs were genetically linked, except for *CsGH3.4* and *CsGH3.5*. The clades of CsIAAs with relatively strong bootstrap support (>90%), such as CsIAA1/CsIAA10, CsIAA14/CsIAA15/CsIAA16, and CsIAA24/CsIAA26, were also located in different

Figure 2 Domain distribution of CsARF, CsIAA, CsGH3, CsSAUR, and CsLBD gene families on their peptide sequences. (a) CsARF contains a DNA-binding domain (DBD), a middle region (MR), and a C-terminus domain (CTD). MRs rich in glutamine (Q-rich) are activator domains (ADs), whereas those rich in proline, serine, and threonine (PST-rich) are repressor domains (RDs). CsARF17 lacks CTD. **(b)** CsIAA proteins consist of four domains, I, II, III, and IV, but several members lack one or more of the four domains. **(c)** CsGH3 proteins contain a highly conservative GH3 domain. **(d)** CsSAUR proteins contain a highly conservative SAUR domain. **(e)** The LOB domain of LBD genes consists of three highly conservative regions: C-domain, GAS block, and coiled coil.

chromosomes or far apart on the same chromosome. Based on these results, we can conclude that the entire genome or the chromosomal segment duplications are the main factors responsible for the expansion of the CsARF, CsIAA, CsGH3, and CsLBD gene families.

Evolutionary analysis of the ARF, Aux/IAA, SAUR, GH3, and LBD gene families
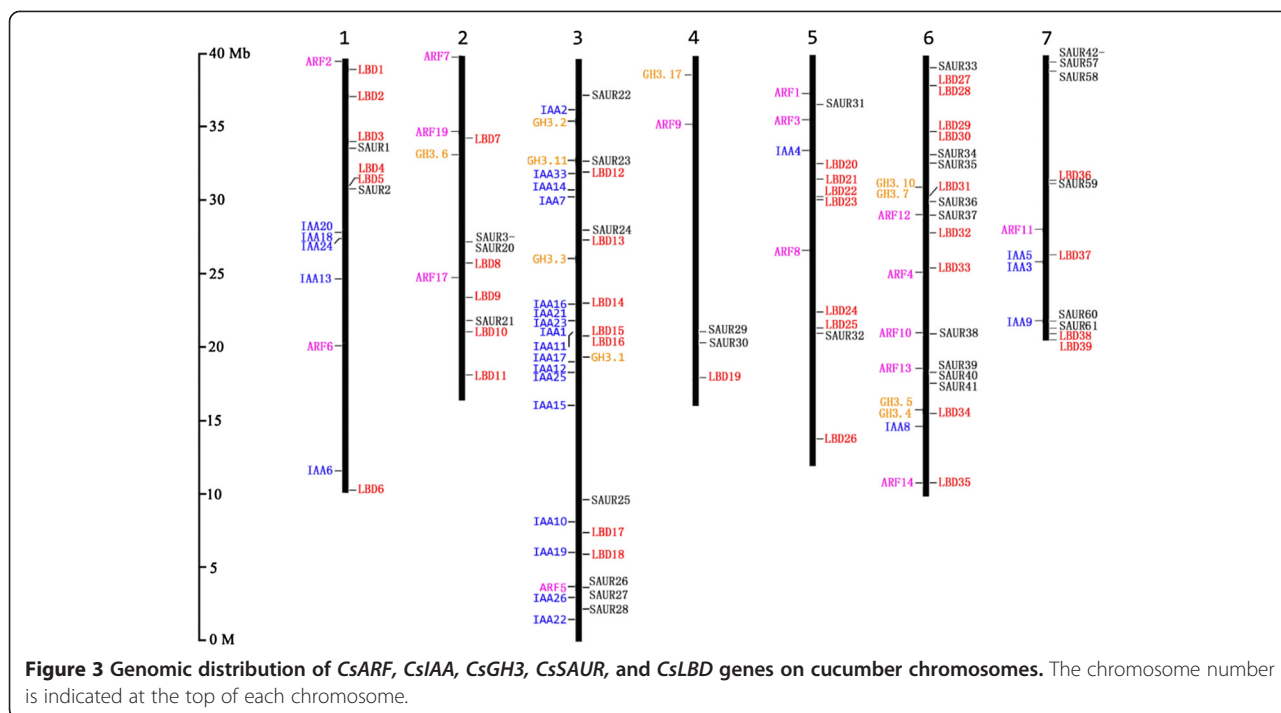
To investigate the evolutionary relationships of the auxin response proteins in different species, the full-length protein sequences of auxin response genes from cucumber and other species, such as *Arabidopsis*, rice, maize, sorghum, and tomato, were used to build the phylogenetic trees. All 140 ARF proteins from the six species (tomato, rice, maize, sorghum, cucumber, and *Arabidopsis*) can be classified into four major groups (classes I to IV). Class I can be further divided into classes 1a-1, 1a-2, and 1-b, whereas class II can also be further divided into classes IIa and IIb (Additional file 2: Figure S1-1). Similar results were found in our previous study [26].

All 144 IAA genes from rice, maize, sorghum, cucumber, and *Arabidopsis* were divided into two classes (classes I and II) in accordance with a previous study [27] (Additional file 2: Figure S1-2). According to previous study [18], 60 GH3 genes from rice, sorghum, cucumber, and *Arabidopsis* were classified into three major classes (classes I to III) (Additional file 2: Figure S1-3). A total of 133 SAUR genes, including 61 CsSAURs and 73 AtSAURs, were divided into two classes based on the phylogenetic relationship and the methods reported in a previous study [19] (Additional file 2: Figure S1-4). Up to 39 CsLBD genes and 42 AtLBD genes were divided into two classes according to the method of Majer and Hochholdinger [20] (Additional file 2: Figure S1-5).

Classification of the auxin response genes from phylogenetic trees revealed that most classes or subclasses contained genes from different species, implying that these genes originated prior to species differentiation (Additional file 2: Figure S1). However, one class of GH3 (class III) and one subclass of ARFs (class Ia-2) (Additional file 2: Figure S1-1; Additional file 2: Figure S1-3) only contained genes from the *Arabidopsis* genome. This result, which is consistent with previous studies [18,26], indicating that these genes were generated over the long-term evolution of *Arabidopsis* and may have species-specific functions. Some clades contained sequence representatives from *Arabidopsis*, tomato, and cucumber, but not from rice and sorghum. The combined phylogenetic analysis revealed eight triplets and four sister pairs of ARF family genes among rice and sorghum, as well as one triplet and six sister pairs among *Arabidopsis*, tomato, and cucumber. However, only one sister pair (OsARF14/AtARF14) was found in the ARF gene family between monocots and dicots, indicating that the auxin response genes experienced significant evolution for a long period after the divergence of monocots and dicots.

Expression profiles of the five gene families

Transcript abundance in particular organs at a given time is an important factor in elucidating the function of a

Figure 3 Genomic distribution of *CsARF, CsIAA, CsGH3, CsSAUR,* and *CsLBD* genes on cucumber chromosomes. The chromosome number is indicated at the top of each chromosome.
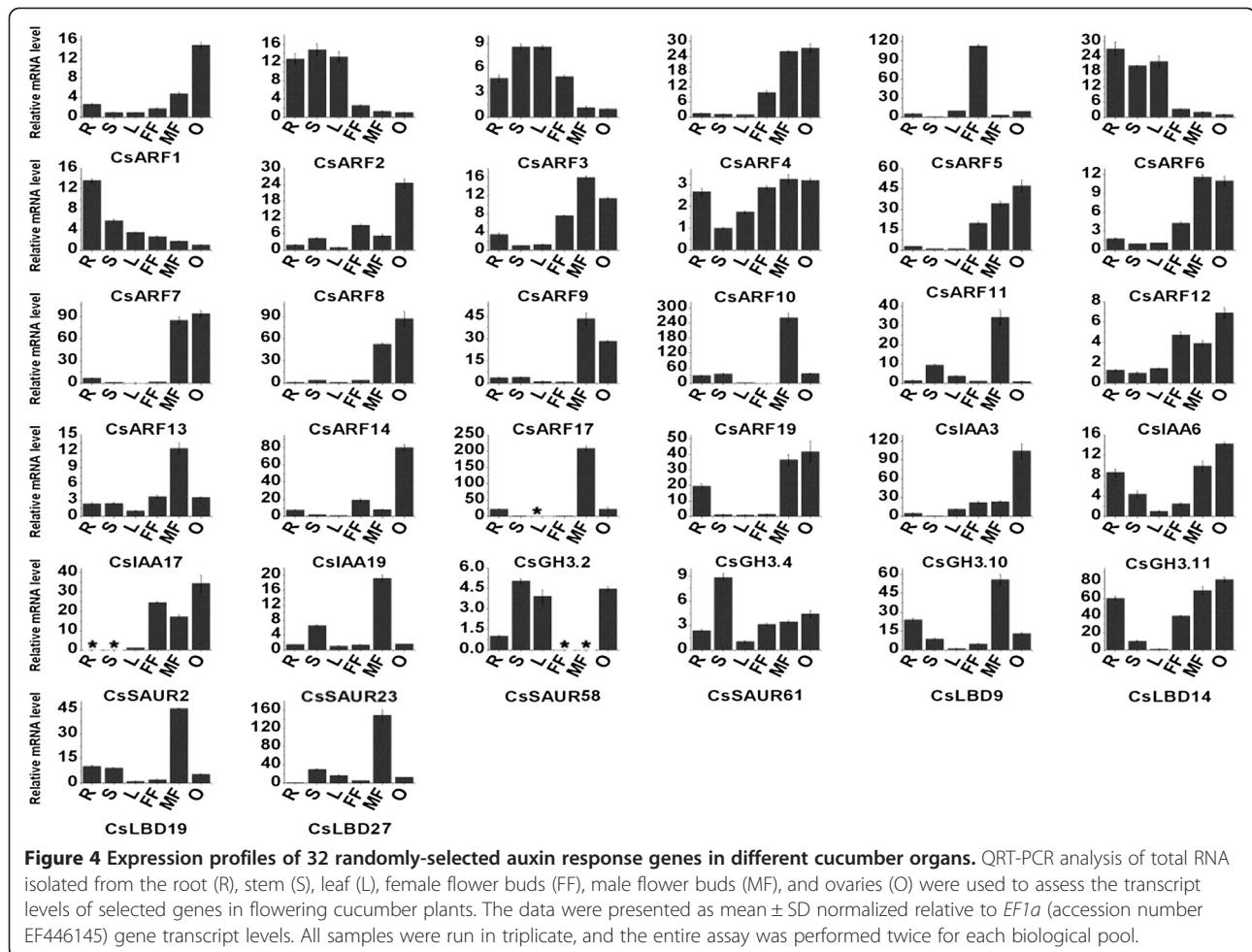
corresponding protein required in developmental, metabolic, and signaling processes. Although the expression of most of the 32 selected auxin response genes can be detected in most selected organs, their expression levels varied considerably. *CsARF1* and *CsGH3.19* were mainly expressed in cucumber ovaries (Figure 4), implying that they might have important functions in the development of the ovary. *CsARF9, CsARF17, CsARF19, CsIAA3, CsIAA17, CsGH3.2, CsSAUR23, CsLBD9, CsLBD19,* and *CsLBD27* might have crucial functions in male flowers because of their higher expression levels in this organ than in other organs (Figure 4). *CsARF5* was mainly expressed in female flowers; thus, it may have a crucial function in the development of female flowers (Figure 4). *CsARF2, CsARF3, CsARF6,* and *CsARF7* may have more important functions during the vegetative growth of cucumber plant because they are mainly expressed in vegetative organs (roots, stems, and leaves) (Figure 4). By contrast, *CsARF4, CsARF9, CsARF11, CsARF12, CsARF13, CsARF14, CsARF17,* and *CsIAA6* may be more important during reproductive growth (Figure 4).

During fruit development, 19 auxin responsive genes, including *CsARF1-8, CsARF10, CsARF14, CsARF19, CsIAA3, CsIAA6, CsGH3.10, CsGH3.11, CsSAUR2, CsSAUR23, CsSAUR61, CsLBD14,* and *CsLBD27* experienced mRNA accumulation during ovary or young fruit development. However, these genes showed a relatively low expression level during the subsequent fruit development (Figure 5). This result indicates that these genes mainly function in ovary or early fruit development. *CsARF9* and *CsGH3.2*

were expressed mainly at 9 days after pollination (DAP). The relative mRNA level of *CsARF17* at 9 DAP was much higher than that at other stages (Figure 5). The three aforementioned genes might have stage-specific functions. *CsARF11, CsARF12, CsARF13, CsARF19, CsIAA17, CsIAA23, CsSAUR58 CsLBD9,* and *CsLBD58* showed relatively high expression levels at all selected stages (Figure 5), implying that they might be functioning during whole fruit development.

Although the ARFs and primary auxin response genes in *Arabidopsis*, rice, sorghum, and tomato are induced by exogenous auxin, they display differential expression patterns [18,19,23,26,27,57,58]. In cucumber, *CsARF3-8, CsARF14, CsIAA3, CsIAA26, CsGH3.4, CsSAUR58,* and *CsSAUR61* were up-regulated by over four-fold, whereas *CsARF1, CsARF19, CsIAA6, CsLBD14,* and *CsLBD27* were drastically down-regulated after IAA treatment in young leaves (Figure 6). Our promoter analysis revealed that two types of auxin-responsive elements, (AuxREs)-S00026 and -S000270, were identified in the promoter region of most of the primary auxin response genes, except in *CsIAA1, CsIAA3, CsIAA10, CsSAUR6, CsSAUR28, CsSAUR31, CsSAUR61, CsLBD17,* and *CsLBD24* (Additional file 4; Table S2). The diversity of numbers and locations of the auxin signaling transduction-related cis-elements may partially account for the different expression patterns of cucumber auxin response genes under IAA treatment. However, although none of the auxin signaling transductions-related cis-elements were found in the promoter regions of *CsIAA3* and *CsSAUR61* (Additional file 4: Tables S2-2 and 4),

Figure 4 Expression profiles of 32 randomly-selected auxin response genes in different cucumber organs. QRT-PCR analysis of total RNA isolated from the root (R), stem (S), leaf (L), female flower buds (FF), male flower buds (MF), and ovaries (O) were used to assess the transcript levels of selected genes in flowering cucumber plants. The data were presented as mean ± SD normalized relative to *EF1a* (accession number EF446145) gene transcript levels. All samples were run in triplicate, and the entire assay was performed twice for each biological pool.

the mRNA levels of *CsIAA3* and *CsSAUR61* significantly increased after IAA treatment (Figure 6).

The mRNA levels of all five GH3 genes of Group II in *Arabidopsis* were up-regulated by exogenous auxin, suggesting that Group II-mediated auxin conjugation is a specific response to auxin application [40,59]. In the present study, the expression level of *CsGH3.4* belonging to Group II increased significantly after IAA treatment (Figure 6). However, the mRNA levels of *CsGH3.2* also belonging to group II showed no obvious change after IAA treatment (Figure 6). These results may reflect the functional divergence in the GH3 gene family between *Arabidopsis* and cucumber.

Increasing evidence proved that the auxin response genes are involved in stress/defense responses and that various environmental signals are integrated into changes in auxin homeostasis, redistribution, and signaling [60,61]. In the present study, promoter region analysis revealed that not only auxin-responsive elements (AuxREs) were found in the promoter regions of ARF, IAA, SAUR, GH3, and LBD family members (Additional file 4: Table S2). That is, many cis-elements in other signaling transduction

pathways, such as drought-, salt-, and heat stress-related cis-elements, light signal transduction related cis-element, Ca^{2+}-responsive cis-element, and calmodulin-binding/CGCG box, were also found. These results imply that these genes might function in connecting the auxin signaling transduction pathway with other signaling transduction pathways.

The GH3s were previously suggested to be linkers among the auxin, JA, and salicylic acid (SA) signal transduction pathways [62]. *AtGH3.11* and *AtGH3.10* are both members of group I. *AtGH3.11* can adenylate JA in vitro, but *AtGH3.10* shows no adenylation activity [21]. Many GH3 genes in *Arabidopsis*, soybean, and tobacco were found to be differentially expressed in various tissues in response to exogenous auxin and light stimuli [1,50,63]. In cucumber, all four selected GH3 genes were slightly induced by JA treatment (Additional file 5; Figure S3a). Only *GH3.11* mRNA was increased by approximately one-fold at 3 h after JA treatment. GH3 genes also affect SA signaling; for instance, GH3.5 in *Arabidopsis* was proposed to be a positive regulator of SA signaling [64,65]. Our research found that the expression
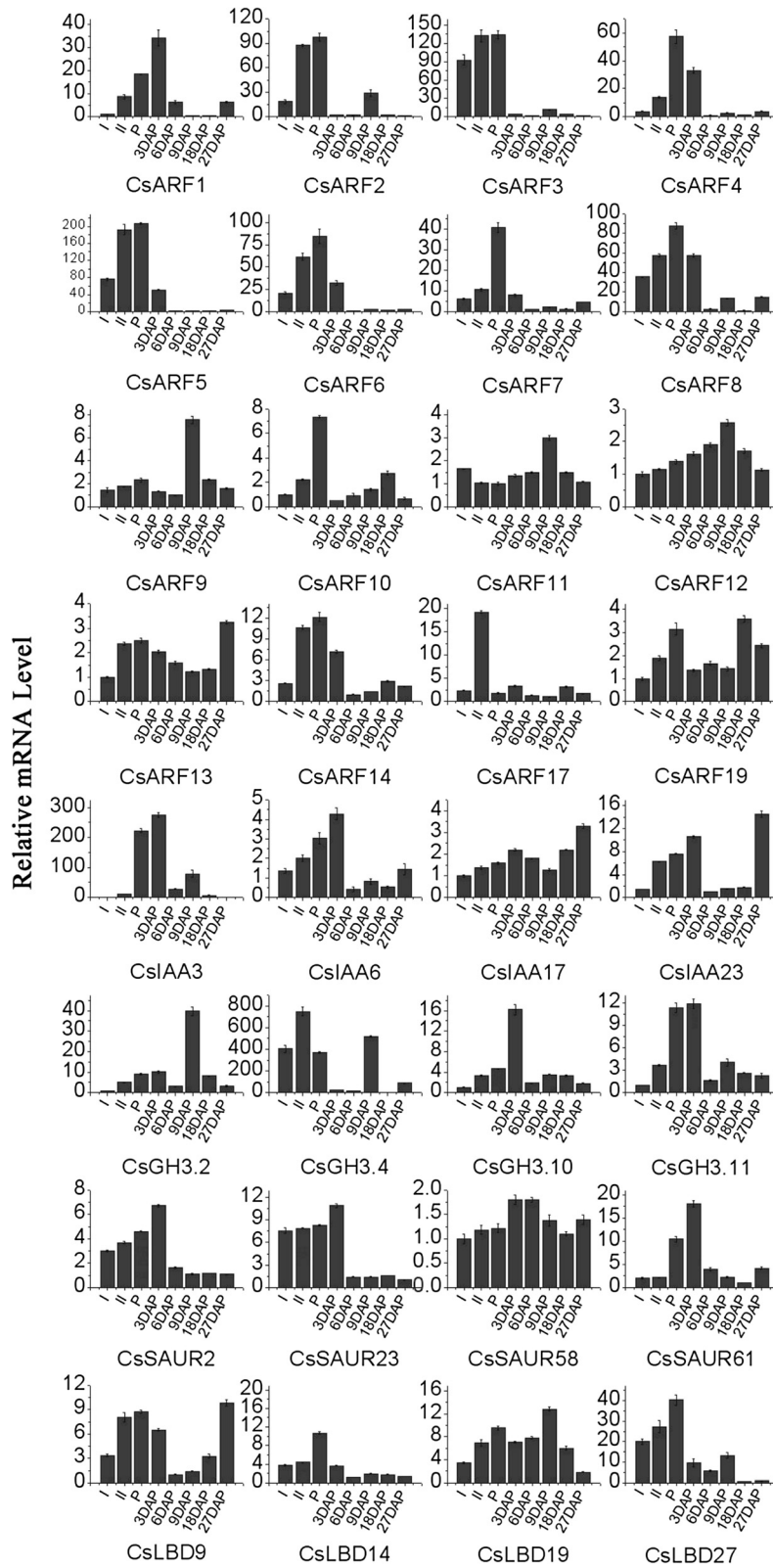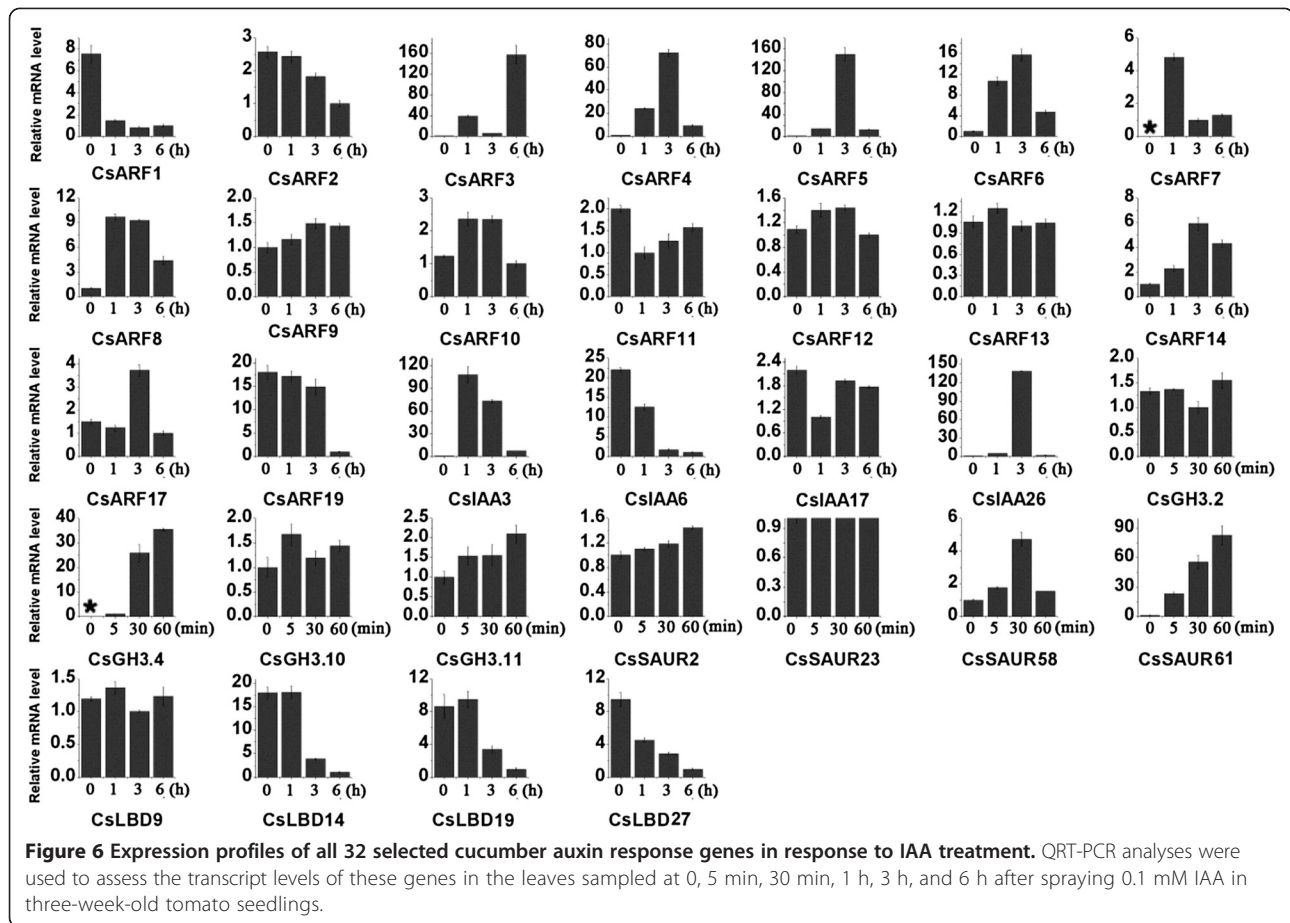
Figure 5 Expression profiles of all the 32 cucumber auxin response genes during cucumber fruit development using qRT-PCR. The ovary and fruits were sampled at eight stages, including two stages before pollination (Stages I and II: The ovary was approximately 0.3 cm and 1.5 cm in length, respectively), the pollination stage (P), and five fruit developmental stages [3, 6, 9, 18, and 27 days after pollination (DAP)]. For more details, see Figure 3.

Figure 6 Expression profiles of all 32 selected cucumber auxin response genes in response to IAA treatment. QRT-PCR analyses were used to assess the transcript levels of these genes in the leaves sampled at 0, 5 min, 30 min, 1 h, 3 h, and 6 h after spraying 0.1 mM IAA in three-week-old tomato seedlings.

level of *CsGH3.4* was up-regulated by more than twofold after SA treatment (Additional file 5; Figure S3b). Considering that *CsGH3.4* can also be up-regulated by auxin treatment (Figure 6), we suggest that *CsGH3.4* plays a specific role in the integration of auxin-SA signaling transduction pathways.

Conclusion

Auxin controls a wide range of plant growth and development processes. In the present study, we carried out a genome-wide survey of auxin response-related gene including ARF, Aux/IAAs, GH3s, SAURs, and LBDs in cucumber (*Cucumis sativus* L.). Their gene structure, phylogenetic relationship, conserved motif, chromosomal location, promoter region and their expression profiles were also presented. Gene structure analysis revealed that most of the auxin-responsive genes had a conserved intron/exon structure, whereas some were more divergent, suggesting the possibility of functional diversification for these genes. Most of these genes possess auxin-responsive elements in their promoter region. Quantitative real-time RT-PCR analysis showed that the *CsARFs*, *CsAUX/IAAs*, *CsGH3s*, *CsSAURs*, and *CsLBDs* genes were expressed in at least one of the cucumber organs or tissues. However, different members of

auxin-response genes displayed distinctive expression patterns in different cucumber organs and tissues. Furthermore, most of the detected auxin response genes were up-regulated during early fruit development. Some were expressed in a developmental stage-specific manner. Most tested genes were up-regulated by exogenous treatment with auxin, JA, or SA. However, the genes showed varying dynamic expression patterns. Our data imply that the auxin response genes may be involved in various vegetative and reproductive developmental processes and may have different functions during plant development. Characterization of selected members of these five families in cucumber is underway in our laboratory so that we can accurately determine the molecular basis of auxin regulation.

Methods

Searching for auxin response genes

To find previously identified and all potential auxin response genes in cucumber, we initially surveyed the cucumber Genomics Database (http://www.icugi.org/cgi-bin/ICuGI/genome/index.cgi?organism=cucumber) through TBLASTN using the protein sequences of the previously known auxin response genes as queries. The query

consisted of 100 ARFs, 91 IAAs, 199 SAURs, and 49 GH3s sequences from *Arabidopsis*, rice, maize, sorghum, and tomato. Meanwhile, 42 AtLBDs and 36 SbLBD from *Arabidopsis* and sorghum were used for searching the LBD family genes. All predicted peptide sequences identified in this initial search were used as query in the BLASTP searches against the Cucumber Genomics Database and NCBI to find their potential functional domains, such as AUX_RESP (PF06507.5), Aux/IAA (PF02309.8), GH3 (PF03321.5), or DUF260 (PF03195.6). The Pfam 26.0 database was used to confirm the presence of auxin response-related domains in the predicted auxin response genes under a E-value level of 1.0 (http://pfam.sanger.ac.uk/). The genes without anticipant domains were removed. Based on the combined results from all of the performed searches, we identified all members of auxin response-related genes in the currently available cucumber genomic databases.

Mapping auxin response genes on cucumber chromosomes

To determine the location of all auxin response genes on chromosomes, the nucleotide sequences of these genes were further used as query sequences for the BLASTN search against cucumber whole genome Scaffolds data (version 2) (http://www.icugi.org/cgi-bin/ICuGI/genome/blast.cgi?organism=cucumber&ver=2). Finally, the locations of all the cucumber auxin response genes were detected.

Subcellular localization prediction of each of these family genes was carried out using the CELLO v2.5 server (http://cello.life.nctu.edu.tw/) [66].

Gene structure analysis, multiple-sequence alignments, and phylogenetic analysis

To detect the intron/exon structure, the coding sequences (CDS) of auxin response-related genes were aligned with their corresponding genomic sequences using spidey tool available on NCBI (http://www.ncbi.nlm.nih.gov/spidey/). The nature of the predicted protein such as PI and molecular weight were predicted by ProtParam tool available on Expert Protein Analysis System (ExPASy) proteomics server (http://web.expasy.org/protparam/). ClustalX v1.81 was used for multiple sequence alignments [67]. Phylogenetic relationship analysis was performed using MEGA 4.1 through the neighbor-joining method [68]. The Multiple Expectation Maximization for Motif Elicitation utility was employed to detect conserved motifs of cucumber auxin response family genes (http://meme.nbcr.net) [69].

To investigate cis-elements in the promoter sequences of cucumber auxin response genes, 2000 bp of genomic DNA sequences upstream of the initiation codon (ATG) were downloaded from the SGN database (Additional file 6: Figure S4). The PLACE website (http://www.dna.affrc.go.jp/PLACE/) was employed in the identification of *cis*-regulatory elements in the promoters [69].

Plant growth conditions in relation to IAA, JA, and SA treatments

The cucumber (*Cucumis sativus* L. cv. Jianyan) plants used for expression analysis were grown in a growth chamber under 28°C/18°C (day/night) with a 16h photoperiod. The roots, stems, leaves, female flower buds (approximately 3 d before anthesis, excluding the ovary), male flower buds (approximately 1.0 cm in length), and ovaries (3 d before anthesis) were collected from flowering cucumber plants.

To analyze the expression patterns of cucumber auxin response genes at different developmental stages, cucumber ovaries or fruits were collected at the following eight developmental stages: ovary initiation stage (approximately 0.3 cm length, stage I), ovary elongating stage (approximately 1.5 cm length, 3 d before pollination, stage II), beginning of fruit development (0day after pollination, DAP, stage III), fruit early growing stage (3 DAP, stage IV), middle developmental stage (6 DAP, stage V), marketable maturing stage (9 DAP, stage VI), seed developmental stage (18 DAP, stage VII), and seed maturing stage (27 DAP, stage VIII, the fruits totally turned yellow). All flowers for each experiment were hand-pollinated on a single date.

Three-week-old cucumber seedlings with three fully opened leaves were sprayed with 0.1 mM IAA, 0.1 mM methyl JA (Sigma-Aldrich, WI, USA), or 1.5 mM SA on the seedling leaves. The plants were sampled at 0 min, 5 min, 30 min, 1 h, 3 h, and 6 h after auxin treatment and 0, 1 h and 3 h after JA and SA treatments. The experiment was repeated three times, and 15 seedlings were used in each treatment in each replication. All materials were stored at −80°C.

Expression analysis of auxin response genes using qRT-PCR

Thirty-two auxin response genes belonging to five families were selected based on the phylogeny trees so that the expression profile of at least one gene of each branch in the phylogeny trees would be checked using qRT-PCR techniques. The primer pairs were listed in Additional file 7: Table S3 and the specificity of each primer to its corresponding gene was checked using the BLASTN program of the cucumber genome database. A sample of cDNA (1 µg) was subjected to RT-PCR in a final volume of 20 µl containing 12.5 µl SYBR Green Master Mix Reagent (Takara, Japan) and specific primers (3 pmol). Two biological and three technical replicates for each sample were performed in the RT-PCR machine (BIO-RAD CFX96, USA). To normalize the total amount of cDNA

present in each reaction, the *EF1a* gene (accession number EF446145) was co-amplified as an endogenous control for the calibration of relative expression, The C_t method of relative gene quantification recommended by Applied Biosystems (PE Applied Biosystems, USA) was used to calculate the expression level of different treatments.

Additional files

Additional file 1: Table S1. Summary of ARF, AUX/IAA, GH3, SAUR and LBD family genes in cucumber.

Additional file 2: Figure S1. Phylogenetic relationships of ARF, AUX/IAA, GH3, SAUR and LBD gene families between cucumber and some other plant species.

Additional file 3: Figure S2. Multiple sequence alignments of the full-length proteins of CsARF, CsAUX/IAA, CsGH3, CsSAUR and CsLBD in cucumber obtained with Clustal and manual correction.

Additional file 4: Table S2. Cis-elements in the promoters of *CsARF, CsAUX/IAA, CsGH3, CsSAUR* and *CsLBD* genes in cucumber.

Additional file 5: Figure S3. Expression profiles of four selected CsGH3 genes in response to JA and SA treatment. QRT-PCR analyses were used to assess the transcript levels of these genes in JA **(a)** and SA **(b)** treated plants. The leaves were sampled at 0 h, 1 h and 3 h after spraying 100 µM MeJA **(a)** and 1.5 mM SA, respectively, in 3-week tomato seedlings.

Additional file 6: Figure S4. Promoter regions of *CsARF, CsAUX/IAA, CsGH3, CsSAUR* and *CsLBD* genes in cucumber.

Additional file 7: Table S3. Primer sequences for qRT-PCR expression analysis.

Abbreviations
ARF: Auxin response factors; Aux/IAA: Auxin/indole-3-acetic acid; AuxRE: Auxinresponsive cis-element; DBD: DNA-binding domain; GH3: Gretchen Hagen 3; LBD: Lateral organ boundaries; MEME: Multiple Expectation Maximization for Motif Elicitation; NLS: Nuclear localization signal; QRT–PCR: Quantitative reverse transcription–PCR; SAUR: Small auxin up mRNA; SGN: Solanaceae Genomics Network; TAIR: The Arabidopsis Information Resource.

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

Authors' contributions
J W performed all the bioinformatics analysis and drafted the manuscript; LSY carried out the qRT-PCR analysis and promoter analysis; XG helped in bioinformatics analysis and data mining; LC finished the hormone treatment; YH helped in expression analysis; J W participated in data analysis and writing the paper; GL designed the study and drafted the manuscript. All authors read and approved the final manuscript.

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