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The *ROSEA1* and *DELILA* transcription factors control anthocyanin biosynthesis in *Nicotiana benthamiana* and *Lilium* flowers

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ABSTRACT

The activity of anthocyanin biosynthesis genes is regulated at the transcriptional level, thus manipulation of transcription factors (TFs) is an ideal strategy to alter the expression of multiple target genes. In this study, we investigated the effect of introducing *ROSEA1* (*ROS1*, a MYB-type) and *DELILA* (*DEL*, a bHLH-type) TFs from snapdragon under control of a flower specific promoter, *Floral Binding Protein 1* (*FBP1*) from petunia into *Nicotiana benthamiana* flowers and *Lilium* tepals. The usefulness of the *FBP1* promoter was demonstrated by the generation of purplish flowers in otherwise normal-growing plants of *N. benthamiana*, while the expression by the *35S* promoter led to the development of stunted plants with anthocyanins in all parts. *N. benthamiana* was successfully transformed by *ROS1* alone and by a combination of *ROS1* + *DEL*. The observed accumulation of delphinidin corresponded to the expression of *NbCHS, NbF3H, NbDFR* and *NbANS*. The effect of *ROS1* + *DEL* on *Lilium* flower colour was investigated using agroinfiltration. A higher cyanidin accumulation was observed in tepals of the Oriental hybrid lily cv. 'Perth', resulting in deeper pink colouration at the infiltrated area. Nevertheless, the introduction of *ROS1* + *DEL* did not produce any phenotypic changes to the white-flowered *L. longiflorum* cv. 'Lincoln' and the white Oriental hybrid lily cv. 'Rialto' due to other deficiencies in their anthocyanin biosynthetic pathway. Co-expression of *ROS1* + *DEL* under control of the *FBP1* promoter together with active structural anthocyanin biosynthetic genes can result in modification of *Lilium* flower colour.

1. Introduction

The genus *Lilium* (Liliaceae) represents a bulbous monocot consisting of more than 100 species (Comber, 1949; Lighty, 1968; Lim et al., 2008). The hybrids are divided into several groups namely, Longiflorum (L), Trumpet (T), Oriental (O) and Asiatic (A) hybrids. The Oriental and Asiatic hybrid lilies have been commercially predominant in the ornamental cut flower industry due to their outstanding flower shape, fragrance and large colour variations (Lim and van Tuyl, 2006; Yamagishi and Akagi, 2013). Lilies from the Longiflorum group nowadays have become more desired among growers and consumers because of several positive traits such as beautiful flowers, early flowering, easy to grow and propagate, and vase life quality (Martens et al., 2003). However, they only exist in purely white-flowered cultivars. Plant breeders have developed various flower colours in the Oriental and Asiatic hybrid lilies using interspecific hybridization (Barba-Gonzalez et al., 2004; Lim and van Tuyl, 2006; Yamagishi and Nakatsuka, 2017). A wide interspecific hybridization between *L. longiflorum* and other lilies from the Asiatic group resulted mainly in sterile F1-hybrids, thus hampering its breeding program (Karlov et al., 1999). Genetic modification offers an alternative approach for the introduction of novel flower colour traits into this plant species. This approach has been successfully developed in another commercially important ornamental crops such as roses and carnations (Chandler and Tanaka, 2007; Chandler and Brugliera, 2011).

Flower colour is an important characteristic that determines the commercial value of ornamental crops. Much interest had been placed in ornamental crops that bear flowers exhibiting large colour variations and hues. Anthocyanins are pigments that provide colours in red, purple and blue to plant organs (Tanaka and Ohmiya, 2008). There are three major groups of anthocyanidins present in higher plants, i.e. cyanidin, pelargonidin and delphinidin (Schwinn and Davies, 2004). Coloured *Lilium* flowers contain cyanidin 3-O-β-rutinoside as a major anthocyanin and cyanidin 3-O-β-rutinoside-7-O-β-glucoside as a minor

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anthocyanin (Nørbæk and Kondo, 1999).

Because the accumulation of anthocyanins during flower development usually, but not always, correlates with the increasing expression of structural genes, the transcription of the structural genes would appear to be the key factor in controlling the production of anthocyanins. Gene expression is mainly controlled at the transcriptional level and is regulated by MBW complexes consisting of members of the *MYB*, basic helix-loop-helix (*bHLH*) and *WD40* transcription factor families (Quattrocchio et al., 1998; Koes et al., 2005). Among these three transcription factors, co-expression of *MYB* and *bHLH* is essential for activation of anthocyanin structural genes (Bovy et al., 2002; Butelli et al., 2008) whereas *WD40* is needed to stabilize the MBW complexes (Hichri et al., 2011). *MYB* has been most extensively studied and was shown to play the most important role in regulating anthocyanin production, as it can bind directly to the target genes (Hichri et al., 2011). In several plant species, the expression of the *MYB* alone is sufficient to stimulate anthocyanin production, as was demonstrated e.g. by overexpression of *PAP1* in arabidopsis (Borevitz et al., 2000; Shi and Xie, 2014), anthocyanin1 (*ANT1*) in tomato (Schreiber et al., 2012), *MYB10* in apple, strawberry and potato (Kortstee et al., 2011) and in gerbera (Elomaa et al., 2003). By contrast, expression of the maize *C1* (*MYB*) failed to stimulate anthocyanin accumulation in tomato without the *LC* transcription factor (*bHLH*; Bovy et al., 2002). The results indicated that some plants require both *MYB*- and *bHLH*-type transcription factors to activate anthocyanin biosynthesis, while some others may require only one of the transcription factors.

Different transcription factors can regulate the expression of different sets of the structural genes (Quattrocchio et al., 1993). This means that selection of transcription factors from suitable sources depending on crops might be an important factor in determining successful genetic engineering strategies. *ROSEA1* (*ROS1*) and *DELILA* (*DEL*) transcription factors from snapdragon interact to control anthocyanin accumulation in flowers (Martin et al., 1991). Expression of *ROS1* affects anthocyanin accumulation in both the corolla lobes and tubes, whereas *DEL* is only effective in the corolla tube (Schwinn et al., 2006). The expression of the late structural genes including *flavanone 3-hydroxylase* (*F3H*), *dihydroflavonol 4-reductase* (*DFR*), *anthocyanidin synthase* (*ANS*) and *UDP-glucose 3-O-flavonoid transferase* (*UFGT*) are thought to be regulated by *DEL* (Martin et al., 1991). On the other hand, *ROS1* controls the expression of *F3H*, *flavonol synthase* (*FLS*), *flavonoid* 3′*-hydroxylase* (*F3′H*), *DFR, leucoanthocyanidin dioxygenase* (*LDOX*) and *UFGT* (Schwinn et al., 2006). In most cases, *DEL* is often required to support the function of *ROS1* (Butelli et al., 2008; Maligeppagol et al., 2013; Outchkourov et al., 2014), although overexpression of *DEL* alone has been reported to induce anthocyanin accumulation in tobacco (Naing et al., 2017).

Floral Binding Protein 1 (*FBP1*) is a flower-specific promoter which is expressed in the petals and stamens of *Petunia hybrida* (Angenent et al. 1993). The *FBP1* promoter has been used to study flower senescence in carnation and herbicide resistance in lily (Bovy et al., 1999; Benedito et al., 2005). The observation that *FBP1* was active in petals and stamens from the primordia stage to mature flowers of carnation indicated that this promoter is still expressed in the later stages of flower development when anthocyanin is produced.

This study investigated the effect of introducing *ROS1* and *DEL* separately or combined on inducing anthocyanin biosynthesis in the flowers of *Nicotiana benthamiana* and *Lilium* spp.. A method for efficient transient transformation by agroinfiltration for lily tepals was developed and optimized. Our study demonstrates that the introduction of *ROS1* alone and a combination of *ROS1* + *DEL* activated delphinidin biosynthesis in the flowers of *N. benthamiana*. Purplish flowers generated from the phenotypically-normal transgenic plants showed the efficacy of the *FBP1* promoter in driving tissue-specific gene expression, while the use of the constitutive cauliflower mosaic virus (CaMV) *35S* promoter led to the generation of stunted plants with anthocyanin in all parts of the plants. Cyanidin accumulated in agroinfiltrated pink tepals of the Oriental hybrid lily cv. 'Perth' showing darker colouration at the infiltrated area. Unfortunately, *ROS1* + *DEL* failed to induce anthocyanin biosynthesis in the white tepals of the Oriental hybrid lily cv. 'Rialto' and of *L. longiflorum* cv. 'Lincoln' indicating more deficiencies in the pathway of those particular genotypes, which warrants further investigation. The results of this study are a first confirmatory step in the genetic modification of flower colour in monocotyledonous plants.

2. Materials and methods

2.1. Plant materials

N. benthamiana was sown in pots (10.5 cm diameter) in the greenhouse. The greenhouse temperature was maintained at around 21 °C during the day and 19 °C during the night (16/8 h photoperiod). The relative humidity was set at 70%. The soil mixture used in this study was composed of 5% of Swedish sphagnum peat, 41% of grinding clay granules, 5% of garden peat, 4% of beam structure, 33% of steamed compost and 12% of PG-Mix 15-10-20. The bulbs of Oriental hybrid lily cultivar 'Perth' (pink tepals) and 'Rialto' (white tepals) and *L. longiflorum* cultivar 'Lincoln' (white tepals) were purchased from De Jong Lelies Holland BV (Andijk, The Netherlands) and were stored at -1 °C. The bulbs were transferred to 4 °C for 24 h before planting in crates (30 cm x 50 cm, 6 bulbs per crate) filled with commercial jiffy substrate (Jiffy Products International B.V., Moerdijk, The Netherlands). The plants were grown in the same greenhouse conditions as mentioned for *N. benthamiana*.

2.2. Construction of expression vectors

Total gDNA of *Petunia hybrida* was isolated using Dneasy® Plant Mini Kit (250) Cat. No. 69106 according to the manufacturer's instruction. The flower specific promoter, *FBP1* was amplified by PCR using genomic DNA of *P. hybrida*, and primers were extended with restriction sites to accommodate the directional cloning as DNA insert. Primers used for *FBP1* cloning are given in Table 1.

The PCR product of *FBP1* was gel purified and cloned into pENTR/D-TOPO vector (Invitrogen). Fragments of *ROS1-t35S* (NcoI-KpnI) and *DEL-t35S* (NcoI-KasI) were digested from pJAM1890 (or known as *35S:ROS1* + *DEL* in this study; Martin et al. 2012) using restriction enzymes. All the digestions were performed at 37 °C in a water bath for 1.5 h. Complete digestion products were separated on agarose gel and the fragments were gel purified. Gel purification was performed using Zymoclean gel extraction kit following the protocol given by the manufacturer (Zymo Research Corp., USA). The *ROS1-t35S* fragment was ligated into the NcoI-KpnI site of pENTR/D-TOPO:*FBP1* to yield pENTR/D-TOPO:*FBP1-ROS1*-t35S. The *DEL-t35S* fragment was ligated into the NcoI-KasI site of pENTR/D-TOPO:*FBP1* to yield pENTR/D-TOPO:*FBP1-DEL-t35S*. Ligation was performed using T4 ligase (Thermo Fisher Scientific) and reaction was incubated overnight at 16 °C. The resultant pENTR/D-TOPO:*FBP1-ROS1*-t35S was further digested with PacI and SbfI, and this fragment was inserted into the PacI-SbfI site of pBinPLUS (van Engelen et al. 1995) to yield *FBP1*:*ROS1* construct. The pENTR/D-TOPO:*FBP1-DEL*-t35S was digested with KpnI and AscI, and this fragment was inserted into the KpnI-AscI site of pBinPLUS to yield *FBP1*:*DEL* construct. Next, *FBP1*:*ROS1* and *FBP1*:*DEL* were combined into a single cloning vector. The pENTR/D-TOPO:*FBP1-DEL*-t35S was digested with KpnI and KasI, and the isolated fragment subsequently inserted into the KpnI-KasI site of pENTR/D-TOPO:*FBP1-ROS1*-t35S to yield pENTR/D-TOPO:*FBP1-ROS1*-t35S-*FBP1-DEL*-t35S. The plasmid obtained was further digested with PacI and SbfI, and the fragment was inserted into PacI-SbfI site of pBinPLUS to yield *FBP1*:*ROS1* + *DEL* construct. Finally, after sequence verification, the resultant plasmids obtained were introduced into *A. tumefaciens* strain AGL0 (Lazo et al., 1991).