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Construction of prokaryotic expression vector and expression of GbMYBFL protein

Liangqiong Ma, Weiwei Zhang*, Feng Xu, Yongling Liao

College of Horticulture and Gardening, Yangtze University, Jingzhou, China

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Abstract

Flavonoids are one of the main secondary metabolites of *Ginkgo biloba*, and MYB is a transcription factor involved in regulation of flavonoid synthesis. In order to construct the prokaryotic expression vector of GbMYBFL from *Ginkgo biloba* and express the target protein, a pair of primers were designed with *Bam* H I and *Eco*R I restriction sites to amplify the ORF region of Ginkgo *GbMYBFL* gene by PCR. The PCR product was digested by *Bam*H I and *Eco*R I, and inserted into the prokaryotic expression vector pET32a. Then the recombinant plasmid was transformed into *E.coli* BL21 (DE3). After induction and expression, the protein was detected by SDS-PAGE. The results showed that the prokaryotic expression recombinant plasmid pET32a-GbMYBFL was successfully constructed. The recombinant plasmid was induced by IPTG in *E. coli* BL21 (DE3), and the size of the protein electrophoresis band is about 48 kDa, which was consistent with the molecular weight of protein predicted by bioinformatics tools. This study laid the foundation for the functional research of *GbMYBFL* in *Ginkgo biloba*.

* **Corresponding Author:** Weiwei Zhang ✉ wwzhangchn@163.com

Introduction

The MYB transcription factor is a type of DNA-binding protein, which is named for its conserved DNA binding region, the MYB domain (Albert *et al.,* 2011). As one of the largest families of plant transcription factors, MYB transcription factors are numerous and diverse. In plants, MYB proteins are involved in different physiological and biochemical processes, including regulating secondary metabolism, controlling cell morphogenesis, regulating flower color (Wang *et al.,* 1997), seed development (Penfield *et al.,* 2001) and cell cycle (Martin and Paz-Are, 1997). The *MYB* genes have been isolated from many plants, such as *Pharbitis nil* (Quattrocchio *et al.,* 1999), *Vitis vinifera* (Deluc *et al.,* 2006), apple (Takos *et al.,* 2006) and pear (Feng *et al.,* 2010), which are involved in the regulation of flavonoid synthesis in plants. The R2R3-MYB gene *VvMYB5a* was isolated from *Vitis vinifera* and expressed correctly in tobacco. It was found that *VvMYB5a* affected the synthesis of anthocyanins, flavonols, tannins and lignin in tobacco (Deluc *et al.,* 2006). The *VvMYB5b* of *Vitis vinifera* is mainly involved in the flavonoid metabolism pathway, and overexpression in tobacco causes an increase in anthocyanin and tannin content (Deluc *et al.,* 2008). Flavonoids from Ginkgo leaves have important physiological activities and medicinal value. The study of MYB is of great significance for revealing the molecular mechanism of ginkgo flavonoid synthesis. Qin (2007) had cloned a transcription factor gene *GbMYB1* from *Ginkgo biloba*. However, overexpression in Arabidopsis indicated that *GbMYB1* is involved in the regulation of lignin. The *GbMYBF2* gene was also cloned from *Ginkgo biloba*, the expression of *GbMYBF2* gene was negatively correlated with the accumulation of flavonoids in *Ginkgo biloba*. Overexpression in *Arabidopsis thaliana* showed that *GbMYBF2* inhibited the accumulation of flavonoids and anthocyanins, indicating that *GbMYBF2* is negative regulators in flavonoid synthesis (Xu *et al.,* 2013). We have isolated the *GbMYBFL* gene from *Ginkgo biloba* (Zhang *et al.,* 2018). In the current study, the prokaryotic expression vector pET32a-GbMYBFL was constructed and expressed in *E. coli* BL21 (DE3), these laid a foundation for the expression and purification of GbMYBFL protein in future.

Materials and methods

Experiment Materials

pET32a, *E. coli* Top10, and *E. coli* BL21 (DE3) used in this experiment were preserved in the molecular biology laboratory of Yangtze University. Taq DNA polymerase, restriction enzymes *Bam*H I and *Eco*R I, T_4 DNA ligase, and plasmid extraction kits were purchased from TaKaRa Co., Ltd (Dalian, China).

Construction of prokaryotic expression vector of pET32a-GbMYBFL

Based on the cDNA sequence of *GbMYBFL* gene, a pair of primers containing *Bam*H I and *Eco*R I restriction sites were designed and synthesized (Upstream primer: GbMYBFLES, 5'-CGGGATCCATGGGCAGGGCTCCTTGCTG-3'; Downstream primer: GbMYBFLEA, 5'-CGGAATTCTCAAAAGATTATTCCCTCTTGACAC-3'). The ORF region of the *GbMYBFL* gene was amplified from the pMD18-T-GbMYBFL recombinant plasmid by PCR. The PCR reaction procedure was as follows: initial denaturation at 94°C for 3 min; 32 cycles (94°C for 30 s, 60°C for 40 s, 72°C for 90 s); extension at 72°C for 10 min. The PCR product was digested with *Bam*H I and *Eco*R I enzymes according to the instructions, and the digested product was purified by TaKaRa Mini BEST Agarose Gel DNA Extraction Kit. The pET32a vector was also digested with *Bam*H I and *Eco*R I. The *GbMYBFL* was inserted into the pET32a under the catalysis of T_4 DNA ligase, and then introduced into prokaryotic expression strain BL21 (DE3) via 42°C heat-shock method.

Resistance screening of transformed strains was performed on LB medium containing ampicillin, positive strains were identified by PCR using two pairs of primers GbMYBFFLES+GbMYBFLEA and T7+T7term (T7: 5'-TAATACGACTCACTATAGGG-3', T7term: 5'-TGCTAGTTATTGCTCAGCGG-3'). Furthermore, the pET32a-GbMYBFL vector was

verified by sequencing, and the constructed positive strains were used for protein expression.

Expression of prokaryotic expression vector pET32a-GbMYBFL

After PCR and sequencing verification, the colony contains recombinant plasmid pET32a-GbMYBFL was inoculated into LB liquid medium containing 50 μg/mL ampicillin, and cultured overnight at 37°C, 200 rpm; the empty vector was also inoculated into LB under the same culture conditions. Cultured colony was inoculated to a fresh LB liquid medium containing 50 μg/mL ampicillin at a dilution ratio of 1:100, and cultured at 37°C, shaking at 200 rpm to make the bacterial solution OD600 ≈ 0.5-0.6. The bacterial liquid was added with IPTG to a final concentration of 1 mM, and cultured at 30°C for 4 h, then 1 mL of the bacterial solution was centrifuged to collect the cells. The cells were suspended in 200 μL of loading buffer, boiled for 5 min, centrifuged at 12,000 rpm for 15 min, and 10 μL of supernatant were analyzed by 12% SDS-PAGE gel electrophoresis. The bacterial liquid without IPTG and the control group were subjected to the same operation. The SDS-PAGE electrophoresis was carried out in accordance with the instrument manual of Mini-PROTEAN® Tetra Cell. The voltages of the sample through the concentrated gel and the separation gel were 80V and 120V, respectively. The staining solution was Coomassie Brilliant Blue R250, and the eluent was methanol/glacial acetic acid solution.

Results

Construction and identification of prokaryotic expression vector pET32a-GbMYBFL

The GbMYBFL gene containing BamH I and EcoR I restriction sites and the vector pET32a were double digested with BamH I and EcoR I. As shown in Fig. 1, the pET32a plasmid became larger after digestion, indicating that the pET32a plasmid was linearized.

Fig. 1. Double Digestion of pET32a plasmid and *GbMYBFL* gene. M1. λ-*Hind* III Marker; 1. pET32a; 2. pET32a after digested; M2. DL2000 DNA Marker; 3. *GbMYBFL*; 4. *GbMYBFL* after digested.

GbMYBFL was also changed in size after digestion. Colony PCR detection showed that the amplified fragment sizes corresponded to the length of the target sequences (Fig. 2).

The results of the sequencing further confirmed that the GbMYBFL gene was inserted into the pET32a and could be used for prokaryotic expression analysis.

Expression of GbMYBFL Protein in E. coli BL21(DE3) After induced by 1 mM IPTG for 4 h, *GbMYBFL* was expressed as a major protein product in the total cellular protein. SDS-PAGE showed that the expressed recombinant protein band was between 66.4 kDa and 44.3 kDa (Fig. 3), the molecular weight was estimated to be about 48 kDa with the His-tag, the size of which was in good agreement with that predicted through bioinformatics. The pET32a-GbMYBFL expression vectors that not induced by IPTG and empty vectors not containing *GbMYBFL* were not expressed in the positions (Fig. 3). The results suggest that *GbMYBFL* gene can express in the *E. coli* correctly.

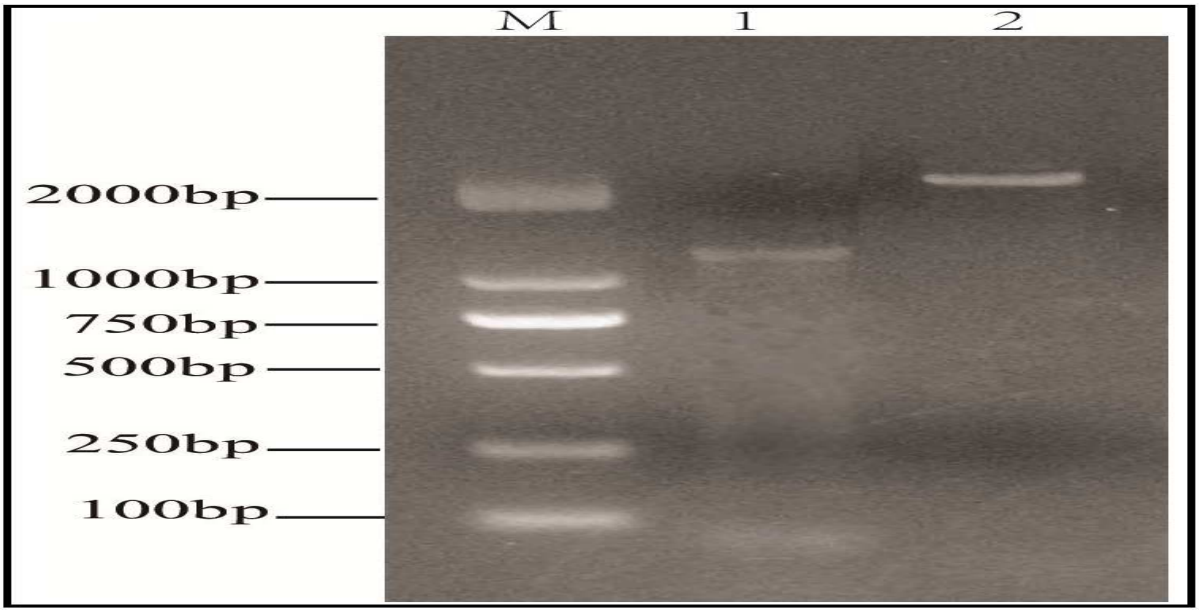

Fig. 2. PCR detection of prokaryotic expression vector *GbMYBFL*. M. DL2000 DNA Marker; 1. Primer pair GbMYBFFLES+GbMYBFLEA amplification results; 2. Primer pair T7+T7term amplification results.

Discussion

Although some research on *MYB* gene in *Ginkgo biloba* have been reported (Xu *et al.,* 2013; Zhang *et al.,* 2018), to clarify the function of MYB transcription factors in plants, further research is needed at the protein level. In this study, the prokaryotic expression vector of pET32a-GbMYBFL was constructed and successfully expressed in expression strain BL21 (DE3) induced by IPTG. SDS-PAGE results confirmed that the molecular mass of GbMYBFL was approximately 48 kDa (Fig. 3), and this protein consistent with the molecular weight predicted by bioinformatics method. Similar to our results, the Arabidopsis ATMYB2 fusion protein was successfully expressed in *Escherichia coli* bound to the conserved MYB recognition sequence (Urao *et al.,* 1993).

In many plants, MYB transcription factors are involved in promoting or negatively regulate the synthesis of flavonoids. For example, *MdMYBA* gene isolated from apples can promote the accumulation of anthocyanins in transgenic tobacco, and can specifically bind to the promoter of the key gene *MdANS* that involved in anthocyanin synthesis (Ban *et al*., 2007).

The *MdMYB10* gene in apple is involved in the regulation of anthocyanin biosynthesis (Espley *et al*., 2007), and the abnormal expression of the *PAP1* gene encoding MYB transcription factor in Arabidopsis can also lead to the accumulation of purple anthocyanins in most organs of Arabidopsis (Borevitz *et al*., 2000).

However, strawberry *FaMYBl* gene negatively regulates the synthesis of anthocyanins, the overexpression of *FaMYBl* in tobacco found that the accumulation of anthocyanins and flavonoids in flowers and stamens was suppressed, and the expression level of some key enzyme genes involved in anthocyanins synthesis pathway is also decreased

accordingly (Aharoni *et al.*, 2001). *GbMYBFL* positively related to flavonoid biosynthesis, and the overexpression of *GbMYBFL* was sufficient to induce flavonoids and anthocyanin accumulation (Zhang *et al.*, 2018). The prokaryotic expression analysis results of *GbMYBFL* created conditions for further protein purification and validation of GbMYBFL protein function.

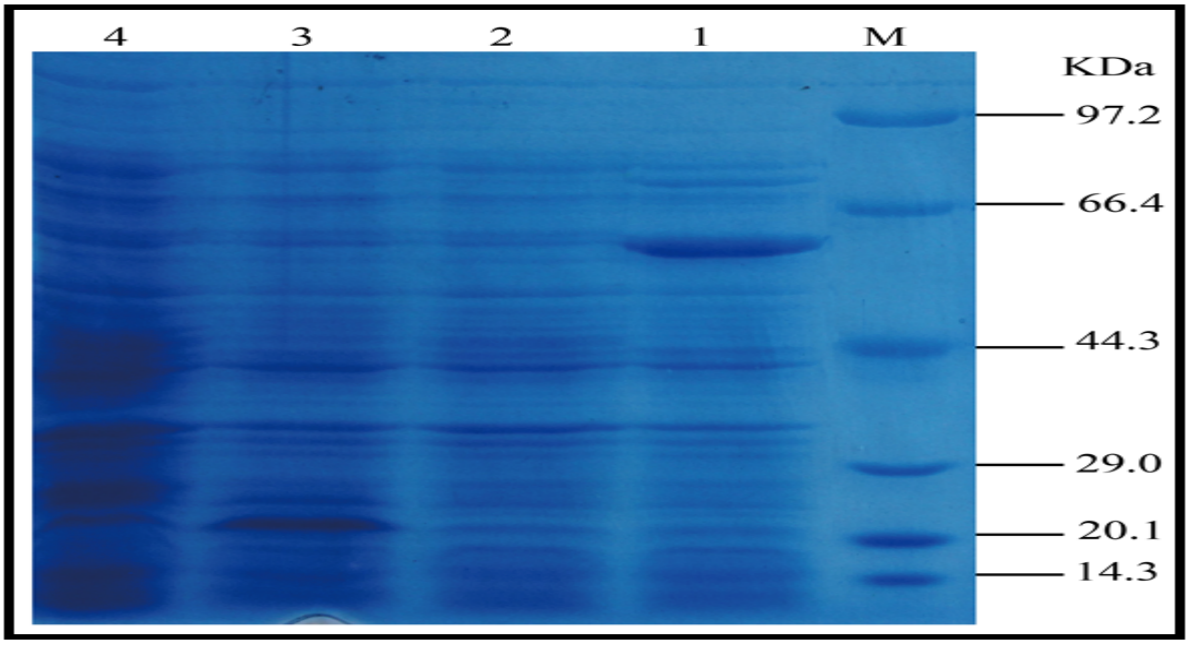

Fig. 3. SDS-PAGE electrophoresis of GbMYBFL expressed in *E.coli* BL21 (DE3). M, protein molecular weight; Lane 1, Proteins of total cells containing pET32a-GbMYBFL after 4 h induction by IPTG; Lane 2, Proteins of total cells containing pET32a-GbMYBFL without IPTG induction; Lane 3, Proteins of total cells containing pET32a after 4 h induction by IPTG; Lane 4, Proteins of total cells containing pET32a without IPTG induction.

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