Phytochemistry 94 (2013) 60–67

Contents lists available at SciVerse ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

The identification of a vacuolar iron transporter involved in the blue coloration of cornflower petals

Kumi Yoshida *, Takashi Negishi

Graduate School of Information Science, Nagoya University, Japan
G-COE in Chemistry, Nagoya University, Furo-cho, Chikusa, Nagoya, Aichi 464-8601, Japan

ARTICLE INFO

Article history:
Received 5 October 2012
Received in revised form 28 February 2013
Available online 6 July 2013

Keywords:
Centaurea cyanus
Asteraceae
Amino acid substitution
Blue flower coloration
Metalloanthocyanin
Protocyanin
Iron distribution
Vacuolar iron transporter

ABSTRACT

The blue petal color of the cornflower (*Centaurea cyanus*) is caused by protocyanin, a kind of metalloanthocyanin, which is a self-assembled supramolecular metal complex pigment. Protocyanin is composed of six molecules of anthocyanin, six molecules of flavone, one ferric ion, and one magnesium ion. The ferric ion is essential for blue color development. Here, we identify the vacuolar iron transporter gene (*CcVIT*) from the blue petals of *C. cyanus* and its function is identified and characterized. The CcVIT transcript was observed only in the petals. Its amino acid sequence is highly homologous to the *Arabidopsis thaliana* (AtVIT1) and *Tulipa gesneriana* (TgVit1) vacuolar iron transporters. Heterologous expression of the *CcVIT* gene in yeast indicated that the corresponding gene product transports ferrous ion into vacuoles. Analysis of purple mutant-line petals clarified that the anthocyanin and flavone components were the same as those found in plants with blue petals, but the amount of iron ions in the colored cells decreased, and consequently the amount of blue protocyanin was reduced. The *CcVIT* gene was expressed even in purple mutant petals, however, an amino acid substitution (A236E) occurred in that case. This change in the CcVIT gene sequence also resulted in loss of iron transport activity. The CcVIT protein thus plays a critical role in the blue coloration of cornflower petals.

1. Introduction

Blue flower coloration is primarily caused by anthocyanins, which are found in the vacuoles of petal epidermal cells (Yoshida et al., 2009). All anthocyanins are able to chemically develop blue colors under alkaline conditions (Willstätter and Everest, 1913; Brouillard, 1982; Goto and Kondo, 1991; Brouillard and Dangles, 1994; Andersen and Monica, 2006; Yoshida et al., 2009). However, vacuolar pH is generally maintained at weakly acidic levels (pH of approximately 5), so that simple anthocyanins, such as mono- and di-glycosylanthocyanins, that are modified with zero to one aliphatic and/or aromatic acyl residue, initially take on the blue anhydrobase form but are quickly decolorized by hydration (Brouillard, 1982; Goto and Kondo, 1991; Brouillard and Dangles, 1994; Andersen and Monica, 2006; Yoshida et al., 2009). Therefore, there must be sophisticated mechanisms that allow the maintenance of the blue anionic form under physiological conditions. Complexation of various divalent and trivalent metal ions, such as Mg^{2+}, Fe^{3+} and Al^{3+}, with catechol groups at the B-ring of the anthocyanidin nucleus results in a blue color under neutral and weakly acidic conditions (Shibata et al., 1919; Bayer et al., 1960; Jurd and Asen, 1966; Dangles et al., 1994). Thus, formation of metal-complexed anthocyanins is a common phenomenon in blue flowers (Goto and Kondo, 1991; Kondo et al., 1992, 2001; Brouillard and Dangles, 1994; Takeda, 2006; Yoshida et al., 2009).

The blue pigment of the cornflower (*C. cyanus*, Fig. 1A) has a long history of controversial chemical studies. In 1913, Willstätter isolated cyanin from blue cornflower petals, and found it to be the same pigment that occurs in red roses. From observation of color change experiments, he proposed the pH theory of blue petal coloration (Willstätter and Everest, 1913). Later in 1958, Bayer isolated a blue pigment from cornflower petals and named it protocyanin (Bayer, 1958). This was composed of complex components, including cyanin, sugars and metal ions, such as Fe^{3+} and Al^{3+}. He proposed a metal complex structure as being the cause of the blue coloration (Bayer, 1958, 1960). After Bayer's research, Hayashi et al. (1961) and Asen and Jurd (1967) independently reported different metal ion compositions and structures of the blue pigment of cornflower. Finally, the entire composition of protocyanin was determined by reconstruction from its individual components (Kondo et al., 1994), including six molecules of anthocyanin (succinylcyanin, Sc, **1**), six molecules of flavone (malonylflavone, Mf, **2**) and one atom each of Fe^{3+} and Mg^{2+} (Fig. 2). The mechanism of the blue coloration caused by protocyanin due to the characteristic absorption peak at 676 nm was verified to be the LMCT (ligand-to-metal charge transfer) band between Sc (**1**) and the

* Corresponding author at: Graduate School of Information Science, Nagoya University, Japan. Tel./fax: +81 52 789 5638.
E-mail address: yoshidak@is.nagoya-u.ac.jp (K. Yoshida).

0031-9422/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.phytochem.2013.04.017

Fig. 1. Different colored petal lines in *C. cyanus*. In cornflower, color of petals in the normal line is blue (A). A mutant line (B) was observed with purple petals, which prompted an investigation into the cause of this abnormal coloration.

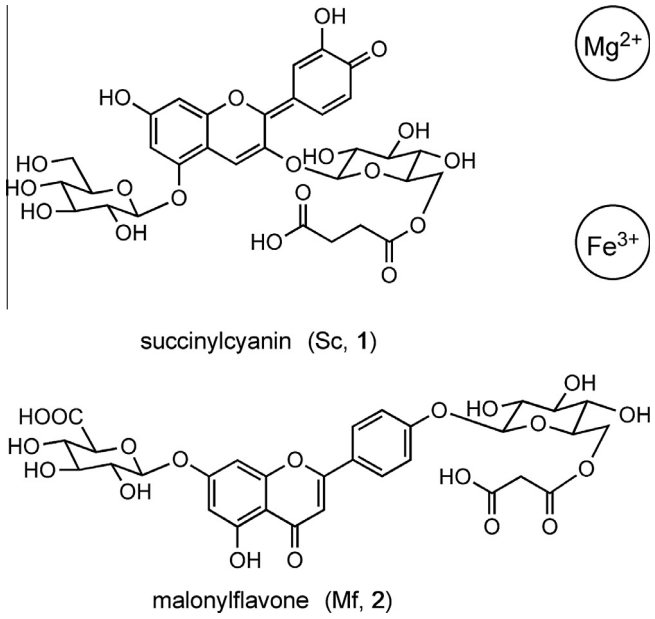

Fig. 2. Components of blue petal pigment of cornflower. In petal vacuoles of *C. cyanus*, protocyanin, which is a self-assembled supramolecular metal complex pigment, forms and provides the color. Protocyanin is a complex of succinylcyanin (**1**), malonylflavone (**2**), Fe^{3+} and Mg^{2+} in a 6:6:1:1 ratio.

ferric ion (Kondo et al., 1998). The fine structure of protocyanin was clarified with X-ray structural determination (Shiono et al., 2005).

Iron is an essential element for all living organisms, but excess iron in cells is toxic because it causes the overproduction of reactive oxygen species (ROS) (Gallego et al., 1996; Kampfenkel et al., 1995). Therefore, delivery and compartmentalization of iron in plants must be strictly regulated (Guerinot and Yi, 1994; Clemens, 2001; Curie and Briat, 2003; Martinoia et al., 2007). One strategy for reducing free iron in the cytosol is its sequestration into vacuoles. However, the vacuolar iron transporter (VIT) that transports cytosolic iron into vacuoles has not yet been fully elucidated. In 2006, isolation of a VIT gene in *Arabidopsis thaliana* seeds (*AtVIT1*) and its characterization was published (Kim et al., 2006). However, no difference in iron concentration was observed throughout the seed (Kim et al., 2006). In 2009, another VIT gene, *TgVit1*, was reported from tulip petals (*Tulipa gesneriana* cv. Murasakizuisho) (Momonoi et al., 2009) that is responsible for the blue color of the inner perianth bottom by specific accumulation of iron (Shoji et al., 2007). Similar iron accumulation causing blue coloration of tulips was also observed in various tulip cultivars (Momonoi et al., 2012).

As described above, iron is essential for the blue color of cornflower petals, indicating that ferric ion accumulates in the blue-colored vacuoles. This fact prompted us to search for VIT in cornflower petals. In the present study, the VIT gene, *CcVIT*, was isolated from the blue petals of the cornflower. A complementation assay was performed in yeast ccc1 mutants (*Δccc1*) that carried *CcVIT*, and it was found that the *CcVIT* gene product exhibits iron transport activity. In addition, a mutant line with a purple petal phenotype was discovered (Fig. 1B), whose blue pigmen amounts decreased relative to normal cornflowers as a result of iron deficiency. Chemical analysis of the purple petals strongly suggested that protocyanin decreased due to iron deficiency, and the gene analysis showed that a single amino acid replacement is involved in creating the purple petal color.

2. Results

2.1. Isolation of CcVIT cDNA and the expression of the gene in tissues

To clone the vacuolar iron transporter (VIT) genes from blue petals of *C. cyanus*, known VIT proteins was first analyzed for their similarities. High similarities in amino acid sequences were observed between AtVIT1 (Kim et al., 2006) and TgVit1 (Momonoi et al., 2009), and a database search indicated that there are several putative VIT genes from *Oryza sativa* (*OsVIT*), *Populus trichocarpa* (*PtVIT*), *Vitis vinifera* (*VvVIT*), *Zea mays* (*ZmVIT*), *Sorghum bicolor* (*SbVIT*), *Physcomitrella patens* (*PpVIT*), *Ricinus communis* (*RcVIT*), *Selaginella moellendorffii* (*SmVIT*) and *Picea sitchensis* (*PsVIT*) that are highly homologous. These results indicated that the VIT gene might be well-conserved in plants. Using this sequence data, a strategy was developed to isolate the cornflower VIT cDNA from the petals. Total RNA was isolated from petals that were in early growth stages (Stages 1, 2), and cDNA libraries were prepared. Full-length *CcVIT* transcripts were obtained using 5′ RACE and 3′ RACE with a combination of degenerate primers based on the conserved sequences of *A. thaliana* VIT1 (*AtVIT1*, At2g01770), *T. gesneriana* Vit1 (*Tg Vit1*, AB500106) and *Oryza sativa* Vit1 (OsVit1, Os04g0463400), respectively.

The putative CcVIT cDNA consisted of 720 base pairs (bp), which encoded a polypeptide of 239 amino acids (Fig. 3). A BLAST search showed that the protein belonged to the CCC1 family, iron and manganese transporters that transport metals from the cytosol into the vacuole in *S. cerevisiae* (Li et al., 2001). Phylogenetic analysis indicated that CcVIT is highly similar to putative plant VIT proteins (Fig. S1A). CcVIT shared 79% amino acid identity with AtVIT1, with the protein having 83% identity with PtVIT (ABK94901, *Populus trichocarpa*), which is the most closely related homolog identified to date.

Secondary structure analysis of CcVIT using the SOSUI program (Fig. 3) (Hirokawa et al., 1998) indicated the presumed presence of four transmembrane domains, not the five that may be present in AtVIT1 and TgVit1. To determine the correlation between *CcVIT* gene expression and blue petal coloration, the amount of *CcVIT* mRNA in cornflower tissues was analyzed by RT-PCR (Fig. 4). *CcVIT* mRNA was only detected in petal tissues, not in other parts of the plant, such as leaves, roots, sepals and stems. In petals, expression levels increased with petal maturation (Fig. 4A), and highest expression was observed at stage 3, which is when petals are fully opened and exhibit their blue coloration.

2.2. Iron transport activity of CcVIT

To determine whether the putative gene product CcVIT exhibits iron transport activity, a complementation assay in yeast was performed using a heterologous expression system. As previously