RESEARCH ARTICLE

Genome-wide identification of the *Capsicum* bHLH transcription factor family: discovery of a candidate regulator involved in the regulation of species-specific bioactive metabolites

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Abstract

Background: The basic helix–loop–helix (bHLH) transcription factors (TFs) serve crucial roles in regulating plant growth and development and typically participate in biological processes by interacting with other TFs. Capsorubin and capsaicinoids are found only in *Capsicum*, which has high nutritional and economic value. However, whether bHLH family genes regulate capsorubin and capsaicinoid biosynthesis and participate in these processes by interacting with other TFs remains unknown.

Results: In this study, a total of 107 CabHLHs were identified from the *Capsicum annuum* genome. Phylogenetic tree analysis revealed that these CabHLH proteins were classified into 15 groups by comparing the CabHLH proteins with *Arabidopsis thaliana* bHLH proteins. The analysis showed that the expression profiles of *CabHLH009*, *CabHLH032*, *CabHLH048*, *CabHLH095* and *CabHLH100* found in clusters C1, C2, and C3 were similar to the profile of carotenoid biosynthesis in pericarp, including zeaxanthin, lutein and capsorubin, whereas the expression profiles of *CabHLH007*, *CabHLH009*, *CabHLH026, CabHLH063* and *CabHLH086* found in clusters L5, L6 and L9 were consistent with the profile of capsaicinoid accumulation in the placenta. Moreover, *CabHLH007*, *CabHLH009*, *CabHLH026* and *CabHLH086* also might be involved in temperature-mediated capsaicinoid biosynthesis. Yeast two-hybrid (Y2H) assays demonstrated that *CabHLH007*, *CabHLH009*, *CabHLH026, CabHLH063* and *CabHLH086* could interact with MYB31, a master regulator of capsaicinoid biosynthesis.

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Conclusions: The comprehensive and systematic analysis of CabHLH TFs provides useful information that contributes to further investigation of CabHLHs in carotenoid and capsaicinoid biosynthesis.

Keywords: BHLH, Peppers, Carotenoids, Capsaicinoids, Temperature, Yeast two-hybrid assays

Background

Peppers (*Capsicum* spp.), which include sweet and hot chili varieties, are important economic crops in the world [1]. Five domesticated *Capsicum* species, namely, *Capsicum annuum*, *Capsicum baccatum*, *Capsicum chinense*, *Capsicum frutescens* and *Capsicum pubescens*, corresponding to a total of 38.1 million tons of hot peppers, are consumed by three-quarters of the world's population (FAO, see www.fao.org) [1]. Capsorubin and capsanthin can be described as carotenoids and are exclusively biosynthesized in peppers. Peppers are beneficial for preventing various diseases, such as eye diseases, cardiovascular diseases and certain cancers [2–4], and provide excellent natural colourants [5]. The accumulation of carotenoid pigments results in colour variations in the ripe pepper fruit. The carotenoids biosynthetic pathway is currently known (Figure S1A) [6, 7]. Capsorubin or capsanthin is transformed by geranylgeranyl pyrophosphate (GGPP) in a series of enzymatic reactions with phytoene synthase (PSY), phytoene desaturase (PDS), lycopene β-cyclase (LCYB), etc. [6, 7]. Moreover, capsaicinoids, the alkaloids specific to *Capsicum*, are responsible for pungency. Capsaicinoids are produced in the fruit placenta of hot peppers [8]. Capsaicinoids mainly contain capsaicin, dihydrocapsaicin and several analogues [9, 10]. Capsaicin and dihydrocapsaicin comprise approximately 91% of the total capsaicinoid content in *Capsicum* species [11]. Numerous studies have demonstrated that capsaicinoids are produced after the condensation of phenylalanine and in fatty acid chain biosynthetic pathways (Figure S1B) [1, 12, 13].

The bHLH family is one of the largest transcription factors (TFs) in plants and is important for plant growth and development [14, 15]. bHLH proteins were originally found in eukaryotic species [14] and contain a basic and a helix–loop–helix (HLH) region. The two regions possess DNA-binding and protein-interacting abilities [16]. The basic region contains 13–17 basic amino acids in the N-terminal domain and provides a DNA-binding region to bind to consensus hexanucleotide E-box (CANNTG) [17, 18], whereas the HLH region includes approximately 40 amino acids in the C-terminal domain with two alpha helixes separated by a loop of variable length [16, 19]. Furthermore, the HLH region promotes the interaction with other bHLH proteins and the formation of homodimers and heterodimeric complexes [18]. Except for the two conserved regions, the bHLH protein sequences are dissimilar, revealing protein evolution [20].

The bHLH TFs have been identified in the majority of plants, including *Arabidopsis thaliana*, rice, and potato [15, 21–30]. WRKY, NAC and APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) TF families have been analysed systematically in peppers [31–34]. The bHLH TFs are closely related to the primary and specialized metabolites of the plant. PHYTOCHROME INTERACTING FACTOR 3 (PIF3), a bHLH TF, positively regulates anthocyanin biosynthesis by activating the transcription of anthocyanin biosynthetic genes in *Arabidopsis thaliana* [35]. SlAN1 and SlPIF1a regulate anthocyanin and carotenoid biosynthesis in tomato, whereas SlPRE2 negatively regulates pigment accumulation in fruit [36–38]. The bHLH proteins typically regulate secondary metabolite biosynthesis by interacting with TFs such as MYB, WD40-repeat (WDR), and ETHYLENE RESPONSIVE FACTOR (ERF) [39–43]. bHLH TFs physically interact with MYB proteins and facilitate their regulation of anthocyanin biosynthesis in plants. PacMYBA physically interacts with several anthocyanin-related bHLH TFs controlling anthocyanin biosynthesis in sweet cherry by activating the promoters of *PacDFR*, *PacANS* and *PacUFGT* [39]. AtTT8 interacts with MYB proteins PRODUCTION OF ANTHOCYANIN PIGMENT1/ PRODUCTION OF ANTHOCYANIN PIGMENT (PAP1/PAP) and TRANSPARENT TESTA2 (TT2) to regulate anthocyanin and proanthocyanidin biosynthesis in *Arabidopsis thaliana* [44, 45]. Rosea1 (ROS1, an MYB type) and Delila (DEL, a bHLH type) collectively regulate anthocyanin biosynthesis in tomato [46]. In peppers, CaMYC combined with CaMYB and CaWD40 regulates anthocyanin biosynthesis through the regulation of the transcription of a synthetic gene [40]. The physical interaction and regulatory synergy between MYB and bHLH TFs are mediated by the R3 domain in MYB proteins and the N-terminal region in bHLH proteins [45, 47–49]. Additionally, bHLH-mediated ERF TFs regulate synthesis of alkaloids, such as terpenoid indoles alkaloids [42], glycoside alkaloids [41], and nicotine [43]. Capsorubin and capsaicinoids are strictly biosynthesized in *Capsicum*, which possesses high economic and nutritional value. Our previous studies confirmed that MYB31, MYB108 and MYB48 were involved in capsaicinoid biosynthesis [1, 12, 50, 51]. Specifically, natural variations of the

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master regulator MYB31 promoter can affect capsaicinoid contents among different *Capsicum* species [1, 50]. However, whether bHLH TFs regulate capsorubin and capsaicinoid biosynthesis in peppers and orchestrate the regulation of capsaicinoid biosynthesis by interacting with MYB or other TFs needs to be addressed.

In this study, bHLH family genes were identified in the *Capsicum annuum* genome. A reference genome of *C. annuum* was sequenced ($2n = 2x = 24$), and its genome size was estimated to be 3.48 Gb by 19-mer analysis [52]. Characteristic analysis of bHLH family members was systematically performed for better understanding the potential biological function of candidate bHLH TFs in regulating species-specific metabolite biosynthesis in peppers. Capsaicinoids biosynthesis is affected by environmental factors, such as water, temperature and light [53]. Higher temperatures are beneficial to the accumulation of total capsaicinoids [53]. bHLH TFs respond to temperature in *Camellia sinensis* [26] and *Arabidopsis thaliana* [54]. Thus, the candidate bHLH TFs were also studied in response to different temperatures. Moreover, the interaction between bHLH TFs and MYB31 was analysed by Y2H. This study provides candidate bHLH TFs in carotenoid and capsaicinoid biosynthetic pathways in peppers.

Results
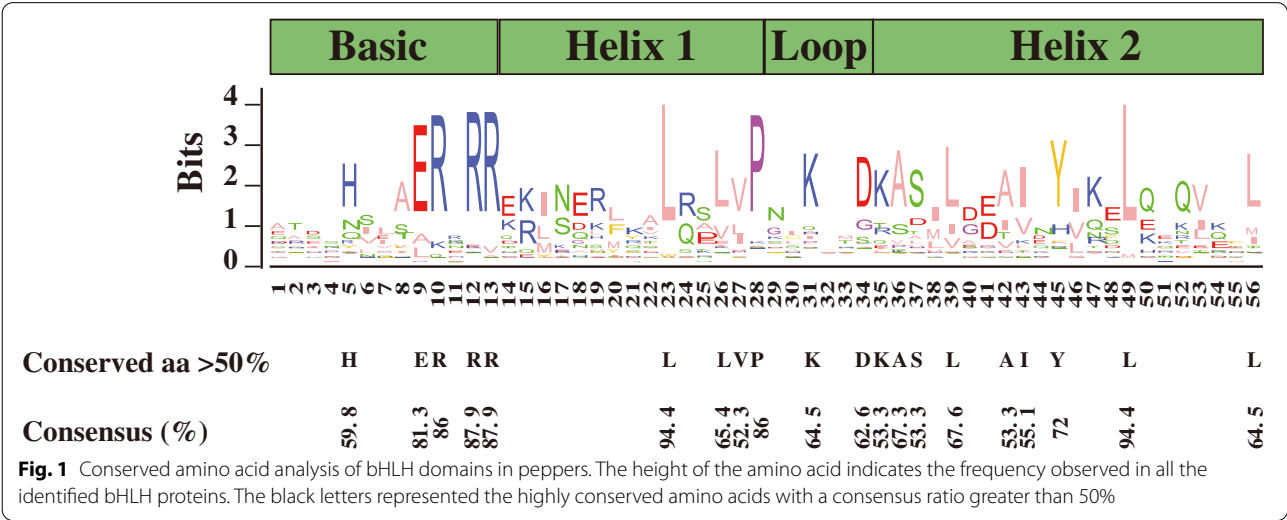### Identification and chromosomal localizations of *bHLHs* in pepper
After excluding redundant sequences, a total of 107 bHLH proteins were identified from the *C. annuum* genome using the Hidden Markov Model (HMM) profile of the HLH domain (PF00010). As shown in Table S1, all identified CabHLH proteins encoded 190–940 amino

residues. The molecular weight (Mw) of these proteins ranged from 21.61 kDa to 106.70 kDa, and the theoretical pI ranged from 4.60 to 9.91.

The 107 *CabHLHs* were renamed as *CabHLH001* to *CabHLH107* and mapped to the pepper chromosomes based on the chromosomal positions (Figure S2). They were distributed on 12 chromosomes. Chromosome 01 contained the largest number of *CabHLH* members (18). Chromosomes 05 and 09 included three *CabHLH* members, separately. However, the genes ranging from *CabHLH092* to *CabHLH107* were not located on any chromosomes. Genes located in some scaffolds were not assembled to chromosomes due to sequencing and assembly technology limitations.

Conserved amino acid residues in the bHLH domain
The amino acid sequences of the bHLH domain were used to perform multiple alignment analyses (Figure S3). The results indicated that bHLH family proteins possessed a conserved bHLH domain, which contained the basic, first helix, loop and second helix regions. As shown in Fig. 1, 20 amino acid residues were conserved with a consensus ratio greater than 50%, and six amino acid residues were conserved with a consensus ratio greater than 75% among the conserved bHLH domains. Five residues (His-5, Glu-9, Arg-10, Arg-12 and Arg-13) were conserved in the basic region. Four residues (Leu-23, Leu-26, Val-27 and Pro-28) were conserved in the first helix region. Lys-31 and Asp-34 were conserved in the loop region, and nine residues (Lys-35, Ala-36, Ser-37, Leu-39, Ala-42, Ile-43, Tyr-45, leu-49 and leu-56) were conserved in the second helix region. The residues Leu-23 and Leu-49 were extremely conserved among the 107 bHLH proteins in pepper.

Fig. 1 Conserved amino acid analysis of bHLH domains in peppers. The height of the amino acid indicates the frequency observed in all the identified bHLH proteins. The black letters represented the highly conserved amino acids with a consensus ratio greater than 50%

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Phylogenetic analysis of the bHLH family proteins

To classify the CabHLH proteins, a phylogenetic tree that contained all of the identified bHLH protein sequences in peppers and those in *Arabidopsis thaliana* was constructed with the neighbour-joining method (Fig. 2). According to the classification of AtbHLHs in a previous study [21], the CabHLH proteins were divided into 15 subfamilies and named groups I to XII. Group II contained the largest numbers of CabHLHs (25) and AtbHLHs (13), whereas group VII contained only one CabHLH and three AtbHLH proteins. The different number of CabHLHs and AtbHLHs proteins in the same group might arise from unequal duplication of the bHLH family during the plant's evolutionary process.

Members of the same group might possess similar biological functions. To preliminarily speculate on the biological functions of CabHLHs, another neighbour-joining phylogenetic tree was constructed using all bHLH proteins in *Arabidopsis thaliana*, tomato, rice and pepper

(Figure S4). The functional characteristics of AtbHLHs and SlbHLHs have been reported and summarized in the literature, and these functional characteristics were used to evaluate the potential function of CabHLH in the same group (Table S2). SlPIF1a included in group VI could regulate carotenoid biosynthesis by a light-dependent mechanism in tomato [38]. SlPIF1a mapped to *CabHLH051* in peppers and was also classified into group VI. These results indicated that the members of group VI might be involved in carotenoid biosynthesis.

Analysis of conserved motifs in CabHLH proteins

To investigate the structural features of CabHLH proteins, the conserved amino acid motifs were analysed and identified using the Multiple EM for Motif Elicitation (MEME) Suite. A total of 15 conserved motifs containing 21–100 residues were found in motifs 1 to 15. The motifs information is provided in Table S3. Motifs 1 and 2 were located in the bHLH domain region, which appeared in
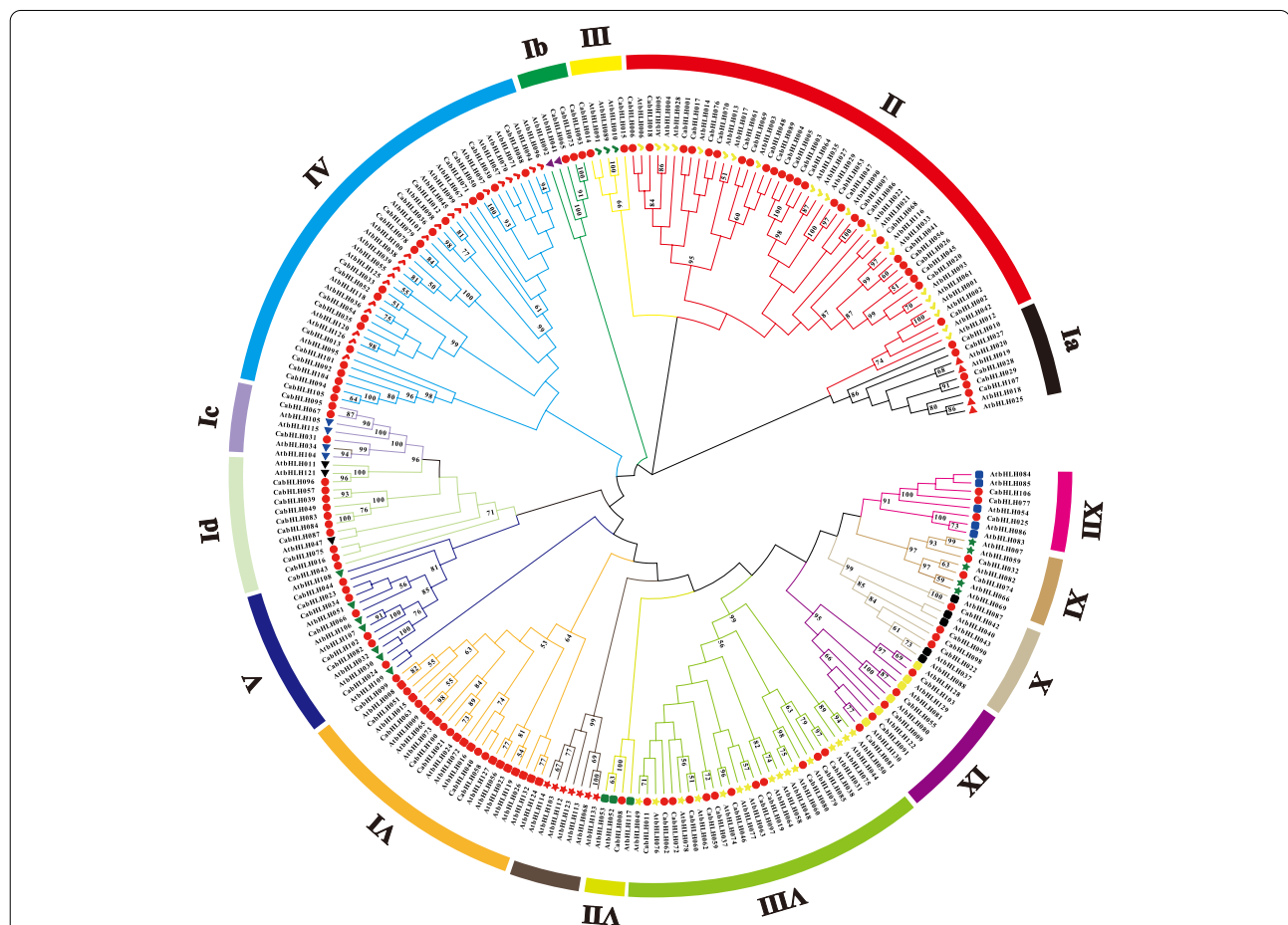

Fig. 2 The phylogenetic tree of the bHLH family members in *Capsicum* and *Arabidopsis thaliana*. The differently coloured branches indicated different subgroups. Red circles represented CabHLH proteins. Different colours and shapes represented different groups of AtbHLH proteins identified in a previous study [21]. The brown branch indicated the absence of CabHLH proteins

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all proteins. Motifs 3 to 15 were distributed outside the bHLH domain region. Motifs 6, 7, 8 and 11 were primarily restricted to group II. Motifs 9, 12 and 15 were specifically identified within group IV. Motifs 10 and 13 were found in group Id, and motif 14 was observed in group V. Generally, most of the same group's proteins possessed common motifs in terms of alignment and position (Fig. 3).

Expression profiles of *CabHLHs* at different developmental stages in pericarp and placenta

Capsorubin and capsaicinoids are found in pepper fruit and closely associated with the biosynthetic genes transcriptional level during developmental stages. To obtain further insight into the potential functions of CabHLHs during capsorubin and capsaicinoid biosynthesis, the expression profiles of *CabHLHs* in different developmental pericarp and placenta were investigated. RNA-Seq raw data were obtained from Kim et al. [52] and included 6, 16, 25, 36, 38, 43 and 48 days post-anthesis (DPA) stages (Figs. 4 and 5). All the raw reads were spliced and remapped to version 2.0 of the *C. annuum* genome.

As shown in Fig. 4b, the expression levels of capsorubin biosynthetic genes gradually increased at 36 DPA, which was consistent with the accumulation profile of capsorubin in pericarp tissue. A total of 20 expressed *CabHLH* genes were not detectable. These genes were probably transcribed at a low level in different developmental pericarp stage. According to the similarity of the expression profiles, all *CabHLH* expression profiles in different developmental pericarp stages were hierarchically clustered and classified into 10 clusters (Fig. 4a). The expression profiles of *CabHLHs* in clusters C1 to C4 maintained good agreement with the expression profiles of capsorubin biosynthetic genes. The members of clusters C1 to C4 might be associated with capsorubin biosynthesis.

Capsaicinoids were abundantly produced from 13 to 25 DPA in the placenta's developmental stages, and the expression levels of capsaicinoid biosynthetic genes were high during this stage (Fig. 5b). Based on the similarity of the expression profiles, the expression of all the CabHLHs in different developmental placenta stages were hierarchically clustered into 10 clusters (Fig. 5a). A total of 16 genes were expressed at a low level that could not be detected. The expression profiles of *CabHLHs* in clusters L5, L6, L8 and L9 were similar to the expression profiles of capsaicinoid biosynthetic genes. Therefore, these results indicated that the members of clusters L5, L6, L8 and L9 might be associated with capsaicinoids biosynthesis.

Additionally, capsorubin and capsaicinoids are produced mainly in the pericarp and placental tissues of peppers. To confirm whether *CabHLHs* were specifically expressed in pericarp and placental tissues, the expression profiles of all identified CabHLHs in different tissues, including the leaf, root, stem, pericarp and placenta, were investigated. However, the RNA-Seq raw data uploaded by Kim et al. did not contain leaf, root or stem tissues from peppers [52]. The RPKM values of these tissues, which were mapped to version 1.5 of the *C. annuum* genome, were directly published online. The heat map indicated that *CabHLHs* were not specifically expressed in certain tissues (Figure S5). Presumably, these TFs orchestrated functions in addition to regulating capsorubin and capsaicinoid biosynthesis.

Validation of candidate bHLH genes involved in capsorubin and capsaicinoids biosynthesis

To further verify the expression profiles of *CabHLHs* in the pericarp and placenta, ten *CabHLHs* from candidate clusters that might be related to capsorubin and capsaicinoid biosynthesis were selected for qRT-PCR analysis. The selected genes were relatively highly expressed in different developmental pericarp or placental tissues. As shown in Fig. 6a, the contents of zeaxanthin and capsorubin increased from the MG stage in pericarp tissue, whereas the lutein content from the branch of the non-synthetic capsorubin decreased. No significant difference in β-carotene content was noted in different developmental pericarp tissues. *CabHLH032*, *CabHLH048*, *CabHLH095* and *CabHLH100* expression was consistent with the accumulation profile of carotenoids (zeaxanthin and capsorubin) in pericarp, whereas *CabHLH009* expression was similar to the accumulation profile of lutein in pericarp. However, these genes were also highly expressed in other tissues (roots, flowers, stems, placentas, leaves and seeds) (Fig. 6b). Thus, it was likely that the members of clusters C1, C2 and C3 were associated with capsorubin biosynthesis but also possessed other specific functions in other tissues.

The capsaicin and dihydrocapsaicin were initially produced at 10 DPA, peaked at 25 DPA in placental tissue, and then gradually decreased (Fig. 7a). *CabHLH007*, *CabHLH009*, *CabHLH026*, *CabHLH063* and *CabHLH086* expression resembled the accumulation profile of capsaicin in different developmental placenta stages. A high expression level of *CabHLH026* was observed in pericarp, seed and placental tissues, and high expression of *CabHLH063* was evident in stem and leaf. *CabHLH007*, *CabHLH009* and *CabHLH086* were highly expressed in certain tissues (Fig. 7b).
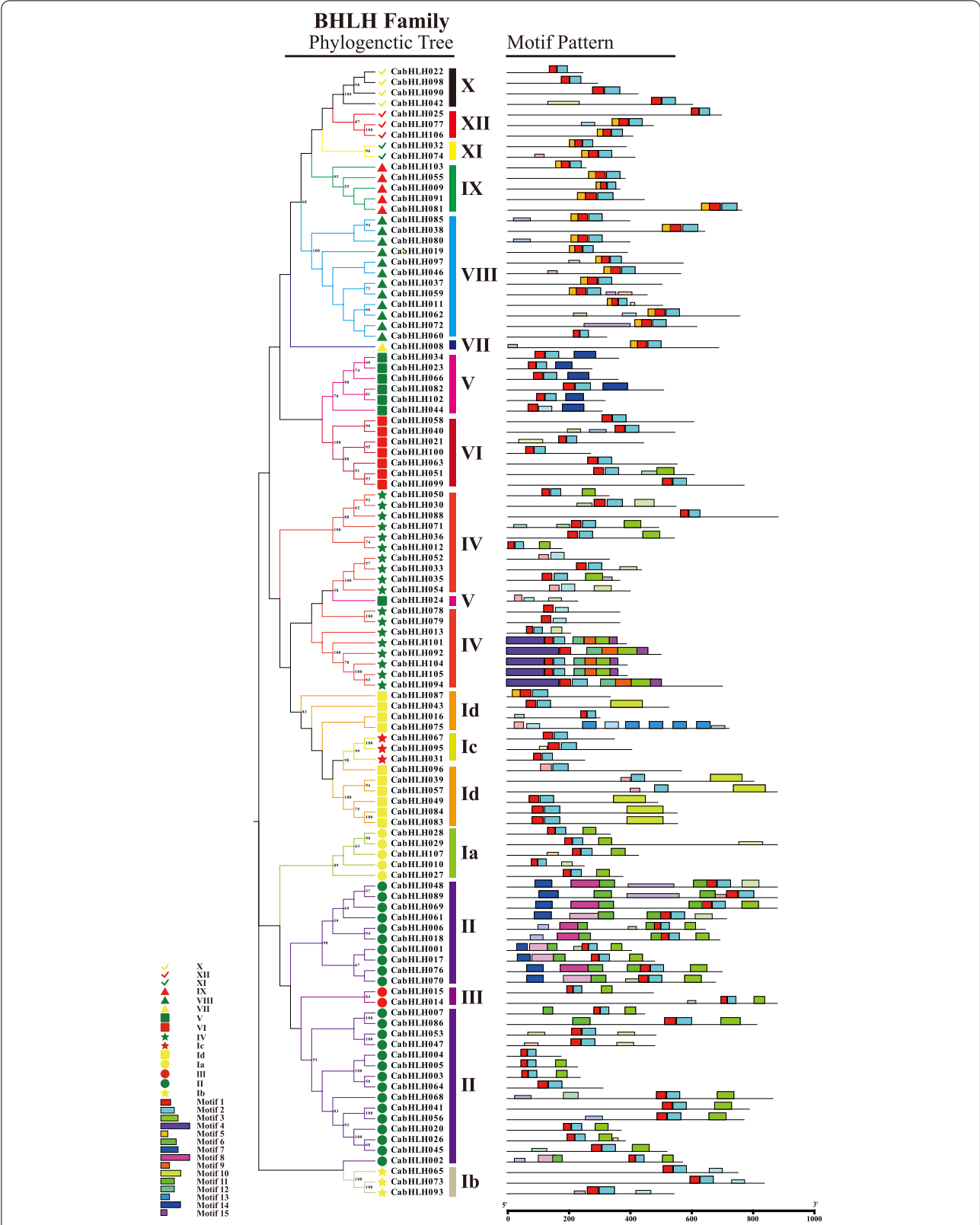
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Fig. 3 Phylogenetic tree and conserved motif distributions of CabHLH proteins. The neighbour-joining phylogenetic tree was constructed with MEGA-X. Different coloured boxes represent different motifs. Box length represents motif length. Differently coloured shapes indicate different groups in the phylogenetic tree of CabHLHs

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Therefore, *CabHLH007*, *CabHLH009*, *CabHLH026*, *CabHLH063* and *CabHLH086* in clusters L5, L6 and L9 might be associated with capsaicinoid biosynthesis.

The expression of candidate *CabHLHs* associated with capsaicinoid biosynthesis in response to different temperatures

To obtain a preliminary understanding of whether capsaicinoid biosynthesis is regulated by *CabHLH* genes in response to different temperatures, the expression of five candidate *CabHLHs* and five important capsaicinoid biosynthetic genes at different temperatures was measured. As shown in Fig. 8a, the capsaicin and dihydrocapsaicin content tended to increase with increasing temperature from T15 to T25. The placenta capsaicin content with T25 treatment was significantly increased compared with that in peppers with T33 treatment. *CabHLH007*, *CabHLH009* and *CabHLH086* expression increased with increasing temperatures, which was similar to the accumulation of dihydrocapsaicin (Fig. 8b). *CabHLH026* was highly expressed in T25, which was consistent with the accumulation of capsaicin and the expression of capsaicinoid biosynthetic genes (*AT3*, *AMT*, *BCKDH* and *KasIa*) (Fig. 8b; c). In contrast, *CabHLH063* expression decreased with increasing temperature, which maintained consistency with the expression profile of the capsaicinoid biosynthetic gene *Acl* (Fig. 8b; c). Thus, *CabHLH007*, *CabHLH009* and *CabHLH086* expression was positively associated with an increase in dihydrocapsaicin content and temperature. *CabHLH063* expression was negatively related to the increase in the capsaicin content and temperature, whereas *CabHLH026* expression was positively related to these factors. These candidate genes might be related to capsaicinoid biosynthesis in response to different temperatures by regulating the transcription of capsaicinoid biosynthetic genes.

The interaction of candidate CabHLHs and MYB31 in yeast and identified bHLH binding sites

bHLH proteins typically bind to E-box binding sites of gene promoter and regulate the transcription. To characterize the potential association of bHLH TFs and the pathway biosynthetic genes, one thousand five hundred base pair nucleotide sequences upstream of the start codon (ATG) from capsorubin and capsaicin biosynthetic genes, including *CCS*, *PSY*, *β-CH*, *β-LCY*, *Acl*, *AMT*,

AT3, *BACT, BCKDH, COMTa, FatA* and *KasIa*, were analysed using the PlantCARE database [55]. As shown in Table S7, multiple bHLH DNA binding sites were detected in the promoters of capsorubin and capsaicin biosynthetic genes, except for *CCS*, *β-CH* and *KasIa*.
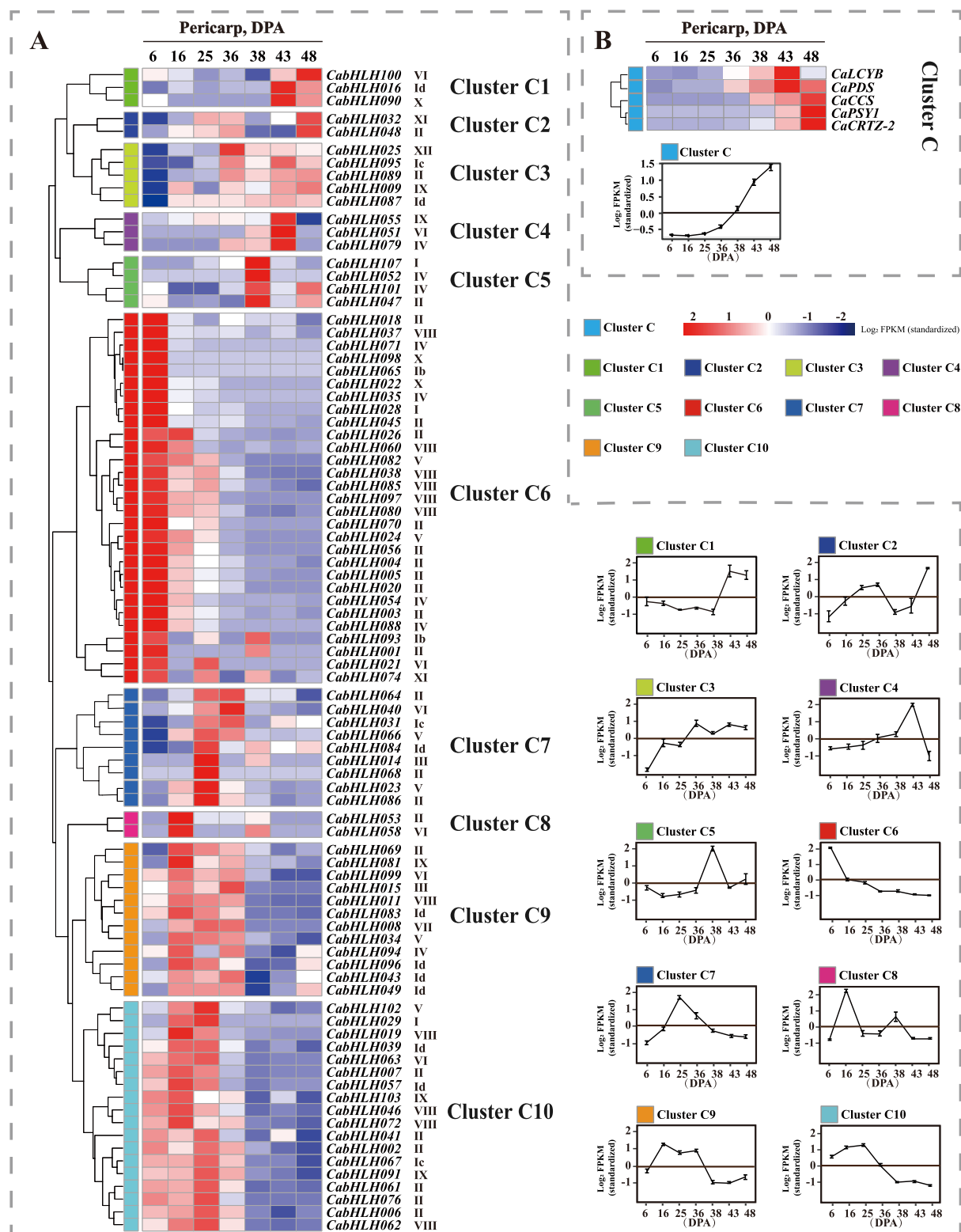
The bHLH protein executes function always by interacting with other TFs, such as MYB. We performed Y2H assay to verify the interaction between candidate Cab-HLHs and CaMYB31. The results indicated that these bHLHs interacted with MYB31 in a gene-dependent manner. CabHLH007, CabHLH009, CabHLH026, Cab-HLH063 and CabHLH086 could interact with MYB31 in a heterologous system. CabHLH026 strongly interacts with CaMYB31 in yeast, whereas only weak interactions were observed in the group of CabHLH063-CaMYB31 and CabHLH086-CaMYB31 (Fig. 9). Therefore, it was likely that CabHLH could regulate capsaicinoid biosynthesis by interacting with CaMYB31, a master regulator of capsaicinoid biosynthesis.

Discussion

The bHLH family has emerged as the second-largest TF family in plants [48]. This family has been successfully identified and investigated in many plants, including *Solanum tuberosum L.* (124) [24], *Camellia sinensis (L.) O. Ktze.* (120) [26], *Solanum lycopersicum* (159) [30], *Zea mays* L. (208) [28], *Brassica rapa pekinensis* (230) [27], *Arabidopsis thaliana* (147) [15], *Glycine max (L)* Merr. (319) [56], *Malus pumila* (188) [57] and *Oryza sativa* L. (167) [22]. In this study, 107 CabHLH genes were identified in the pepper genome. The conserved bHLH domain consisted of the basic, first helix, loop and second helix regions, which contained approximately 60 amino acids [22]; this structure was also observed in peppers (Figure S3). Twenty amino acid residues were conserved with a consensus ratio greater than 50% (Fig. 1), consistent with a previous study [21]. Glu-9, Arg-12 and Arg-13 were conserved with a consensus ratio greater than 80%, whereas Leu-23 and Leu-49 were extremely conserved among 107 CabHLH proteins (Fig. 1). A previous study had confirmed that Glu-13 and Arg-16/Arg-17 (corresponding to Glu-9 and Arg-12/Arg-13 in this study; Fig. 1) played an important role in DNA binding [58]. Leu-27 and Leu-61 (corresponding to Leu-23 and Leu-49 in this study; Fig. 1) are necessary for dimerization [23]. Glu-13 and Arg-16 (corresponding to Glu-9 and Arg-12 in this study; Fig. 1) recognize the E-box

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Fig. 4 Expression profiles of the genes in the pericarp at different developmental stages. **a**. Expression profiles of *CabHLH* genes at different developmental stages of pericarp. **b**. Expression profiles of the capsorubin biosynthetic genes at different developmental stages of pericarp. The name of each gene and the short name of the phylogenetic group were included to the right of the heat map. Log2 values of fragments per kilobase of exon per million fragments mapped (FPKM) were used to construct the heat map based on the hierarchical clustering analysis. Line charts were generated using the mean value for the whole cluster. The letter "C" in cluster C indicates the pericarp

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(5'-CANNTG-3'), whereas the His/Lys-9, Glu-13 and Arg-17 (corresponding to His-5, Glu-9 and Arg-13 in this study; Fig. 1) were required for binding G-Box (5'-CAC GTG-3') [19, 59].

According to a previous study [21], the phylogenetic tree was divided into 15 subgroups (Fig. 2), which was similar to the classification of potato [24]. Motifs 1 and 2 located in the bHLH domain were included in all Cab-HLH proteins (Fig. 3), which was consistent with the observation of MdbHLH proteins [57]. Motifs 1 and 2 containing some conserved amino residues played important roles in DNA binding and protein dimerization [23, 58]. The motifs from 3 to 15 were distributed outside the bHLH domain and randomly arranged in CabHLH proteins (Fig. 3). The results indicated that the members of the same group had the same motif arrangements in most case.

Homologous genes generally possessed a similar function in plant growth and development processes [60]. Some SlbHLHs and AtbHLHs included in groups II and VI were functionally identified (Table S2). MYC2 interacted with MYB TFs to regulate glucosinolate biosynthesis [61] and respond to abscisic acid [62], jasmonic acid [63], and light signalling [64] in *Arabidopsis thaliana*. TRANSPARENT TESTA8 (TT8), GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) combined with MYB and WDR TFs could regulate anthocyanin biosynthesis and trichome development in *Arabidopsis thaliana* [65–67]. These proteins were in group II (Figure S4). Some SlbHLHs in group II were also involved in anthocyanin biosynthesis [36] and trichome development [68] and responded to cold, osmotic and salt stress [69]. Furthermore, MYC2 interacted with ERF TFs to regulate the biosynthesis of alkaloids such as steroidal glycoalkaloids [41], terpenoid indole alkaloids [42], and nicotine biosynthesis [43]. The homologous genes of MYC2 in pepper were mapped to *CabHLH006* and *CabHLH018*, and all of the proteins were classified into group II (Figure S4). These results indicated that group II might be related to secondary metabolites, development and signal response processes in plants. In addition, PIF3 combined with bZIP TFs could regulate anthocyanin biosynthesis [35]. PIFs served as cellular signalling hubs that function by integrating multiple signals to regulate the transcriptional network during plant growth and development [70]. SlPIF1a was included in group VI and regulated carotenoid biosynthesis in tomato [38]. Homologous genes of these PIFs were mapped to *CabHLH063* and *CabHLH051* in pepper, and the proteins were located in group VI. The CabHLH protein members in groups II and VI probably regulated secondary metabolite biosynthesis by interacting with other TFs.

The expression profiles of *CabHLHs* in clusters C1, C2, C3 and C4 maintained good agreement with the expression of capsorubin biosynthetic genes (Fig. 4). The expression profiles of *CabHLH009*, *CabHLH032*, *CabHLH048*, *CabHLH095* and *CabHLH100* from these clusters aligned with the accumulation profile of carotenoids, including zeaxanthin, lutein and capsorubin, in pericarp (Fig. 6). Therefore, it was likely that the members of these clusters were related to capsorubin biosynthesis. The expression profiles of *CabHLHs* in clusters L5, L6, L8 and L9 were similar to the expression profiles of capsaicinoid biosynthetic genes (Fig. 5). *CabHLH007*, *CabHLH009*, *CabHLH026*, *CabHLH063* and *CabHLH086* expression profiles were consistent with the accumulation profile of capsaicinoids in placenta (Fig. 7). These results indicated that the members of clusters L5, L6 and L9 might regulate capsaicinoid biosynthesis. Moreover, the similar transcript levels of the genes indicated that they might perform similar functions in some cases. CaMYC combined with CaMYB and CaWD40 to regulate anthocyanin biosynthesis [40] and was mapped to *CabHLH0016* in cluster C1 (Fig. 4). Numerous studies have reported that MYC2 typically regulated alkaloid biosynthesis by interacting with ERF TFs [41–43], and ERF TFs were associated with capsaicinoid biosynthesis in *Capsicum* [34, 71]. MYC2 was mapped to two homologous genes CabHLH006 and CabHLH018 that were classified into clusters L8 and L7, respectively. The expression profile of cluster L8 maintained good agreement with the profile of capsaicinoid biosynthesis. SlPIF1a was related to carotenoid biosynthesis in tomato [38] and mapped to *CabHLH051* in the pepper, which was classified into cluster C4. The members of cluster C4 were candidate regulators for carotenoid biosynthesis in this study. The members of clusters C1, C2, C3, C4, L5, L6, L8 and L9 were probably related to carotenoid and capsaicinoid biosynthesis.

Environmental factors are essential for secondary metabolite biosynthesis. Previous studies have demonstrated that bHLH TFs were responsive to a heat response in *Camellia sinensis* and *Arabidopsis thaliana* [26, 72]. PIFs acted as a signalling centre that

(See figure on next page.)
Fig. 5 Expression profiles of the genes in the placenta at different developmental stages. **a**. Expression profiles of *CabHLH* genes at different developmental stages of placenta. **b**. Expression profiles of capsaicinoid biosynthetic genes at different developmental stages of placenta. The name of each gene and the short name of the phylogenetic group appear to the right of the heat map. Log2 values of FPKM were used to construct the heat map with hierarchical clustering analysis. Line charts were prepared using the mean value for the whole cluster. The letter "L" in cluster L indicates the placenta
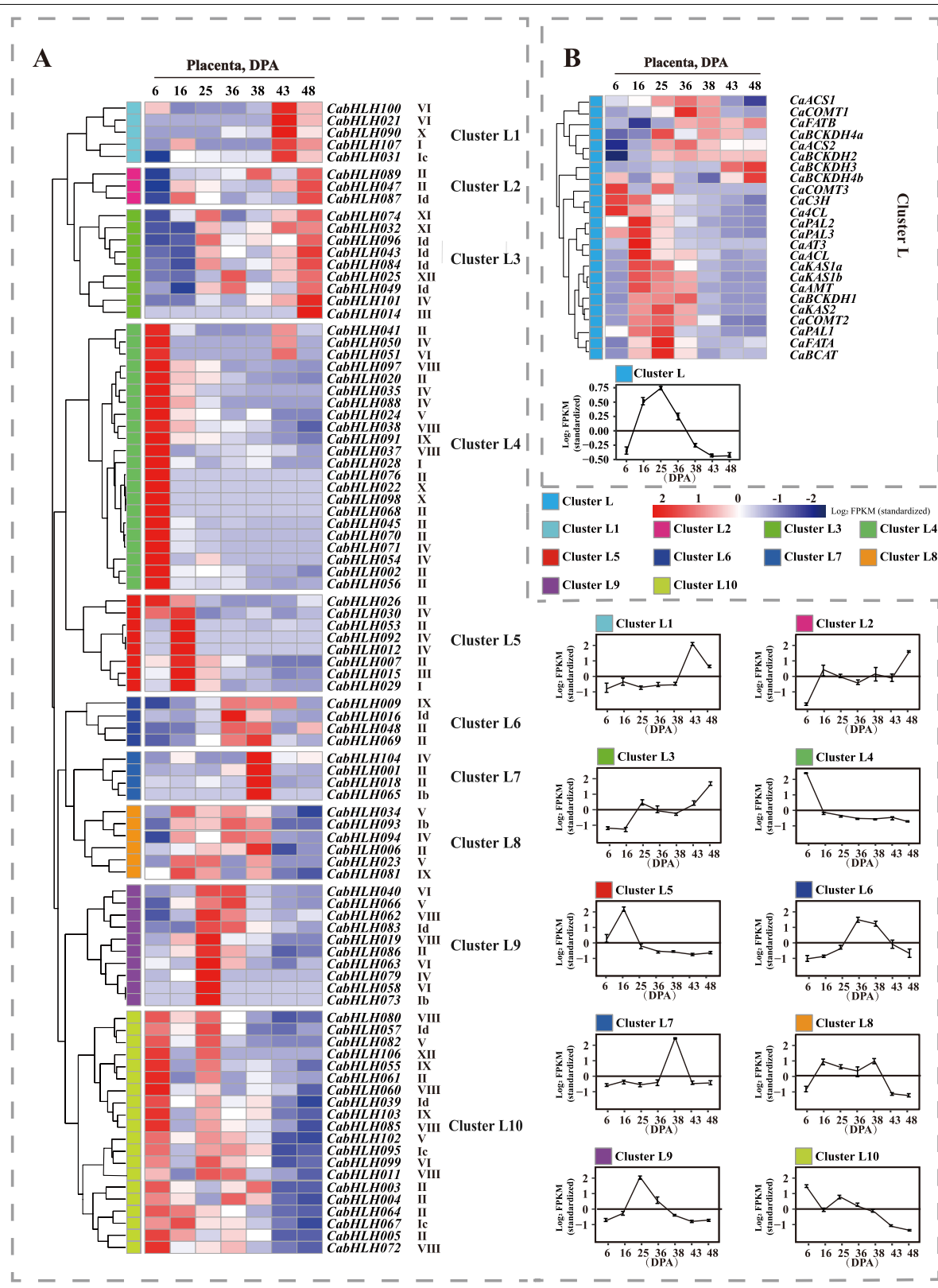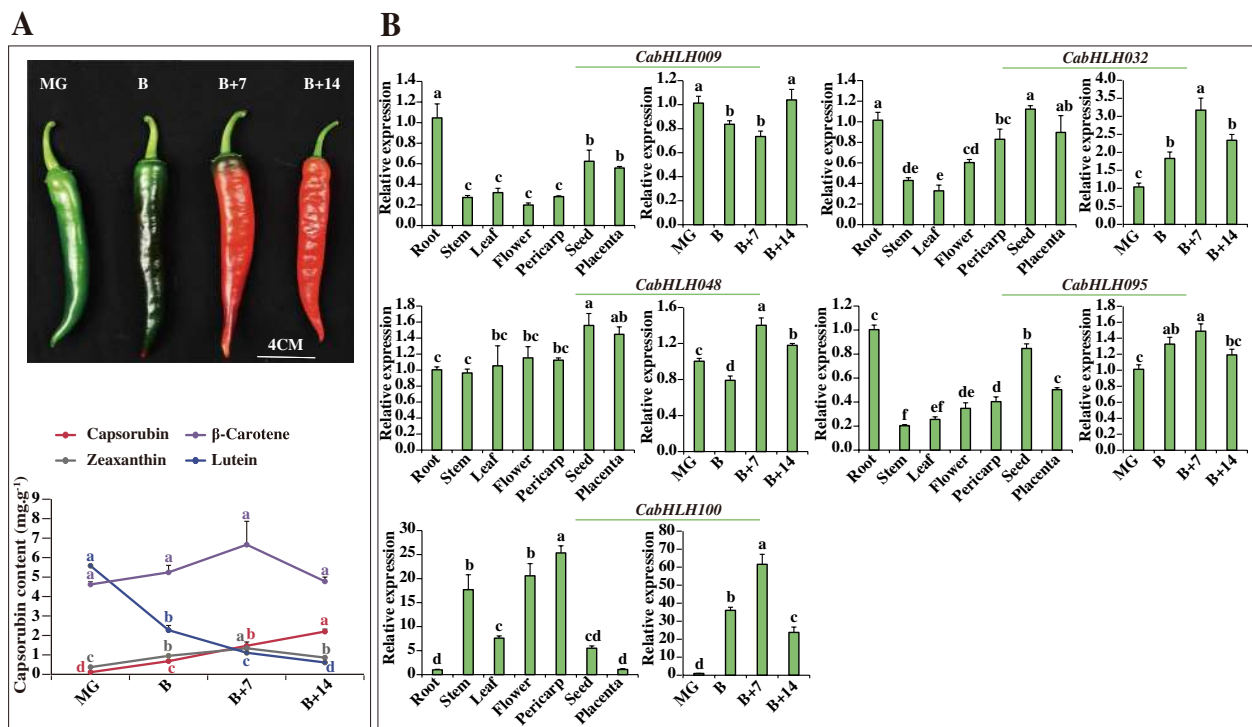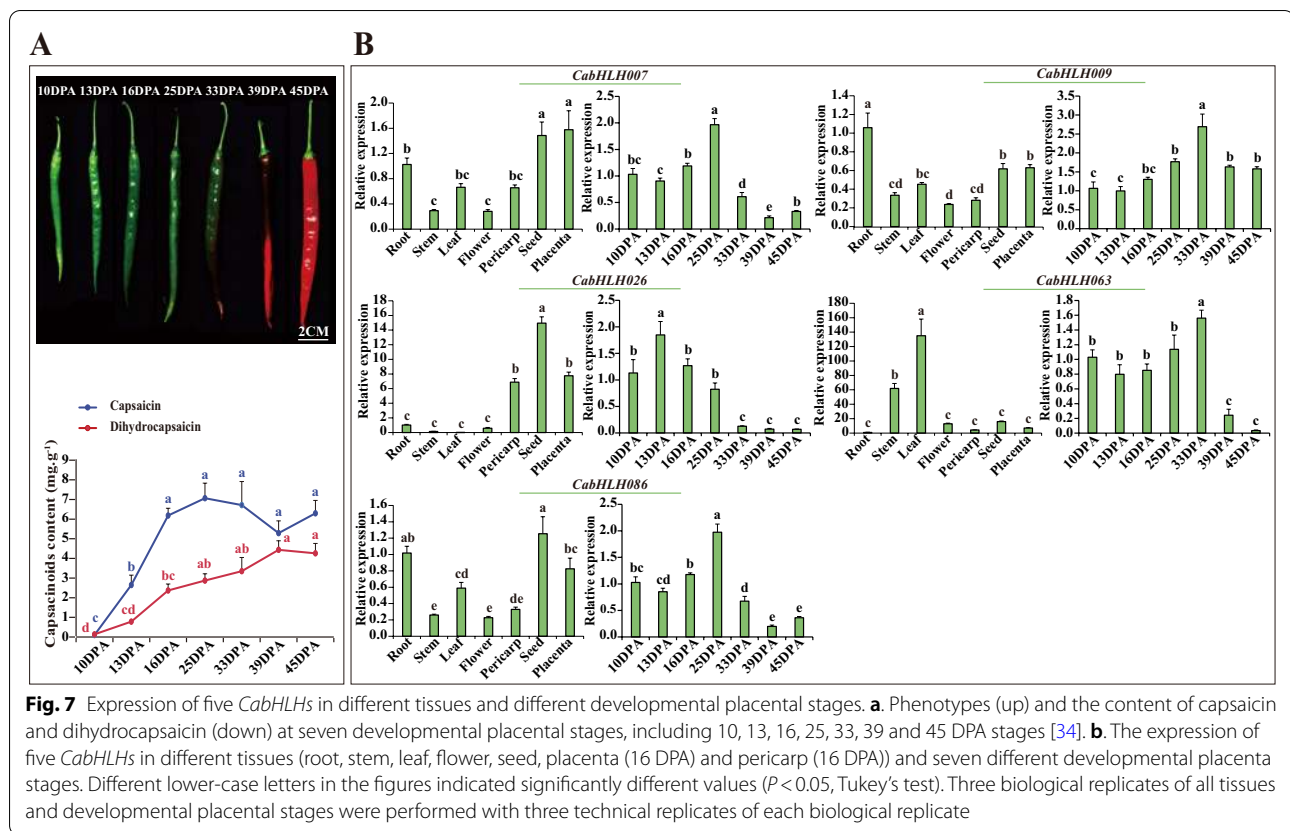
Fig. 6 Expression profiles of five *CabHLHs* in different tissues and developmental stages of pericarp. **a**. Phenotypes and the contents of β-carotene, zeaxanthin. lutein and capsorubin at four developmental pericarp stages, namely, the mature green (MG), breaker (B), breaker plus 7 days (B + 7) and breaker plus 14 days (B + 14) stages [34]. **b**. The expression of five *CabHLHs* in different tissues (stem, root, seed, flower, leaf, pericarp (B) and placenta (16 DPA)) and four different pericarp stages. Different letters in the figures indicate significantly different values (*P* < 0.05, Tukey's test). Three biological replicates of all tissues and developmental pericarp stages were performed with three technical replicates of each biological replicate

integrated multiple signals to regulate plant growth and development, including high temperature and light [70]. The expression of CabHLH063, a homologous gene of PIF, and *Acl* decreased with increasing temperature. The expression of *CabHLH026* and four capsaicinoid biosynthetic genes (i.e., *AT3, AMT, BCKDH* and *KasIa*) peaked in T25, which was similar to capsaicin accumulation (Fig. 8c). These results indicated that the members of clusters L5, L6 and L9 might possess different functions in regulating capsaicinoid biosynthesis in response to different temperatures. In addition, bHLH proteins were typically involved in plant metabolic pathways by interacting with other TFs [39, 41–46]. MYB TFs played a key role in capsaicinoid biosynthesis as evidenced in previous studies [1, 12, 50, 51]. Y2H assays revealed that CabHLH007, CabHLH009, CabHLH026, CabHLH063 and CabHLH086 could interact with MYB31 in a heterologous system. These results implied that CabHLH007, CabHLH009, CabHLH026, CabHLH063 and CabHLH086 might be involved in capsaicinoid biosynthesis by interacting with MYB31.

Conclusions

A total of 107 CabHLH proteins were identified in peppers. These proteins were divided into 15 groups according to the classification of *Arabidopsis thaliana*. The bHLH conserved domain containing motifs 1 and 2 appeared in all CabHLHs. The expression profiles showed that clusters C1, C2, C3, C4, L5, L6, L8 and L9 were candidates for the regulation of carotenoid and capsaicinoid biosynthesis. *CabHLH009, CabHLH032, CabHLH048, CabHLH095, CabHLH100, CabHLH007, CabHLH009, CabHLH026, CabHLH063* and *CabHLH086* were selected from the candidate clusters because they might contribute to carotenoid and capsaicinoid biosynthesis. *CabHLH007, CabHLH009, CabHLH026, CabHLH063* and *CabHLH086* might be responsive to different temperatures to mediate capsaicinoid biosynthesis. CabHLH007, CabHLH009, CabHLH026, CabHLH063 and CabHLH086 likely control capsaicinoid biosynthesis by interacting with MYB31. However, further studies showing how these candidate bHLHs regulate carotenoid and capsaicinoid biosynthesis are required.

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Fig. 7 Expression of five *CabHLHs* in different tissues and different developmental placental stages. **a**. Phenotypes (up) and the content of capsaicin and dihydrocapsaicin (down) at seven developmental placental stages, including 10, 13, 16, 25, 33, 39 and 45 DPA stages [34]. **b**. The expression of five *CabHLHs* in different tissues (root, stem, leaf, flower, seed, placenta (16 DPA) and pericarp (16 DPA)) and seven different developmental placenta stages. Different lower-case letters in the figures indicated significantly different values (*P* < 0.05, Tukey's test). Three biological replicates of all tissues and developmental placental stages were performed with three technical replicates of each biological replicate

Methods

Identification of *bHLHs* in peppers and their chromosomal locations

The bHLH protein sequences were retrieved from *C. annuum* 'CM334' genome [73] using the HMM profile of the HLH domain (PF00010) obtained from the PFAM database [74]. Redundant sequences were filtered with HMMER software [75], SMART database [76] and NCBI Conserved Domain Search Service (CD Search) [77]. The ExPASy server [78] was used to predict the full length of amino acid sequences, MW, PI and instability index of the proteins. The corresponding information is provided in Table S1. The chromosomal locations of gene loci were acquired from version 2.0 of the pepper genome. According to the chromosomal positions, all the identified *CabHLHs* were renamed consecutively.

Multiple alignments and phylogenetic analysis

Multiple sequence alignments were performed using conserved bHLH domain sequences of four plant species, including peppers, rice, tomato and *Arabidopsis thaliana*. The alignments were performed with ClustalX 2.1 with the default parameters. The bHLH protein sequences in *Arabidopsis thaliana* (131) were obtained from The Arabidopsis

Information Resource (TAIR) database [79], whereas the bHLH protein sequences in rice (144) and tomato (145) were acquired from Plant TF Database version 4.0 [80]. All of these bHLH protein sequences were renamed and listed in Table S4. The neighbour-joining phylogenetic trees were generated using MEGA X with 1000 bootstrap replications [81] and visualized using EvolView v3 [82].
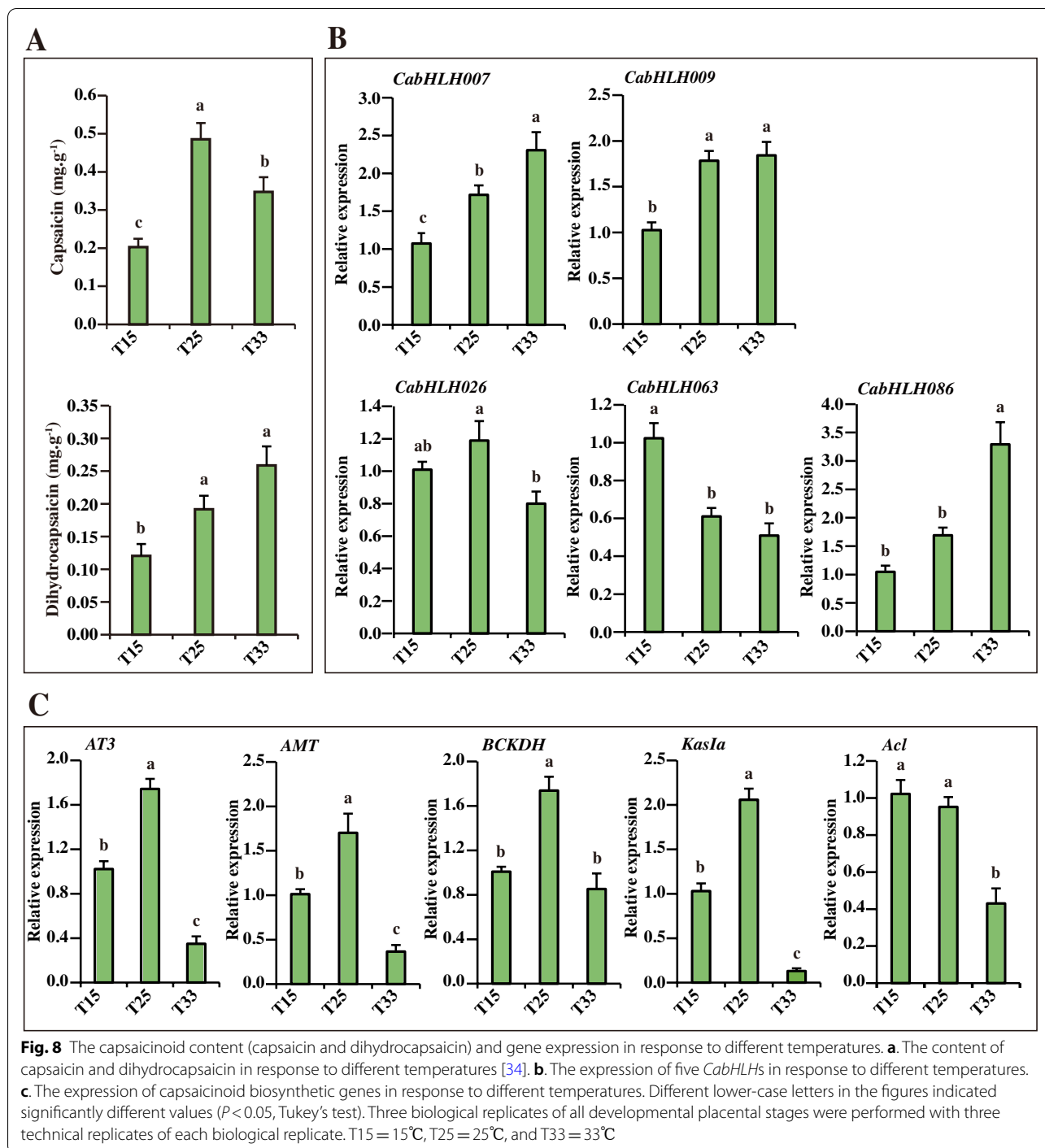
Protein motif analysis

Conserved and functional motifs were analysed using the MEME tool [83] with parameters according to Song et al. [34].

RNA-Seq data and expression profiles of *CabHLHs*

FPKM values of candidate CabHLHs were obtained from the pepper RNA-Seq raw data (GenBank: AYRZ00000000) [84, 85]. Heat maps showing the gene expression profiles in seven different developmental placenta and pericarp tissues (6 DPA to 48 DPA) were drawn by R language.

Plant materials and temperature treatments

The 59 inbred line (*Capsicum annuum*) authenticated by Professor Jianjun Lei (College of Horticulture, South China Agricultural University) was used for the experiments [1]. The variety rights of this breed belong to

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Fig. 8 The capsaicinoid content (capsaicin and dihydrocapsaicin) and gene expression in response to different temperatures. **a**. The content of capsaicin and dihydrocapsaicin in response to different temperatures [34]. **b**. The expression of five *CabHLHs* in response to different temperatures. **c**. The expression of capsaicinoid biosynthetic genes in response to different temperatures. Different lower-case letters in the figures indicated significantly different values (*P* < 0.05, Tukey's test). Three biological replicates of all developmental placental stages were performed with three technical replicates of each biological replicate. T15 = 15℃, T25 = 25℃, and T33 = 33℃

our lab. If somebody needs this inbred line, they can acquire from the corresponding authors. All plant materials used in this study were provided by South China Agricultural University. According to institutional, national and international guidelines, these samples do not require specific permissions for research purposes. The seeds were sown using mixture substrate (peat, perlite and coir pith) in the plastic greenhouse under the following environment conditions: 35℃/23℃(day/night); 12 h/12 h (light/dark cycle), and 300–1000 µmol·m^{-2}·s^{-1} of photosynthetic photon flux density (PPFD) at 12:00 AM. The sample used for RNA extraction was collected according to the methods of our previous study [34].
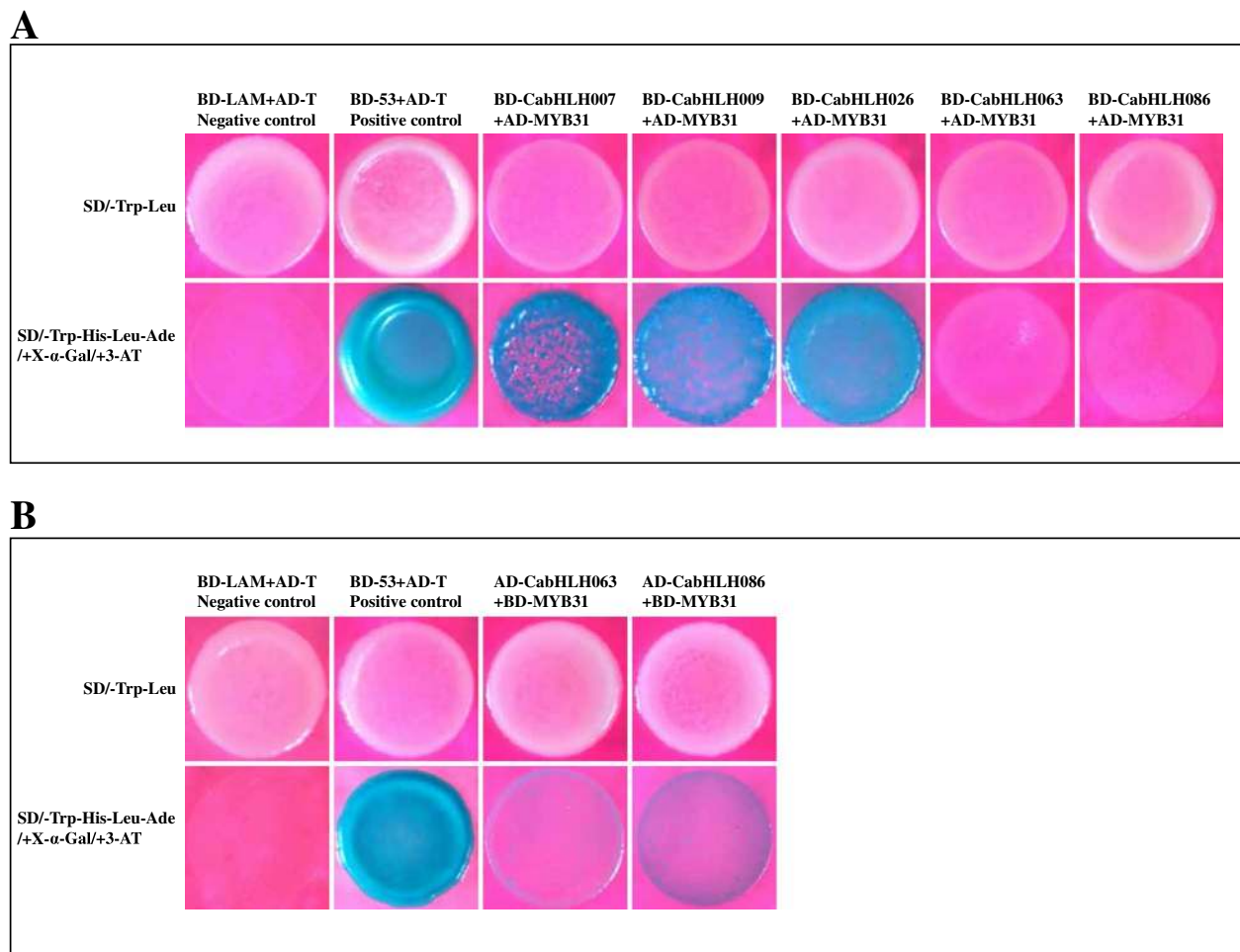
Fig. 9 Yeast two-hybrid assays of the protein interactions between candidate CabHLHs and CaMYB31. **a**. Yeast two-hybrid analysis of protein–protein interactions between CabHLHs and CaMYB31. **b**. Reverse yeast two-hybrid analysis of protein–protein interactions between CabHLHs and CaMYB31. AD and BD represented empty pGADT7 and pGBKT7 vectors. SD/-Trp-Leu, the synthetic dextrose (SD) medium lacked tryptophan and leucine; the SD/-Trp-His-Leu-Ade medium lacked tryptophan, histidine, leucine and adenine

Peppers used for temperature treatment experiment were also cultivated using mixture substrate as described above. The temperature treatment and sample collection were performed as previously described [34]. All the samples were performed with three biological replicates and one technical replicate for each biological replicate.

Quantitative Real-time PCR (qRT-PCR) analysis
The isolation and extraction of total RNA and RNA reverse transcription were performed according to Song et al. [34]. The primers for qPCR were designed using Primer 5.0 (Table S5). Ploy (A)-binding protein (*CA00g52140*) and ubiquitin extension protein (*CA12g20490*) were used as housekeeping genes, given that transcriptome studies revealed that their transcription levels were fairly stable among different tissues and plant different development stages in *Capsicum* [52].

qRT-PCR was performed according to Wang et al. [86]. The relative expression of each *CabHLH* was calculated using the $2^{-\Delta\Delta Ct}$ method [87]. Three technical replicates of each biological replicate and three biological replicates were performed for all samples. The Dunnett's *t*-test was used to determine the results of significant differences by using SPSS 22.

Yeast two-hybrid assay
The full-length cDNAs of *CabHLH007*, *CabHLH009*, *CabHLH026*, *CabHLH063* and *CabHLH086* were cloned into a pMD19-T vector (6013, TaKaRa, China). The amplified full-length fragments of *CabHLH007*, *CabHLH009*, *CabHLH026*, *CabHLH063* and *CabHLH086* were ligated into the pGADT7 and pGBKT7 vectors using one-step cloning (C112, Vazyme, China), separately. Negative and positive plasmids and plasmids

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containing different CabHLH were transformed into the AH109 yeast strain (YC1010, Weidi Biotechnology, China). The Y2H assay was performed according to the manufacturer's instructions (Clontech). Image processing was performed using Adobe Illustrator CS2020. All the primers were listed in Table S6.

Promoter *cis*-element discovery

The basis region of the bHLH protein could bind to E-box (CANNTG). One thousand five hundred base pair nucleotide sequences upstream of the start codon in the biosynthetic genes of capsorubin (*CCS, PSY, β-CH, β-LCY*) and capsaicin (*Acl, AMT, AT3, BACT, BCKDH, CoMTa, FatA* and *KasIa*) were retrieved from *C. annuum* genome and applied to E-box discovery. The *cis*-elements were identified by using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Abbreviations

TFs: Transcription factors; Chr: Chromosome; FPKM: Fragments per kilobase of transcript per million mapped reads; bHLH: Basic helix–loop–helix; HLH: Helix–loop–helix; MEME: Multiple EM for Motif Elicitation; HMM: Hidden Markov model; GGPP: Geranylgeranyl pyrophosphate; PSY: Phytoene synthase; PDS: Phytoene desaturase; LCYB: Lycopene β-cyclase; Pal: Phe ammonia-lyase; COMT: Caffeic acid O-methyltransferase; AT3: A putative acyltransferase; CCS: Capsanthin/capsorubin synthase; CS: Capsaicin synthase; ZDS: ζ-Carotene desaturase; CRTISO: Carotenoids isomerase; LCYE: Lycopene ε-cyclase; CrtZ-2: β-Carotene hydroxylase-2; PAL: Phenylalanine ammonia-lyase; C4H: Cinnamate 4-hydroxylase; 4CL: 4-Coumaroyl-CoA ligase; HCT: Hydroxycinnamoyl transferase; C3H: p-Coumaroyl shikimate/quinate 3-hydroxylase; COMTa: Caffeoyl-CoA 3-O-methyltransferase; HCHL: Hydroxycinnamoyl-CoA hydratase lyase; AMT: Aminotransferase; BCAT: Branched-chain amino acid aminotransferase; Kas: Ketoacyl-ACP synthase; ACL: Acyl carrier protein; FatA: Acyl-ACP thioesterase; DPA: Days post-anthesis; MG: Mature green stage; B: Breaker; B + 7: Breaker plus 7 days; B + 14: Breaker plus 14 days; WDR: WD40-repeat; ERF: Ethylene responsive factor; PAP1/PAP: Production of anthocyanin pigment1/production of anthocyanin pigment; TT2: Transparent testa2; AP2/ERF: Apetala2/ethylene response factor; PIF3: Phytochrome interacting factor 3; TT8: Transparent testa8; GL3: GLABRA3; EGL3: Enhancer of glabra3; Y2H: Yeast two-hybrid assays; PPFD: photosynthetic photon flux density.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-021-03004-7.

Additional file 1: Figure S1. Carotenoid (A) and capsaicinoid (B) biosynthetic pathways. **Figure S2.** Chromosomal localizations of *CabHLHs*. **Figure S3.** Multiple alignment analysis of the bHLH domain in pepper bHLH proteins. **Figure S4.** Phylogenetic tree of the bHLH family in *Capsicum annuum*, *Arabidopsis thaliana*, tomato and rice. **Figure S5.** The expression profiles of CabHLH genes in different tissues. **Figure S6.** Diagram of yeast two-hybrid assays experiments

Additional file 2: Table S1. The corresponding information of 107 *CabHLHs*. **Table S2.** Functionally characterized partial bHLH proteins from tomato and *Arabidopsis thaliana*. **Table S3.** Features of the CabHLH proteins motifs. **Table S4.** List of the bHLH genes in rice, tomato and *Arabidopsis thaliana*. **Table S5.** List of the primer for real-time quantitative PCR. **Table S6.** List of the primer for yeast two-hybrid assays. **Table S7.** Putative *cis*-elements of capsorubin and capsaicin biosynthetic genes promoters.

Declarations

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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