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The Basic/Helix-Loop-Helix Transcription Factor Family Gene RcbHLH112 Is a Susceptibility Gene in Gray Mould Resistance of Rose (Rosa Chinensis)

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Abstract: The basic/helix–loop–helix (*bHLH*) family is a major family of transcription factors in plants. Although it has been reported that *bHLH* plays a defensive role against pathogen infection in plants, there is no comprehensive study on the *bHLH*-related defence response in rose (*Rosa* sp.). In this study, a genome-wide analysis of *bHLH* family genes (*RcbHLHs*) in rose was carried out, including their phylogenetic relationships, gene structure, chromosome localization and collinearity analysis. Via phylogenetic analysis, a total of 121 *RcbHLH* genes in the rose genome were divided into 21 sub-groups. These *RcbHLHs* are unevenly distributed in all 7 chromosomes of rose. The occurrence of gene duplication events indicates that whole-genome duplication and segmental duplication may play a key role in gene duplication. Ratios of non-synonymous to synonymous mutation frequency (Ka/Ks) analysis showed that the replicated *RcbHLH* genes mainly underwent purification selection, and their functional differentiation was limited. Gene expression analysis showed that 46 *RcbHLHs* were differentially expressed in rose petals upon *B. cinerea* infection. It is speculated that these *RcbHLHs* are candidate genes that regulate the response of rose plants to *B. cinerea* infection. Virus-induced gene silencing (VIGS) confirmed that *RcbHLH112* in rose is a susceptibility factor for infection with *B. cinerea*. This study provides useful information for further study of the functions of the rose *bHLH* gene family.

Keywords: *bHLH*; transcription factor; *Botrytis cinerea*; phylogenetic analysis; expression pattern

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1. Introduction

Transcription factors have been extensively studied in plant growth, development, metabolism and stress response due to their important roles in transcriptional regulation [1]. Transcription factors usually consist of at least DNA-binding domains, transcriptional regulatory domains, oligomerisation sites and nuclear localisation signals [2]. The *bHLH* gene family is one of the most important transcription factor families in plants. Since the discovery of basic/helix–loop–helix (bHLH) motifs [3] with the ability to bind DNA, members of the bHLH protein superfamily have been found to have more and more functions in the basic physiology and development of animals and plants [4–8]. The bHLH domain consists of about 60 amino acids and has two regions with different functions, i.e., the basic domain and the HLH domain. The basic domain is located at the N-terminus of the bHLH domain and acts as a DNA-binding motif. It consists of about 15 amino acids, usually including 6 basic residues. The HLH region contains two amphiphilic alpha helices and a variable-length linker. Two amphiphilic alpha helices of bHLH proteins can interact to form homodimers or heterodimers [9,10]. Some bHLH proteins have been shown

to bind to sequences containing a common core element called the E-box (5′-CANNTG-3′). In addition, nucleotides flanking the core elements may also play a role in binding specificity [11].

bHLH transcription factors are involved in the regulation of various plant processes, including growth, development and response to biotic and abiotic stresses. The function of *bHLHs* in disease resistance has been characterized in Arabidopsis and many other crops. For example, the wheat *bHLH* transcription factor gene *TabHLH060* increases the susceptibility of transgenic Arabidopsis to *Pseudomonas aeruginosa* [12]. In tomato, *SlybHLH131* increases resistance to yellow leaf curl virus by controlling cell death [13]. Overexpression of jasmonate-responsive *OsbHLH034* in rice results in the induction of bacterial blight resistance via an increase in lignin biosynthesis [14]. In addition, *bHLHs* are also associated with abiotic stress in plants. For example, *MdbHLH130* is the drought response bHLH protein in apple that confers drought tolerance in transgenic tobacco [15]. Overexpression of a *bHLH* gene from *Tamarix hispida* in Arabidopsis can improve salt and drought tolerance by increasing osmotic potential and reducing the accumulation of reactive oxygen species [16]. In Arabidopsis, *bHLH122* is important for drought and osmotic stress resistance and repressing ABA catabolism [17].

Recent research has shown that plant bHLHs can act as a susceptibility gene, negatively regulating plant disease resistance. Zhang and co-authors found that loss of function of the bHLH transcription factor Nrd1 in tomato enhances resistance to *Pseudomonas syringae*. The mutant plants showed increased immunity due to the suppression of a defence gene, Agp1, by Nrd1. This enhanced immunity is independent of the activation of other immunity-associated genes, indicating that Nrd1 plays a specific role in regulating Agp1 expression and susceptibility to Pseudomonas syringae in tomato [18].

Roses (*Rosa* sp.) are commercially the most important ornamental plant, generating tens of billions of dollars in value each year [19]. Grey mould disease of roses caused by *Botrytis cinerea* causes huge losses. There are no reports on the involvement of *bHLH* transcription factors in rose grey mould resistance. To better understand the involvement of the *bHLH* genes in rose resistance against *B. cinerea*, we performed a genome-wide analysis of the *bHLH* family in rose. We further performed RNA-Seq analysis and showed that a large number of genes encoding *bHLH* transcription factors were significantly upregulated upon *B. cinerea* infection, implying that they were involved in the resistance of rose to *B. cinerea* [20]. Importantly, virus-induced gene silencing (VIGS) further confirmed that *RcbHLH112* plays an important role in resistance to *B. cinerea* as a susceptibility gene.

2. Results

2.1. Identification of RcbHLH Genes in Rose

In the process of identifying *bHLH* family genes in the rose genome, we used the *bHLH* Hidden Markov Model (HMM) file (PF00010) to perform a Hmmsearch search in the rose genome database, and a total of 136 candidate RcbHLH proteins were obtained. MEME (https://meme-suite.org/meme/) (accessed on 11 July 2022) and Pfam database comparison further confirmed that the extracted protein domain was consistent with the characteristics of the family, and finally 121 *RcbHLH* gene members were identified in the rose genome, as these 121 protein sequences had a domain profile consistent with a typical bHLH transcription factor. All *RcbHLH* family genes can be mapped to chromosomes and named *RcbHLH1* to *RcbHLH121* according to their order on chromosomes (Figure 1).

There is a significant difference in the protein size of these *RcbHLHs*. Among the 121 *RcbHLHs*, *RcbHLH25* has the longest amino acid sequence with 1275 amino acids, while the shortest *RcbHLH85* has only 151 amino acids. The average length of RcbHLH protein is 385 aa. The details of all *RcbHLH* genes are listed in Table 1.

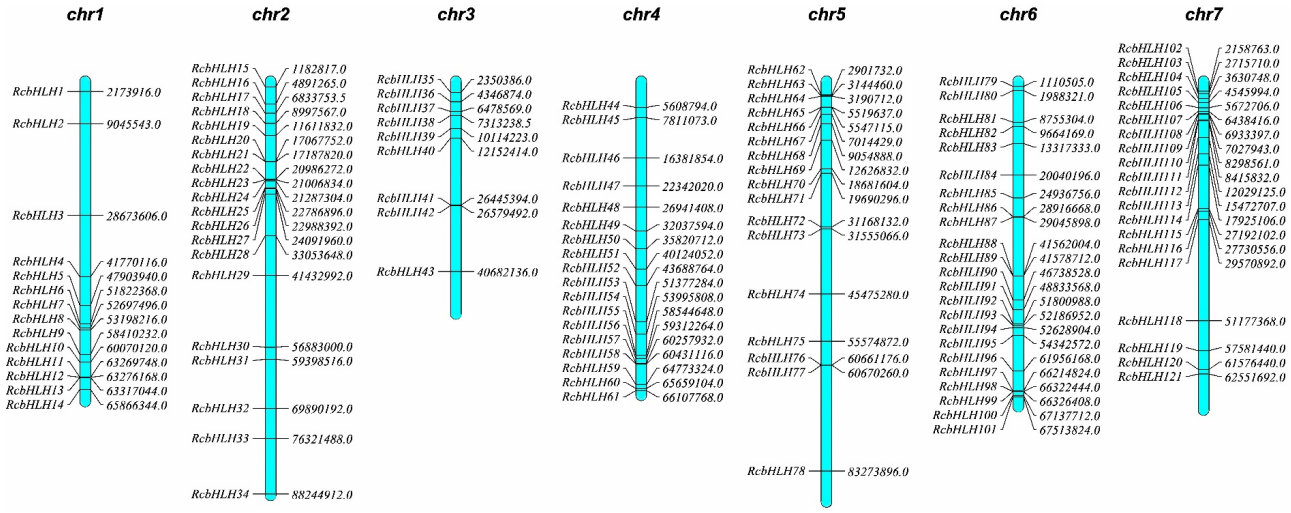

Figure 1. Chromosome localization of rose *bHLH* family members. The physical distribution of each *RcbHLH* gene is listed on the seven chromosomes of *Rose chinensis*.

Table 1. Members of the *RcbHLH* gene family as predicted in *R. chinensis* genome sequence.

Gene	Accession Number [1]	Chr. [2]	Position [3]	Intron	Extron	CDS (bp)	Amino Acids	Clade
RcbHLH1	RchiOBHm_Chr1g0314891	1	2,173,916	7	8	1296	432	IX
RcbHLH2	RchiOBHm_Chr1g0321521	1	9,045,543	1	2	708	236	Ib
RcbHLH3	RchiOBHm_Chr1g0337011	1	28,673,606	2	3	1371	457	II
RcbHLH4	RchiOBHm_Chr1g0348781	1	41,770,117	5	6	1404	468	VII
RcbHLH5	RchiOBHm_Chr1g0355211	1	47,903,940	7	8	1944	648	III(d+e+f)
RcbHLH6	RchiOBHm_Chr1g0360001	1	51,822,366	1	2	1851	617	III(d+e+f)
RcbHLH7	RchiOBHm_Chr1g0360811	1	52,697,497	0	1	1464	488	III(d+e+f)
RcbHLH8	RchiOBHm_Chr1g0361191	1	53,198,215	3	4	1254	418	Ia
RcbHLH9	RchiOBHm_Chr1g0368211	1	58,410,232	1	2	708	236	Ib
RcbHLH10	RchiOBHm_Chr1g0370561	1	60,070,116	1	2	795	265	Vb
RcbHLH11	RchiOBHm_Chr1g0376001	1	63,269,742	1	2	918	306	Ib
RcbHLH12	RchiOBHm_Chr1g0376011	1	63,276,165	1	2	570	190	Ib
RcbHLH13	RchiOBHm_Chr1g0376061	1	63,317,042	1	2	711	237	Ib
RcbHLH14	RchiOBHm_Chr1g0380101	1	65,866,341	2	3	1098	366	Ia
RcbHLH15	RchiOBHm_Chr2g0085911	2	1,182,817	6	7	1263	421	XI
RcbHLH16	RchiOBHm_Chr2g0091241	2	4,891,265	6	7	1158	386	X
RcbHLH17	RchiOBHm_Chr2g0093571	2	6,833,754	6	7	1743	581	IVd
RcbHLH18	RchiOBHm_Chr2g0096091	2	8,997,567	6	7	1293	431	XI
RcbHLH19	RchiOBHm_Chr2g0099391	2	11,611,832	4	5	954	318	IVb
RcbHLH20	RchiOBHm_Chr2g0105811	2	17,067,752	0	1	753	251	VIII(a+b+c)
RcbHLH21	RchiOBHm_Chr2g0105931	2	17,187,821	7	8	1128	376	VII
RcbHLH22	RchiOBHm_Chr2g0109611	2	20,986,271	3	4	1056	352	IVa
RcbHLH23	RchiOBHm_Chr2g0109621	2	21,006,834	4	5	1062	354	IVa
RcbHLH24	RchiOBHm_Chr2g0109941	2	21,287,305	3	4	1101	367	III(a+b+c)
RcbHLH25	RchiOBHm_Chr2g0111201	2	22,786,895	2	3	3825	1275	Orphan
RcbHLH26	RchiOBHm_Chr2g0111351	2	22,988,391	1	2	624	208	Vb
RcbHLH27	RchiOBHm_Chr2g0112221	2	24,091,959	2	3	996	332	Ia
RcbHLH28	RchiOBHm_Chr2g0120331	2	33,053,648	0	1	849	283	VIII(a+b+c)
RcbHLH29	RchiOBHm_Chr2g0126861	2	41,432,994	2	3	678	226	Ib
RcbHLH30	RchiOBHm_Chr2g0139261	2	56,883,003	8	9	1026	342	X
RcbHLH31	RchiOBHm_Chr2g0141851	2	59,398,516	0	1	1308	436	VIII(a+b+c)
RcbHLH32	RchiOBHm_Chr2g0152511	2	69,890,195	8	9	1617	539	VII
RcbHLH33	RchiOBHm_Chr2g0160481	2	76,321,485	0	1	912	304	VIII(a+b+c)
RcbHLH34	RchiOBHm_Chr2g0176421	2	88,244,910	3	4	1503	501	III(a+b+c)
RcbHLH35	RchiOBHm_Chr3g0451111	3	2,350,386	6	7	999	333	XI
RcbHLH36	RchiOBHm_Chr3g0454211	3	4,346,874	2	3	1020	340	Ia
RcbHLH37	RchiOBHm_Chr3g0457291	3	6,478,569	1	2	591	197	XVI
RcbHLH38	RchiOBHm_Chr3g0458701	3	7,313,238	8	9	2184	728	VII

Table 1. *Cont.*

Gene	Accession Number [1]	Chr. [2]	Position [3]	Intron	Extron	CDS (bp)	Amino Acids	Clade
RcbHLH39	RchiOBHm_Chr3g0462431	3	10,114,223	0	1	768	256	VIII(a+b+c)
RcbHLH40	RchiOBHm_Chr3g0465361	3	12,152,414	9	10	2079	693	XIII
RcbHLH41	RchiOBHm_Chr3g0480621	3	26,445,394	5	6	1032	344	IX
RcbHLH42	RchiOBHm_Chr3g0480751	3	26,579,491	6	7	1077	359	XII
RcbHLH43	RchiOBHm_Chr3g0493491	3	40,682,138	4	5	891	297	VIII(a+b+c)
RcbHLH44	RchiOBHm_Chr4g0390311	4	5,608,794	2	3	792	264	IVd
RcbHLH45	RchiOBHm_Chr4g0392401	4	7,811,073	3	4	678	226	IVa
RcbHLH46	RchiOBHm_Chr4g0399211	4	16,381,854	4	5	735	245	III(a+b+c)
RcbHLH47	RchiOBHm_Chr4g0403251	4	22,342,021	5	6	465	155	XII
RcbHLH48	RchiOBHm_Chr4g0405961	4	26,941,409	5	6	501	167	X
RcbHLH49	RchiOBHm_Chr4g0409001	4	32,037,594	5	6	1302	434	IX
RcbHLH50	RchiOBHm_Chr4g0412071	4	35,820,711	7	8	1662	554	XII
RcbHLH51	RchiOBHm_Chr4g0415421	4	40,124,051	4	5	1341	447	Ia
RcbHLH52	RchiOBHm_Chr4g0418301	4	43,688,763	2	3	609	203	Ia
RcbHLH53	RchiOBHm_Chr4g0425781	4	51,377,284	0	1	681	227	XVI
RcbHLH54	RchiOBHm_Chr4g0429161	4	53,995,807	6	7	558	186	XII
RcbHLH55	RchiOBHm_Chr4g0434901	4	58,544,648	5	6	720	240	XII
RcbHLH56	RchiOBHm_Chr4g0435901	4	59,312,260	3	4	1062	354	Vb
RcbHLH57	RchiOBHm_Chr4g0437041	4	60,257,934	8	9	1647	549	XII
RcbHLH58	RchiOBHm_Chr4g0437281	4	60,431,122	6	7	1008	336	Va
RcbHLH59	RchiOBHm_Chr4g0443741	4	64,773,328	7	8	1464	488	III(a+b+c)
RcbHLH60	RchiOBHm_Chr4g0445091	4	65,659,106	5	6	825	275	XII
RcbHLH61	RchiOBHm_Chr4g0445691	4	66,107,770	6	7	1578	526	X
RcbHLH62	RchiOBHm_Chr5g0004471	5	2,901,732	4	5	747	249	IVa
RcbHLH63	RchiOBHm_Chr5g0004791	5	3,144,460	3	4	816	272	III(a+b+c)
RcbHLH64	RchiOBHm_Chr5g0004831	5	3,190,712	7	8	1329	443	X
RcbHLH65	RchiOBHm_Chr5g0008581	5	5,519,637	3	4	573	191	Ib
RcbHLH66	RchiOBHm_Chr5g0008601	5	5,547,115	2	3	495	165	Ib
RcbHLH67	RchiOBHm_Chr5g0010631	5	7,014,429	1	2	789	263	Vb
RcbHLH68	RchiOBHm_Chr5g0013411	5	9,054,888	0	1	741	247	XIV
RcbHLH69	RchiOBHm_Chr5g0018101	5	12,626,832	1	2	861	287	VIII(a+b+c)
RcbHLH70	RchiOBHm_Chr5g0024601	5	18,681,604	1	2	711	237	VIII(a+b+c)
RcbHLH71	RchiOBHm_Chr5g0025741	5	19,690,297	3	4	633	211	III(a+b+c)
RcbHLH72	RchiOBHm_Chr5g0036871	5	31,168,132	6	7	1356	452	X
RcbHLH73	RchiOBHm_Chr5g0037201	5	31,555,063	4	5	699	233	IVa
RcbHLH74	RchiOBHm_Chr5g0048491	5	45,475,282	9	10	2886	962	XIII
RcbHLH75	RchiOBHm_Chr5g0053301	5	55,574,872	1	2	792	264	Vb
RcbHLH76	RchiOBHm_Chr5g0056871	5	60,661,176	3	4	987	329	III(a+b+c)
RcbHLH77	RchiOBHm_Chr5g0056881	5	60,670,256	3	4	1101	367	III(a+b+c)
RcbHLH78	RchiOBHm_Chr5g0077341	5	83,273,897	6	7	1314	438	IX
RcbHLH79	RchiOBHm_Chr6g0245181	6	1,110,505	5	6	1020	340	IVb
RcbHLH80	RchiOBHm_Chr6g0246251	6	1,988,321	2	3	975	325	IVa
RcbHLH81	RchiOBHm_Chr6g0253641	6	8,755,304	4	5	1017	339	VIII(a+b+c)
RcbHLH82	RchiOBHm_Chr6g0254731	6	9,664,169	4	5	855	285	X
RcbHLH83	RchiOBHm_Chr6g0257881	6	13,317,333	2	3	1095	365	XIV
RcbHLH84	RchiOBHm_Chr6g0264701	6	20,040,197	7	8	1272	424	XII
RcbHLH85	RchiOBHm_Chr6g0268091	6	24,936,757	1	2	453	151	III(d+e+f)
RcbHLH86	RchiOBHm_Chr6g0270891	6	28,916,670	2	3	732	244	Ib
RcbHLH87	RchiOBHm_Chr6g0271001	6	29,045,898	2	3	2796	932	Orphan
RcbHLH88	RchiOBHm_Chr6g0278441	6	41,562,004	8	9	1653	551	III(a+b+c)
RcbHLH89	RchiOBHm_Chr6g0278471	6	41,578,713	7	8	1890	630	III(a+b+c)
RcbHLH90	RchiOBHm_Chr6g0283511	6	46,738,527	6	7	1650	550	XII
RcbHLH91	RchiOBHm_Chr6g0285491	6	48,833,566	7	8	1533	511	VII
RcbHLH92	RchiOBHm_Chr6g0288541	6	51,800,989	10	11	2190	730	XIII
RcbHLH93	RchiOBHm_Chr6g0288981	6	52,186,951	1	2	1434	478	III(d+e+f)
RcbHLH94	RchiOBHm_Chr6g0289601	6	52,628,904	5	6	882	294	XI
RcbHLH95	RchiOBHm_Chr6g0291161	6	54,342,571	5	6	2109	703	III(d+e+f)
RcbHLH96	RchiOBHm_Chr6g0301601	6	61,956,169	6	7	1434	478	X
RcbHLH97	RchiOBHm_Chr6g0308101	6	66,214,825	0	1	750	250	VIII(a+b+c)
RcbHLH98	RchiOBHm_Chr6g0308241	6	66,322,449	1	2	633	211	XIV
RcbHLH99	RchiOBHm_Chr6g0308251	6	66,326,408	5	6	1002	334	VII
RcbHLH100	RchiOBHm_Chr6g0309431	6	67,137,708	3	4	1137	379	VIII(a+b+c)

Table 1. *Cont.*

Gene	Accession Number [1]	Chr. [2]	Position [3]	Intron	Extron	CDS (bp)	Amino Acids	Clade
RcbHLH101	RchiOBHm_Chr6g0310101	6	67,513,823	8	9	1293	431	XII
RcbHLH102	RchiOBHm_Chr7g0180121	7	2,158,763	5	6	816	272	XII
RcbHLH103	RchiOBHm_Chr7g0181001	7	2,715,710	4	5	750	250	IVb
RcbHLH104	RchiOBHm_Chr7g0182341	7	3,630,748	4	5	1092	364	VII
RcbHLH105	RchiOBHm_Chr7g0183781	7	4,545,994	11	12	1701	567	Va
RcbHLH106	RchiOBHm_Chr7g0185551	7	5,672,706	5	6	855	285	XII
RcbHLH107	RchiOBHm_Chr7g0186541	7	6,438,416	9	10	2337	779	XIII
RcbHLH108	RchiOBHm_Chr7g0187141	7	6,933,397	1	2	1407	469	III(d+e+f)
RcbHLH109	RchiOBHm_Chr7g0187261	7	7,027,943	1	2	1350	450	III(d+e+f)
RcbHLH110	RchiOBHm_Chr7g0188921	7	8,298,561	3	4	1593	531	III(a+b+c)
RcbHLH111	RchiOBHm_Chr7g0189021	7	8,415,832	7	8	1041	347	XII
RcbHLH112	RchiOBHm_Chr7g0193761	7	12,029,125	2	3	573	191	Ib
RcbHLH113	RchiOBHm_Chr7g0197531	7	15,472,708	7	8	1920	640	III(d+e+f)
RcbHLH114	RchiOBHm_Chr7g0199961	7	17,925,106	1	2	765	255	Ib
RcbHLH115	RchiOBHm_Chr7g0209751	7	27,192,104	0	1	2082	694	III(d+e+f)
RcbHLH116	RchiOBHm_Chr7g0210101	7	27,730,558	2	3	963	321	Ia
RcbHLH117	RchiOBHm_Chr7g0212241	7	29,570,891	1	2	1392	464	III(d+e+f)
RcbHLH118	RchiOBHm_Chr7g0227911	7	51,177,367	4	5	672	224	IX
RcbHLH119	RchiOBHm_Chr7g0233161	7	57,581,440	3	4	1068	356	III(a+b+c)
RcbHLH120	RchiOBHm_Chr7g0236841	7	61,576,445	1	2	645	215	Vb
RcbHLH121	RchiOBHm_Chr7g0237511	7	62,551,696	1	2	1083	361	XIII

[1] Available at https://lipm-browsers.toulouse.inra.fr/pub/RchiOBHm-V2/ (accessed on 5 July 2022). [2] Chromosome. [3] Starting position (b).

2.2. Chromosomal Locations, Whole-Genome Duplication and Microsynteny

The 121 *RcbHLH* genes identified are unevenly distributed across 7 rose chromosomes (Figure 1). Chromosome 6 has the most *RcbHLH* genes with 23. There are 20 *RcbHLH* genes on chromosomes 2 and 7. Chromosome 3 has the fewest *RcbHLH* genes, only 9. Meanwhile, 12.37% and 13.22% of *RcbHLH* genes are located in the upper and middle parts of chromosomes 2 and 7, respectively; 9. 92% of the *RcbHLH* genes are located in the upper and middle parts of chromosome 5; 9.09% and 10.74% of the genes are distributed in the middle and lower parts of chromosomes 1 and 4, respectively; and 19.01% of the *RcbHLH* genes are distributed on chromosome 6.

Tandem and segmental duplication play an important role in the expansion of gene families and the generation of new gene functions. On further examination of the repetitive events, we found that there were 16 gene pairs in this family, all of which were whole-genome duplication (WGD) or segmental duplication, while there were gene pairs on different chromosomes, indicating that these genes were paralogous genes. The microsynteny of these *RcbHLH* genes is shown in Figure 2.

To investigate the selective constraints between duplicated *RcbHLH* genes, the ratios of non-synonymous mutation frequency (Ka) to synonymous mutation frequency (Ks) of 16 gene pairs were calculated (Table 2). In general, Ka/Ks > 1 is consistent with positive selection, whereas Ka/Ks < 1 indicates purifying selection. The Ka/Ks ratio of all 16 repetitive gene pairs is less than 1 (Table 2), indicating limited functionally divergent purifying selection during the evolutionary history of the repetitive *RcbHLH* genes.

Table 2. Duplication analysis of the *RcbHLH* gene family.

Sequence 1	Sequence 2	Ka	Ks	Ka/Ks	Effective Len	Average S-Sites	Average N-Sites
RcbHLH7	*RcbHLH109*	0.443081	NaN	NaN	1275	291.6667	983.3333
RcbHLH11	*RcbHLH114*	0.462303	NaN	NaN	597	133.8333	463.1667
RcbHLH20	*RcbHLH97*	0.328431	2.687639	0.1222	636	140	496
RcbHLH21	*RcbHLH99*	0.48712	1.774278	0.274545	885	202.0833	682.9167

Table 2. *Cont.*

Sequence 1	Sequence 2	Ka	Ks	Ka/Ks	Effective Len	Average S-Sites	Average N-Sites
RcbHLH24	*RcbHLH119*	0.287838	1.620301	0.177645	1032	222.1667	809.8333
RcbHLH25	*RcbHLH87*	0.457511	2.840734	0.161054	2733	555.5833	2177.417
RcbHLH26	*RcbHLH120*	0.59898	1.798421	0.333059	555	133.8333	421.1667
RcbHLH29	*RcbHLH65*	0.455457	4.125944	0.110389	537	122.5	414.5
RcbHLH34	*RcbHLH110*	0.370849	2.185043	0.169722	1443	325.9167	1117.083
RcbHLH37	*RcbHLH53*	0.392481	1.639823	0.239344	588	153.5833	434.4167
RcbHLH54	*RcbHLH111*	0.438746	NaN	NaN	552	122.75	429.25
RcbHLH55	*RcbHLH106*	0.435383	1.727525	0.252027	681	142.3333	538.6667
RcbHLH58	*RcbHLH105*	0.367022	2.17115	0.169045	981	223	758
RcbHLH60	*RcbHLH102*	0.205017	1.25652	0.163163	753	174.1667	578.8333
RcbHLH62	*RcbHLH73*	0.230957	1.156031	0.199784	693	159.3333	533.6667
RcbHLH64	*RcbHLH72*	0.369998	1.720924	0.215	1137	258.75	878.25

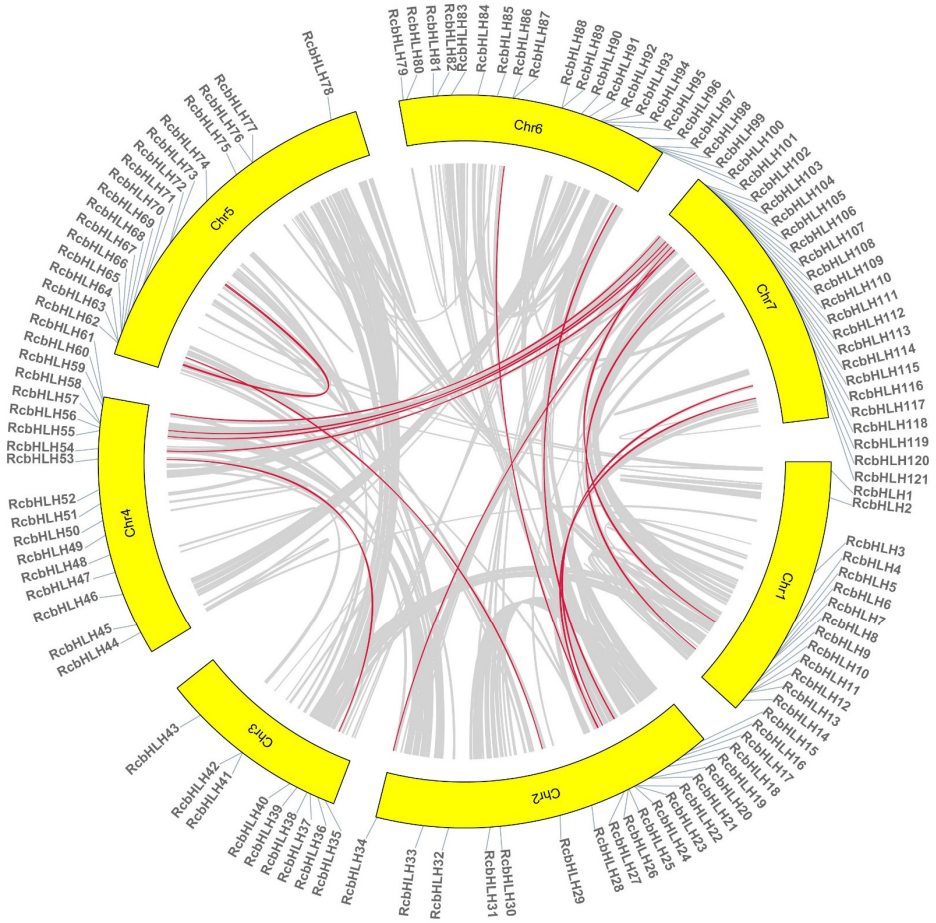

Figure 2. Microsyntenic analyses of the rose *bHLH* transcription factors in the *Rose chinensis* genome. Circular visualization of rose *bHLH* transcription factors is mapped onto different chromosomes using Circos [21]. The red lines indicate rose *bHLH* genes with a syntenic relationship. The grey lines represent all syntenic blocks in the genome of *Rose chinensis*.

2.3. Phylogenetic and Exon-Intron Structural Analysis of Rose bHLH Genes

We used the neighbour-joining method (NJ) method to reconstruct the phylogeny of all *RcbHLH* genes and constructed a phylogenetic tree. The results of the follow-up analysis of the exon–intron structure are consistent with those of the phylogenetic analysis (Figure 3). Most genes clustered in the same group have similar genetic structures, especially in terms of the number of introns, such as *RcbHLH10*, *RcbHLH26* and *RcbHLH120*. However, there

were some exceptions. For example, *RcbHLH58* and *RcbHLH105* contain different numbers of introns. In addition, their intron length is very variable, ranging from tens to thousands of nucleotides. These results indicate that there is a highly conservative structure in the *RcbHLH* subfamily and that there is sequence diversity between different *RcbHLH* groups.

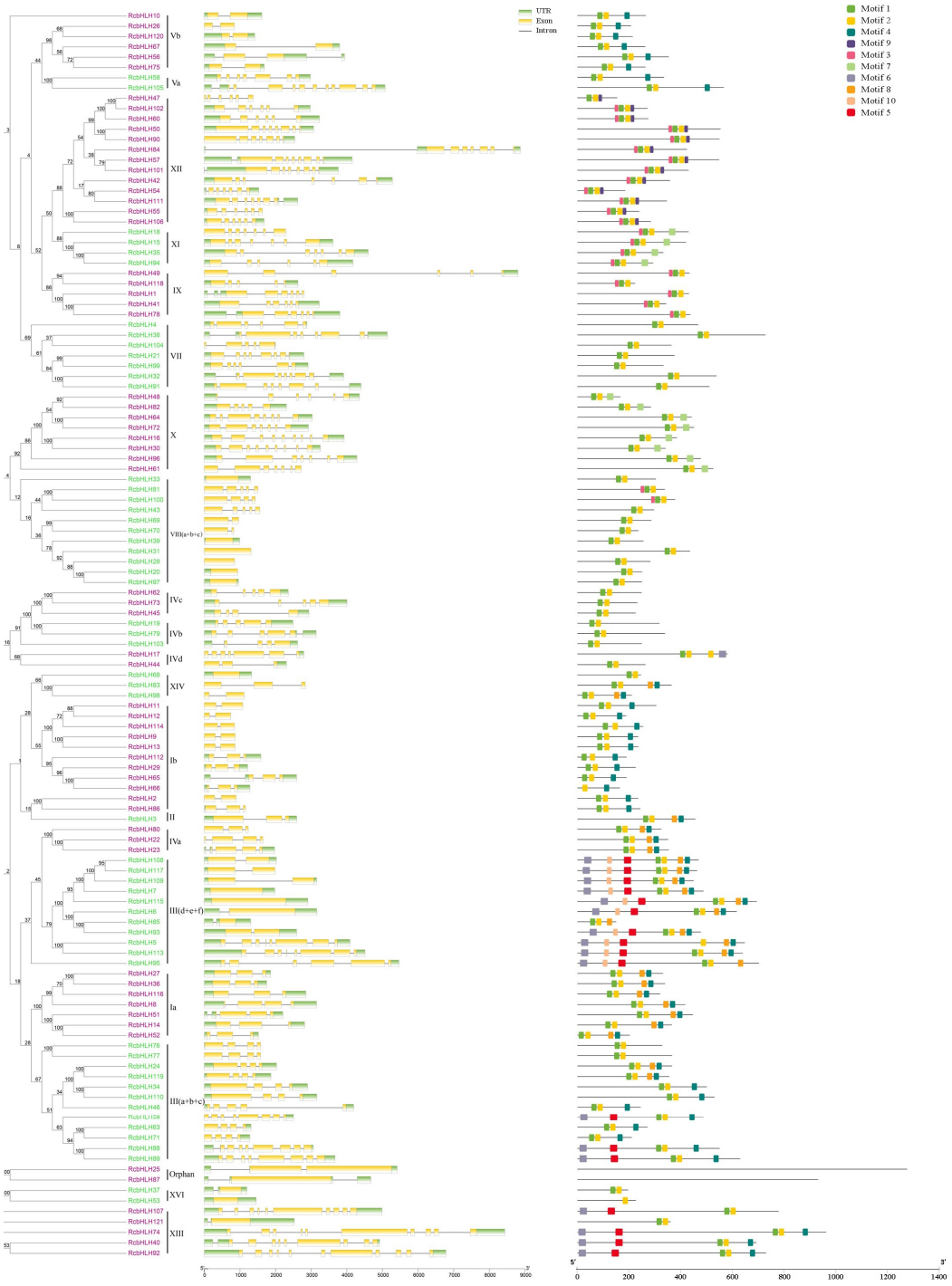

Figure 3. Phylogenetic analyses, DNA structures and protein motifs of the *bHLH* gene family in rose. Complete alignments of all rose *bHLH* proteins were used to construct a phylogenetic tree using the neighbour-joining method. The left represents gene structures. The green boxes, yellow boxes and grey lines in the exon–intron structure diagram represent UTRs, exons and introns, respectively. The right represents protein motifs in the *bHLH* members. The colourful boxes delineate different motifs (unit: aa). The scale on the bottom is provided as a reference.

In addition, there is increasing evidence that *bHLH* transcription factors play a key role in disease resistance in various plant species (Table 3). To assess the evolutionary relationship between *RcbHLHs* and *AtbHLHs* genes, we constructed a composite phylogenetic tree (Figure 4). According to The Arabidopsis Information Resources (TAIR) (http://www.arabidopsis.org/) (accessed on 11 July 2022), there are 158 *AtbHLH* genes in Arabidopsis. These members can be divided into 21 different groups. The results confirmed the previously proposed classification of the *bHLH* family. The subfamily VIII (a+b+c) contains 31 proteins, and the subfamily IVb contains 3 proteins. The bootstrap values of some branches in the phylogenetic tree are low, which may be due to the short bHLH domain and relatively little information other than highly conserved information.

Table 3. Plant *bHLH* family genes involved in disease resistance.

Gene Name	Gene ID	Species	Pathogens	References
SlybHLH131	Solyc06g051550.2.1	*Solanum lycopersicum*	*Tomato yellow leaf curl virus*	[13]
FAMA	AT3G24140	*Arabidopsis thaliana*	*Botrytis cinerea*	[22]
AtMYC2	At1g32640	*Arabidopsis thaliana*	*Botrytis cinerea*	[23]
AtbHLH13	At1g01260	*Arabidopsis thaliana*	*Botrytis cinerea*	[24]
OsbHLH6	Os04g23550	*Oryza sativa*	*Magnaporthe oryzae*	[25]
OsbHLH034	Os02g49480	*Oryza sativa*	*Xanthomonas oryzae pv. oryzae*	[14]

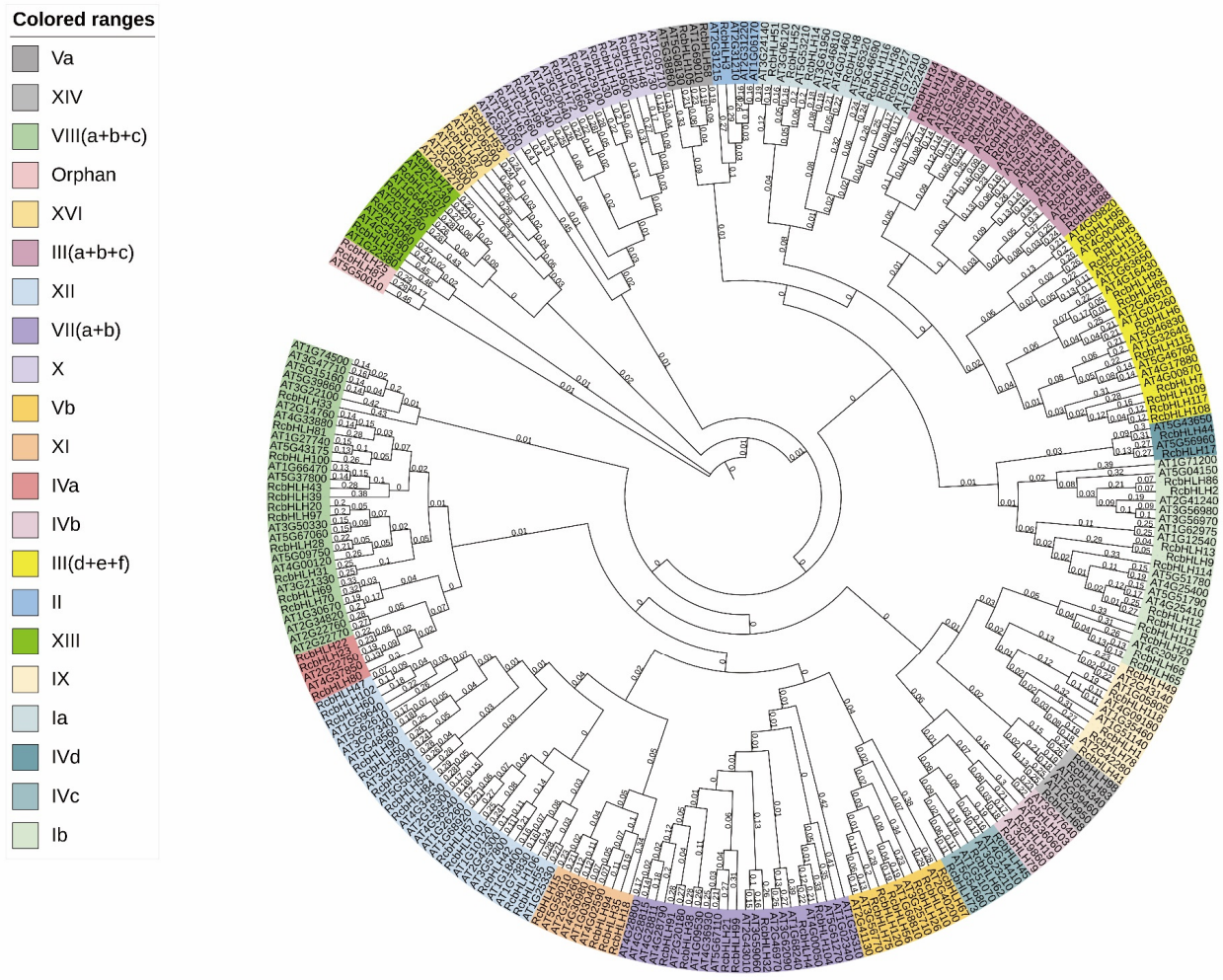

Figure 4. Phylogenetic analyses of the rose *bHLH* transcription factors. Composite phylogenetic tree of rose and Arabidopsis *bHLH* transcription factors. The bootstrap values are indicated on the nodes of the branches.

2.4. Expression of RcbHLH Genes in Response to B. cinerea Infection

A growing body of evidence from different plant species indicates that plant *bHLH* transcription factors play an important role in pathogen response. To investigate the role of *bHLH* genes in *B. cinerea* resistance in rose, we analysed transcriptome data from rose petals inoculated with the pathogen at 30 and 48 hpi. The 30 hpi time point represents the early response to infection, whereas the 48 hpi time point corresponds to the late response (Table 4). The log_2Ratio transformed expression profiles were obtained from the RNA-seq dataset [20]. A total of 21 *RcbHLH* genes (*RcbHLH17*, *RcbHLH21*, *RcbHLH29*, *RcbHLH34*, *RcbHLH40*, *RcbHLH44*, *RcbHLH46*, *RcbHLH59*, *RcbHLH62*, *RcbHLH67*, *RcbHLH72*, *RcbHLH75*, *RcbHLH80*, *RcbHLH90*, *RcbHLH99*, *RcbHLH101*, *RcbHLH106*, *RcbHLH108*, *RcbHLH111*, *RcbHLH112* and *RcbHLH115*) were upregulated, suggesting that they may be the key regulators of *B. cinerea* infection and influence the disease resistance of rose. To further verify the expression profile of RNA-seq, the expression of 4 *RcbHLHs* was analysed by RT-qPCR. The results of the RT-qPCR analysis were consistent with those of the transcriptome analysis (Figure 5).

Table 4. Expression patterns of *RcbHLH* genes under infection of *B. cinerea*.

Gene [2]	Accession Number	Group	log_2Ratio 30 hpi	log_2Ratio 48 hpi
RcbHLH4	RchiOBHm_Chr1g0348781	VII	−1.02302	−1.36247
RcbHLH8	RchiOBHm_Chr1g0361191	Ia	−1.05954	−1.91491
RcbHLH16	RchiOBHm_Chr2g0091241	X	0	−1.13271
RcbHLH17	**RchiOBHm_Chr2g0093571**	**IVd**	**3.07535**	**4.92649**
RcbHLH21	**RchiOBHm_Chr2g0105931**	**VII**	**0**	**1.03517**
RcbHLH29 *	**RchiOBHm_Chr2g0126861**	**Ib**	**0**	**6.04668**
RcbHLH32	RchiOBHm_Chr2g0152511	VII	0	−16.01
RcbHLH34	**RchiOBHm_Chr2g0176421**	**III(a+b+c)**	**1.07031**	**1.74663**
RcbHLH37	RchiOBHm_Chr3g0457291	XVI	−1.36188	--
RcbHLH39	RchiOBHm_Chr3g0462431	VIII(a+b+c)	−1.48345	--
RcbHLH40	**RchiOBHm_Chr3g0465361**	**XIII**	**1.40229**	**2.20545**
RcbHLH42	RchiOBHm_Chr3g0480751	XII	0	−1.48859
RcbHLH44	**RchiOBHm_Chr4g0390311**	**IVd**	**2.8578**	**4.76511**
RcbHLH46	**RchiOBHm_Chr4g0399211**	**III(a+b+c)**	**1.25346**	**3.44205**
RcbHLH50	RchiOBHm_Chr4g0412071	XII	−1.46896	−1.77088
RcbHLH53	RchiOBHm_Chr4g0425781	XVI	0	−1.61078
RcbHLH55	RchiOBHm_Chr4g0434901	XII	0	−2.09156
RcbHLH57	RchiOBHm_Chr4g0437041	XII	1.04454	--
RcbHLH59	**RchiOBHm_Chr4g0443741**	**III(a+b+c)**	**0**	**1.92286**
RcbHLH60 *	RchiOBHm_Chr4g0445091	XII	−1.22975	−4.12905
RcbHLH62	**RchiOBHm_Chr5g0004471**	**IVa**	**0**	**2.03271**
RcbHLH67	**RchiOBHm_Chr5g0010631**	**Vb**	**1.17478**	**1.30658**
RcbHLH72	**RchiOBHm_Chr5g0036871**	**X**	**0**	**2.48493**
RcbHLH75	**RchiOBHm_Chr5g0053301**	**Vb**	**−2.3709**	**−1.67965**
RcbHLH78	RchiOBHm_Chr5g0077341	IX	−1.05564	−1.12357
RcbHLH80 *	**RchiOBHm_Chr6g0246251**	**IVa**	**−3.29702**	**−2.05486**
RcbHLH84	RchiOBHm_Chr6g0264701	XII	0	−1.21958
RcbHLH90	**RchiOBHm_Chr6g0283511**	**XII**	**1.64315**	**2.09937**
RcbHLH91	RchiOBHm_Chr6g0285491	VII	0	−1.17746
RcbHLH92	RchiOBHm_Chr6g0288541	XIII	0	−1.37089
RcbHLH94	RchiOBHm_Chr6g0289601	XI	0	−1.06842
RcbHLH99	**RchiOBHm_Chr6g0308251**	**VII**	**1.31529**	**2.89317**
RcbHLH101	**RchiOBHm_Chr6g0310101**	**XII**	**0**	**1.23509**

Table 4. *Cont.*

Gene [2]	Accession Number	Group	log_2Ratio 30 hpi	log_2Ratio 48 hpi
RcbHLH102	RchiOBHm_Chr7g0180121	XII	0	−1.80483
RcbHLH104	RchiOBHm_Chr7g0182341	VII	0	−1.28473
RcbHLH105	RchiOBHm_Chr7g0183781	Va	−1.38441	--
RcbHLH106	**RchiOBHm_Chr7g0185551**	**XII**	**0**	**1.81513**
RcbHLH108	**RchiOBHm_Chr7g0187141**	**III(d+e+f)**	**0**	**2.74536**
RcbHLH109	RchiOBHm_Chr7g0187261	III(d+e+f)	−3.56492	--
RcbHLH110	RchiOBHm_Chr7g0188921	III(a+b+c)	0	−1.77728
RcbHLH111	**RchiOBHm_Chr7g0189021**	**XII**	**1.34134**	**2.0827**
RcbHLH112 *	**RchiOBHm_Chr7g0193761**	**Ib**	**1.22723**	**4.82187**
RcbHLH115	**RchiOBHm_Chr7g0209751**	**III(d+e+f)**	**0**	**1.34433**
RcbHLH116	RchiOBHm_Chr7g0210101	Ia	0	−3.38838
RcbHLH118	RchiOBHm_Chr7g0227911	IX	0	−1.0994
RcbHLH121	RchiOBHm_Chr7g0237511	XIII	0	−1.02865

The log2 transformed expression profiles were obtained from the RNA-seq dataset [20]. [2] RcbHLHs upregulated are shown in bold. The genes validated by qPCR were marked with asterisks.

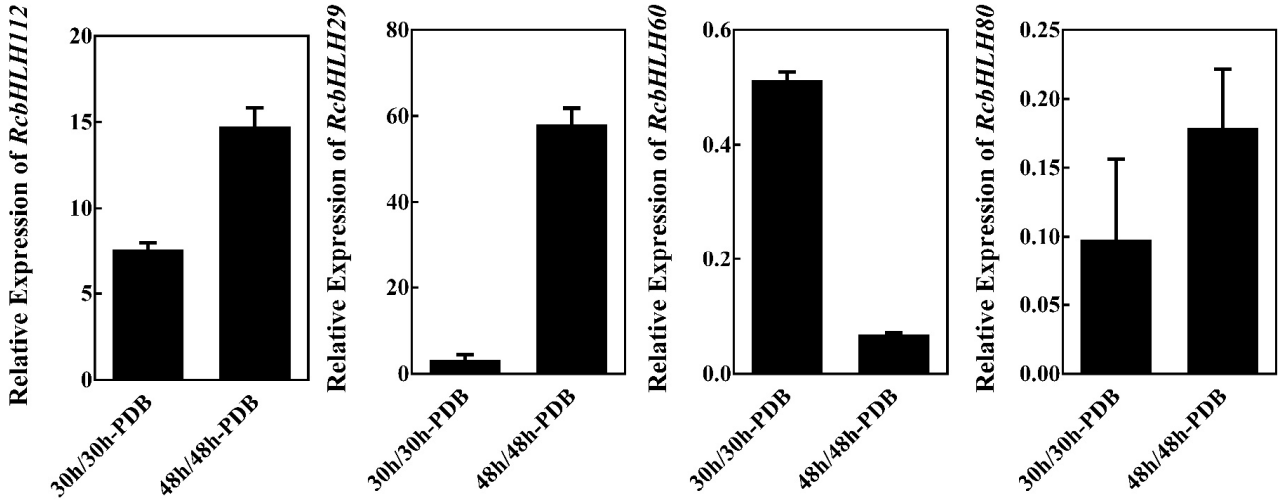

Figure 5. Validation of RNA-Seq results using qRT-PCR. RhUbi was used as an internal control. Expression profile data of four *RcbHLH* genes at 30 hpi and 48 hpi after *B. cinerea* inoculation were obtained using qRT-PCR. Values are the means of three replicates ± SD. The primers used are listed in Table 5.

Table 5. List of primers used in this study.

Gene Name	Accession Number	Primer Sequence (5′-3′)	Amplicon Length	Ta	Tm	Amplification Efficiency
RcbHLH29	RchiOBHm_Chr2g0126861	F: GGTTCCACCCTAGAGGTTGTT R: CTGCACGGACTAGGTGAAGT	110 bp	60 °C	81.69	2.005
RcbHLH60	RchiOBHm_Chr4g0445091	F: CGATGAGTTTGGACCACCGA R: CCTCAGCTTTGGCCTCAAGA	116 bp	60 °C	84.1	1.972
RcbHLH80	RchiOBHm_Chr6g0246251	F: ACACAAACCAAGTGGGGGTT R: GTTCCCTGACTGGCCTTCAA	102 bp	60 °C	85.27	1.968
RcbHLH112	RchiOBHm_Chr7g0193761	F: CGATCTTGCAGCCTCCTACA R: CAACCTTGATCCGACCACCA	120 bp	60 °C	82.43	2.024
RcUBI2	RchiOBHm_Chr1g0359561	F: GCCCTGGTGCGTTCCCAACTG R: CCTGCGTGTCTGTCCGCATTG	82 bp	60 °C	82.43	2.024

Ta: amplification temperature; Tm: melting temperature.

2.5. RcbHLH112 Is a Susceptibility Gene to B. cinerea in Rose

To further investigate the potential role of *B. cinerea*-induced *RcbHLH* genes in pathogen resistance, we used VIGS to knock down the expression of *RcbHLH112* in rose petals. The reason for selecting *RcbHLH112* for this VIGS study was that *RcbHLH112* is one of the most upregulated *RcbHLHs* after *B. cinerea* infection (Table 4). To silence *RcbHLH112* in rose petals, we cloned the 256 bp fragment of *RcbHLH112* into the tobacco rattle virus (*TRV2*) vector to generate *TRV-RcbHLH112*. Agrobacterium tumefaciens carrying *TRV-RcbHLH112* and *TRV1* were co-infiltrated into rose petals to produce rose petals with *RcbHLH112* silencing. The infiltrated rose petals were then inoculated with *B. cinerea*. Compared with the control petals (*TRV-GFP*) inoculated with *TRV* with a GFP sequence, petals inoculated with *TRV-RcbHLH112* showed attenuation of disease symptoms, with a significant reduction in the size of the lesion (Figure 6A,B). In addition, we used RT-qPCR to verify the silencing efficiency of VIGS (Figure 6C). These results show that *RcbHLH112* is a susceptibility factor for rose resistance against *B. cinerea* and that its silencing increases resistance to *B. cinerea* in rose.

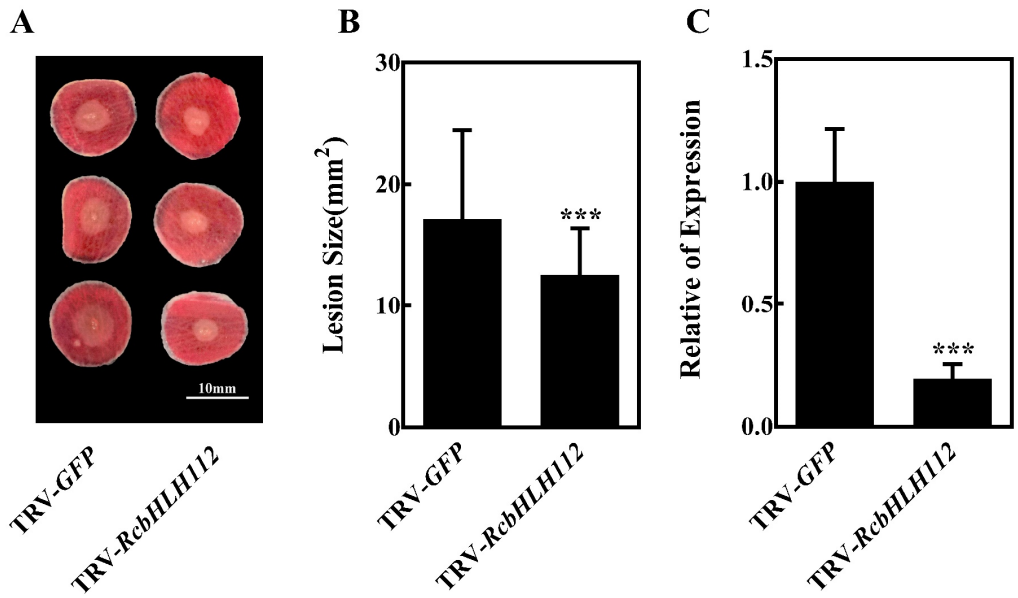

Figure 6. Functional analysis of rose *bHLH* transcription factor gene *RcbHLH112*. (**A**) Compromised *B. cinerea* resistance upon silencing of *RcbHLH112* at 60 hpi post inoculation. A recombinant tobacco rattle virus (*TRV*) targeting *RcbHLH112* (*TRV-RcbHLH112*) was used for the gene silencing, and a *TRV* with GFP sequence (*TRV-GFP*) was used as the control. (**B**) Quantification of *B. cinerea* disease lesions on *TRV-RcbHLH112*- and *TRV-GFP*-inoculated rose petal discs. The graph shows the lesion size from three biological replicates (n = 48) with the standard deviation. (**C**) Expression of *RcbHLH112* relative to that in the control at 6 days post silencing, before the infection with *B. cinerea* (0 hpi). All statistical analyses were performed using Student's t-test; *** $p < 0.001$.

3. Discussion

The *bHLH* genes play important roles in plant growth, development and defence. In this study, we comprehensively analysed the *RcbHLH* family, including phylogeny, gene structure, chromosome localization, gene duplication events, sequence characteristics and expression profile analysis. We demonstrated that *RcbHLH112* is involved in the regulation of resistance to *B. cinerea* in rose.

It was found that the number of *RcbHLH* genes in rose (121) was lower than that in Arabidopsis (158), rice (167), potato (124) and maize (208) [26–28], indicating that the *bHLH* gene has expanded to different degrees in different plants. Gene replication plays a very important role in gene family expansion. In this study, 16 replication events were identified in 56 *RcbHLHs*, all of which involved segmental duplication. The Ka/Ks ratio of the 16 *RcbHLH* repeats indicates that the *RcbHLH* gene family is under purifying selection,

suggesting a highly conserved evolution. The phylogenetic relationship of *bHLH* between rose and Arabidopsis showed that most evolutionary branches contained different numbers of AtbHLH and RcbHLH proteins, indicating that the two species showed conservative evolution. These results suggest that the species-specific *bHLH* gene was lost in rose or gained in the Arabidopsis phylogeny after divergence from the most recent common ancestor.

The role of *RcbHLH* in *B. cinerea* resistance is still unclear. In this study, we constructed a phylogenetic tree of known resistance-related *bHLHs* and found that the *bHLHs* involved in disease resistance were distributed in groups Ia, Ib, IVb, IVc and III(d+e+f). According to the expression in response to *B. cinerea* infection, we identified 21 *RcbHLHs* that could be involved in *B. cinerea* resistance in rose petals. Interestingly, most of the *RcbHLH* genes induced by *B. cinerea* are associated with segmental duplication events. The *RcbHLH112* belonging to Ib was on the same evolutionary branch as the *B. cinerea* resistance-related *bHLH* found in many different species and was significantly induced by *B. cinerea* at 30 hpi and 48 hpi. Therefore, *RcbHLH112* should be considered as an important candidate gene involved in the regulation of disease resistance in rose. The results of VIGS in rose petals showed that silencing of *RcbHLH112* improved resistance to *B. cinerea*, indicating that it is a susceptibility factor of rose in *B. cinerea* infection process.

4. Materials and Methods

4.1. Identification and Characteristics of the bHLH Genes in Rose Genome

The complete genome data were downloaded from the Rosa chinensis 'Old Blush' genome website https://lipm-browsers.toulouse.inra.fr/pub/RchiOBHm-V2/ (accessed on 5 July 2022) for local alignment and analysis. To identify the non-redundant *bHLH* genes in the rose genome, first, the common protein sequence of the *bHLH* Hidden Markov Model (HMM) (PF00010) was downloaded from the Pfam website (http://pfam.xfam.org) (accessed on 11 July 2022). Then, using the HMM profile as a query, the rose genome was searched using the hmmblast function and all sequences were identified as containing bHLH domains with an E-value of $<1 \times 10^{-3}$ in rose. Finally, the protein and DNA sequences of the above rose *bHLH* members were extracted using the TBTools tool, and all candidate *RcbHLHs* were verified using the functional structure identified by MEME (https://meme-suite.org/meme/) (accessed on 11 July 2022) and the Pfam database to determine the final family members.

4.2. Mapping bHLH Genes on Rose Chromosomes

The physical locations of 121 genes were extracted from the genomic gff3 annotation file of rose. Mapchart 2.2 software was used to visualise the distribution of *bHLH* genes on 7 rose chromosomes [29].

4.3. Phylogenetic Analyses and Structure Analysis

A total of 158 Arabidopsis bHLH protein sequences were collected from TAIR (http://www.arabidopsis.org/) (accessed on 11 July 2022). The bHLH protein sequences of Arabidopsis thaliana and rose were compared using ClustalW. The bHLH sequence alignments were used for phylogenetic analysis. The phylogenetic tree was constructed using MEGA6 software, calculating the advance distance via p-distance, estimating the amino acid substitution at each site, performing 1000 bootstrap sampling steps and constructing the phylogenetic tree via the NJ method [30]. The gene structure map and functional structure map of *RcbHLH* were completed using TBtools [31].

4.4. Collinearity Analyses and Calculation of Ratios of Non-Synonymous (Ka) to Synonymous (Ks) Nucleotide Substitution

We used TBtools to analyse the collinearity of *bHLH* members and calculate the ratio of Ka/Ks [32].

4.5. Expression of RcbHLHs in Response to B. cinerea

The RNA-Seq data of rose petals infected with *B. cinerea* can be obtained from the National Center for Biotechnology Information (NCBI) database, accession number PRJNA414570. Clean sequencing reads were mapped to the rose reference genome. Reads per kb per million reads (RPKM) were used to obtain gene expression level. The gene expression level of *RcbHLH* was calculated as reads per kb per million reads. Differential expression analysis based on Log2 fold change was analysed using DEseq2. To verify the results of RNA-Seq, quantitative PCR (qPCR) was used to analyse the expression of 4 *RcbHLH* genes. Therefore, total RNA was extracted from rose petals 30 and 48 h after inoculation using the hot borate method [33]. First-strand cDNA was synthesised using HiScript II Q Select RT SuperMix (Vazyme) in 20 µL reaction volume, and 1 µg DNase-treated RNA was used. SYBR Green Master Mix (Takara, Dalian, China) was used for the qPCR reaction, and detection was performed on a StepOnePlus real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). RcUBI2 was used as an internal control. Expression was analysed via the delta–delta–CT method. All primers used for qPCR are listed in Table 5.

4.6. VIGS and B. cinerea Inoculation Assays

To generate the *TRV-RcbHLH112* constructor, the 256 bp fragment of *RcbHLH112* was amplified using a pair of primers, *RcbHLH112-F*(5′-GGGGGACAAGTTTGTACAAAAAAGCAGGCTTCTGAGGAAGAAGGAGCCGAAG-3′) and *RcbHLH112-R*(5′-GGGGGACCACTTTGTACAAGAAAGCTGGGTCCTCAGCTTAGCCTTGTGGAGT-3′).

The VIGS process involved taking individual petals from the outermost whorls of the rose at the second stage of flowering. A 15 mm disc was then cut from the centre of each petal. Agrobacterium tumefaciens cultures containing constructs expressing *TRV1* and *TRV2* were mixed 1:1 and infiltrated into the petal disc under vacuum [34]. On day 6 after infection, the petal disc was inoculated with *B. cinerea*. A minimum of 48 petal discs were used for genes, and VIGS was repeated at least three times. After inoculation with *B. cinerea*, Student's *t*-test was performed to determine the significance of lesion size.

5. Conclusions

In this study, a genome-wide analysis of the *RcbHLH* family genes was performed, including phylogenetic relationship, collinearity and expression analysis. A total of 121 non-redundant *bHLH* family members were identified. These *RcbHLH* family genes were classified into 21 groups based on phylogeny and conserved domains. Expression analysis showed that the transcriptional regulation of some *RcbHLH* family genes was induced by *B. cinerea* infection in rose petals. Furthermore, plant *bHLHs* involved in disease resistance tended to cluster on the same branch of the phylogenetic tree. Based on these analyses, we used VIGS to further demonstrate that *RcbHLH112* is a susceptibility factor of rose in *B. cinerea* infection process. The information provided by these results can promote further functional analysis of the *RcbHLH* gene in rose.

Author Contributions: C.D., X.H. and Z.Z. conceived and designed the experiments. C.D., J.G., S.Z., D.S. and Z.Z. analysed the data and wrote the paper. X.H., S.Z. and N.J. performed the experiments. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable. Our research did not involve any human or animal subjects, material or data. The plant materials used in this study were provided by the China Agricultural University and are freely available for research purposes, following institutional, national and international guidelines.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used and/or analysed during the current study has been included within supplemental data. The plant materials are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

hpi	hours post inoculation;
bHLH	basic/helix–loop–helix;
Ka/Ks	Ratios of non-synonymous to synonymous mutation frequencies;
ABA	abscisic acid;
VIGS	virus-induced gene silencing;
HMM	Hidden Markov Model;
WGD	whole-genome duplication;
Ka	non-synonymous mutation frequency;
Ks	synonymous mutation frequency;
NJ	neighbour-joining method;
TAIR	The Arabidopsis Information Resources;
TRV2	tobacco rattle virus;
NCBI	National Center for Biotechnology Information;
RPKM	reads per kb per million reads.

References

1. Dou, L.; Zhang, X.; Pang, C.; Song, M.; Wei, H.; Fan, S.; Yu, S. Genome-wide analysis of the WRKY gene family in cotton. *Mol. Genet. Genom.* **2014**, *289*, 1103–1121. [CrossRef] [PubMed]
2. Cao, Y.; Han, Y.; Li, D.; Lin, Y.; Cai, Y. MYB Transcription Factors in Chinese Pear (*Pyrus bretschneideri* Rehd.): Genome-Wide Identification, Classification, and Expression Profiling during Fruit Development. *Front. Plant Sci.* **2016**, *7*, 577. [CrossRef] [PubMed]
3. Murre, C.; McCaw, P.S.; Vaessin, H.; Caudy, M.; Jan, L.; Jan, Y.; Cabrera, C.V.; Buskin, J.N.; Hauschka, S.D.; Lassar, A.B.; et al. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **1989**, *58*, 537–544. [CrossRef]
4. Ledent, V.; Vervoort, M. The basic helix-loop-helix protein family: Comparative genomics and phylogenetic analysis. *Genome Res.* **2001**, *11*, 754–770. [CrossRef] [PubMed]
5. Toledo-Ortiz, G.; Huq, E.; Quail, P.H. The Arabidopsis basic/helix-loop-helix transcription factor family. *Plant Cell* **2003**, *15*, 1749–1770. [CrossRef]
6. Skinner, M.K.; Rawls, A.; Wilson-Rawls, J.; Roalson, E.H. Basic helix-loop-helix transcription factor gene family phylogenetics and nomenclature. *Differentiation* **2010**, *80*, 1–8. [CrossRef]
7. Mateo, J.L.; Berg, D.L.v.D.; Haeussler, M.; Drechsel, D.; Gaber, Z.B.; Castro, D.S.; Robson, P.; Lu, Q.R.; Crawford, G.E.; Flicek, P.; et al. Characterization of the neural stem cell gene regulatory network identifies OLIG2 as a multifunctional regulator of self-renewal. *Genome Res.* **2015**, *25*, 41–56. [CrossRef]
8. Imayoshi, I.; Kageyama, R. bHLH factors in self-renewal, multipotency, and fate choice of neural progenitor cells. *Neuron* **2014**, *82*, 9–23. [CrossRef] [PubMed]
9. Ellenberger, T.; Fass, D.; Arnaud, M.; Harrison, S.C. Crystal structure of transcription factor E47: E-box recognition by a basic region helix-loop-helix dimer. *Genes Dev.* **1994**, *8*, 970–980. [CrossRef]
10. Nesi, N.; Debeaujon, I.; Jond, C.; Pelletier, G.; Caboche, M.; Lepiniec, L. The TT8 gene encodes a basic helix-loop-helix domain protein required for expression of DFR and BAN genes in Arabidopsis siliques. *Plant Cell* **2000**, *12*, 1863–1878. [CrossRef]
11. Massari, M.E.; Murre, C. Helix-loop-helix proteins: Regulators of transcription in eucaryotic organisms. *Mol. Cell. Biol.* **2000**, *20*, 429–440. [CrossRef] [PubMed]
12. Wang, F.; Lin, R.; Feng, J.; Qiu, D.; Chen, W.; Xu, S. Wheat bHLH transcription factor gene, TabHLH060, enhances susceptibility of transgenic Arabidopsis thaliana to Pseudomonas syringae. *Physiol. Mol. Plant Pathol.* **2015**, *90*, 123–130. [CrossRef]
13. Wang, J.; Hu, Z.; Zhao, T.; Yang, Y.; Chen, T.; Yang, M.; Yu, W.; Zhang, B. Genome-wide analysis of bHLH transcription factor and involvement in the infection by yellow leaf curl virus in tomato (*Solanum lycopersicum*). *BMC Genom.* **2015**, *16*, 39. [CrossRef] [PubMed]
14. Onohata, T.; Gomi, K. Overexpression of jasmonate-responsive OsbHLH034 in rice results in the induction of bacterial blight resistance via an increase in lignin biosynthesis. *Plant Cell Rep.* **2020**, *39*, 1175–1184. [CrossRef] [PubMed]

15. Zhao, Q.; Fan, Z.; Qiu, L.; Che, Q.; Wang, T.; Li, Y.; Wang, Y. MdbHLH130, an Apple bHLH Transcription Factor, Confers Water Stress Resistance by Regulating Stomatal Closure and ROS Homeostasis in Transgenic Tobacco. *Front. Plant Sci.* **2020**, *11*, 543696. [CrossRef]
16. Ji, X.; Nie, X.; Liu, Y.; Zheng, L.; Zhao, H.; Zhang, B.; Huo, L.; Wang, Y. A bHLH gene from *Tamarix hispida* improves abiotic stress tolerance by enhancing osmotic potential and decreasing reactive oxygen species accumulation. *Tree Physiol.* **2016**, *36*, 193–207. [CrossRef] [PubMed]
17. Liu, W.; Tai, H.; Li, S.; Gao, W.; Zhao, M.; Xie, C.; Li, W. bHLH122 is important for drought and osmotic stress resistance in Arabidopsis and in the repression of ABA catabolism. *New Phytol.* **2014**, *201*, 1192–1204. [CrossRef]
18. Zhang, N.; Hecht, C.; Sun, X.; Fei, Z.; Martin, G.B. Loss of function of the bHLH transcription factor Nrd1 in tomato enhances resistance to Pseudomonas syringae. *Plant Physiol.* **2022**, *190*, 1334–1348. [CrossRef]
19. Liorzou, M.; Pernet, A.; Li, S.; Chastellier, A.; Thouroude, T.; Michel, G.; Malécot, V.; Gaillard, S.; Briée, C.; Foucher, F.; et al. Nineteenth century French rose (*Rosa* sp.) germplasm shows a shift over time from a European to an Asian genetic background. *J. Exp. Bot.* **2016**, *67*, 4711–4725. [CrossRef]
20. Liu, X.; Cao, X.; Shi, S.; Zhao, N.; Li, D.; Fang, P.; Chen, X.; Qi, W.; Zhang, Z. Comparative RNA-Seq analysis reveals a critical role for brassinosteroids in rose (*Rosa hybrida*) petal defense against Botrytis cinerea infection. *BMC Genet.* **2018**, *19*, 62. [CrossRef]
21. Krzywinski, M.; Schein, J.; Birol, I.; Connors, J.; Gascoyne, R.; Horsman, D.; Jones, S.J.; Marra, M.A. Circos: An information aesthetic for comparative genomics. *Genome Res.* **2009**, *19*, 1639–1645. [CrossRef] [PubMed]
22. Li, X.; Yang, R.; Chen, H. The Arabidopsis thaliana Mediator subunit MED8 regulates plant immunity to Botrytis Cinerea through interacting with the basic helix-loop-helix (bHLH) transcription factor FAMA. *PLoS ONE* **2018**, *13*, e0193458. [CrossRef] [PubMed]
23. Lorenzo, O.; Chico, J.M.; Saénchez-Serrano, J.J.; Solano, R. JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. *Plant Cell* **2004**, *16*, 1938–1950. [CrossRef]
24. Huang, H.; Gao, H.; Liu, B.; Fan, M.; Wang, J.; Wang, C.; Tian, H.; Wang, L.; Xie, C.; Wu, D.; et al. bHLH13 Regulates Jasmonate-Mediated Defense Responses and Growth. *Evol. Bioinform.* **2018**, *14*, 1176934318790265. [CrossRef] [PubMed]
25. He, Q.; Lu, H.; Guo, H.; Wang, Y.; Zhao, P.; Li, Y.; Wang, F.; Xu, J.; Mo, X.; Mao, C. OsbHLH6 interacts with OsSPX4 and regulates the phosphate starvation response in rice. *Plant J.* **2021**, *105*, 649–667. [CrossRef] [PubMed]
26. Li, X.; Duan, X.; Jiang, H.; Sun, Y.; Tang, Y.; Yuan, Z.; Guo, J.; Liang, W.; Chen, L.; Yin, J.; et al. Genome-wide analysis of basic/helix-loop-helix transcription factor family in rice and Arabidopsis. *Plant Physiol.* **2006**, *141*, 1167–1184. [CrossRef] [PubMed]
27. Wang, R.; Zhao, P.; Kong, N.; Lu, R.; Pei, Y.; Huang, C.; Ma, H.; Chen, Q. Genome-Wide Identification and Characterization of the Potato bHLH Transcription Factor Family. *Genes* **2018**, *9*, 54. [CrossRef] [PubMed]
28. Zhang, T.; Lv, W.; Zhang, H.; Ma, L.; Li, P.; Ge, L.; Li, G. Genome-wide analysis of the basic Helix-Loop-Helix (bHLH) transcription factor family in maize. *BMC Plant Biol.* **2018**, *18*, 235. [CrossRef]
29. Voorrips, R.E. MapChart: Software for the graphical presentation of linkage maps and QTLs. *J. Hered.* **2002**, *93*, 77–78. [CrossRef]
30. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* **2013**, *30*, 2725–2729. [CrossRef]
31. Chen, C.; Xia, R.; Chen, H.; He, Y. TBtools, a toolkit for biologists integrating various HTS-data handling tools with a user-friendly interface. *bioRxiv* **2018**, *1*, 289660.
32. Wang, Y.; Tang, H.; DeBarry, J.D.; Tan, X.; Li, J.; Wang, X.; Lee, T.-H.; Jin, H.; Marler, B.; Guo, H.; et al. MCScanX: A toolkit for detection and evolutionary analysis of gene synteny and collinearity. *Nucleic Acids Res.* **2012**, *40*, e49. [CrossRef]
33. Wu, L.; Ma, N.; Jia, Y.; Zhang, Y.; Feng, M.; Jiang, C.-Z.; Ma, C.; Gao, J. An Ethylene-Induced Regulatory Module Delays Flower Senescence by Regulating Cytokinin Content. *Plant Physiol.* **2017**, *173*, 853–862. [CrossRef]
34. Liu, Y.; Schiff, M.; Dinesh-Kumar, S.P. Virus-induced gene silencing in tomato. *Plant J.* **2002**, *31*, 777–786. [CrossRef]