ETHYLENE RESPONSE FACTOR 070 inhibits flowering in Pak-choi by indirectly impairing BcLEAFY expression

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Abstract

APETALA2/ethylene responsive factors respond to ethylene and participate in many biological and physiological processes, such as plant morphogenesis, stress resistance, and hormone signal transduction. Ethylene responsive factor 070 (BcERF070) is important in flowering. However, the underlying molecular mechanisms of BcERF070 in floral transition in response to ethylene signaling have not been fully characterized. Herein, we explored the function of BcERF070 in Pak-choi [Brassica campestris (syn. Brassica rapa) ssp. chinensis]. Ethylene treatment induced BcERF070 expression and delayed flowering in Pak-choi. Silencing of BcERF070 induced flowering in Pak-choi. BcERF070 interacted with major latex protein-like 328 (BcMLP328), which forms a complex with helix-loop-helix protein 30 (BcbHLH30) to enhance the transcriptional activity of BcbHLH30 on LEAFY (BcLFY), ultimately promoting flowering. However, BcERF070 impaired the BcMLP328–BcbHLH30 complex activation of LEAFY (BcLFY), ultimately inhibiting flowering in Pak-choi. BcERF070 directly promoted the expression of the flowering inhibitor gene B-box 29 (BcBBX29) and delayed flowering by reducing FLOWERING LOCUS T (BcFT) expression. These results suggest that BcERF070 mediates ethylene-reduced flowering by impairing the BcMLP328–BcbHLH30 complex activation of BcLFY and by directly promoting the gene expression of the flowering inhibition factor BcBBX29 to repress BcFT expression. The findings contribute to understanding the molecular mechanisms underlying floral transition in response to ethylene in plants.

Introduction

Flowering is a biological process that transforms vegetative growth into generative growth. The main flowering regulation pathways include photoperiod, vernalization, gibberellin, and autonomous pathways (Fornara et al. 2010; Song et al. 2015). The photoperiod pathway includes an internal system (biological clock) to detect light (photoreceptors) and measure time, which forms the main GIGANTEA (GI)–FLAVIN-BINDING KELCH-REPEAT F-box 1 (FKF1)–CYCLING DOF FACTORS (CDFs) model. The pathway also controls the expression and protein stability of CONSTANS (CO) and further controls the expression of FLOWERING LOCUS T (FT) (Mathieu et al. 2007). Long periods of light increase CO expression. High CO expression activates FT expression and accelerates flowering by directly binding to the FT promoter (Mathieu et al. 2007; Lazaro et al. 2015). The BBX family comprises typical photoperiodic response genes involved in the regulation of flowering. BBX28 and BBX29 delay flowering by interacting with CO to inhibit its transcriptional activity in FT (Liu et al. 2020; Wang et al. 2021a, 2021b). The vernalization pathway refers to the flowering ability of plants after long-term exposure to cold temperatures. The pathway contains two key genes: FRIGIDA and FLOWERING LOCUS C (FLC). These genes function as flowering inhibitors (Song et al. 2012; Tao et al. 2012; Airoldi et al. 2015; Huang et al. 2018; Fu et al. 2021). LEAFY (LFY) is also
involved in promoting flowering by controlling flowering time and floral organ development (Nilsson et al. 1998; Lamb et al. 2002; Wang et al. 2004). The gibberellin pathway is an important pathway that promotes flowering under noninduced photoperiod conditions. Gibberellic acid (GA) promotes flowering by activating the MADS-box genes and LFY (Eriksson et al. 2006; Achard et al. 2007). In addition, other hormones, such as salicylic acid, abscisic acid, and ethylene, are reportedly involved in the regulation of flowering (Martínez et al. 2004; Finkelstein 2006; Lin et al. 2009).

Ethylene plays an irreplaceable role in plant growth and development, including flowering, seed germination, leaf shedding, sex differentiation, fruit ripening, and stress and pathogen responses (Pierik et al. 2007; Abeles et al. 2012). In Arabidopsis thaliana, ethylene reduces bioactive GA levels to enhance the accumulation of DELLA and delays flowering by repressing the floral meristem-identity genes LFY and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) (Achard et al. 2007). Another study showed that the 1-amincyclopropane-1-carboxylic acid ethylene biosynthesis precursor delays flowering by downregulating the expression of FLOWERING LOCUS D, thereby increasing the level of H3K4me2 and activating the expression of FLC (Xu et al. 2023). As a downstream response factor of ethylene, APETAL2/ethylene response factors (AP2/ERF) are activated after receiving a dimer signal formed by upstream Ethylene Insensitive 3/ETHYLENE-INSENSITIVELIKE 1 (EIN3/EIL1), which respond to ethylene signaling and regulates the transcript levels of downstream genes (Solano et al. 1998; Giovannoni 2007).

AP2/ERF are a large group of factors mainly reported in plants. The functions of this family include plant morphology, growth and development, floral transformation, and resistance to biotic and abiotic stressors (Feng et al. 2020). Some members of the AP2/ERF family reportedly activate flowering. In Chrysanthemum morifolium, CmERF110 induces early flowering through multiple mechanisms (Schaller 2012; Xing et al. 2019). Overexpressed CmERF110 can increase APETALA1 (AP1) expression to promote early flowering (Xing et al. 2019). Another regulatory mechanism was proposed in which CmERF110 interacts with CmFLK to regulate flowering (Huang et al. 2022). Moreover, overexpression of SIERF36 in Solanum lycopersicum causes flowers to flower earlier than wild-type (WT) plants, which may take part in the photoperiod pathway (Upadhyay et al. 2014).

However, some members of the AP2/ERF family act as flowering repressors (Song and Galbraith 2006). For example, AtERF1 directly binds to the FT promoter to suppress its expression (Chen et al. 2021). In rice (Oryza sativa), OsERF1 also delays flowering (Hu et al. 2008). The AP2/ERF gene DREB1 can delay flowering by inhibiting GA20ox expression to decrease GA biosynthesis (Liu et al. 1998; Magome et al. 2004; Qin et al. 2007; Achard et al. 2008; Magome et al. 2008). Moreover, ERF070 regulates root development and phosphate starvation-mediated responses and inhibits flowering transformation (Ramaiah et al. 2014). However, the mechanism through which ERF070 inhibits flowering has not been investigated. Therefore, the molecular mechanisms of ERFs responding to ethylene to regulate flowering remain unclear.

Pak-choi (Brassica rapa ssp. chinensis) is a leafy vegetable that originated in China and has gradually been cultivated worldwide. However, premature flowering limits Pak-choi leaf development and decreases its yield accumulation (Huang et al. 2018; Zhang et al. 2022). Therefore, understanding the mechanisms regulating flowering is important for Pak-choi. This study explored the molecular mechanism of BcERF070 response to ethylene to regulate flowering in Pak-choi. Exogenous ethylene treatment increased BcERF070 expression and delayed flowering in Pak-choi. We also demonstrate that BcERF070 delays flowering by impairing the activation of the MLP328–bHLH30 complex to LFY expression and directly promoting transcription of the flowering inhibitor BcBBX29 to suppress FT expression. This study provides insights into how ethylene mediates flowering in Pak-choi.

Results

BcERF070 responds to ethylene and delays flowering in Pak-choi

Ethylene reportedly mediates flowering time in plants (Achard et al. 2006). To explore the role of ethylene in the flowering time of Pak-choi, an exogenous ethylene treatment was applied. Pak-choi treated with 0.1 mM ethylene showed delayed flowering and increased rosette leaf number (Fig. 1, A to C; Supplementary Fig. S1). A previous study demonstrated that the AP2/ERF family of factors respond to ethylene signaling to regulate plant growth (Jin et al. 2018). To verify whether BcERF070 can respond to ethylene, its expression was measured in the ethylene-treated and control groups. The RNA level of BcERF070 was induced and reached a peak 2 h after ethylene treatment compared with the control (Fig. 1D). Moreover, the transcript level of BcERF070 gradually decreased throughout the vegetative growth period and was expressed at its lowest level during the reproductive period (Fig. 1E). Taken together, these results suggest that BcERF070 is involved in the ethylene-mediated regulation of flowering.

To explore the role of BcERF070 in flowering regulation, a VIGS assay was performed on Pak-choi (Supplementary Fig. S2, A to C). The expression of BcERF070 decreased significantly in BcERF070-silenced plants compared with the expression in control plants (Supplementary Fig. S2D). As expected, the buds appeared earlier in the BcERF070-silenced group than in the control group (Fig. 1F). Days to flowering were earlier in the BcERF070-silenced plants than in the control plants (Fig. 1G; Supplementary Fig. S2E). The rosette leaf numbers in BcERF070-silenced plants were lower than those in the control plants (Fig. 1G). The expression of BcFT was induced by silencing BcERF070 (Supplementary Fig. S2F). BcERF070-overexpressing Arabidopsis also exhibited...
Figure 1. Silencing of BcERF070 promotes flowering and responds to ethylene in Pak-choi. A) Exogenous ethylene can delay flowering in Pak-choi. Bar = 2 cm. ET, ethylene treatment. B, C) Days to flowering (B) and rosette leaf numbers (C) in control (CK) and ethylene treatment (ET) sprayed plants. D) Expression of BcERF070 in control and ET sprayed plants. E) Expression levels of BcERF070 at the growth stage for 10, 15, 20, and 25 d at the vegetative period and 30 d at the reproductive period. Error bars are ± SD from three biological replicates (n = 3 plants for each replicate). Letters above the bars indicate significant differences determined by one-way ANOVA (P < 0.05). F) BcERF070-silenced plants showed earlier bolting and flowering in Pak-choi. Bar = 2 cm. G) Days to flowering (up) and rosette leaf numbers (bottom) in control and BcERF070-silenced plants with or without ET treatment. H) Flowering phenotype in control and BcERF070-silenced plants under ET treatment. Bar = 2 cm. Error bars are ± SD from three biological replicates (n = 3 plants for each replicate). For (B to D, G), significant differences were determined by t-test (*P < 0.05, **P < 0.01, ***P < 0.001).
significantly delayed flowering (Supplementary Fig. S3). These results demonstrate the negative role of BcERF070 in the regulation of flowering in Pak-choi.

To investigate the potential function of BcERF070 in ethylene-reduced flowering in Pak-choi, BcERF070-silenced and control plants were treated with 0.1 mM ethylene. After ethylene treatment, the flowering time of silenced plants was significantly advanced, and the number of rosette leaves was significantly reduced (Fig. 1, H and G). The flowering time of BcERF070-silenced plants under ethylene treatment was later than that of the untreated plants (Fig. 1G). The findings indicate that BcERF070 is not the only pathway involved in the regulation of flowering in Pak-choi.

BcERF070 interacts with BcMLP328

To explore the flowering regulatory mechanism of BcERF070 in Pak-choi, proteins that potentially interact with BcERF070 were screened using the Y2H assay (Supplementary Table S2). BcMLP328 interacted with BcERF070 in both the N- and C-terminal regions (Fig. 2A). To validate the BcERF070-BcMLP328 interaction, we performed BiFC using YFPn-BcERF070 and YFPc-BcMLP328 in Nicotiana benthamiana leaves. BcERF070 and BcMLP328 interacted in the nucleus (Fig. 2B). Luciferase complementation imaging (LCI) revealed co-infiltration of nLUC-BcERF070 and cLUC-BcMLP328 in N. benthamiana leaves and obvious LUC signals (Fig. 2C). The findings confirmed the interaction between BcERF070 and BcMLP328. In the GST pull-down assay, the band from His-tagged BcMLP328 was visualized with GST-tagged BcERF070, whereas no His-tagged BcMLP328 band was observed in the control, indicating that BcERF070 interacts with BcMLP328 in vitro (Fig. 2D). Co-IP also showed that His-tagged BcMLP328 was immunoprecipitated with FLAG-tagged BcERF070 (Fig. 2E; Supplementary Fig. S12, A to C, and E). These results indicate that BcERF070 interacts with BcMLP328 both in vivo and in vitro. However, the mechanism through which BcERF070 and BcMLP328 interact to regulate flowering remains unclear.

BcMLP328 promotes flowering in Pak-choi

To analyze the function of BcMLP328 in Pak-choi, we first determined whether BcMLP328 responds to ethylene. The expression of BcMLP328 decreased under ethylene treatment compared with that in the control (Fig. 3A). The expression levels of BcMLP328 in the different organs were also measured. BcMLP328 was expressed at high levels exclusively in the flower organs, suggesting that BcMLP328 may be related to the flowering process in Pak-choi (Fig. 3B).

To investigate the potential effect of BcMLP328 on flowering, we first generated 35S:BcMLP328-GFP lines (#11 and #20) in Pak-choi through Agrobacterium-mediated transformation, which was confirmed using western blotting and qPCR assays (Supplementary Fig. S4). Overexpression of BcMLP328 promoted flowering in Pak-choi (Fig. 3C). As expected, BcERF070-silenced plants flowered earlier and had fewer rosette leaves than the control plants (Fig. 3, D and E).

These results suggest that the overexpression of BcMLP328 induces early flowering in Pak-choi. To explain the potential causes of the early flowering phenotype of 35S:BcMLP328-GFP Pak-choi, we measured the relative expression levels of the downstream flowering time regulators BcSOC1, BcSPL15, BcFT, BcAP3, and BcTEM in 35S: BcMLP328-GFP Pak-choi. The mRNA levels of BcSOC1, BcSPL15, BcFT, and BcAP3 were significantly higher in 35S: BcMLP328-GFP plants than in the control (Fig. 3F), whereas that of the flowering suppressor BcTEM was lower in 35S:BcMLP328-GFP plants than in the control (Fig. 3F), consistent with the early flowering phenotype. The overexpression of BcMLP328 in Arabidopsis promoted flowering and affected the expression of flowering-related genes (Supplementary Fig. S5).

BcMLP328-silenced ‘Suzhouqing’ Pak-choi (pTY-BcMLP328) was generated using VIGS. The control (pTY) was injected with an empty pTY vector. Compared with pTY-S plants, BcMLP328-silenced plants flowered later (Fig. 3, G and H). Accordingly, the number of days to flowering in pTY-BcMLP328 was longer than that in pTY-S, and the number of rosette leaves was greater in pTY-BcMLP328 than in pTY-S (Fig. 3, I and J). Moreover, the relative expression levels of the flowering time regulators BcSOC1, BcSPL15, BcFT, BcAP3, and BcTEM were measured in BcMLP328-silenced Pak-choi. BcSOC1, BcSPL15, BcFT, and BcAP3 expressions decreased in BcMLP328-silenced Pak-choi (Fig. 3K), while the expression of BcTEM increased in silenced plants (Fig. 3K). We also silenced BcMLP328 in ‘Caixin,’ which similarly delayed flowering (Supplementary Fig. S6, A and B). The days to flowering were later, whereas rosette leaf numbers were higher in pTY-BcMLP328 plants than in controls (Supplementary Fig. S6, C and D). Taken together, these results indicate that BcMLP328 promotes flowering in Pak-choi. However, the mechanism through which BcMLP328 promotes flowering remains unclear.

BcMLP328 enhances transcriptional activity of BcbHLH30 to BcLFY

To elucidate the molecular mechanism of BcMLP328 in promoting flowering, the Y2H assay was performed using BcMLP328 as prey (Supplementary Table S3). BcbHLH30, a gene homologous to Arabidopsis bHLH30 that promotes flowering (An et al. 2014), interacted with BcMLP328 (Fig. 4A). An LCI assay was performed to confirm the interaction between BcMLP328 and BcbHLH30. BcMLP328-cLUC and BcbHLH30-nLUC co-infiltrated N. benthamiana leaves. These results indicate that BcMLP328 interacts with BcbHLH30 (Fig. 4B). Additionally, the BiFC assay revealed the interaction of BcMLP328 with BcbHLH30 in the nucleus (Fig. 4C). Co-IP confirmed that BcMLP328-His could be immunoprecipitated with BcbHLH30-FLAG (Fig. 4D; Supplementary Fig. S12, D and F). Therefore, these results demonstrate that BcMLP328 interacts with BcbHLH30 in vivo and in vitro.
We also studied the effect of BcbHLH30 on Pak-choi flowering using a VIGS-mediated silencing system. BcbHLH30-silenced pak-choi displayed delayed flowering (Fig. 4, E and F). The days to flowering were later, and the rosette leaf number was higher in pTY-BcbHLH30 plants than in pTY-S plants (Fig. 4, G and H). In addition, the flowering regulators BcFT and BcLFY were expressed at lower levels in pTY-BcbHLH30 plants than in pTY-S plants (Fig. 4I; Supplementary Fig. S7). These results suggest that BcbHLH30 is involved in the positive regulation of Pak-choi flowering.

Although bHLH30 promotes flowering in Arabidopsis (An et al. 2014), its direct target genes are unknown. To investigate the potential targets of BcbHLH30 in flowering...
**Figure 3.** BcMLP328 responses to ethylene (ET) and promotes flowering in Pak-choi. 

A) Expression of BcMLP328 in CK (control) and ET (ethylene treatment) sprayed Pak-choi. BcACTIN was used as the reference gene. 

B) Expression patterns of BcMLP328 in different tissues of Pak-choi (root, stem, leaf, bud, and flower organs). Error bars are ±SD from three biological replicates (n = 3 plants for each replicate). Letters above the bars indicate significant differences determined using one-way ANOVA (P < 0.05). 

C) Flowering phenotype of WT and 35S:BcMLP328 lines in Pak-choi. Bar = 2 cm. 

D, E) Days to flowering (D) and rosette leaf numbers (E) in control (WT) and BcMLP328-overexpressing (35S: BcMLP328-GFP) plants. 

F) Expressions of BcSOC1, BcSPL15, BcFT, BcAP3, and BcTEM in WT and BcMLP328-overexpression plants. 

G) Phenotypes of pTY-S and BcMLP328-silencing (pTY-BcMLP328) plants. Bar = 2 cm. Image was digitally extracted for comparison. 

H) BcMLP328 silencing efficiency in BcMLP328-silencing plants detected using qPCR. 

I, J) Rosette leaf numbers (I) and days to flowering (J) in control and BcMLP328-silenced plants. 

K) Expressions of BcSOC1, BcSPL15, BcFT, BcAP3, and BcTEM in WT and BcMLP328-silenced plants. Error bars are ±SD from three biological replicates (n = 3 plants for each replicate). For (A, D to F, H to K), significant difference was determined using t-test (*P < 0.05, **P < 0.01, ***P < 0.001).
Figure 4. BcbHLH30 interacts with BcMLP328 and promotes flowering in Pak-choi. A) Y2H assay showing interaction of BcMLP328 with BcbHLH30. The positive group is pGADT7-T and pGBK7-T7. The negative group is pGADT7-T and pGBK7-T7-Lam. B) Luciferase complementation assay of BcbHLH30 and BcMLP328 interaction in plants. The four quadrants show different transformation combinations; only the mixture of BcbHLH30 and BcMLP328 displays fluorescence. C) BiFC assay confirmed interaction between BcbHLH30 and BcMLP328. Bar = 10 μm. D) Co-IP assay of BcMLP328–BcbHLH30 interaction. BcbHLH30-FLAG and BcMLP328-His were transiently expressed in N. benthamiana leaves. Total protein was extracted 3 d later. Immunoprecipitation was performed with anti-FLAG antibody. Anti-FLAG and anti-His antibodies were used to detect BcbHLH30-FLAG and BcMLP328-His protein, respectively. E) Silencing of BcbHLH30 delayed flowering in Pak-choi. Bar = 2 cm. F) Expression of BcERF070 in pTY-S and pTY-BcbHLH30 in Pak-choi using qPCR. BcACTIN was used as an internal control gene. G, H) Days to flowering (G) and rosette leaf numbers (H) in pTY-S and pTY-BcbHLH30 plants. I) Expression of BcLFY in pTY-S and pTY-BcbHLH30 in Pak-choi using qPCR. Error bars are ± sd from three biological replicates (n = 3 plants for each replicate). For (B, F to I), *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t-test). YFP, yellow fluorescent protein; BF, bright field image; Merge, overlay of YFP and bright field images.
regulation, flower-related genes (BcFT, BcLFY, BcFLC, and BcTSF) were cloned to perform the yeast one-hybrid (Y1H) assay. BcbHLH30 directly bound to the promoter of BcLFY (Fig. 5A; Supplementary Fig. S8A). The β-galactosidase activity in BcbHLH30-proBcLFY was significantly higher than that in the control (Fig. 5A), further demonstrating that BcbHLH30 directly bound to the BcLFY promoter. A LUC assay was performed to confirm the direct binding of BcbHLH30 to the BcLFY promoter. The LUC fluorescence intensity of the BcbHLH30 and proBcLFY combination was stronger than that of the control (Fig. 5B), indicating that BcbHLH30 directly activated the expression of BcLFY. ChIP-qPCR assay showed that the P2 fragment containing the CATTGG-box was significantly enriched in 35S:FLAG-BcbHLH3030F Pak-choi (Fig. 5C; Supplementary Fig. S8B). In addition, the EMSA experiment confirmed that BcbHLH30 could bind to the CATTGG-box in the BcLFY promoter (Fig. 5D). These results indicate that BcbHLH30 induces BcLFY expression by directly binding to its promoter.

Because BcMLP328 interacts with BcbHLH30 (Fig. 4, A to D), we assumed that BcMLP328 might affect the activation of BcbHLH30 to BcLFY. We confirmed this assumption by co-transforming 35S:BcMLP328, 35S:BcbHLH30, and proBcLFY-LUC into N. benthamiana leaves. A LUC-reporting system revealed stronger fluorescence signaling in 35S: BcMLP328, 35S:BcbHLH30, and proBcLFY-LUC compared with the combination of 35S:BcbHLH30 and proBcLFY-LUC (Fig. 5E), suggesting that BcMLP328 enhanced the activation ability of BcbHLH30 to BcLFY. In addition, using heterologous overexpression in Arabidopsis, the effects of BcMLP328 and BcbHLH30 on flowering were explored. The flowering time of co-overexpression of BcMLP328 and BcbHLH30 was earlier than that of BcbHLH30 overexpression lines, both of which were earlier than that of the WT (Supplementary Fig. S9, A to D). Accordingly, AtLFY, AtFT, and AtSOC1 expressions in plants co-expressing BcMLP328 and BcbHLH30 were significantly higher than the expression in BcbHLH30 overexpression plants (Supplementary Fig. S9E). These results demonstrate that BcMLP328 can form a complex with BcbHLH30 to enhance the transciptional activity of BcbHLH30 toward BcLFY and induce flowering.

BcERF070 impairs the activation of the BcMLP328–BcbHLH30 complex to BcLFY

The results that BcMLP328 interacts with BcERF070 (Fig. 2) and forms a complex with BcbHLH30 (Fig. 4) suggest that BcERF070 affects the interaction between BcMLP328 and BcbHLH30. To test this suggestion, LCI was performed. 35S: BcERF070, BcMLP328-LUC, and BcbHLH30-nLUC co-infiltrated N. benthamiana leaves. As expected, fluorescence was evident in the BcMLP328 and BcbHLH30 groups (Fig. 6A). However, the fluorescence intensity was significantly reduced with the addition of BcERF070 (Fig. 6A). EMSA was used to provide confirmation. The binding complex was most evident in the presence of BcMLP328, BcbHLH30, and BcLFY biotin probes (Fig. 6B). However, this binding complex was absent when BcERF070 was added (Fig. 6B). These results suggest that BcERF070 negatively affects the interaction between BcMLP328 and BcbHLH30, which may decrease the transcriptional activation of BcMLP328–BcbHLH30 complex to BcLFY.

In addition, the above data indicate that pTY-BcERF070 plants flowered earlier than pTY-5 plants (Fig. 1F), while pTY-BcMLP328 plants show later flowering than the control (Fig. 3G; Supplementary Fig. S6). To explore this further, we generated double-silenced Pak-choi (pTY-BcMLP328 and pTY-BcERF070). If BcERF070 delays flowering by disrupting the BcMLP328–BcbHLH30 complex, the flowering times of pTY-BcMLP328 and pTY-BcMLP328/pTY-BcERF070 should be similar. Unexpectedly, we found that the double-silenced (pTY-BcMLP328/pTY-BcERF070) Pak-choi flowered earlier than the pTY-BcMLP328 plants (Fig. 6, C to E). The number of days to flowering was approximately 28 d in pTY-BcERF070 plants, 35 d in pTY-BcMLP328/pTY-BcERF070 plants, and 38 d in pTY-BcMLP328 plants (Fig. 6F). The number of rosette leaves was approximately five in pTY-BcERF070 plants, eight in pTY-BcMLP328/pTY-BcERF070 plants, and 10 in pTY-BcMLP328 plants (Fig. 6F). These results imply that BcERF070 may also affect flowering through pathways other than disruption of the BcMLP328–BcbHLH30 complex.

BcERF070 enhances BcBBX29 expression by directly binding to its promoter

To further study other pathways of BcERF070 in regulating flowering, a DAP-seq assay was performed using the BcERF070 protein. Stronger BcERF070-binding peaks were observed in the promoter region of BcBBX29 according to IGV visualization using DAP-seq analysis (Supplementary Fig. S10A). We also noted the presence of possible binding regions on the promoter of BcBBX29 (Supplementary Fig. S10B). YIH assays were performed to verify the binding of BcERF070 to the BcBBX29 promoter (Fig. 7A). β-Galactosidase activity in the experimental group of BcERF070-proBcBBX29 was significantly higher than that of the control groups (Fig. 7B). Dual-LUC reporter assays were then performed. The signals of proBcBBX29-LUC and 35S: BcERF070 were stronger than those in the control group, suggesting that BcERF070 induces BcBBX29 expression (Fig. 7C).

Furthermore, EMSA confirmed that BcERF070 could bind specifically to the cis-element in BcBBX29 promoter (Fig. 7D). These results suggest that BcERF070 binds to the promoter of BcBBX29 to enhance its expression.

The effect of BcBBX29 on flowering was determined using the VIGS assay. Compared with the control, BcBBX29-silenced plants showed an earlier flowering phenotype (Fig. 7, E and F). The number of days to flowering in pTY-BcBBX29 plants was earlier, whereas the rosette leaf number in pTY-BcBBX29 plants was lower than that in the control plants (Fig. 7, G and H). BcFT expression was induced after silencing of BcBBX29 in Pak-choi (Fig. 7I). We also
**Figure 5.** BcHLH30 binds to the BcLFY promoter and enhances its expression. **A)** Y1H assay and β-galactosidase activity assays demonstrating the binding of BcHLH30 to the promoter of BcLFY. pLacZi-proBcLFY and pJG-BcHLH30 were used as the test groups. pLacZi, pJG-BcHLH30, pLacZi-proBcLFY, pJG, pLacZi, and pJG were used as controls. Only the group with pLacZi-proBcLFY and pJG-BcHLH30 displayed β-galactosidase activity. Images were digitally extracted for comparison. **B)** Transient expression assay demonstrating that BcHLH30 could bind to the promoter of BcLFY and enhance BcLFY expression. LUC, firefly luciferase; REN, Renilla luciferase. **C)** ChIP-qPCR assay showing the relative amounts of BcLFY fragments in 35S: FLAG-BcHLH30 seedlings. **D)** Electrophoretic mobility shift assay showing that the BcHLH30 protein directly binds to the BcLFY promoter at the P2 site. For the mutant probe, the putative E-box was replaced with AAAAA. 10× and 100× represent the competitor rates. **E)** Transient expression assay demonstrating that BcMLP328 can enhance the binding ability of BcHLH30 to BcLFY promoter. Error bars indicate the standard deviation of approximately three plants. Error bars are ±sd from three biological replicates (n = 3 plants for each replicate). For (A to C, E), *P < 0.05, ***P < 0.001 (t-test).
Figure 6. BcERF070 impairs the activation activity of BcMLP328–BcbHLH30 complex. A) BcERF070 affects the activation activity of BcMLP328–BcbHLH30 complex in N. benthamiana leaves. Luciferase complementation imaging assay revealed reduced luciferase activity when BcERF070 was present in the BcMLP328–BcbHLH30 complex. Error bars are ±SD from three biological replicates (n = 3 plants for each replicate). Significant difference was determined using t-test (**P < 0.01, ***P < 0.001). B) Electrophoretic mobility shift assay indicated that BcERF070 cannot bind to the promoter of BcLFY, but impairs BcMLP328/BcbHLH30 activation to BcLFY. C) The flowering phenotype of pTY-S, pTY-BcERF070, pTY-BcMLP328, pTY-BcERF070/pTY-BcMLP328 Pak-choi. Bar = 2 cm. D) Expression levels of BcERF070 in pTY-S, pTY-BcERF070, and pTY-BcERF070/pTY-BcMLP328 Pak-choi. E) Expression levels of BcMLP328 in pTY-S, pTY-BcMLP328, and pTY-BcERF070/pTY-BcMLP328 Pak-choi. F) Days to flowering (top) and rosette leaf numbers (bottom) in pTY-S, pTY-BcERF070, pTY-BcMLP328, pTY-BcERF070/pTY-BcMLP328 Pak-choi. Error bars are ±SD from three biological replicates (n = 3 plants for each replicate). For D to F, the letters above the bars indicate significant differences determined using one-way ANOVA (P < 0.05).
observed overexpression of BcBBX29 caused later flowering in Arabidopsis, which had more rosette leaves and days to flowering (Supplementary Fig. S11, A to D). These results suggest that BcERF070 delays flowering by directly activating BcBBX29 transcription.

Discussion

Floral transformation plays a key role in the shift from vegetative to reproductive growth. Studying the molecular mechanism of flowering increases crop yield and promotes crop quality (Krieger et al. 2010; Wei et al. 2010). Ethylene is a plant
hormone that affects plant growth and development, including seed germination, flower and leaf shedding, sex differentiation, and pathogen response. Ethylene reportedly inhibits blooming in Arabidopsis through the repression of LFY and SOC1 (Achard et al. 2006, 2007). In short-day chrysanthemum plants, ethylene increases the number of late flowering rosette leaves, which might downregulate the expression of AP1/FRUITFUL-like (Konishi et al. 1985; Cheng et al. 2023a, 2023b). Consistent with this, the flowering time of Pak-choi was significantly delayed, and rosette leaf numbers increased under ethylene treatment (Fig. 1, A to C; Supplementary Fig. S1). This implies that ethylene-inhibited flowering is conserved in both long- and short-day plants.

In the present study, ethylene induced the expression of BcERF070 to delay flowering in Pak-choi (Fig. 1). A previous study found that GA promotes flowering in Arabidopsis overexpressing ERF070 (Ramiah et al. 2014). Ethylene and GA have opposite effects on ERF070 expression. This may be related to the antagonism between ethylene and GA. Ethylene reduces the expression of EATB to inhibit ent-kaurene synthase 2, which biosynthesizes GA. Furthermore, low GA content reduces the degradation of DELLA proteins (Qi et al. 2011; Wang et al., 2023). These findings suggest that ERF070 may be a key node in the response of the two hormonal regulatory regulations to flowering.

Silencing of BcERF070 increased flowering time in Pak-choi (Fig. 1, F and G). By contrast, a previous study reported that silencing BcERF070 in Pak-choi resulted in delayed flowering (Yuan et al. 2020). These different results might be related to the different growth conditions. The silenced plants were grown at a lower temperature (10 °C) in the previous study, whereas the BcERF070-silencing plants were grown in a chamber at a suitable temperature of 24 °C in this study. Furthermore, overexpression of BcERF070 could affect the development of pistils and stamens in Arabidopsis (Supplementary Fig. S3). AP2 controls the period of endosperm cellularization and restricts endosperm development by limiting the elongation of the cell wall of the seed coat body during the early stages of seed development. The overexpression of BABY BOOM can reduce fertility in N. benthamiana, which might affect cell proliferation (Srinivasan et al. 2007). In Trifolium alexandrinum, T2 seeds showed lower fertility in HARDY-overexpressing plants due to the limited efficiency of hand tripping in achieving self-pollination and genetic barriers (Abogadallah et al. 2011).

Y2H revealed the interaction of BcERF070 with BcMLP328 (Fig. 2). BcMLP328 is a major latex-like protein that encodes the Bet_v_1 motif. Overexpression of BcMLP328 induced flowering, whereas silencing of BcMLP328 reduced flowering in Pak-choi (Fig. 3; Supplementary Fig. S6). The expression of flower promoter genes was upregulated (BcSPL15, BcFT, BcSOC1, BcAP3, and BcLFY), while that of BcTEM was downregulated in BcMLP328-OE plants (Fig. 3F; Supplementary Fig. S5E). These findings demonstrate that BcMLP328 acts as a flowering promoter in Pak-choi. We transferred BcMLP328 into Arabidopsis using heterologous overexpression. The results showed that the flowering of BcMLP328-OE plants occurred significantly earlier in both Arabidopsis and Pak-choi (Supplementary Fig. S5). In Arabidopsis, the overexpression of AtMLP328 delays flowering (Gao et al. 2013). This suggests that homologous genes in different crops have different functions. In rice, OsPAD4 induces the accumulation of the phytoalexin momilactone through the jasmonic acid pathway to inhibit the growth of the rice fungal pathogen M. oryzae. This differs from the AtPAD4 function, which acts as a defense mechanism via the salicylic acid pathway (Ke et al. 2014). OsBG2/OsBAK1 regulated grain size and number and functioned differently in Indica and Japonica rice backgrounds. This may be related to a nonsynonymous mutation in the kinase domain (Yuan et al. 2017).

The B-box family is an important gene family involved in the regulation of flowering. Many transcription factors regulate plant flowering via the B-box family. In chrysanthemums, CmERF3 delays flowering by interacting with CmBBX8 to reduce the expression of CmFT1 (Cheng et al. 2023a, 2023b). Another study reported that CmRCD1 repressed flowering by directly interacting with CmBBX8 in summer chrysanthemum (Wang et al. 2021a, 2021b). Herein, BcERF070 directly bound to the promoter of the B-box gene BcBBX29 to induce its expression (Fig. 6; Supplementary Fig. S11). In Arabidopsis, BBX29 interacts with CO and reduces the activation activity of CO on FT, ultimately repressing flowering (Liu et al. 2020; Wang et al. 2021a, 2021b). Similarly, overexpression of BcBBX29 caused a late flowering phenotype and decreased the expression of AtFT in Arabidopsis (Supplementary Fig. S11E). Silencing BcBBX29 promoted flowering and increased the expression of BcFT (Fig. 7I). These findings indicate that the flowering regulation mechanism of BBX29-FT may be conserved in Pak-choi.

In the present study, we conclusively demonstrate a potential model for the regulation of ethylene during late flowering in Pak-choi (Fig. 8). BcERF070 is a downstream response and is induced by ethylene. The regulation of BcERF070 on flowering may involve several processes in Pak-choi. First, BcERF070 inhibits flowering by impairing the activation of the BcMLP328–BcbHLH30 complex to BcLFY in Pak-choi. Second, BcERF070 directly promotes the expression of the flowering inhibitor BcBBX29 and delays flowering by reducing BcFT expression. Our research provides a mechanistic framework for ethylene-mediated late flowering and lays the foundation for the future application of ethylene to regulate crop production.

Materials and methods

Plant materials and growing conditions

Brassica rapa ssp. chinensis (Pak-choi) ‘Suzhouqing’ and ‘Caixin’ were used in the following assays. ‘Suzhouqing’ sprouting seeds were stored at 4 °C for at least 2 wk. Briefly, ‘Suzhouqing’ seeds were first soaked in water for 8 h and then left to vernalize at 4 °C for more than 2 wk.
Caixin' seeds did not need to be vernalized. The seeds were germinated and planted in pots containing a soil mixture in a chamber with a 16-h photoperiod (15,000 lx) at 24 °C and 8 h of darkness at 18 °C.

*N. benthamiana* and Arabidopsis (*A. thaliana*) (Col-0) were grown in plant chambers (16 h light at 24 °C and 8 h dark at 18 °C). The flowering time was scored as the number of days from germination to flowering. The number of rosette leaves was then counted.

**Expression pattern assay and treatments**

For gene expression pattern analysis, leaves were collected at 10, 15, 20, and 25 d in the vegetative period and 30 d in the reproductive period after germination and used to analyze the expression of BcERF070. All samples were put in liquid nitrogen and stored at −80 °C.

To study the response of BcERF070 to ethylene in Pak-choi, 2-wk-old seedlings were sprayed with 0.1 mM ethylene (Solarbio, Beijing, China). Ethephon powder was first dissolved in water to make a mother liquor with a concentration of 1 mM and then diluted with water at a ratio of 1:10 to make the 0.1 mM ethylene solution. Water was used as a control. Leaves were harvested at 0, 2, 6, 8, 12, and 24 h. The samples were stored at −80 °C for further analysis.

To study the effect of ethylene on the flowering time of Pak-choi, 2-wk-old seedlings were sprayed with 0.1 mM ethylene solution every 2 d.

**Yeast two-hybrid assay**

Yeast two-hybrid (Y2H) assays were performed using the Matchmaker Gold Yeast Two-Hybrid System kit (Clontech Laboratories Inc., Palo Alto, CA, USA). The open reading frame (ORF) of BcMLP328 was subcloned into the pGADT7 vector using NdeI and EcoRI restriction sites. ORFs of BcERF070 and BcbHLH30 were cloned into the pGBKT7 vector using NdeI and BamHI restriction sites (primers are listed in Supplementary Table S1). The recombinant plasmid pGADT7-BcMLP328 was co-transformed into Y2H Gold cells, together with pGBKT7-BcERF070 and pGBKT7-BcbHLH30. The transformed cells were grown on SD/-Trp/-Leu media at 28 °C for 3 d, then placed on the SD/-Trp/-Leu/-His/-Ade media for 3 d. Single-positive clones were diluted 100× and 1,000×. pGBK7-Lam and pGADT7-T were used as negative controls. Positive controls were pGADT7-T and pGBKT7-53.

**LCl assay**

Primers for nLUC-BcERF070-F/R, nLUC-BcbHLH30-F/R, and cLUC-BcMLP328-F/R with XbaI and SpeI sites were used to introduce BcERF070, BcbHLH30, and BcMLP328 into the final pCB1300 vectors (primers are listed in Supplementary Table S1). The recombinant plasmids were transformed into Agrobacterium tumefaciens strain GV3101. Different combinations of plasmids, nLUC-BcERF070, cLUC-BcMLP328, nLUC-BcbHLH30, and cLUC-BcMLP328, were coexpressed in 4-wk-old *N. benthamiana* leaves. The empty
pCB1300-nLUC and pCB1300-cLUC vectors were used as negative controls. Transformed N. benthamiana leaves were placed in the chamber for 1 d in the dark and light for 2 d. Before observing the fluorescent signal using a charge-coupled device camera, the leaves were sprayed evenly with 100 mM D-luciferin and kept in the dark for 5 min (Yeasen, Shanghai, China). Solis image analysis software (Andor Technology, Belfast, UK) was used to calculate luciferase activity per pixel per exposure time (Lu et al. 2017).

Bimolecular fluorescence complementation (BiFC) assay

For the BiFC assay, BcMLP328 was fused to the C-terminal yellow fluorescent protein (YFP) using BamHI and XbaI restriction sites. ORFs of BcERF070 and BcbHLH30 were fused to the N-terminal YFP using BamHI and XbaI sites (primers are listed in Supplementary Table S1). The plasmids were introduced into GV3101 cells. Two pairs of constructs, YFPc-BcMLP328 and YFPn-BcERF070, and YFPc-BcMLP328 and YFPn-BcbHLH30, were co-infiltrated into 4-wk-old N. benthamiana leaves. YFP fluorescent signals were visualized by confocal microscopy using an LSM780 microscope (Carl Zeiss, Jena, Germany) after incubation for 60 h (lasers: Ar ion laser, 514 nm, intensity: 2%; collection bandwidth: 525–570 nm, and gains: 700 V). The images were processed using Zeiss LSM780 software.

Pull-down assay

The full-length ORF of BcERF070 was cloned into pGEX-4T-1 (GST) with EcoRI and XhoI sites (primers are listed in Supplementary Table S1). The ORF of BcMLP328 was recombined into pET-28a (His) with EcoRI and SacI sites (primers are listed in Supplementary Table S1). The constructs were transformed into Escherichia coli Rosetta 2 and induced with 100 μM isopropyl β-D-1-thiogalactoside at 16 °C for 12 h. The target proteins were purified by ultrasonic crushing. GST-BcERF070 and BcMLP328-His were mixed, and empty GST and BcMLP328-His were used as controls. Proteins were incubated at 4 °C for 2 h, and 100 μL of supernatant was used as the input. The remaining cells were incubated with GST beads at 4 °C for 12 h and then washed three times. Finally, the eluent was used as the output. Immunoblot analysis was performed to detect proteins using anti-His antibodies (Abcam, Shanghai, China).

Co-immunoprecipitation (Co-IP) assay

For the Co-IP assay, 35S:BcERF070-FLAG, 35S:BcbHLH30-FLAG, and 35S:BcMLP328-HIS were constructed (the primers are listed in Supplementary Table S1). The assay was performed according to a previously described method (He et al. 2016). The fusion proteins were used to infect 4-wk-old N. benthamiana leaves. After 60 h, total protein was extracted with lysis buffer and immunoprecipitated with anti-FLAG M2 magnetic beads (Sigma-Aldrich, St. Louis, MO, USA) following a previously described protocol (Muñoz and Castellano 2018). The samples were analyzed using western blotting. Anti-FLAG (Sigma-Aldrich) and anti-His (Abcam) antibodies were used to detect BcERF070-FLAG, BcbHLH30-FLAG, and BcMLP328-His, respectively.

Genetic transformation of A. thaliana

The ORF sequences of BcERF070, BcMLP328, and BcBBX29 were introduced into the pCB1302 vector and fused to the 3′-end of the green fluorescent protein (GFP) reporter gene in-frame under the control of the CaMV 35S promoter (primers are listed in Supplementary Table S1). The resulting binary vectors 35S:BcERF070, 35S:BcMLP328, and 35S:BcBBX29 were transformed into A. tumefaciens strain GV3101, which was used to transform Co-0 using the floral dip method (Clough and Bent 1998). Transgenic plants were selected using 16 mg/L hygromycin (Solarbio). The ORF of BcbHLH30 was constructed using the pCB3302 vector and transformed into GV3101 cells using the same method (primers are listed in Supplementary Table S1). Co-overexpressing plants were generated from BcMLP328-overexpressing Arabidopsis plants. Transgenic plants were selected using 25 mg/L Basta (Yeasen). The flower organs and seeds of overexpressing and the WT plants were observed using a model M165FC stereo fluorescence microscope (Leica, Wetzlar, Germany). Transgenic plants were confirmed using western blotting and qPCR.

Genetic transformation of Pak-choi

The construction methods of 35S:BcMLP328 were followed as described previously for Arabidopsis transformation (Clough and Bent 1998). Pak-choi seeds were sterilized with 75% alcohol for 2 min and 10% sodium hypochlorite for 18 min, washed five times with distilled deionized water and grown on 1/2 MS medium for 6 d. Cotyledons with stalks without shoots were cut off and placed in preculture medium for 3 d. The explants were then infected by Agrobacterium harboring BcMLP328 and planted on the co-medium in the dark. After incubating for 3 d, the explants were grown in differentiating medium for 20 to 30 d and then transferred to selection medium for 2 wk. Finally, regenerated plants were grown in rooting medium for at least 10 d. The transgenic Pak-choi and the WT plants were cultivated in a chamber using (16 h of light at 24 °C and 8 h dark at 18 °C) for further analysis. Details of the medium have been reported previously (Guo et al. 2021).

Virus-induced gene silencing (VIGS) in Pak-choi

A 40-bp sequence of BcMLP328 and BcbHLH30 was designed and then reverse-complemented to obtain an 80-bp palindrome sequence (5′-GTGACGGAGGTCCATTGAAAGGACGATGGAGAAACACTAGTGTTTCTCCATGCTTTTTTAATTGGAACCTCCGTCAC-3)′. The silent sequence of BcBBX29 was designed following the same rules.
(5'-GTAGGAGTGGGGCGAATGGTCTGAGTLCAGAC-CAGGGCCCTGTTCTGACTCAGAACTTCTGGCACA-

CCCTCACA-3'). The silent sequences are listed in Supplementary Table S1. The BcMLP328-silencing vector (pTY-BcMLP328), BcbHLH30-silencing vector (pTY-BcbHLH30), and BcBBX29-silencing vector (pTY-BcBBX29) were synthesized and constructed using GenScript (Nanjing, China) according to a previous study (Yu et al. 2018). The silencing vector of BcERF070 has been previously described (Yuan et al. 2020). The empty pTY vector served as the negative control, whereas the pTY-BcPDS vector acted as the positive control. Two-wk-old Pak-choi seedlings were used for the VIGS assay. pTY-BcERF070, pTY-BcMLP328, pTY-BcBBX29, pTY-BcPDS, and pTY-S empty vector (5 μg) were coated with gold powder and then bombarded into Pak-choi leaves with PDS1000/He gene-gun (Bio-Rad, Hercules, CA, USA) as previously described (Hamada et al. 2017). One month later, qPCR was performed to confirm the silencing effect of BcERF070, BcMLP328, BcbHLH30, and BcBBX29 in Pak-choi.

Yeast one-hybrid assay
The BcBBX29 promoter was cloned into the pLacZi vector at the EcoRI and Xhol sites, and the coding frame of BcERF070 was cloned into the pG vector with EcoRI and Xhol sites. The combined vectors pLacZi-proBcBBX29 and pG-BcERF070 were transformed into EGY48 using a previously described method (Gao et al. 2013). Negative controls were pLacZi-proBcBBX29, pG, pLacZi, pG-BcERF070, pLacZi, and pG. The transformed cells were subsequently incubated on SD-Trp/-Ura media for 3 d at 28 °C and then placed on SD-Trp/-Ura/Raf/X-Gal (20 μg/mL) until blue color development. β-Galactosidase Assay Kit (Beyotime, Nanjing, China) was used to measure the β-galactosidase activity.

For BcLFY, the promoter was cloned into a pLacZi vector for the Y1H assay. The pLacZi-proBcLFY and pG-BcbHLH30 vectors were transformed into EGY48. The transformed cells were incubated on SD-Trp/-Ura medium for 3 d at 28 °C and then selected with SD-Trp/-Ura/Raf/X-Gal (20 μg/mL) media. β-Galactosidase activity was detected using a β-galactosidase Assay Kit (Beyotime).

Transient dual-luciferase assay
The promoter regions of BcBBX29 and BcLFY were constructed in the pGreenII 0800-LUC vector as reporters with Kpn I and Xho I restriction sites, respectively (primers are listed in Supplementary Table S1). To determine whether BcERF070 could bind to the BcBBX29 promoter, the 35S: BcERF070 empty vector and proBcBBX29-LUC were transiently co-expressed in 4-wk-old N. benthamiana leaves, as previously described (Yuan et al. 2022). To study whether BcMLP328 affects BcLFY transcription by interacting with BcbHLH30, we designed three groups, empty vector and proBcLFY-LUC, 35S:BcbHLH30 and proBcLFY-LUC, as well as 35S:BcMLP328, 35S:BcbHLH30, and proBcLFY-LUC, to express in a N. benthamiana leaf. After 60 h, the transformed N. benthamiana leaves were sprayed evenly with beetle luciferin in the dark for 10 min, and the fluorescent luciferase signal was observed using a charge-coupled device camera. The activities of firefly luciferase (LUC) and Renilla (REN) were measured according to a Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI, USA).

Electrophoretic mobility shift assay (EMSA)
For the EMSA, 18-bp promoter fragments of BcBBX29 were synthesized with or without a biotin label. The ORF of BcERF070 was introduced into pET28a with EcoR I and Sac I restriction sites, fused with His, and transformed into E. coli BL21 cells for protein purification. Three fragments of BcLFY promoter were synthesized and labeled with biotin. BcbHLH30 was fused with His and introduced into E. coli BL21 cells. Then, 100 μM isopropyl β-D-1-thiogalacto-pyranoside was used to induce His-fusion protein. The recombinant protein was purified using a HisSeq Ni-NTA Agarose gel (Beyotime). EMSA was performed according to the manufacturer’s protocol of the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Waltham, MA, USA).

DNA-affinity purification sequencing (DAP-seq)
DAP-seq binding assays were performed as previously described (O’Malley et al. 2016; Bartlett et al. 2017). Fresh Pak-choi leaves were ground into a fine powder to isolate nuclear and genomic DNA (Bluescape, Heibei, China). Genomic DNA was fragmented to nearly 200 bp using a model M220 device. (Covaris, Woburn, MA, USA) to prepare the DNA library. The coding sequencing of BcERF070 was cloned into the pFN19K T7 SP6 Flexi expression vector with a halo tag. The expressed proteins were directly captured using Magne Halo tag beads (Promega). The protein-bound beads were incubated with 50 ng of adapter-ligated gDNA fragments on a rotator for 1 h at 22 °C in 50 μL wash/bind buffer. The beads were washed three times with the same wash buffer to remove unbound DNA fragments. Reads were mapped to the Pak-choi genome sequence (http://tbir.njau.edu.cn/NhCCDbHubs/) using BOWTIE2 (Langmead and Salzberg 2012). Peak calling was done using Macs2 (Zhang et al. 2008). The association of DAP-seq peaks located within 1 kb upstream or downstream of the transcription start site was analyzed using Homer (Heinz et al. 2010). The FASTA sequences were obtained using BEDTools for motif analysis (Quinlan and Hall 2010). Motif discovery was performed using MEME-ChIP suite 5.0.5 (Machanick and Bailey 2011). The data were then visualized using the Integrative Genomics Viewer (IGV, v 2.8.3). DAP-seq data were downloaded from the National Center for Biotechnology Information SRA database (https://www.ncbi.nlm.nih.gov/bioproject; ID: PRJNA942073).

Chromatin immunoprecipitation (ChIP) assay
The chromatin immunoprecipitation (ChIP) assay was performed as previously described (Zhang et al. 2017; Yang et al. 2023). Briefly, fresh leaves from BcbHLH30-overexpressing
Pak-choi were treated with 0.1% formaldehyde to cross-link protein–DNA complexes. After isolation, sonication, and centrifugation (16,000 × g, 45 min, 4 °C), the supernatants were reserved for incubation with FLAG antibody, as well as "INPUT". After immunoincubation of the protein–DNA complex, cross-linked DNA reversal and DNA purification were performed using the EpiQuikTM Plant ChIP Kit (Base Catalog # P-2014-24; Epigentek, Farmingdale, NY, USA). Quantitative data for IgG were used as the negative controls. The primers used are listed in Supplementary Table S1.

Real time quantitative polymerase chain reaction (qPCR) expression analysis
Total RNA from Pak-choi and Arabidopsis was extracted using the RNA Simple Total RNA Kit (Tiangen, Beijing, China). Reverse transcription was performed with Evo M-MLV RT Kit with gDNA Clean for qPCR (AG, Shanghai, China) using 1 μg RNA as a template. Hieff qPCR SYBR Green Master Mix (Low ROX Plus) was used for qPCR with the obtained cDNA as template (Yeasen). All primers used for qPCR are listed in Supplementary Table S1. The PCR program was 40 cycles of 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 30 s using Bio-Rad iQ5. The 2−ΔΔCT method was used to calculate the data (Livak and Schmittgen 2001). Relative gene expression was determined using three independent biological replicates.

Statistical analysis
Statistical significance was determined by t-test or one-way ANOVA with Tukey’s test analysis using the IBM SPSS Statistics 25.0 software (IBM SPSS, Chicago, USA). Significant differences were indicated by *P < 0.05, **P < 0.01, or ***P < 0.001.

Accession numbers
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: BcERF070 (BraC07g014310); BcMLP328 (BraC01g045390); BcbHLH30 (BraC07g030490); BcLFY (BraC02g045610); BcBBX29 (BraC02g014130). Sequence data used in this article can be found in the Arabidopsis Information Resource (https://www.arabidopsis.org/index.jsp) under the following accession numbers: AtFT (AT2G03220), AtLFY (AT5G61850), AtAP3 (AT3G54340), AtSOC1 (AT2G45660), AtSPL15 (AT3G57920) and AtTEM (AT1G25560).

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Author contributions
Z.H.Y. designed the research and wrote the manuscript. X.S.C. analyzed data. Y.L., S.H.A.H., D.X., and J.J.W. revised the manuscript. Y.L. and T.K.L. advised on the design, results and edited the manuscript. All authors read and approved the final manuscript.

Supplementary data
The following materials are available in the online version of this article.

Supplementary Figure S1. Effect of ethylene (ET) on Pak-choi flowering.

Supplementary Figure S2. Flowering phenotypes in pTY-S and pTY-BcPDS Pak-choi through TYMV-mediated silencing system and silencing effect of BcERF070.

Supplementary Figure S3. Heterologous expression of BcERF070 delays flowering in Arabidopsis.

Supplementary Figure S4. Identification of BcMLP328-OE Pak-choi using western blotting and qPCR.

Supplementary Figure S5. Heterologous expression of BcMLP328 promotes flowering in Arabidopsis.

Supplementary Figure S6. Silencing of BcMLP328 in Pak-choi delays flowering.

Supplementary Figure S7. Expression of BcFT in pTY-S and pTY-BcBHLH30 Pak-choi.

Supplementary Figure S8. BcBHLH30 does not bind to the promoters of BcFT, BcTSF, and BcFLC, and BcBHLH30 protein accumulates in BcBHLH30-OE lines.

Supplementary Figure S9. Heterologous expression of BcBHLH30 and BcMLP328 promote flowering in Arabidopsis.

Supplementary Figure S10. IGV browser of BcERF070 binding to BcBBX29 target gene.

Supplementary Figure S11. Heterologous expression of BcBBX29 delays flowering in Arabidopsis.

Supplementary Figure S12. Purified proteins and western blots in Co-IP.

Supplementary Table S1. Primers used in this article.

Supplementary Table S2. Potential interacting protein of BcMLP328.

Potential interacting protein of BcMLP328.

Supplementary Table S3. Potential interacting protein of BcMLP328.

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Conflict of interest statement. The authors declare no conflict of interest.

Data availability
The DNA DAP-seq data have been deposited in the NCBI database with the accession code PRJNA942073. All data can be found in the main text.
References


