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Protein Expression of Flavonol Synthase from Fragrant Wild Cyclamen (*Cyclamen purpurascens*)

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Short Communication

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ABSTRACT

To aid the development of DNA markers and new flower-color mutants, we previously isolated and analyzed the flavonoid biosynthesis-related genes involved in flower coloration in fragrant wild cyclamen (*Cyclamen purpurascens*). Two flavonol synthase genes (*CpurFLS1* and *CpurFLS2*) were subsequently isolated and *CpurFLS2* found to be related to flower coloration. As a next step, *in vitro* observations of enzymatic activity are necessary; therefore, expression analysis of *CpurFLS2* protein was conducted. In this study, we provide the optimal conditions for recombinant soluble *CpurFLS2* protein production in *Escherichia coli*.

INTRODUCTION

Three cultivars of fragrant cyclamen are currently available, all of which were created by crossing the cyclamen cultivar *Cyclamen persicum* with the scented species *C. purpurascens* [1]. Our overall aim is to create new fragrant cultivars with novel petal coloration using ion-beam irradiation. Because ion-beam irradiation tends to cause large DNA rearrangement [2], PCR screening is useful in selecting desired mutants from a mutagenized cyclamen population before flowering. Clarification of the mechanism of flower coloration and identification of the genes involved in flower coloration in cyclamen are therefore necessary.

Cyclamen flower color is controlled by an accumulation of major plant pigment-related compounds known as flavonoids. The main flavonoids in *C. purpurascens* flowers are a single anthocyanin, malvidin 3,5-diglucoside (Mv3,5dG), and two flavanols, quercetin and kaempferol [3,4]. Anthocyanins are well-known plant pigments responsible for strong flower coloration. Numerous anthocyanin biosynthesis-related genes have been isolated from a number of plant species including cyclamen [5-8]. However, the gene(s) for Flavanol Synthase (FLS) in cyclamen has yet to be isolated. Flavanols occasionally modify flower color when combined with anthocyanins, a process known as co-pigmentation [9]. For example, reduced FLS leads to reddening of flower color in petunia [10]. Determining the combination of anthocyanin and flavanols is therefore important in understanding flower coloration. We recently isolated two *FLS* genes from *C. purpurascens* (*CpurFLS1* and *CpurFLS2*) [10], and in a complement experiment using an *fls* mutant of *Arabidopsis thaliana*, found that both genes function in flavonol synthase. Differential expression of these two genes was also revealed, with constitutive expression of *CpurFLS1* in young petals and other organs **(Figure 1)**. In contrast, *CpurFLS2* expression was strong in young petals but weak in anthers, leaves and petioles. Moreover, no expression of *CpurFLS2* was observed in open petals **(Figure 1)**. These patterns suggest that *CpurFLS1* and *CpurFLS2* possess functional diversity, with a correlation between *CpurFLS2* and flower coloration in *C. purpurascens*. As a next step, it is therefore important to determine the functional differences between these two genes, including substrate specificity. In this report, we therefore examine the optimal conditions for *CpurFLS2* protein expression and purification [10].

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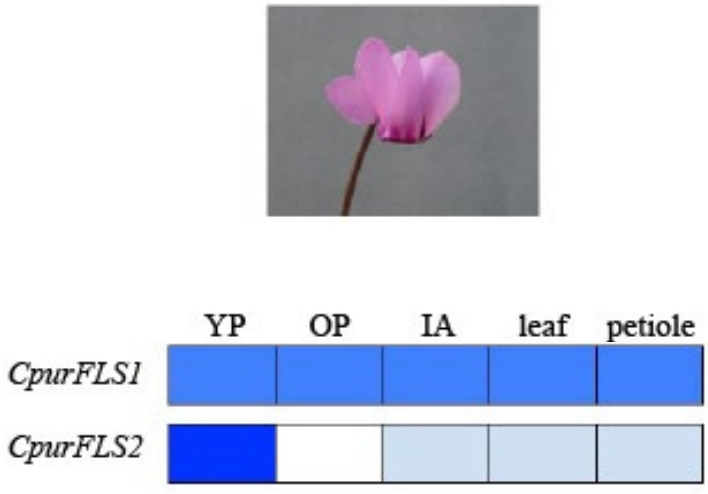

Figure 1. Flower of *Cyclamen purpurascens* and the expression patterns of *CpurFLS1* and *CpurFLS2* shown as blue density. YP: Young Petal; OP: Open Petal; IA: Immature Anther.

MATERIALS AND METHODS

Protein Expression and Purification

The full-length open reading frame (ORF) of *CpurFLS2* cDNA (accession number: LC210073) was amplified by PCR using a forward primer with the *Nde*I site and reverse primer with the *Xho*I site. The amplified fragments were subcloned into the pGEM-T Easy vector (Promega) and digested with *Nde*I and *Xho*I. Then, the excised fragment was ligated into the *Nde*I–*Xho*I site of the His-tagged pET16b expression vector (Merck) to yield pET16b-CkmOMT2. The insertion was sequenced carefully using T7 primer sets to verify that no mutations occurred. To produce recombinant proteins with a His-tag at the N-terminus, the resulting plasmid pET16b-CkmOMT2 was used to transform *Escherichia coli* strain BL21 (DE3) (Merck). *E. coli* harboring pET16b-CkmOMT2 was cultivated in 2 ml of lysogeny broth (Difco) supplemented with 100 μgmL^{-1} ampicillin until reaching an OD_{600} of 0.4–0.5. After addition of isopropyl *β*-D-thiogalactopyranoside (IPTG) to a final concentration of 0–0.1 mM, the cells were further cultured at 20°C to 37°C for 616 h. They were then resuspended in FastBreakTM Cell Lysis Reagent (Promega) and the fusion protein purified using the HisLinkTM Spin Protein Purification System (Promega) according to the manufacturer's protocol.

RESULTS AND DISCUSSION

Our aim was to examine *CpurFLS2* protein production in recombinant *E. coli* under various conditions. At an incubation temperature of 20°C, recombinant *CpurFLS2* protein expression was observed after addition of 10 μM IPTG, with increasing expression under 50 μM IPTG **(Figure 2)**. Furthermore, expression was higher with an incubation time of 8 h compared to 6 h **(Figure 2)**. However, with an incubation time of more than 10 h, *CpurFLS2* expression was the same as that under no IPTG. We subsequently adjusted the incubation temperature (25°C, 28°C, 30°C, and 37°C, respectively) and similarly analyzed the effect on expression. Under all conditions, an increase in *CpurFLS2* protein was observed; however, in most cases, the protein was insoluble (data not shown). As a result, we determined the optimal conditions for recombinant soluble *CpurFLS2* protein production in *E. coli* as 1) a final concentration of IPTG of 10 μM, 2) an incubation temperature of 20°C, and 3) an incubation time of 8 h.

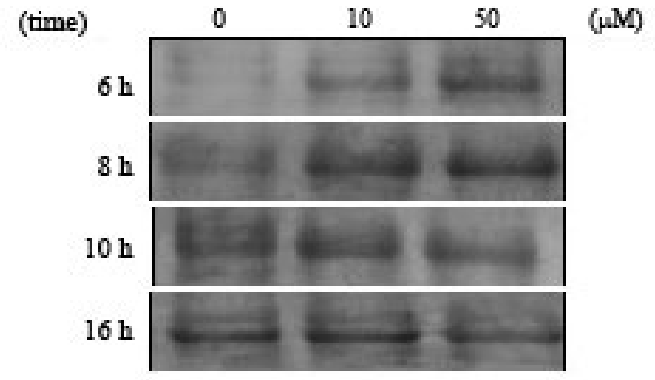

Figure 2. Protein expression of recombinant CpurFLS2 under various culture conditions. The incubation temperature was kept constant at 20°C.

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We subsequently attempted to purify the recombinant *CpurFLS2* protein using a His-tagged expression vector. As a result, using the above optimal culture conditions, N-terminus His-tagged *CpurFLS2* protein was successfully produced in recombinant *E. coli*. The isoelectric point of *CpurFLS2* was 6.15 and the putative molecular weight was 37.7 kDa (data not shown). The molecular mass of the purified protein was consistent with the expected mass of the fusion protein including the additional His-tag of 2 kDa **(Figure 3)**. These results suggested successful purification of recombinant soluble *CpurFLS2*. The next step is to carry out an enzymatic assay of *CpurFLS2* to determine enzymatic activity and substrate specificity. Two FLS proteins were recently isolated from different colored onions [11], with only three amino acid differences between AcFLS-H6 (isolated from red onion) and AcFLS-HRB (isolated from yellow onion). Despite the relatively high amino acid similarity, the catalytic efficiency of AcFLS-HRB was approximately twice that of AcFLS-H6 when dihydroflavonol was used as a substrate. Comparisons of the predicted amino acid sequences of *CpurFLS1* and *CpurFLS2* revealed approximately 50 differences **(Figure 4)**, suggesting differences in enzymatic activity and/or substrate specificity. To examine enzymatic activity, we therefore expressed *CpurFLS1* under the above culture conditions. As a result, strong expression of recombinant *CpurFLS1* was observed similar to *CpurFLS2*; however, all were insoluble (data not shown).

CONCLUSION

These results suggest that the optimal conditions for production of recombinant soluble protein differ between *CpurFLS1* and *CpurFLS2*. Further clarification of the optimal conditions for expression of soluble recombinant *CpurFLS1* are therefore necessary. We previously purified anthocyanin-*O*-methyltransferase (CkmOMT2) from the fragrant cyclamen cultivar 'Kaori-no-mai' [7]. The optimal conditions for CkmOMT2 production were 1) a final concentration of IPTG of 100 μM, 2) an incubation temperature of 28°C, and 3) an incubation time of 18 h. Thus, despite the proteins being isolated from cyclamen, the optimal culture conditions for protein expression differed depending on the protein. In a future study, we will examine the question of why the protein expression conditions differ. These findings will aid research on flower coloration in cyclamen.

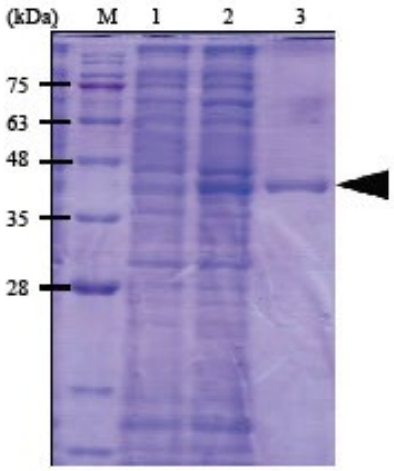

Figure 3. Purification of recombinant CpurFLS2. Lane M: Protein Marker; Lane 1: E. coli Lysate without IPTG; Lane 2: E. coli Lysate with 10 μM IPTG; Lane 3: The Purified Recombinant Protein. The purified CpurFLS2 recombinant protein is indicated by an arrowhead.

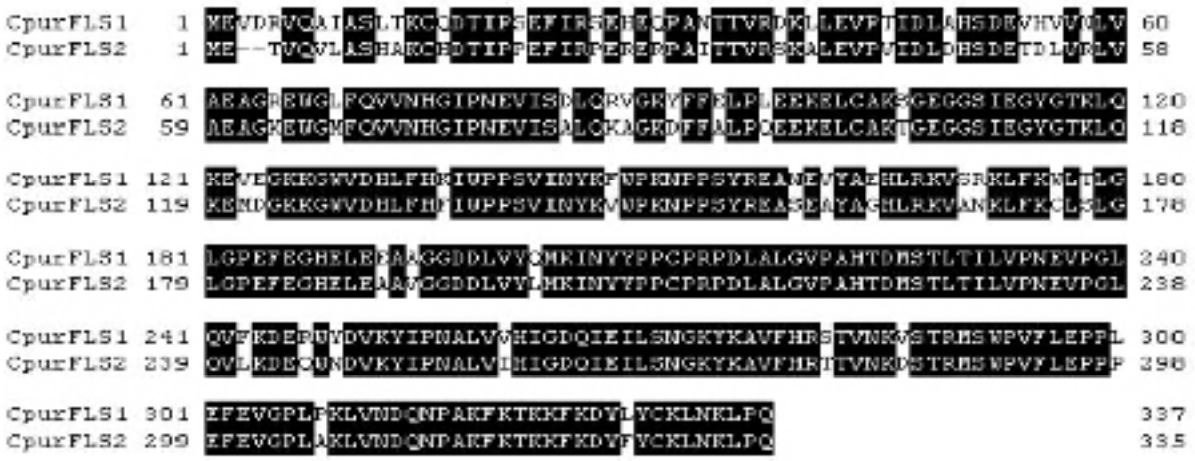

Figure 4. Comparison of the deduced amino acid sequences of *CpurFLS1* and *CpurFLS2*. Amino acid residues identical in the two sequences are indicated by a black background.

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REFERENCES

1. Ishizaka H. Interspecific hybridization by embryo rescue in the genus Cyclamen. Plant Biotechnol. 2008;25:511-519.
2. Shikazono N, et al. Analysis of mutations induced by carbon ions in *Arabidopsis thaliana*. J Exp Bot. 2005;412:587-596.
3. Takamura T, et al. Flower color and anthocyanins in the petal of *Cyclamen* species. J Jpn Soc Hort Sci. 1997;66:508-509.
4. Takamura T, et al. Inheritance of flower color pigment in crosses between cyclamen cultivars and *Cyclamen purpurascens*. Acta Hortic. 2005;673:437-441.
5. Boase MR, et al. Isolation and antisense suppression of flavonoid 3', 5'-hydroxylase modifies flower pigments and colour in cyclamen. BMC Plant Biol. 2010;10:107.
6. Akita Y, et al. Comparative analysis of floral pigmentation between wild-type and white-flowered varieties of *Cyclamen graecum*. J Hortic Sci Biotechnol. 2010;84:437-443.
7. Akita Y, et al. Isolation and characterization of the fragrant cyclamen *O*- methyltranseferase involved in flower coloration. Planta. 2011;234:1127-1136.
8. Hase Y, et al. Development of an efficient mutagenesis technique using ion beams: Toward more controlled mutation breeding. Plant Biotechnol. 2012;29:193-200.
9. Forkmann MA. Flavonoids as flower pigments: The formation of the natural spectrum and its extension by genetic engineering. Plant Breed. 1991;106:1-26.
10. Folton TA, et al. Cloning and expression of flavonol synthase from *Petunia hybrida*. Plant J. 1993;4:1003-1010.
11. Akita Y, et al. Identification of functional *flavonol synthase* genes from fragrant wild cyclamen (*Cyclamen purpurascens*). J Plant Biochem. Biotechnol. 2017;1-9.
12. Park S, et al. Comparative analysis of two flavonol synthases from different-colored onions provides insight into flavonoid biosynthesis. J Agric Food Chem. 2017;65:5283-5298.