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Genome-wide analysis of the bHLH gene family in Chinese jujube (*Ziziphus jujuba* Mill.) and wild jujube

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Abstract

Background: The bHLH (basic helix-loop-helix) transcription factor is one of the largest families of transcription factors in plants, containing a large number of members with diverse functions. Chinese jujube (*Ziziphus jujuba* Mill.) is the species with the highest economic value in the family Rhamnaceae. However, the characteristics of the bHLH family in the jujube genome are still unclear. Hence, *ZjbHLHs* were first searched at a genome-wide level, their expression levels under various conditions were investigated systematically, and their protein-protein interaction networks were predicted.

Results: We identified 92 *ZjbHLHs* in the jujube genome, and these genes were classified into 16 classes according to bHLH domains. Ten ZjbHLHs with atypical bHLH domains were found. Seventy *ZjbHLHs* were mapped to but not evenly distributed on 12 pseudo- chromosomes. The domain sequences among *ZjbHLHs* were highly conserved, and their conserved residues were also identified. The tissue-specific expression of 37 *ZjbHLH* genes in jujube and wild jujube showed diverse patterns, revealing that these genes likely perform multiple functions. Many *ZjbHLH* genes were screened and found to be involved in flower and fruit development, especially in earlier developmental stages. A few genes responsive to phytoplasma invasion were also verified. Based on protein-protein interaction prediction and homology comparison, protein-protein interaction networks composed of 92 ZjbHLHs were also established.

Conclusions: This study provides a comprehensive bioinformatics analysis of 92 identified *ZjbHLH* genes. We explored their expression patterns in various tissues, the flowering process, and fruit ripening and under phytoplasma stress. The protein-protein interaction networks of ZjbHLHs provide valuable clues toward further studies of their biological functions.

Keywords: *ZjbHLHs*, Chinese jujube, Tissue-specific expression, Flower and fruit development, Phytoplasma, Protein-protein interaction

Background

Transcription factors (TFs) are important regulatory factors in eukaryotes that interact with cis-elements to regulate the expression of specific genes in response to environmental stresses [1]. According to the sequence of arginine and lysine residues in the DNA binding region, the TFs of higher plants can be divided into four categories: zinc finger [2], helix-turn-helix (HTH) [3], basic leucine zipper (bZIP) [4], and helix-loop-helix (HLH) [5].

The basic helix-loop-helix (bHLH) family is one of the largest TF families in plants [6]. The bHLH domain is composed of approximately 50–60 conserved amino acid sequences and contains two functional regions: one is the basic amino acid region with a length of approximately 15 amino acids at the N-terminal, and the other is the HLH region at the C-terminal [7]. The basic region of approximately 15 amino acids is responsible for binding to the E-box (CANNTG) element. Studies have shown that two

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helices of the same transcription factor or different transcription factors interact to form homologous or heterologous dimers, which can combine with different parts of the gene promoter to regulate the target gene [8]. Moreover, some atypical bHLHs with a less basic region that is critical for DNA binding were further identified and characterized in Arabidopsis [9–11].

As an increasing number of genome sequences are being released, a variety of bHLH superfamily genes have been identified and analyzed in a wide range of plant species, such as Arabidopsis [12], pear [13], peach [1], apple [14], grape [15] and cotton [16]. Furthermore, the functions of many bHLH proteins in plants have been studied in detail. bHLHs play various roles in plant development [16–18], signal transduction [8], tolerance [19–21] and secondary metabolite production [22]. Identification of *bHLHs* at a genome-wide level is the first step toward further studies into their biological functions. However, the bHLH transcription factors in Chinese jujube (*Ziziphus jujuba* Mill.) have not been reported before and the related functions of *ZjbHLHs* are still unknown.

Chinese jujube is a species with high economic value in the family Rhamnaceae and is also one of the most representative national fruit trees in China. Wild jujube, the wild relative species of Chinese jujube, usually has smaller trees and fruits than the Chinese jujube. Both jujube and wild jujube trees have many agronomic advantages, such as early fruit production, long flowering season and high tolerance to various biotic and abiotic stresses. And the bHLH gene family has a variety of functions such as flower and fruit development and is necessary for the normal growth and development in many plants [8, 16–22]. Compared with other TFs, bHLHs are involved in more reaction pathways and acted as some co-regulators on gene expression together with many other proteins. Therefore, we want to figure out the functions of this gene family in jujube. The jujube genome database [23, 24] provided the possibilities and resources for searching the crucial gene families related to its biological characteristics at the genome level. For the wide and diverse biological roles of *bHLHs* in plant development, the gene number, classification, and gene structure of this family in the jujube genome and their expression under various conditions in jujube and wild jujube were systematically analyzed in this study, and the protein-protein interaction networks were also predicted. The results provide valuable clues for further revealing the functions of this family in jujube growth and development.

Results

Identification of *ZjbHLHs*

A total of 92 nonredundant putative *bHLH* transcripts (Table 1) were identified in the jujube genome sequence (https://www.ncbi.nlm.nih.gov/genome/?term=jujube) [23]. To verify the reliability of each sequence, 92 protein sequences were analyzed using the online CD-search and SMART tools, and 82 bHLH proteins were found to have a typical bHLH structure except for the 10 bHLH proteins with an atypical bHLH domain (Additional file 1: Figure S1). They were named from *ZjbHLH1* to *ZjbHLH92* according to their gene structure and motifs, and the *ZjbHLH2*, *7*, and *54* sequences were new genes with no information in NCBI. The ORF length for *ZjbHLH* genes ranged from 285 bp (*ZjbHLH59*) to 2676 bp (*ZjbHLH57*), and the genes encoded proteins ranging from 94 (*ZjbHLH59*) to 891 (*ZjbHLH57*) amino acids (aa) in length, with predicted pIs ranging from 4.62 (*ZjbHLH1*) to 9.86 (*ZjbHLH41*). The proteins with an isoelectric point of less than 7 accounted for 66% of the total, which means that most of the *ZjbHLH* genes were weakly acidic. Based on their physical and chemical properties, the family members have different characteristics, indicating that they likely have multiple functions.

Previous genome evolution studies showed that Chinese jujube is closely related to species of the Rosaceae family [23], so the number of bHLHs from three Rosaceae species (apple, pear and peach), grape, cotton and Arabidopsis were compared with ZjbHLHs (Additional file 2: Table S1). There were similar gene numbers in the bHLH family of jujube and peach, and this result was consistent with a previous study on the MADS-box family [25]. Compared with the number of *bHLH* genes found in other plant species, the number of *bHLH* genes found in jujube and peach was lower. The gene numbers may be related to evolutionary differences, genome replication, or the genome size of these plants [1].

Phylogenetic analysis of ZjbHLHs

A phylogenetic tree of the ZjbHLH proteins was constructed by aligning multiple domain sequences (Fig. 1). A phylogenetic tree of *bHLH* genes of Arabidopsis and jujube was established, and *ZjbHLH*s were divided into 16 categories (Fig. 1). And the bHLH phylogenetic trees of peach [1] and jujube (Additional file 3: Figure S2) was constructed, which further verified the above classification result. The *bHLH* genes of the six species (jujube, apple, pear, peach, grape, cotton and Arabidopsis) showed a mixed pattern on the evolutionary tree (Additional file 4: Figure S3). These results indicated that the *bHLH* genes were present before the divergence of various plant species and then expanded in each species independently.

Multiple sequence alignment and conserved motifs in ZjbHLHs

Comparison of multiple sequences showed that most of the *ZjbHLH* genes had the same conserved domain structure except for the VI, VII and VIII *ZjbHLH* genes (Fig. 2). The domain sequences in the ZjbHLH gene family were

Table 1 The information of bHLH gene family in Chinese jujube

Gene Name	NCBI Reference	Chromosomes	ORF (bp)	Size (aa)	MW(Da)	PI	Basic motif	Helix1	Loop	Helix2	Group	Exon number
ZjbHLH11	XM_016019005.1	2	981	326	36445.69	4.62	TX₂ERRR	L19, L22, P24	K26, D30	A32, D35, V39, Y41, Q43, L45	I a	4
ZjbHLH02			1533	510	56060.85	5.72	NX₂ERRR	L19, V22, P24	K26, D30	A32, L35, I39, Y41, K43, L45	I a	
ZjbHLH03	XM_016014356.2	Un	1650	549	59884.45	4.96	NX₂ERRR	L19, V22, P24	K26, D30	A32, L35, I39, Y41, K43, L45	I a	4
ZjbHLH04	XM_016027914.2	5	1617	538	58694.45	5.26	NX₂ERRR	L19, V22, P24	K26, D30	A32, L35, I39, Y41, K43, L45	I a	4
ZjbHLH05	XM_016012189.2	Un	1038	345	39641.65	4.75	NX₂ERRR	L19, I22, P24	K26, D30	T32, L35, I39, Y41, K45, L45	I a	4
ZjbHLH06	XM_016035036.2	8	1032	343	38402.09	8.43	NX₂ERRR	L19, I22, P24	K26, D30	T32, L35, I39, Y41, K43, L45	I a	4
ZjbHLH07			1158	358	44202.02	5.27	NX₂ERKER	L19, L22, P24	K26, D30	A32, L35, I39, Y41, K43, L45	I a	
ZjbHLH08	XM_016035052.2	10	1503	500	56753.94	7.78	NX₂HERR	L19, L22, P24	K26, D30	A32, L35, V39, F41, E43, L45	I a	8
ZjbHLH09	XM_016040850.2	11	1641	546	60885.74	6.61	HX₂ERQRR	L19, V22, P24	K28, D30	A32, D35, V39, Y41, K43, L45	I b1	2
ZjbHLH10	XM_016041437.2	11	2145	714	78284.35	5.56	HX₂ERQRR	L19, V22, P24	K28, D30	A32, L35, I39, Y41, N43, L45	I b1	1
ZjbHLH11	XM_016036019.2	9	1506	501	54916.21	6.62	HX₂ERQRR	L19, V22, P24	K28, D30	A32, L35, I39, Y41, T43, L45	I b2	1
ZjbHLH12	XM_016033238.2	8	1839	612	67862.13	6.54	HX₂ERQRR	L19, V22, P24	K28, D30	A32, L35, I39, Y41, N43, L45	I b2	1
ZjbHLH13	XM_016036628.2	9	2118	705	78667.72	5.26	HX₂ERRER	L19, L22, P24	K26, D30	A32, L35, I39, Y41, K43, L45	I c	8
ZjbHLH14	XM_016014727.2	Un	1854	618	68801.54	5.03	HX₂ERRER	L19, L22, P24	K28, D30	I32, L35, I39, Y41, K43, L45	I c	8
ZjbHLH15	XM_016033177.2	7	1932	643	72790.07	5.63	CX₂VKYDK	L19, K22, P24	E28, D30	A32, L35, I39, Y41, K43, L45	I c	8
ZjbHLH16	XM_016013705.1	Un	1089	362	40988.57	6.28	HX₂ERKER	L19, V22, P24	K26, D30	A32, L35, I39, F41, K45, L45	II a	4
ZjbHLH17	XM_016034962.2	8	1113	370	41128.11	6.13	HX₂ERKER	L19, V22, P24	K28, D30	A32, L35, I39, Y41, K43, L45	II a	4
ZjbHLH18	XM_016037809.2	9	1023	340	38687.71	8.39	HX₂ERKER	L19, I22, P24	K28, D30	A32, L35, I39, Y41, K43, L45	II a	4
ZjbHLH19	XM_025076595.1	8	744	247	28332.01	9.28	HX₂ERKER	L19, I22, P24	K28, D30	S32, L35, I39, Y41, K43, L45	II a	4
ZjbHLH20	XM_016034892.1	8	1014	337	37568.3	5.35	HX₂ERKER	L19, V22, P24	K28, D30	S32, L35, I39, Y41, K43, L45	II a	3
ZjbHLH21	XM_016034893.2	8	561	186	21095.49	8.46	HX₂ERKER	L19, I22, P24	K28, D30	A32, L35, I39, Y41, K43, L45	II a	4
ZjbHLH22	XM_016037950.2	9	996	331	36750.68	5.43	KX₂EKLRR	L19, A22, D24	P28, D31	A33, L36, I40, V42, K44, L46	II b	5
ZjbHLH23	XM_016042854.2	1	711	236	25847.31	7.68	KX₂EKLRR	L19, I22, E24	P28, D32	A34, L37, V41, M43, N45, L47	II b	5
ZjbHLH24	XM_016025582.2	4	717	238	26730.46	8.25	KX₂EKLRR	L19, V22, D24	L28, D32	G34, L37, V41, I43, T45, L47	II b	5
ZjbHLH25	XM_016013471.2	Un	327	108	11695.26	9.19	KX₂EKLRR	L19, V22, E24	P28, D32	P34, L37, M41, V45, N45, L47	II b	3
ZjbHLH26	XM_016015873.2	Un	609	202	23497.72	6.86	KX₂EKLRR	L19, V22, E24	P28, D32	P34, L37, M41, V45, N45, L47	II b	5
ZjbHLH27	XM_016030375.2	1	711	236	26241.78	6.13	KX₂EKMRR	L19, I22, Y24	P28, D32	G34, L37, V41, I43, T45, L47	II b	5
ZjbHLH28	XM_016034154.2	8	981	326	35961.67	6.26	KX₂EKLRR	L19, T22, D24	P28, D31	A33, L36, I40, V42, K44, L46	II b	5
ZjbHLH29	XM_016039844.2	1	1521	506	56840	5.37	QX₂ DRQRR	L19, L22, P24	K28, D30	A32, V35, I39, Y41, R43, L45	III	3
ZjbHLH30	XM_016029783.2	6	1119	372	41295.5	6.02	HX₂ERNER	L19, L22, P24	V28, D32	A34, I37, I41, F43, K45, L47	IV a	3
ZjbHLH31	XM_016035243.2	8	1002	333	37069.55	5.87	HX₂ERNER	L19, M22, P24	V28, D32	A34, I37, I41, F43, K45, L47	IV a	3
ZjbHLH32	XM_016041401.2	11	1071	356	40102.02	9.46	HX₂ERNER	L19, L22, P24	V28, D32	A34, V37, I41, F43, K45, L47	IV a	3
ZjbHLH33	XM_016033408.2	8	1314	437	48732.35	5.7	HX₂ERNER	L19, L22, P24	I28, D32	A34, V37, I41, F43, K45, L47	IV a	4
ZjbHLH34	XM_016032903.2	7	1218	405	45959.38	5.64	HX₂ERNER	L19, L22, P24	V28, D32	A34, I37, I41, F43, R45, L47	IV a	3
ZjbHLH35	XM_016023719.2	3	1080	359	39299.08	5.16	HX₂ERNER	L19, L22, P24	V28, D32	A34, I37, V41, V43, N45, L47	IV a	3
ZjbHLH36	XM_016032784.2	7	615	204	23088.8	8.3	HX₂ERNER	L19, L22, P24	I28, D32	A34, I37, I41, F43, K45, M47	IV a	3
ZjbHLH37	XM_016028792.2	6	858	285	31042.07	6.81	HX₂ERER	L19, L22, P24	P28, D31	S33, V36, V40, Y42, K44, L46	IV b	3
ZjbHLH38	XM_016022498.2	3	585	194	21643.74	9.02	HX₂ERQRR	L19, Q22, P24	I28, S33	S35, I38, V42, H44, N46, L48	IV c	3
ZjbHLH39	XM_016044860.2	un	759	252	28809.29	5.82	HX₂ERQRR	I19, L22, P24	L28, S33	S35, M38, A42, Y44, K46, L48	IV c	3
ZjbHLH40	XM_016022485.2	3	726	241	28060.02	9.18	RX₂ERQRR	L19, L22, P24	I28, S33	S35, M38, V42, Y44, K46, L48	IV c	3
ZjbHLH41	XM_016022496.2	3	762	253	28200.2	9.86	HX₂ERLRR	L19, L22, P24	I28, S33	S35, I38, V42, Y44, K46, L48	IV c	3
ZjbHLH42	XM_016013488.2	Un	561	186	20985.57	9.49	RX₂ERNRR	L19, L22, P24	P28, S36	Q38, L41, A45, Y47, K49, L51	IV c	3
ZjbHLH43	XM_016045494.2	Un	672	223	25721.37	6.33	RX₂EKNRR	L19, L22, P24	S28, T33	P35, I38, I42, Y44, K46, L48	IV c	3
ZjbHLH44	XM_016042524.2	12	774	257	28597.39	7.04	HX₂EKRER	L19, L22, P24	K28, D30	A32, L35, V38, R41, R43, L45	I a	2
ZjbHLH45	XM_016040199.2	10	1149	382	43003.34	6.19	HX₂EHRER	L19, L22, P24	K28, D30	A32, L35, I39, H41, K43, L45	I a	3
ZjbHLH46	XM_016025838.2	4	747	248	27458.93	6.98	HX₂EKRER	L19, L22, P24	K28, D30	A32, L35, I39, H41, K43, L45	I a	2
Gene Name	**NCBI Reference**	**Chromosomes**	**ORF (bp)**	**Size (aa)**	**MW(Da)**	**PI**	**Basic motif**	**Helix1**	**Loop**	**Helix2**	**Group**	**Exon number**
ZjbHLH47	XM_016030498.2	6	744	247	27114.73	7.65	HX₂ERRER	L19, L22, P24	K28, D30	A32, L35, V39, H41, K43, L45	I a	3
ZjbHLH48	XM_016011540.2	1	948	315	35183.85	9.25	HX₂EKRER	L19, L22, P24	K28, D30	A32, L35, I39, Q41, K43, L45	I a	2
ZjbHLH49	XM_016011483.2	Un	1008	335	36958.88	6.02	HX₂EQRRR	L19, L22, P24	Q28, D31	A33, L36, I40, Y42, Q44, L46	I b	7
ZjbHLH50	XM_016024758.2	4	933	310	34400.21	5.78	HX₂EQRER	L19, L22, P24	Q28, D31	A33, L36, I40, Y42, R44, L46	I b	7
ZjbHLH51	XM_016027768.2	5	1704	567	62082.87	7.69	HX₂EQRER	L19, L22, P24	Q28, D31	A33, L36, I40, Y42, Q44, L46	I b	11
ZjbHLH52	XM_016011308.2	Un	1464	487	52721.69	6.43	PX₂FKVRK	L19, L22, S24	K28, D30	A32, L35, I39, Y41, K43, L45	VI	7
ZjbHLH53	XM_016031291.2	1	1458	485	52506.77	5.2	PX₂FKVRK	L19, L22, S24	K28, D30	A32, L35, I39, Y41, K43, L45	VI	7
ZjbHLH54			729	242	27253.23	9.56	PX₂FKVRK	L19, L22, S24	K28, D30	A32, L35, I39, Y41, K43, L45	VI	
ZjbHLH55	XM_016042755.2	12	1212	403	43899.34	6.56	TX₂FKVRK	L19, L22, S24	K28, D30	A32, L35, I39, Y41, K43, L45	VI	9
ZjbHLH56	XM_016037332.2	9	798	265	29655.73	5.97	TX₂PAKRK	L19, L22, S24	K28, D30	A32, L35, M39, Y41, K43, L45	VI	5
ZjbHLH57	XM_016040549.2	11	2676	891	97447.25	5.08	SX₂RPKDR	L19, I22, P24	K28, S30	D32, L35, I39, Y41, L43, L45	VII	10
ZjbHLH58	XM_016035994.2	9	2241	746	82628.62	6.02	NX₂RPRDR	L19, L22, P24	K28, S30	D32, L35, I39, E41, L43, M45	VII	11
ZjbHLH59	XM_016015590.2	Un	285	94	10601.85	7.94	SX₂SATD	L19, L22, P24	N28, S35	S37, L40, C44, Y46, R48, L50	VIII	2
ZjbHLH60	XM_016023864.2	4	333	110	12559.45	8.93	SX₂SRSD	L18, L21, P23	Q27, S34	S36, L39, C43, Y45, R47, L49	VIII	2
ZjbHLH61	XM_016036107.2	9	1221	406	45007.79	6.02	AX₂ORHRR	L19, L22, P24	E28, G30	A32, L35, I39, Y41, K43, L45	IX	8
ZjbHLH62	XM_016021607.2	1	741	246	27420.19	5.31	SX₂SERKR	L19, I22, P24	Q28, N30	V32, L35, V39, Y41, K43, L45	X	1
ZjbHLH63	XM_016019322.2	2	2190	729	77782	5.64	HX₂ERRER	L19, L22, P24	K28, D30	A32, L35, I39, Y41, K43, L45	XI	7
ZjbHLH64	XM_016029850.2	6	1191	396	42608.05	5.17	HX₂EKRER	L19, L22, P24	K28, D30	A32, L35, I39, Y41, K43, L45	XI	6
ZjbHLH65	XM_016011251.2	Un	1392	463	51090.15	5.93	HX₂EHVER	L19, L22, P24	K28, S31	A33, L36, I40, Y42, Q44, L46	XII	7
ZjbHLH66	XM_016026781.2	5	1077	358	40468.29	7.12	HX₂ERVER	L19, L22, P24	K28, G31	A33, L36, I40, Y42, Q44, L46	XII	7
ZjbHLH67	XM_016040429.2	10	990	329	36619.13	8.1	HX₂ERARR	L19, L22, P24	K28, G31	A33, L36, I40, Y42, Q44, L46	XII	7
ZjbHLH68	XM_016029648.2	6	1305	434	47105.32	5.7	HX₂ERVER	L19, L22, P24	K28, G31	A33, L36, I40, Y42, Q44, L46	XII	8
ZjbHLH69	XM_016039783.2	10	1695	564	60554.48	5.84	HX₂ERVRR	L19, L22, P24	K28, G31	A33, L36, I40, Y42, Q44, L46	XII	8
ZjbHLH70	XM_016027226.2	5	855	284	30283.45	5.55	HX₂ERARR	L19, L22, P24	K28, G31	A33, L36, I40, Y42, Q44, L46	XII	6
ZjbHLH71	XM_016016330.2	Un	948	315	34821.01	8.54	HX₂ERARR	L19, L22, P24	K28, G31	A33, L36, I40, Y42, Q44, L46	XII	5
ZjbHLH72	XM_016043537.2	12	1107	368	40650.2	5.46	HX₂ERARR	L19, L22, P24	K28, G31	A33, L36, I40, I042, Q44, L46	XII	7
ZjbHLH73	XM_016027494.2	5	816	271	30400.24	6.11	HX₂ERVRR	L19, L22, P24	K28, G31	A33, L36, I40, Y42, Q44, L46	XII	6
ZjbHLH74	XM_016036807.2	9	1713	570	61416.22	6.09	HX₂ERVRR	L19, L22, P24	K28, G31	A33, L36, I40, Y42, Q44, L46	XII	8
ZjbHLH75	XM_016014544.1	Un	1500	499	53674.68	9.17	HX₂ERVRR	L19, L22, P24	K28, G31	A33, L36, I40, Y42, Q44, L46	XII	9
ZjbHLH76	XM_016029857.2	4	720	239	27443.08	7.75	HX₂ERLRR	L19, L22, P24	K28, G31	T33, L36, I40, Y42, H44, L46	XII	6
ZjbHLH77	XM_016012786.2	Un	801	266	30128.94	5.45	HX₂ERVER	L19, L22, P24	K28, G31	A33, L36, I40, Y42, Q44, L46	XII	6
ZjbHLH78	XM_016035984.2	9	963	320	34132.62	6.33	HX₂ERLRR	L19, L22, P24	K28, D30	A32, L35, V39, Y41, K43, L45	XIII	6
ZjbHLH79	XM_016019938.2	2	1053	350	36568.63	5.68	HX₂ERLRR	L19, L22, P24	K28, D30	A32, L35, V39, Y41, K43, L45	XIII	7
ZjbHLH80	XM_016037030.2	1	1476	491	51580.13	6.04	HX₂ERLRR	L19, L22, P24	K28, R30	N32, F35, Y39, Y41, K43, E45	XIII	6
ZjbHLH81	XM_016020533.2	2	1239	412	45023.68	6.72	RX₂ERVRR	L19, L22, P24	K28, N31	S33, L36, V40, Y42, K44, L46	IIY	6
ZjbHLH82	XM_016031637.2	7	1089	362	38812.9	8.66	HX₂EHERR	L19, L22, P24	K28, S31	A33, L36, V40, H42, K44, L46	IIY	6
ZjbHLH83	XM_016032495.2	1	1272	423	46808.67	6.38	RX₂ERVER	L19, L22, P24	K28, S31	A33, L36, V40, Y42, K44, L46	SIY	6
ZjbHLH84	XM_016025580.1	4	393	130	14762.3	9.79	SX₂ARQRR	L19, L22, P24	K28, N30	A32, L35, V39, Y41, K43, L45	XV a	1
ZjbHLH85	XM_016026786.2	1	912	303	34132.22	5.19	QX₂ARERR	L19, L22, P24	K28, N30	A32, F35, S39, Y41, K43, L45	XV a	2
ZjbHLH86	XM_016028831.2	6	750	249	27492.33	8.67	QX₂ARHRR	L19, L22, P24	K28, D30	A32, L35, I39, Y41, K43, L45	XV b	1
ZjbHLH87	XM_016034652.2	8	852	283	31340.52	8.93	QX₂ARHRR	L19, L22, P24	K28, D30	A32, L35, A39, Y41, K43, L45	XV b	1
ZjbHLH88	XM_016042118.2	11	582	193	21549.95	7.16	QX₂ARERR	L19, L22, P24	K28, D30	V32, L35, I39, Y41, K43, L45	XV b	1
ZjbHLH89	XM_016024785.2	4	888	295	32819.1	6.78	QX₂ARNRR	L19, L22, P24	K28, D30	V32, L35, I39, Y41, K43, L45	XV c1	5
ZjbHLH90	XM_016029540.1	6	1047	348	38744.24	5.22	QX₂ARKRR	L19, L22, P24	K28, D30	S32, L35, V39, Y41, K43, M45	XV c2	5
ZjbHLH91	XM_016046616.1	Un	1140	379	41370.69	5.02	QX₂ARKRR	L19, L22, P24	K28, D30	S32, L35, V39, Y41, K43, L45	XV c2	5
ZjbHLH92	XM_016018705.2	2	1080	359	39296.58	4.7	LX₂KRMKR	L19, I22, P24	G28, D30	I32, L35, I39, Y41, K43, L45	SY1	1

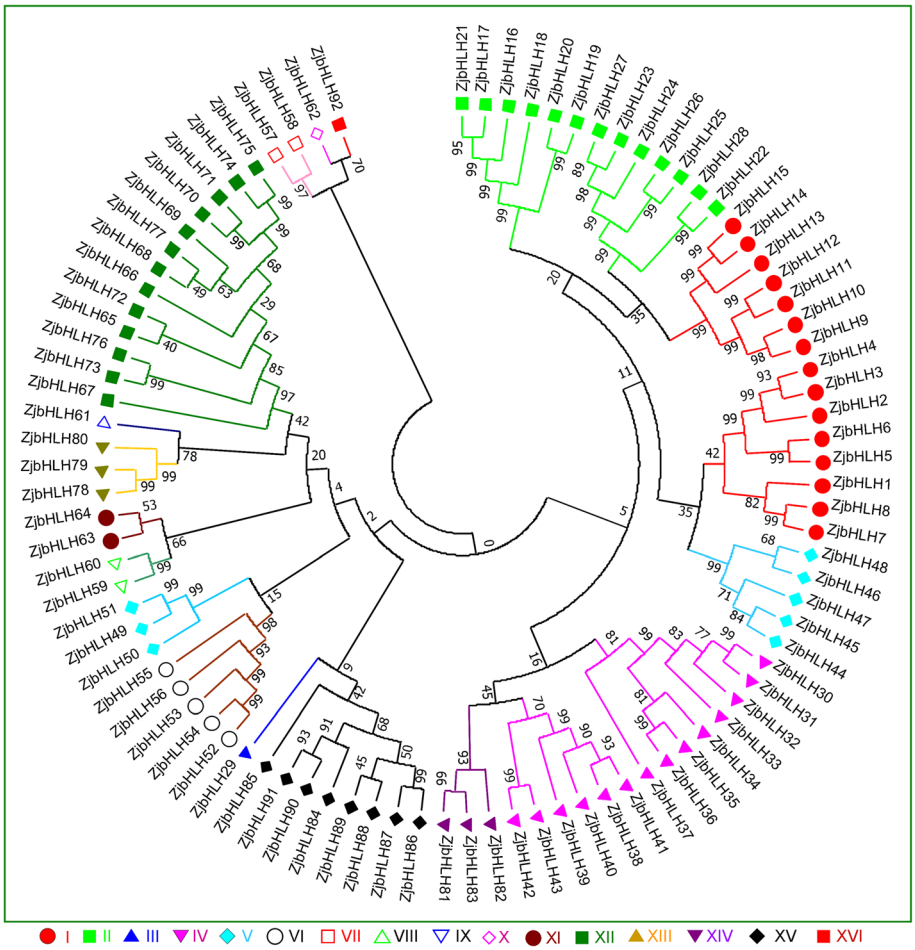

Fig. 1 The phylogenetic analysis of ZjbHLHs. The NJ tree was constructed from the protein sequences of ZjbHLHs using MEGA7 with 1000 bootstrap copies

highly conserved (Additional file 1: Figure S1). Five residues (His-1, Glu-5, Arg-6, Arg-8, and Arg-9), three residues (Leu-19, Leu-22, and Pro-24), two residues (Lys-28 and Asp-30) and six residues (Ala-32, Leu-35, Ile-39, Tyr-41, Lys-43, and Leu-45) made up the basic region, the first helix region, the loop region and the second helix region, respectively. There were 6 motifs among ZjbHLHs, and proteins in the same group had similar numbers and types of motifs (Additional file 5: Figure S4). The bHLH domains Motif 1 and Motif 2 were highly conserved among the 92 proteins (Additional file 6: Figure S5), and only ten (*ZjbHLH15, ZjbHLH52~ZjbHLH60*) of them contained variations. *ZjbHLH59* and *ZjbHLH60* were identified as atypical *bHLH* genes by the Conserved Domain Search Service (CD Search) [26].

The chromosomal location and gene structure of *ZjbHLHs*

Of the 92 *ZjbHLH* genes, 70 were mapped to 12 pseudochromosomes in the jujube genome (Fig. 3), 17 genes were located on 12 scaffolds, and 5 genes were uncommented. *ZjbHLHs* were not evenly distributed across the 12 chromosomes. Nine *ZjbHLH* genes (9.8%) were on Chr. 1, 8, and 9, and 7 *ZjbHLHs* (7.6%) were located on Chr. 4 and 6. Furthermore, some *ZjbHLHs* concentrated on part of the chromosome, and some relatively high-density *bHLH* genes were observed in some chromosomal regions. Some genes were tightly packed into clusters to form tandem repeats (*ZjbHLH27* and *53*; *ZjbHLH38, 40* and *41*; *ZjbHLH64* and *37*; *ZjbHLH17, 20* and *21*). A previous study analyzed repeated events in rice and Arabidopsis [27], indicating that some *bHLH* subfamily members are most likely derived from repetitive events.

Additionally, the gene structure was highly conserved within each group (Fig. 4), except for the 3 uncommented *ZjbHLHs* (2, 7 and 54). We found that Group VI, VII, IX, XI, XII, XIII, and XIV genes contained more introns and were more complicated than genes in the other groups (Fig. 4).

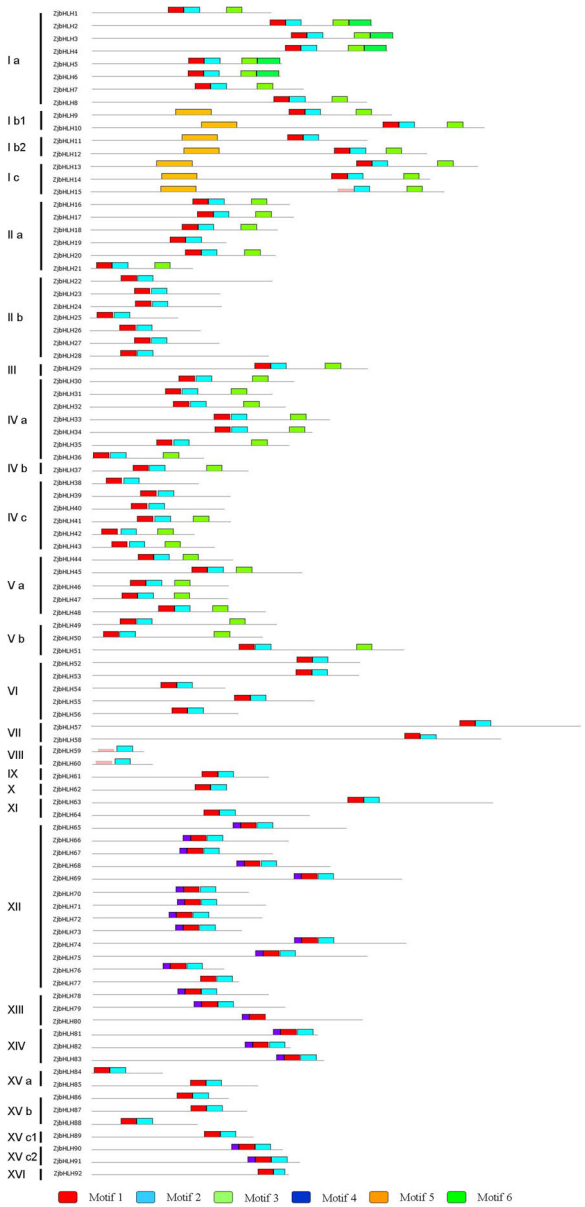

Fig. 2 Conserved motifs of ZjbHLH proteins. The motifs in ZjbHLHs were identified by using Multiple Em for Motif Elicitation (MEME). In these ZjbHLH proteins, six conserved motifs were identified and displayed in different colors

Expression patterns of *ZjbHLHs* in various tissues/organs

To explore the tissue-specific expression of *ZjbHLHs*, their expression patterns were determined in various tissues by semiquantitative PCR. The expression patterns of most examined *ZjbHLHs* were similar in jujube and wild jujube (Fig. 5), except for 8 *ZjbHLHs* (17, 21, 54, 60, 63, 79, 83, and 87). Some genes were mainly expressed in vegetative organs (*ZjbHLH1, 2, 3, 4, 11, 63, 65, 81, 83, and 87*) or reproductive organs (*ZjbHLH60*). In particular, *ZibHLH62* was stably expressed in various organs of both jujube and wild jujube and can be used as a housekeeping gene. These results showed that most of the *ZjbHLHs* had diverse tissue-specific expression patterns, indicating that they play multiple roles in various organs.

In addition, the expression of *ZjbHLH8* and *19* genes in the branches and leaves of jujube was significantly weaker than that in wild jujube. This differential expression indicated that some *ZjbHLHs* may have different functions between jujube and wild jujube.

ZjbHLHs involved in flower and fruit development

Based on the tissue-specific expression, the expression of *ZjbHLHs* was further detected at four floral developmental stages (Fig. 6a). Among them, *ZjbHLH62* and *ZjbHLH53* were expressed stably at the four stages in jujube and wild jujube. The expression levels of *ZjbHLH4, 12, 23, 78* and *87* genes decreased gradually with flower development, and *ZjbHLH92* had a high expression level at the later stages in both jujube and wild jujube. It is remarkable that the expressions of *ZjbHLH4*, *12*, *34*, *60*, *62*, *78*, *79* and *83* genes in four stages showed opposite trends between jujube and wild jujube and four genes (*ZjbHLH4*, *12*, *60* and *78*) showing significantly different expression were screened out (Additional file 9: Figure S6), indicating that they may perform different functions during flower development in jujube and its wild-type species. Through protein-protein interaction prediction and homology comparison, it is predicted that *ZjbHLH2, 4, 65, 83,* and *87* genes have crucial functions during flower development (Fig. 6b).

During jujube fruit development, some genes (*ZjbHLH2, 4, 12, 15, 23, 62, 63, 78* and *83*) were highly expressed at the first two stages and then significantly decreased at later stages (Fig. 7a). However, *ZjbHLH60* was mainly expressed at the late stages. The protein interaction prediction and homology comparison also indicated that *ZjbHLH15* and *63* might play important roles in fruit development (Fig. 7b). The above results indicated that some *ZjbHLHs* were truly involved in jujube flower and fruit development.

ZjbHLHs participated in jujube-phytoplasma interactions

JWB caused by phytoplasma is a destructive disease in jujube production. Since *bHLH* genes have multiple functions in plants, whether they participate in jujube-phytoplasma interactions remains unclear. Hence, their expression changes were investigated in jujube under phytoplasma stress. Among the 23 *ZjbHLH* genes detected, the expression of *ZjbHLH12, 18, 23, 24, 34, 53,* and *62* genes in diseased leaves was significantly lower than the expression in healthy leaves (Fig. 8a). *ZjbHLH49, 63, 79, 83,* and *88* genes were highly expressed in diseased leaves (Fig. 8b). These results suggested that some *ZjbHLHs* participate in jujube-phytoplasma interactions.

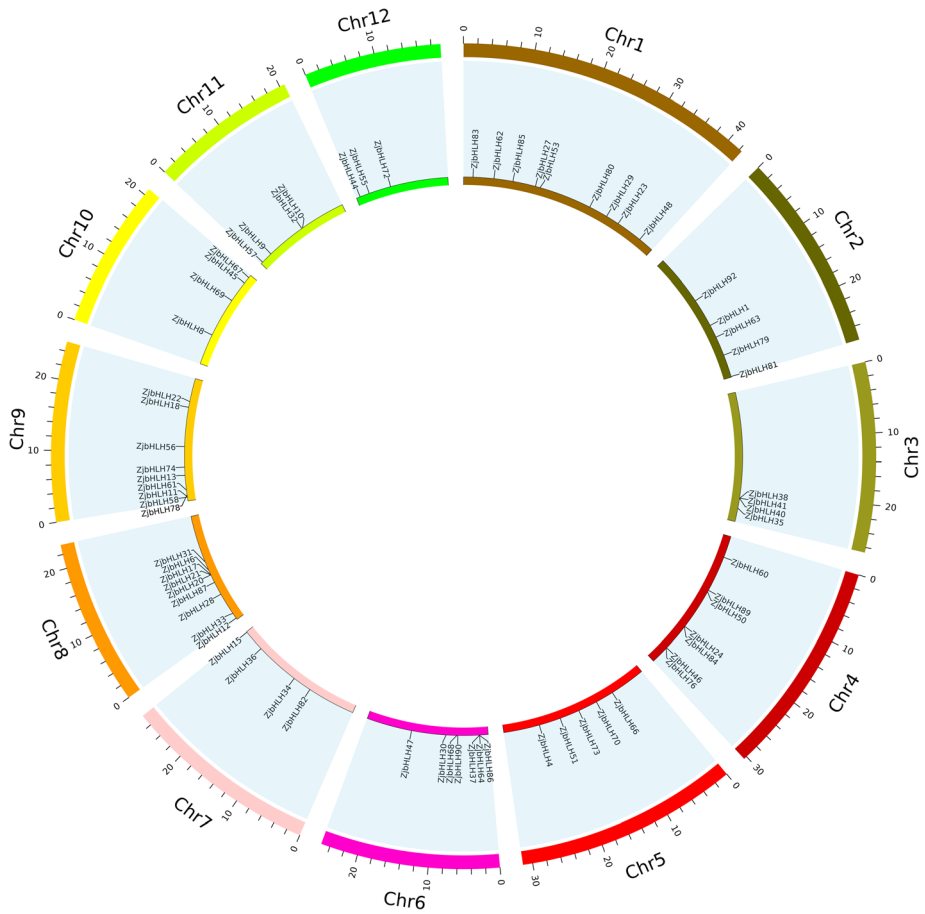

Fig. 3 The chromosomal location of 70 *ZjbHLH* genes. Genes are mapped to jujube chromosomes by the Circos tool. The chromosomes of jujube are arranged in a circle

ZjbHLH protein-protein interaction network prediction

Based on the orthologs in Arabidopsis (Additional file 7: Table S2), it was predicted by STRING that many ZjbHLH proteins interacted with each other (Fig. 9), which is in accord with previous reports that the binding activity of bHLH proteins depends upon the formation of homodimers or heterodimers among bHLH proteins [28, 29]. Overall, several important interactions were predicted in Fig. 9. Both FBH4 (homolog of ZjbHLH81 and 83) and CIB1 (homolog of ZjbHLH65) were involved in the regulation of flowering time [30, 31], and HEC (homolog of ZjbHLH86 and 87) could interact with SPT (homolog of ZjbHLH64) to jointly regulate pistil development by regulating cytokinins and other hormones [32]. ICE1 (homolog of ZjbHLH2, 3, and 4) could interact with FMA (homolog of ZjbHLH34), SPCH (homolog of ZjbHLH35) and MUTE (homolog of ZjbHLH36) could regulate stomatal differentiation [33]. Moreover, ICE1 also regulated lateral bud growth and plant stress response [34, 35], and LRL1 (homolog of ZjbHLH80), RHD6 (homolog of ZjbHLH89) and RSL2 (homolog of ZjbHLH90) were involved in the regulation of root hair development [36, 37]. These results further proved the functional diversity of *ZjbHLH* genes. In addition, we also found that the functions of those genes contained more introns were mostly related to flower and root development (Additional file 7: Table S2). The predicted network provides some useful clues for functional studies, further experimental evidences should be needed.

Discussion

In this study, a total of 92 *bHLH* genes were identified in the jujube genome. Based on phylogenetic analysis, intron-exon gene structure, conserved protein motifs and amino acid physical and chemical property prediction, these ZjbHLHs were divided into 16 categories; most sequences have the same conservative sequence except for the VI, VII, and VIII groups. Among them, there are 10 atypical sequences, and this trait has also been demonstrated in other plants [25]. Furthermore, our BLAST results strongly supported our classifications

Fig. 4 The gene structure of *ZjbHLHs* in Chinese jujube. Introns and exons are represented by black lines and red boxes respectively and upstream/ downstream are represented by green boxes

of the ZjbHLHs, and detailed information about these orthologs was also summarized (Additional file 7: Table S2).

Many ZjbHLH proteins are involved in jujube flower development. *ZjbHLH2* and *ZjbHLH4* were expressed at higher levels at earlier flower development stages. These two genes belong to the ICE1 branch, which can regulate lateral bud growth [35]. A similar function was also confirmed by homologous protein interactions (Fig. 6B-a). ICE1 can interact with the HOS1 protein, which is an important regulator of flowering time [38]. ZjbHLH65, 83 and 87 proteins were homologous to CIB4, FBH4 and HEC2, respectively, and were three key regulatory factors in flower development (Fig. 6B-b, c, d). *ZjbHLH83* is the

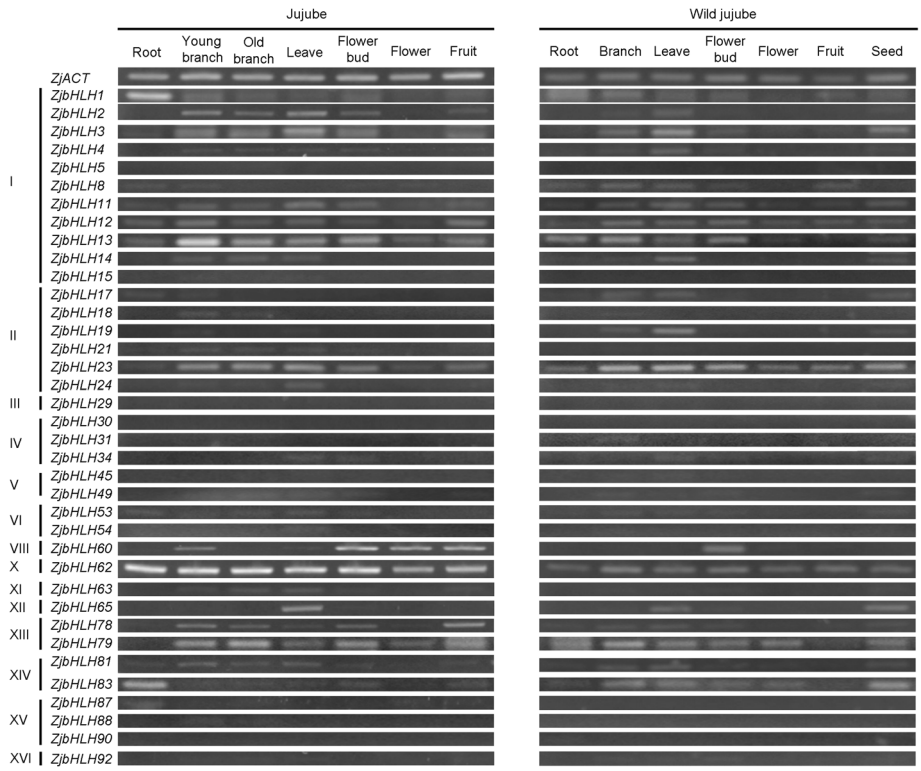

Fig. 5 Expression patterns of 37 *ZjbHLH* genes in seven tissues of jujube and wild type jujube by RT-PCR. *ZjACT* was used as an internal control. Left: jujube, from left to right: root, young branch, old branch, leaf, flower bud, flower, and fruit. Right: wild jujube, from left to right: root, branch, leaf, flower bud, flower, fruit and seed

homologous gene of FBH4 (At2g42280). Overexpression of FBH4 in Arabidopsis drastically elevated CO expression and caused early flowering regardless of the photoperiod [30]. Here, *ZjbHLH83* showed high expression at earlier flower development stages (Fig. 6a) and was also predicted to interact with CO (Fig. 6B-c). In addition, a series of CIB genes (CIB1, 2, 3, 4, and 5) in Arabidopsis can activate FT transcription by interacting with CRY2 protein and mediate the regulation of flowering time [31]. There are also a series of CIB homologous genes in jujube, namely, *ZjbHLH65* (homologs of CIB1), *ZjbHLH74* and *75* (homologs of CIB3), *ZjbHLH68* (homologs of CIB4), and *ZjbHLH69* (homologs of CIL1). Since CIB genes have been proven to be conserved among plants [31], they are likely to have a similar function in flower development.

In fruit development, the expression patterns of most *ZjbHLH* genes detected in the two cultivars were in line with each other, which indicated that their functions in fruit development might be conserved among jujube varieties. *ZjbHLH63* expression was significantly higher at the early stage of fruit development (Fig. 7a), which is the period of fruit enlargement. ZjbHLH63 is the homolog of AtPIF3 (Fig. 9), a key factor affecting light morphogenesis [39]. The homologous comparison and protein interaction prediction (Fig. 7B-b) also indicated that it might be involved in fruit enlargement. In addition, ZjbHLH15 homologous protein in Arabidopsis was predicted to be involved in fruit anthocyanin synthesis (Fig. 7B-a), but in this study, its expression decreased significantly at the fruit coloring stages (Fig. 7a). Therefore, we hypothesized that jujube fruit color changes might not correlate with the accumulation of anthocyanin.

In addition, the expression patterns of some bHLH genes in jujube and wild jujube were not the same, indicating that *ZjbHLH* genes participate in different regulation pathways between jujube and wild jujube, especially in flower development. Further studies are needed to elucidate the detailed interaction network of the growth and development of jujube and wild jujube.

Conclusions

This study described the bHLH gene family of Chinese jujube at the genome level. Their gene structure, chromosomal distribution, phylogenetic relationship, and tissue-specific expression patterns were presented. Ten ZjbHLHs with atypical bHLH domains were identified. Many *ZjbHLH* genes were confirmed to involve in flower and fruit development and responsive to phytoplasma stress. An integrated ZjbHLHs protein-protein interaction network was also predicted. These results are

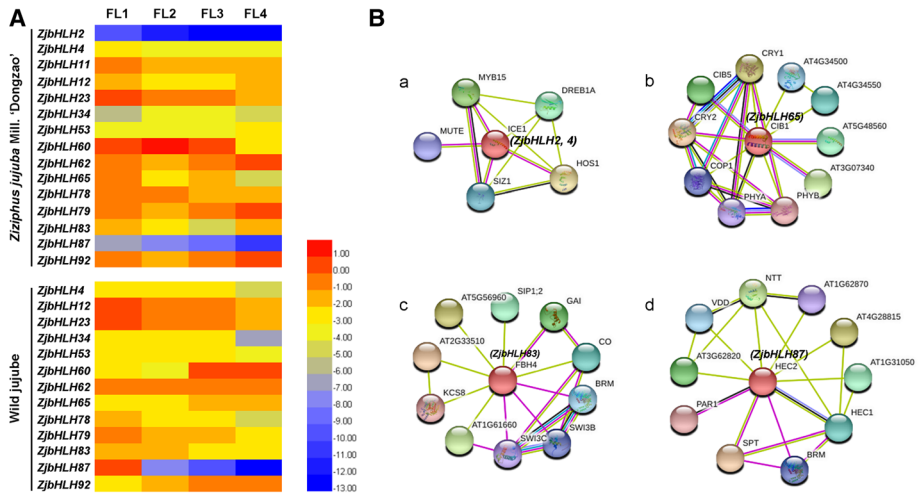

Fig. 6 **a** Heat maps of the relative expression of *ZjbHLH* genes during flower development. FL1, bud emergence stage; FL2, inflorescence emergence stage; FL3, yellow bud stage; FL4, petal spread stage. Scaled log2 expression values based on qRT-PCR data are shown from blue to red, indicating low to high expression. **b** The protein-protein interaction analysis of four ZjbHLHs by STRING database

very meaningful to the future functional analysis of ZjbHLHs.

Methods

Plant materials

Chinese jujube and wild jujube trees used in this study are cultivated in the Experimental Station of Chinese Jujube, Hebei Agricultural University. No specific permits are required for the sample collection. They are not endangered or protected species.

Seven tissue types (roots, young branches, old branches, leaves, flower buds, flowers and young fruits) collected from three jujube trees and three wild jujube trees were used for organ-specific expression analysis. The flowers of jujube and wild jujube were used for qRT-PCR analysis. The four development stages sampled were the bud emergence stage (FL1), inflorescence emergence stage (FL2), yellow bud stage (FL3) and petal spread stage (FL4). The fruits of two jujube cultivars ('Lizao' and 'Yazao') were used to investigate the expression pattern of *ZjbHLHs*. Five developmental stages, including the young fruit stage (Y), early white mature fruit stage (EWM), white mature fruit stage (WM), half-red fruit stage (HR) and full-red fruit stage (FR), were sampled. Each treatment was collected from three biological replicates.

Three kinds of tissues representing disease symptoms of different severity of Jujube witches' broom (JWB) disease (apparently normal leaves (ANL), phyllody leaves (PL), and witches' broom leaves (WBL)) from diseased trees and healthy leaves (HL) from healthy trees were collected in four periods (June, July, August and September). All treatments were conducted with three biological replicates.

Identification and protein structure analysis of ZjbHLHs

The hidden Markov model (HMM) file of the bHLH domain (PF00010) was downloaded from the Pfam database (http://pfam.xfam.org/), and HMMER 3.1b2 software was used to find the ZjbHLH protein sequences in the jujube genome [23]. To further confirm our sequences, we used the online CD-search tool (NCBI database), the SMART tool (http://smart.embl-heidelberg.de/) and the website of PlantTFDB to screen sequences. Truncated and false genes were excluded from our analysis. The number of amino acids, molecular weight, and theoretical pI of *ZjbHLH* genes were predicted by NCBI and ProtParam (https://web.expasy.org/compute_pi/). The conserved motifs of ZjbHLH proteins were detected by MEME (http://meme-suite.org/) [40].

The chromosomal location and gene structure of *ZjbHLHs*

To determine the chromosomal location of the *ZjbHLH* genes, their gene sequences were used as query sequences in BLASTN searches against the jujube genome. Each *ZjbHLH* gene was mapped to the jujube genome according to its genome coordinates. Tandem duplications were identified as previously described [41].

The website GSDS (http://gsds.cbi.pku.edu.cn/) was used to predict the number of exons from the coding domain sequences (CDS) and DNA sequences of the *ZjbHLH* genes [42].

Multiple sequence alignment and phylogenetic tree construction

Multiple sequence alignment was analyzed by using ClustalX2 and edited by BioEdit. A phylogenetic tree of 92 ZjbHLHs was constructed based on their conserved domains. bHLH proteins of six other species (*Arabidopsis*

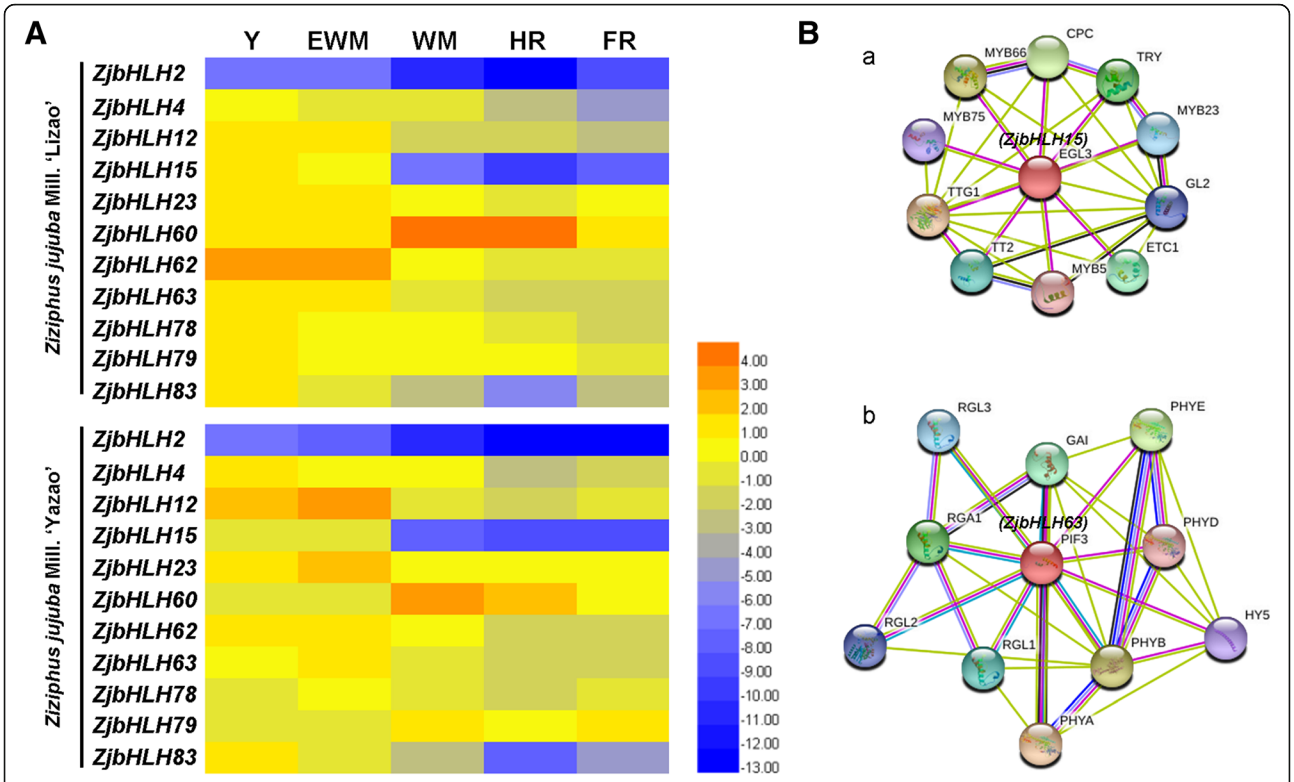

Fig. 7 **a** Heat maps of the relative expression of *ZjbHLH* genes during fruit ripening in 'Lizao' and 'Yazao'. Y, young fruit; EWM, early white mature fruit; WM, white mature fruit; HR, half-red fruit; FR, full red fruit. Scaled log2 expression values based on qRT-PCR data are shown from blue to red, indicating low to high expression. **b** The protein-protein interaction analysis of two ZjbHLHs by STRING database

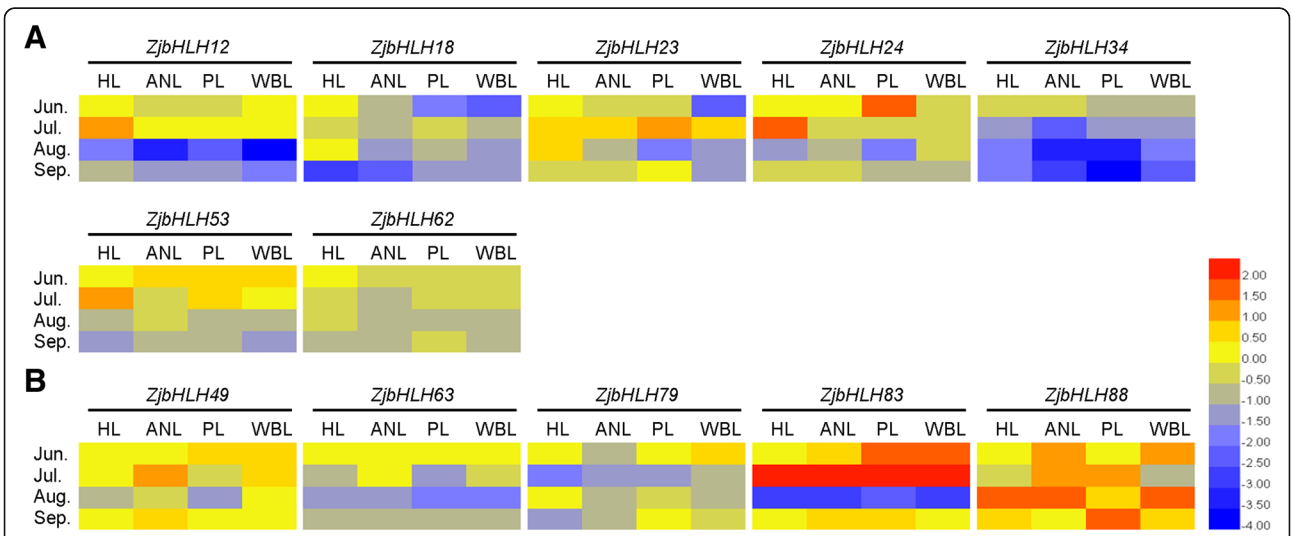

Fig. 8 Heat maps of the relative expression of *ZjbHLH* genes under phytoplasma stress. **a** The gene expression in diseased leaves was significantly lower than that in healthy leaves. **b** The gene expression in diseased leaves was significantly higher than that in healthy leaves. Scaled log2 expression values based on qRT-PCR data are shown from blue to red, indicating low to high expression, respectively

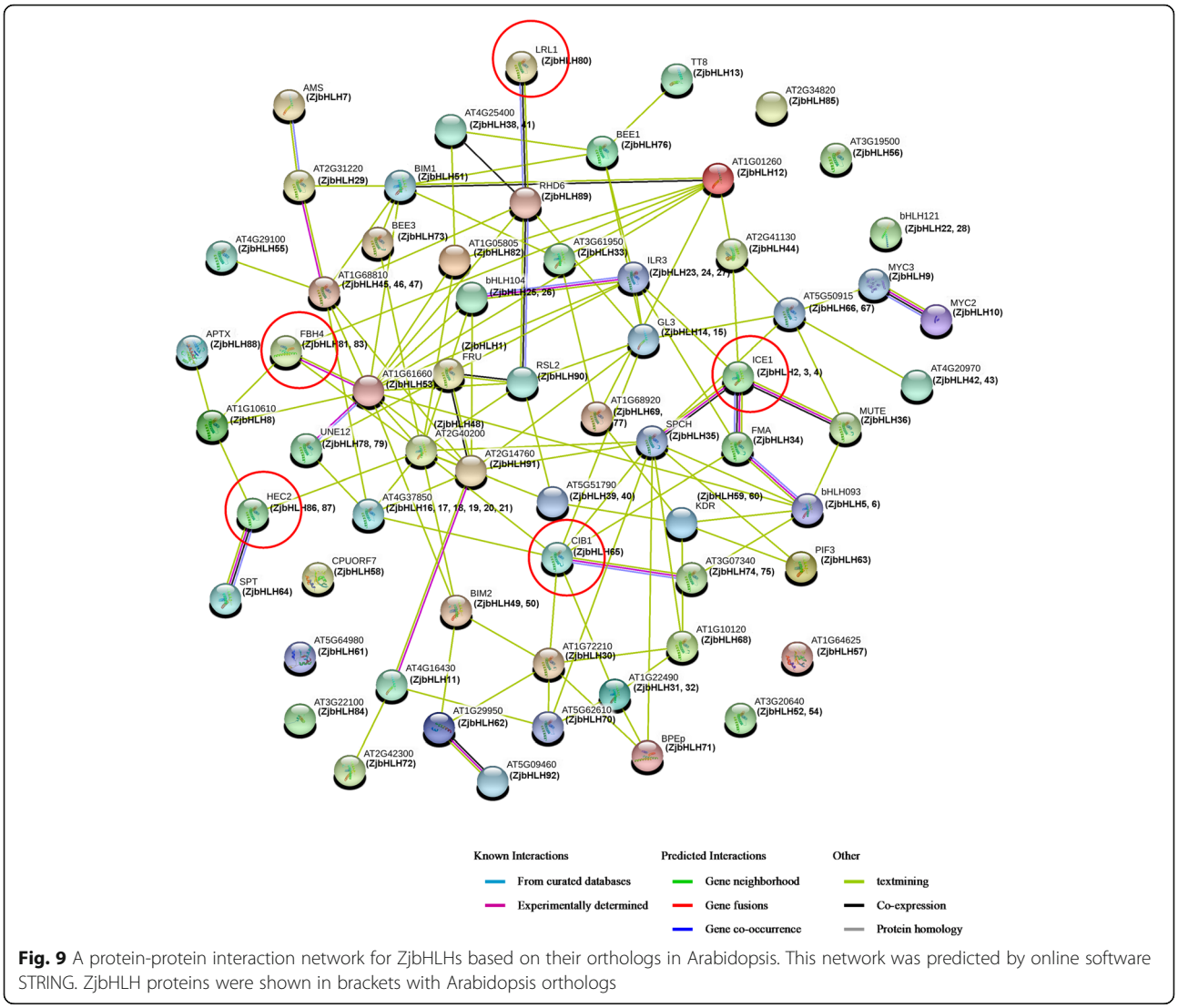

Fig. 9 A protein-protein interaction network for ZjbHLHs based on their orthologs in Arabidopsis. This network was predicted by online software STRING. ZjbHLH proteins were shown in brackets with Arabidopsis orthologs

thaliana, *Prunus persica*, *Malus domestica*, *Pyrus bretschneideri*, *Vitis vinifera* L. and *Anemone vitifolia* Buch.) were downloaded from NCBI. MEGA 7 software and the neighbor-joining statistical method were used to construct a rooted phylogenetic tree [43, 44]. The evolutionary distances were obtained using the p-distance method, and these distances were used to estimate the number of amino acid substitutions per site. The reliability of each phylogenetic tree was established by conducting 1000 bootstrap sampling iterations.

RNA isolation and expression analysis

Total RNA was extracted using an RNAprep Pure Plant Kit (TIANGEN) according to the manufacturer's protocol. After genomic DNA was removed by RNase-free DNase I (TIANGEN), the RNA concentration and purity were checked on a NanoDrop2000 spectrophotometer. First-strand cDNA was synthesized by reverse transcribing 500 ng of total RNA with a FastQuant RT Super Mix Kit (TIANGEN). The cDNA was used as the template for gene expression analysis.

Gene expression was detected by semiquantitative PCR and qRT-PCR. The primers used in this study are listed in Additional file 8: Table S3. PCR products were amplified in triplicate using Bio-Rad iQ™5 with TransStart Top Green qPCR SuperMix AQ131 (TransGen Biotech, China) in 20 μL reactions. Each reaction contained 10 μL of 2 × TransStart® Top Green qPCR SuperMix, 0.4 μL each of 10 μM primers, 8.2 μL of ddH_2O and 1 μL of cDNA. The thermal profile for RT-qPCR was as follows: preincubation for 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, 10 s at 53–58 °C, and 10 s at 72 °C. Three biological replicates were performed for each treatment. Threshold cycle values were calculated using iCycler software, and *ZjACT* was used as an internal control [45]. Relative transcript levels were calculated according to the $2^{-\Delta\Delta CT}$ method [46].

Protein-protein interaction network prediction

Ninety-two ZjbHLH protein sequences were used as queries, and protein-protein interactions were predicted by the STRING website (https://string-db.org/). The orthologs of *Arabidopsis thaliana* were selected as references. After completing the BLAST step, the network was constructed using the highest score gene (bitscore). Finally, an interaction network among ZjbHLHs was constructed in this study.

Additional files

Additional file 1: Figure S1. The multiple sequence alignment in ZjbHLH proteins. (DOC 3607 kb)

Additional file 2: Table S1. Number of bHLH gene family from Chinese jujube and other six species. (DOC 50 kb)

Additional file 3: Figure S2. The phylogenetic analysis of bHLH proteins of *Ziziphus jujuba* and *Persica prunu*. The NJ tree was constructed from the protein sequences of ZjbHLHs and PpbHLHs using MEGA7 with 1000 bootstrap copies. (DOC 483 kb)

Additional file 4: Figure S3. The phylogenetic analysis of bHLH proteins of *Ziziphus jujuba*, *Arabidopsis thaliana, Persica prunus, Malus domestica, Pyres bretschneideri, Vitis vinifera* and *Gossypium raimondii*. There are 16 categories in total, and I, IV, XI and XV are selected for display. (DOC 1096 kb)

Additional file 5: Figure S4. The amino acid sequences of 6 motifs among ZjbHLH proteins. (DOC 240 kb)

Additional file 6: Figure S5. The major functional domain of ZjbHLH proteins. (DOC 384 kb)

Additional file 7: Table S2. Summary information for 92 ZjbHLH proteins in STRING database. (XLS 95 kb)

Additional file 8: Table S3. The primers of *ZjbHLH* genes used in this study. (DOC 142 kb)

Additional file 9: Figure S6. Expression patterns of four *ZjbHLH* genes in flower development stage of jujube and wild jujube. FL1, bud emergence stage; FL2, inflorescence emergence stage; FL3, yellow bud stage; FL4, petal spread stage. The expression levels of eight treatments (four development stages in jujube and wild jujube, respectively) were compared and analyzed either between different stages of the same species or between different species of the same stage. All statistical analyses were performed with SPSS software 17.0. Duncan's multiple range tests were used to assess differences between treatments. Different letters mean significant difference at 0.05 levels between the corresponding treatments. (DOC 319 kb)

Abbreviations

ANL: Apparently normal leaves; bHLH: Basic helix-loop-helix; CDS: Coding domain sequences; EWM: Early white mature fruit stage; FR: Full-red fruit stage; HL: Healthy leaves; HMM: Hidden Markov model; HR: Half-red fruit stage; JWB: Jujube witches' broom disease; PL: Phyllody leaves; qRT-PCR: Quantitative real-time PCR; TFs: Transcription factors; WBL: Witches'-broom leaves; white WM: mature fruit stage; Y: Young fruit stage

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Not applicable.

Authors' contributions

JZ designed the research; HL, WG, CX, YZ and JZ performed the experiments, analyzed the data and wrote the paper. ZL, ML and YZ participated in the data analysis. YZ and XM performed RT-PCR and RT-qPCR experiments. All authors read and approved the final the manuscript.

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Availability of data and materials

All data and materials are presented in the main manuscript and additional supporting file.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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