Expression of *BjuBMYB28-2* in mustard and its functional analysis in pest and disease resistance

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2026-08-12

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Abstract

To investigate the functions of mustard MYB28 family members in defense against biotic stress, this study used six BjuMYB28s homologous genes cloned from leaf mustard to determine their subcellular localization and transcriptional activation activity, and focused on the expression pattern of BjuBMYB28-2 and its response characteristics to Spodoptera exigua and Sclerotinia sclerotiorum. The results showed that: (1) BjuAMYB28-1, BjuBMYB28-1, and BjuBMYB28-3, consistent with the previously studied BjuAMYB28-2 and BjuAMYB28-3, were all localized in the nucleus and cytoplasm, whereas BjuBMYB28-2 was localized only in the nucleus, and all six BjuMYB28s possessed transcriptional activation activity. (2) BjuBMYB28-2 was mainly specifically expressed in vascular tissues; after feeding by Spodoptera exigua and infection by Sclerotinia sclerotiorum for 12–24 h, GUS signals in leaf veins weakened, and RT-qPCR further verified that its overall expression level decreased after Sclerotinia sclerotiorum infection. (3) Heterologous expression of BjuBMYB28-2 significantly enhanced the resistance of Arabidopsis to Spodoptera exigua and Sclerotinia sclerotiorum, as manifested by reduced larval weight after feeding and smaller lesions after Sclerotinia sclerotiorum infection. (4) Transcriptome analysis showed that multiple key genes in the three stages of glucosinolate biosynthesis were upregulated in the overexpression lines. In summary, BjuBMYB28-2 may increase glucosinolate content by coordinately upregulating the expression of key genes in the glucosinolate biosynthesis pathway, thereby enhancing resistance to Spodoptera exigua and Sclerotinia sclerotiorum. This study provides a theoretical basis for understanding the functional differentiation of mustard MYB28 family members and their roles in insect and disease resistance.

Full Text

DOI:10.11931/guihaia.gxzw202603046

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Abstract: To investigate the functions of members of the *MYB28* family in biological defense, this study used six *BjuMYB28s* homologous genes cloned from leaf mustard, clarified their subcellular localization and transcriptional activation activity, and focused on the expression pattern of *BjuBMYB28-2* and its response characteristics to beet armyworm and *Sclerotinia sclerotiorum*. The results showed: (1) BjuAMYB28-1, BjuBMYB28-1, and BjuBMYB28-3, consistent with the previously studied BjuAMYB28-2 and BjuAMYB28-3, were all

localized in the nucleus and cytoplasm, whereas BjuBMYB28-2 was localized only in the nucleus; all six BjuMYB28s possessed transcriptional activation activity. (2) *BjuBMYB28-2* was expressed mainly and specifically in vascular tissues; after beet armyworm feeding and *Sclerotinia sclerotiorum* infection, the GUS signal in leaf veins weakened at 12–24 h, and RT-qPCR further verified that its overall expression level decreased after *Sclerotinia sclerotiorum* infection. (3) Heterologous expression of *BjuBMYB28-2* significantly improved the resistance of Arabidopsis to beet armyworm and *Sclerotinia sclerotiorum*, manifested as reduced body weight of feeding larvae and smaller disease lesions caused by *Sclerotinia sclerotiorum* infection. (4) Transcriptome analysis showed that multiple key genes in the three stages of glucosinolate biosynthesis were upregulated in the overexpression lines. In summary, *BjuBMYB28-2* may increase glucosinolate content by coordinately upregulating the expression of key genes in the glucosinolate biosynthesis pathway, thereby enhancing resistance to beet armyworm and *Sclerotinia sclerotiorum*. This study provides a theoretical basis for understanding the functional differentiation of members of the mustard *MYB28* family and their roles in pest and disease resistance.

Keywords: mustard, *BjuBMYB28-2*, glucosinolate, beet armyworm, *Sclerotinia sclerotiorum*, biotic stress

Chinese Library Classification No.: Q943 **Document code:** A **Article No.:** Blank

Expression of *BjuBMYB28-2* in mustard (*Brassica juncea*) and its functional analysis in pest and disease resistance

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Abstract: To investigate the functional roles of the *MYB28* family members in biotic stress defense in mustard (*Brassica juncea*), this study utilized six *BjuMYB28s* homologous genes cloned from leaf mustard. Their subcellular localization and transcriptional activation activities were systematically characterized, with particular emphasis on the expression patterns of *BjuBMYB28-2* and its responsiveness to *Spodoptera exigua* (beet armyworm) herbivory and *Sclerotinia sclerotiorum* infection. Our experimental results revealed several key findings. (1) Regarding subcellular distribution, BjuAMYB28-1, BjuBMYB28-1, and BjuBMYB28-3 were localized in both the nucleus

Funding projects: National Natural Science Foundation of China General Program (32072557); Zhejiang Provincial Natural Science Foundation Project

(LY24C150003).
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and the cytoplasm, consistent with previously reported patterns for BjuAMYB28-2 and BjuAMYB28-3. In contrast, BjuBMYB28-2 exhibited an exclusive nuclear localization, suggesting potential functional specialization among these homologs. Notably, all six BjuMYB28 proteins demonstrated transcriptional activation activity in yeast assays, indicating their capacity to function as transcriptional regulators. (2) Tissue-specific expression analysis showed that *BjuBMYB28-2* was predominantly expressed in vascular tissues. Interestingly, GUS staining intensity in leaf veins markedly decreased at 12–24 h post-treatment with either insect feeding or fungal inoculation, implying a rapid down-regulation of promoter activity under stress conditions. This observation was further corroborated by RT-qPCR analysis, which confirmed a significant reduction in overall transcript abundance of *BjuBMYB28-2* following *S. sclerotiorum* infection. (3) Heterologous overexpression of *BjuBMYB28-2* in *Arabidopsis thaliana* conferred significantly enhanced resistance to both insect pests and fungal pathogens. Transgenic lines exhibited reduced larval body weights of *S. exigua* fed on overexpression plants, alongside diminished lesion areas resulting from *S. sclerotiorum* inoculation, compared to wild-type controls. (4) Transcriptomic profiling of overexpression lines revealed coordinated up-regulation of multiple key genes involved in all three stages of glucosinolate biosynthesis, including core structural and regulatory genes. Collectively, these findings strongly suggest that *BjuBMYB28-2* reinforces plant defense by synergistically up-regulating the glucosinolate biosynthetic pathway, leading to increased glucosinolate accumulation, which in turn contributes to enhanced resistance against both chewing insects and necrotrophic fungi. This comprehensive investigation not only provides mechanistic insights into how *BjuBMYB28-2* modulates biotic stress tolerance but also establishes a theoretical foundation for understanding the functional divergence among *MYB28* family members in *B. juncea*, with potential implications for breeding programs aimed at improving pest and disease resistance in *Brassica* crops.

Key words: *Brassica juncea*, *BjuBMYB28-2*, glucosinolate, *Spodoptera exigua*, *Sclerotinia sclerotiorum*, biotic stress

Mustard greens (*Brassica juncea*) are an important vegetable, oilseed, and condiment crop in China and across Asia. They are rich in types and play an important role in ensuring the year-round supply of vegetables, enriching dietary structure, and generating foreign exchange through exports (Wan Zhengjie et al., 2025). However, mustard production is often seriously threatened by pests and diseases such as beet armyworm (*Spodoptera exigua*) and *Sclerotinia sclerotiorum* (Huang Jianchao et al., 2021; Khan et al., 2021). Therefore, in-depth

investigation of the resistance mechanisms of mustard against major pests and diseases, together with the identification of key resistance genes, is of great theoretical and practical significance for breeding new insect- and disease-resistant mustard varieties and ensuring the sustainable development of the industry.

Glucosinolates are sulfur-containing glycosides (abbreviated GSLs), a class of secondary metabolites unique to cruciferous plants. In intact plant tissues, they are stored in vacuoles and are spatially separated from myrosinases in the cytoplasm. When tissues are damaged, the two come into contact and undergo hydrolysis to generate active substances such as isothiocyanates, which have broad-spectrum antibacterial, insecticidal, and feeding-deterrent activities and are key components of chemical defense in cruciferous plants (Bones & Rossiter, 1996; Lv et al., 2022). Glucosinolate biosynthesis mainly consists of three stages: side-chain elongation, core structure synthesis, and secondary side-chain modification (Sønderby et al., 2010a; Nguyen et al., 2020). This process is precisely regulated by multiple transcription factors, among which R2R3-MYB family transcription factors play a core role (Seo & Kim, 2017; Mitreiter &

Gigolashvili, 2021). Therefore, elucidating the mechanism by which R2R3-MYB family transcription factors regulate glucosinolate biosynthesis is an important prerequisite for mining the intrinsic resistance genes of mustard greens and for developing resistance breeding.

In the model plant *Arabidopsis thaliana*, the R2R3-MYB family transcription factors AtMYB28, AtMYB29, and AtMYB76 are key positive regulators of aliphatic glucosinolate biosynthesis. The three form a complex regulatory network, in which AtMYB28 plays a dominant role (Sønderby et al., 2010b). All three can directly activate the promoters of key genes in glucosinolate biosynthesis, and AtMYB28 has the strongest activation ability (Gigolashvili et al., 2007, 2008). *AtMYB28* expression is transiently induced by mechanical injury; its overexpression can significantly increase glucosinolate content and enhance resistance to the diamondback moth, whereas long-chain glucosinolates are almost absent in the *myb28* mutant, and short-chain glucosinolates are also significantly reduced (Gigolashvili et al., 2007; Beekwilder et al., 2008). *AtMYB28* and *AtMYB29* are partially functionally redundant; in the double mutant, all aliphatic glucosinolates are completely abolished, and the expression levels of glucosinolate-biosynthesis-related genes decrease sharply (Beekwilder et al., 2008; Sønderby et al., 2010b). Sønderby et al. (2010b) further found that, although *AtMYB76* has no significant effect in the single mutant, its overexpression in the *myb28 myb29* double mutant can still induce aliphatic glucosinolate biosynthesis, demonstrating that it has functions independent of *AtMYB28* and *AtMYB29* and may participate in network integration by regulating *AtMYB29*.

Unlike the model plant *Arabidopsis thaliana*, Brassica crops have all lost *MYB76* during evolution, but retain multiple *MYB28* and *MYB29* homologs (Seo & Kim, 2017). Three *BrMYB28* homologs were isolated from Chinese cabbage; *BrMYB28.1* and *BrMYB28.2* show the highest expression in leaves and are not expressed in seeds, stamens, or roots, whereas *BrMYB28.3* expression was

not detected; however, overexpression of any of them can significantly increase the total glucosinolate content of Chinese cabbage (Seo et al., 2016). Yin et al. (2017) found that *BoaMYB28* cloned from Chinese kale is expressed most highly in stems, and that it specifically increases aliphatic glucosinolate content by positively regulating the key genes *MAM1/3* and *CYP79F1/2* in the aliphatic glucosinolate biosynthetic pathway. A total of six *MYB28* homologs have been identified in *Brassica napus*, with differences in expression patterns; among them, *BnA9.MYB28* co-localizes with a major-effect QTL for seed glucosinolate content, and its overexpression can significantly increase glucosinolate content (Long et al., 2016). Another study confirmed that *BnaC2.MYB28* positively regulates glucosinolate accumulation by directly activating multiple glucosinolate biosynthetic genes, and is the major-effect gene controlling seed glucosinolate content in *Brassica napus*; this gene has been subjected to strong artificial selection in low-glucosinolate breeding (Zhou et al., 2023). In mustard greens, Augustine et al. (2013) identified four *MYB28* homologs from oilseed mustard, and functional complementation experiments confirmed that all of them could restore the glucosinolate-deficient phenotype of the *Arabidopsis thaliana myb28* mutant, indicating that their function in the regulation of glucosinolate biosynthesis is conserved. Using RNAi technology to silence multiple *MYB28* homologous genes, transgenic lines with significantly reduced total seed aliphatic glucosinolate content were successfully obtained, providing important germplasm resources for low-glucosinolate breeding. Yang et al. (2021) mapped key loci controlling glucosinolate accumulation on the A02 and A09 chromosomes of mustard greens and found that variation in the copy number of the *MYB28* gene is an important reason for differences in glucosinolate accumulation among mustard-green populations. In the early stage of our research project, six *MYB28* homologs were isolated and identified from leaf mustard (*BjuAMYB28-1/2/3* and *BjuBMYB28-1/2/3*, collectively referred to as *BjuMYB28s*), all of which show the highest expression in leaves. Preliminary studies of *BjuAMYB28-2* (*BjuAMYB28a*) and *BjuAMYB28-3* showed that the proteins encoded by both are localized in the nucleus and cytoplasm, are induced by mechanical injury, and that heterologous expression of *BjuAMYB28-3* in *Arabidopsis thaliana* can significantly increase aliphatic glucosinolate content (Xia Rui, 2023; Wang Jiachen et al., 2026).

Although the above progress has been made, it remains unclear whether functional differentiation exists among the proteins encoded by the homologous genes of mustard-green *BjuMYB28s* in terms of subcellular localization and transcriptional activation activity, as well as how members of this family respond to biological stress and what specific functions they have in response to insect feeding and fungal infection. These are key scientific questions that require further in-depth investigation. Therefore, on the basis of the six mustard-green *BjuMYB28s* homologs obtained in our previous work, this study systematically analyzed the subcellular localization characteristics and transcriptional activation activity of their encoded proteins, with a focus on *BjuBMYB28-2*. Through promoter-driven *GUS* expression, its spatiotemporal expression pattern and its

response to the diamondback moth were analyzed.

responses to diamondback moth feeding and *Plasmodiophora brassicae* infection; further construct *BjuBMYB28-2* overexpression transgenic *Arabidopsis thaliana*, evaluate its resistance levels to diamondback moth and *P. brassicae*, and analyze, by transcriptome sequencing, changes in the expression of genes related to the regulation of glucosinolate biosynthesis.

1 Materials and Methods

1.1 Experimental Materials

In this experiment, the leaf mustard cultivar 'Jiannan Shuidong' was used as the material; seeds were purchased from Guangzhou Xiangqi Vegetable Seed Co., Ltd. Transgenic tobacco carrying the H2B-RFP nuclear localization marker and wild-type *Arabidopsis thaliana* (*Col-0*) seeds were preserved in the laboratory. All plant materials were planted in the artificial-climate cultivation room of the College of Horticulture, Zhejiang A&F University. The cultivation conditions were as follows: photoperiod, 14 h light/10 h dark; daytime temperature, (24±2) °C; nighttime temperature, (20±2) °C; light intensity, 8 000 lx. The cultivation substrate was prepared at a volume ratio of peat:vermiculite:perlite = 3:2:1.

1.2 Main Reagents

Restriction endonucleases and related reagents were purchased from TaKaRa; high-fidelity polymerase, homologous recombination kits, plasmid extraction and RNA extraction kits, etc., were purchased from Vazyme; GUS staining kits were purchased from Beijing Huayueyang Biotechnology; the pFGC-eGFP, pGBKT7, and pBI121 vectors were preserved in the laboratory; *Escherichia coli* DH5α, *Agrobacterium tumefaciens* GV3101, and yeast strain AH109 were purchased from Shanghai Weidi Biotechnology Co., Ltd.; the *P. brassicae* strain was preserved in the laboratory; diamondback moth larvae were purchased independently. Primers were synthesized by Hangzhou Youkang Biotechnology Co., Ltd., and sequencing was completed by Nanjing GenScript Biotechnology Co., Ltd.

1.3 Vector Construction

The CDS sequence of leaf mustard *BjuMYB28s* had been obtained and cloned in earlier work (Xia Rui, 2023). Using the plasmid containing this sequence as the template, the target fragments were amplified by PCR and, through homologous recombination, ligated separately into the pFGC-eGFP and pGBKT7 vectors to obtain the subcellular-localization and transcriptional-activation analysis vectors.

Based on the BRAD (http://brassicadb.cn) database, a promoter sequence of approximately 2 500 bp upstream of the start codon of BjuVB04G02480

(*BjuAMYB28-2*) was obtained. Using 'Jiannan Shuidong' leaf mustard genomic DNA as the template for amplification, the fragment was ligated into the pBI121 vector by homologous recombination to obtain the *GUS* reporter-gene fusion vector pBI121-ProBjuBMYB28-2::GUS. In addition, the CDS fragment of *BjuBMYB28-2* was amplified and inserted into pBI121 by homologous recombination to obtain the overexpression vector pBI121-CaMV35S::BjuBMYB28-2 driven by the *CaMV35S* promoter.

1.4 Transient Expression in Tobacco

The pFGC-eGFP-BjuMYB28s vector was transformed into *Agrobacterium tumefaciens* GV3101, resuspended to an OD_{600} of approximately 1.0, and activated at 28 °C for 2 h. Leaves of 4-week-old tobacco were injected; after dark cultivation for 2 d, the tissue surrounding the injection site was sampled, and fluorescence signals were observed with a laser confocal microscope (Leica SP8 MP, Germany).

1.5 Transcriptional Activation Assay

The pGBKT7-BjuMYB28s, pGBKT7-AD, and pGBKT7 vectors were separately transformed into AH109 yeast and plated on SD/-Trp medium, followed by culture at 28 °C for 3 d. Six single colonies were selected, resuspended, and spotted onto SD/-Trp and SD/-Trp-Ade-His media; after culture at 28 °C for 3 d, photographs were taken for observation.

1.6 Transformation of *Arabidopsis thaliana* by the Floral-Dip Method and Screening and Identification of Transgenic Plants

The *Agrobacterium tumefaciens* floral-dip method was used to transform pBI121-ProBjuBMYB28-2::GUS and pBI121-CaMV35S::BjuBMYB28-2 into [text continues]

of *Arabidopsis thaliana*, kanamycin was used to screen resistant seedlings, and PCR was used to identify positive plants; total RNA was extracted from overexpression lines, and after reverse transcription, RT-qPCR was used to detect expression levels.

1.7 Expression analysis of *BjuBMYB28-2*

GUS staining was performed on *Arabidopsis thaliana* transformed with the *BjuBMYB28-2* promoter-*GUS* transgene, including 7 d cotyledon-stage seedlings and rosette leaves, cauline leaves, stems, inflorescences, and siliques at the bolting and flowering stage. At the rosette-leaf stage, treatments with diamondback moth larvae and *Sclerotinia sclerotiorum* inoculation were conducted separately: each plant was inoculated with three second-instar

diamondback moth larvae, or a fungal block was placed at the center of a leaf; samples were collected for staining at 0, 12, 24, and 36 h. In the kale inoculation experiment, *Sclerotinia sclerotiorum* was inoculated at the four-leaf to one-heart stage; at the same time points, leaves were collected for RNA extraction, and after reverse transcription, RT-qPCR analysis was performed using *Actin* as the internal reference. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with three biological replicates.

1.8 Feeding by diamondback moth larvae and *Sclerotinia sclerotiorum* infection assay

For the rosette-leaf stage, *BjuBMYB28-2* overexpression lines (3 lines) and wild-type plants were selected, with 11 plants of each. Each plant was inoculated with three second-instar diamondback moth larvae. To reduce bias caused by larval escape or death, only plants on which all three larvae remained intact and alive were retained for statistical analysis. After 7 d of feeding, larval body weight was measured, and the plants were observed under a stereomicroscope and photographed.

Sclerotinia sclerotiorum was inoculated onto potato dextrose agar (PDA) medium plates and cultured in the dark at 22 °C for 3–5 d. A 5 mm hole punch was used to cut mycelial plugs from the colony margin as inocula. Three *BjuBMYB28-2* overexpression lines with consistent growth at the rosette-leaf stage and six wild-type plants were selected. A mycelial plug was placed at the center of the 3rd–5th rosette leaves of each plant, with the mycelial surface facing downward. After moisturizing, the plants were cultured at 22 °C under 16 h light/8 h dark. Photographs were taken 24 h after inoculation, and lesion area (mm^2) was measured using ImageJ software to evaluate differences in resistance.

1.9 Transcriptome sequencing

At the rosette-leaf stage, leaves of *BjuBMYB28-2*-overexpressing *Arabidopsis thaliana* and wild-type plants were collected and entrusted to Wuhan MetWare Biotechnology Co., Ltd. for Illumina transcriptome sequencing. The *Arabidopsis thaliana* reference genome (TAIR10) was used as the reference, and the screening criteria for differentially expressed genes were $|\log_2 FC| > 1$ and $P < 0.05$.

1.10 Data analysis

GraphPad Prism 8.0.2 software was used for statistical analysis. Comparisons among groups were performed using one-way analysis of variance (one-way ANOVA). If the overall test result was significant, the least significant difference (least significant difference, LSD) method was further used for post hoc multiple comparisons, with $P < 0.05$ considered statistically significant.

2 Results and Analysis

2.1 Subcellular localization and transcriptional activation activity of BjuMYB28s

To analyze the subcellular localization of BjuMYB28s, expression vectors fusing BjuAMYB28-1, BjuBMYB28-1, BjuBMYB28-2, and BjuBMYB28-3 with eGFP, pFGC-eGFP-BjuMYB28s (Figure 1), were constructed and transiently expressed by Agrobacterium injection in tobacco epidermal cells expressing the nuclear localization marker H2B-RFP. The results (Figure 2) showed that the green fluorescence of eGFP-BjuBMYB28-2 completely overlapped with the red fluorescence, indicating that it localized only to the nucleus; whereas BjuAMYB28-1, BjuBMYB28-1, and BjuBMYB28-3 all showed green fluorescence in both the nucleus and cytoplasm, indicating that they localized simultaneously to the nucleus and cytoplasm.

A. PCR amplification products of CDS sequences; **B.** Validation of *Agrobacterium*-mediated transformation of subcellular localization expression vectors by colony PCR; **C.** Schematic diagram of the subcellular localization expression vector; **1, 2, 9.** *BjuBMYB28-3*; **3, 4, 10.** *BjuAMYB28-1*; **5, 6, 11.** *BjuBMYB28-1*; **7, 8, 12.** *BjuBMYB28-2*; **M.** DL2000 DNA Marker.

A. PCR amplification products of CDS sequences; **B.** Validation of *Agrobacterium*-mediated transformation of subcellular localization expression vectors by colony PCR; **C.** Schematic diagram of the subcellular localization expression vector; **1, 2, 9.** *BjuBMYB28-3*; **3, 4, 10.** *BjuAMYB28-1*; **5, 6, 11.** *BjuBMYB28-1*; **7, 8, 12.** *BjuBMYB28-2*; **M.** DL2000 DNA Marker.

Fig. 1 Amplification of the CDS sequences of *BjuMYB28s* and construction of subcellular localization expression vectors

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Bright. Bright field; **GFP.** Green fluorescent signal; **H2B-RFP.** Nuclear-localized red fluorescent signal; **Merge.** Merged image. Bars = 50 μm.

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Fig. 2 Subcellular localization of *BjuMYB28s* fusion proteins

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2.2 Transcriptional Activation Activity of BjuMYB28s

The CDS sequences of six *BjuMYB28s* were amplified and constructed into the pGBKT7 vector. After verification by PCR and sequencing, vectors in which BjuMYB28s were fused to the GAL4 DNA-binding domain, pGBKT7-BjuMYB28s, were obtained (Fig. 3: A).

Using pGBKT7-AD as the positive control and pGBKT7 as the negative control, the pGBKT7-BjuMYB28s vectors were separately transformed into AH109 yeast. The results showed that all transformants grew on SD/-Trp medium; on SD/-Trp-His-Ade medium, except for the negative control, all others grew normally (Fig. 3: B). These results indicate that the proteins encoded by the six *MYB28* homologous genes of pak choi, BjuMYB28s, all possess transcriptional activation activity, consistent with the characteristics of transcription factors.

A. Schematic diagram of the transcriptional activation expression vector (**GAL4 DNA-BD.** GAL4 DNA-binding domain; **GAL4-AD.** GAL4 transcriptional activation domain; **BjuMYB28s.** CDS sequences of the corresponding genes); **B.** Growth status of transformed yeast strains on different selective media.

A. Schematic diagram of the transcriptional activation expression vector (**GAL4 DNA-BD.** GAL4 DNA-binding domain; **GAL4-AD.** Transcriptional activation domain of GAL4; **BjuMYB28s.** The CDS sequences of the corresponding genes); **B.** Growth status of transformed yeast strains on different selective media.

Fig. 3 Transcriptional activation activity assay of BjuMYB28s

Fig. 3 Transcriptional activation activity assay of BjuMYB28s

2.3 Successful Construction of *BjuBMYB28-2* Promoter-*GUS* Fusion Transgenic Plants

From the *Brassica rapa* plant genome database BRAD, the promoter reference sequence of approximately 2 500 bp upstream of the start codon of the *BjuBMYB28-2* (BjuVB04G02480) gene was obtained. Specific primers were designed accordingly, and a promoter sequence 1 852 bp in length was obtained by PCR amplification and sequencing (Fig. 4: A). Using homologous recombination, this promoter fragment was used to replace the *CaMV35S* promoter in the pBI121 vector. After PCR and sequencing verification, the expression vector pBI121-ProBjuBMYB28-2::GUS, in which the *GUS* reporter gene was driven by the *BjuBMYB28-2* promoter, was successfully constructed. This vector was introduced into wild-type *Arabidopsis thaliana* by the *Agrobacterium*-mediated floral-dip method. After kanamycin-resistance screening and PCR identification, six positive transgenic lines were ultimately obtained (Fig. 4: B).

A. Amplification of the *BjuBMYB28-2* promoter sequence; **B.** PCR validation of individual T_1 generation transgenic *Arabidopsis thaliana* plants; **M.** DL2000 DNA Marker; **1.** PCR products of the *BjuBMYB28-2* promoter; **2.** ddH_2O; **3.** Wild-type *Arabidopsis thaliana*; **4–9.** PCR products of transgenic *Arabidopsis thaliana*.

Fig. 4 Amplification of the *BjuBMYB28-2* promoter and identification of transgenic *Arabidopsis thaliana* plants

2.4 Analysis of the spatiotemporal expression pattern of *BjuBMYB28-2*

GUS histochemical staining analysis was performed on the above-mentioned positive transgenic plants under normal growth conditions. The results showed that, in seedlings at the cotyledon stage, the GUS signal was concentrated in the cotyledon veins and the upper end of the hypocotyl, with no expression in the roots (Fig. 5: A). In plants at the flowering stage, obvious GUS expression was observed in the primary and secondary veins of rosette leaves and cauline leaves (Fig. 5: B, C), whereas no signal was detected in stems (Fig. 5: D). GUS activity was pronounced in flower buds, mainly distributed in the pedicels, flower stalks, filaments, and floral veins (Fig. 5: E), and weak activity was also present in the silique stalks (Fig. 5: F). These results indicate that the *BjuBMYB28-2* promoter can drive tissue-specific expression of the *GUS* gene in vascular and transport tissues, including the cotyledon veins, leaf veins, pedicels, flower stalks, filaments, floral veins, and silique stalks.

A–F. Transgenic *Arabidopsis thaliana* expressing *GUS* driven by the *BjuBMYB28-2* promoter; **G–L.** Wild-type *Arabidopsis thaliana*; **A, G.** Seedlings; **B, H.** Rosette leaves; **C, I.** Cauline leaves; **D, J.** Stems; **E, K.** Inflorescences; **F, L.** Siliques. Bars = 400 μm.

Fig. 5 Analysis of the spatiotemporal expression pattern of *BjuBMYB28-2*

2.5 Analysis of the expression pattern of *BjuBMYB28-2* induced by biotic stress treatment

To investigate whether *BjuBMYB28-2* responds to biotic stress, rosette-stage *Arabidopsis thaliana* leaves were treated by inoculation with diamondback moth larvae and *Sclerotinia sclerotiorum*. GUS staining showed that, before insect feeding, *BjuBMYB28-2* was mainly expressed in leaf veins. At 12 h and 24 h after insect feeding, the GUS signal in the leaf veins was significantly weakened; by 36 h, the signal was diffusely distributed in the mesophyll tissue (Fig. 6: A),

This indicated that feeding by beet armyworm larvae could suppress its expression in leaf veins and might induce its expression in leaf mesophyll tissues. Inoculation with *Sclerotinia sclerotiorum* showed a similar trend: at 12 h and 24 h after inoculation, the GUS signal in leaf veins was weakened, while a weaker signal appeared in the mesophyll; at 36 h after inoculation, relatively strong expression was visible in the secondary veins at the leaf margin (Fig. 6B), indicating that infection by *S. sclerotiorum* likewise suppressed the expression of *BjuBMYB28-2* in leaf veins and might induce its expression in the mesophyll.

To further verify the effect of *S. sclerotiorum* inoculation on the expression of *BjuBMYB28-2*, RT-qPCR analysis was performed on mustard leaves at 12 h, 24 h, and 36 h after inoculation. The results (Fig. 7) showed that the overall expression level after inoculation was lower than that of the control group. At 12 h after inoculation, expression decreased slightly but did not reach a significant

level; at 24 h, expression was significantly lower than that of the control group; at 36 h, although expression recovered somewhat, it remained lower than that of the control. This further confirmed the inhibitory effect of *S. sclerotiorum* treatment on *BjuBMYB28-2* expression.

A. GUS staining of *ProBjuBMYB28-2::GUS* after feeding by beet armyworm larvae; **B.** GUS staining after infection by *Sclerotinia sclerotiorum*. Bars = 1 mm.

Fig. 6 Expression patterns of *BjuBMYB28-2* in response to insect herbivory and *Sclerotinia sclerotiorum* infection

* indicates significant difference ($P < 0.05$). The same below.

Fig. 7 Relative expression levels of *BjuBMYB28-2* in mustard leaves at different time points after inoculation with *Sclerotinia sclerotiorum*

2.6 Construction and Identification of *BjuBMYB28-2* Overexpression Transgenic Plants

A *BjuBMYB28-2* overexpression vector driven by the *CaMV35S* promoter was successfully constructed, and after transformation into *Arabidopsis thaliana*, five positive plants were obtained (Fig. 8A). RT-qPCR results showed that the expression levels of *MYB28* (the sum of endogenous *AtMYB28* and exogenous *BjuBMYB28-2*) in the transgenic plants were all significantly higher than those in the wild type. Among them, the expression levels in the lines *BjuBMYB28-2^OE*-1 and *BjuBMYB28-2^OE*-3 were more than 10-fold higher than that of the wild type, and that in the *BjuBMYB28-2^OE*-5 line was approximately 7.5-fold higher (Fig. 8B), indicating that *BjuBMYB28-2* overexpression transgenic *Arabidopsis thaliana* had been successfully obtained.

2.7 Overexpression of *BjuBMYB28-2* Enhanced the Resistance of *Arabidopsis thaliana* to Feeding by *Plutella xylostella* and to *Sclerotinia sclerotiorum* Infection

To investigate the function of *BjuBMYB28-2* in biotic stress responses, overexpression transgenic *Arabidopsis thaliana* and the wild type were used as materials for larval feeding experiments with *Plutella xylostella* and infection experiments with *Sclerotinia sclerotiorum*. After 7 d of feeding by *P. xylostella* larvae, the body size of larvae feeding on transgenic plants was smaller than that of larvae feeding on wild-type plants (Fig. 9A). Body-weight statistics showed that the average body weight of larvae feeding on transgenic plants was lower than that of the wild-type group, among which the larval body weight in the *BjuBMYB28-2^OE*-5 line was significantly reduced (Fig. 9B). At 24 h after inoculation with *S. sclerotiorum*, wild-type leaves began to show yellowing and water-soaked symptoms, with relatively large lesion areas; leaves of *BjuBMYB28-2^OE* transgenic plants suffered less damage, and lesion expansion

was limited (Fig. 9C). Measurement of lesion areas showed that the average lesion area of transgenic plants was smaller than that of the wild type, among which the lesion area of the *BjuBMYB28-2^OE*-3 line was significantly smaller than that of the wild type (Fig. 9D). These results indicate that overexpression of *BjuBMYB28-2* can enhance the resistance of *Arabidopsis thaliana* to feeding by *P. xylostella* and to *S. sclerotiorum* infection.

A. PCR identification of transgenic positive seedlings; **M.** DL2000 DNA Marker; **1.** ddH_2O; **2.** WT; **3–7.** *BjuBMYB28-2^OE*; **B.** Relative expression analysis of *MYB28*. ** indicates an extremely significant difference ($P < 0.01$), the same below.

A. PCR identification of transgenic positive seedlings; **M.** DL2000 DNA Marker; **1.** ddH_2O; **2.** WT; **3–7.** *BjuBMYB28-2^OE*; **B.** Relative expression analysis of *MYB28*. ** indicates extremely significant difference ($P < 0.01$), the same below.

Fig. 8 Identification and expression-level analysis of T_1-generation *Arabidopsis thaliana* positive seedlings overexpressing *BjuBMYB28-2*

Fig. 8 Identification and expression level analysis of T_1 generation *Arabidopsis thaliana* positive seedlings overexpressing *BjuBMYB28-2*

A. Growth status of beet armyworm larvae after feeding on wild-type and *BjuBMYB28-2^OE* plants for 7 days, bars = 5 mm; **B.** Statistical analysis of larval weights; **C.** Lesion phenotypes of wild-type and *BjuBMYB28-2^OE* plant leaves at 24 h post-inoculation with *Sclerotinia sclerotiorum*, bars = 1 cm; **D.** Statistical analysis of lesion areas.

Fig. 9 Overexpression of *BjuBMYB28-2* enhances resistance of *Arabidopsis thaliana* to beet armyworm larvae feeding and *Sclerotinia sclerotiorum* infection

2.8 *BjuBMYB28-2* coordinately up-regulates the expression of genes related to glucosinolate biosynthesis

To investigate the effect of overexpression of *BjuBMYB28-2* on the transcriptome level of plants, RNA-seq analysis was performed on leaves of *BjuBMYB28-2^OE*-3 and the wild type. Using $|\log_2 \mathrm{FC}| > 1$ and $P < 0.05$ as the screening criteria, a total of 121 differentially expressed genes were identified, including 86 up-regulated and 35 down-regulated genes (Fig. 10: A). GO enrichment analysis showed (Fig. 10: B) that the differentially expressed genes were mainly enriched in terms related to redox activity, such as glutathione oxidoreductase activity (GO:0097573) and iron–sulfur cluster binding (GO:0051536), with down-regulated genes predominating. They were also enriched in auxin response-related terms, including response to toxic substance (GO:0009636) and detoxification (GO:0098754), with up-regulated genes predominating. In the related terms of glutamine catabolic process (GO:0006536) and dicarboxylic

acid catabolic process (GO:0043649), all differentially expressed genes were up-regulated. KEGG pathway enrichment analysis showed that the differentially expressed genes were significantly enriched in the alanine, aspartate and glutamate metabolism pathway (ko00250), involving five differentially expressed genes, of which four were up-regulated and one was down-regulated.

Notably, the expression changes of genes in the glucosinolate biosynthesis pathway did not reach the differential threshold of $|\log_2 \mathrm{FC}| > 1$; however, in the *BjuBMYB28-2^OE*-3 plants, the expression levels of many genes were generally higher than those in the wild type. Among them, 7 of the 9 genes in the side-chain elongation stage showed an up-regulation trend, including *MAM1/3*, *BCAT4*, *IPMI1/2*, *IIL1* and *IMDH1* (Fig. 11: A); 16 of the 18 genes in the core-structure biosynthesis stage showed an up-regulation trend, namely *CYP79F1/2*, *CYP79B2/3*, *CYP83A1*, *CYP83B1*, *CYTB5C*, *GGP1*, *GSTF9*, *GSTU20*, *SUR1*, *UGT74B1*, *UGT74C1* and *SOT16/17/18* (Fig. 11: B); and 8 of the 12 genes in the side-chain modification stage showed an up-regulation trend, including *CYP81F2*, *IGMT1/5*, $FMO_{*\text{GS-OX1/2/3/7*}}$ and *GSL-OH* (Fig. 11: C). These results indicate that *BjuBMYB28-2* may, through broad but weak transcriptional activation, coordinately up-regulate the expression of multiple key genes in the glucosinolate biosynthesis pathway, thereby cumulatively enhancing glucosinolate accumulation and consequently increasing the resistance of *Arabidopsis thaliana* to beet armyworm larval feeding and *Sclerotinia sclerotiorum* infection.

A. Volcano plot of differentially expressed genes; **B.** GO enrichment analysis of differentially expressed genes. From outside to inside are the GO terms, the number of genes in the category among the background genes and the FDR (the longer the bar, the more genes; the redder the bar color, the more significant the enrichment); the proportion of up- and down-regulated genes (light red indicates the proportion of up-regulated genes, light blue ···

is the proportion down-regulated, with the specific values labeled below).

A. Volcano plot of differentially expressed genes; **B.** GO enrichment circle plot of differentially expressed genes. From outer to inner circles: GO terms; the number of genes in each category in the background genome and FDR values (longer bars indicate more genes, redder colors indicate more significant enrichment); the proportion of up-regulated and down-regulated genes (light red for the up-regulated proportion, light blue for the down-regulated proportion, with specific values displayed below).

Fig. 10 Transcriptome differential expression analysis of *BjuBMYB28-2* overexpression *Arabidopsis thaliana* leaves

A

B

C

A. Side chain elongation stage; **B.** Core structure synthesis stage; **C.** Side chain modification stage. Data shown are mean FPKM values for each gene ($n = 3$).

Fig. 11 Expression analysis of genes related to glucosinolate biosynthesis in *BjuBMYB28-2* overexpression *Arabidopsis thaliana* leaves

Fig. 11 Expression analysis of glucosinolate biosynthesis-related genes in leaves of *Arabidopsis thaliana* heterologously expressing *BjuBMYB28-2*

3 Discussion and Conclusion

Leaf mustard is an allo-tetraploid (AABB), and its genome was formed by hybridization and chromosome doubling between Chinese cabbage (AA) and black mustard (BB), resulting in multiple homologous copies for most genes (Yang et al., 2021). In the preliminary work of this research group, six *MYB28* homologous genes were identified and cloned from leaf mustard, more than the four reported in oilseed mustard (Augustine et al., 2013; Lu Junxing et al., 2017). Among them, *BjuAMYB28-3* and *BjuBMYB28-1* were newly identified members in leaf mustard, while the other four genes differ from the corresponding sequences in Indian oilseed mustard, indicating that genetic differentiation may exist at the sequence level among members of the *MYB28* gene family in different varieties of mustard.

Phylogenetic analysis showed that all six BjuMYB28s possess typical R2R3-MYB structural domains and are highly homologous to AtMYB28, suggesting functional conservation (Xia Rui, 2023). Transcriptional activation activity is a key attribute for transcription factors to exert regulatory functions. In *Arabidopsis thaliana*, AtMYB28, AtMYB29, and AtMYB76 all have transcriptional activation activity, among which AtMYB28 has the strongest activation capacity (Gigolashvili et al., 2007, 2008; Sønderby et al., 2010b). In Brassica plants, the transcriptional activation function of MYB28 is likewise conserved. Chinese cabbage BrMYB28, broccoli BoaMYB28, and rutabaga-type rapeseed BnA9.MYB28 can all upregulate the expression of glucosinolate biosynthesis-related genes (Long et al., 2016; Seo et al., 2016; Yin et al., 2017); BnaC2.MYB28 not only has activation ability, but also shows functional differences among allelic genes, with the allele from the high-glucosinolate parent G120 exhibiting significantly stronger activation of some target genes than the allele from the low-glucosinolate parent ZY50 (Zhou et al., 2023). This study found that all six BjuMYB28s in leaf mustard have transcriptional activation activity, further indicating that MYB28, as a typical R2R3-MYB transcription factor in Brassica plants, possesses the basic function of regulating downstream target-gene expression; however, whether differences in activation capacity exist among the members remains to be investigated. In terms of subcellular localization, previous studies found that BjuAMYB28-2 and BjuAMYB28-3 are localized in the nucleus and cytoplasm (Wang Jiaceng et al., 2026; Xia Rui, 2023). This study further found that BjuBMYB28-2 is localized only in the nucleus, whereas the remaining three members (*BjuAMYB28-1*, *BjuBMYB28-1*,

and *BjuBMYB28-3*) are simultaneously localized in the nucleus and cytoplasm. Previous studies have reported that AtMYB28 is localized in the nucleus in *Arabidopsis* protoplasts (Gigolashvili et al., 2007), and BjuB.MYB28.2 (corresponding to BjuBMYB28-3 in this study) is also localized only in the nucleus in onion epidermal cells (Augustine et al., 2013). The localization differences described above may be related to differences in experimental materials or detection systems, and may also reflect genuine functional differentiation. Notably, BjuBMYB28-2 exhibited a unique nuclear localization pattern in tobacco epidermal cells, suggesting that it may exert transcriptional regulatory functions only in the nucleus, whereas the other five members may have both nuclear transcriptional regulatory functions and unknown biological functions in the cytoplasm.

Previous studies have shown that the contributions of members of the mustard *MYB28* family to glucosinolate biosynthesis differ. Augustine et al. (2013) predicted, based on expression levels and cellular specificity, that *MYB28* in the A subgenome of oilseed mustard may play a stronger role. Yang et al. (2021), through GWAS and CNV analyses, directly demonstrated that *MYB28* on chromosomes A02 and A09 is the major locus controlling glucosinolate accumulation. In *Arabidopsis thaliana*, *AtMYB28* is mainly expressed in vascular tissues and is a core regulator of aliphatic glucosinolate biosynthesis (Gigolashvili et al., 2007; Sønderby et al., 2010). *AtMYB29* and *AtMYB76* also participate in regulation, but their contributions are relatively small; among them, *AtMYB76* requires an extremely high overexpression level to achieve an effect similar to that of *AtMYB29* (Gigolashvili et al., 2008; Sønderby et al., 2010). In Brassica plants, the contribution of *MYB28* to glucosinolates likewise differs. In Chinese cabbage, *BrMYB28.1* contributes the most, and overexpression can increase total glucosinolate content by approximately 4.7-fold; *BrMYB28.2* ranks second, while *BrMYB28.3* is not expressed under normal conditions but remains functional after overexpression (Seo et al., 2016); in rutabaga-type rapeseed,

BnaC2.MYB28 and *BnaA9.MYB28* are both major genes controlling glucosinolate accumulation (Long et al., 2016; Zhou et al., 2023). This study focused on *BjuBMYB28-2* on chromosome B04 of leaf mustard and found that it is mainly expressed in vascular tissues, including cotyledon veins, leaf midribs, and secondary leaf veins. This pattern is similar to the expression of *AtMYB28* in vascular bundles of *Arabidopsis thaliana* (Gigolashvili et al., 2007), but differs from the expression of the homologous gene *BjuB.MYB28.1* in oilseed mustard, which is expressed at the leaf margin and in mesophyll, with no signal in the leaf veins (Augustine et al., 2013). Given that glucosinolates are mainly synthesized in vascular tissues (Nintemann et al., 2017), differences in the expression patterns of homologous genes among different *Brassica juncea* accessions may indicate functional differentiation. In addition, *BjuBMYB28-2* was strongly expressed in the pedicel, filament, and petal veins, suggesting that it may play a role in defense during reproductive growth.

The study found that feeding by *Spodoptera litura* significantly altered the ex-

pression pattern of *BjuBMYB28-2*. At the early stage of feeding, the GUS signal in leaf veins weakened, consistent with transcriptome data showing that *BjuMYB28s* are generally downregulated (Xia et al., 2023). *BjuMYB29s*, which are also regulators of aliphatic glucosinolates, were instead induced after feeding (Zhang et al., 2025), indicating that members of the *MYB28* and *MYB29* families may play different roles in response to insect herbivory. In contrast, studies have shown that Chinese cabbage *MYB28* is upregulated after feeding by *S. litura*; its expression level increases significantly 1 h after feeding and reaches a peak after 48 h, while glucosinolate-biosynthesis-related genes are also upregulated synchronously, promoting glucosinolate accumulation (Hao Jiaojiao et al., 2023). The results of this study differ from the above report, possibly due to functional differentiation among species, or because the first sampling time in this study was 12 h, so the instantaneous upregulation at the early feeding stage could not be captured. Nevertheless, the expression of *BjuBMYB28-2* in mesophyll tissues increased during the later stage after feeding. This dynamic change may be related to the "source–sink"transport mechanism of glucosinolates (Nour-Eldin & Halkier, 2009). In *A. thaliana*, glucosinolates are synthesized in vascular tissues (sources) and then transported to mesophyll and leaf margins (sinks) for storage (Shroff et al., 2008). When leaves are attacked by insects or infected by pathogens, glucosinolates stored in the mesophyll are rapidly hydrolyzed, releasing active substances to resist herbivory (Halkier & Gershenzon, 2006). It can therefore be inferred that the enhanced expression of *BjuBMYB28-2* in mesophyll tissues during the later stage after herbivory may be a feedback regulation in response to glucosinolate consumption in the mesophyll, thereby initiating new glucosinolate synthesis. Regarding *Sclerotinia sclerotiorum* infection, this study found that *BjuBMYB28-2* expression decreased after infection. As a necrotrophic pathogen, *S. sclerotiorum* is primarily defended against by plants through the jasmonic acid (JA) and ethylene (ETH) signaling pathways rather than the salicylic acid (SA) pathway, and antagonism exists between SA and JA (Zhang et al., 2025). In *Brassica napus* of the canola type, Chi Peng et al. (2020) found that SA can inhibit the expression of *BnaMYB28-2*, and that this gene is also downregulated after infection by *Plasmodiophora brassicae*. In this study, *BjuBMYB28-2* was also downregulated after infection by *S. sclerotiorum*, and the two showed similar trends. It is therefore speculated that pathogen infection may suppress the expression of specific *MYB28* members by activating the SA signaling pathway. Different members of *BnaMYB28* show differentiated responses to SA and *P. brassicae*: SA induces the expression of *BnaMYB28-1* and *BnaMYB28-3* but suppresses *BnaMYB28-2*; *P. brassicae* infection induces *BnaMYB28-1* throughout the entire infection process and induces *BnaMYB28-3* at the early stage, whereas *BnaMYB28-2* remains lowly expressed and is suppressed (Chi Peng et al., 2020). Given that six homologous members of *BjuMYB28s* exist in *Brassica juncea*, it is speculated that similar response differentiation may also occur among different members, which deserves further investigation. In addition, because the first sampling was performed 12 h after infection, the expression dynamics at the early stage of infection could not be evaluated, and the early-stage change patterns need to be verified in

subsequent experiments with denser time points.

Overexpression of *BjuBMYB28-2* enhanced the resistance of *A. thaliana* to feeding by *S. litura*, consistent with reports that overexpression of *AtMYB28* in *A. thaliana* enhances insect resistance (Gigolashvili et al., 2007; Beekwilder et al., 2008). Gene expression analysis further revealed that, in *BjuBMYB28-2* overexpression lines, multiple key genes in the three stages of glucosinolate biosynthesis were all upregulated. This broad activation pattern is highly similar to the gene network regulated by *AtMYB28* (Sønderby et al., 2010b), indicating that the two in

functionally highly conserved. The hydrolysis product of glucosinolate, isothiocyanate, has broad-spectrum antibacterial activity and can inhibit the growth of pathogenic fungi such as *Alternaria brassicicola* (Zeng et al., 2025). In this study, overexpression of *BjuBMYB28-2* likewise enhanced resistance to *Sclerotinia sclerotiorum*, suggesting that glucosinolates or their hydrolysis products may participate in defense against this pathogen. This study provides scientific evidence for understanding the functional differentiation of mustard MYB28 family members and their roles in resistance to insects and diseases.

This study clarified the transcriptional activation activity and differences in subcellular localization of six BjuMYB28s in leaf mustard, and for the first time revealed the spatiotemporal expression characteristics of *BjuBMYB28-2* in response to feeding by the beet armyworm and infection by *Sclerotinia sclerotiorum*. It also confirmed that its overexpression can enhance the resistance of *Arabidopsis thaliana* to both biotic stresses by coordinately upregulating key genes in the glucosinolate biosynthetic pathway. These findings reveal subcellular localization divergence among members of the mustard MYB28 family, among which BjuBMYB28-2 has a unique nuclear localization pattern. Its tissue-specific expression and dynamic changes under stress provide new perspectives for understanding the regulation of glucosinolate metabolism, and also provide candidate genes for breeding cruciferous crops with resistance to insects and *Sclerotinia* disease. It should be noted that the subcellular localization and resistance evaluation were mainly based on heterologous expression systems, and their authentic functions still require further verification using the genetic transformation system of mustard itself. In addition, the time points for stress treatments were relatively sparse, and the dynamic expression patterns at the early stages of insect feeding or fungal infection remain unclear. Subsequent studies should set denser sampling time points and further analyze its directly regulated target genes and molecular interaction networks.

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