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MYC2 INVOLVES IN ERUCIC ACID SYNTHESIS BY NEGATIVELY REGULATION *FAE1* TRANSCRIPTION ACTIVITY IN *BRASSICA NAPUS*

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Abstract

Erucic acid is a typical long-chain fatty acid, which is considered to human health but extensively applied in industry and other fields. MYC2 is the crucial regulator in the JA signaling pathway, which participates in plant response to insect, pathogenic bacteria and other stress. However, the involvement of MYC2 in other biological processes is unclear. In this study, we reported that BnMYC2 took part in erucic acid synthesis by directly binding to promoter of *BnFAE1* a major gene during the erucic acid synthesis in *Brassica napus*. GUS histochemistry assay showed that staining was observed in all detected tissues, especially in seed. The subcellular localization indicated that BnMYC2 was located in the nucleus. Yeast one-hybridization and double luciferase experiments indicated that *BnFAE1* transcription activity was regulated by BnMYC2 *In vitro* and *In vivo*, respectively. The results of genetic transformation verified that compared with WT, the expression level of *AtFAE*1 gene was significantly enhanced in the mutant *myc2-2*, and the same tendency was also found in mutant background. Unexpectedly, the opposite trend was observed in *BnMYC2*-overexpression Arabidopsis. Collectively, these results revealed that BnMYC2 played a essential role in erucic acid synthesis in oilseed rape. Our research provided a preliminary understanding of how erucic acid synthesis was modulated by JA signaling in *Brassica napus*.

Key words: *Brassica napus*; *BnMYC*2; *BnFAE*1; Synthesis; Erucic acid.

Abbreviations: CDS, Coding sequence; JA, Jasmonic acid; MeJA, Methyl Jasmonate; GFP, Green fluorescent protein; CaMV, Cauliflower mosaic virus; AbA, Aureobasidin A; Hyg B, Hygromycin B; NIR, Near-infrared spectroscopy; PCR, Polymerase chain reaction; qRT-PCR, Quantitative real-time PCR.

Introduction

As second biggest crucial oil crops in the world (Teng, 2006), rape has three major cultivation types, and *Brassica napus* is the type with the highest yield and the largest planting area among three types. For a long time, cross breeding has been the typical method of rapeseed variety selection and quality improvement. However, the effectiveness of conventional method depends largely on the observation and judgment of the scientists, and the main drawback is the long breeding cycle. Furthermore, linkage relationship between genes generally results in both favorable and unfavorable traits in a variety. Modern biotechnology has been widely applied in rice, corn, wheat and other food crops. In oilseed rape, quality improvements are performed mainly through marker assisted selection on location of the major effect genes to enhancing oil content (Tang *et al*., 2022; Li *et al*., 2022; Song *et al*., 2022; Zhang *et al*., 2023) and enhancement or declination main components levels in fatty acid (Liu *et al*., 2014; Chen *et al*., 2011; Yang *et al*., 2011; Behnke *et al*., 2018; Yang *et al*., 2018; Yan *et al*., 2022; Hu *et al*., 2021; Tang *et al*., 2019).

Erucic acid is an important component of long-chain fatty acid. Animal and clinical experimental results demonstrated that long-term consumption of rapeseed oil with high erucic acid content would enhance the probability of cardiovascular diseases (Tian *et al*., 2015), and induce related metabolic diseases in rats (Gao, 2021), or change the intestinal structure of grass carp by destroying the integrity of the intestinal cell structure of grass carp, thereby reducing the production performance of grass carp in the middle growth period (Gan, 2022). Therefore, reduction of the erucic acid content is required in the rapeseed oil for human consumption. On the other hand, erucic acid, as an essential chemical raw material is widely applied in chemical industry, metallurgy, medicine and other fields (Piazza & Foglia, 2015).

Jasmonic acid is a crucial plant hormone that modulates plants response for several of stresses in a timely manner (Chen *et al*., 2012). Currently, some progresses have been made through research on model plants, but few can be found in oilseed rape. Previous report showed that the contents of palmitic acid, palmitoleic acid, linoleic acid and eicosadienoic acid were decreasd in the seeds of the JA signal mutant *coi1*, while the contents of oleic acid, corunduric acid and erucic acid were enhanced in the same background, suggesting that JA signal might regulate the metabolism of fatty acids through the mechanism of feedback regulation (Niu *et al*., 2009). In rice, the total amount of fatty acids was significantly enhanced in the grains of *OsMED25* gene silencing lines, and the proportion of unsaturated fatty acid and linolenic acid components were also changed in mutant lines (Wang, 2018). Thus, JA signal plays an essential role in modulating the synthesis and metabolism of fatty acids in seeds. In previous work,

we obtained the transgenic *BnMYC*2-overexpression Arabidopsis plants, and found that its resistance to *Spodoptera litura* was obviously enhanced than that in wild type and mutant (Jiang *et al*., 2022). Here, we cloned the promoter sequence of *BnMYC2* located on chromosome No. 6 of C sub-genome from seed of oilseed rape variety 'Zhongyou 821'. To determine whether BnMYC2 involves in regulating the synthesis of erucic acid in seeds; we amplified the promoter sequence of *BnFAE1* gene, which is well-known to modulate erucic acid synthesis in plants, then, analyzed expression pattern of *BnMYC2* and localization of the protein in tobacco leaves cells. Both molecular biological and genetic methods were also carried out for studying the regulatory mechanism of BnMYC2 on transcription activity in *BnFAE1*. The research provides a basis to further study the mechanism of how erucic acid synthesis was regulated by JA signaling.

Material and Methods

Plant materials and growth conditions: The Oilseed rape seed was the 'Zhongyou 821' (*B. napus*). The wild type Col-0 and mutant *myc2-2* (Salk_083483, ABRC) of Arabidopsis (*Arabidopsis thaliana*) were sown in a cultural medium (peat soil: vermiculite= 2:1) and grown in an artificial greenhouse at 22℃ under a long-day condition at proper humidity.

DNA extraction, RNA isolation and qRT-PCR: Genomic DNA of 'Zhongyou 821' seeds was extracted by CTAB method according to previous described (Dugdale *et al*., 2013). DNA quality and concentration were tested by 1% agarose gel electrophoresis. Total RNA was extracted from *Brassica napus* fruits (siliqua) after pollination 45 d using Total RNA Kit (Vazyme, Nanjing, China) and cDNA was synthesized with M-MLV (H-) Reverse Transcriptase Kit (Vazyme, Nanjing, China). Quantitative PCR was done with ChamQ SYBR Color qPCR Master Mix (Vazyme, Nanjing, China). The primers were listed in Supporting Information Table S1. *BnACTIN* was considered as the internal reference.

Spraying treatment of MeJA: To analyze the transcriptional levels of *BnMYC2* and *BnFAE1* in response to Methyl Jasmonate treatment, oilseed rape fruits/siliqua in the late flowering stage were treated with MeJA in different concentration, with clear water as the control, then, matured seeds were harvested for further analysis. Spraying frequency was one time every two days, five times were performed in total. Matured seeds were frozen in liquid nitrogen for following assays.

GUS histochemistry: The ~2.0 kb promoter sequence of *BnMYC2* derived from NCBI website (https://www.ncbi.nlm.nih.gov/gene/106439012) was amplified from genomic DNA. To generate $BnMYC2_{pro}$*: GUS* recombination construct, *BnMYC2* promoter sequence was inserted into digested vector *pCAMBIA1301-GUS* to replace *CaMV 35S* promoter. GUS staining was performed as reported, with minor modifications (Liu *et al*., 2021). Several tissues of Arabidopsis were fixed with 90% ice-cold acetone for 30 minutes. Then, materials were cleaned with sterile water. The tissues and whole plants were collected and stained overnight at 37℃ with staining solution [0.5M Phosphate buffer, pH 7.2, 0.1% Triton X-100, 0.1 M $K_3Fe(CN)_6$, 0.5 M EDTA, 2 mM X-Gluc] to visualize GUS activity. After staining, the tissues were decolorized with 70% ethanol.

Construction of the GFP fusion plasmids and subcellular localization of BnMYC2: The coding sequence of *BnMYC2* was cloned into Pcambia1302-GFP to create GFP fusion constructs. Then, indicated constructs and empty vector *35S: GFP* were transformed into *Agrobacterium* strain GV3101, respectively. The MMA impregnation solution (10 mM MES, pH 5.6, 10 mM $MgSO_4$, 150 μM acetosyringone) was prepared and the mycelium was re-suspended. The OD value of the transformation solution was 0.5-0.6 and stewed 2-3 hours in the dark. *Agrobacterium* containing recombinant vector and empty vector were injected into the *Nicotiana benthamiana* tobacco leaves that have grown for 30 days, respectively. The infected tobacco was cultured overnight at 25℃ in the dark, then, transferred to the normal culture under the light condition the next day. The location of the protein was observed with the Zeiss fluorescence inversion microscope (Axio Imager M2, Germany) within 48 hours.

Bioinformatics analysis of BnMYC2 CDS and BnFAE1 promoter in Brassica napus: The cloned sequence of *BnMYC2* CDS and *BnFAE1* promoter were aligned with corresponding sequence derived from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), respectively. The possible *cis-elements* of *BnFAE1* promoter sequence were analyzed by promoter regulatory element prediction website Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html).

Yeast one-hybrid experiment: The cloned *BnFAE1* promoter or three truncated fragments, named *BnFAE1-Pro-1*, *BnFAE1-Pro-2*, *BnFAE1-Pro-3*, with sequence lengths of 570 bp, 527 bp, and 542 bp, respectively, inserted into *pAbAi*, which was digested with restriction endonuclease *BstB*I. The CDS of *BnMYC2* was inserted into *pGADT7*. Recombination constructions of promoter and truncated versions were transformed into yeast competent cell Y1H Gold. The trans-formants were grown on SD/-Ura nutrient deficient medium 2-3 days. To eliminate the possibility in transcription activity of marker gene was induced by endogenous proteins, AbA with different concentrations were added into SD/-Ura medium for ascertain appropriate AbA inhibited concentration. *pGADT7-BnMYC2* was transformed into the competent cells, generated from the yeast bait receptor strain to obtain corresponding co-transformed strains, and then transforms were grown on SD/- Leu containing the optimal AbA. The plasmid *pGADT7-53* and *pGADT7* were transformed as positive and negative controls, respectively, and all groups were cultured at 30℃ for 3-5 days.

Supporting Information Table 1. List of primers used in this study.

Primer name	Primer sequence (5'-3')	Function
BnFAE1	F: GAGAAGGATGTAAATAGTTGGGAAGT R: GGAGAATAGTAGAGGAAGCGATGAG	Gene cloning for *BnFAE1* promoter
BnMYC2-Pro-GUS	F: GACCTGCAGGCATGCGGATCCAAGCTTGGCACTGGC R: TTACCCTCAGATCTACCATGGCCATGGTCAAGAGTC	GUS Staining
1302-BnMYC2-GFP	F: ACTCTTGACCATGGTAGATCTATGACGGAGCCGACGATGAAT R: AAGTTCTTCTCCTTTACTAGTACCAATCTTTGAGATTA	Subcellular localization of BnMYC2
pGADT7-BnMYC2	F: GTACCAGATTACGCTCATATGATGACGGAGCCGACGATGAAT R: CAGCTCGAGCTCGATGGATCCTTAACCAATCTT TGAGATTA	Construction vector of *BnMYC2* for yeast one-hybrid
pAbAi-BnFAE1-Pro	F: CTTGAATTCGAGCTCGGTACCGAGAAGGATGTA AATAGTTGGGAAGT R: ATACAGAGCACATGCCTCGAGGGAGAATAGTA GAGGAAGCGATGAG	Construction vector of full-length of *BnFAE1* promoter for yeast one-hybrid
pAbAi-BnFAE1-Pro-1	F: CTTGAATTCGAGCTCGGTACC GAGAAGGATGTA AATAGTTGGGAAGT R: ATACAGAGCACATGCCTCGAGCGAAGACCGACT CCGCCTTCTC	Construction vector of truncation 1 of *BnFAE1* promoter for yeast one-hybrid
pAbAi-BnFAE1-Pro-2	F: CTTGAATTCGAGCTCGGTACCGGTCTTCGGTTTC GGCCGAGAA R: ATACAGAGCACATGCCTCGAGCATCATAAACTCATTGCC	Construction vector of truncation 2 of *BnFAE1* promoter for yeast one-hybrid
pAbAi-BnFAE1-Pro-3	F: CTTGAATTCGAGCTCGGTACCGATCGGTAATAACCTTTC R: ATACAGAGCACATGCCTCGAGGGAGAATAGTA GAGGAAGCGATGAG	Construction vector of truncation 3 of *BnFAE1* promoter for yeast one-hybrid
62SK-BnMYC2	F: CGCTCTAGAACTAGTGGATCCATGACGGAGCCG ACGATGAAT R: GTCGACGGTATCGATAAGCTTTTAACCAATCTTTGAGATTA	Construction vector of *BnMYC2* for dual luciferase analysis
BnFAE1-Pro-LUC	F: CTATAGGGCGAATTGGGTACCGAGAAGGATGTAAAT AGTTGGGAAGT R: CGCTCTAGAACTAGTGGATCCGGAGAATAGTA GAGGAAGCGATGAG	Construction vector of full-length of *BnFAE1* promoter for dual luciferase analysis
BnFAE1-Pro-1-LUC	F: CTATAGGGCGAATTGGGTACCGAGAAGGATGTAAATAGTTGGGAAGT R: CGCTCTAGAACTAGTGGATCCCGAAGACCGACT CCGCCTTCTC	Construction vector of truncation 1 of *BnFAE1* promoter for dual luciferase analysis
BnFAE1-Pro-2-LUC	F: CTATAGGGCGAATTGGGTACCGGTCTTCGGTTTC GGCCGAGAA R: CGCTCTAGAACTAGTGGATCCCATCATAAACTCATTGCC	Construction vector of truncation 2 of *BnFAE1* promoter for dual luciferase analysis
BnFAE1-Pro-3-LUC	F: CTATAGGGCGAATTGGGTACCGATCGGTAATAACCTTTC R: CGCTCTAGAACTAGTGGATCCGGAGAATAGTA GAGGAAGCGATGAG	Construction vector of truncation 3 of *BnFAE1* promoter for dual luciferase analysis
QAtFAE1	F: ATCGTAACCCGACCCAATCC R: TGCCACGTTCCGTGAAGAAG	Expression analysis of *AtFAE1*
QBnMYC2	F: CGGATCAGGAGTACAGGA R: GAAAAACCATTCCGTATCCGTC	Expression analysis of *BnMYC2*
QBnFAE1	F: AACCTCATAACCATCGCTCC R: TCAAGAAGTCAAGCCACGAC	Expression analysis of *BnFAE1*
QBnACTIN	F: TGCTCTTCCTCACGCTATCCTC R: GCTCGTAGTTCTTCTCCACCG	Keep house gene for expression analysis of *BnFAE1* and *BnMYC2*
QAtTUB2	F: ATCCGTGAAGAGTACCCAGAT R: AAGAACCATGCACTCATCAGC	Keep house gene for expression analysis of *AtFAE1*

Double luciferase analysis: The full-length of *BnMYC2* CDS were cloned into *pGreenII 62-SK* as effectors, and full-length of *BnFAE1* promoter or three truncated versions were inserted into *pGreen II 0800-LUC* as reporter plasmids, respectively. *Agrobacterium tumefaciens* GV3101 containing the recombinant constructs was cultured to OD_{600} = 0.7. The *Agrobacterium tumefaciens* suspensions of the recombinant vectors *BnFAE1$_{Pro}$: LUC*, *BnFAE1$_{Pro-1}$: LUC*, *BnFAE1$_{Pro-2}$: LUC*, *BnFAE1$_{Pro-3}$: LUC* and *35S: BnMYC2* were mixed in a ratio of 1: 9 and injected into *Nicotiana benthamiana* leaves according to different combination of these constructs. After the injected tobacco was incubated at 25°C for 3 days, the fluorescence values of LUC and REN were surveyed using Thermo Scientific TM Varioskan TM LUX (Thermo Fisher, USA) and the fluorescence activity was determined by calculating the ratio.

Genetic transformation: To generate transgenic plants, genetic transformation in Arabidopsis was done by floral dip method as reported by Clough & Bent (1998). To harvest positive seedlings, T_0 generation seeds were selected in 1/2 MS medium plates adding 20 μg/ml Hyg B. T_1 generation seedlings were grown on the same condition to screen root length separation ratio whether demand standard of 3:1. To generate pure line plants, selected lines were screened further till T_3 generation.

***BnMYC2* heterologous expression regulates the erucic acid synthesis:** The expression level of *AtFAE1* was analyzed in the *BnMYC2* transgenic *Arabidopsis thaliana* as described (Jiang *et al*., 2022), and the fatty acid compositions in transgenic Arabidopsis thaliana were monitored using Foss B291 KMAT NIR transport system. The method is briefly described as follows. Pure over-expression plants seeds approximately 6 grams each time per sample type were tested by NIR, then data were automatically calculated according to pre-designed parameters and formulae were set by manufacturer.

Statistical data analysis

The obtained data were done via one-way analysis of variance (ANOVA). Letters indicate statistical differences (p<0.05). Significance difference analysis of root length separation ratio in overexpressed offspring was detected with the chi-square test.

Results

Bioinformatics analysis of BnFAE1 promoter in oilseed rape: The prediction results online indicated that, both basic elements such as CAAT-box and TATA-box, a light-induced response element I-box and a low-temperature induced response element LTR were found in the promoter. In addition, multiple hormone response elements were also identified, for instance, the auxin response element TGA, the gibberellin response element GARE, the abscisic acid response element ABRE, and the MYC2 transcription factor binding G-box and G-box like (Table 1).

Expression analysis *BnMYC2* and *BnFAE1*: Different concentration of MeJA was sprayed in initial stage of formation of oilseed rape fruit/ siliqua, mature seeds were collected and expression levels were analyzed by RT-qPCR. The results indicated that compared with untreated control, the transcriptional level of *BnMYC2* was enhanced in different concentration of MeJA (Fig. 1A). However, the transcriptional level of *BnFAE1* was declined in the same conditions (Fig. 1B).

Expression pattern of *BnMYC2*: The Arabidopsis homozygous lines overexpressing *BnMYC2$_{pro}$: GUS* was obtained and GUS staining was performed. The results exhibited the GUS staining in the border of stem and root, flower, stem, leaf, pod and seeds in overexpression plants (Fig. 2B-G). However, little GUS staining was observed in wild-type Col-0 (Fig. 2A).

Subcellular localization of *BnMYC2*: To ascertain distribution of BnMYC2 in the cell, we generated full-length *BnMYC2-GFP* fusions under control of the 35S cauliflower mosaic virus (CaMV; *35S::BnMYC2-GFP*) promoter. The constructs was transiently expressed in epidermal cells of tobacco leaves. Fluorescence of *35S::GFP*, which was used as a negatively control, was found at location both in the cytoplasm and nucleus. Fluorescence of *35S:: BnMYC2-GFP* was only detected in the nucleus. Thus, these results demonstrated that the BnMYC2 protein was localized in the nucleus (Fig. 3).

Table 1. Analysis of the cis-elements of *BnFAE1* promoter.

Regulation element	Functional annotation	Number
W box	Induced response element	1
RY	Cis-acting regulatory element involved in seed-specific regulation	1
MBS	MYB-binding site	2
I box	Light response element	2
LTR	cis-acting element involved in low-temperature responsiveness	3
ABRE	Cis-acting element involved in the abscisic acid responsiveness	2
GARE	Gibberellin-responsive element	2
TGA	Auxin-responsive element	2
G box	MYC2-binding site	1
G box-like	MYC2 binding-like site	4
CAAT box	Common cis-acting element in promoter and enhancer regions	14
TATA box	Core promoter element around -30 of transcription start	17

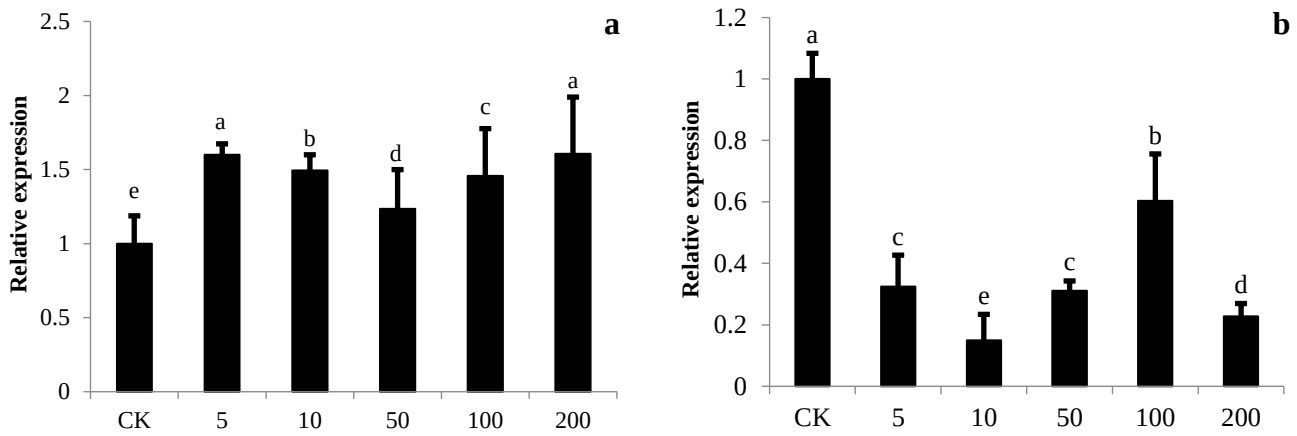


Fig. 1. Relative expression level of *BnMYC2* and *BnFAE1* in MeJA treatment. Mock indicates clean water treatment; Arabic numerals indicates different concentrations of MeJA. Different letter on the top of error bar indicates remarkable differences between treatments (p<0.05). Transcript levels of *BnMYC2* (left) and *BnFAE1* (right) were determined by RT-qPCR. Relative gene expression value in the mock regarded as 1. Data show means ± SD (n=3). (a) Relative expression level of *BnMYC2* in MeJA treatment. (b) Relative expression level of *BnFAE1* in MeJA treatment.

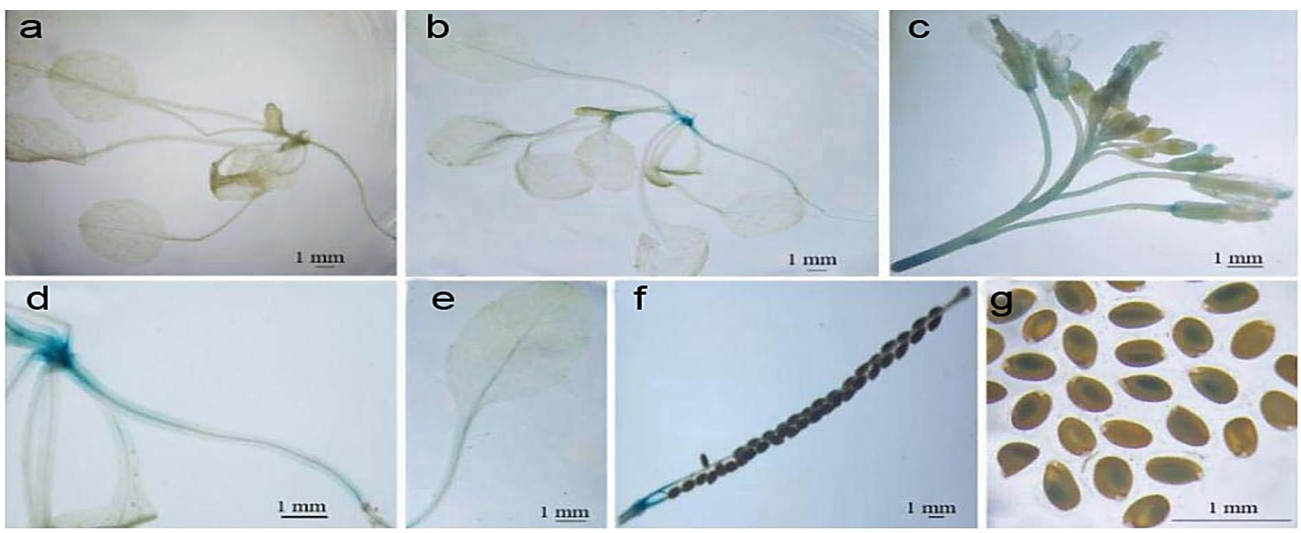


Fig. 2. Reporter gene expression in Col-0 and *BnMYC2$_{pro}$: GUS* Plants. (a) 7 to 10-d-old seedlings of wild-type Col-0. Bar =1 mm. (b) 7 to 10-d-old seedlings of *BnMYC2$_{Pro}$: GUS* plants. Bar = 1 mm. (c) Flower. Bar =1 mm. (d) Border of stem and root. Bar =1 mm. (e) Leaf. Bar =1 mm. (f) Pod. Bar = 1 mm. (g) Seeds. Bar = 1 mm.

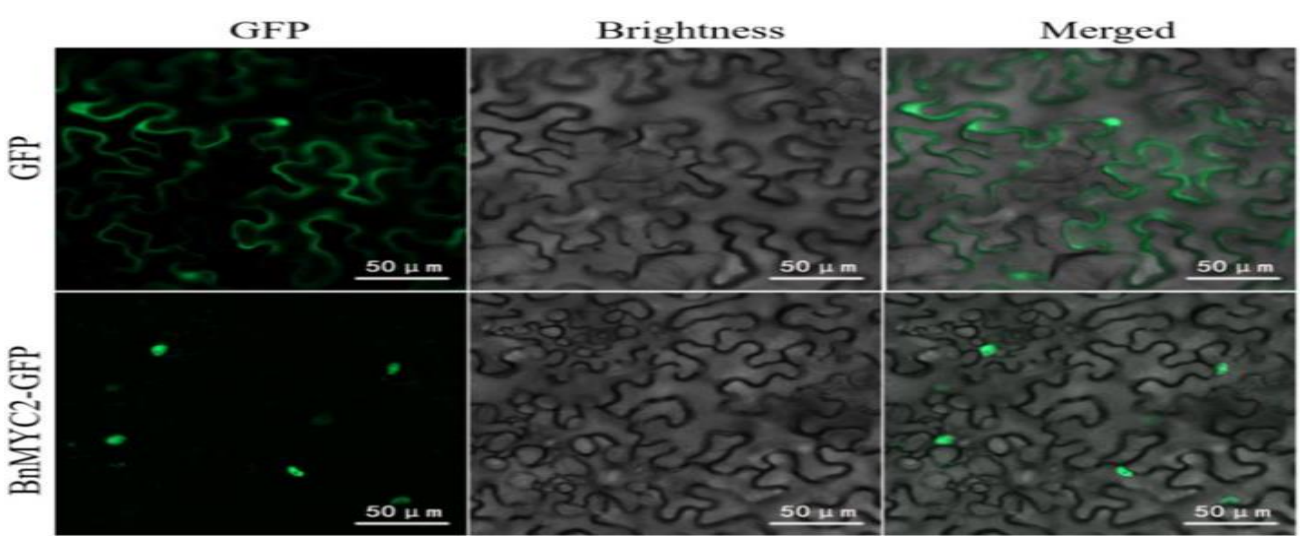
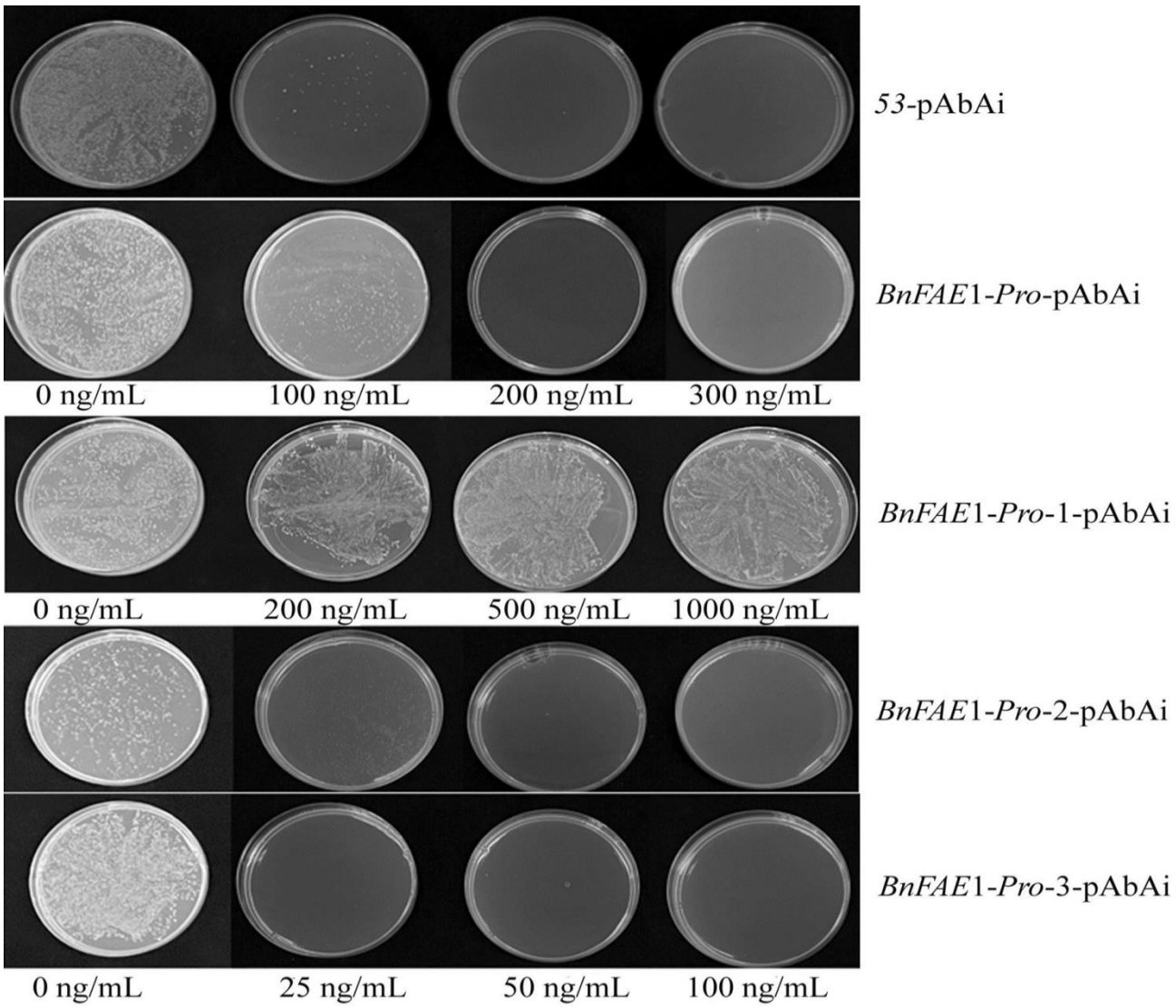


Fig. 3. Subcellular localization of BnMYC2. Green indicates GFP fluorescence (left row), brightness indicates tobacco epidermal cell status (middle row), merged indicates regions where the two types mentioned above merged (right row). GFP signals from the empty vector *pCAMBIA1302-GFP* is as control (upper line), nuclear localization signal of BnMYC2 is shown (bottom line). Bar = 50 μm.



Supplemental Fig. 1. The screening of the optimum Aba concentration for Yeast bait expression vector.

Yeast one-hybrid: Yeast bait receptor strains *p53-AbAi*, *BnFAE1-Pro-pAbA*i, *BnFAE1-Pro-1-pAbAi*, *BnFAE1-Pro-2-pAbAi*, and *BnFAE1-Pro-3-pAbAi* were grown on SD/- Leu medium containing AbA. The results indicated that the optimal AbA concentrations for *p53-AbAi* and *BnFAE1-Pro-pAbAi* was 200 ng/mL, for *BnFAE1-Pro-2-pAbAi*, and *BnFAE1-Pro-3-pAbAi* were 50 ng/mL and 25 ng/mL, respectively. Unexpectedly, *BnFAE1-Pro-1-pAbAi* was found to normally grow on an Aba concentration of 1000 ng/mL (Supplemental Fig. 1). The combinations *pGADT7-BnMYC2*/*BnFAE1-Pro-pAbAi*, *pGADT7-BnMYC2*/*BnFAE1-Pro-2-pAbAi*, *pGADT7-BnMYC2*/*BnFAE1-Pro-3-pAbAi* and positive control *pGADT7-53*/*p53-AbAi* were able to grow on SD/-Leu/AbA* medium, while negative control *pGADT7*/*BnFAE1-Pro-pAbAi* failed to grow in the same conditions (Fig. 4).

Double luciferase analysis: To further verify BnMYC2 binding to *BnFAE1* promoter *In vivo*, we performed double luciferase analysis in tobacco leaves. Co-transformed with *35S::BnMYC2* and *BnFAE1* promoter, the relative luminescence activity was notably lower than that of the control, which indicated that the BnMYC2 transcription factor inhibited *BnFAE1* transcription activity through combining *cis-acting* elements. Among the three truncated fragments of the *BnFAE1* promoter, the luminescence activities of co-transformed with *35S::BnMYC2* and *BnFAE1-Pro-1: LUC* or *35S::BnMYC2* and *BnFAE1-Pro-2: LUC* were remarkably lower than that of the control, but the luminescence activity of co-transformed with *35S::BnMYC2* and *BnFAE1-Pro-3: LUC* was not obviously diversity from that of the control (Fig. 5A and 5B).

***BnMYC2* involved in erucic acid synthesis on transgenic Arabidopsis:** To analyze BnMYC2 participates erucic acid synthesis in plant, *AtFAE1* expression levels was detected on the *BnMYC2* over-expression Arabidopsis. The lowest transcriptional level of *AtFAE1* was found in *BnMYC2-OE*, followed by that in wild-type Col-0, and the highest expression level of *AtFAE1* was observed in *myc2-2* (Fig. 6). To further confirm these results, the fatty acid composition of Arabidopsis seeds in different genetic backgrounds were detected and the results indicated that the eicosenoic acid (C20:1) and erucic acid (C22:1) of *BnMYC2* over-expression Arabidopsis were significantly lower than those of the wild type, but the opposite trend was observed in the *myc2-2* mutant (Fig. 7).

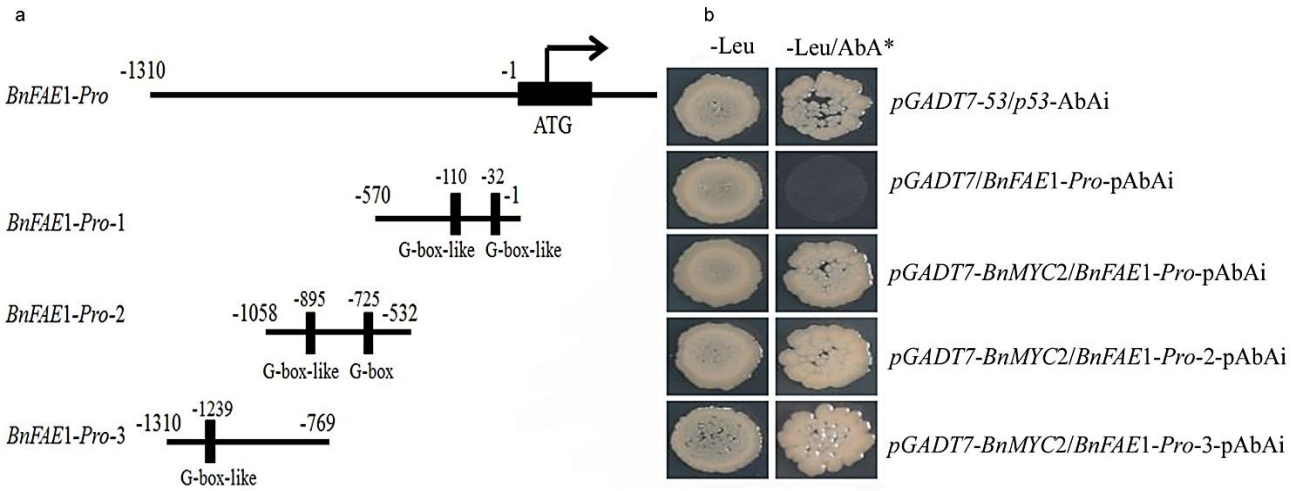


Fig. 4. Y1H of BnMYC2 with full-length and truncated *BnFAE1* promoter. (a) The full-length of *BnFAE1* promoter and truncated fragments diagrams. (b) BnMYC2 binds to *BnFAE1* promoter and truncated versions, respectively.

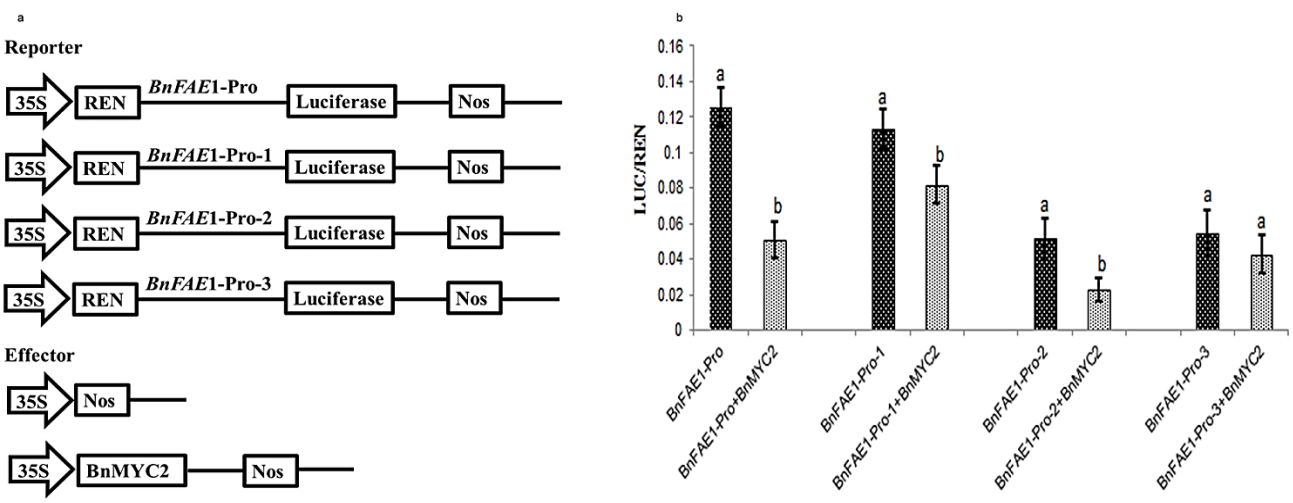


Fig. 5. BnMYC2 binding to motif in the *BnFAE1* promoter in plant. (a) The Reporter and Effector diagrams. (b) BnMYC2 inhibits *BnFAE1* transcript activity. LUC/REN shows the ratio of the signal tested for firefly luciferase (LUC) versus Renilla reniformis luciferase (REN) activity. Error bars represent standard deviation for three replicates. The different letters above each bar indicate that the means differ significantly by one-way ANOVA and Turkey's multiple comparison test at $p<0.05$.

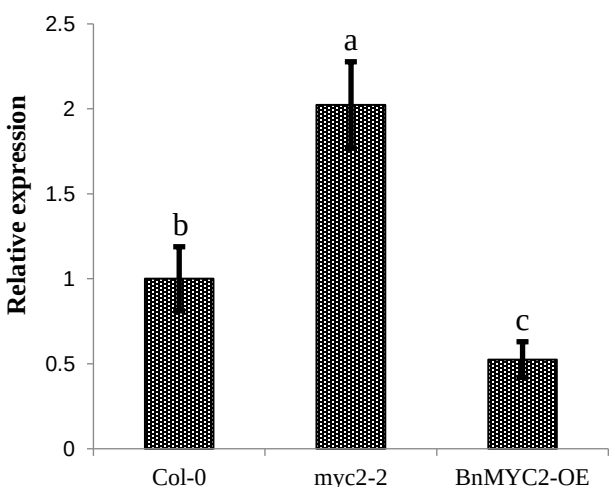


Fig. 6 Expression analysis of *AtFAE1*. Error bars represent standard deviation for three replicates. The different letters above each bar indicate that the means differ significantly by one-way ANOVA at $p<0.05$.

Discussion

Transcription factors are well-known class of important regulators in eukaryotes, which directly activate or repressed down-stream gene expression by bind to *cis-acting* elements of target gene promoters or indirectly modulate through interact with regulators in the nucleus (Jin *et al*., 2014). In this work, recombination vector of *35S::BnMYC2* was constructed and transiently expressed in tobacco leaves. The result verified that BnMYC2 was located in the nucleus of cells (Fig. 3), which was similar to the results of previous reports (Chini *et al*., 2009; Yue *et al*., 2023; Uji *et al*., 2016).

Fatty acids, including erucic acid, are mainly synthesized in seeds (Tang *et al*., 2020). To determine whether BnMYC2 involved in erucic acid synthesis, the expression levels of *BnMYC2* and *BnFAE1* in seed were tested by the RT-qPCR. Compared with mock, the transcriptional level of *BnMYC2* was respond to different concentrations of MeJA treatments. However, the

expression level of *BnFAE1* was declined under the same treatment conditions (Fig. 1). GUS staining also indicated that the expression of *BnMYC2* also was observed in nearly all tested tissues, especially in seed. These results suggested the possibility of BnMYC2 modulated the transcription of *BnFAE1*. MYC2 regulates expression of downstream gene by binding to the target gene promoter and thereby affecting the transcription of the latter. For instance, GmMYC2a modulated seed weight in soybean by directly enhancement transcriptional activity of cytokinin oxidase gene *G. max CYTOKININ OXIDASE 3-4* (*GmCKX3-4*) (Hu *et al*., 2023). In maize, ZmMYC2s involves in benzoxazinoid biosynthesis by directly binding to promoters of biosynthesis genes, such as *IGPS1/3*, *BX10/11/12/14*, *TPS10/2/3/4/5/8* (Ma *et al*., 2023). Using genetic and molecular biology assays, a direct target gene of MYC2, *SlJIG,* exhibited playing role in terpene biosynthesis (Cao *et al*., 2022). To confirm that BnMYC2 regulated *BnFAE1* transcription activity, we performed bioinformatics analysis of *BnFAE1* gene promoter (Table 1). Then, yeast one-hybrid and dual luciferase assays were done to verify whether BnMYC2 modulated *BnFAE1* transcription activity *In vitro* and *In vivo*, respectively. Y1H results indicated that BnMYC2 was binding to the full-length of the promoter and the truncated versions *BnFAE1-Pro-2* and *BnFAE1-Pro-3* (Fig. 4). Double luciferase assay results also demonstrated that the BnMYC2 inhibited transcription activity of *BnFAE1* by binding to the full-length of promoter and the truncated fragments *BnFAE1-Pro-1* and *BnFAE1-Pro-2* (Fig. 5). However, the intense self-activation of truncated *BnFAE1-Pro-1* was observed in yeast one-hybrid system, we cannot be fully ascertain BnMYC2 binding to *BnFAE1-Pro-1*, other experiments need to be performed to analyze possibility of combination. At present, the results of experiments were performed *In vitro* and *In vivo* indicated that BnMYC2 repressed *BnFAE1* transcription activity by binding to the *BnFAE1-Pro-2* fragment of the gene promoter.

To further confirm the results of molecular biology, transcription level of *AtFAE1* was analyzed in different genetic background. The result indicated that the highest transcriptional level was detected in *myc2-2* mutant, the next was in Col-0, and the lowest was observed in *BnMYC2*-overexpression plant (Fig. 6). We also analyzed the fatty acid composition in these materials, and found that the content of long chain fatty acids declined and the content of oleic acid was enhanced in *BnMYC2*-overexpression Arabidopsis, while the opposite trend was observed in *myc2-2* mutants (Fig. 7). Previous reports have demonstrated that the content of oleic acid in the seeds of *coi1* is higher than that of the wild type (Niu *et al.*, 2009). COI1 and MYC2 are widely involved in other signaling pathways and interact with different hub regulator (Nakata *et al*., 2013; Wild *et al*., 2012; Pan *et al*., 2020; Mei *et al*., 2023; Song *et al*., 2014; Hong *et al*., 2012), we speculated JA signal receptor COI1 and regulator MYC2 may play different roles on the fatty acids composition synthesis. Furthermore, different regulators interact with COI1 and MYC2, respectively, which suggested the fatty acid was regulated by other hormones in addition to JA. The synthesis of fatty acids is a complex network of gene modulation and enzyme catalysis processes, and fatty acid components can also interplay each other. This study will play an essential role in in-depth understanding of the complex mechanism of JA involves in erucic acid synthesis.

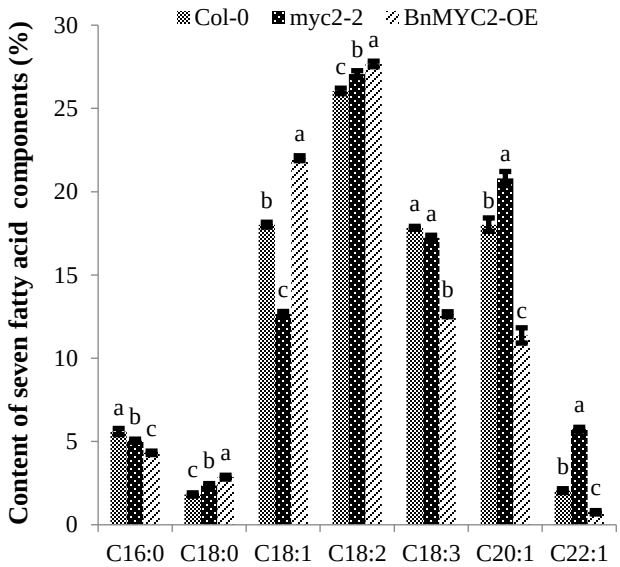


Fig. 7. Fatty acid components in *BnMYC*2 overexpression in Arabidopsis seeds. C16:0 indicates palmitic acid, C18:0 indicates stearic acid, C18:1 indicates oleic acid, C18:2 indicates linoleic acid, C18:3 indicates linolenic acid, C20:1 indicates eicosenoic acid, C22:1 indicates erucic acid. Error bars represent standard deviation for three replicates. The different letters above each bar indicate that the means differ significantly by one-way ANOVA and Turkey's multiple comparison test at $p<0.05$.

Conclusions

In summary, BnMYC2 located in the nucleus of *Nicotiana benthamiana* tobacco leaf cells, identified as the transcription factor to regulate expression of downstream gene. Spraying treatment and expression pattern analysis of *BnMYC2* suggested that expression level of *BnFAE1* was modulated by BnMYC2, which was demonstrated by yeast one-hybrid system and double luciferase analysis. We also obtained similar results by analysis *AtFAE1* expression and fatty acid components in *BnMYC2* over-expression transgenic Arabidopsis. Our study provided valuable clues for revealing the molecular mechanism of how erucic acid synthesis was influenced by JA signaling.

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References

Behnke, N., E. Suprianto and C. Möllers. 2018. A major QTL on chromosome Co5 significantly reduces acid detergent lignin (ADL) content and increases seed oil and protein content in oilseed rape (*Brassica napus* L.) *Theor. Appl. Genet*., 131: 2477-2492.

Cao, Y.Y., L. Liu, K.S. Ma, W.J. Wang, H.M. Lv, M. Gao, X.M. Wang, X.C. Zhang, S.X. Ren, N. Zhang and Y.D. Guo. 2022. The jasmonate-induced bHLH gene *SlJIG* functions in terpene biosynthesis and resistance to insects and fungus. *J. Integ. Plant Biol*., 64(5): 1102-1115.

Chen, R., H.L. Jiang, L. Li, Q.Z. Zhai, L.L. Qi, W.K. Zhou, X.Q. Liu, H.M. Li, W.G. Zheng, J.Q. Sun and C.Y. Li. 2012. The Arabidopsis mediator subunit MED25 differentially regulates jasmonate and abscisic acid signaling through interacting with the MYC2 and ABI5 transcription factors. *Plant Cell*, 24(7): 2898-2916.

Chen, W., C. Fan, J. Qin, Z.H. Guo, T.D. Fu and Y.M. Zhou. 2011. Genetic improvement of oleic and linolenic acid content through marker-assisted selection in *Brassica napus* seeds. *Mol Breed.*, 9: 123-127.

Chini, A., M. Boter and R. Solano. 2009. Plant oxylipins: COI1/JAZs/MYC2 as the core jasmonic acid-signaling module. *FEBS J*., 276(17): 4682-4692.

Clough, S.J. and A.F. Bent. 1998. Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J*., 16(6): 735-743.

Dugdale, B., C.L. Mortimer, M. Kato, T.A. James, R.M. Harding and J.L. Dale. 2013. In plant activation: an inducible, hyper-expression platform for recombinant protein production in plants. *Plant Cell*, 25(7): 2429-2443.

Gan, L. 2022. Effect of erucic acid on growth and intestinal structure of on-growing grass carp (Ctenopharyngodon idella) and involved mechanism. Dissertation

Gao, T. 2021. Effect of erucic acid on fatty acid metabolism in rat liver and mechanism study. Dissertation

Hong, G.J., X.Y. Xue, Y.B. Mao, L.J. Wang and X.Y. Chen. 2012. Arabidopsis MYC2 interacts with DELLA proteins in regulating sesquiterpene synthase gene expression. *Plant Cell*, 24(6): 2635-2648.

Hu, D.D., Y.S. Zhao, J.X. Shen, X.X. He, Y.K. Zhang, Y. Jiang, R. Snowdon, J.L. Meng, J.C. Reif and J. Zou. 2021. Genome-wide prediction for hybrids between parents with distinguished difference on exotic introgressions in *Brassica napus*. *Crop J*., 9: 1169-1178.

Hu, Y., Y. Liu, J.J. Tao, L. Lu, Z.H. Jiang, J.J. Wei, C.M. Wu, C.C. Yin, W. Li, Y.D. Bi, Y.C. Lai, W. Wei, W.K. Zhang, S.Y. Chen and J.S. Zhang. 2023. GmJAZ3 interacts with GmRR18a and GmMYC2a to regulate seed traits in soybean. *J. Integ. Plant Biol*., 65(8): 1983-2000.

Jiang, Q., Y. Yang, S.X. Guo, Y.C. Wu and Z. Li. 2022. Study of BnMYC2 cloning and resistance to insect stress. *Acta Bot. Boreali-Occidentalia Sinica*., 42: 201-209.

Jin, J., H. Zhang, L. Kong, G. Gao and J. Luo. 2014. Plant TFDB 3.0: A portal for the functional and evolutionary study of plant transcription factors. *Nucl. Acids Res*., 42 (D1): D1182-D1187.

Li, Y.Q., U. Ali, Z.X. Cao, C.H. Zeng, M.Y. Xiao, F. Wei, X. Yao and S.P. Lu and L. Guo. 2022. Fatty acid exporter 1 enhances seed oil content in Brassica napus. *Mol. Breed*., 42: 75.

Liu, H.L., Y.G. Li, Y.L. Hu, Y.H. Yang, W.B. Zhang, M. He, X.M. Li, C.Y. Zhang, F.J. Kong, X. Liu and X.L. Hou. 2021. EDS1-interacting J protein 1 is an essential negative regulator of plant innate immunity in Arabidopsis. *Plant Cell*, 33: 153-171.

Liu, L.Z. and J.N. Li. 2014. QTL mapping of oleic acid, linolenic acid and erucic acid content in Brassica napus by using the high density SNP genetic map. *Sci. Agri. Sinica*., 47: 24-32.

Ma, C.R., R.Y. Li, Y. Sun, M. Zhang, S. Li, Y.X. Xu, J. Song, J. Li, J.F. Qi, L. Wang and J.Q. Wu. 2023. ZmMYC2s play important roles in maize responses to simulated herbivory and jasmonate. *J. Integ. Plant Biol*., 65(4): 1041-1058.

Mei, S., M.H. Zhang, J.W. Ye, J.C. Du, Y.J. Jiang and Y.R. Hu. 2023. Auxin contributes to jasmonate-mediated regulation of abscisic acid signaling during seed germination in Arabidopsis. *Plant Cell*, 35: 1110-1133.

Nakata, M., N. Mitsuda, M. Herde, A.J. Koo, J.E. Moreno, K. Suzuki, G.A. Howe and M.O. Takagi. 2013. A bHLH-Type Transcription Factor, ABA-Inducible BHLH-Type Transcription Factor/JA-Associated MYC2-Like1, acts as a repressor to negatively regulate jasmonate signaling in Arabidopsis. *Plant Cell*, 25(5): 1641-1656.

Niu, Y., G.Z. Wu, R. Ye, W.H. Lin, Q.M. Shi, L.J. Xue, X.D. Xu, Y. Li, G. Du and H.W. Xue. 2009. Global analysis of gene expression profiles in *Brassica napus* developing seeds reveals a conserved lipid metabolism regulation with *Arabidopsis thaliana*. *Mol. Plant*, 2(5): 1107-1122.

Pan, J.J., Y.R. Hu, H.P. Wang, Q. Guo, Y.N. Chen, G.A. Howe and D.Q. Yu. 2020. Molecular mechanism underlying the synergetic effect of jasmonate on abscisic acid signaling during seed germination in Arabidopsis. *Plant Cell*, 32: 3846-3865.

Piazza, G. and T. Foglia. 2015. Rapeseed oil for oleochemical usage. *Eur. J. Lipid Sci. Tech*., 103: 450-454.

Song, J.M., Y.T. Zhang, Z.W. Zhou, S.P. Lu, W. Ma, C.F. Lu, L.L. Chen and L. Guo. 2022. Oil plant genomes: current state of the science. *J. Exp. Bot*., 73(9): 2859-2874.

Song, S.S., H. Huang, H. Gao, J.J. Wang, D.W. Wu, X.L. Liu, S.H. Yang, Q.Z. Zhai, C.Y. Li, T.C. Qi and D.X. Xie. 2014. Interaction between MYC2 and Ethylene Insensitive3 modulates antagonism between jasmonate and ethylene signaling in Arabidopsis. *Plant Cell*, 26(1): 263-279.

Tang, M.Q., Y.Y. Zhang, Y.Y. Liu, C.B. Tong, X.H. Cheng, W. Zhu, Z.Y. Li, J.Y. Huang and S.Y. Liu. 2019. Mapping loci controlling fatty acid profiles, oil and protein content by genome-wide association study in *Brassica napus*, *Crop J*., 7: 217-226.

Tang, S, D.X. Liu, S.P. Lu, L.Q. Yu, Y.Q. Li, S.L. Lin, L. Li, Z.L. Du, X. Liu, X. Li, Q.Y. Yang and L. Guo. 2020. Development and screening of EMS mutants with altered seed oil content or fatty acid composition in *Brassica napus*. *Plant J*., 104: 1410-1422.

Tang, S., N. Guo, Q.Q. Tang, F. Peng, Y.H. Liu, H. Xia, S.P. Lu and L. Guo. 2022. Pyruvate transporter BnaBASS2 impacts seed oil accumulation in *Brassica napus*. *Plant Biotechnol. J*., 20(12): 2406-2417.

Teng, H.Y. 2006. Changes in the production and trade of major grain and oil crops in the world. *World Agri.,* 8: 31-33.

Tian, D., S. Wang, L. Wang, J. Wang and F. Li. 2015. The biosynthesis and metabolic engineering of very long-chain monounsaturated fatty acid. *Biotechnol. Bull*., 31: 42-49.

Uji, Y., S. Taniguchi, D. Tamaoki, H. Shishido, K. Akimitsu and K. Gomi. 2016. Overexpression of OsMYC2 results in the up-regulation of early JA-responsive genes and bacterial blight resistance in rice. *Plant Cell Physiol*., 57: 1814-1827.

Wang, W. 2018. The mechanism of mediator subunit OsMED25 regulates fatty acid biosynthesis in Rice seeds. Dissertation

Wild, M., J.M. Davière, S. Cheminant, T. Regnault, N. Baumberger, D. Heintz, R. Baltz, P. Genschik and P. Achard. 2012. The Arabidopsis DELLA RGA-LIKE3 is a direct target of MYC2 and modulates jasmonate signaling. *Plant Cell*, 24(8): 3307-3319.

Yan, S.X., H.X. Li, H.B. Chao, J.J. He, Y.R. Ding, W.G. Zhao, K. Zhang, Y.Y. Xiong, K. Chen, L.B. Zhang and M.T. Li. 2022. Refinement of four major QTL for oil content in Brassica napus by integration of genome resequencing and transcriptomics. *Crop J*., 10: 627-637.

Yang, S.J., B. Zhang, G. Liu, B.H. Hong, J.S. Xu, X. Chen, B. Wang, Z.K. Wu, F. Hou, X.P. Yue, J. Wang, Q.H. Zhang, G.J. King and K.D. Liu. 2018. A comprehensive and precise set of intervarietal substitution lines to identify candidate genes and quantitative trait loci in oilseed rape (*Brassica napus* L.) *Theor Appl Genet*., 131: 2117-2129.

Yang, Y. Y., S.Q. Yang, Z.H. Chen, C.Y. Guan, S.Y. Chen and Z.S. Liu. 2011. QTL analysis of 18-C unsaturated fatty acid contents in zero-reucic rapeseed (*Brassica napus* L.) *Acta Agron. Sinica*., 37: 1342-1350.

Yue, P., Z. Jiang, Q. Sun, R. Wei, Y. Yin, Z. Xie, R. Larkin, J. Ye, L. Chai and X. Deng. 2023. Jasmonate activates a CsMPK6-CsMYC2 module that regulates the expression of β-citraurin biosynthetic genes and fruit coloration in orange (*Citrus sinensis*). *Plant Cell*, 35: 1167-1185.

Zhang, L.B., L. Liu, H.X. Li, J.J. He, H.B. Chao, S.X. Yan, Y.T. Yin, W.G. Zhao and M.T. Li. 2023. 3D genome structural variations play important roles in regulating sed oil content of *Brassica napus*. *Plant Comm.*, 100666: 1-15.

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