

Genome-wide identification and expression analysis of *CsAIL* gene family in cucumber

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Abstract

AINTEGUMENTA-LIKE (*AIL*) genes encode key transcriptional regulators governing plant growth. However, little is known of *CsAIL* genes in cucumber (*Cucumis sativus* L.). Here, six *CsAIL* genes in the cucumber genome were identified. Bioinformatic analyses revealed high evolutionary conservation of gene structures, protein domains, and motifs among *CsAIL* members. Expression profiling revealed tissue-specific patterns of *CsAIL*s, hinting at their functional diversification in cucumber growth and development. Notably, *CsAIL1* exhibited higher expression levels in ovaries, fruit spines, and seeds, implying its specialized role in these tissues, whereas no functions of *AIL1* homologs were reported in other plant species. *CsAIL* members were also involved in abiotic stress responses, as evidenced by altered expression patterns under salt treatment. Additionally, *CsAIL1* protein was localized in the nucleus and was predicted to interact with the ethylene response factor *CsERF13*. These findings provide a foundational understanding of the *CsAIL* gene family and contribute valuable insights towards the functional characterization of *CsAIL* genes in cucumber.

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Introduction

The external environmental conditions and internal regulatory factors interactively influence growth and development during the plant life cycle. Elucidating the underlying molecular mechanism of plant growth is necessary to facilitate the utilization of more gene resources in modern breeding engineering. Over the past several decades, numerous pieces of evidence have demonstrated that *AINTEGUMENTA-LIKE* (*AIL*) transcription factors (TFs) play a crucial role in regulating plant growth and development processes^[1,2]. The *AIL* genes, which are members of the *APETALA 2/ETHYLENE RESPONSE FACTOR* (*AP2/ERF*) superfamily, have been extensively studied in plants^[1]. The *AIL* proteins typically share two *AP2/ERF* domains connected by a linker region^[3]. In *Arabidopsis*, eight *AIL* genes, including *AINTEGUMENTA* (*ANT*), *AIL1*, *AIL2/BABY BOOM* (*BBM*)/*PLETHORA4* (*PLT4*), *AIL3/PLT1*, *AIL4/PLT2*, *AIL5/PLT5*, *AIL6/PLT3*, and *AIL7/PLT7*, have been identified^[4–8]. Additionally, the genome-wide identification of the *AIL* gene family has been conducted in various plant species, including pumpkin, apple, *Medicago truncatula*, and *Brassica* species^[9–12].

The *AIL* genes play major roles in embryogenesis, maintenance of meristems, and organ positioning and growth^[1,2]. Among them, *ANT* is specifically required for initiating and maintaining early floral primordia development and regulating organ size^[4,5,13]. Loss-of-function of *ANT* shows smaller organ size, fewer floral organs, abnormal integument, and ovule development, while ectopic expression of *ANT* causes the formation of enlarged organs^[4,5,13–17]. Also, *ANT* acts together with polarity factor *FIL*, a *YABBY* TF, to be involved in organ polarity establishment^[18]. During flower development, *ANT* functions redundantly with *AIL6* in regulating floral organ initiation, identity specification, growth, and patterning^[19,20]. *ANT*, *AIL6*, and *AIL7* maintain the cell division and differentiation capacity of the shoot apical meristem; the *ant ail6 ail7* triple mutant shoots are prematurely terminated^[21]. Moreover, the *BBM*, *AIL4*, and *AIL6* genes are expressed in the roots and play primary roles in root stem cell

niche (*SCN*) pattern formation, meristem maintenance, and embryogenesis^[6–8,22]. The *ail3 ail4 ail6* triple mutant exhibits a rootlessness phenotype, and the *bbm ail4* double mutant results in embryo lethality^[8]. In contrast, overexpression of *AIL4* causes ectopic root identity^[7]. Overexpression of *BBM* or *AIL5* can induce ectopic embryo formation on meristems, cotyledons, and leaves^[6,23–26]. Importantly, *BBM* also contributes to improving the transformation efficiency^[27–29]. In addition, *AIL5*, *AIL6*, and *AIL7* are also responsible for phyllotaxy stability and lateral root initiation^[30–32]. These results suggest that *AIL* gene functions are diverse but highly conserved in a partially overlapping manner in plant growth and development.

Cucumber, which is consumed as a tender fruit, serves as an important horticultural crop with higher economic value. Given its allogamy characteristic, the cucumber has been used as a model plant for studying sex determination^[33–35]. The shape and size of cucumber fruit vary across different geographic populations, but they tend to have a faster growth trait, resulting in a shorter growth cycle^[36–38]. Hence, the cucumber also serves as a model species for research on fruit development within the *Cucurbitaceae* family. Although increasing studies focus on important traits in cucumber, such as sex determination, fruit development, and the brightness of the wart and peel, more work is also required to elucidate the molecular and genetic mechanisms underlying its overall growth and development.

The *AIL* genes have been extensively characterized in various plant species, especially in *Arabidopsis*, but their features and functions remain largely unexplored in cucumber. Here, a genome-wide analysis of the *CsAIL* gene family in cucumber was conducted. Six *CsAIL* members were identified, and their phylogenetic relationships, gene structures, conserved domains, and motif compositions were systematically characterized. The evolutionary relationships of *AIL* genes between cucumber and *Arabidopsis* was also analyzed. Furthermore, the expression profiles of *CsAIL* genes were well investigated in different tissues and/or the ovaries/fruits at various developmental stages of cucumber. These findings provided

important insights into the potential functions of *CsAILs* during the growth and development processes in cucumber.

Materials and methods

Plant materials

The cucumber local cultivar 'Xintaimici' and the *Nicotiana benthamiana* were used in this study. The cucumber plants were cultured in a solar greenhouse, and the *Nicotiana benthamiana* seedlings were grown in a growth chamber at Shandong Agricultural University (Tai'an, Shandong, China) (36°18' N, 117°17' E). Management of cucumber and *Nicotiana benthamiana* plants were conducted according to standard practices.

Identification and characterization of the *CsAIL* gene family

The eight *Arabidopsis* AIL proteins were used as queries to identify the *CsAIL* genes using the BLASTP program in the cucumber genome (<http://cucurbitgenomics.org/v2>) (accessed on 12 November 2023). The search parameters were set as follows: 'Num Descriptions = 5', 'Num Alignments = 5', and 'e-Value (Expected Threshold) = 1e-2'. Furthermore, all predicted *CsAIL* sequences were manually confirmed by examining the conserved AP2 domain using Conserved Domain Database (CDD)^[39] (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and SMART^[40] (<http://smart.embl.de>). Chromosomal locations of the *CsAIL* genes were retrieved from the cucumber genome. Physicochemical properties of *CsAIL* proteins, including molecular weights (MW) and isoelectric points (pI), were predicted via the ProtParam tool on the ExPASy server^[41] (<https://web.expasy.org/protparam>). The eight *Arabidopsis* AIL protein data were obtained from TAIR (www.arabidopsis.org) (accessed on 12 November 2023).

Phylogenetic and synteny analyses of *CsAIL* genes

The protein sequences of *CsAILs* and *Arabidopsis* AILs were aligned by ClustalW. A phylogenetic tree was constructed from the multiple sequence alignment with the MEGA 11 software under the Neighbor-Joining method, applying the following parameters: 1,000 bootstrap replicates, Poisson model, and complete deletion. The tree was visualized using the Interactive Tree Of Life (iTOL) (<https://itol.embl.de>) (accessed on 12 November 2023). The synteny analysis was conducted using the Multiple Collinearity Scan toolkit (MCScanX)^[42].

Analysis of gene structures, conserved motifs, and protein domains

Gene structures of *CsAILs* were visualized by the Gene Structure Display Server^[43] (<http://gsds.gao-lab.org>). Conserved motifs of *CsAIL* proteins were predicted via the MEME online tool^[44] (<https://meme-suite.org/meme/tools/meme>). Following the multiple sequence alignment of *CsAIL* proteins, the conserved domains were highlighted by Jalview^[45] (www.jalview.org).

Transcriptome analysis of *CsAIL* genes in cucumber

The cucumber transcriptome data analyzed in this study were obtained from the RNA-Seq projects in the Cucurbit Genomics Database (http://cucurbitgenomics.org/cgi-bin/RNA_project) (accessed on 15 November 2023). The datasets included:

PRJNA80169: roots, stems, leaves, male flowers, female flowers, ovaries, and tendrils of *Cucumis sativus* var. *sativus* line 9,930.

PRJNA258122: ovaries of about 2–4 cm in length from cucumber hermaphrodite line 408 (long fruit), and its near-isogenic line 409 (short fruit).

PRJNA431715: ovaries at 6 d before flowering (DBF), and pericarps at 0 DBF from cucumber inbred lines NCG122 (numerous fruit spines), and NCG121 (few fruit spines).

PRJNA319011: seeds at 28, 37, 45, and 60 d after pollination (DAP).

PRJNA271595: growing tips of seedling primary roots of *Cucumis sativus* Gy14, including meristematic zone (MZ), differentiation zone (DZ), and elongation zone (EZ).

PRJNA437579: root apices (0–5 mm from the tips) of hydroponically grown seedlings (5-day-old) of cucumber cv. Jinchun No. 2 under control conditions and 75 mM NaCl stress for 24 h.

Expression patterns of *CsAIL* genes were analyzed using the above transcriptome data, with significance thresholds set at false discovery rate (FDR)-adjusted $p < 0.05$ and fold change (FC) ≥ 1.5 . Values represent the means \pm standard deviation (SD).

RT-qPCR analysis

Samples of ovaries or fruits at different developmental stages were taken into liquid nitrogen and then pulverized. The total RNA was extracted by the Quick RNA Isolation Kit (Huayueyang, Beijing, China), and the cDNA was synthesized from the extracted RNA template using the FastKing gDNA Dispelling RT SuperMix (TianGen, Beijing, China). RT-qPCR was carried out on an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, USA) with the Ultra-SYBR Mixture (Cwbio, Beijing, China). The *UBIQUITIN* (*UBI*) gene served as the internal control. Three biological replicates (each consisting of three technical replicates) were performed for these experiments. Values were the means \pm SD. The primers are listed in Supplementary Table S1.

Subcellular localization

The CDS of *CsAIL1* was cloned and constructed into the Super1300: *CsAIL1*-EGFP vector. The Super1300-EGFP and Super1300: *CsAIL1*-EGFP vectors were introduced into *Arobacterium tumefaciens* GV3101 (pSoup-P19), respectively, and subsequently were co-expressed with the cell nucleus marker NF-YA4-mCherry in young *Nicotiana benthamiana* leaves. The GFP signals (at 488 nm) and mCherry signals (at 561 nm) were detected using a Nikon A1 laser confocal scanning microscope and captured by the NIS-Elements software (Supplementary Table S2).

Prediction of protein interaction network

The *CsAIL1* protein sequence was analyzed using the STRING database (<https://cn.string-db.org>) to predict the potential interaction network. Active interaction data were integrated from multiple sources, including curated databases, experimental determination, gene co-occurrence, text mining, and protein homology.

Results

Identification of the *CsAIL* gene family in cucumber

The *Arabidopsis* eight AIL proteins were employed as queries to identify the *CsAIL* homologs in the cucumber genome using the BLASTP program. In total, six *CsAIL* genes, including *CsAIL1*, *CsBBM*, *CsAIL3*, *CsAIL5*, *CsAIL6*, and *CsANT* were obtained in cucumber after further confirmation of the highly conserved AP2 domain via SMART and CDD analysis (Table 1). The *CsAIL* genes were named according to the highest protein sequence identification values to the closest *Arabidopsis* homologs (Table 1, Fig. 1). These six *CsAIL* genes are distributed across five cucumber chromosomes (chrs) except for chr 5 and chr 7. The sequences of *CsAIL* proteins ranged from 466 to 650 amino acids (AA), accompanied by predicted molecular weights (MW) that varied from 51.43 to 70.01 kDa. Moreover, the predicted isoelectric point (pI) values ranged from 5.88 to 8.64; notably, *CsAIL5* displayed a basic pI (8.64) which differed from the acidic or near-neutral values of other members (Table 1).

Table 1. The classification of *CsAIL* gene family in cucumber.

Gene name	Gene ID	Chromosome location	Amino acid length (AA)	MW (kDa)	pI	<i>Arabidopsis</i> ortholog
<i>CsAIL1</i>	CsaV3_3G031350	chr3: 27057687...27061567 (–)	533	59.07	5.88	<i>AIL1</i>
<i>CsBBM</i>	CsaV3_2G010320	chr2: 7285623...7289916 (+)	650	70.01	6.23	<i>BBM</i> (<i>AIL2</i> , <i>PLT4</i>)
<i>CsAIL3</i>	CsaV3_4G024120	chr4: 14070320...14073714 (–)	615	67.27	6.34	<i>AIL3</i> (<i>PLT1</i>)/ <i>AIL4</i> (<i>PLT2</i>)
<i>CsAIL5</i>	CsaV3_1G031650	chr1: 18716067...18719027 (+)	466	51.43	8.64	<i>AIL5</i> (<i>PLT5</i>)
<i>CsAIL6</i>	CsaV3_3G006900	chr3: 6134843...6139602 (–)	517	57.00	6.28	<i>AIL6</i> (<i>PLT3</i>)/ <i>AIL7</i> (<i>PLT7</i>)
<i>CsANT</i>	CsaV3_6G044340	chr6:26200005...26204175 (+)	629	69.60	7.08	<i>ANT</i>

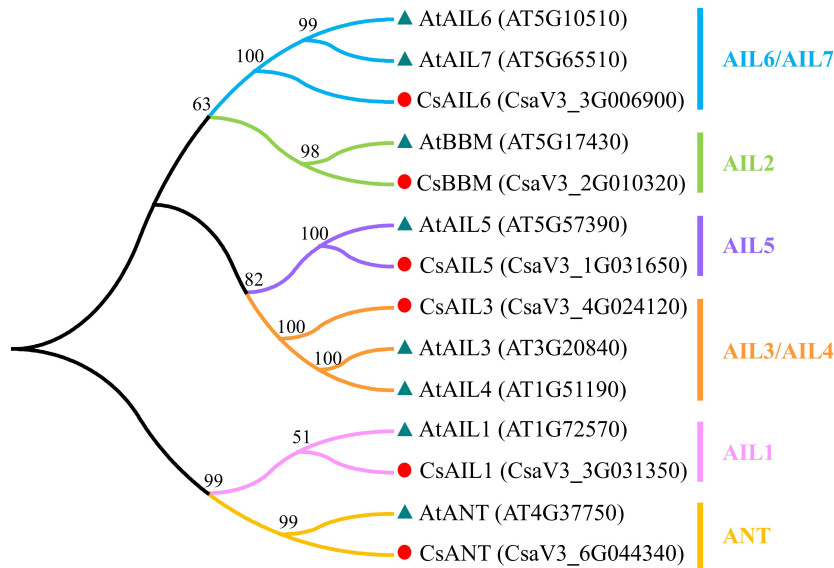


Fig. 1 Phylogenetic analysis of the AIL proteins between cucumber and *Arabidopsis*. Red circle and blue-grey triangle represent the AIL proteins from cucumber and *Arabidopsis*, respectively. Cs, cucumber; At, *Arabidopsis*.

Phylogenetic and synteny analyses of the *CsAIL* genes

To elucidate evolutionary relationships among *CsAIL* genes, full-length protein sequences were recruited to construct a phylogenetic tree between cucumber and *Arabidopsis* using MEGA 11 software. Similar to *Arabidopsis*, the six *CsAILs* were divided into six subclades, including AIL1, AIL2, AIL3/AIL4, AIL5, AIL6/AIL7, and ANT, in which each subclade has only one *CsAIL* gene (Fig. 1). Notably, *CsAIL3* was closest to AIL3 and AIL4 in *Arabidopsis*, and *CsAIL6* showed the closest relationship with AIL6 and AIL7 in *Arabidopsis* (Fig. 1). These results suggest that the *CsAIL* genes of cucumber possibly share conserved evolutionary relationships with *Arabidopsis* AIL genes.

Segment and tandem duplications are key mechanisms reflecting plant genome evolution and facilitating gene family expansion^[46]. Here, a synteny analysis of the AIL gene family between cucumber and *Arabidopsis* was conducted. Four *CsAIL* genes (*CsAIL1*, *CsAIL3*, *CsAIL5*, and *CsAIL6*) have syntenic relationships with *Arabidopsis* AIL genes. Among them, *CsAIL5* and *CsANT* had one syntenic gene pair, respectively (Fig. 2). *CsAIL3* and *CsAIL6* showed two syntenic gene pairs, respectively (Fig. 2), suggesting an absence of any tandem/segment duplication of the two genes in cucumber compared to *Arabidopsis*. However, no synteny relationship was present between *CsAIL1* and *AtAIL1*, or *CsBBM* and *AtBBM*, respectively (Fig. 2), indicating that these two genes between cucumber and *Arabidopsis* may not undergo the segment and/or tandem duplication events.

Analysis of gene structures and conserved motifs/domains in the *CsAIL* genes

Gene structure diversity and conserved motifs/domains are related to the evolutionary history of a gene family and also reflect

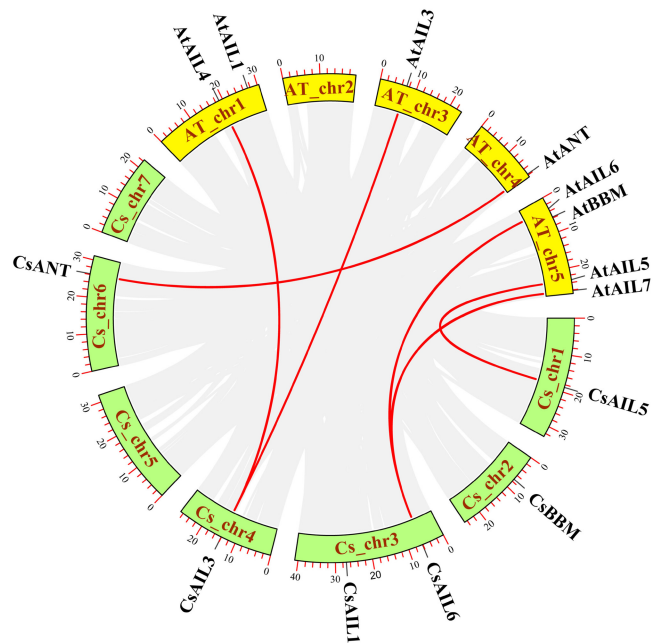


Fig. 2 Synteny analysis of AIL genes between cucumber and *Arabidopsis*. The synteny gene pairs are highlighted in the red lines. Cs, cucumber; At, *Arabidopsis*.

the conservation or divergence of gene function to some extent^[47]. A comprehensive analysis, which consists of an unrooted evolutionary tree, gene structure characterized by exon-intron distribution, and conserved motifs were conducted. The evolutionary analysis

divided *CsAILs* into three subgroups: (i) *CsBBM* and *CsAIL6*; (ii) *CsAIL3* and *CsAIL5*; and (iii) *CsAIL1* and *CsANT* (Fig. 3a), but the genes in same subgroup (e.g., i and ii) did not have a similar exon/intron organization pattern, while the genes in subgroup (iii) showed the same distribution of nine exons and eight introns, respectively (Fig. 3b). Totally, all *CsAIL* genes displayed differentiation with 7–9 exons and 6–8 introns (Fig. 3b), which is in line with other plant species *AIL* genes, implying that the cucumber *CsAIL* gene structures are probably conserved during evolution. Furthermore, the conserved motifs of *CsAILs* were predicted by MEME analysis. It was observed that most *CsAILs* share the same motif composition, except that *CsAIL5* lacks motif 8 (Fig. 3c, Supplementary Fig. S1). Meanwhile, the multiple sequence alignment results showed that all *CsAIL* proteins possess two highly conserved AP2 domains (designated AP2-R1 and AP2-R2), which are separated by a conserved linker region (Fig. 4a). Each AP2 domain is composed of YRG and

RAYD (which contains an amphipathic α -helix) elements. No significant differences in amino acid residues of AP2 domains existed among all *CsAIL* proteins (Fig. 4b). Collectively, these results suggest that the *CsAIL* genes may have conserved functions during the evolution process in cucumber.

Expression profiles of *CsAILs* in cucumber

To understand the potential functions of *CsAILs* during cucumber growth and development, their expression patterns were analyzed across various tissues of cucumber, including roots, stems, leaves, tendrils, male and female flowers, and ovaries. The *CsAIL* genes exhibited different expression patterns across the examined tissues. As shown in Fig. 5, *CsBBM*, *CsAIL3*, and *CsAIL6* were specifically expressed in the roots, and no expression was detected in other tissues. *CsAIL5* and *CsANT* were also expressed in the roots. Moreover, *CsAIL1*, *CsAIL5*, and *CsANT* showed higher expression levels in the leaves and ovaries. Meanwhile, *CsAIL1* expression was also

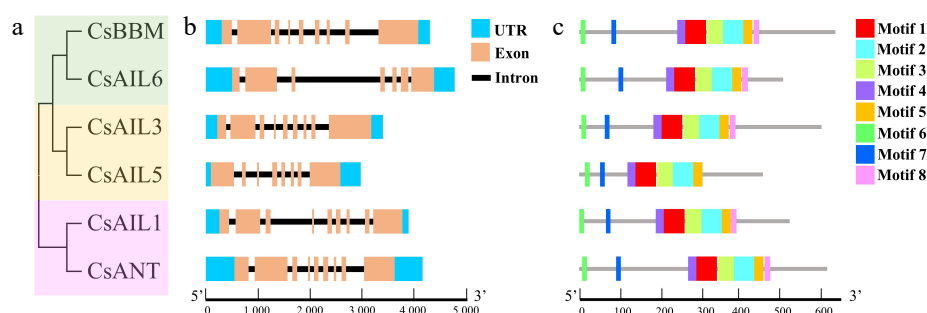


Fig. 3 Phylogenetic clustering, gene structures, and conserved motifs of *CsAIL* genes. (a) Unrooted phylogenetic tree of *CsAIL* proteins. (b) Gene structures of *CsAIL* genes. Untranslated regions (UTR), exons, and introns are indicated by blue boxes, light-brown boxes, and black lines, respectively. (c) Conserved motifs of *CsAIL* proteins. Distinct motifs are represented by different colored boxes.

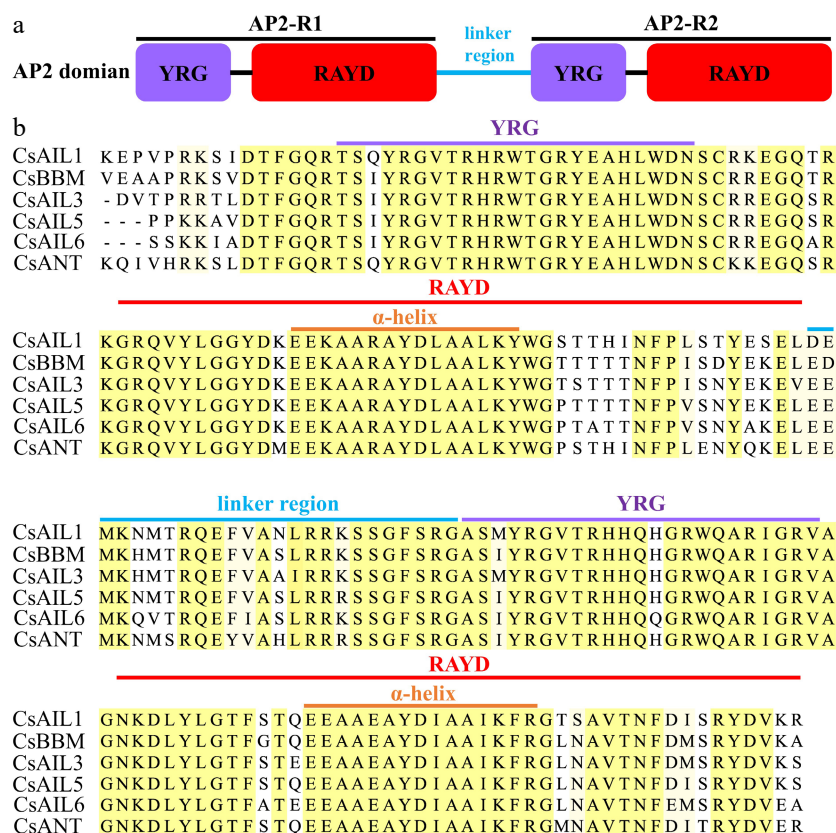


Fig. 4 The conserved AP2 domain of *CsAIL* proteins. (a) Model of AP2 domain. (b) Typical amino acid residues within AP2 domain.

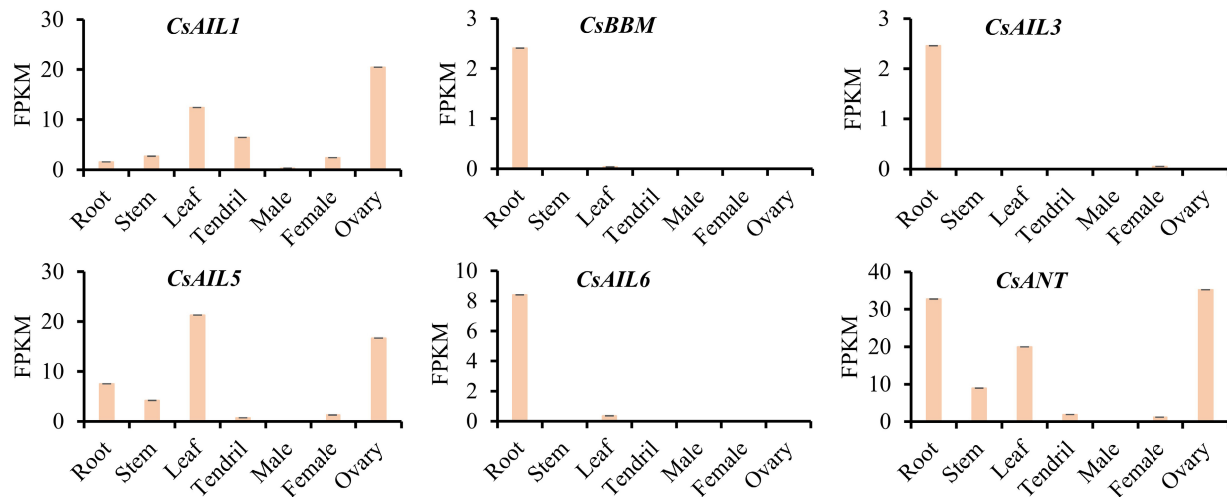


Fig. 5 Expression patterns (FPKM) of *CsAIL* genes in different tissues of cucumber. The expression data were derived from the PRJNA80169 transcriptome dataset.

detected in the tendrils, while *CsAIL5* and *CsANT* were also expressed in the stems. These results imply that *CsAIL* genes may be involved in the regulation of diverse developmental processes in cucumber.

***CsAIL* genes may participate in the development of ovaries, fruit spines, and seeds**

To further explore the potential roles of *CsAIL* genes during cucumber ovary/fruit development, their expression levels in two cucumber varieties that have differences in fruit length (408 has a long fruit phenotype, 409 exhibits a short fruit phenotype) were analyzed. Only *CsAIL1*, *CsAIL5*, and *CsANT* were expressed in the ovaries of 408 and 409 (Fig. 6a), and this result is in line with that in Fig. 5. In addition, the three genes showed lower expression levels in 409 than in 408 (Fig. 6a), hinting at their potential roles in fruit elongation. Furthermore, we examined the expression patterns of *CsAIL1*, *CsAIL5*, and *CsANT* in cucumber ovaries/fruits at different developmental stages. As shown in Fig. 6b–d, the three genes showed consistently high expression levels in the ovaries at various developmental stages, but not in the fruits. Given massive cell proliferation at the ovary stage of cucumber^[48,49], these results indicate that *CsAIL1*, *CsAIL5*, and *CsANT* possibly contribute to fruit development via cell proliferation.

In the two cucumber varieties, NCG121 and NCG122, NCG121 has few fruit spines, while NCG122 fruit harbors numerous fruit spines. We noted that *CsAIL5* and *CsANT* exhibited higher and non-differential expression levels in the ovaries of NCG121 and NCG122, both at the 6 DBF and 0 DBF stages (Fig. 7a). While *CsAIL1* was more highly expressed in the ovaries than *CsAIL5* and *CsANT* at 6 DBF stage (Fig. 7a). Interestingly, *CsAIL1* expression was also significantly higher in NCG121 ovaries at 6 DBF stage compared to NCG122 (Fig. 7a), implying that *CsAIL1* may negatively regulate fruit spine initiation or development in cucumber.

In plants, *AIL* genes have been well documented to play crucial roles in embryogenesis^[6–8]. Here, the *CsAIL* gene expression patterns were examined during different stages of seed development (28, 37, 45, and 60 DAP). At the four tested stages, the expressions of *CsAIL1* and *CsAIL5* progressively increased along with seed development; *CsAIL6* exhibited sustained expression, and *CsBBM* showed a slight expression level. In contrast, no expression of *CsAIL3* and *CsANT* was detected (Fig. 7b). These data indicate that *CsAIL1*, *CsAIL5*, and *CsAIL6* may participate in controlling embryogenesis during seed development.

Involvement of *CsAILs* in root development and salt stress response

Previous studies showed that *AIL* genes control root development and rhizotaxis formation^[7,8]. Therefore, the expression patterns of *CsAIL* genes in distinct root zones of cucumber, including the differentiation zone (DZ), the elongation zone (EZ), and the meristematic zone (MZ) were explored. *CsBBM*, *CsAIL3*, and *CsAIL6* were highly expressed in MZ, and *CsAIL6* also exhibited higher expression levels in DZ and EZ (Fig. 8a). These results suggest that *CsBBM*, *CsAIL3*, and *CsAIL6* may be responsible for meristem maintenance in the roots. Accordingly, *CsBBM* and *CsAIL3* were sensitive to salt stress, as evidenced by their decreased expression levels following salt treatment (Fig. 8b), implying potential roles for these genes in abiotic stress, especially in salt stress. However, *CsAIL6* was insensitive to salt stress, as no expression difference of *CsAIL6* was found between salt-treated and control plants (Fig. 8b).

Subcellular localization and predicted interaction network of *CsAIL1* protein

Given the potential roles of *CsAIL1* with its higher expression level in the ovaries, fruit spines, and seeds, the localization of *CsAIL1* in cells and the potential interaction network were further explored. When *CsAIL1* was expressed in *N. benthamiana* leaves, the *CsAIL1*-GFP fusion protein localized exclusively to the cell nucleus (Fig. 9a). To further understand how *CsAIL1* functions during plant growth and development, the possible interaction factors of *CsAIL1* were predicted. Among all predicted interaction factors, it was found that *CsAIL1* potentially interacts with an ethylene response factor, *CsERF13*, for which there are no reports in fruit development (Fig. 9b). The *CsAIL1*-*CsERF13* module may provide a new avenue for research into fruit development.

Discussion

The master regulatory factors, *AIL* proteins, are involved in multiple developmental processes throughout the plant life cycle^[1,2]. However, the functions of *CsAILs* in the important Cucurbitaceae crop, cucumber, have not been well studied. Herein, six *CsAIL* members were identified in the cucumber genome; however, the total number of *CsAIL* genes is fewer than those in *Arabidopsis* (Table 1). Since gene duplication can contribute to gene family expansion^[46], it was speculated that the fewer gene duplication events of *CsAIL3* and *CsAIL6* might be responsible for the reduced

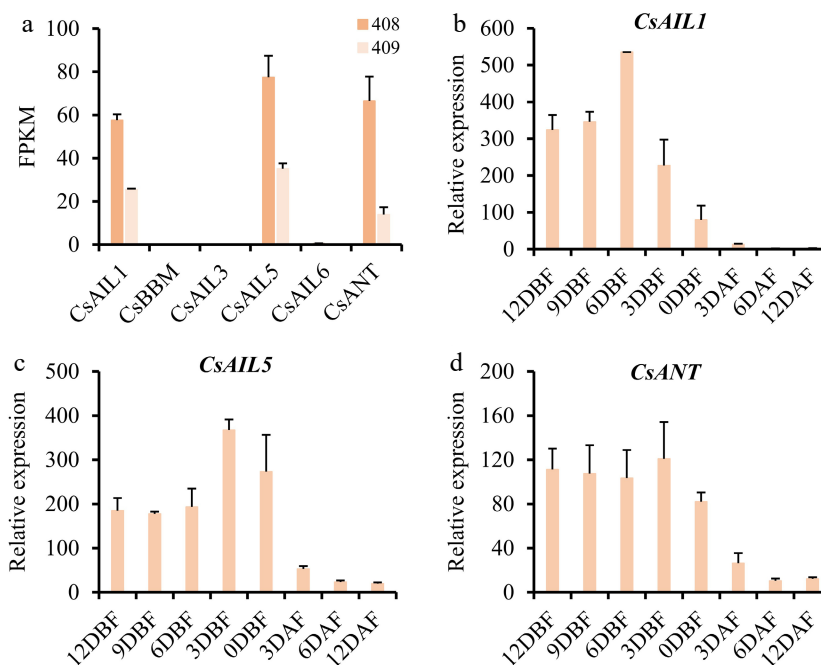


Fig. 6 Expression profiles of *CsAIL* genes in different stages of ovaries and fruits in cucumber. (a) Expression abundance (FPKM) of *CsAILs* in the ovaries from long-fruit (408), and short-fruit (409) cucumber lines. Expression data were obtained from the PRJNA258122 transcriptome dataset. (b)–(d) Relative expression levels of *CsAIL1*, *CsAIL5*, and *CsANT* at different developmental stages of ovaries and fruits as determined by RT-qPCR. Error bars represent the SD of three biological replicates. DBF, days before flowering; DAF, days after flowering.

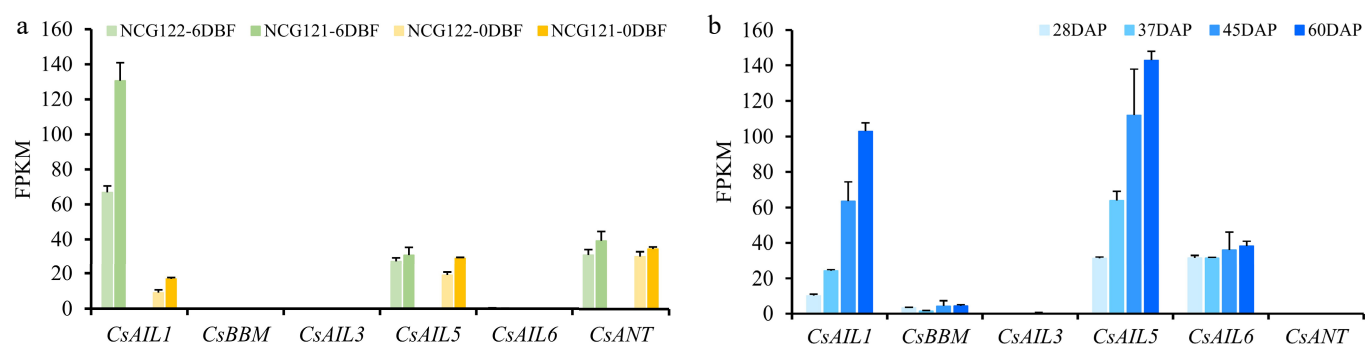


Fig. 7 Expression profiles of *CsAIL* genes in cucumber ovaries and seeds across developmental stages. (a) Expression levels (FPKM) of *CsAILs* in ovaries of the NCG121 (few fruit spines), and NCG122 (numerous fruit spines) varieties at different developmental stages. Expression data were obtained from the PRJNA431715 transcriptome dataset. (b) Expression patterns (FPKM) of *CsAILs* during seed development. Expression data from the PRJNA319011 transcriptome dataset were used in this analysis. Error bars indicate the SD of three biological replicates. DAP, days after pollination.

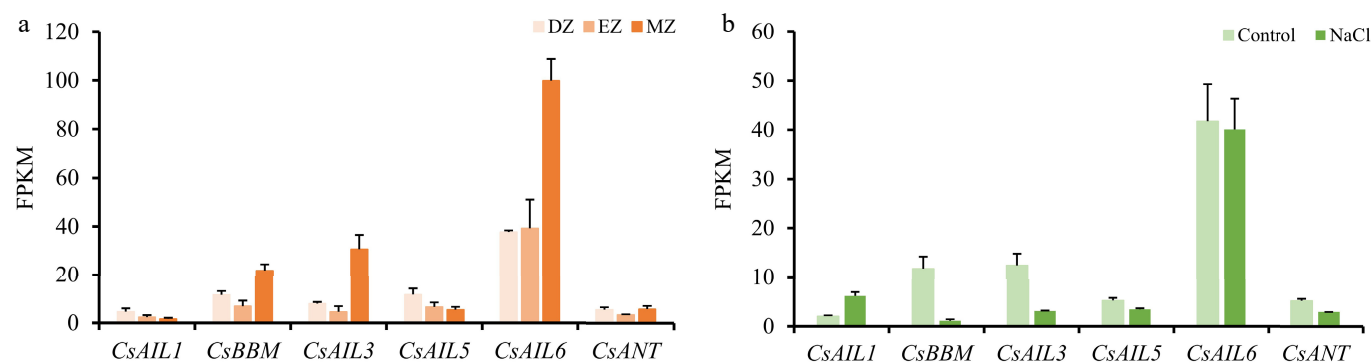


Fig. 8 Expression patterns of *CsAIL* genes in cucumber roots and under salt stress. (a) Expressions (FPKM) of *CsAILs* in different root developmental regions. Data were derived from the PRJNA271595 transcriptome dataset. DZ, differentiation zone; EZ, elongation zone; MZ, meristematic zone. (b) Expression levels (FPKM) of *CsAILs* in roots under control conditions and NaCl treatment. Data from the PRJNA437579 transcriptome dataset were used in this analysis. Error bars represent the SD of three biological replicates.

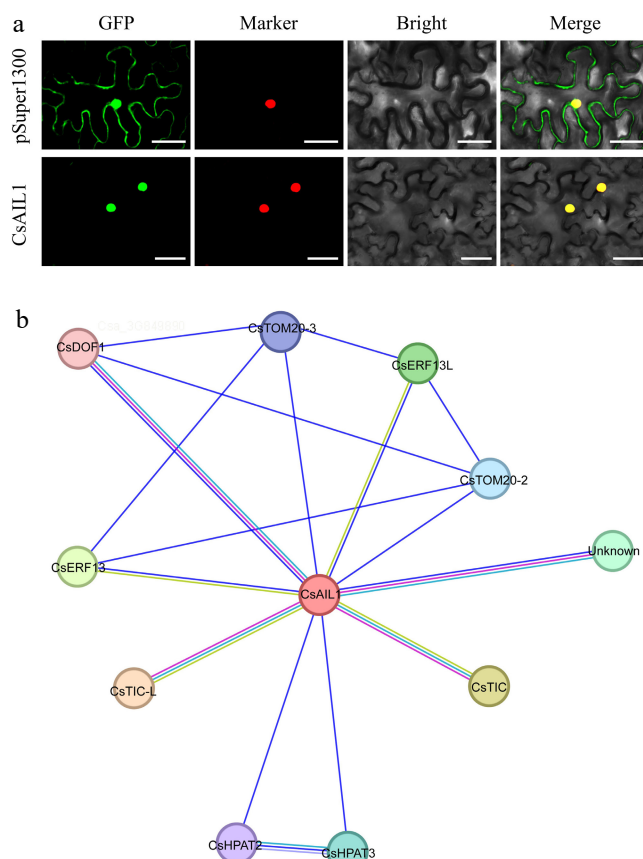


Fig. 9 Subcellular localization and interaction network of CsAIL1 protein. (a) Subcellular localization of CsAIL1 in epidermal cells of *N. benthamiana* leaves. Bars = 50 μ m. (b) Predicted interaction network of CsAIL1 in cucumber. Data sources of predicted interactions are color-coded as follows: purple line, experimentally determined; pearl blue line, from curated databases; blue line, gene co-occurrence; light green line, textmining; light blue-violet line, protein homology.

number of *CsAIL* genes in cucumber compared to *Arabidopsis*. Furthermore, the synteny analysis results showed that no gene duplication events are observed between *CsAIL1* and *Arabidopsis AIL1*, and between *CsBBM* and *Arabidopsis BBM*, respectively (Fig. 2), hinting that the *AIL1* or *BBM* gene is not derived from a common ancestor between cucumber and *Arabidopsis*, although they are in the same corresponding phylogenetic clade, respectively (Fig. 1). The conserved domains and motifs, which are involved in protein interactions, DNA binding and transcription activity^[50], mainly determine the function of the gene. It was found that all *CsAIL* proteins consist of two highly conserved AP2 domains connected by a linker region (Fig. 4), and share eight conserved motifs except *CsAIL5*, which lacks motif 8 (Fig. 3). The highly conserved motifs and AP2 domains suggested that the *CsAILs* might have conserved functions during cucumber growth and development.

To investigate the possible functions of *CsAIL* genes, the expression patterns of these genes were examined across multiple cucumber tissues. Among the six *CsAIL* genes, *CsAIL1*, *CsAIL5*, and *CsANT* showed higher expression levels in the ovaries and leaves of cucumber (Fig. 5). The expression pattern of *CsANT* aligns well with the function of the *ANT* gene in other plants, suggesting a potentially conserved function of the *ANT* gene^[4,5,13,14,16]. However, no evidence describes the function of the *AIL1* gene in plants and the function of *AIL5* in ovary or fruit development^[1]. Thus, these results suggested that *CsAIL1*, *CsAIL5*, and *CsANT* might control the phyllotaxy and

ovary development in cucumber. Moreover, the expression levels of *CsAIL1*, *CsAIL5*, and *CsANT* in the ovaries of 408, which harbors short fruits, are lower than those in 409, which shares long fruits (Fig. 6a). In addition, during the different developmental stages of ovary and fruit, the three genes are mainly expressed in the ovary developmental stage but not in the fruit developmental stage (Fig. 6b–d). Cell proliferation primarily occurred at the ovary developmental stage, and cell expansion appeared during the fruit developmental stage in cucumber^[48,49]. Hence, these data further confirmed the potential function of *CsAIL1*, *CsAIL5*, and *CsANT* in cell proliferation during the ovary growth and developmental stage in cucumber.

Numerous pieces of evidence have confirmed that *BBM*, *AIL3*, *AIL4*, and *AIL5* play essential roles in embryogenesis^[6–8]. However, no expression of *CsAIL3* and a relatively lower expression of *CsBBM* were detected during the seed developmental stages after pollination (Fig. 7b). Previous studies have demonstrated that *AIL4* and *BBM* have shared roles in early embryo development^[8,25]. Given that the expression analysis was based on the transcriptome data of seeds at 28 DAP (not the early embryo stage) in this study, and *CsAIL3* in cucumber is homologous to *AIL3* and *AIL4* in *Arabidopsis* (Table 1 and Fig. 1), it was thus speculated that *CsAIL3* and *CsBBM* also might regulate early embryo development in cucumber, but require further functional verification. In addition, the expression levels of *CsAIL1* and *CsAIL5* gradually increased except for *CsAIL6*, with a higher and stable expression level (Fig. 7b), hinting that the *CsAIL1*, *CsAIL5*, and *CsAIL6* possibly fulfil the predominant roles in the cucumber seed later developmental stage. Interestingly, the *CsAIL1* gene also exhibited higher expression in the ovaries at 6 DBF stage in the NCG122 cucumber variety, which has numerous fruit spines, compared to the NCG121 variety, which has fewer fruit spines (Fig. 7a). The fruit spine (also known as trichome) can influence the appearance quality of cucumber fruit and is a fundamental commercial trait in cucumber breeding^[51]. Hence, the *CsAIL1* might be a promising candidate gene for investigating fruit spine development in cucumber.

Similar to the functions of *BBM*, *AIL3*, *AIL4*, and *AIL6* in root development^[7,8], their homologs also exhibited specific expression patterns in the roots of cucumber (Fig. 5). Further analysis revealed that *CsBBM*, *CsAIL3*, and *CsAIL6* are highly expressed in the DZ, EZ, and MZ, especially in the MZ (Fig. 8a), suggesting their potential roles in cell proliferation of the roots. Whereas *CsANT* had a lower expression level in the three zones (Fig. 8a), hinting at a non-vital function of *CsANT* in root development of cucumber. Previous studies have suggested that *ANT* is involved in response to abiotic stress^[52,53]. However, in this study, *CsBBM* and *CsAIL3*, but not *CsANT*, were found to be salt-tolerant genes, with their transcript levels decreasing under salt stress. In contrast, *CsAIL6* maintained expression levels comparable to the control despite showing higher expression than *CsBBM* and *CsAIL3* following salt treatment (Fig. 8b). Thus, these results provided valuable insights into the potential applications of *CsAIL* genes for enhancing salt tolerance in cucumber breeding.

Conclusions

In this study, a genome-wide characterization of the *CsAIL* gene family in cucumber was conducted. Six *CsAIL* genes were identified and their gene structures, conserved motifs and domains, evolutionary relationships, and gene duplication events were described in detail. Expression profiles of the *CsAIL* genes in cucumber were similar to those of their orthologs in other plants, indicating their potentially conserved functions, including leaf development, ovary and fruit development, root development, embryogenesis, and salt

stress response. Importantly, *CsAIL1* was identified as a candidate regulator of ovary, fruit spine, and seed development in cucumber. This speculation diverges from other plants and requires further functional verification. Moreover, *CsAIL* genes exhibited distinct expression profiles under salt stress, implicating their potential involvement in abiotic stress responses. Subcellular localization confirmed the nuclear presence of *CsAIL1* protein, and interaction network prediction suggested a potential interaction with the ethylene response factor *CsERF13*. Altogether, these findings lay a promising foundation for future functional studies and targeted breeding strategies, potentially enabling the fine-tuning of cucumber development and stress resilience through *CsAIL*-mediated regulatory pathways.

Author contributions

The authors confirm their contributions to the paper as follows: conceived, designed, wrote and revised the manuscript: Yin S, Zhang Y; performed the experiments: Yin S, Chen C; analyzed the data: Yin S, Liu J, Lou A. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed in the current study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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