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Functional analysis of mango *MiFCA2* gene involved in the regulation of flowering and stress response in *Arabidopsis*

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ABSTRACT

FLOWERING CONTROL LOCUS A (FCA), as a core component of the autonomous flowering time pathway, can also regulate flowering time in response to environmental temperature. However, the function of *FCA* in *Mangifera indica* (mango) remains unclear. Elucidating the regulatory pathways of *FCA* genes under temperature and abiotic stress could improve mango flowering and resilience under unfavourable environmental conditions. In this study, two *FCA2* genes were isolated from two mango varieties with different flowering habits and named *MiFCA2-SJ* and *MiFCA2-JH*. Both proteins contain two RRM domains and one WW domain, along with a relatively long glycine-rich region. The expression of *MiFCA2-SJ* peaked during early flowering, whereas that of *MiFCA2-JH* peaked towards the end of flowering. Both genes were expressed primarily in leaves. In transgenic *Arabidopsis*, *MiFCA2-SJ* plants exhibited early flowering at 13 °C/31 °C but no significant phenotypic change at 22 °C, whereas *MiFCA2-JH* plants flowered earlier at all three temperatures. Furthermore, *MiFCA2-SJ/JH* participated in the ABA signalling pathway and responded differently to drought

and salt stress. MiFCA2-SJ/JH were localized to the nucleus. Protein interaction assays demonstrated that MiFCA2-SJ interacts with MiFY, the stress-related proteins MiPP2C25B and MiZFP4. In summary, *MiFCA2* may play a key role in the temperature-regulated flowering and stress adaptation of mango.

Keywords: *FLOWERING CONTROL LOCUS A*; Mango; Functional analysis; Flowering time; Abiotic stress

1 Introduction

In woody plants, the progression to flowering occurs through three sequential phases: floral induction, followed by bud differentiation and culminating in organ development. The initial stage, floral induction, is particularly crucial as it is precisely controlled by integrated environmental cues and internal plant factors (Higgins et al. 2010; Abou-Elwafa et al. 2011; Sun et al. 2022). In *Arabidopsis thaliana*, several distinct regulatory pathways, including the photoperiod, vernalization, gibberellin, temperature, autonomous and age pathways, have been identified under vernalization and facultative long-day conditions (He and Amasino 2005; Srikanth and Schmid 2011; Song et al. 2018; Zhao et al. 2025). While external environmental cues can induce flowering under favourable conditions, plants also possess an endogenous flowering mechanism that is triggered once a certain developmental stage is reached, independent of external signals. This endogenous mechanism is referred to as the autonomous flowering time pathway (Liu et al. 2015). The autonomous pathway promotes flowering primarily by repressing the expression of the central flowering inhibitor and vernalization regulator *FLOWERING LOCUS C (FLC)* (Michaels et al. 2003; Yan et al. 2010; Li et al. 2018). Known components of this pathway include *FLOWERING CONTROL LOCUS A (FCA)*, *FLOWERING LOCUS D (FLD)*, *FLOWERING LOCUS K (FLK)*, *FLOWERING LOCUS PA (FPA)*, *FLOWERING LOCUS VE (FVE)*, *FLOWERING LOCUS Y (FY)*, and *LUMINIDEPENDENS (LD)* (Simpson et al. 2004;

Marquardt et al. 2006; Srikanth and Schmid 2011; Amara et al. 2023). Although mutations in these genes typically lead to delayed flowering under both long-day (LD) and short-day (SD) conditions, this phenotypic defect can be effectively reversed by vernalization treatment (Abou-Elwafa et al. 2011).

As a core component of the autonomous flowering pathway in plants, the RNA-binding protein FLOWERING CONTROL LOCUS A (FCA) was among the first to be identified (Macknight et al. 1997). It accelerates flowering by suppressing *FLC* transcription (Bäurle and Dean 2006). *FCA* also autoregulates its expression by promoting premature transcription termination of its own gene (Simpson et al. 2003; Quesada et al. 2003). In *Arabidopsis thaliana*, the expression of *FCA* is subject to post-transcriptional regulation via alternative splicing of its pre-mRNA, which yields four distinct isoforms (α , β , γ , and δ) through differential processing of intron 3, of these, only the fully spliced γ isoform is functional in controlling flowering time (Macknight et al. 2002). Impairment of FCA function results in delayed flowering. Conversely, its overexpression promotes early flowering, a consequence observed consistently under both long-day (LD) and short-day (SD) photoperiods (Tian et al. 2019). In addition to flowering regulation, *FCA* integrates ambient temperature signals into the thermosensitive flowering pathway (Moon et al. 2005; Lee et al. 2017). Elevated temperatures upregulate *FCA* expression; *FCA* then binds to the primary transcript of microRNA172 (miR172), promoting its processing and thereby modulating *FT*-mediated flowering (Jung et al. 2012). The observed results indicate that FCA might be central to plant adaptation under variable temperature regimes. As abiotic stress critically influences growth and development, multiple genes in the autonomous flowering pathway are known to participate in responses to environmental challenges like cold, drought, and high salinity. A case in point is *FVE*, which mediates the perception of intermittent cold and regulates cold-responsive genes via an FLC-independent mechanism (Kim et al. 2004). *FLK* acts as a m6A reader, repressing *FLC* by reducing its mRNA stability and affecting splicing (Amara et al. 2023). Recent

studies have shown that *FLK* and *HOS5* cooperatively negatively regulate stress-responsive genes; corresponding double mutants in *Arabidopsis* exhibit increased tolerance to salt and oxidative stress (Rodríguez-Cazorla et al. 2025).

Mangifera indica (mango) is a globally important tropical fruit with high economic value. Flowering and fruit set are key determinants of mango yield and profitability. Mango floral bud differentiation is influenced by multiple external factors, and in recent years, frequent extreme climate events have caused substantial losses in mango production. Investigating the function of *FCA*, a central component of the autonomous flowering pathway, in mango could improve our understanding of how endogenous developmental cues regulate flowering in this species. In *Arabidopsis*, ambient temperature influences flowering through the *FT*-mediated pathway, with multiple flowering-related genes, including *FCA*, participating in this process (Capovilla et al. 2015; Choi et al. 2025). Temperature also markedly affects the duration of mango floral bud differentiation (Wang et al. 2019). ‘JinHuang’ flowers only once a year, usually in March, a process typically induced by exposure to low temperatures. In contrast, the ‘SiJiMi’ cultivar can bloom multiple times a year and naturally even in the hot summer. However, the molecular mechanisms underlying temperature-regulated flowering in mango remain poorly understood. Therefore, elucidating how *FCA* genes respond to temperature and abiotic stress and exploring the potential role of the *FCA* gene in shaping floral phenotypic differences would help mitigate adverse environmental impacts on flowering and improve mango yield. In this study, ‘SiJiMi’ and ‘JinHuang’ mango were used as experimental materials. The functions of *MiFCA2-SJ* and *MiFCA2-JH* were characterized in transgenic *Arabidopsis*.

2 Materials and methods

2.1 Materials

Two mango cultivars, ‘SiJiMi’ and ‘JinHuang’, were used in this study. The plants

were maintained in the Fruit Tree Resource Nursery of the College of Agriculture, Guangxi University, located in Nanning, Guangxi Province, China. For tissue-specific expression analysis, leaf, stem, and flower samples were collected from six-year-old trees of both cultivars during the peak flowering stage on March 15, 2025. To examine temporal expression patterns, mature leaves from six-year-old trees were sampled monthly on the 15th from November 2024 to March 2025, yielding five time points in total. Temperature expression profiles were assessed via two-year-old potted plants of both cultivars placed in climate-controlled chambers under three temperature regimes: 13 °C, 22 °C, and 31 °C, all under a 16 h light/8 h dark photoperiod. Mature leaves were collected at 0, 3, 6, 9, and 12 days under each condition, with three biological replicates per treatment. For the stress treatments, one-year-old seedling roots of both cultivars were irrigated with either 30% PEG 6000 (to simulate drought) or 300 mM NaCl (to simulate salt stress), and distilled water was used as a control. Leaves were harvested at 0, 12, 24, 48, and 72 h after treatment, with three replicates per time point and treatment. All the collected plant materials were immediately frozen in liquid nitrogen and stored at −80 °C until further use. *A. thaliana* Columbia ecotype was used for genetic transformation.

2.2 Sequence and phylogenetic analysis

The DNA and cDNA sequences of MiFCA2-SJ and MiFCA2-JH were obtained from the transcriptomic and genomic data of ‘SiJiMi’ mango and ‘JinHuang’ mango, respectively (unpublished). Gene structures were predicted via GSDS 2.0 (<https://gsds.gao-lab.org>). The FCA protein sequences used for phylogenetic analysis were retrieved from the Rosaceae Genome Database (GDR, <https://www.rosaceae.org>), the Citrus Pan-Genome Breeding Database (CPBD, <http://citrus.hzau.edu.cn/>), and the National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov/>). Multiple sequence alignment was performed with DNAMAN software, and phylogenetic trees were constructed via MEGA 11.0. In addition, cis-regulatory

elements within the 2-kb region upstream of the start codon of *MiFCA2-SJ* and *MiFCA2-JH* were predicted via PlantCARE, and the results were visualized with TBtools.

2.3 RNA extraction and expression analysis

Total RNA was isolated using a commercial kit (Magen, Guangzhou, China). Following extraction, first-strand cDNA was generated with the M-MLV reverse transcriptase kit (Takara, Dalian, China). For quantitative reverse transcription PCR (qRT-PCR) analysis, reactions were carried out on an ABI 7500 system (Applied Biosystems, Foster City, USA) using SYBR Premix Ex Taq II (TaKaRa, Dalian, China), with the *MiActin1* gene serving as the internal control (Luo et al. 2013). Each sample was run in three technical replicates, and the $2^{-\Delta\Delta C_t}$ method was employed to determine relative expression levels. All primer sequences are provided in Supplementary Table S1.

2.4 Subcellular location

The coding sequences (CDSs) of *MiFCA2-SJ* and *MiFCA2-JH*, without their stop codons, were subsequently cloned and inserted into the pBI221 vector. The resulting fusion constructs, 35S::MiFCA2-SJ-GFP and 35S::MiFCA2-JH-GFP, were generated via in-frame fusion with the GFP reporter gene. Using *Agrobacterium tumefaciens* (GV3101), the appropriate construct was transformed into onion epidermal cells. Fluorescence was observed under a laser scanning confocal microscope (TCS-SP8MP, Leica, Germany). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). An empty GFP vector was used as a negative control.

2.5 Transformation of *Arabidopsis* with *MiFCA2-SJ/JH* and phenotypic analyses

The recombinant vectors pBI121-*MiFCA2-SJ* and pBI121-*MiFCA2-JH*, which were constructed via *EcoR* I and *BamH* I double digestion, were subsequently introduced into *Agrobacterium* EHA105. Wild-type Arabidopsis (WT) plants were transformed via inflorescence infection to generate T₀ seeds. Homozygous T₃ plants, confirmed by PCR and antibiotic selection, were used for subsequent analyses. Flowering time and plant height were recorded under three temperature regimes (all with a 16 h light/8 h dark photoperiod): 13 °C, 22 °C, and 31 °C. WT plants and those transformed with the empty pBI121 vector served as controls. Additionally, RNA was extracted from *MiFCA2-SJ*-overexpressing (OE), *MiFCA2-JH*-overexpressing (OE), and WT seedlings. The expression levels of flowering-related endogenous genes were quantified via qRT-PCR using *AtACTIN2* (AT3G18780) as an internal reference.

2.6 Analysis of abiotic stress and hormone responses in *MiFCA2-SJ/JH*-OE plants

Seeds of *MiFCA2-SJ*-OE, *MiFCA2-JH*-OE, and WT were surface sterilized, vernalized at 4 °C for 2 d, and germinated on 1/2 MS media at 23 °C for 3 d. Uniform seedlings were then transferred to fresh 1/2 MS media for vertical culture. For the chemical and hormonal treatments, the seedlings were grown for 5 d on media supplemented with NaCl (100 and 150 mM), mannitol (300 and 400 mM), or ABA (5 and 10 µM), after which the length of the roots was measured. To assess temperature stress responses, the seedlings were incubated for 5 d at 13 °C, 22 °C, or 31 °C, after which their growth phenotypes were recorded. For the soil-based assays, 10-d-old plants were subjected to drought stress (by withholding water for 12 d, followed by rewatering to determine the survival rate) or salt stress (by irrigation with 300 mM NaCl for 14 d before survival was scored). To examine the ABA response, the plants were irrigated with 20 µM ABA, and the flowering time was measured.

Leaves from 26 d *MiFCA2-SJ*-OE, *MiFCA2-JH*-OE, and WT plants were subjected to histochemical staining as follows: 3 h with 0.5 mg/mL NBT, 24 h with 1

mg/mL DAB, and 2 h with 2.5% Evans blue. 26 d *MiFCA2-SJ*-OE, *MiFCA2-JH*-OE and WT plants were treated with 150 mM NaCl or 10% PEG6000 for 2 d, with distilled water serving as the control. The levels of malondialdehyde (MDA), superoxide dismutase (SOD), and proline (Pro) were determined via kits (Solarbio Science and Technology, Beijing, China). Additionally, the concentration of H₂O₂ was assessed via a kit (Grace Biotechnology, Jiangsu, China). For gene expression analysis under stress, RNA was extracted from *MiFCA2-SJ*-OE, *MiFCA2-JH*-OE, and WT plants after 24 h of treatment with 150 mM NaCl, 10% PEG6000, or 20 μM ABA. The transcript levels of stress-responsive endogenous genes were analysed via qRT-PCR with *AtActin2* as an internal reference.

2.7 Yeast two-hybrid (Y2H) verification

The coding sequences of *MiFCA2-SJ* and *MiFCA2-JH* were subsequently cloned and inserted into the pGBKT7 vector via the *Nde* I and *EcoR* I restriction sites. Self-activation of the bait constructs was tested via the pGBKT7-T positive control. The prey proteins *MiFY*, *MiZFP4*, and *MiPP2C25B* were similarly cloned and inserted into the pGADT7 vector. Y2H experiment was conducted to assess the interaction between *MiFCA2-SJ* and non-self-activated yeast.

2.8 Bimolecular fluorescence complementation (BiFC) assay

MiFCA2-SJ and *MiFCA2-JH* were constructed on the puc-sPYNE vector, and *MiFY*, *MiZFP4*, and *MiPP2C25B* were constructed on the puc-sPYCE vector. The recombinant plasmids were subsequently introduced into *Agrobacterium* GV3101. Positive colonies were grown overnight at 28 °C in LB media supplemented with appropriate antibiotics and then used for transient transformation of onion epidermal cells. After 24 h, the fluorescence signals were visualized and imaged via a laser scanning confocal microscope (TCS-SP8MP; Leica, Germany).

220

221 **2.9 Data analysis**

222 All the experiments were performed with three independent biological replicates.
223 The data were analysed via IBM SPSS Statistics 26.0. Differences among treatments
224 were evaluated by Duncan's multiple range test, with a threshold of $p < 0.05$ considered
225 statistically significant. Figures were prepared via GraphPad Prism 9.

226

227 **3 Results**

228 **3.1 Sequence analysis of MiFCA2-SJ/JH**

229 The whole open reading frames (ORFs) of *MiFCA2-SJ* and *MiFCA2-JH* are 2319
230 bp and 2352 bp long, encoding 773 and 784 amino acids, respectively. Both genes share
231 an identical structure, comprising 19 exons and 18 introns. These two variants
232 primarily diverge in the C-terminal region, exhibiting differences spanning several
233 dozen amino acids in length. (Fig. 1a). At the protein level, MiFCA2-SJ and MiFCA2-
234 JH each contain two RNA recognition motifs (RRM domains) and one WW domain.
235 Notably, the RRM and WW domains are highly conserved across the species listed in
236 the diagram (Fig. 1c). Sequence alignment revealed that two mango MiFCA2s
237 presented increased amino acid sequence similarity to *Pistacia vera* (Supplementary
238 Fig. S1). Additionally, the N-terminal region of MiFCA2 contains a relatively long
239 glycine-rich segment. A polyglutamine (polyQ) tract is located at the C-terminus of
240 MiFCA2; its length varies among species, with mango MiFCA2 possessing a longer
241 polyQ region than Arabidopsis does (Fig. 1c). Analysis of the cis-acting elements within
242 the promoters revealed that both *MiFCA2* genes harbor numerous ABA-responsive
243 elements, followed by high-temperature, salt, drought, and low-temperature responsive
244 elements (Fig. 1b). These data implicate the MiFCA2 genes in having a potential role
245 during abiotic stress.

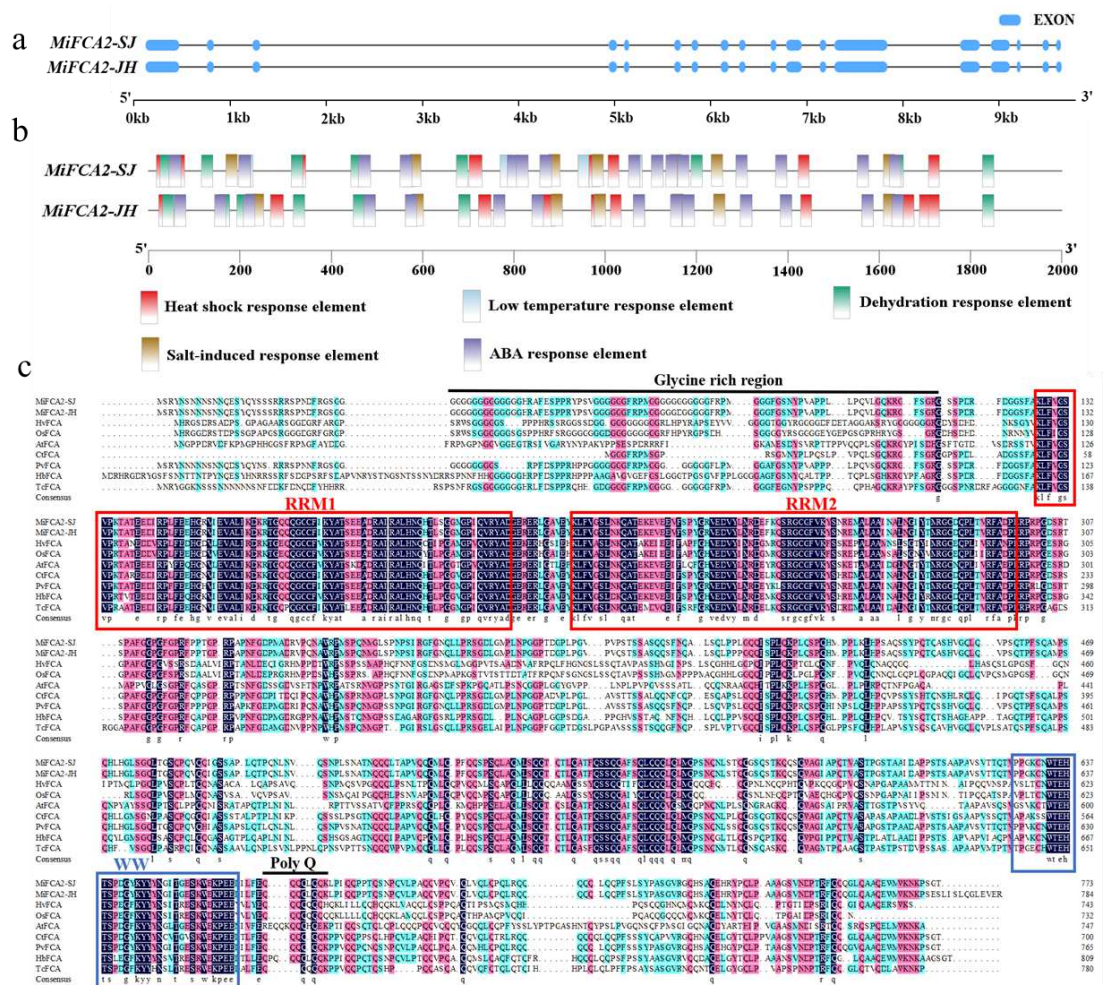


Figure 1 Identification of the *MiFCA2-SJ* and *MiFCA2-JH* genes. (a) *MiFCA2-SJ* and *MiFCA2-JH* gene structure. (b) A visualization map of the cis-regulatory element-based promoter analysis of the *MiFCA2-SJ* and *MiFCA2-JH* genes. (c) Alignment of the *MiFCA2-SJ* and *MiFCA2-JH* protein amino acid sequences; the FCA protein information (species and gene names) is as follows: *MiFCA2-SJ* (*M. indica*), *MiFCA2-JH* (*M. indica*), *HvFCA* (*Hordeum vulgare*), *OsFCA* (*Oryza sativa*), *AtFCA* (*A. thaliana*), *CtFCA* (*Citrus trifoliata*), *PvFCA* (*P. vera*), *HbFCA* (*Hevea brasiliensis*), and *TcFCA* (*Theobroma cacao*). The locus IDs of FCA genes from different plants were listed in Supplementary Table S2

3.2 Expression patterns of the *MiFCA2-SJ/JH* genes in mango

Expression analysis of the two *MiFCA2* genes across different mango developmental stages revealed that the transcript levels of both *MiFCA2-SJ* and *MiFCA2-JH* gradually decreased from the vegetative growth phase to the early stages of flower development. However, during flowering, their expression patterns diverged: *MiFCA2-SJ* peaked at flowering initiation, whereas *MiFCA2-JH* peaked at the end of

the flowering stage (Fig. 2a, b). Both genes were predominantly expressed in leaves (Fig. 2c, d). Under salt and drought stress, both genes presented differential expression patterns (Fig. 3a, b). Under temperature treatments, the expression of both genes exhibited a marked transient increase at 31 °C, followed by a decrease. The changes at 13 °C and 22 °C were more subtle than 31 °C (Fig. 3a, b).

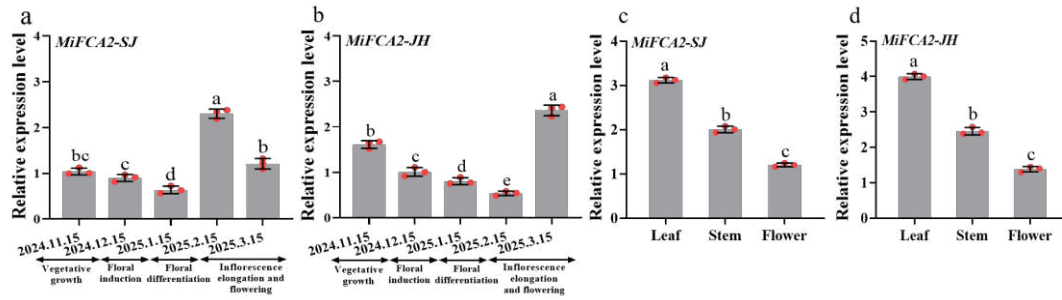


Figure 2 Expression analysis of *MiFCA2-SJ* and *MiFCA2-JH* in different mango tissues. (a), (b) Temporal expression analyses of the *MiFCA2-SJ* and *MiFCA2-JH* genes in leaves. (c), (d) *MiFCA2-SJ* and *MiFCA2-JH* gene expression analysis in tissues during peak flowering; p < 0.05 indicated significant differences

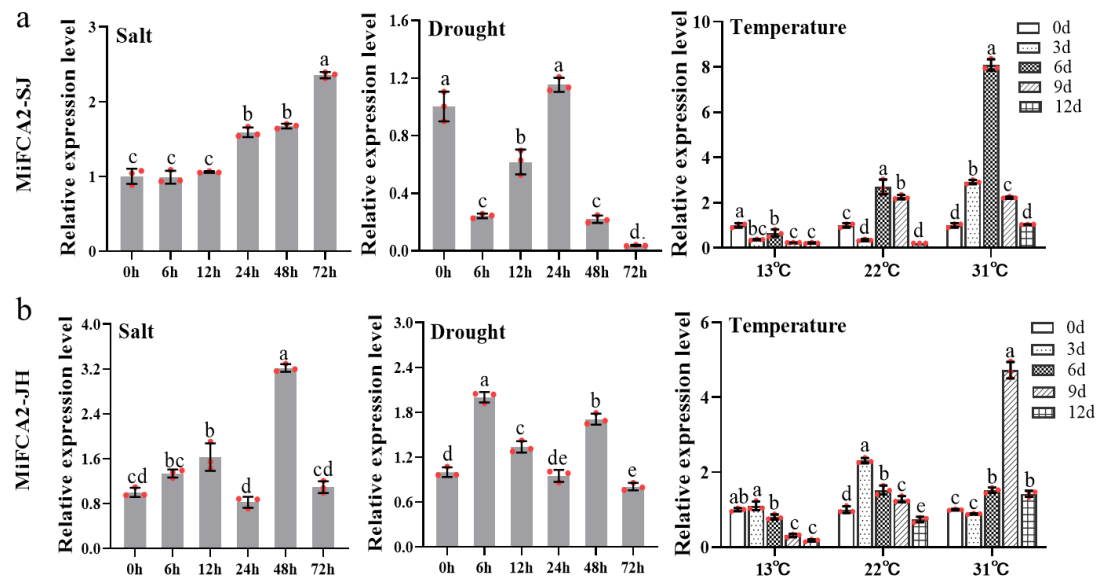


Figure 3 Expression analyses of the *MiFCA2-SJ* and *MiFCA2-JH* genes in mango under different treatments. (a) *MiFCA2-SJ* gene expression analysis in response to salt, drought and different temperature treatments. (b) *MiFCA2-JH* gene expression analysis in response to salt, drought and different temperature treatments; p < 0.05 indicated significant differences

3.3 Subcellular localization

Transient expression in onion epidermal cells revealed that 35S::GFP control fluorescence localized to both the cell wall and the nucleus. Conversely, MiFCA2-SJ and MiFCA2-JH were present only in the nucleus, as determined by DAPI staining (Fig. 4).

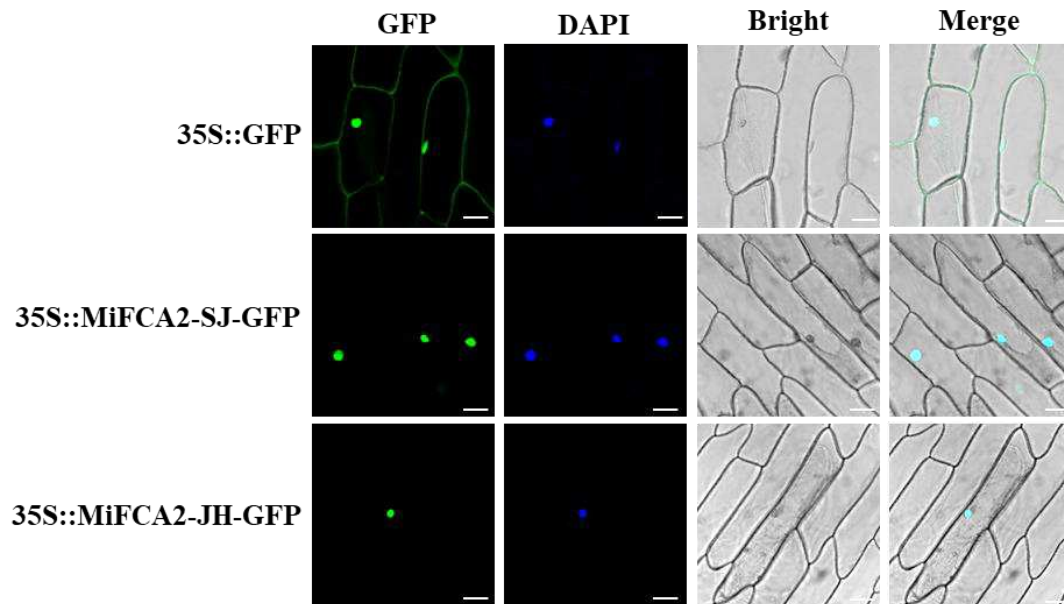


Figure 4 Subcellular localization of MiFCA2-SJ and MiFCA2-JH. Bars = 100 μ m

3.4 Phenotypic analysis of *MiFCA2-SJ/JH* in *Arabidopsis* at different temperatures

To examine the potential role of *MiFCA2-SJ* and *MiFCA2-JH* in temperature-dependent flowering regulation, this study generated and selected three independent overexpression lines for each gene in *Arabidopsis* (*MiFCA2-SJ*-OE: OE2, OE6, OE7; *MiFCA2-JH*-OE: OE3, OE5, OE9). Compared with those in WT plants, the transcript levels of the corresponding transgenes in these lines were significantly greater (Fig. 5). Phenotypic evaluation under three temperature regimes (13 °C, 22 °C, and 31 °C) revealed distinct flowering and growth responses. Compared with WT plants, *MiFCA2-SJ*-OE plants flowered earlier at 13 °C and 31 °C but presented no significant difference in flowering time at 22 °C (Fig. 6a). In contrast, *MiFCA2-JH*-OE plants presented accelerated flowering at all three temperatures (Fig. 7a). Analysis of downstream

flowering-related gene expression via qRT-PCR indicated that overexpression of *MiFCA2-SJ* (Fig. 6d, e, f) and *MiFCA2-JH* (Fig. 7d, e, f) generally promoted the expression of *AtFT* and *AtSOC1* while suppressing *AtFLC* across most temperatures, except at 22 °C, where *MiFCA2-SJ* overexpression had no significant effect on these genes.

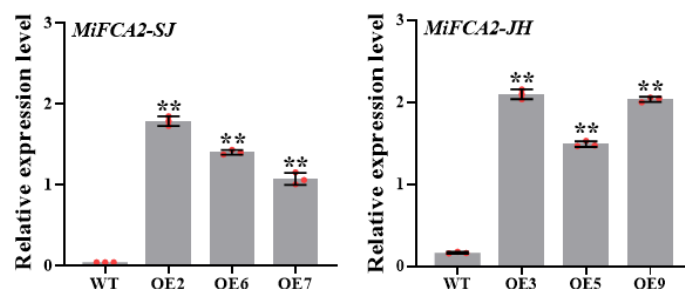


Figure 5 Expression levels of *MiFCA2-SJ/JH* in transgenic and WT leaves. $p < 0.05$ indicated significant differences

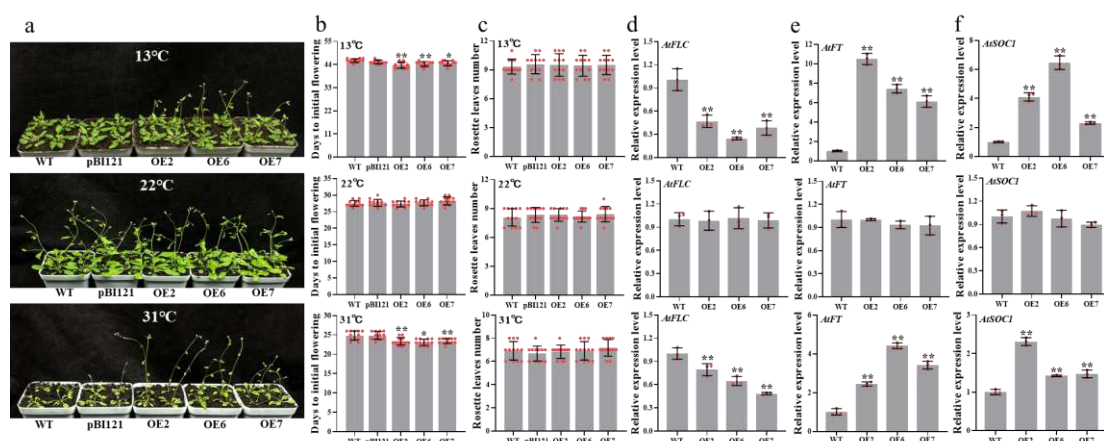


Figure 6 Phenotypes of *MiFCA2-SJ* transgenic Arabidopsis under different temperature conditions. (a) Flowering phenotypic analysis of *MiFCA2-SJ* transgenic and control lines under different temperature conditions. (b), (c) Number of rosette leaves and days to flowering. (d)-(f) Expression analysis of flowering-related endogenous genes of *MiFCA2-SJ* transgenic and WT plants at 13 °C, 22 °C and 31 °C. $p < 0.05$ indicated significant differences

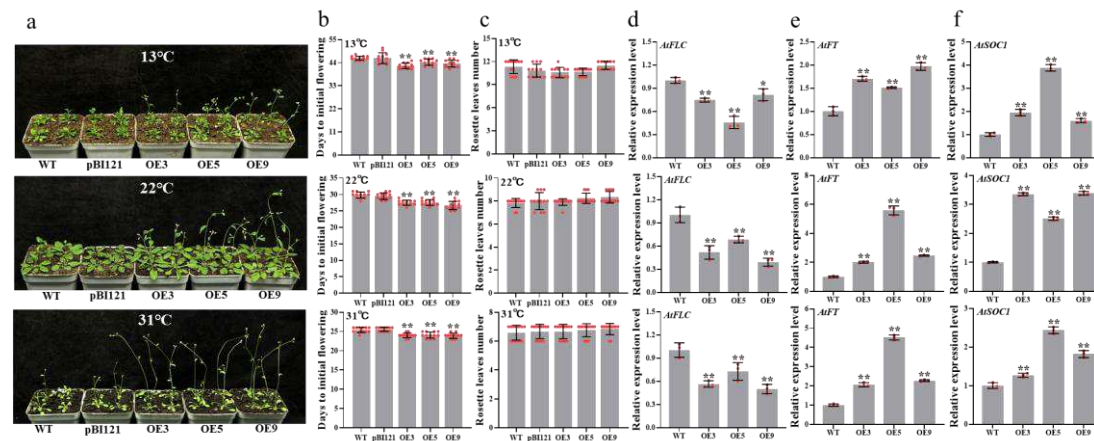


Figure 7 Phenotypes of *MiFCA2-JH* transgenic Arabidopsis plants under different temperature conditions. (a) Flowering phenotypic analysis of *MiFCA2-JH* transgenic and control lines under different temperature conditions. (b), (c) Number of rosette leaves and days to flowering of the *MiFCA2-JH* and WT lines under different temperature conditions. (d)-(f) Expression analysis of flowering-related endogenous genes in *MiFCA2-JH* transgenic and WT plants at 13 °C, 22 °C and 31 °C. p < 0.05 indicated significant differences

3.5 *MiFCA2-SJ/JH* overexpression enhances *Arabidopsis* tolerance to abiotic stress

To assess the potential roles of *MiFCA2-SJ* and *MiFCA2-JH* in abiotic stress tolerance, this study subjected the overexpression lines to salt and drought treatments. Under control conditions, *MiFCA2-SJ*-OE plants presented significantly longer roots than did WT plants (Fig. 8a, c). After treatment with 100 mM or 150 mM NaCl, the inhibition rates of root elongation in WT plants (52.51% and 79.19%, respectively) were consistently greater than those in the three *MiFCA2-SJ*-OE lines (100 mM: 47.57%, 40.57%, 49.44%; 150 mM: 70.37%, 68.20%, 71.65%), indicating that *MiFCA2-SJ* overexpression alleviated salt-induced growth suppression (Fig. 8c). In contrast, under drought stress simulated by 300 mM or 400 mM mannitol, root growth inhibition was more pronounced in *MiFCA2-SJ*-OE plants (300 mM: 48.23%, 44.07%, 49.01%; 400 mM: 66.42%, 67.70%, 63.18%) than in WT plants (34.47% and 53.90%, respectively), suggesting increased sensitivity to drought conditions (Fig. 8c). For *MiFCA2-JH*-OE, the root length under control conditions did not differ from that under

WT conditions (Fig. 9a, c). However, under salt stress (100 mM and 150 mM NaCl), the average root lengths of *MiFCA2-JH*-OE plants were 1.96 times and 1.75 times greater than those of WT plants (Fig. 9c). Similarly, under mannitol-induced drought stress (300 mM and 400 mM), the root length was 1.44 times and 1.40 times longer than that of the WT (Fig. 9c), preliminarily indicating that *MiFCA2-JH* may increase both salt tolerance and drought tolerance.

Physiological and histochemical analyses supported these observations. After 150 mM NaCl treatment, Evans blue, NBT, and DAB staining revealed less tissue damage and reactive oxygen species (ROS) accumulation in both *MiFCA2-SJ*-OE and *MiFCA2-JH*-OE leaves than in WT leaves (Fig. 8e, 9e). Following 10% PEG 6000 treatment, reduced staining was also evident in *MiFCA2-JH*-OE leaves (Fig. 9e). Similarly, under salt stress, the MDA and H₂O₂ levels were significantly lower, and the SOD activity and proline content were markedly greater in both overexpression lines than in the WT (Fig. 8g, 9g). Under PEG-induced drought stress, *MiFCA2-JH*-OE plants also presented lower MDA and H₂O₂ contents and higher SOD activity and proline content than WT plants did (Fig. 9g).

In whole-plant stress assays, although *MiFCA2-SJ*-OE, *MiFCA2-JH*-OE, and WT plants presented similar growth rates under normal conditions, the survival rates of the transgenic plants were significantly greater than those of the WT plants after recovery from chronic salt stress (300 mM NaCl applied every 3 d) (Fig. 8b, 9b). Moreover, under rehydration conditions following drought treatment, the survival rate of *MiFCA2-JH*-OE plants was significantly greater than that of wild-type plants (Fig. 9b). To explore the underlying molecular mechanism, we examined the expression of six stress-responsive genes: *AtNHX1*, *AtDREB2A*, *AtSOS1*, *AtKIN1*, and *AtCOR15A*. Under normal conditions, their expression did not differ between the WT and overexpression lines. However, after 150 mM NaCl treatment, the transcript levels of *AtNHX1*, *AtDREB2A*, and *AtSOS1* were significantly greater in *MiFCA2-SJ*-OE plants than in WT plants (Fig. 8f). In *MiFCA2-JH*-OE plants, all six genes were strongly induced

under either salt or drought stress, with expression levels that exceeded those of the WT plants (Fig. 9f). These results suggest that *MiFCA2-SJ* and *MiFCA2-JH* enhance stress tolerance, at least in part, by upregulating key stress-responsive genes.

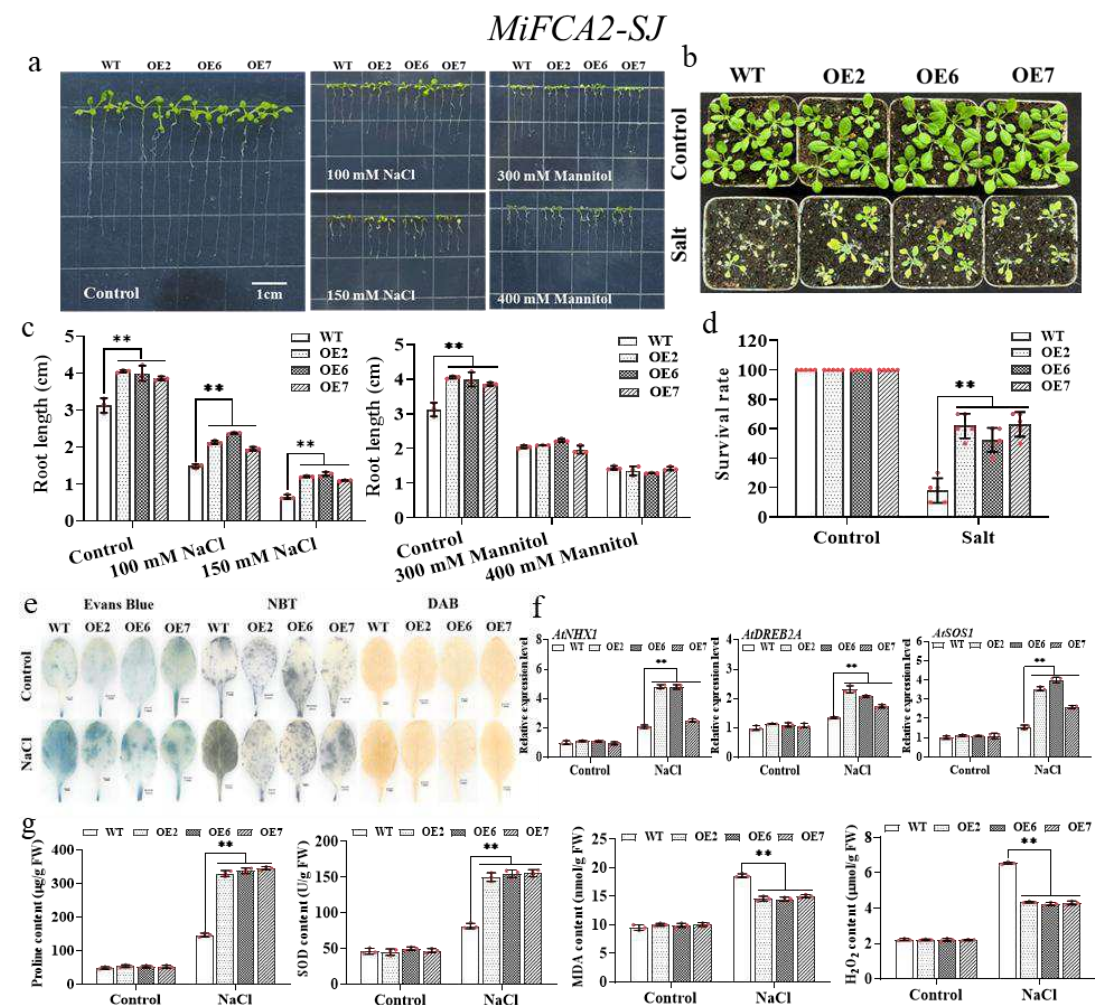


Figure 8 Functional detection of *MiFCA2-SJ* in transgenic Arabidopsis under stress treatment. (a) Root length phenotypes. Bar= 1.0 cm. (b) Phenotypes under salt stress. Control, 300 mM NaCl. (c) Root length. (d) Survival rates of the WT and *MiFCA2-SJ* transgenic plants. (e) DAB, Evans blue, and NBT staining of the WT and *MiFCA2-SJ* transgenic lines. (f) Relative expression levels of stress-related genes in *MiFCA2-SJ* transgenic and WT plants after treatment. (g) The contents of MDA, Pro, and H₂O₂ and the enzymatic activities of SOD in the WT and *MiFCA2-SJ* transgenic lines. p < 0.05 indicated significant differences

MiFCA2-JH

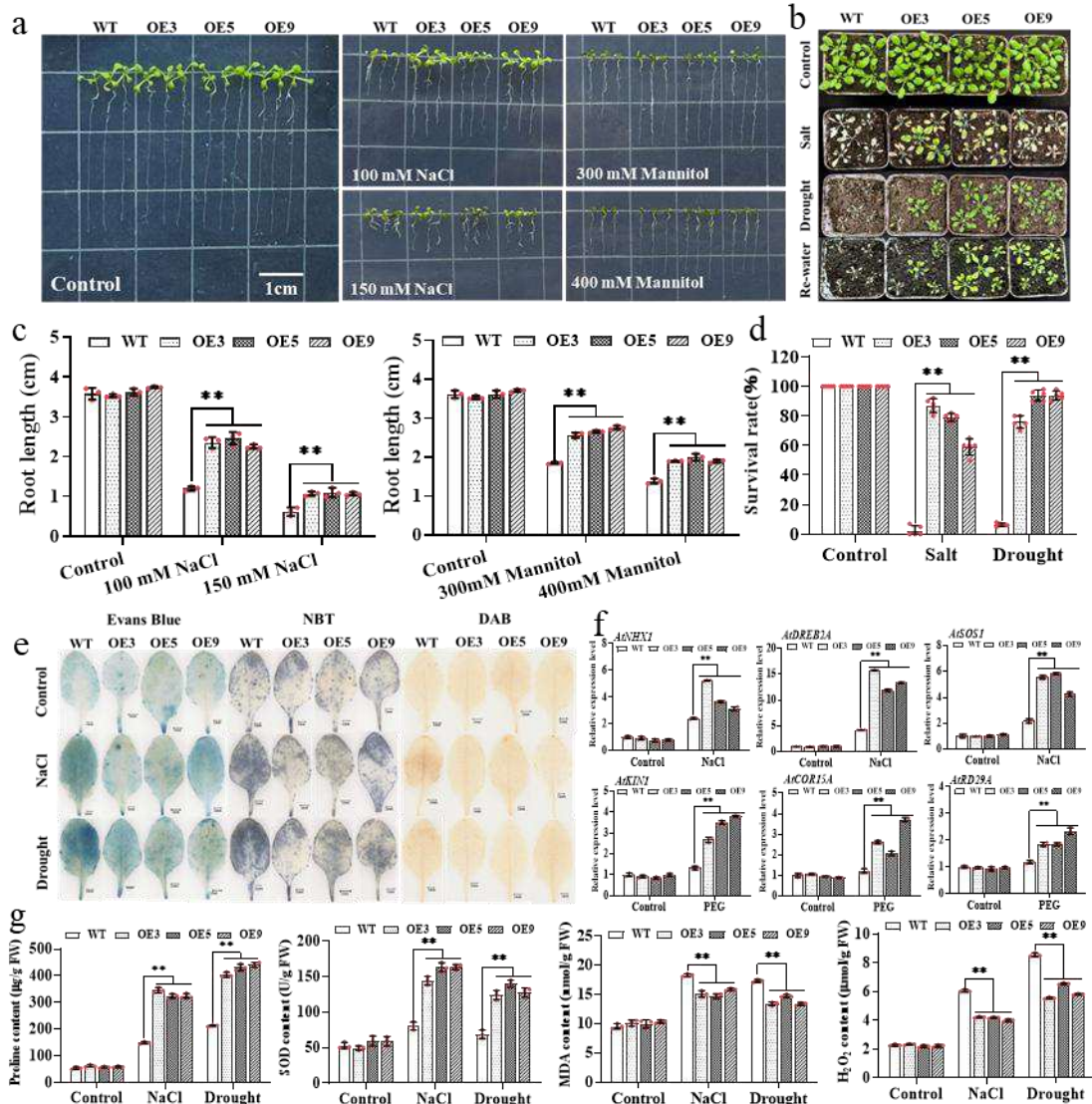


Figure 9 Functional detection of *MiFCA2-JH* in transgenic *Arabidopsis* under stress treatment. (a) Root length phenotypes. Bar= 1.0 cm. (b) Phenotypes under drought and salt stress. Control, 300 mM NaCl. (c) Root length. (d) Survival rates of the WT and *MiFCA2-JH* transgenic plants. (e) DAB, Evans blue, and NBT staining of the WT and *MiFCA2-JH* transgenic lines. (f) Relative expression levels of stress-related genes in *MiFCA2-JH* transgenic and WT plants after treatment. (g) The contents of MDA, Pro, and H₂O₂ and the enzymatic activities of SOD in the WT and *MiFCA2-JH* transgenic lines. $p < 0.05$ indicated significant differences

3.6 Overexpression of *MiFCA2-SJ/JH* enhances the response to ABA in *Arabidopsis*

To investigate the potential involvement of *MiFCA2-SJ* and *MiFCA2-JH* in ABA

signalling, this study analysed root growth, the flowering phenotype, and the expression of ABA-responsive genes in overexpression lines under ABA treatment. Under control conditions, *MiFCA2-SJ*-OE plants presented significantly longer roots than WT plants did. Following treatment with 5 μ M or 10 μ M ABA, the inhibition of root elongation was less pronounced in *MiFCA2-SJ*-OE lines than in WT plants. The root inhibition rates in WT plants were 56.46% (5 μ M ABA) and 33.94% (10 μ M ABA), exceeding the rates in the three *MiFCA2-SJ*-OE lines (5 μ M: 42.30%, 44.41%, 39.15%; 10 μ M: 31.28%, 30.88%, 28.52%) (Fig. 10b, d). Similarly, under the same ABA treatments, the average root length of *MiFCA2-JH*-OE plants was 1.21 times and 1.35 times greater than that of WT plants, respectively (Fig. 10g, i). Compared with WT plants, both *MiFCA2-SJ*-OE and *MiFCA2-JH*-OE plants presented an early-flowering phenotype after exogenous ABA application (Fig. 10c, h). Gene expression analysis further revealed that, relative to those in the WT, the expression of the negative ABA signalling regulator *AtABII* was downregulated in both overexpression lines, whereas the expression of the positive regulators *AtABI5* and *AtNCED3* was upregulated (Fig. 10e, j). Collectively, these results suggest that *MiFCA2-SJ* and *MiFCA2-JH* may function within the ABA signalling pathway.

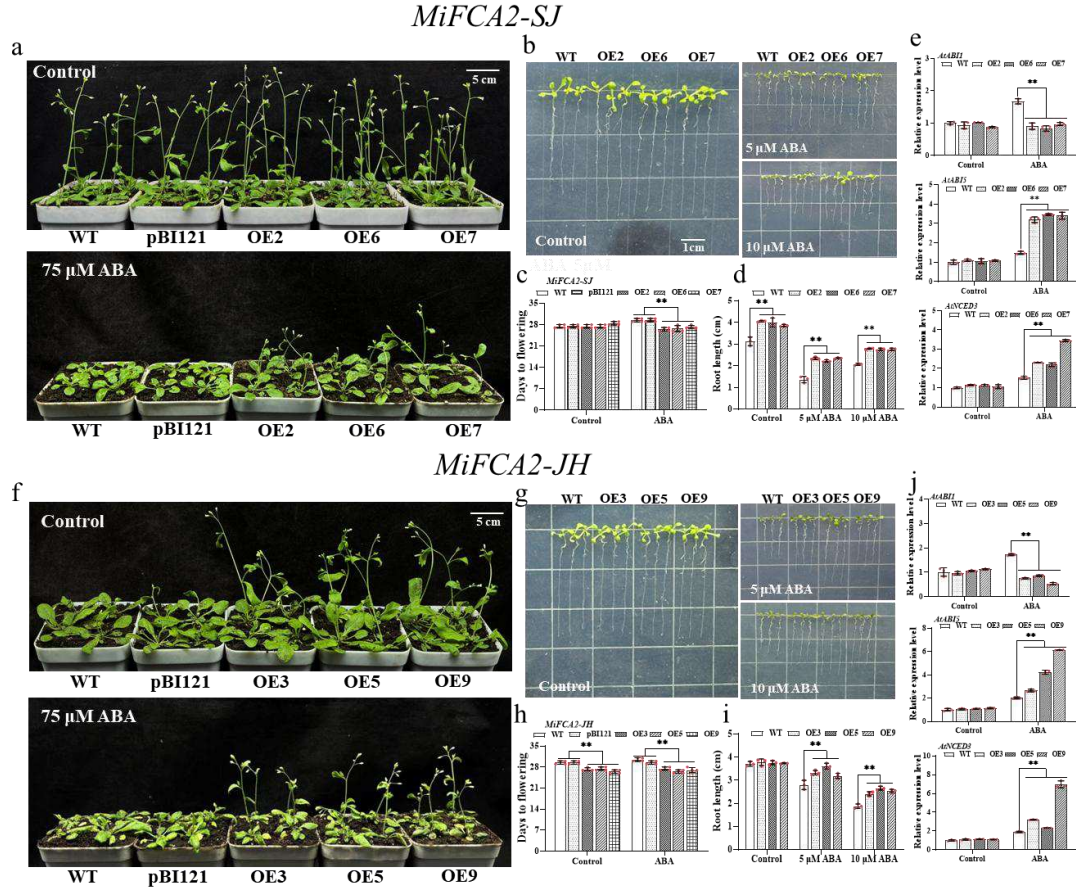


Figure 10 Functional detection of *MiFCA2-SJ* and *MiFCA2-JH* transgenic Arabidopsis under ABA treatment. (a), (f) Flower phenotype analysis of *MiFCA2-SJ* and *MiFCA2-JH* transgenic Arabidopsis plants treated with 20 μ M ABA. (b), (g) Phenotypes of *MiFCA2-SJ* and *MiFCA2-JH* transgenic and WT plants under ABA. (c), (h) Phenotype of days to flowering. (d), (i) Root length of *MiFCA2-SJ* and *MiFCA2-JH* transgenic and WT plants under ABA treatment. (e), (j) Relative expression levels of ABA-related genes in *MiFCA2-SJ* and *MiFCA2-JH* transgenic and WT plants under ABA treatment. $p < 0.05$ indicated significant differences

3.7 Protein interaction analysis of MiFCA2

To identify potential interacting partners, a yeast two-hybrid (Y2H) screen was performed. The pGBKT7-MiFCA2-JH construct displayed autonomous activation (Fig. 11a), which could not be suppressed by aureobasidin A (AbA) concentrations up to 500 ng/mL, precluding its use in further Y2H screens. In contrast, pGBKT7-MiFCA2-SJ showed no self-activation activity (Fig. 11a). Subsequent Y2H and bimolecular fluorescence complementation (BiFC) assays confirmed that MiFCA2-SJ interacts with MiFY, a core component of the autonomous flowering pathway (Fig. 11b, c).

Furthermore, novel interactions were identified between MiFCA2-SJ and two abiotic stress-related proteins: MiPP2C25B (a protein phosphatase) and MiZFP4 (a zinc finger protein).

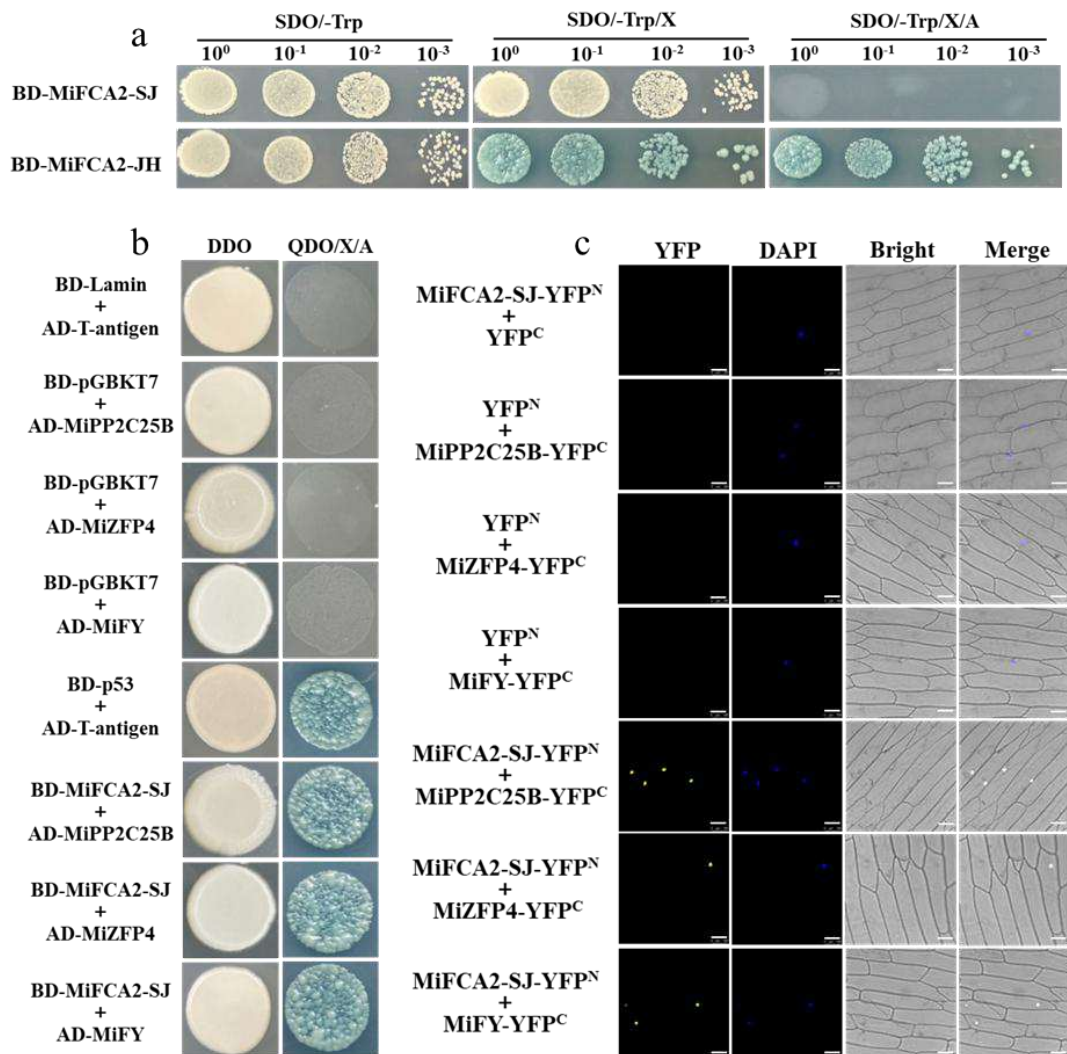


Figure 11 Identification of MiFCA2-SJ-interacting proteins. (a) Transcriptional activation activity. (b) The positive control was BD-53+AD-T, and the negative control was BD-Lam+AD-T. Yeast two-hybrid assay validation of MiFCA2-SJ proteins. (c) BiFC validation of MiFCA2-SJ. MiFCA2-SJYFP^N+YFP^C, YFP^N+MiPP2C25BYFP^C, YFP^N+MiZFP4YFP^C, and YFP^N+MiFY YFP^C were employed as negative controls. Scale bars = 100 μ m

4 Discussion

RNA binding proteins (RBPs) are key mediators of post-transcriptional regulation and play essential roles in plant growth, development, and stress responses (Simpson and Filipowicz 1996; Lorković 2009; Jung et al. 2013; Fan et al. 2024). Canonical RBPs

typically contain one or more RNA recognition motif (RRM) domains, often located at the N- or C-terminus, and may additionally harbor auxiliary motifs such as glycine-rich regions (Burd and Dreyfuss 1994; Albà and Pagès 1998; Yan et al. 2022). In this study, two MiFCA2 proteins isolated from the ‘SiJiMi’ and ‘JinHuang’ mango cultivars were found to possess two RRM domains and one WW domain. Sequence alignment revealed high similarity between MiFCA2 and its homologues in *A. thaliana*, *C. trifoliata* and *O. sativa* (Fig. 1c), supporting the evolutionary conservation of FCA across plant species. Previous reports suggest that the structure and function of FCA diverged during the evolution of monocots and dicots (Kumar et al. 2011; Ai et al. 2016). Consistent with this, mango *FCA2* exhibited greater sequence identity to citrus FCA (64%) and *Arabidopsis* FCA (42%) than to barley (35%) or rice FCA (36%). Phylogenetic analysis further revealed that mango FCA2 clusters more closely with homologues from pistachio and citrus (Supplementary Fig. S1). Interestingly, an N-terminal glycine-rich region, previously reported only in monocots such as barley and rice, was also identified in mango and appears to be extended in length. This domain may influence protein folding and modulate G protein signalling (Mumby et al. 1990; Krieger et al. 2005). Downstream of the WW domain in FCA homologues, polyglutamine (polyQ) tracts of variable length are present. In MiFCA2, the polyQ region is shorter than that in Citrus, barley, and rice but longer than that in *Arabidopsis* (Fig. 1c). Polymorphisms in the polyQ tract of FCA proteins may reflect plant adaptation through selection on flowering time and morphology (Lindqvist et al. 2007). The mango genome harbors two homologous *FCA* genes, designated *MiFCA1* and *MiFCA2*. In the ‘JinHuang’ cultivar, *MiFCA1* undergoes alternative splicing, albeit via a pattern distinct from that observed in *Arabidopsis* (unpublished data). In *Arabidopsis*, *FCA* locus generates four transcripts through alternative splicing. Comparison of the amino acid sequences of the two MiFCA2 transcripts and the four *Arabidopsis* FCA splice variants (Supplementary Fig. S2) revealed that the two MiFCA2, along with the full-length AtFCA- γ transcript, exhibit high sequence similarity and share a conserved

domain architecture, each containing two RRM domains and one WW domain. In contrast, they show substantial sequence divergence from the other three *AtFCA* transcripts. These structural variations likely contribute to distinct regulatory mechanisms and functional diversification of *FCA* across plant species.

In contrast to the findings of high expression in the flowers of *Brassica campestris* (Xiao et al. 2010) and in the roots of *C. trifoliata* (Ai et al. 2016), the *MiFCA2* gene studied herein exhibited high expression in the leaves of the ‘SijiMi’ and ‘JinHuang’ mangoes (Fig. 2b). Similar to the findings of studies on *Eriobotrya japonica* (Wu et al. 2021), from vegetative growth to early floral development, *MiFCA2-SJ* and *MiFCA2-JH* expression gradually decreased until flowering, when *MiFCA2* expression began to rise. *MiFCA2-SJ* peaked at the onset of flowering, whereas *MiFCA2-JH* peaked at the end of flowering (Fig. 2a, b). Previous studies have indicated that *FCA* genes regulate floral bud differentiation in response to temperature changes in *Arabidopsis* (Anderson et al. 2011; Cho et al. 2012), *Citrus* (Ai et al. 2016), and *Osmanthus fragrans* (Wu et al. 2020). In this study, the expression of both *MiFCA2* genes significantly increased, followed by a decrease at 31 °C, as the duration of treatment increased. In contrast, the expression levels at 13 °C and 22 °C presented less pronounced changes (Fig. 3). Furthermore, both *MiFCA2* genes presented differential expression responses to drought and salt (Fig. 3) treatments, mirroring findings in citrus and barley plants (Kumar et al. 2011).

In *Arabidopsis*, *FCA* plays a critical role in flowering regulation, where its overexpression leads to early flowering (Lee et al. 2005; Sun et al. 2012). Within the autonomous pathway, *FCA* controls flowering through the modulation of polyadenylation and histone demethylation at the *FLC* locus (Macknight et al. 1997; Quesada et al. 2003; Liu et al. 2007; Liu et al. 2010). Under elevated temperatures, *FCA* promotes flowering in *Arabidopsis* by inducing *FT* expression, which is a mechanism independent of its repression of *FLC* in the autonomous pathway (Moon et al. 2005; Capovilla et al. 2015). In this study, transgenic *Arabidopsis* plants expressing *MiFCA2*

from ‘SiJiMi’ (*MiFCA2-SJ*) flowered earlier under alternating 13 °C/31 °C conditions than did WT plants did. This phenomenon was accompanied by downregulation of the late-flowering gene *AtFLC* and upregulation of the flowering promoters *AtFT* and *AtSOC1*. At 22 °C, however, the flowering time and expression levels of these genes remained unchanged relative to those of the WT (Fig. 6). Previous studies have suggested that gene redundancy may exist between the *FCA* gene and other members of the autonomous pathway, thereby leading to a lack of pronounced flowering phenotypes in mutant *Arabidopsis thaliana* (Cheng et al. 2017). In contrast, transgenic lines expressing *MiFCA2* from ‘JinHuang’ (*MiFCA2-JH*) flowered earlier at all three temperatures tested, consistently showing reduced *AtFLC* expression and elevated *AtFT* and *AtSOC1* levels (Fig. 7). Together, these results indicate that the two mango *MiFCA2* alleles differentially regulate flowering in a temperature-dependent manner. However, the specific regulatory mechanisms require further investigation.

In addition to its well-established role in flowering, *FCA* is involved in abscisic acid (ABA) signalling and environmental stress responses. In *Arabidopsis*, *FCA* modulates antioxidant metabolism under high temperature through an ABA-dependent pathway (Lee et al. 2015). In citrus (Ai et al. 2016) and barley (Kumar et al. 2011), *FCA* has also been demonstrated to be a response gene in the ABA signalling pathway. Furthermore, the *FCA* gene may also respond to drought and salt stress, suggesting that the *MiFCA2* gene may participate in stress responses. This study first analysed the promoter sequences of *MiFCA2-SJ* and *MiFCA2-JH* (Fig. 1b), which revealed an abundance of ABA-responsive elements, along with motifs associated with high temperature, salt, and drought stress. Under ABA treatment (Fig. 10), both *MiFCA2-SJ-OE* and *MiFCA2-JH-OE* transgenic plants presented longer roots and earlier flowering than did WT plants did. Expression analysis of key ABA signalling components revealed that *AtABI1* (a central negative regulator) was downregulated in the transgenic lines, whereas *AtABI5* (a major ABA-responsive transcription factor) and *AtNCED3* (involved in ABA biosynthesis) were upregulated (Fig. 10e, j) (Cutler et al.

2010). These results indicate that *MiFCA2* likely functions within the ABA signalling pathway in mango. Under salt stress, both *MiFCA2-OE* lines presented greater tolerance than did the WT. Phenotypically, both *MiFCA2-OE* lines presented longer roots (Fig. 8c, 9c) and higher survival rates (Fig. 8d, 9d). Physiological and biochemical assays revealed decreased cell death, reduced Evans blue uptake, diminished accumulation of O_2^- and H_2O_2 (as indicated by lighter NBT and DAB staining) (Fig. 8e, 9e), elevated proline content and SOD activity, and decreased levels of MDA and H_2O_2 (Fig. 8g, 9g). These findings suggest that *MiFCA2* enhances salt tolerance by increasing the reactive oxygen species scavenging capacity and improving osmotic adjustment. At the molecular level, the expression of salt-related endogenous genes (*AtNHX1*, *AtDREB2A*, *AtSOS1*) was significantly upregulated in both transgenic lines compared with the WT (Fig. 8f, 9f). Under mannitol treatment, root growth in *MiFCA2-SJ-OE* plants was more inhibited than that in WT plants, suggesting increased drought sensitivity. In contrast, *MiFCA2-JH-OE* plants presented longer roots than did WT plants under the same conditions. Subsequent drought tolerance assays focused on *MiFCA2-JH-OE* plants confirmed their enhanced survival, favourable physiological metrics, and upregulation of drought-related endogenous genes, demonstrating that *MiFCA2-JH* confers drought tolerance.

To further elucidate the regulatory network through which *MiFCA2* mediates abiotic stress responses, this study performed protein interaction assays between *MiFCA2* and flower-related, stress-related candidate proteins previously identified from mango cDNA libraries in our laboratory (Fig. 11). Since the *MiFCA2-JH* protein displayed self-activation activity in yeast (Fig. 11a), subsequent yeast two-hybrid (Y2H, Fig. 11b) and bimolecular fluorescence complementation (BiFC, Fig. 11c) experiments were conducted with only the *MiFCA2-SJ* variant. These assays confirmed the interaction of *MiFCA2-SJ* with both *MiPP2C25B* and *MiZFP4*. Given that *PP2C25B* and *ZFP4* homologues are known regulators of ABA signalling and plant stress adaptation (Bhaskara et al. 2012; Joseph et al. 2014; Zhao et al. 2024), these results

suggest that MiFCA2-SJ may participate in mango ABA signalling and stress responses through physical association with MiPP2C25B and MiZFP4. FCA is a central component of the autonomous flowering pathway in plants. Its core function is to act as an RNA-binding protein, cooperating with its partner FY protein to specifically promote the premature processing of the flowering repressor FLC transcript at cryptic polyadenylation sites. This leads to the production of unstable truncated mRNAs, thereby silencing FLC expression and promoting flowering (Simpson et al. 2003). The interaction between FCA and FY is evolutionarily conserved across plant species (Winichayakul et al. 2005; Jang et al. 2009; Ai et al. 2016). In mango, MiFY contains two PPLPP domains (Fan et al. 2021), which are predicted to mediate binding to the WW domain of FCA. Consistent with these findings, our study revealed a direct interaction between MiFCA2-SJ and MiFY.

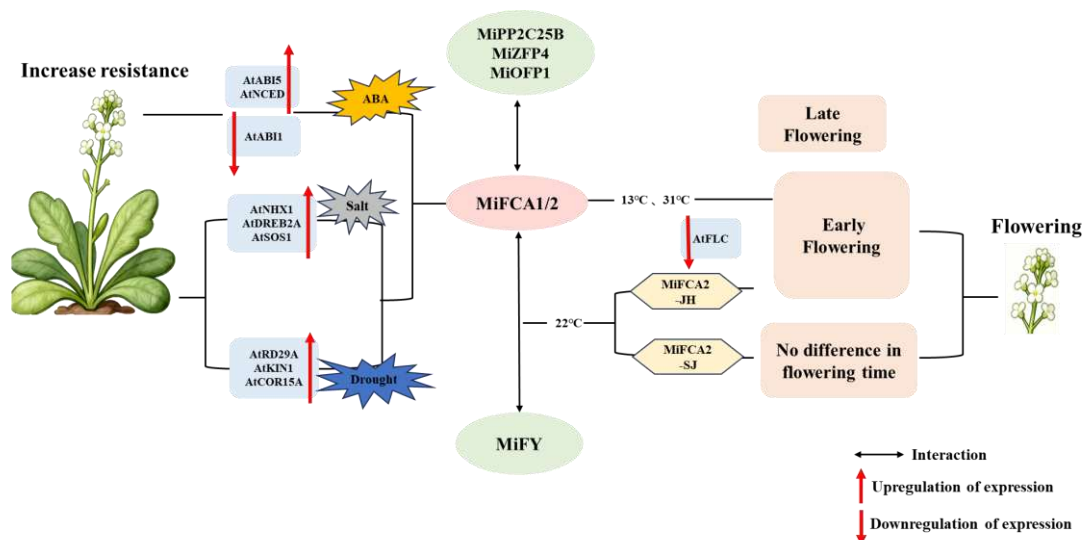


Figure 12 Diagram of the regulatory network of MiFCA2

5 Conclusion

MiFCA2-SJ and *MiFCA2-JH* were isolated from the mango cultivars ‘SiJiMi’ and ‘JinHuang’, respectively. The *MiFCA2* gene is involved in regulating both flowering time and abiotic stress responses (Fig. 12). Interestingly, *MiFCA2-SJ* exhibited a temperature-dependent effect, inducing early flowering at 13 °C and 31 °C but showing

no significant phenotypic alteration at 22 °C. ABA treatment significantly promoted the early flowering of MiFCA2-SJ transgenic plants. MiFCA2-SJ is also involved in regulating the autonomous pathway of mango flowering through its interaction with MiFY. Moreover, MiFCA2-SJ transgenic lines exhibit no drought tolerance, whereas MiFCA2-JH lines confer enhanced drought resistance. But the underlying molecular mechanisms remain elusive. Future studies will focus on generating stable transgenic mango lines to validate these functions.

Data availability

Data will be made available on request.

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Ethics declarations

Ethical approval

Not applicable.

Competing interests

The authors declare no competing interests.

Supplementary information

801 Supplementary file1| Primers used in this work and the following genes and their
802 accession numbers were used

803 Supplementary file 2| A phylogenetic tree of FCA proteins and amino acid
804 sequence alignment

Supplementary Files

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