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Genome-wide identification of the *LRX* gene family in Cucurbitaceae and expression analysis under salt and drought stress in Cucumber

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Abstract

Leucine-Rich Repeats Extensins (LRX) are a type of cell wall protein that participate in the formation of the plant cell wall and play a crucial role in plant growth and development. However, the study of *LRX* genes in Cucurbitaceae has not been reported. Here, we identified 40 *LRX* genes from seven cucurbit species in the Cucurbitaceae database, including cucumber (*Cucumis sativus*), wax gourd (*Benincasa hispida*), watermelon (*Citrullus lanatus*), pumpkin (*Cucurbita maxima*), moschata pumpkin (*Cucurbita moschata*), bottle gourd (*Lagenaria siceraria*), and melon (*Cucumis melon*). These *LRX* genes were divided into two subfamilies: *LRX*, controlling vegetative growth, and *PEX*, controlling reproductive growth. The gene structure, domain, and motif were relatively conserved, indicating that genes within each subfamily have similar functions. The differences in the number of *LRX* genes among the seven cucurbit species indicate the presence of gene loss or duplication events during evolution. Analysis of cis-regulatory elements showed that these *LRX* genes may be involved in plant growth and development, phytohormone response, and biotic and abiotic stress responses. In addition, the expression pattern of *CsLRX* genes in different tissues of cucumber and its expression analysis under salt stress and drought stress were detected by real-time quantitative PCR (qRT-PCR). The results showed that *CsLRX* genes showed organ-specific expression pattern in cucumber and responded to adversity stress. In summary, our results provide a reference for further understanding the role of these genes in cell wall formation and the growth and development of Cucurbitaceae crops.

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Introduction

Leucine-Rich Repeats Extensins (LRX) are a class of cell wall-anchored proteins, with an N-terminal leucine-rich repeat (LRR) domain and a C-terminal extensin domain. In plants, LRR domains are typically present in receptor proteins that can recognize and bind various external signaling molecules, such as hormones and pathogenic proteins. This binding triggers downstream signaling pathways, thereby regulating plant growth, development, and responses to the environment[1–3]. Extensins are highly glycosylated cell wall proteins, with multiple repetitive sequences and hydroxyproline-rich domains[4–6]. These structural features enable extensins to interact with other components within the cell wall, forming a cross-linking network, thereby increasing the strength and stability of the cell wall and playing an important role in cell wall formation and mechanical support for cells[7,8]. There are 11 *LRX* genes identified in *Arabidopsis*, and based on their tissue-specific expression patterns, they can be roughly divided into two subfamilies. The first subfamily includes *LRX1* to *LRX7*, which are highly expressed in rapidly growing tissues such as root tips, shoot tips, and young leaves[9,10]. *LRX* genes play an essential role in plant cell wall formation and cell elongation processes, suggesting their involvement in the regulation of plant development. Specifically, LRX1 is crucial for root hair cell wall formation[11]. Additionally, *LRX3/4/5* are crucial for salt tolerance in *Arabidopsis*. *LRX* interacts with Rapid Alkalinization Factors (RALF) peptides RALF22/23, which in turn interact with the plasma membrane-localized receptor-like protein kinase FERONIA (FER), forming the LRX3/4/5-RALF22/23-FER module. This module controls plant growth and salt stress responses by regulating hormone homeostasis and reactive oxygen species (ROS) accumulation[12–14]. The second subfamily includes *LRX8* to *LRX10* (also known as *AtPEX1-4*, Pollen extension-like), which are expressed in specific reproductive tissues such as flowers and pollen[15–17]. Plant reproduction relies on tightly regulated pollen tube growth to deliver the sperm. This process is controlled by secreted RALF peptides, which have been shown to be perceived by Catharanthus roseus RLK1-like (CrRLK1Ls) receptor-like kinases/LORELEI-like GLYCOLPHOSPHATIDYLINOSITOL (GPI)-anchored protein (LLG) complexes or leucine-rich repeat extensins. *LRX8* to *LRX11* can interact with RALF4/19 on the pollen tube cell wall and enter the signaling pathway mediated by ANNEXIN1/2 (ANX1/2), thus regulating pollen germination and pollen tube growth[18–20]. Disruption of LRX protein function leads to unstructured pectin deposition and changes in wall mechanical properties, resulting in pollen tube defects[18].

In addition, in contrast to the tissue specificity of the *Arabidopsis PEX* genes in reproductive organs, rice *OsPEX1*, besides being expressed outside of reproductive tissues, also exerts negative regulation on root growth in gibberellin-medi-

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ated pathways and regulates root growth by influencing cell wall plasticity[21]. The LRR domain of maize and tomato PEX proteins is highly conserved, and the tomato *PEX* gene exhibits pollen specificity[1]. Comparison of *PEX* genes in maize, tomato, tobacco, or *Arabidopsis* revealed that *PEX* genes not only play a role as structural proteins in polarized pollen tube growth, but may also act as gender recognition factors in the process of interacting with the pistil[22].

The Cucurbitaceae plants are a pivotal group of economic crops, including many species of agricultural and horticultural significance such as cucumber, pumpkin, and watermelon. These crops are widely distributed across the world, particularly in tropical and subtropical regions of Asia, Africa, and the Americas[23,24]. Cucurbitaceae plants are rice in nutritional and medicinal value[25], containing substances such as cellulose, vitamins, minerals, and others[26–28], and they are used in traditional medicine to treat various diseases, including cancer[29], inflammation, diabetes, and detoxification[30]. In recent years, an increasing number of researches on demonstrating that gene families play an essential role in the adaptive changes and physiological function of plants. Although the *LRX* gene families has been discovered in some plants, research on the *LRX* gene families in Cucurbitaceae plants remains relatively limited.

Here, we first identified the *LRX* family members from seven cucurbit species in Cucurbitaceae at a genome-wide level and obtained a total of 40 *LRX* genes. We systematically analyzed the gene structure, conserved motifs, conserved domains, chromosome localization, cis-acting elements and phylogeny of these 40 *LRX* genes. The synteny relationship of *LRX* genes between cucumber and other six cucurbit species was also discussed. Finally, the expression patterns of *CsLRX* genes in different tissues and under drought stress and salt stress in cucumber were analyzed by qRT-PCR. The results of this study will provide a theoretical basis for the functional identification of *LRX* genes in Cucurbitaceae crops.

Materials and Methods

Gene identification and chromosomal localization analysis

Download the LRX protein sequences of *Arabidopsis* and rice from the Ensemble Plants database (https://plants.ensembl.org/index.html). The *AtLRX1 (*At1G12040) gene in *Arabidopsis* was used as a template to search for homologous genes of seven cucurbit species in the Cucurbitaceae database (http://cucurbitgenomics.org/search/genome/2), and further identified by BLAST and HMM Search in TBtools[31]. In addition, perform a BLAST search of these *LRX* genes in the TAIR database (https://www.Arabidopsis.org/) to obtain their homologous genes. The molecular weight (MW) and Isoelectric point (PI) of the identified proteins were studied by ExPASy online software (https://web.expasy.org/compute_pi/). The chromosome distribution map of *LRX* genes was drawn by TBtools[32].

Phylogenetic analysis

There are 11 *LRX* genes known in *Arabidopsis*[33] and 8 in rice[34]. To understand the evolutionary relationship of *LRX* genes in Cucurbitaceae, multiple sequence alignment was performed using ClustalW tool for LRX proteins in Cucurbitaceae, as well as LRX proteins in *Arabidopsis* and rice. A phylogenetic tree was generated by the neighbor-joining (NJ) method with default parameter settings: bootstrap method setting to 1000, Poisson model, and Pairwise deletion in MEGA 11[35]. Further visualization and refinement of the tree were performed via Interactive Tree Of Life (iTOL) (https://itol.embl.de/)[36].

Gene structure analysis, identification of conserved domains and conserved motifs

Gene structure analysis was performed by Tbtools[32], extract the protein sequences of 40 *LRX* genes from seven species in Cucurbitaceae, and submit them to the NCBI-CDD (https://www.ncbi.nlm.nih.gov/cdd) and MEME (https://meme-suite.org/meme/tools/meme) websites for predicting conserved domains and motifs[37,38]. Merge and visualize the results by TBtools[32].

Synteny Analysis

Download the genome and annotation files of *Arabidopsis*, rice, and seven cucurbit species. The intraspecific collinearity analysis of the cucumber *CsLRX* gene family and the synteny analysis between the cucumber *CsLRX* genes and the *LRX* genes of other six cucurbit species were plotted using TBtools. In addition, TBtools was used to draw the synteny nalysis between cucumber *CsLRX* genes and rice and *Arabidopsis*[32].

Analysis of cis-acting elements

The genomic DNA sequences of 2000 base pairs (bp) upstream of the transcription start site of 40 *LRX* genes were extracted from the reference genomes of seven cucurbit species by TBtools, and then submitted to the PlantCare database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to identify possible cis-regulatory elements[39]. Visualize, classify and analyze the prediction results. The heat map of cis-acting element analysis is generated by TBtools[32].

Plant Materials

The North China type (Chinese Long) cucumber inbred line 3667 was grown in a greenhouse of the China Agricultural University in Beijing. Samples from different tissues such as roots, stems, young leaves, tendrils, female flowers, and male flowers were collected. The samples were rapidly frozen in liquid nitrogen and stored at −80 °C.

RNA extraction and qRT-PCR Analysis

For analysis of *CsLRX* genes expression patterns, including salt stress and drought stress. The sample RNA was extracted using the Quick RNA Isolation Kit (Huayueyang, Beijing, China). The FastKing gDNA Dispelling RT SuperMix (TianGen Biotech, Beijing, China) was applied to synthesize the first-strand cDNA with the extractive RNA template.qRT-PCR was performed using the UltraSYBR Mixture (Low ROX) (Cwbio, Beijing, China) on an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City , CA, USA). The UBIQUITIN EXTENSION PROTEIN (UBI-EP) gene was used as a reference gene. Three biological and three technical replicates were carried out for expression dynamics analysis. The significant differences were analyzed by Student's t-tests ($p < 0.05$). The primers were listed in Supplemental Table S2.

Subcellular localization

Cloning of CsLRX1 and CsLRX3 coding sequences without stop codons and construct it into the pSuper-1300 vector containing the green fluorescent protein (GFP) to create a fusion protein. After selecting the correct vector through

sequencing, extract the plasmid and transform it into the GV3101 Agrobacterium strain. Resuspend the Agrobacterium liquid, which contains the target vector, a nuclear marker vector, and P19, in infection solution to an OD_{600} of approximately 1.0, then mix and let it stand for 3 hours. Select 4-week-old *N. benthamiana* leaves for infiltration, and after a dark treatment of 12-24 hours followed by a light treatment of 48 hours, observe the GFP fluorescence using a confocal microscope. The subcellular localization primers listed in Supplemental Table S2.

Results

Whole-genome identification of *LRX* genes in seven cucurbit species and their chromosomal distribution

The known *LRX* genes (*AtLRX1*, At1G12040) protein sequences of *Arabidopsis* were used as templates to search their homologous genes in the genome database (http://cucurbitgenomics.org/search/genome/2) of seven cucurbit species, respectively. BLAST and HMM Search in Tbtools were used to further identify and finally 40 *LRX* genes were obtained in Table 1, including 6 from cucumber (*C.sativus*), 5 from wax gourd (*B.hispida*), 4 from watermelon (*C.lanatus*), 8 from pumpkin (*C.maxima*), 8 from moschata pumpkin (*C. moschata*), 5 from bottle gourd (*L. siceraria*), and 4 from melon (*C. melon*) (Table 1). The coding sequence lengths ranged from 936 to 4956 bp. The physical characteristics of these LR X proteins were predicted, revealing significant differences among the 40 LRX family proteins (Table 1). The average amino acid length was 646.4 aa, with predicted protein molecular weights ranging from 42123.14 to 166407.81 Da, and theoretical Isoelectric points (PI) ranging from 3.7 to 10.08 (Table 1). The identified genes are named based on their chromosomal locations rather than following uniform naming conventions.

To clarify the physical locations of *LRX* genes in the genomes of these cucurbit species, we generated chromosome distribu-

Table 1. Genome-wide identification of *LRX* gene family members in seven cucurbit species

Gene Name	Gene ID	Gene Position		CDS (bp)	AA	MW (Da)	PI	*Arabidopsis* Homology
		Start	End (+/−)					
CsLRX1	*Csa1G383520.1*	14493464	14496369 (+)	2328	776	42123.14	4.84	*AT4G13340*
CsLRX2	*Csa2G004760.1*	814446	817377 (+)	2298	766	82435.4	6.5	*AT4G13340*
CsLRX3	*Csa3G146350.1*	9732797	9733957 (+)	1161	387	43772.94	5.71	*AT3G22800*
CsPEX1	*Csa6G005160.1*	473143	474726 (−)	1584	528	42813.05	4.68	*AT2G15880*
CsPEX2	*Csa6G006180.1*	487825	489724 (−)	1854	618	76300.69	6.17	*AT2G15880*
CsPEX3	*Csa6G006190.1*	491897	494318 (+)	2286	762	41120.04	5.01	*AT2G15880*
BhiLRX1	*Bhi01M000558*	14563184	14564624 (+)	1158	386	89384.76	6.46	*AT3G22800*
BhiLRX2	*Bhi02M000924*	25237963	25240855 (−)	2304	768	82573.58	5.98	*AT3G24480*
BhiLRX3	*Bhi06M001733*	55954895	55964421 (+)	1170	390	76954.31	5.4	*AT4G18670*
BhiPEX1	*Bhi09M001722*	56817604	56820319 (−)	1176	392	57276.43	5.83	*AT3G19020*
BhiPEX2	*Bhi12M001681*	60171798	60173997 (+)	2100	700	120050.2	7.96	*AT3G19020*
ClaLRX1	*Cla97C05G086020.1*	4563471	4564598 (+)	1128	376	41548.56	4.58	*AT3G22800*
ClaLRX2	*Cla97C06G119550.1*	20517876	20520416 (+)	2541	847	148150.13	5.41	*AT3G24480*
ClaLRX3	*Cla97C11G208980.1*	2574856	2577150 (+)	2295	765	166407.81	3.7	*AT4G13340*
ClaPEX1	*Cla97C06G112900.1*	3979734	3981893 (−)	2160	720	81584.74	6.46	*AT3G19020*
CmaPEX1	*CmaCh01G018740.1*	12291107	12293909 (−)	1590	530	54054.38	5.13	*AT4G33970*
CmaLRX1	*CmaCh05G013340.1*	10085979	10089368 (−)	3390	1130	74989.78	4.43	*AT3G24480*
CmaLRX2	*CmaCh06G013780.1*	8987856	8988998 (+)	1143	381	42903.13	5.18	*AT3G22800*
CmaPEX2	*CmaCh08G008980.1*	5563777	5568808 (−)	4275	1425	40235.09	4.75	*AT4G33970*
CmaPEX3	*CmaCh09G001990.1*	826607	831562 (+)	4956	1652	35186.52	4.96	*AT3G19020*
CmaLRX3	*CmaCh10G006020.1*	2780772	2788301 (−)	2328	776	52221.21	5.8	*AT4G13340*
CmaLRX4	*CmaCh14G017400.1*	12740774	12742300 (−)	1527	509	88647.32	6.16	*AT3G22800*
CmaPEX4	*CmaCh17G004460.1*	2660615	2662696 (+)	2082	694	83972.54	5.88	*AT2G15880*
CmoPEX1	*CmoCh01G019320.1*	13801421	13802672 (−)	1182	394	40916.77	4.78	*AT3G19020*
CmoLRX1	*CmoCh06G013900.1*	10200801	10201904 (+)	1104	368	85186.25	4.81	*AT3G22800*
CmoPEX2	*CmoCh08G008690.1*	5609995	5611160 (−)	936	312	82799.35	6.04	*AT3G19020*
CmoPEX3	*CmoCh08G008700.1*	5621196	5622985 (−)	1413	471	82892.28	6.06	*AT3G19020*
CmoLRX2	*CmoCh10G006420.1*	2945225	2947750 (−)	2526	842	42186.25	4.73	*AT4G18670*
CmoLRX3	*CmoCh12G012710.1*	11268328	11273318 (−)	2328	776	55986.22	4.62	*AT4G13340*
CmoLRX4	*CmoCh14G017780.1*	13715371	13717483 (−)	1128	376	66641.59	5.19	*AT3G22800*
CmoPEX4	*CmoCh17G004250.1*	2835184	2837965 (+)	2355	785	79908.67	4.65	*AT2G15880*
LsiPEX1	*Lsi04G016020.1*	23544642	23546786 (−)	1944	648	67872.59	5.61	*AT2G15880*
LsiLRX1	*Lsi05G016310.1*	23953552	23954682 (−)	1131	377	41040.03	4.92	*AT3G22800*
LsiLRX2	*Lsi06G008900.1*	18515443	18517854 (+)	2412	804	85115.97	6.11	*AT4G13340*
LsiPEX2	*Lsi09G015830.1*	23966231	23967994 (−)	1764	588	62720.9	4.87	*AT2G15880*
LsiLRX3	*Lsi11G002700.1*	2740543	2743776 (+)	2226	742	79910.5	5.85	*AT4G13340*
MELOLRX1	*MELO3C004550.2.1*	28466831	28470698 (−)	1770	590	null	null	*AT3G24480*
MELOLRX2	*MELO3C006506.2.1*	3799835	3800974 (+)	1122	374	40873.81	4.84	*AT3G22800*
MELOPEX1	*MELO3C021195.2.1*	31428500	31430511 (−)	1947	649	70632.34	6.37	*AT3G19020*
MELOPEX2	*MELO3C034935.2.1*	31442588	31444077 (+)	1446	482	52356.41	5.13	*AT3G19020*

Note: CDS: coding sequence, AA: the number of amino acids, MW: Molecular weight, PI: Theoretical isoelectric point

tion maps of these genes (Supplemental Fig. S1). Specifically, in cucumber, 6 *LRX* genes were distributed on chromosomes 1, 2, 3, and 6; in wax gourd, 5 were found on chromosomes 1, 2, 6, 9, and 12; in watermelon, 4 were located on chromosomes 5, 6, and 11; in pumpkin, 8 were distributed on chromosomes 1, 5, 6, 8, 9, 10, 14, and 17; in moschata pumpkin, 8 were present on chromosomes 1, 6, 8, 10, 12, 14, and 17; in bottle gourd, 5 were found on chromosomes 4, 5, 6, 9, and 11; and in melon, 4 were located on chromosomes 5, 6, and 11 (Supplemental Fig. S1).

Phylogenetic analysis of the *LRX* genes in Cucurbitaceae

In *Arabidopsis*, there are known to be 11 *LRX* genes[33], while rice contains 8 *LRX* genes[34]. To further explore the phylogenetic relationships and evolutionary patterns of *LRX* genes in the Cucurbitaceae family, this study utilized the 11 *LRX* genes in *Arabidopsis*, the 8 *LRX* genes in rice, and the 40 *LRX* gene members from seven cucurbit species to construct a phylogenetic tree using the neighbor-joining method (Fig. 1). In *Arabidopsis*, *LRX* genes are typically divided into two subfamilies, with genes highly expressed in vegetative tissues represented by *LRX*, and genes highly expressed in reproductive organs represented by *PEX*[34]. Consistent with previous studies, all *LRX* genes were divided into two clades, with the first clade containing 34 *LRX* homologues (including 7 *AtLRX*, 5 *OsLRX*, 3 *CsLRX*, 3 *BhiLRX*, 3 *ClaLRX*, 4 *CmaLRX*, 4 *CmoLRX*, 3 *LsiLRX*, 2 *MELOLRX*), while the remaining 25 *LRX* homologues belonged to the second clade (including 4 *AtPEX*, 3 *OsPEX*, 3 *CsPEX*, 2 *BhiPEX*, 1 *ClaPEX*, 4 *CmaPEX*, 4 *CmoPEX*, 2 *LsiPEX*, 2 *MELOLRX*) (Fig. 1). The evidence shows that these *LRX* genes are closely related in their evolutionary relationships, with no significant genetic differences.

Analysis of gene structure, conserved motifs, and conserved domains of *LRX* genes in Cucurbitaceae

The gene structure provides important clues for its functional diversification and can also reflect the evolutionary history of the gene family. Therefore, the exon-intron structure

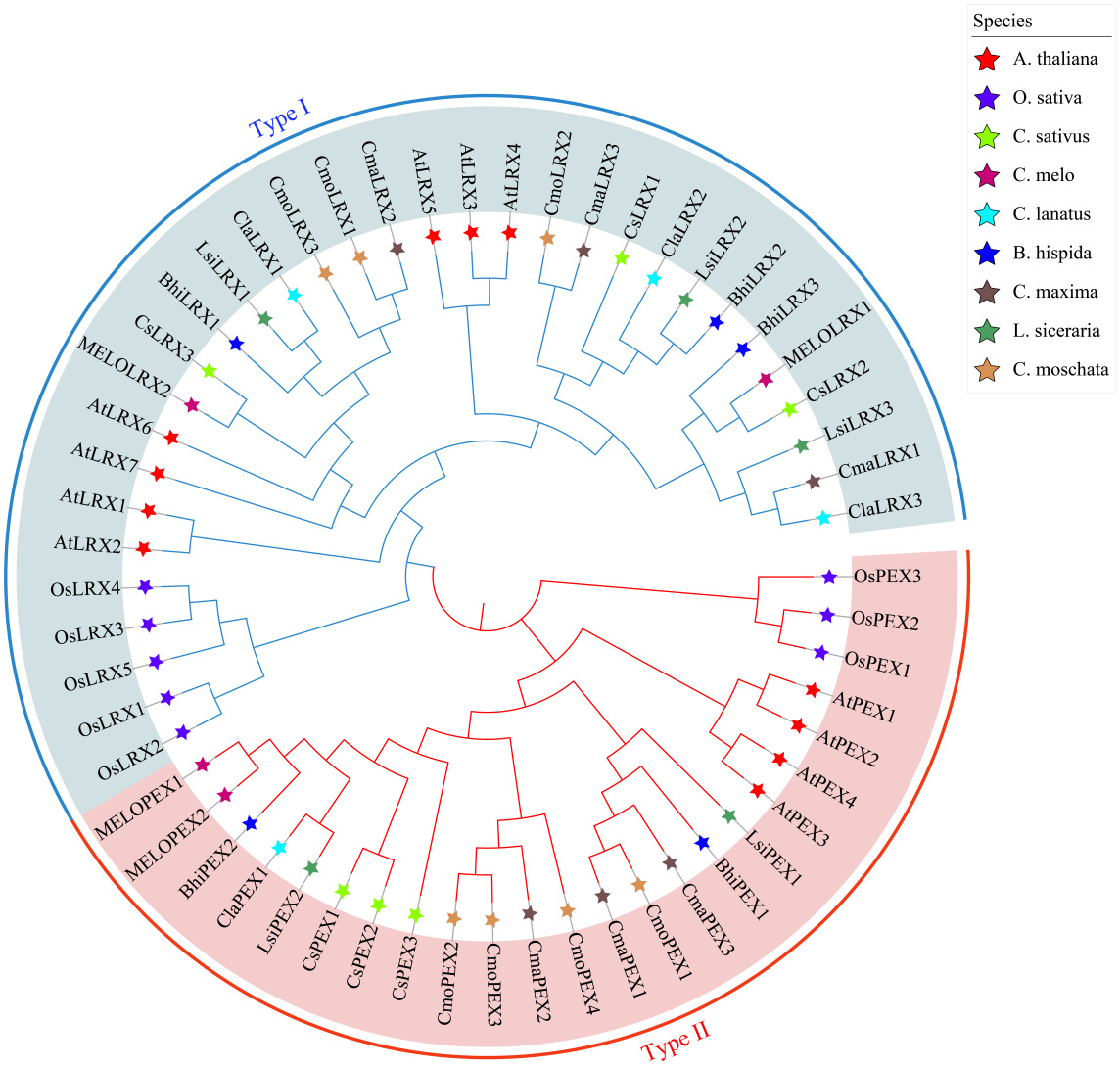

Fig. 1 Phylogenetic tree of the LRX proteins from *Arabidopsis*, rice, and seven cucurbit species. Red star, purple star, light green star, deep red star, sky blue star, deep blue star, deep brown star, deep green star and light brown star represent *Arabidopsis thaliana* (*A. thaliana*), rice (*O. sativa*), cucumber (*C. sativus*), melon (*C. melon*), watermelon (*C. lanatus*), wax gourd (*B. hispida*), pumpkin (*C. maxima*), bottle gourd (*L. siceraria*) and moschata pumpkin (*C. moschata*), respectively.

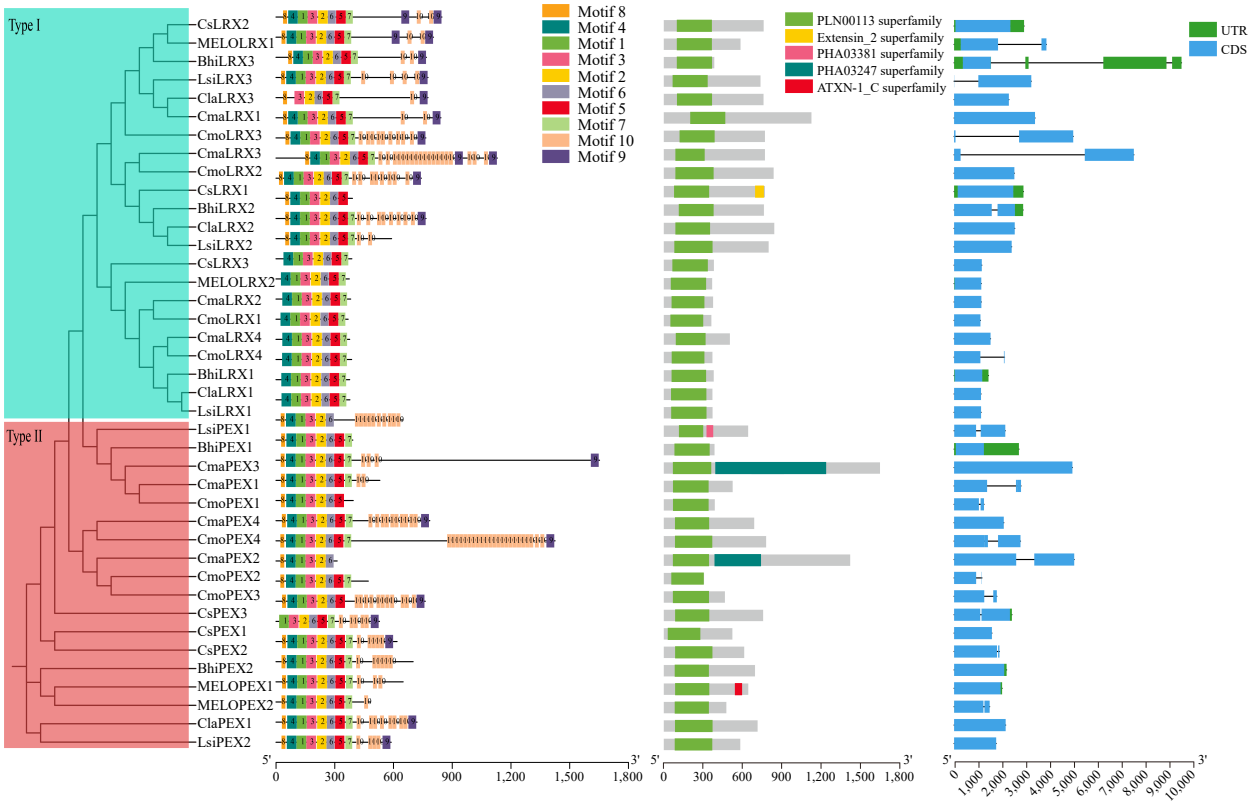

Fig. 2 Phylogenetic clustering, conserved motifs, conserved domains and gene structures of seven cucurbits *LRX* genes. Left one: The phylogenetic tree of *LRX* genes. Light green and brownish red represent *LRX* and *PEX* subfamilies, respectively. Left two: the conserved motif of *LRX* genes, different colors represent different motifs; right one: *LRX* gene structure analysis, green and blue represent the UTR region and the CDS region, respectively; right two: the conserved domain of *LRX* genes, different colors represent different domains.

of these *LRX* genes was further analyzed (Fig. 2). Gene structure analysis showed that most genes lack a 5' untranslated region (UTR) or a 3' UTR region, containing 1 to 2 exons, with most genes lacking introns. For example, all 4 *LRX* genes in watermelon contain only one exon structure, without introns and UTR regions. Among the 6 *LRX* genes in cucumber, only two genes each contain one intron, while the remaining four have no introns (Fig. 2). This is consistent with the prediction of their small number of transcripts. Although the UTR regions of *LRX* genes within the same subfamily exhibit differences in size and structure, suggesting their involvement in different regulatory processes and potentially functioning in different biological functions. Overall, genes within the same subfamily show a similar gene structure, indicating a higher degree of conservation during the evolutionary process. This conservation may reflect the similar biological functions performed by these genes across different species or the presence of similar evolutionary pressures.

Subsequently, the MEME software was used to predict the conserved motifs in these *LRX* genes (Fig. 2, Supplemental Fig. S2). Among them, Motif1 to Motif7 are conserved motifs, shared by the vast majority of *LRX* genes and mainly located at the N-terminus. Motif10 appears most frequently and is located at the C-terminal, with 22 tandem repeats in *CmaLRX1*, suggesting that this repetition indicates the importance of this motif for gene function. Motif 9 was not found in the *LRX* genes of melon, but is present in the other six species of cucurbits, suggesting that the *LRX* genes in melon have undergone specific changes in function or regulation, which may also reflect the diversity of this motif among different species or individuals (Fig. 2). Considering that LRX is a class of cell wall proteins containing a conserved N-terminal leucine-rich repeat domain[16] and a typical C-terminal extensin protein domain[4], we visualized the conserved domains of 40 LRX proteins and found that all *LRX* genes contain these two conserved sequences (Fig. 2, Supplemental Fig. S3), indicating the high functional and structural conservation of these genes.

Synteny analysis of *LRX* genes

The existence of collinearity can provide important clues for gene evolution and evolutionary relationships. By comparing genes with collinearity, it is possible to reveal their common ancestors and evolutionary history, and to infer the origin and expansion process of gene families[40]. To better understand the amplification patterns of *LRX* genes during the evolutionary process, we used TBtools software to conduct collinearity analysis of *LRX* genes in cucumber and six other cucurbit species (Fig. 3a). The results indicated that cucumber shares collinear genes with 4, 6, 8, 7, 6, and 6 genes in wax gourd, watermelon, pumpkin, moschata pumpkin, bottle gourd, and melon, respectively, suggesting that cucumber, wax gourd, watermelon, pumpkin, moschata pumpkin, bottle gourd, and melon may have common ancestral genes, and have retained similar gene structures and functions during the evolutionary process (Supplemental Table S1). 83% of cucumber *CsLRX* genes showed synteny relationship with moschata pumpkin, while 75% of cucumber *CsLRX* genes showed synteny relation-

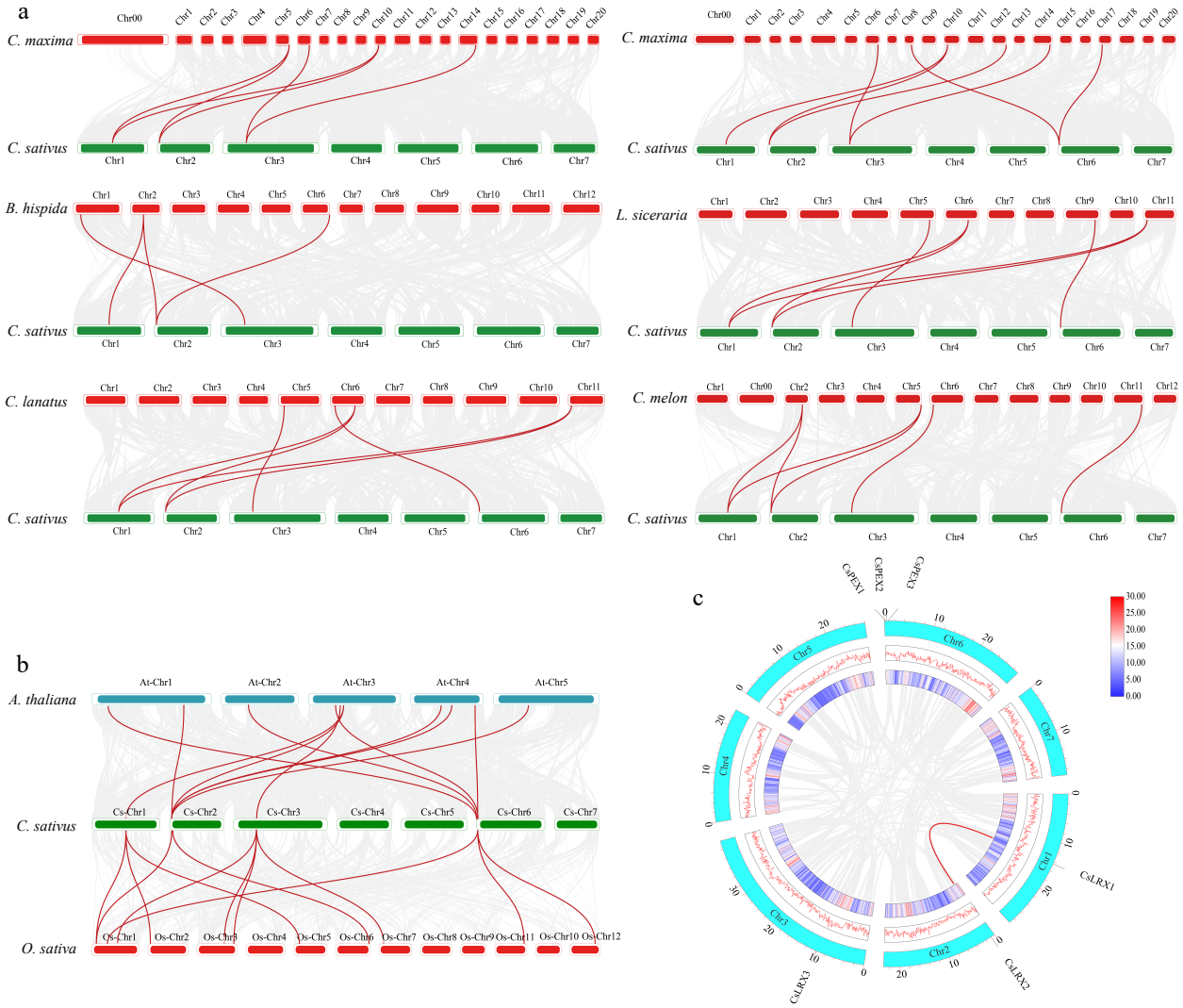

Fig. 3 Synteny analysis of *LRX* genes. (a) Synteny analysis of *LRX* genes between cucumber and other six cucurbits. (b) Synteny analysis of *LRX* genes in cucumber, *Arabidopsis thaliana (A. thaliana)* thaliana and rice (*O. sativa*). (c) Colinearity analysis of *CsLRX* genes in cucumber species. The red and blue lines represent gene pairs with collinearity.

ship with watermelon, bottle gourd, and melon, with the least relationship found between wax gourd and pumpkin, accounting for 50% (Fig. 3a). Interestingly, *CsLRX1* and *CsLRX2* in cucumber both show collinearity with *LRX* genes in the other six cucurbit species, and each has two pairs of collinear gene pairs with melon, watermelon, bottle gourd, and pumpkin. In addition, *CsLRX1* and *CsLRX2* replicate each other (Fig. 3c), promoting the expansion of the cucumber *LRX* gene family. The different numbers of collinearity relationships between cucumber *LRX* genes and *LRX* genes in other cucurbit species may indicate gene rearrangements, insertions, or deletions during the evolution of these crops, leading to differences in the number of collinear gene pairs in their genomes.

Rice and *Arabidopsis* are representative model plants in the plant kingdom, and we conducted synteny analysis among cucumber, *Arabidopsis*, and rice to reveal their evolutionary relationships and genetic changes (Fig. 3b). We identified 11 gene pairs between cucumber and *Arabidopsis*, and 12 gene pairs between cucumber and rice, a similarity in number that may imply a certain degree of conservation and similarity between cucumber and *Arabidopsis*, as well as between cucumber and rice. Gene duplication is usually caused by repeat events in the genome, such as whole-genome duplication, segmental duplication, *etc*., and it can help us understand the diversity and conservation of gene functions[40,41]. Through intraspecific collinearity analysis, we further explored segmental duplication events of *CsLRX* genes in cucumber. We identified one pair of duplicated genes in the cucumber genome: *CsLRX1* and *CsLRX2*, which provides the potential for the diversity and emergence of new gene functions through duplication (Fig. 3c).

Cis-acting elements analysis of Cucurbitaceae *LRX* genes promoter regions

To better understand the transcriptional regulation and potential functions of *LRX* cucumber (*C. sativus*), wax gourd (*B. hispida*), watermelon (*C. lanatus*), pumpkin (*C. maxima*), moschata pumpkin (*C. moschata*), bottle gourd (*L. siceraria*), and melon (*C. melon*) genes, we predicted cis-regulatory elements in their promoters. Core elements of promoters such as TATA-box and CAAT-box were found in all *LRX* genes. The

functional cis-regulatory elements in the promoters can be mainly divided into three categories, including biotic and abiotic stress response, plant hormone response, and plant growth and development (Fig. 4). Multiple stress-responsive element (SRE) binding promoter elements ARE were found in the promoters of 38 *LRX* genes, consistent with the function of LRX as a cell wall protein involved in maintaining cell wall integrity to reduce cell damage caused by external stress. Multiple TAACCA (CCAAT-box) elements, which is bound by transcription factors and regulate gene transcription activity and stress response processes, were also discovered[42]. Additionally, low-temperature-responsive (LTR) elements and stress responsiveness (TC-rich repeats) elements were found in individual *LRX* genes. Interestingly, there are MYB elements in the *LRX* genes of seven cucurbits, and some MYB transcription factors are involved in the response of plants to abiotic stresses such as drought, high salt and low temperature. They enhance plant tolerance by regulating gene expression related to stress response[31]. Numerous hormone-related elements were also discovered in the promoter regions of these *LRX* genes, including multiple ethylene response elements (ERE) in 34 *LRX* genes and other hormone response elements such as methyl jasmonate response motifs, including CGTCA and TGACG; gibberellin response elements, such as GARE and P-box. *LRX* genes also contains plant development-related elements, such as G-box. There are also some widely functional cis-regulatory elements such as the binding site W-box for the WRKY family of transcription factors, which are involved in regulating various biological processes including growth and development, hormone signal transduction, and stress response[43,44]. All of the above indicate that *LRX* plays a crucial regulatory role in physiological processes in plant growth and development, abiotic stress, and hormone signaling.

Expression analysis of *LRX* genes in Cucurbitaceae different tissues and qRT-PCR analysis in Cucumber

We plotted the heat map based on the published RNA-seq data (Fig. 5a). *CsLRX1* in cucumber was specifically expressed in stems, *CsLRX2* was highly expressed in stems, and was also highly expressed in male flowers, roots and ovaries. *CsLRX3* was specifically expressed in roots, while *CsPEX1*, *CsPEX2* and *CsPEX3* were specifically expressed in male flowers. These findings are consistent with previous reports that *LRX* is highly expressed in vegetative organs and *PEX* is highly expressed in reproductive organs[15,17]. The *CmoLRXs* in moschata pumpkin were specifically expressed in different tissues. The expression of *CmaLRXs* in pumpkin was low in fruit and specifically expressed in leaves, stems and roots. In bottle gourd, *LsiLRXs* had low expression in leaves and specific expression in other tissues. *MELOLRX2*, *MELOPEX1* and *MELOPEX2* are strongly expressed in male flowers of melon, indicating that they play an important role in male flower development.

Furthermore, we further validated the expression patterns of 6 *CsLRX* genes in different cucumber tissues (root, stem, leaf, tendril, male flower, female flower) using qRT-PCR (Fig. 5b). The data results showed minor differences compared to the public RNA-seq data, which could be attributed to variations in plant growth environment, sampling methods, and sampling time. However, the overall expression patterns were similar. For example, *CsLRX1* and *CsLRX2* genes showed relatively high expression in the stem as well as in the root, male flower, and female flower, while *CsLRX3* exhibited specific expression in the roots. *CsPEX1* to *CsPEX3* genes were highly expressed in male flowers, suggesting functional redundancy in regulating male flower development. Additionally, the expression levels of *CsLRX* genes in cucumber leaves and tendrils were relatively low, especially with almost undetectable levels in tendrils. In conclusion, our results suggest that these genes play a promi-

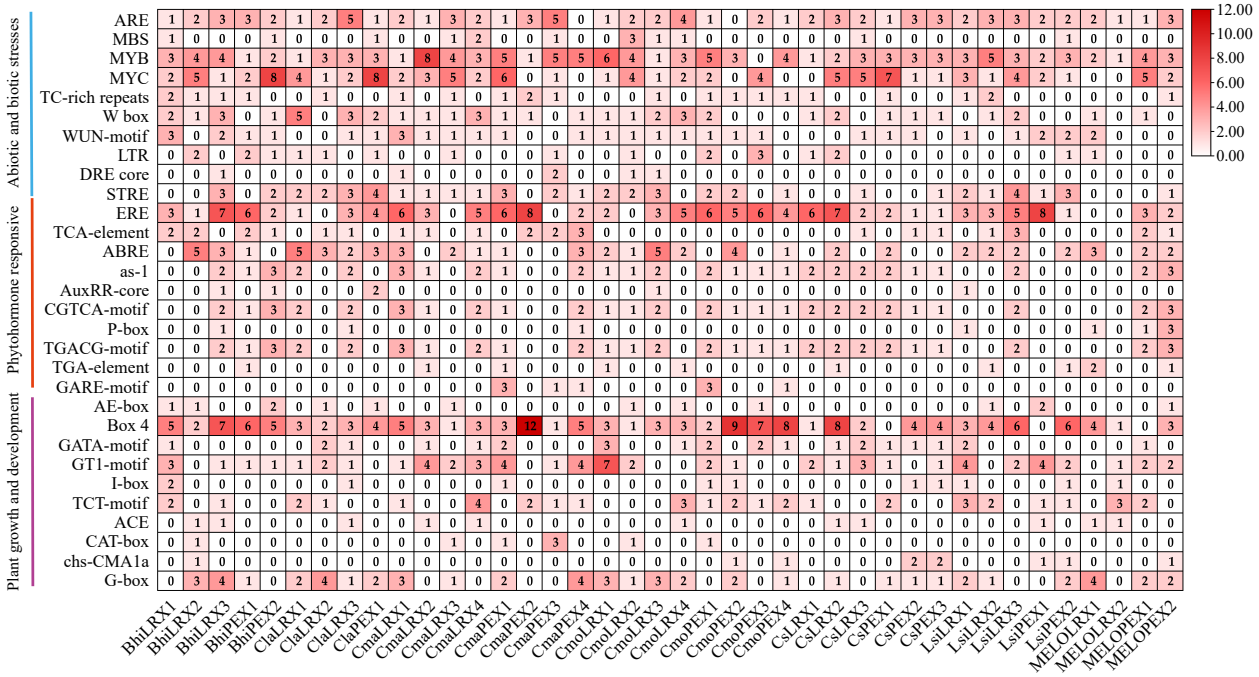

Fig. 4 Cis-acting elements in the promotors of *LRX* genes in seven cucurbit species. The colors indicate the different cis-elements numbers. Values indicate the statistical number of cis elements.

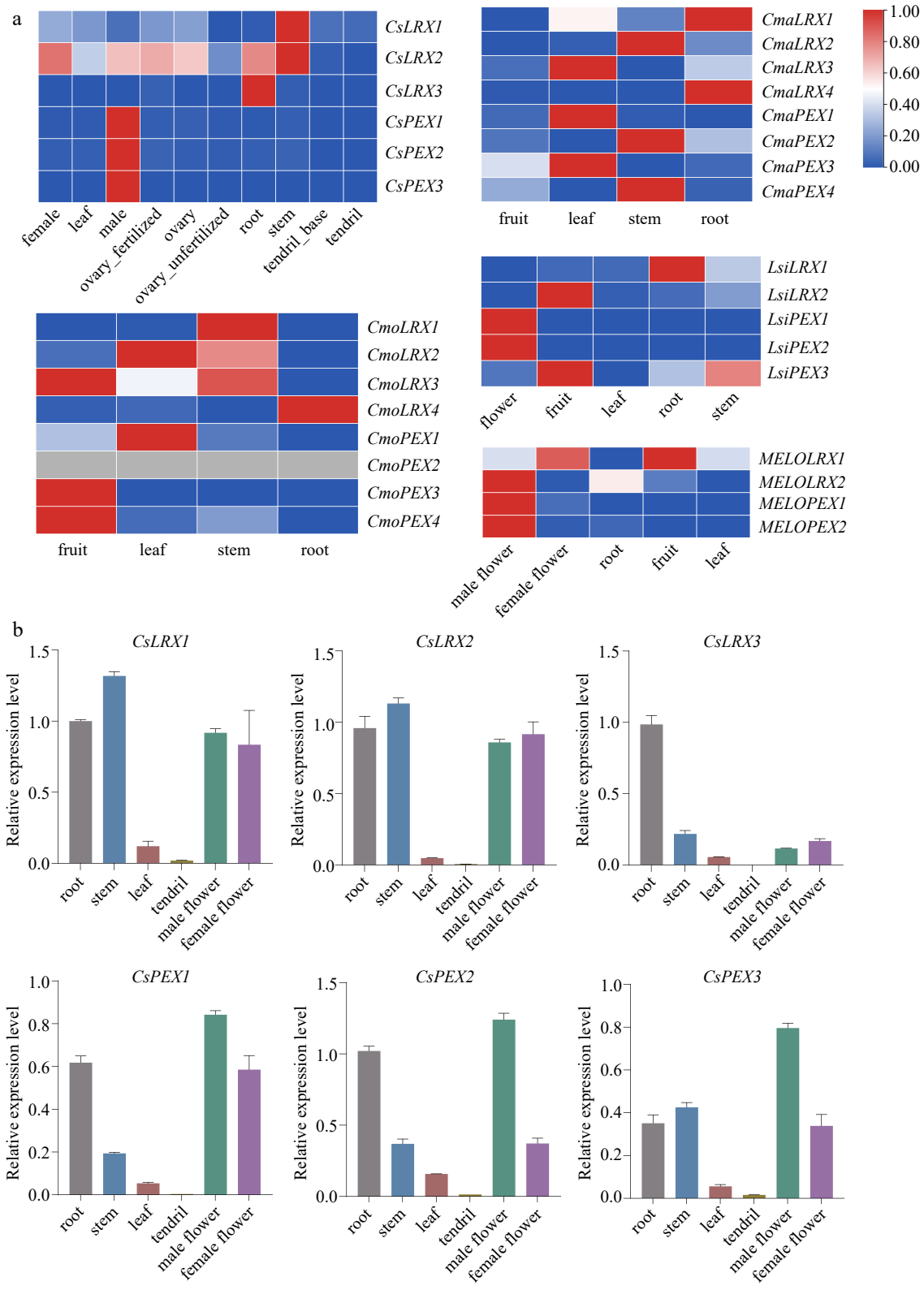

Fig. 5 The expression pattern of *LRX* genes in different Cucurbitaceae plant tissues. (a) The expression of public RNA-seq data of *LRX* genes in different tissues of Cucurbitaceae; (b) qRT-PCR analysis of *CsLRX* genes in different tissues of cucumber. Values are means ± SD of three biological replicates.

nent role in the growth of plant vegetative organs and in regulating male flower development.

The *CsLRX* genes responds to salt stress and drought stress in Cucumber

Plants need to regulate growth and respond to stress by

sensing and transmitting cell wall signals. In *Arabidopsis*, *LRX3/4/5* are involved in plant responses to salt stress[12]. In order to explore whether the *CsLRX* genes in cucumber is induced by salt stress and drought stress, we treated the roots of cucumber with 100 mM NaCl and 10% PEG6000, and sampled at 0 h, 3 h, 6 h, 9 h, 12 h and 24 h for qRT-PCR to detect its expression (Fig. 6a). The results showed that *CsLRX1*, *CsLRX2* and *CsLRX3* involved in vegetative growth responded to salt stress and drought stress. Under salt stress, *CsLRX1*, *CsLRX2* and *CsLRX3* all showed a downward trend at 3 h, and then continued to rise to the highest value and then showed a downward trend. The difference is that *CsLRX1* and *CsLRX2* have the highest expression at 12 h, while *CsLRX3* has a peak at 9 h (Fig. 6b). They may help plants maintain the integrity and stability of cell walls in high-salt environments by regulating the morphological and mechanical properties of cell walls. Under drought stress, they all showed an upward trend (Fig. 6c). In fact, cucumbers had wilted after 9 hours of treatment, indicating that these genes played an important regulatory role in cucumber response to drought stress. The different expression patterns of *LRX* genes in cucumber under salt stress and drought stress reflect the different physiological response strategies of plants to these two different types of abiotic stresses. The expression changes of these genes reveal that plants optimize their survival strategies by regulating the expression of cell wall proteins in the face of environmental stress.

To detect the subcellular localization of these genes, we removed the stop codon of these three genes in cucumber and constructed them into p1300 vector containing GFP tag, and transiently expressed them in *N. benthamiana* leaves. Because the CDS sequence of CsLRX2 contains many tandem repeat structures, it is impossible to clone the complete CDS region. Therefore, only CsLRX1 and CsLRX3 proteins were explored. The results showed that GFP fluorescence was observed in cell wall, cell membrane and cytoplasm (Fig. 6d). This is consistent with the fact that LRX protein is a kind of plant cell wall protein, which participates in the growth and development of plants by regulating the morphology and properties of cell wall[10].

Interaction network prediction of CsLRX protein in cucumber

In order to further explore the biological function of CsLRX protein in cucumber, this study used the STRING database to search and analyze its potential interacting proteins. It has been found that many proteins containing LRR domain may interact with CsLRX protein. The LRR domain has attracted much attention due to its key role in the interaction between various proteins, suggesting that CsLRX protein may play a role in many aspects of plant growth. In addition, according to the expression pattern of *LRX* gene, it can be divided into LRX proteins expressed in vegetative growth stage and PEX proteins expressed in reproductive growth stage[34]. In particular, most of the proteins interacting with LRX proteins are closely related to the maintenance of cell wall integrity. For example, RLP12 (*Csa7G032260*), a member of the receptor-like protein family, is crucial for the jasmonic acid (JA) signaling pathway induced by coronatine and is involved in the maintenance of cell wall integrity[45]. On the other hand, proteins interacting with PEX proteins are mainly involved in anther development. BAME1 (*Csa6G425100*) kinase, for example, affects cell division and differentiation by regulating intercellular communication during early anther development, thereby forming cell layers[46]. In summary, CsLRX protein may play a key role in regulating plant vegetative growth and reproductive growth by interacting with proteins containing LRR domain.

Discussion

The *LRX* genes play a significant role in plant cell wall formation and plant growth and development[33]. The proteins encoded by *LRX* genes contain abundant leucine-rich repeat sequences, which form a helical structure and participate in cell-cell interactions and signal transduction[10], regulating the synthesis and remodeling of plant cell walls[5]. *LRX* genes also possess a unique Extensin domain, rich in amino acids such as leucine, glutamic acid, and cysteine, forming a cross-linked polymer that contributes to the formation and stability of plant cell walls[5]. They are chimeric proteins that are insoluble in the cell wall and form a protein-protein interaction platform[6]. LRX protein regulates cell wall expansion by binding to RALF peptide hormones and directly interacts with the transmembrane receptor FERONIA, thereby participating in cell growth regulation[6] In addition, LRX protein also plays an important role in pollen tube growth and pollen germination. Especially in Arabidopsis, LRX protein interacts with RALF4/19 polypeptide to control the integrity and growth of pollen tubes[18,33]. Studies have shown that mutations in the LRX protein can lead to defects in cell wall structures such as root hairs, further confirming its importance in cell wall development[18]. Additionally, *LRX* genes are involved in responses to stress conditions such as salt stress. In *Arabidopsis*, *LRX* has been reported to be involved in regulating various biological processes, including root hair development and resistance to salt stress[12]. Therefore, LRX protein plays an indispensable role in proper plant development and growth regulation.

Cucurbitaceae crops include many important fruit and vegetable that are widely distributed in tropical and subtropical regions and have high economic value[23]. Cucurbit species have various forms and uses, such as food, medicinal, and ornamental purposes, and are extensively cultivated and utilized[25]. In recent years, with the development of high-throughput sequencing technologies, genomic research on cucurbits has been conducted, and the genome sequences of several cucurbit crops have been published[47–52]. Furthermore, ongoing genomic studies are being conducted on other cucurbit crops such as bitter gourd and Buddha's hand fruit[53]. The release of these genome sequences will facilitate a deeper understanding of the genetics, biology, and agricultural applications of cucurbit plants. Research on Cucurbitaceae crops has received significant attention, involving various aspects such as growth and development, stress resistance, gene regulation, and more. However, there is currently no reported research on *LRX* genes in the Cucurbitaceae family. In this study, we identified and characterized the *LRX* genes in cucurbit plants based on these published whole-genome sequences. We identified 6, 5, 4, 8, 8, 5, and 4 *LRX* genes in cucumber, wax gourd, watermelon, pumpkin, moschata pumpkin, bottle gourd, and melon, respectively (Table 1). The distribution of these genes on chromosomes is random (Supplemental Fig. S1). Similar to the 11 *LRX* genes in *Arabidopsis*, we found that the *LRX* genes in these seven cucurbit species are also divided into two subfamilies, *LRX* and *PEX* (Fig. 1). It is worth noting that the number of *LRX*

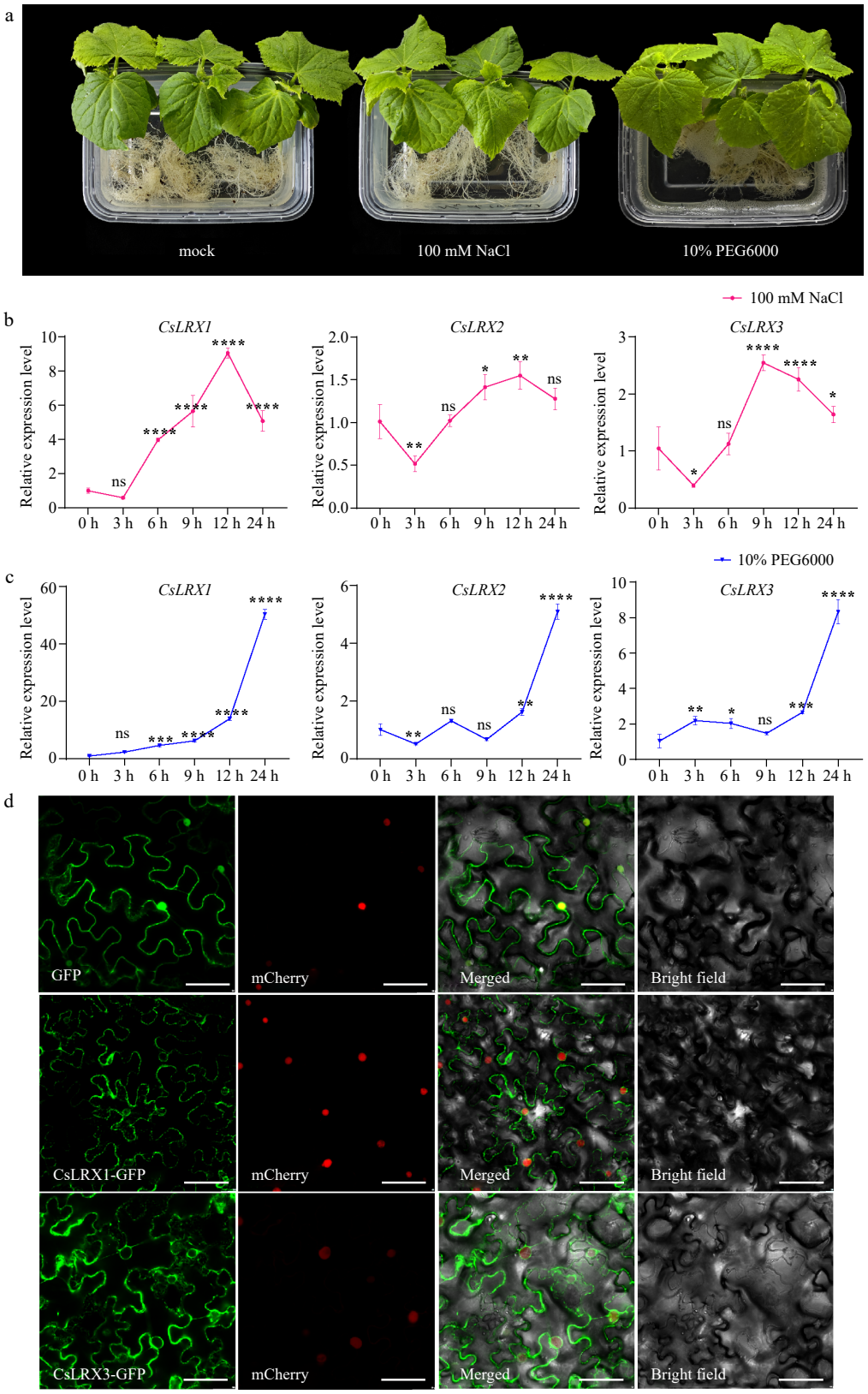

Fig. 6 Expression analysis of *CsLRX* genes in cucumber. (a) Cucumber roots were treated with 100 mM NaCl and 10% PEG6000, and water was used as a control (mock). (b-c) The *CsLRX* genes in cucumber roots treated with 100 mM NaCl and 10 % PEG6000 for different time was analyzed by qRT-PCR. Values are means ± SD of three biological replicates. Significant differences between 0 h and other time points are indicated by asterisks (ns: no significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, Student's t test). (d) Subcellular localization of CsLRX1 and CsLRX3. The empty vector was used as a control. These indicated structures were transiently expressed in *N. benthamiana* leaves. Bar, 50 μm.

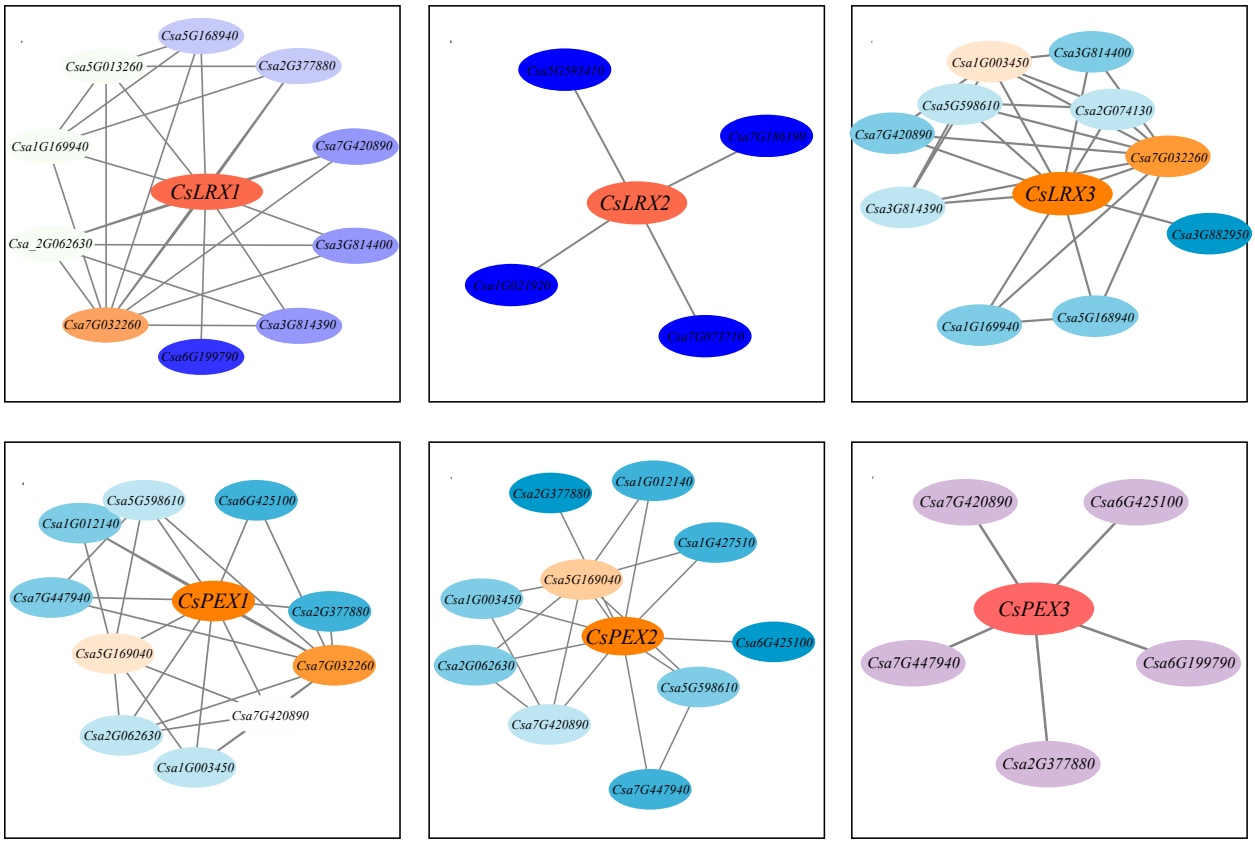

Fig. 7 Interaction network prediction of CsLRX protein in cucumber.

genes in these seven cucurbit species varies, but they are less than *LRX* genes in *Arabidopsis*. We speculate that the differences in the number of *LRX* genes between different cucurbit species may be related to gene duplication or loss during the evolutionary process, as segmental and tandem duplications promote the expansion of gene families[40]. These *LRX* genes have highly similar structures, with 1 to 2 exons, and the majority do not have introns (Fig. 2). Most *LRX* genes share conserved motifs Motif1 to Motif7, mainly located at the N-terminus. Motif10 appears most frequently. In general, the repeated occurrence of conserved motifs may indicate their crucial role in regulating gene expression or protein structure, suggesting their importance and diversity in gene function. Conserved domain analysis revealed an LRR domain at the N-terminus and an extensin domain at the C-terminus (Fig. 2), which is consistent with previous reports[33].

To better understand the amplification patterns of genes during the evolutionary process, we conducted synteny analysis of *LRX* genes in cucumber and six cucurbit species were 4, 6, 8, 7, 6, and 6, respectively (Fig. 3a; Supplemental Table S1). Interestingly, *CsLRX1* and *CsLRX2* in cucumber have collinearity with *LRX* genes of other six cucurbits, and there are two pairs of collinearity gene pairs with melon, watermelon, bottle gourd and pumpkin respectively. Furthermore, *CsLRX1* and *CsLRX2* replicated each other in cucumber (Fig. 3c), promoting the expansion of the cucumber *LRX* gene family[40]. These differences in quantity may reflect various genetic changes, including gene rearrangements, insertions, or deletions, that these cucurbit species experienced during the evolutionary process[54]. These differences may be associated with genetic diversity in growth, development, stress resistance, and quality traits. The cis-acting elements on the promoter can reveal the mechanism of gene expression regulation, cell signal transduction network and the influence of gene-environment interaction[55]. Generally, transcription factors bind to specific cis-acting elements to regulate the expression of target genes. The analysis of cis-acting elements in the promoters of 40 *LRX* genes showed that these genes may be related to the growth and development of cucurbitaceae plants, phytohormone response and response to biotic and abiotic stresses. A large number of stress response elements and hormone response elements were identified, such as ARE, ERE, GARE, P-box and so on. These elements have been reported to be directly involved in plant growth and development and stress (Fig. 4).

LRX gene plays an important role in plant response to drought stress and salt stress. The three genes *LRX3*, *LRX4* and *LRX5* in the LRX protein family are essential for plant salt tolerance. When these three genes were mutated at the same time, the plants showed a phenotype of short growth and very sensitive to salt stress[12]. This indicates that the *LRX* gene plays a role by participating in the regulation of plant growth and salt stress response, and works together with RALF22/23 and FER[14]. Our results also indicate that CsLRX in cucumber responds to salt stress (Fig. 6b). In addition, the LRXs-RALFs-FER module controls plant growth and salt stress response by regulating a variety of phytohormone. Cell wall integrity is a key factor affecting cell wall integrity and determines the expression pattern of stress response genes. Although there is little evidence directly mentioning the response of *LRX* genes to drought stress, there is evidence that achieving the best

balance between stress response and plant growth is essential for survival in the field environment[56]. Considering the role of LRX genes in regulating plant growth and responding to salt stress, it can be speculated that these genes may also play a role in plant response to drought stress, our results have confirmed that the CsLRX gene in cucumber is induced by drought stress (Fig. 6c). Interestingly, the expression trend of *LRX* genes was not consistent under salt stress and drought stress (Fig. 6b, c). Under salt stress conditions, plants first enhance the integrity of the cell wall by increasing the expression of cell wall proteins to resist salt-induced cell damage. This may be the reason why the expression of *LRX* genes increased first. Over time, if plants fail to effectively eliminate accumulated salt or other harmful substances, their physiological state may deteriorate, resulting in a decrease in the expression of cell wall proteins, thereby showing a downward trend in *LRX* genes expression[14]. In contrast, under drought stress, plants need to quickly adjust their physiological state to adapt to the water shortage environment. In this case, plants may continue to increase the expression of cell wall proteins to maintain the integrity of the cell wall and maintain water. Since drought stress is usually more severe, plants may not be able to return to normal physiological state in time, so the expression of *LRX* genes continues to rise throughout the stress process[12,14]. In summary, plants adapt to these challenges by regulating the expression of cell wall proteins in the *LRX* gene family when facing different types of environmental stresses.

Finally, the STRING database was used to search and analyze the potential interacting proteins of CsLRX in cucumber (Fig. 7). Many proteins containing LRR domain had the possibility of interacting with CsLRX protein. The LRR domain has attracted much attention due to its key role in a variety of protein-protein interactions, which may involve a variety of biological functions, including but not limited to signal transduction, cell death, and response to environmental stress. These functions may make CsLRX protein play an important role in cucumber growth and development and response to environmental stress. Through these studies, we not only improved the understanding of the genome structure of cucurbitaceae, but also provided an important molecular basis for the resistance improvement of cucumber.

Conclusions

In this study, 40 *LRX* genes were first identified from seven cucurbit species. We conducted a comprehensive analysis of these genes, including chromosome localization, gene structure, conserved motifs, conserved domains, cis-regulatory elements, evolutionary relationships, and gene duplications. The results revealed that *LRX* genes in the Cucurbitaceae family can be classified into two subfamilies. Gene duplications were observed in *CsLRX1* and *CsLRX2* genes in cucumber as well as *LRX* genes in the other six cucurbit species, leading to an expansion of the *LRX* gene family in these plants. Additionally, *CsLRX1* and *CsLRX2* genes underwent reciprocal duplication within the cucumber species. Promoter cis-regulatory element analysis suggested that these *LRX* genes may participate in plant growth and development, phytohormone responses, as well as responses to biotic and abiotic stresses. In addition, the *CsLRX* genes in cucumber is involved in response to salt stress and drought stress. our study provides crucial insights and references for further understanding the functional roles of *LRX* genes in cell wall formation and growth and development processes in Cucurbitaceae crops.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Fan S; writing the first version of the manuscript: Fan S; manuscript revision: Yang S, Shi K, Yang L, An M, Wang F, Qi Y, Feng M, Wang M, Gen P, Liu X, Ren H. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed during this study are included in this article and its supplementary information files.

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Conflict of interest

The authors declare that they have no conflict of interest.

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