Pak. J. Bot., 56(6): DOI: http://dx.doi.org/10.30848/PJB2024-6(25)

CLONING OF *RhBEE1-LIKE* GENE AND THE EFFECT ON PETAL SENESCENCE IN ROSE

WENYU WANG, TINGTING DU, SIWEN HAN, JINGJING ZHANG, ZHE QIN AND HAIXIA PEI*

School of Life Science and Technology, Inner Mongolia University of Science and Technology, 014010, Baotou, Inner Mongolia, China
**Corresponding author's email: phx2003@126.com*

Abstract

Rosa hybrida is one of the most important cut flowers in the world, the premature senescence of its petals seriously affects its quality and economic value. In order to explore the senescence mechanism of rose petals and provide gene reserve for the creation of anti-senescence rose varieties through molecular breeding and other means, a bHLH transcription factor (TF) was screened based on our previous transcriptome data. bHLH TFs have been shown to play important roles in the plant growth and development, and in response to stress response. In this study, we investigated the function of bHLH TF in petal senescence of cut rose 'Carola'. We cloned a gene named *RhBEE1-like* belonging to bHLH TF family. Quantitative RT-PCR (qRT-PCR) showed that the expression level of *RhBEE1-like* increased significantly with the opening of flowers in rose and was significantly induced by ethylene. Transient overexpression of RhBEE1-*like* in tobacco mesophyll cells showed that the GFP signal of RhBEE1-like coincided with the nuclear signal of DAPI through confocal microscope, indicating that RhBEE1-like was located in the nucleus. Silencing *RhBEE1-like* in rose petals by virus-induced gene silencing (VIGS) can accelerate the senescence of rose petals, and at the same time, the expression level of senescence marker gene *RhSAG12*, the ion leakage rate and fading rate of rose petals were significantly increased. To summarize, our study showed that *RhBEE1-like* has antagonistic effect on petal senescence in rose.

Key words: Rose, Senescence, bHLH transcription factor, *RhBEE1-like*.

Introduction

Rosa hybrida is important cut flowers in the world, with high economic value and ornamental value, but the senescence of petals seriously affects the ornamental value of cut flowers, and then reduces the economic value of rose. Therefore, it is always a hot spot to explore the molecular and physiological mechanism of rose petal senescence in the postharvest research.

The bHLH gene family is widespread in eukaryotes and is one of the largest families of transcription factors (TF) in plants (Riechmann *et al*., 2000; Zhang *et al*., 2020). bHLH is named for its highly conserved basic region and helix-loop-helix functional region (HLH region) (Pires & Dolan, 2010; Zhang *et al*., 2018). The basic region located at the N-terminal of the domain can recognize and specifically bind to the E-Box in the promoter sequence of the target gene (Feller *et al*., 2011). The HLH region located at the C-terminal of the domain, consists of two α-helices containing conserved hydrophobic residues and is separated by a variable-length ring. The homologous or heterologous dimers can be formed by interaction of two α-helices, and that can bind to different regions of the target gene promoter to regulate gene transcription (Lang & Liu, 2020).

Currently, the bHLH TF family has been identified and analyzed in many plants and crops, such as *Arachis hypogaea* (Li *et al*., 2021), *Arabidopsis thaliana* (Hao *et al*., 2021), *Cucumis sativus* (Li *et al*., 2020), and *Camellia sinensis* (Liu *et al*., 2021). Previous studies have shown that bHLH transcription factors can be involved in regulating many biological processes such as plant photoporphogenesis (Akmakjian *et al*., 2021), anthocyanin accumulation (Zhao *et al*., 2019) and stress response (Sun *et al*., 2019). For example, overexpression of *SlbHLH22* can regulate the flowering time of tomato and accelerate fruit ripening (Waseem *et al*., 2019). *StbHLH1* can respond to high temperature by regulating anthocyanin biosynthesis in potato (Liu *et al*., 2019). Overexpression of *GhbHLH171* can activate JA synthesis and improve the tolerance to fungi in cotton (He *et al*., 2018). In rose, *RcbHLH112* is a susceptibility factor for rose resistance against *B. cinerea* and that its silencing increases resistance to *B. cinerea* (Ding *et al*., 2023). RcbHLH59-RcPRs module enhances salinity stress tolerance by balancing Na^+/K^+ through callose deposition (Su *et al*., 2022).

In this study, *RhBEE1-like*, as a member of bHLH TF family genes, has attracted our attention because of higher expression level at the middle and late stages in the opening progress of rose flower. Through bioinformatics analysis, amino acid sequence alignment, evolutionary tree analysis, and subcellular localization, the basic characteristics of RhBEE1-like were preliminarily understood. On this basis, through gene silencing, phenotype observation and determination of related physicochemical index, it was found that silencing *RhBEE1-like* could accelerate the senescence of rose petals. This indicates that *RhBEE1-like* has antagonistic effect on petal senescence in rose. This study provides some new clues for understanding the effects of bHLH TFs on petal senescence in rose, and also provides useful genetic resource for improving the ornamental quality of rose flowers.

Material and Methods

Plant materials: The experimental material was cut rose 'Carola', which was harvested at the opening stages 2 from the flower planting park in Baotou City, Inner Mongolia Autonomous Region, China. The fresh disease-free rose flowers were immediately taken back to the laboratory after cutting, then they were cut the branches to 45 cm and placed

in a bucket filled with deionized water for rehydration with 1-2 h at around 18℃. After rehydration, roses were used for subsequent experiments at room temperature.

RNA extraction and quantitative real-time PCR: Rose flowers were divided into 0-6 stages from bud no red to full bloom and senescence (Ma *et al*., 2005). The extraction of total RNA in petals was carried out according to the kit instructions (pBIOZOL Plant Total RNA extraction Kit, Beijing Bomase Technology Development Co., LTD., China). Using RNA (1 μg) as template, cDNA was synthesized by reverse transcription of total RNA according to Kit instructions (Evo M-MLV RT Kit with gDNA Clean for qPCR kit, Ecoray BioEngineering Co., LTD., China). Primer Premier 5.0 software was used to design primers (Table 1). SYBR® Green Premix Pro Taq HS qPCR Kit (Ecore Bioengineering Co., LTD.) was used to perform qRT-PCR to detect gene expression in different stages of rose petals. *RhUBI2* (Gene ID: LOC112181994) was used as the internal reference gene, and the sequences of the internal reference gene primer were shown in Table 1. The expression was calculated by $2^{-\Delta\Delta CT}$.

Cloning of *RhBEE1-like* gene and vector construction: The sequence of *RhBEE1-like* gene (Gene ID: LOC112196479) was obtained from the rose genome database (https://www.ncbi.nlm.nih.gov/genome/11715). The PCR reaction system was set as follows: 1 μL cDNA as template, 2 μL (10 μmol·L^{-1}) per primer, 5 μL 5*Prime STAR GXL buffer, 0.3 μL 5*Prime STAR GXL, 2 μL dNTP Mix, adding ddH_2O to make the final volume of 25 μL. The PCR reaction procedure was set as follows: pre-denaturation at 95℃ for 5 min, denaturation at 95℃ for 30 s, annealing at 50℃ for 30 s, extension at 72℃ for 30 s, set 30 cycles, and finally extension at 72℃ for 7 min. The amplified products were recovered using a centrifugal-column common agarose gel DNA recovery kit (Tiangen Biochemical Technology Co., LTD., China).

The plasmids of pYY13 (TRV1, used for VIGS) and pJG186 (subcellular localization) vectors were extracted by SteadyPure universal genomic DNA extraction kit (Ecoril Bioengineering Co., LTD., China), and the plasmids were digested by restriction endonuclease *Pst* I and *Apa* I. They were purified using a universal DNA purification recovery kit (Tiangen Biochemical Technology Co., LTD., China). The target gene was linked to the purified vector using EasyGeno Rapid Recombinant Cloning kit (Tiangen Biochemical Technology Co., LTD., China). The recombinant vectors were transferred into *E. coli DH5α* receptor cells, and were sequenced in Qingke (Beijing Qingke Biotechnology Co., LTD., China). Finally, the correctly sequenced recombinant vectors were transferred to *Agrobacterium GV3101* receptor cells.

Bioinformatics analysis: BLAST tool in NCBI database was used to compare homologous amino acid sequences. DNAMAN 6.0 software was used to analyze homologous sequences. MEGA 11 software was used to build the phylogenetic tree. ExPASy tool and ProtScale were used to predict the physicochemical properties and hydrophilicity of proteins. The potential phosphorylation sites of protein were predicted by NetPhos tool and signal peptide sequences were predicted by SignalP online software (Schiffrin *et al*., 2020).

Protein subcellular localization assays: The constructed pJG186-*RhBEE1-like* was transferred into *Agrobacterium GV3101* receptor cells and mixed with p19-*GV3101* at the ratio 1:1 of bacterial solution volume. The recombinant bacteria were collected by centrifugation and were resuspended with infection solution to $OD_{600} \approx 1.2$. Subsequently, the bacterial solution was left in darkness for 4-6 h. Bunsen tobacco with good growth for about 45 days was injected. After then, the tobaccos were cultured for 2 days and observed by laser confocal scanning microscope.

Virus-induced gene silencing and phenotypic observation: The transformed *Agrobacterium* strain *GV3101* carrying pYY13-*RhBEE1-like*, pYY13 (TRV1) and pYL192 (TRV2) were cultured in LB medium supplemented with 50 μg/mL kanamycin, rifampicin and gentamicin at 28℃ (180 rpm) for 18 h. After the Agrobacterium cells were collected by centrifuge, an infection solution (10 mM $MgCl_2$, 200 mM acetosyringone, 10 mM MES, pH = 5.6) was used to reinsert Agrobacterium to $OD_{600} \approx 1.5$. The *Agrobacterium* cells carrying pYY13-*RhBEE1-like* and pYY13 (TRV1) were mixed at the same volume (1:1) with pYL192 (TRV2) separately. At the room temperature，incubate it in the dark for 3-4 hours to subsequent experiments. The 8 mm punches were used to punch the petals of roses in stage 2 to obtain petal discs. The discs were immersed in infiltration buffer to be vacuumed (-25 kPa) twice (each for 1 min), then washed with deionized water and placed in a petri dish with two layers of filter papers for 3 days under dark conditions at 4℃. After 3 days, the discs were transferred to a culture room (22-23℃, light (12 h) / darkness (12 h)) to observe the phenotype. The detection of each indicator under different treatments were 180 discs, which were repeated 3 times.

Determination of ion leakage rates: The fifteen rose petal discs were randomly selected as a sample for mixed detection in each treatment. That was repeated at least 3 times. Rose petal discs were placed in 20 mL ddH_2O at 28℃, 180 rpm, and cultured for 30 min under shock. The initial conductivity (E_0) was measured with the conductivity meter. After measuring E_0, they were boiled in boiling water for 30 min. After cooling, their conductivities were measured again, denoted as E_1. E_0/E_1 is the ion leakage rate.

Table 1. Sequences of primers used in this investigation.

Gene	Primer F(5'-3')	Primer R (5'-3')	Usage
RhUBI2	GCCCTGGTGCGTTCCCAACTG	CCTGCGTGTCTGTCCGCATTG	qPCR primer
RhBEE1-like	GCGAGGCCAAGCTACTGATA	GTTGCTTCAAACTGTGGCCTT	qPCR primer
RhBEE1-like	ATGGAACTGATAGACAGTCT	TCAGTCTTATGTAGTAATGG	VIGS primer
RhSAG12	AAGACCAATCCAAAAGCAAC	AATCAAAACCACCTCCTTCA	qPCR primer

Determination of color fading rate: Image pro plus software was used to determine the area of faded petals in the photo. Color fading rate = (faded petal area / total area of petal circle) ×100%. Each treatment was observed and counted with 36 rose petal discs, which were repeated at least 3 times.

Statistical analysis: Statistical analysis of the data was performed by SPSS. T-test was used to analyze the difference significance, Bar value represents standard deviation. The image was generated using Origin 2018. Photoshop CC is used for image composition and annotation.

Results and Analysis

Expression of *RhBEE1-like* increased with petal senescence: In order to clarify the expression changes of *RhBEE1-like* in rose petals at the different opening stages, qRT-PCR was used to detect the expression level of *RhBEE1-like*, and *RhUBI2* (Gene ID: LOC112181994) was used as the internal reference gene. Compared with the control treatment at stage 0, the relative expression level of *RhBEE1-like* gradually increased at stage 3 (4.80-fold), stage 4 (7.04-fold), stage 5 (8.17-fold), and reached a peak of 9.63-fold at stage 6 (Fig. 1A). The expression levels of *RhBEE1-like* in petals gradually increased with the opening of the rose and were significantly higher than stage 0, indicating that *RhBEE1-like* may be involved in the regulation of senescence in rose petals. In addition, it was found that the expression level of *RhBEE1-like* in rose petals significantly increased in 6 (9.09-fold), 12 (21.45-fold), 18 (38.18-fold), and 24 h (52.84-fold), with the treatment of ethylene, indicating that *RhBEE1-like* could also be induced by ethylene (Fig. 1B).

Cloning and protein structure analysis of *RhBEE1-like* gene: According to the ORF sequence of RhBEE1-like gene obtained from NCBI, we successfully cloned *RhBEE1-like* with a full length of 720 bp (Fig. 2A). The PCR products of the gene clones were purified, then ligated to the VIGS vector pYY13 and the localization vector pJG186 respectively (Fig. 2B, C). The recombinant vectors were transferred into *E. coli DH5α*. Positive clones were analyzed by PCR, and sent to DNA sequencing subsequently.

The *RhBEE1-like* gene encodes a protein containing 239 amino acids (Fig. 3). Its isoelectric point is 6.12, molecular weight is 26799.7 Da, and its instability coefficient is 47.31, which is greater than 40, therefore it is speculated to be an unstable protein.

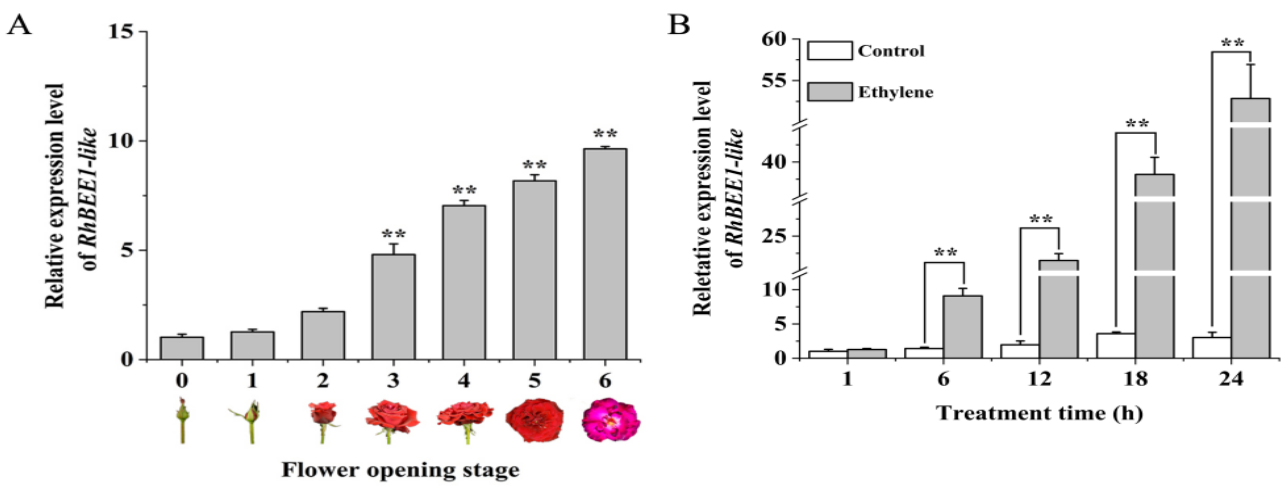

Fig. 1. The expression levels of *RhBEE1-like* in rose petals during the state of nature opening stage and ethylene treatment (* indicates $p<0.05$, ** indicates $p<0.01$)

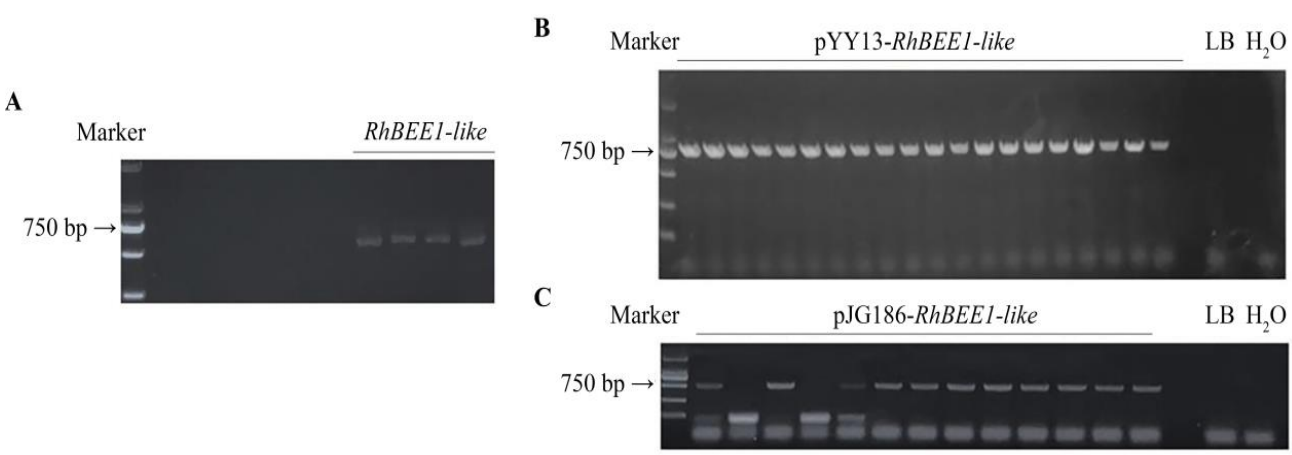

Fig. 2. Construction of *RhBEE1-like* recombinant vectors.

```
1    ATGGAACTGATAGACAGTCTCTGTGAGTTGGAGAGTCTCGGCGCCAGTCATCATCATGGA
1     M  E  L  I  D  S  L  C  E  L  E  S  L  G  A  S  H  H  H  G
61   ATCATGGCTCTCTCCACTGACAGCTTTACTCCTCATCATCATCATGATCAGCAACAACCT
21    I  M  A  L  S  T  D  S  F  T  P  H  H  H  H  D  Q  Q  Q  P
121  CAAGTCCCTGCAGCTATGAACTTCAGCACTGATCGCAGTCATGATATTCAGAGATTTTCT
41    Q  V  P  A  A  M  N  F  S  T  D  R  S  H  D  I  Q  R  F  S
181  CATTCTCATGATCGAAATGATGATGATCATCATCATGTCCTAACTGCTCAATCAACAGCT
61    H  S  H  D  R  N  D  D  D  H  H  H  V  L  T  A  Q  S  T  A
241  GCAGATTTTGGCGAACAAAGCCAAATGGAAAAGCCATTGGGAGGAAGAAAGAGAAAGAGA
81    A  D  F  G  E  Q  S  Q  M  E  K  P  L  G  G  R  K  R  K  R
301  AGCAATAGTGATAAAGATATGGACAAACCAAAGGAGGTCATTCATGTGAGAGCTAAGCGA
101   S  N  S  D  K  D  M  D  K  P  K  E  V  I  H  V  R  A  K  R
361  GGCCAAGCTACTGATAGCCACAGTTTGGCAGAAAGGGTAAGAAGAGAAAAAATAAATGAA
121   G  Q  A  T  D  S  H  S  L  A  E  R  V  R  R  E  K  I  N  E
421  AGGCTGAGATGCCTGCAAAATCTTGTTCCAGGGTGCTATAAGACAATGGGAATGGCAGTA
141   R  L  R  C  L  Q  N  L  V  P  G  C  Y  K  T  M  G  M  A  V
481  ATGCTGGATGTGGTTATCAGTTACGTTCAGTCACTGCAGAATCAAATTGAGTTTCTCTCT
161   M  L  D  V  V  I  S  Y  V  Q  S  L  Q  N  Q  I  E  F  L  S
541  ATGAAGCTTTCTGCAGCAAGCTTGTATTATGATTTCAATTCACCAGGTCATGCAGATCCT
181   M  K  L  S  A  A  S  L  Y  Y  D  F  N  S  P  G  H  A  D  P
601  GTTGACACAACGCAGGGGACAAATGCATATGAGGTACAAGAGAATATGGTAAGATCAGAG
201   V  D  T  T  Q  G  T  N  A  Y  E  V  Q  E  N  M  V  R  S  E
661  CAAGGGTATGGAGGAGGAGGAGGACTTTCTTTTAGCCAATCAACATCGTGGCCTCTTTGA
221   Q  G  Y  G  G  G  G  G  L  S  F  S  Q  S  T  S  W  P  L  *
```

Fig. 3. ORF nucleotide sequence and coding amino acid sequence of *RhBEE1-like.*

This protein is a hydrophilic protein with an average hydrophilic coefficient of -0.766 (Fig. 4A). It has 38 serine, 5 threonine and 2 tyrosine phosphorylation sites (Fig. 4B), no signal peptide sequence (Fig. 4C). Swiss-model was used to predict the three-dimensional structure of proteins, and the results showed that RhBEE1-like was dominated by alpha helices and random curls, containing conserved HER motifs, and the side chains of these residues could form perfect clip-like structures for DNA binding (Fig. 4D). The protein also has no transmembrane domain (TMD) (Fig. 4E).

Phylogenetic tree and homology analysis of RhBEE1-like protein: At present, the research on *AtbHLH* gene of model plant *Arabidopsis thaliana* is relatively clear, so amino acid sequence of 140 AtbHLH proteins were selected as classification reference to participate in the construction of the phylogenetic evolutionary tree of *RhbHLH* transcription factor family (Toledo-Ortiz *et al*., 2003). According to the topological structure of phylogenetic tree and the classification of AtbHLH subfamily, 95 RhbHLH family members including *RhBEE1-like* were classified. The results showed that 95 RhbHLH family members were distributed in 21 subfamilies and *RhBEE1-like* belongs to the 18th subfamily and was highly homologous to *Arabidopsis ATBEE1* (AT1G18400), which may be related to plant growth (Fig. 5A). Protein sequence analysis revealed that RhBEE1-like contains a conserved bHLH domain containing conserved HER motifs and several residues that are critical for DNA binding and dimer stabilization (Pires & Dolan, 2010) (Fig. 5B).

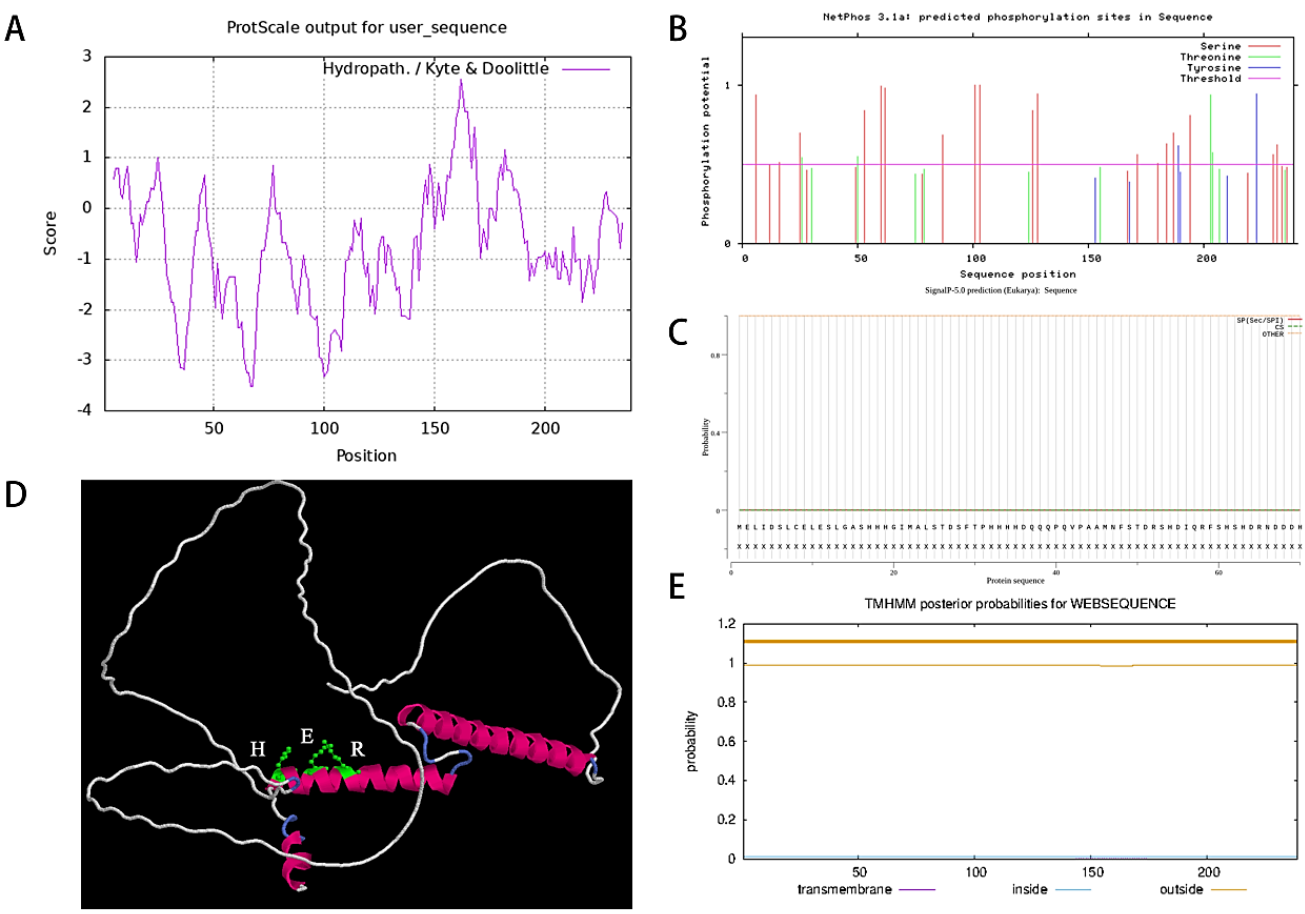

Fig. 4. Bioinformatics analysis of RhBEE1-like protein.

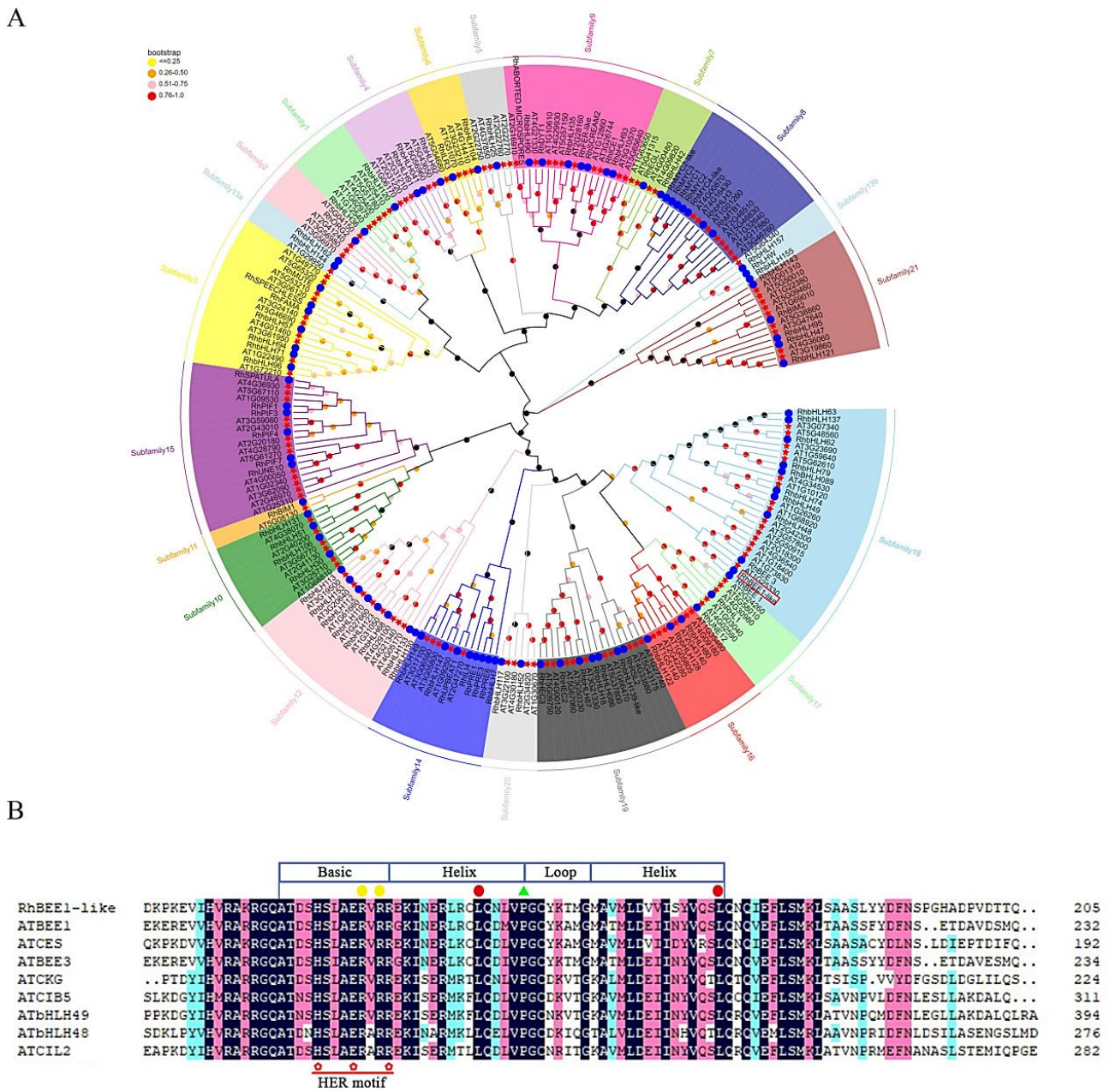

Fig. 5. Phylogenetic tree and homology analysis of RhBEE1-like protein
(A: Blue round label for rose, red five-pointed star for *Arabidopsis*; B: Sequence comparison of bHLH domain of Rose RhBEE1-like and *Arabidopsis* bHLH protein. The rectangular box represents the bHLH domain. The red pentagon represents HER theme. The circles and triangles represent highly conserved amino acids in the bHLH family).

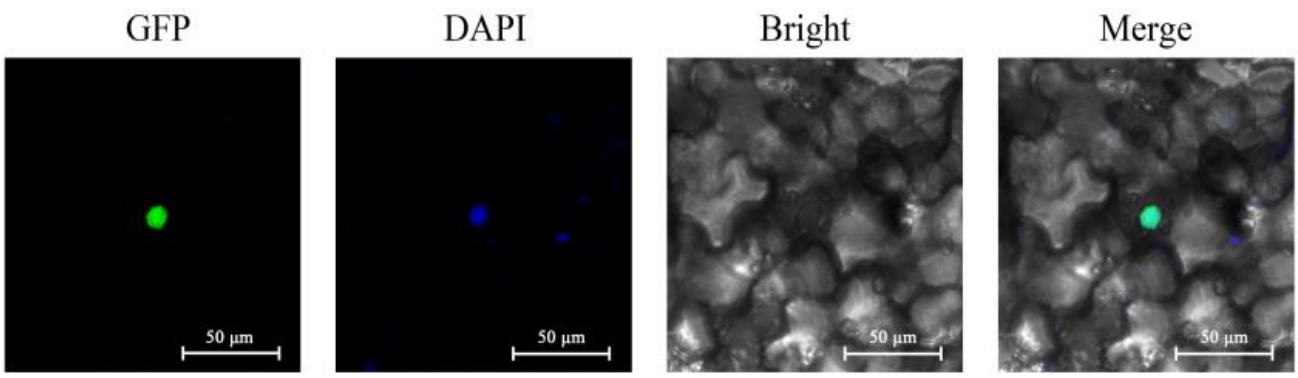

Fig. 6. Subcellular localization of RhBEE1-like protein
(GFP: pJG186-*RhBEE1-like*; DAPI: nuclear signal; Merge: Merge GFP, DAPI, and Bright. Scale: 50 μm).

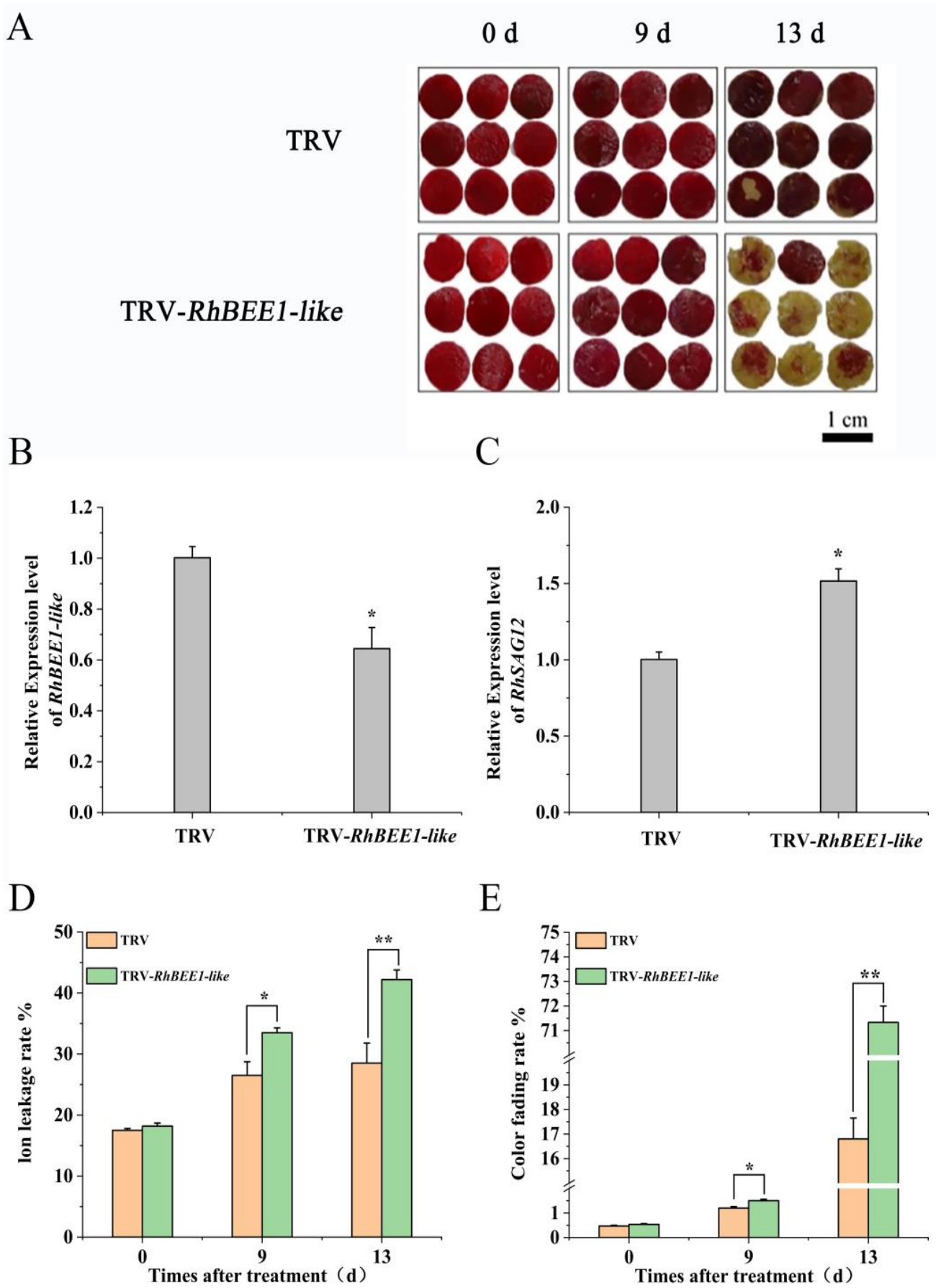

Fig. 7. Phenotype and related physicochemical indexes of rose petal discs after *RhBEE1-like* gene silencing
(A: Phenotype of TRV (Control) and TRV-*RhBEE1-like* (Silencing of *RhBEE1-like*) petal discs; B: Expression level of *RhBEE1-like*; C: Expression level of *RhSAG12*; D: Ion leakage rate; E: Color fading rate; * indicates $p<0.05$, ** indicates $p<0.01$).

Subcellular localization analysis of RhBEE1-like protein: After transient expression of RhBEE1-like-GFP protein in *N. benthamiana* leaves, the fluorescence signal pattern of GFP was found to be consistent with the localization of DAPI nuclear signals, indicating that RhBEE1-like protein was localized in the nucleus (Fig. 6).

Silencing of *RhBEE1-like* delays petal senescence: In order to further confirm the role of *RHBEE1-like* gene on rose petal senescence, virus-induced gene silencing (VIGS) was used to silence the *RhBEE1-like* gene. It was found that on the 9th day, the color of rose petals in TRV-*RhBEE1-like* group was deepened and the hardness of rose petals was softer than that in TRV control group. On the 13th day, almost all petals of TRV-*RhBEE1-like* group were nearly discolored, however, only a few rose petal discs in the TRV control group showed significant discoloration (Fig. 7A).

According to the results of qRT-PCR, the expression of *RhBEE1-like* was significantly lower in the *RhBEE1-like* -silenced discs than that in TRV control discs ($p<0.01$, Fig. 7B). The expression of *RhSAG12* is usually increased with the increase of senescence degree of plant leaves or petals, and it is often used as a marker gene for the detection and indication of plant senescence. *RhSAG12* in *RhBEE1-like* silenced samples was detected by qRT-PCR. The expression of *RhSAG12* in petals of *RhBEE1-like* silenced rose was significantly higher than that in TRV control group ($p<0.05$, Fig. 7C). In addition, the ion leakage and color fading rate were also measured. The results showed that compared with TRV control group, the ion leakage significantly higher in *RhBEE1-like* silenced discs on the 9 and 13 days (Fig. 7D). Moreover, the silenced discs also presented significantly higher color fading rate than the TRV control discs (Fig. 7E).

In conclusion, the expression of senescence related gene *RhSAG12* was significantly increased, and the ion leakage and petal color fading rate were also significantly increased in *RhBEE1-like* silenced rose petals. These results all proved that silencing *RhBEE1-like* gene accelerated senescence of rose petals.

Discussion

bHLH TFs work synergistically with other transcription factor members to play important roles in plant physiological metabolism, secondary metabolite synthesis, stress-related regulatory networks and signal transduction pathways (Amoutzias *et al*., 2007). For example, bHLH transcription factor *GhPAS1* can regulate cotton plant development and structure by mediating BR signaling in cotton (Wu *et al*., 2021). Silencing *SmbHLH92* gene can significantly increase the content of phenolic acid in hairy roots of *Salvia miltiorrhiza*, improve the transcription level of key enzymes related to tanshinone biosynthesis pathway and negatively regulate its biosynthesis (Zhang *et al*., 2020). In rose, the silencing of *RcbHLH112* increases resistance to *B. cinerea* (Ding *et al*., 2023), and RcbHLH59-RcPRs module enhances salinity stress tolerance by balancing Na^+/K^+ through callose deposition (Su *et al*., 2022).

BEE1 belongs to the bHLH transcription factor family and is also a positive regulator of BRs signal transduction (Friedrichsen *et al*., 2002). BEE1 is involved in the regulation of various plant growth and development processes such as cell growth (Mora-Garcia *et al*., 2004), stem branching (Wang *et al*., 2013), plant resistance (Jiang *et al*., 2012) and cell division (Jiang *et al*., 2012) by BRs hormone. Meanwhile, *BEE1* gene expression is also regulated by ABA. For example, overexpression of *BEE1* can reduce ABA response (Friedrichsen *et al*., 2002). In *Arabidopsis*, *AtBEE1* can also interact with *BES1* to activate FLOWERING LOCUS T (FT) transcription and promote flowering (Wang *et al*., 2019). *AtBEE1* can also promote shade avoidance syndrome in seedlings (Cifuentes-Esquivel *et al*., 2013).

Currently, there are few reports of *BEE1* affecting flower senescence. In our study, we screened a bHLH TF family gene *RhBEE1-like*. Interestingly, the results showed that *RhBEE1-like* gene had antagonistic effect on rose senescence. The expression level of *RhBEE1-like* increased significantly with the opening of flowers in rose, and was significantly induced by ethylene (Fig. 1). However, the silencing of *RhBEE1-like* gene can accelerate the senescence of rose petals (Fig. 7). It was found that the expression of *BREVIPEDICELLUS (BP)/KNAT1* was induced during petal senescence of *Arabidopsis thaliana*, and the deletion of *bp* mutant accelerated petal shedding, indicating that BP/KNAT1 played an antagonistic role during petal shedding (Shi *et al.*, 2011). In sunflower, the expression of *HaHB-4* increased with the senescence of flowers, but overexpression of *HaHB-4* inhibited the senescence of flowers (Manavella *et al.*, 2006). In rose, *RhPR10.1* was significantly induced by ethylene. The transcript levels of *RhPR10.1* in all the floral organs were increasing from the partially opened flower bud to the onset of petal wilting. However, the decrease of *RhPR10.1* expression by VIGS could promote the senescence of rose petals. The role of *RhPR10.1* on senescence is antagonistic in rose (Wu *et al.*, 2017), and the function of *RhBEE1-like* is similar to this in rose senescence. These studies suggest that antagonism may be an important mechanism to prevent premature senescence in plants. In addition, we hypothesized that the antagonistic function of *RhBEE1-like* in senescence of rose may be dependent on the interaction of ethylene and BRs, and further research is needed to confirm this speculation.

Acknowledgments

This work was supported by IMAR (Inner Mongolia Autonomous Region) Natural Science Foundation (grant nos. 2023MS03055), Basic Scientific Research Foundation for Universities Directly under the Inner Mongolia Autonomous Region (grant nos. 0406082215), and the National Natural Science Foundation of China (grant nos. 31860572).

References

Akmakjian, G.Z., N. Riaz and M.L. Guerinot. 2021. Photoprotection during iron deficiency is mediated by the bHLH transcription factors PYE and ILR3. *Proc. Natl. Acad. Sci. U S A.*, 118(40): e2024918118.

Amoutzias, G.D., A.S. Veron, J. Weiner, M. Robinson-Rechavi, E. Bornberg-Bauer, S.G. Oliver and D.L. Robertson. 2007. One billion years of bZIP transcription factor evolution: conservation and change in dimerization and DNA-binding site specificity. *Mol. Biol. Evol.*, 24(3): 827-835.

Cifuentes-Esquivel, N., J. Bou-Torrent, A. Galstyan, M. Gallemí, G. Sessa, M. Salla Martret, I. Roig-Villanova, I. Ruberti and J.F. Martínez-García. 2013. The bHLH proteins BEE and BIM positively modulate the shade avoidance syndrome in *Arabidopsis* seedlings. *Plant J*., 75(6): 989-1002.

Ding, C., J.Z. Gao, S.Y. Zhang, N. Jiang, D.T. Su, X.Z. Huang and Z. Zhang. 2023. The basic/helix-loop-helix transcription factor family gene *RcbHLH112* is a susceptibility gene in gray mould resistance of rose (*Rosa chinensis*). *Int. J. Mol. Sci*., 24(22): 16305.

Feller, A., K. Machemer, E.L. Braun and E. Grotewold. 2011. Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. *Plant J*., 66(1): 94-116.

Friedrichsen, D.M., J. Nemhauser, T. Muramitsu, J.N. Maloof, J. Alonso, J.R. Ecker, M. Furuya and J. Chory. 2002. Three redundant brassinosteroid early response genes encode putative bHLH transcription factors required for normal growth. *Genetics*, 162(3): 1445-1456.

Hao, Y.Q., X.M. Zong, P. Ren, Y.Q. Qian and A.G. Fu. 2021. Basic helix-loop-helix (bHLH) transcription factors regulate a wide range of functions in *Arabidopsis*. *Int. J. Mol. Sci*., 22(13): 7152.

He, X., L.F. Zhu, G.M. Wassan, Y.J. Wang, Y.H. Miao, M. Shaban, H.Y. Hu, H. Sun and X.L. Zhang. 2018. GhJAZ2 attenuates cotton resistance to biotic stresses via the inhibition of the transcriptional activity of *GhbHLH171*. *Mol. Plant Pathol*., 19(4): 896-908.

Jiang, Y.H., L. Bao, S.Y. Jeong, S.K. Kim, C.G. Xu, X.H. Li and Q.F. Zhang. 2012. XIAO is involvedin the control of organ size by contributing to the regulation of signaling andhomeostasis ofbrassinosteroids and cell cycling in rice. *Plant J. Cell Mol. Biol.*, 70: 398-408.

Jiang, Y.P., F. Cheng, Y.H. Zhou, X.J. Xia, W.H. Mao, S. Kai, Z. Chen and J.Q. Yu. 2012. Cellularglutathione redox homeostasis plays an important role in the brassinosteroid-inducedincrease in CO_2 assimilation in *Cucumis sativus*. *New Phytol*., 194: 932-943.

Lang, Y.H and Z. Liu. 2020. Basic helix-loop-helix (bHLH) transcription factor family in yellow horn (*Xanthoceras sorbifolia* Bunge): genome-wide characterization, chromosome location, phylogeny, structures and expression patterns. *Int. J. Biol. Macromol*., 160: 711-723.

Li, C.J., C.X. Yan, Q.X. Sun, J. Wang, C.L. Yuan, Y.F. Mou, S.H. Shan and X.B. Zhao. 2021. The bHLH transcription factor *AhbHLH112* improves the drought tolerance of peanut. *BMC Plant Biol*., 21(1): 540.

Li, J.L., T. Wang, J. Han and Z.H. Ren. 2020. Genome-wide identification and characterization of cucumber bHLH family genes and the functional characterization of *CsbHLH041* in Nacl and ABA tolerance in *Arabidopsis* and cucumber. *BMC Plant Biol*., 20(1): 272.

Liu, R.J., Y.Y. Wang, S. Tang, J.R. Cai, S.Q. Liu, P. Zheng and B.M. Sun. 2021. Genome-wide identification of the tea plant bHLH transcription factor family and discovery of candidate regulators of trichome formation. *Sci. Rep*., 11(1): 10764.

Liu, Y.H., K. Lin-Wang, R.V. Espley, L. Wang, Y.M. Li, Z. Liu, P. Zhou, L.H. Zeng, X.J. Zhang, J.L. Zhang and A.C. Allan. 2019. *StMYB44* negatively regulates anthocyanin biosynthesis at high temperatures in tuber flesh of potato. *J Exp Bot*., 70(15): 3809-3824.

Ma, N., L. Cai, W.J. Lu, H. Tan and J.P. Gao. 2005. Exogenous ethylene influences flower opening of cut roses (*Rosa hybrida*) by regulating the genes encoding ethylene biosynthesis enzymes. *Sci. China Life Sci*., 48(5): 434-44.

Manavella, P.A., A.L. Arce, C.A. Dezar, F. Bitton, J.P. Renou, M. Crespi and R.L. Chan. 2006. Cross-talk between ethylene and drought signalling pathways is mediated by the sunflower *Hahb-4* transcription factor. *Plant J*., 48: 125-137.

Mora-García, S., G. Vert, Y.H. Yin, A. Caño-Delgado, H. Cheong and J. Chory. 2004. Nuclear protein phosphatases with Kelch-repeat domains modulate the response to brassinosteroids in *Arabidopsis*. *Genes Dev*., 18(4): 448-60.

Pires, N and L. Dolan. 2010. Origin and diversification of basic-helix-loop-helix proteins in plants. *Mol. Biol. Evol*., 27(4): 862-74.

Riechmann, J.L., J., Heard, G. Martin, L. Reuber, C. Jiang, J. Keddie, L. Adam, O. Pineda, O.J. Ratcliffe, R.R. Samaha, R. Creelman, M. Pilgrim, P. Broun, J.Z. Zhang, D. Ghandehari, B.K. Sherman, and G. Yu. 2000. *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes. *Science*, 290(5499): 2105-2110.

Schiffrin, B., S.E. Radford, D.J. Brockwell and A.N. Calabrese. 2020. PyXlinkViewer: A flexible tool for visualization of protein chemical crosslinking data within the PyMOL molecular graphics system. *Protein Sci*., 29(8): 1851-1857.

Shi, C.L., G.E. Stenvik, A.K. Vie, A.M. Bones, V. Pautot, M. Proveniers, R.B. Aalen and M.A. Butenko. 2011. *Arabidopsis* class I KNOTTED-like homeobox proteins act downstream in the IDA-HAE/HSL2 floral abscission signaling pathway. *Plant Cell*, 23: 2553-2567.

Su, L., Y.C. Zhang, S. Yu, L.F. Geng, S. Lin, L. Ouyang and X.Q. Jiang. 2022. RcbHLH59-RcPRs module enhances salinity stress tolerance by balancing Na^+/K^+ through callose deposition in rose (*Rosa chinensis*). *Hort. Res*., 10(3):uhac291.

Sun, K.L., H.Y. Wang and Z.L. Xia. 2019. The maize bHLH transcription factor *bHLH105* confers manganese tolerance in transgenic tobacco. *Plant Sci*., 280: 97-109.

Toledo-Ortiz, G., E. Huq and P.H. Quail. 2003. The *Arabidopsis* basic/helix-loop-helix transcription factor family. *Plant Cell*, 15(8): 1749-1770.

Wang, F., Y.S. Gao, Y.W. Liu, X. Zhang, X.X. Gu, D.B. Ma, Z.W. Zhao, Z.J. Yuan, H.W. Xue and H.T. Liu. 2019. BES1-regulated BEE1 controls photoperiodic flowering downstream of blue light signaling pathway in *Arabidopsis*. *New Phytol*., 223(3): 1407-1419.

Wang, Y., S.Y. Sun, W.J. Zhu, K.P. Jia and X.L. Wang. 2013. Strigolactone/MAX2-induced degradationof brassinosteroid transcriptional effector BES1 regulates shoot branching. *Dev Cell.*, 27(6): 681-688.

Waseem, M., N. Li, D. Su, J. Chen and Z. Li. 2019. Overexpression of a basic helix-loop-helix transcription factor gene, *SlbHLH22*, promotes early flowering and accelerates fruit ripening in tomato (*Solanum lycopersicum* L.). *Planta*, 250(1): 173-185.

Wu, H.H., Z.Y. Ren, L. Zhang, M.Z. Guo, J.Y. Yang, L.Y. Hou, G. Qanmber, F.G. Li and Z.R. Yang. 2021. The bHLH transcription factor *GhPAS1* mediates BR signaling to regulate plant development and architecture in cotton. *The Crop J*., 9(5): 1049−1059.

Wu, L., N. Ma, Y.C. Jia, Y. Zhang, M. Feng, C.Z. Jiang, C. Ma and J.P. Gao. 2017. An ethylene-induced regulatory module delays flower senescence by regulating cytokinin content. *Plant Physiol*., 173(1):853-862.

Zhang, J.H., H.Z. Lv, W.J. Liu, A.J. Ji, X. Zhang, J.Y. Song, H.M. Luo and S.L. Chen. 2020. bHLH transcription factor *SmbHLH92* negatively regulates biosynthesis of phenolic acids and tanshinones in *Salvia miltiorrhiza*. *Chin Herb. Med*., 12(3): 237-246.

Zhang, T.T., W. Lv, H.S. Zhang, L. Ma, P.H. Li, L. Ge and G. Li. 2018. Genome-wide analysis of the basic helix-loop-helix (bHLH) transcription factor family in maize. *BMC Plant Biol*., 18(1): 235.

Zhang, Z.S., J. Chen, C.L. Liang, F. Liu, X.L. Hou and X.X. Zou. 2020. Genome-wide identification and characterization of the bHLH transcription factor family in pepper (*Capsicum annuum* L.). *Front. Genet*., 11: 570156.

Zhao, M.R., J. Li, L. Zhu, P. Chang, L.L. Li and L.Y. Zhang. 2019. Identification and characterization of MYB-bHLH-WD40 regulatory complex members controlling anthocyanidin biosynthesis in blueberry fruits development. *Genes (Basel)*., 10(7): 496.

(Received for publication 7 December 2023)