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ORIGINAL PAPER

Cloning and expression analysis of *Cs-TIR1/AFB2*: the fruit development-related genes of cucumber (*Cucumis sativus* L.)

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Abstract Phytohormone auxin plays an important role in fruit development and is perceived by the TIR1/AFB family of F-box proteins as auxin receptors involved in auxin signal pathway. Cucumber (*Cucumis sativus* L.) fruit development is either parthenocarpic or non-parthenocarpic. However, little is known on *TIR1* and *AFB* participation in the early stage of cucumber fruit development. In present study, *TIR1* and *AFB2* were isolated from cucumber. *CsTIR1* and *CsAFB2* were highly expressed in leaves and ovaries. Their transcript levels decreased in parthenocarpic and pollinated fruits, but continuously up-regulated in aborted fruits, indicating that down-regulation of *CsTIR1* and *CsAFB2* may be in favor of cucumber fruit set and development. The transcript levels of *CsTIR1* and *CsAFB2* were significantly induced in leaves by NAA, 6-BA, GA_3, ABA, and ethephon. The expression levels were up-regulated by ABA and ethephon treatments. This expression patterns was accordant with the aborted fruits. Thus, *CsTIR1* and *CsAFB2* may be important regulators during cucumber fruit development.

Keywords Cucumber · Parthenocarpy · Fruit development · *CsTIR1* · *CsAFB2*

Abbreviations

CPPU	*N*-(2-ehloro-4-Pyidyl)-*N*′-Phenylurea
TIR1	Transport inhibitor response 1
AFB	Auxin signaling F-box
NAA	a-Naphthalene acetic acid
6-BA	6-Benzylaminopurine
GA_3	Gibberellin
ABA	Abscisic acid
ORF	Open reading frame
UTR	Untranslated region
SKP1	S-Phase kinase-associated protein 1
SAPK7	Serine/threonine-protein kinase 7

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Introduction

Cucumber (*Cucumis sativus* L.) belongs to the Cucurbitaceae family and is an important vegetable crop (Jeffrey 2008). After tomato (*Solanum lycopersicum*), the recently completed cucumber genome has been added to the existing crop database and a new model is being used for fruit development (Huang et al. 2009). Cucumber is an excellent plant to study fruit set and development, as its genotypes have different parthenocarpic capacities. Fruit development can be uncoupled from fertilization and seed development, and parthenocarpic fruits are seedless (Fos and Nuez 1996; Talon et al. 1992; Varoquaux et al. 2000). Parthenocarpy is a potentially desirable trait for many commercially grown fruits to avoid undesirable changes in structure, flavor, or nutrition proprieties (Martinelli et al. 2009).

Applying auxin and cytokinin to unpollinated cucumber ovaries (Kim et al. 1992; Fu et al. 2010), or overexpression of auxin biosynthesis gene in ovaries can promote the formation of fertilization-independent or parthenocarpic fruit (Yin et al. 2006). The increase in IAA content in cucumber ovaries is closely related to parthenocarpic fruit set (Kim et al. 1992). In addition, Auxin transport

inhibitors induce parthenocarpy in cucumber by blocking the normal movement of auxin out of the ovary, thereby causing auxin accumulation within the ovary, which is sufficient to trigger parthenocarpy (Beyer and Quebedeaux 1974). Parthenocarpy has been staged by altering auxin signaling at different steps, indicating that auxin controls fruit development.

Auxin plays a key role in plant fruit development. Since decades, auxin signaling is known to be involved in transcriptional regulation by members of the Aux/IAA and ARF protein families, which are key regulators of auxin-modulated gene expression (Guilfoyle et al. 1998a, b; Leyser 2002; Walker and Estelle 1998). A significant advance in our understanding of the auxin-dependent mechanism underlying fruit set came from transgenic manipulation of *Aux/IAA* and *ARF* genes leading to parthenocarpic fruit in both tomato and Arabidopsis. Transgenic tomato lines with down-regulated *SlIAA9* and decreased *SlARF7* expression result in parthenocarpic fruit development before fertilization (Wang et al. 2005; De Jong et al. 2009b). In addition, inactivation of ARF8 causes parthenocarpic fruit development in tomato (Goetz et al. 2006, 2007). TIR1/AFB F-box proteins were later reported as auxin receptors that facilitate interaction with Aux/IAA proteins in an auxin-dependent manner (Dharmasiri et al. 2005b; Mockaitis and Estelle 2008). Aux/IAAs are targeted for degradation by ubiquitination and catalyzed by an SCF-type ubiquitin-protein ligase (Gray et al. 2001). TIR1 and its paralogs, AFB1, 2, 3, and 4 are the F-box subunits of the SCF E3-ubiquitin ligase complex and function as auxin receptors. The TIR1/AFB F-box protein acts as an auxin receptor and directly links auxin detection of binding to $SCF^{TIR1/AFB}$ to the degradation of the Aux/IAA proteins (Dharmasiri et al. 2005a, b; Kepinski and Leyser 2005b). Recently, overexpression of *SlTIR1* results in parthenocarpic fruit formation in tomato (Ren et al. 2011). These findings suggest that auxin regulates most aspects of plant growth and development by TIR1-dependent degradation of Aux/IAA proteins. However, the auxin receptor molecular mechanism has remained elusive in cucumber fruit development.

In this study, we isolated *CsTIR1* and *CsAFB2* mRNA sequences from cucumber fruit and the characteristics of the two genes were analyzed using a bioinformatics program. The expression levels of *CsTIR1* and *CsAFB2* in different tissues and early fruit developmental stages under different treatments indicated that these two genes are important regulators during fruit development. In particular, the declining levels of *CsTIR1* and *CsAFB2* played an important role in fruit initiation in natural parthenocarpic fruit. The expression levels in leaves using NAA, 6-BA, GA3, ABA and ethephon treatments were analyzed. The results indicated that the transcript levels of *CsTIR1* and *CsAFB2* were regulated by these hormones.

Materials and methods

Plant materials

Five cucumber cultivars, EC1, L8, S12533, and S12535 (strong parthenocarpic lines) and 8419s-1 (non-parthenocarpic line) were used in the experiments. Seedlings were grown in a greenhouse [12-h photoperiod, mean daily air temperatures, 29/17 °C (day/night), relative humidity, 85 %, photosynthetic photo flux density 800 µmol m^{-2} s^{-1}] at Nanjing Agricultural University. All tissues (including root, stem, leaf, flower, and fruit) were collected from 10-week-old cucumber plants. The ovaries at the 12–15th node of the main stem were isolated to prevent pollen contamination on the day before anthesis. Ovaries of the parthenocarpy cultivars were isolated only for sampling. The experiments with cultivar 8419s-1 included three treatments: non-pollination, pollination, and 100 mg L^{-1} CPPU treatment of unpollinated ovaries according to Fu et al. (2010). Other cultivars were not pollinated. Except unpollinated ovaries of L8, S12533, and S12535 were sampled at 2 days after anthesis (DAA), and other samples were harvested at 0, 2, 4, 6, and 8 DAA (8419s-1 unpollinated fruits were not obtained at 8 DAA). Samples were frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction.

Plant hormone treatment

8419s-1 seedlings were treated with different exogenous hormones. Germinated seeds were planted in nutrition pots, and 3-week-old seedlings were subjected to hormone treatments. Hormones treatments were carried out by spraying the leaves of the seedling with NAA (5, 10, and 50 µM), 6-BA (10 µM), GA_3 (10 µM), ABA (10 µM), or ethephon (10 µM) and the plants were sampled at 0, 3, 6, and 9 h after spraying. Leaf samples were frozen in liquid nitrogen and stored at −80 °C.

Cloning the full-length cDNA of *CsTIR1* and *CsAFB2* and sequence analysis

The *CsTIR1* and *CsAFB2* CDS sequences in the cucumber genome database were Blast-searched with the *SlTIR1* (GQ370812.1) and *AtTIR1* (AAF78487) sequences, and genes ID of the homologous fragments were obtained. *Csa01802* and *Csa015043* have the highest identity with *SlTIR1* and *AtAFB2*, respectively. We designed two primer pairs to amplify the full-length cDNA sequences of *Csa01802* and *Csa015043* (Table 1). The PCR products were directly sequenced (Invitrogen).

Searches for nucleotide and protein sequence similarities were completed with the BLAST algorithm at the National Center for Biotechnology Information website (http://www.

Table 1 The primer sequences of *CsTIR1* and *CsAFB2*

Primer	Primer sequence	Description
CsTIR1-F	F:CAAACCGAAATTAGGGCAACA	Gene cloning
CsTIR1-R	R:TCGATCCTCGGTTTGGTG	Gene cloning
CsAFB2-F	F:TTGGATGCTGAGAAACGATG	Gene cloning
CsAFB2-R	R:AAATTCAAAAGCCTTGATGGA	Gene cloning
CsTIR1-q-F	F:GTGCCATTGTTGAGTATTGC	qRT-PCR
CsTIR1-q-R	R:CTCCAGCTTGCGAAGACTAT	qRT-PCR
CsAFB2-q-F	F:GTCCTGCGAGATTACTCTTG	qRT-PCR
CsAFB2-q-R	R:AGACTTCTGACTGACCATTCT	qRT-PCR
Actin-F	F:TTCTGGTGATGGTGTGAGTC	qRT-PCR
Actin-R	R:GGCAGTGGTGGTGAACATG	qRT-PCR

ncbi.nlm.nih.gov/BLAST/) (Altschul et al. 1997). Sequences were aligned using the DNAMAN program, v6.0. Structural domains were annotated using Smart (http://smart.embl-heidelberg.de/; Letunic et al. 2012). The phylogenetic tree, based on homology, was constructed with the MEGA program v4.0. The TIR1/AFB protein sequences used in the phylogenetic tree can be found at GenBank under the following accession numbers: *Populus trichocarpa* PtFBL1 (XP_002123035.1), PtFBL2 (XP_002321336.1), PtFBL3 (XP_002300140.1), PtFBL4 (XP_002328871.1), *Ricinus communis* RcTIR1 (XP_002520681.1), *Vitis vinifera* VvTIR1 (XP_002269127.1), *Dimocarpus longan* DiTIR1(ACX31301.2), *Gossypium hirsutum* GhTIR1 (ABG46343.1), *Nicotiana tabacum* NtTIR1 (ACT53268.1), *S.lycopersicum* SlTIR1 (GQ370812.1), *Arabidopsis thaliana* AtTIR1 (NP_567135.1), AtAFB2 (NP_566800.1), AtAFB3 (NP_563915.1), AtAFB5 (NP_568718.1), AtAFB18 (AAK76473.1), AtGRH1 (NP_567255.1), AtCOl1 (NP_565919.1), *Oryza sativa* OsTIR1 (AAV32196.1), *Zea mays* ZmTIR1 (NP_001148131.1), *Arabidopsis lyrata* subsp. *lyrata* AIF-box2 (XP_002875343.1), AIF-box3 (XP_002892715.1), AlAFB5 (XP_002865768.1), *S. lycopersium* SlCOl1 (AAR82926.1).

RNA extraction and qRT-PCR for gene expression analysis

Total RNA was extracted from different cucumber tissues using Trizol reagent (Invitrogen), according to manufacturer's instructions. The RNA was treated with DNase I (Fermentas, UK) for 30 min at 25 °C and purified according to manufacturer's instructions. The first-strand cDNA synthesis was performed using 2 μg of total RNA and a Fermentas Reverse Transcription Kit. The expression levels of *CsTIR1* and *CsAFB2* were determined by real-time RT-PCR. The primer sequences used are listed in Table 1. For the quantification of the PCR products, *Cs-Actin* was used as control (GenBank Accession No. AB010922). Relative fold differences were calculated based on the comparative Ct method. The formula is calculated as follows: $2^{-\Delta\Delta Ct}$ according to the previous report by Audran–Delalande et al. (2012).

Determination of IAA and ZR concentration

The extraction and purification method for IAA and ZR were modified from those described by Yang et al. (2001). Samples of 8419s-1 fruits were ground in an ice-cooled mortar in 8 mL of 80 % (v/v) methanol extraction medium containing 1 mM butylated hydroxytoluene as an antioxidant. The extracts were incubated at 4 °C for 4 h and centrifuged at 3,500 rpm for 8 min at 4 °C. The supernatants were combined and passed through Chromosep C18 columns (C18 Sep-Park Cartridge, Waters Corp., Milford, MA, USA), prewashed with 10 mL of 100 % and 5 mL of 80 % (v/v) methanol, respectively. The hormone fractions were eluted with 10 mL of 100 % (v/v) methanol and 10 mL of ether and collected and dried under N_2 gas. The residues were dissolved in 2 mL phosphate buffer saline (PBS) containing 0.1 % (v/v) Tween 20 and 0.1 % (w/v) gelatin (pH 7.5) for ELISA.

The antibodies against IAA and ZR horseradish peroxidase used in ELISA were produced at the Phytohormones Research Institute (China Agricultural University). ELISA was performed in a 96-well microtitration plate. Each well was coated with 100 μL coating buffer (1.5 g L^{-1} Na_2CO_3, 2.93 g L^{-1} $NaHCO_3$, and 0.02 g L^{-1} NaN_3, pH 9.6) containing 0.25 μg mL^{-1} antigen against the hormone. The coated plates were incubated for 4 h at 37 °C and overnight at 4 °C for IAA and ZR, and then kept at room temperature for 30 min. After washing four times with PBS + Tween 20 [0.1 % (v/v)] buffer (pH 7.4), each well was filled with 50 μL of either extract IAA or ZR standards (0–200 ng L^{-1} dilution range) and 50 μL of 20 μg mL^{-1} antibody against IAA or ZR. The plates were incubated at 37 °C for 30 min, and then washed as described above. In each well, 100 μL of 1.25 μg mL^{-1} IgG-horseradish peroxidase substrate was added and incubated for 30 min at 37 °C. The plates were rinsed four times with PBS + Tween 20 buffer, and 100 μL color-appearing solution containing 1.5 mg mL^{-1} 0-phenylenediamine and 0.008 % (v/v) H_2O_2 was added to each well. The reaction was stopped by adding 50 μL 6 N H_2SO_4 per well when the 200 ng mL^{-1} standard developed a pale color, and the 0 ng mL^{-1} standard had a deep color. Color development in each well was detected using an ELISA Reader (Model EL301, Bio-TEK, Winooski, VT, USA) at optical density of A490. IAA and ZR contents were calculated following the report by Weiler et al. (1981). The results are presented as mean ± SE of three replicates.

Statistics

CsTIR1 and *CsAFB2* mRNA levels were determined as mean ± SE. Differences among treatments were analyzed by one-way ANOVA. *P* values ≤0.05 and ≤0.01 was considered significant according to Tukey's multiple range tests.

Results

Isolation of the full-length *CsTIR1* and *CsAFB2* cDNA and genome sequence

A 2,243 bp full-length cDNA of the putative *SlTIR1*-like gene and a 2,479 bp full-length cDNA of the putative *AtAFB2*-like gene (*CsTIR1* and *CsAFB2*, respectively) were obtained by sequencing and were deposited in GenBank under accession numbers JX901282 and JX901283, respectively. The *CsTIR1* and *CsAFB2* cDNAs contained 1,755- and 1,764-bp open reading frames and encoded proteins of 587 and 620 amino acid residues, respectively. The genomic organizations of *CsTIR1* and *CsAFB2* were analyzed (Figs. 1a and 2a). The result showed that *CsTIR1* contained an F-box region and six leucine-rich repeat (LRR) domains (Fig. 1b), while *CsAFB2* contained an F-box and seven LRR domains (Fig. 2b). A phylogenetic tree comprising 25 TIR1 sequences from different species was constructed (Fig. 3). Phylogenetic analysis indicated that CsTIR1 and CsAFB2 were the most similar to SlTIR1 and PtFBL3, closely followed by AtTIR1 and AtAFB2, respectively.

Fig. 1 Sequence analysis of cucumber *CsTIR1*. **a** Genomic structure of *CsTIR1*. *Black lines* represent introns, *gray boxes* are exons, and *white boxes* are untranslated regions (*UTRs*). **b** Alignment of predicted amino acid sequences of AtTIR1, SlTIR1, and CsTIR1. *Numbers* at the *right* indicate the positions of the amino acid residues. *Shaded black* regions indicate the identical amino acids. The putative F-box motif and conserved LRR domains are indicated by *thin lines*

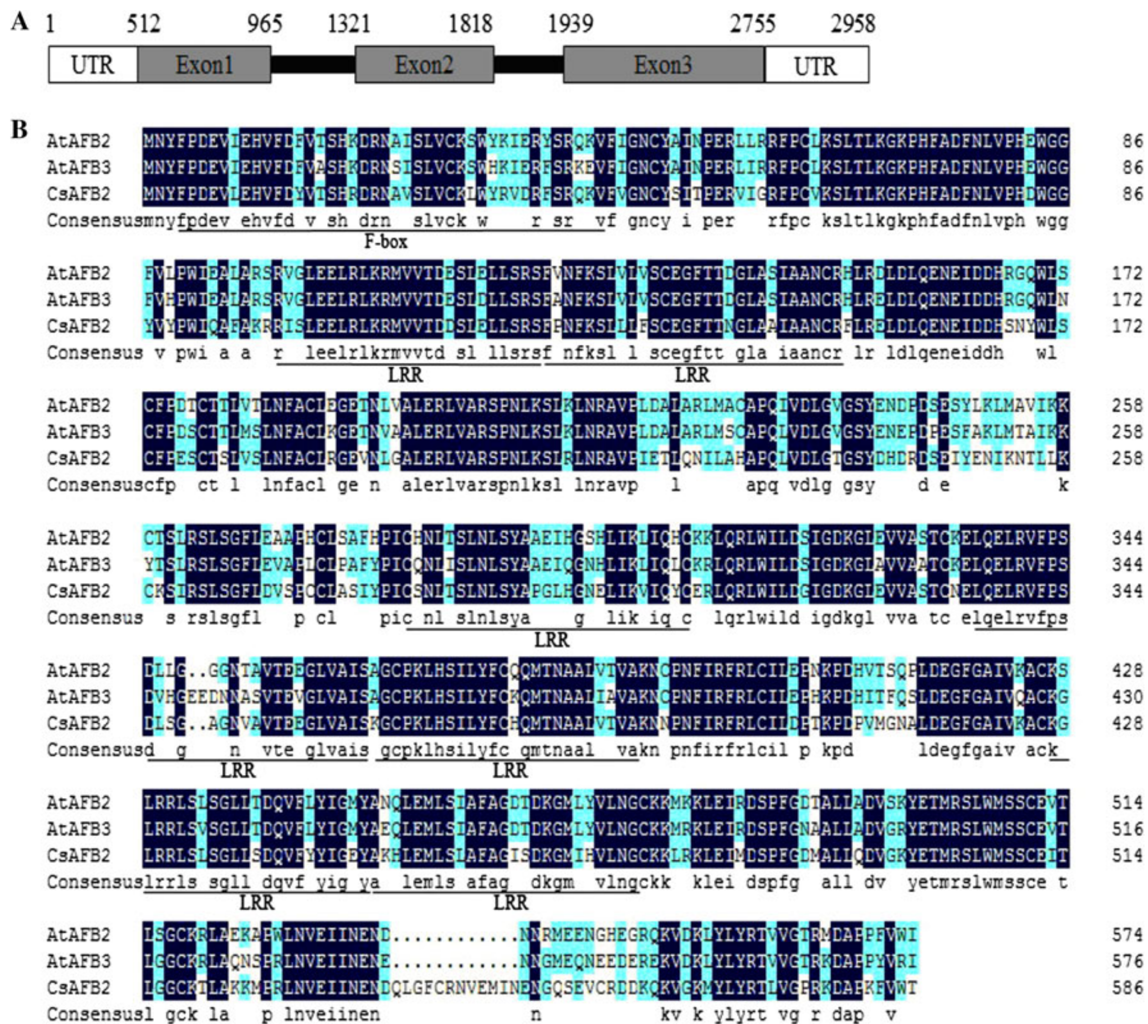

Fig. 2 Sequence analysis of cucumber *CsAFB2*. **a** Genomic structure of *CsAFB2*. *Black lines* represent introns, *gray boxes* are exons, and *white boxes* are UTRs. **b** Alignment of predicted amino acid sequences of AtAFB2, AtAFB3, and CsAFB2. *Numbers* at the *right* indicate the positions of the amino acid residues. *Black* shows the identical amino acids. The putative F-box motif and conserved LRR domains are indicated by *thin lines*

The expression patterns of *CsTIR1* and *CsAFB2* in different cucumber organs

CsTIR1 and *CsAFB2* transcript levels were assessed in different cucumber organs to explore their potential role during plant development. The expression analysis performed by qRT-PCR showed that *CsTIR1* and *CsAFB2* were expressed in all organs (Fig. 4), but differences among organs were observed. *CsTIR1* was expressed at its highest levels in leaves, followed by ovaries, anthers, and roots. The expression level was similar in the young fruit (2 days after pollination) and root. *CsTIR1* expression in the stem was very low (Fig. 4a). Although *CsAFB2* is an F-box protein, its expression pattern was different with that of *CsTIR1*. The highest transcript level of *CsAFB2* was observed in the ovaries, followed by the leaves, anthers, roots, stems, and young fruits (Fig. 4b).

The expression analysis of *CsTIR1* and *CsAFB2* in early developmental fruit stages

We further investigated the expression levels of the two genes during early fruit development under different treatments (Fig. 5). First of all, the *CsTIR1* transcript level was analyzed at different fruit developmental stages (Fig. 5a–d). The expression level was gradually declined during natural parthenocarpic fruit development (EC1 unpollinated) from 0 to 8 DAA (Fig. 5a). This result was consistent with *CsTIR1* expression in induced parthenocarpic and pollinated fruits (Fig. 5b and c). In contrast, *CsTIR1* transcript level was increased in aborted fruits, which were non-parthenocarpic fruits under apoptosis (Fig. 5d). Then, expression level of *CsAFB2* during early fruit development was performed. It was agreed with expression level *CsTIR1* (Fig. 5a–d), except for the highest

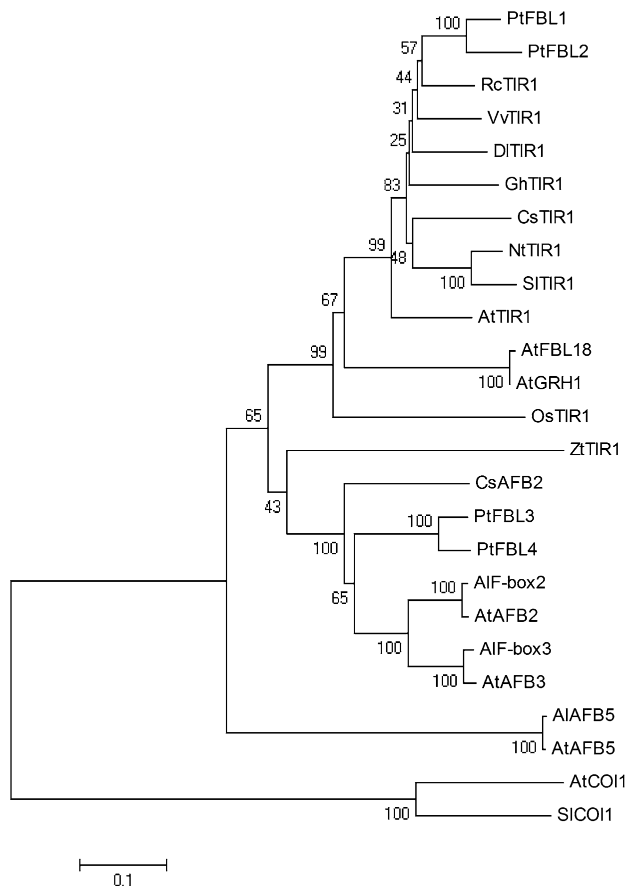

Fig. 3 CsTIR1 belongs to a distinct subfamily of the auxin receptor TIR1 protein family. The phylogenetic tree was created using the neighbor-joining approach and MEGA4 software. The phylogenetic tree was constructed with protein sequences from the following species: *Populus trichocarpa* PtFBL1, PtFBL2, PtFBL3, and PtFBL4; *Ricinus communis* RcTIR1; *Vitis vinifera* VvTIR1; *Dimocarpus longan* DiTIR1; *Gossypium hirsutum* GhTIR1; *Nicotiana tabacum* NtTIR1; *S. lycopersicum* SlTIR1; *Arabidopsis thaliana* AtTIR1; AtAFB2, AtAFB3, AtAFB5, AtAFB18, AtGRH1, and AtCOl1; *Oryza sativa* OsTIR1; *Zea mays.* L. ZmTIR1; *Arabidopsis lyrata subsp. Lyrata* AIF-box2, AIF-box2, AlAFB5; and *S. lycopersicum* SlCOl1

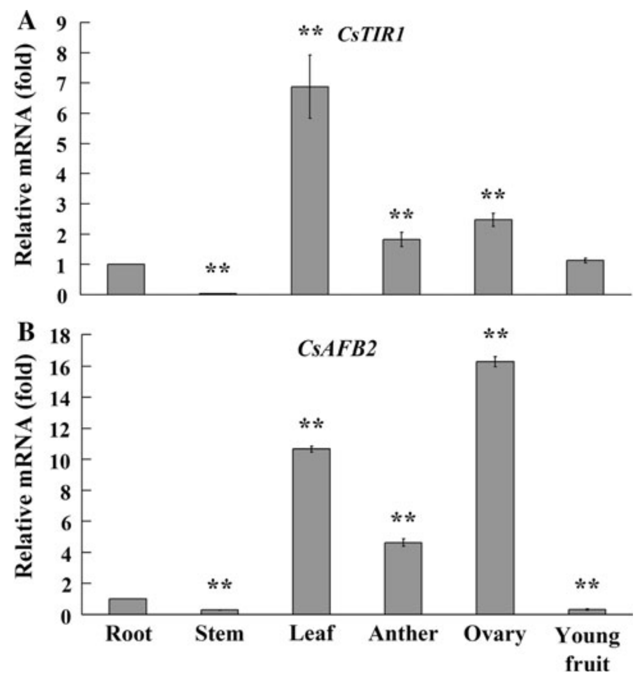

Fig. 4 Expression analysis of *CsTIR1* and *CsAFB2* in different cucumber organs. **a**, **b** Expression levels of *CsTIR1* and *CsAFB2* in roots, stems, leaves, anthers, ovaries, and young fruits. qRT-PCR analyses of total RNA isolated from roots, stems, leaves, anthers, ovaries, and young fruit pollinated after 2 days, which was used to assess *CsTIR1* and *CsAFB2* transcript levels. Data are mean ± SD normalized relative to *Cs-Actin* gene transcript level. All samples were run in triplicate. *Asterisks* indicate significant differences (*t* test; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$)

expression level during pollinated fruit development occurred 4 days after pollination (Fig. 5c). The expression levels of *CsTIR1* and *CsAFB2* declined significantly from the ovarian stage to fruit formation, but increased in aborted fruit. This dynamic expression pattern suggests that *CsTIR1* and *CsAFB2* might play important roles in cucumber fruit development and that pollination might stimulate its quick expression followed by a decrease when the fruit has successfully set.

We next evaluated whether *CsTIR1* and *CsAFB2* expression levels were similar to those in other developing parthenocarpic fruit which were sampled at 2 DAA. *CsTIR1* and *CsAFB2* mRNA levels in parthenocarpic ovaries were significantly lower compared with aborted ovaries (Fig. 6). The results indicated that the expression levels of *CsTIR1* and *CsAFB2* were down-regulated in early developing cucumber fruit.

Changes in IAA and cytokinin content during cucumber fruit development

IAA content was measured in developing cucumber ovaries and young fruits to investigate the relationship between endogenous IAA and expression levels of *CsTIR1* and *CsAFB2* (Fig. 7a). IAA concentration decreased in pollinated and induced parthenocarpic fruits (8419s-1) at 2 days after pollination (DAP), then increased at 4 DAP. However, IAA concentration increased in aborted fruit at 2 DAP, after that decreased. These results were in contrast with *CsTIR1* and *CsAFB2* expression levels. This showed that increased endogenous IAA can not up-regulate the transcript levels of both genes in early stage of cucumber fruit development.

ZR content was detected in 8419s-1 young fruits and aborted fruits for exploring the roles of *CsTIR1* and *CsAFB2* in fruit development (Fig. 7b). ZR concentration decreased in pollinated and induced parthenocarpic fruits (8419s-1) at 2 DAP, then increased in pollinated fruits at 4

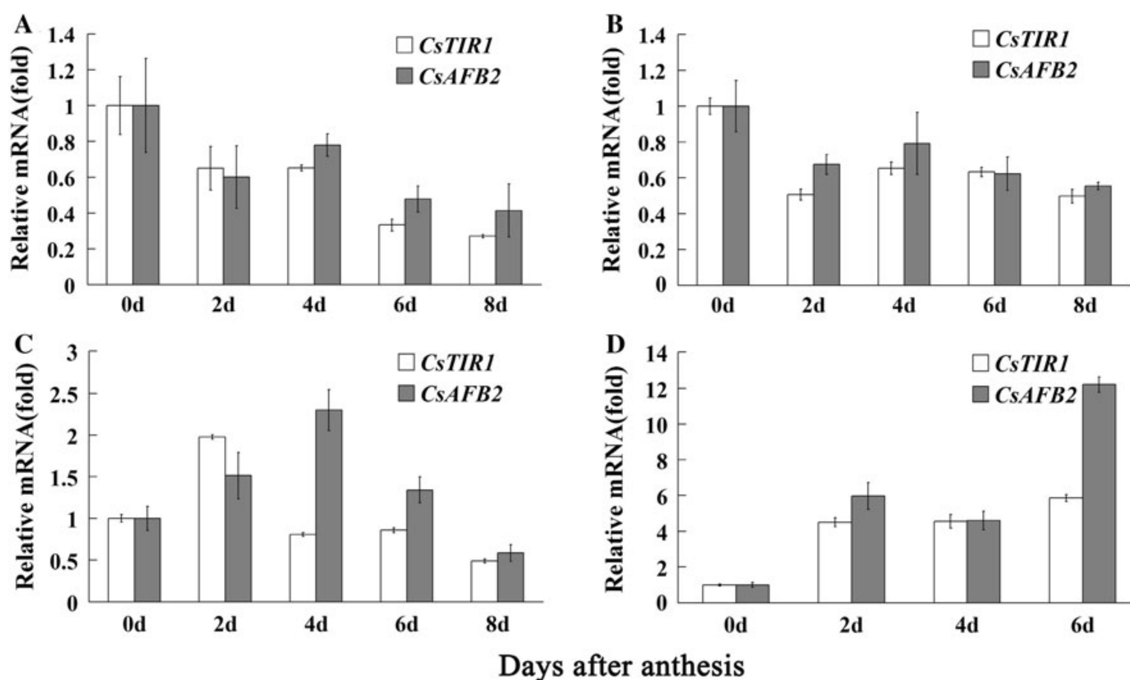

Fig. 5 The expression levels of *CsTIR1* and *CsAFB2* during early cucumber fruit development stages. *CsTIR1* and *CsAFB2* expression patterns in EC1 unpollinated fruits (**a**). 8419s-1 fruits treated with CPPU (**b**). 8419s-1 pollinated fruits (**c**). 8419s-1 non-pollination fruits (**d**). QRT-PCR analyses were preformed using RNA generated from differently treated cucumber ovaries at 0, 2, 4, 6, and 8 days after anthesis (the 8419s-1 non-pollination ovary at 8 days was not obtained). All samples were run in triplicate. *Asterisks* indicate significant differences (*t* test; $*P \leq 0.05$; $**P \leq 0.01$)

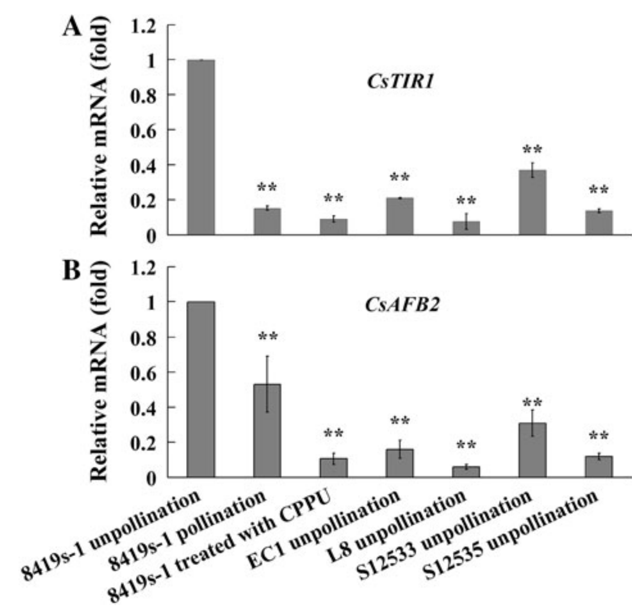

Fig. 6 Transceipt levels of *CsTIR1* and *CsAFB2* in different parthenocarpy cultivars. **a**, **b** *CsTIR1* and *CsAFB2* expression levels in different parthenocarpic fruits and pollinated fruit. Unpollinated fruit of 8419s-1 was the control, indicating that fruit set was not successful. Ovaries that were pollinated and treated with CPPU began to format fruits. EC1, L8, S12533, and S12535 are the natural parthenocarpic cucumber lines. Data are expressed as relative values. Each value represents mean ± SE of three replicates

DAP and in induced parthenocarpic fruits at 6 days after CPPU treatment. ZR content has been declining in aborted fruits. However, ZR concentration was the highest at 4 DAP, and the expression level of *CsAFB2* in pollinated at 4DAA was also the highest. The ZR level in aborted fruits was decreased from 0 to 6 days after anthesis. However, the expression patterns of *CsTIR1* and *CsAFB2* appeared the opposite situation. The result indicated that cytokine level may affect the transcript of *CsAFB2* in fruit development.

The expression of *CsTIR1* and *CsAFB2* in responses to hormones

To investigate the *CsTIR1* and *CsAFB2* expression in responses to hormones, the expression levels were further analyzed in leaves treated with different concentrations of NAA. *CsTIR1* and *CsAFB2* were induced slowly following auxin treatment, and transcript levels were highest at 6 h after treatment, following declined at 9 h (Fig. 8), indicating that *CsTIR1* and *CsAFB2* expression induced by exogenous auxin and their expression patterns were similar in response to 5–50 μM NAA. In addition, we further carried out real-time quantitative PCR analyses. The result showed that *CsTIR1* and *CsAFB2* were induced by other hormones, including: 6-BA, GA_3, ABA, and ethephon

(Fig. 9). *CsTIR1* was just up-regulated in the leaves by 6-BA at 3 h, then declined (Fig. 9a). Exogenous GA_3 application caused a rapid up-regulation expression of *CsTIR1*, the highest level appeared at 6 h. Figure 9a shows *CsTIR1* expression in response to ABA and ethephon, which were identical and significantly higher than those of the control. The *CsAFB2* transcript increased significantly at 3 and 6 h after 6-BA treatment, and then declined (Fig. 9b). The *CsAFB2* expression level in response to GA_3 increased by 3 h and remained high 9 h after treatment. *CsAFB2* expression in response to ABA was similar to that of *CsTIR1* (Fig. 9b). Taken together, these results indicated that *CsTIR1* and *CsAFB2* were induced by exogenous hormones except 6-BA, which down-regulated expression. This was consistent with those of CPPU-induced parthenocarpic fruits.

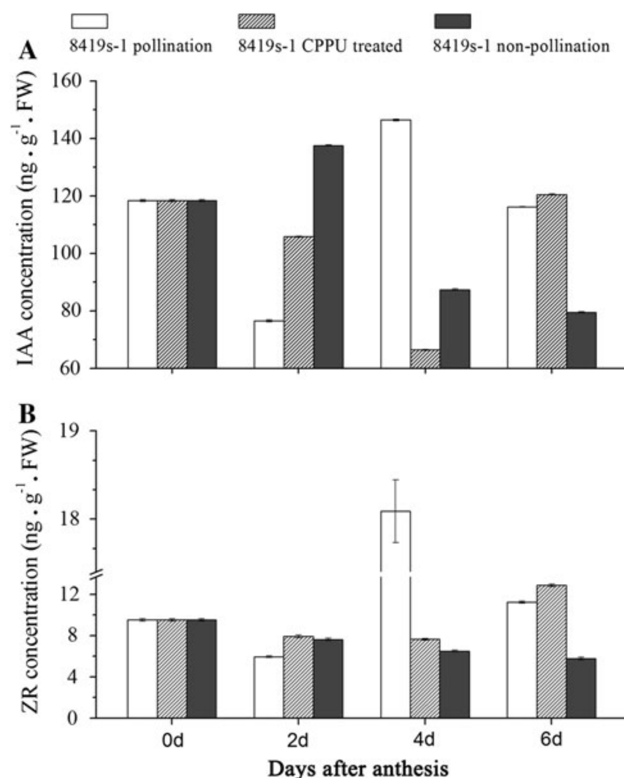

Fig. 7 Analysis of endogenous hormone IAA and ZR in cucumber young fruit. Analysis of endogenous IAA concentration in cucumber young fruits (**a**). Analysis of endogenous ZR concentration in cucumber young fruits (**b**). Treatments included the pollinated, non-pollination and treated with CPPU of 8419s-1 fruits. Samples were collected at 0, 2, 4 and 6 days after anthesis. Data are mean ± 1 SE of three replicates

Discussion

Fruit development is closely related to auxin (de Jong et al. 2009a; Pandolfini et al. 2007; Serrani et al. 2008). *TIR1* and *AFB2* play important roles as auxin receptors in regulating fruit development. Cucumber is an important economic crop with important research value. In this study, *CsTIR1* and *CsAFB2* genes were isolated from cucumber. Sequence analysis of the CsTIR1 protein indicated that it contained one conserved F-box and six LRR domains (Figs. 1a and 2a), which was the same as that of the SlTIR1 structural domain (Ren et al. 2011). Protein interactions were predicted from the *Arabidopsis* protein interaction database (http://arabidopsis.org/). The prediction showed that CsTIR1 interacts with SKP1, Cullin, SAPK7, and auxin-induced protein 22D in cucumber. Pull-down assay had shown that SKP1 links the F-box protein to Cullin to form SCF-ubiquitin ligase complexes (Bai et al. 1996; Dharmasiri et al. 2005a; Kepinski and Leyser 2005a), indicating that *TIR1* may be a conserved gene in plants.

In tomato, the expression level of *SlTIR1* was the highest in flower, down-regulated in immature green fruit (Ren et al. 2011). Our results also showed that the expression levels of *CsTIR1* and *CsAFB2* were high at

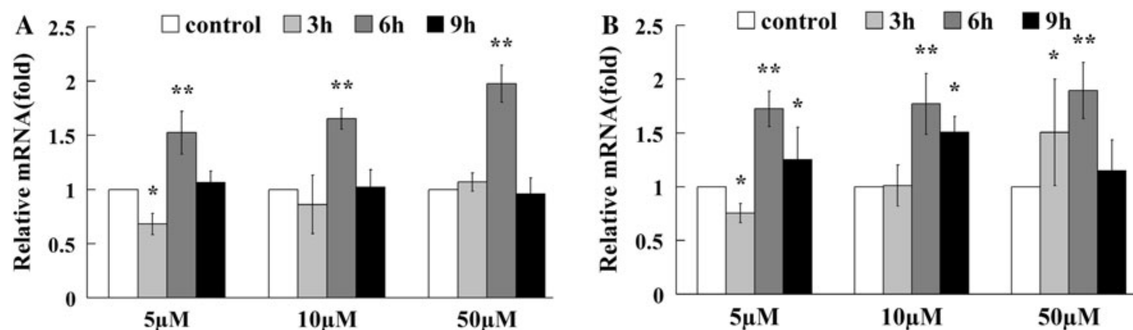

Fig. 8 Quantitative RT-PCR analysis of *CsTIR1* and *CsAFB2* in response to the different concentrations of NAA. **a**, **b** *CsTIR1* and *CsAFB2* expression patterns in leaves of 8419s-1 treated with different concentrations of NAA (5, 10, and 50 μM). QRT-PCR analyses were performed using RNA generated from cucumber leaves after treatments at different times (0, 3, 6, and 9 h). The control was 0 h. Data are mean ± SD normalized relative to *Cs-Actin* gene transcript level. All samples were run in triplicate. *Asterisks* indicate significant differences (*t* test; *$P \leq 0.05$; **$P \leq 0.01$)

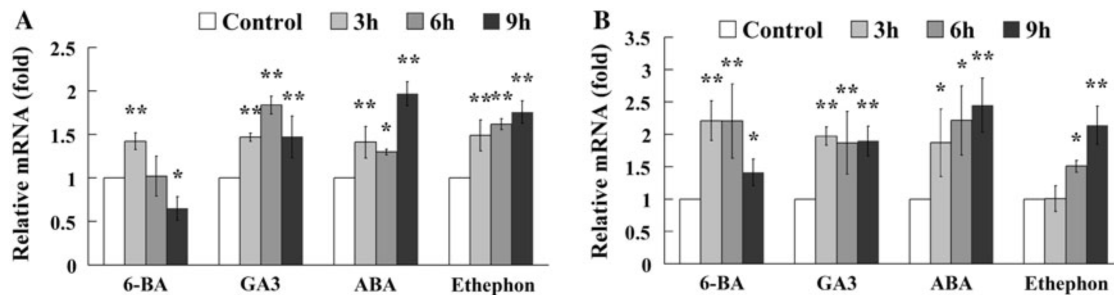

Fig. 9 The expression analysis of *CsTIR1* and *CsAFB2* in cucumber leaves under the treatments of exogenous hormones or hormone analogs **a**, **b** Expression levels of *CsTIR1* and *CsAFB2* under different treatments, including: 6-BA, GA_3, ABA, and ethephon. The control was 0 h. Data are mean ± SD normalized relative to *Cs-Actin* gene transcript level. All samples were run in triplicate. *Asterisks* indicate significant differences (*$P \leq 0.05$; **$P \leq 0.01$)

flower day and decreased during the early stage of natural parthenocarpic cucumber fruit (2–8 DAA) (Fig. 5). We further analyzed the expression patterns of *CsTIR1* and *CsAFB2* in induced parthenocarpic fruit. The *CsTIR1* and *CsAFB2* transcripts were also down-regulated in the induced parthenocarpic fruit of cucumber, which uncoupled fruit initiation without pollination and fertilization and gave rise to parthenocarpic fruits (Fig. 5b). The result was consistent with decline in the expression levels of *CsTIR1* and *CsAFB2* in pollinated fruit (Fig. 5c). This suggested that the fruit set and development may be accompanied by down-regulated *CsTIR1* and *CsAFB2* expression. However, Ren et al. (2011) reported that overexpression *SlTIR1* resulted in parthenocarpic fruit formation in tomato. This is in contradiction with our study that expression levels of *CsTIR1* and *CsAFB2* were down-regulated in parthenocarpic fruit. The reason may be that *TIR1* gene plays an important role at the stages of flower-to-fruit transition; we just analyzed the expression levels of *CsTIR1* and *CsAFB2* in parthenocarpic fruit after anthesis.

Genetic studies have shown that the *TIR1* and *AFB* functions were in a partially redundant manner to mediate the auxin response. A triple and quadruple *tir/afb* mutant analysis demonstrated that these genes have overlapping functions and are collectively essential for *Arabidopsis* growth and development (Dharmasiri et al. 2005b). This is possible reason that *CsAFB2* and *CsTIR1* have similar expression characteristics in different organs. However, the expression levels of *CsTIR1* and *CsAFB2* during early fruit development were different in pollinated fruits. The highest expression level of *CsTIR1* was observed at 2 days earlier than that of *CsAFB2*, which appeared at 4 days after pollination. Thus, the result indicated that *CsTIR1* and *CsAFB2* may have different responses to pollination/ fertilization.

In cucumber, pollination increases the levels of natural IAA and zeatin, which are essential to fruit development (Boonkorkaew et al. 2008). The increase in IAA concentration in cucumber young fruits is closely related to fruit development (Kim et al. 1992; Beyer and Quebedeaux 1974). However, the expression levels of *CsTIR1* and *CsAFB2* were down-regulated at the relevant fruit development stage when endogenous IAA level increased during fruit development (Fig. 7a). The result indicated that down-regulation expression levels of *CsTIR1* and *CsAFB2* and increase endogenous IAA content could help to fruit set and growth. The maximum content of IAA and ZR in pollinated fruits appeared at 4 DAP (Fig. 7), and the highest expression level of *CsAFB2* was also at 4 DAP (Fig. 5c). This indicated that *CsAFB2* may be induced by hormones from pollinated fruit. The hypothesis was verified in cucumber leaves treated by exogenous hormones.

Some studies have found that synthetic auxin analogs, such IAA, NAA, and 2, 4-dichlorophenoxyacetic acid could promote the binding of Aux/IAA proteins to the TIR1 F-box protein to enhance TIR1-Aux/IAA interaction (Dharmasiri et al. 2005a; Kepinski and Leyser 2005a; Tan et al. 2007). This is coincident with that *CsTIR1* and *CsAFB2* transcripts were accumulated in leaves following 5–50 μM NAA treatment (Fig. 8). Kepinski and Leyser (2005a, b) reported that 0.5 μM natural IAA resulted in a greatly enhanced co-purification of TIR1-Myc with the Aux/IAA domain II peptide, the synthetic auxin NAA was also able to promote the interaction, but with lower activity, it has a clear promotive effect at 10 μM. However, any changes in *TIR1/AFB2* RNA levels were not detected even after treatment with high concentration of IAA (1 μM) in Arabidopsis root (Parry et al. 2009). In this study, the 5, 10 and 50 μM NAA were chose for detecting the *CsTIR1* and *CsAFB2* responses to exogenous auxin, and found that the expression levels of *CsTIR1* and *CsAFB2* in treated leaves at 6 h were gradually up-regulated with the increase of NAA concentration. The result indicated that exogenous auxin can regulate the transcript of *CsTIR1* and *CsAFB2* in cucumber leaves.

In conclusion, this study indicated that *CsTIR1* and *CsAFB2* may play an important role in cucumber fruit development. Down-regulated *CsTIR1* and *CsAFB2*

transcripts may have promotive effect on cucumber parthenocarpic fruit development. The transcripts of *CsTIR1* and *CsAFB2* were induced by exogenous hormones. Future studies should utilize transgenic tools to verify *CsTIR1* and *CsAFB2* functions in cucumber.

Author contribution Li Cui was responsible for experimental design and results interpretation, and wrote the paper. Tinglin Zhang and Ji Li performed cloning of *CsTIR1* and *CsAFB2* and qRT-PCR studies. Qunfeng Lou was involved in bioinformatic analysis. Jinfeng Chen helped revise the manuscript. Li Cui was responsible for experimental design and results interpretation, and wrote the paper. Tinglin Zhang and Ji Li performed cloning of *CsTIR1* and *CsAFB2* and qRT-PCR studies. Qunfeng Lou was involved in bioinformatic analysis. Then, Li Cui was responsible for revising manuscript. Jinfeng Chen helped revise the manuscript and approved manuscript final version.

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Conflict of interest The authors declare that they have no conflict of interest.

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