

1 **Identification of two 6'-deoxychalcone 4'-glucosyltransferase genes in dahlia (*Dahlia***
2 ***variabilis*)**

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16 **Main conclusion:**

17 Two glycosyltransferase genes belonging to UGT88 family were identified to have 6'-
18 deoxychalcone 4'-glucosyltransferase activity in dahlia.

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20 **Author Contribution statement**

21 SO conceived the study. SO and MD designed the experiments. KM, HY and SO
22 conducted the experiments. KM and SO wrote the manuscript. All the authors read and
23 approved the manuscript.

26 **Abstract**

27 6'-deoxychalcones (isoliquiritigenin and butein) are important pigments for yellow and
28 orange to red flower color. 6'-deoxychalcones are glucosylated at the 4'-position *in vivo*,
29 but the genes encoding 6'-deoxychalcone 4'-glucosyltransferase have not yet been
30 identified. In our previous study, it was indicated that snapdragon (*Antirrhinum majus*)
31 *chalcone 4'-O-glucosyltransferase* (*Am4'CGT*) has isoliquiritigenin 4'-glucosylation
32 activity. Therefore, to identify genes encoding 6'-deoxychalcone 4'-glucosyltransferase
33 in dahlia (*Dahlia variabilis*), genes expressed in ray florets that shared high homology
34 with *Am4'CGT* were explored. As a result, *c34671_gl_i1* and *c35662_gl_i1* were
35 selected as candidate genes for 6'-deoxychalcone 4'-glucosyltransferases in dahlia. We
36 conducted transient co-overexpression of three genes (*c34671_gl_i1* or *c35662_gl_i1*,
37 dahlia *aldo-keto reductase1* (*DvAKR1*) or soybean (*Glycine max*) *chalcone reductase5*
38 (*GmCHR5*), and chili pepper (*Capsicum annuum*) MYB transcription factor (*CaMYBA*)
39 in *Nicotiana benthamiana* by agroinfiltration. Transient overexpression of *c34671_gl_i1*,
40 *DvAKR1* and *CaMYBA* resulted in increase of accumulation of isoliquiritigenin 4'-
41 glucosides; isoliquiritigenin 4'-*O*-glucoside and isoliquiritigenin 4'-*O*-[6-*O*-(malonyl)-
42 glucoside]. However, transient overexpression of *c35662_gl_i1*, *DvAKR1* and *CaMYBA*
43 did not increase accumulation of isoliquiritigenin 4'-glucosides. Using *GmCHR5* instead
44 of *DvAKR1* showed similar results suggesting that *c34671_gl_i1* has isoliquiritigenin 4'-
45 glucosyltransferase activity. In addition, we conducted co-overexpression of four genes
46 (*c34671_gl_i1*, *c35662_gl_i1* or *Am4'CGT*, *DvAKR1* or *GmCHR5*, *CaMYBA* and
47 *chalcone 3-hydroxylase* from dahlia). Accumulation of butein 4'-*O*-glucoside and butein
48 4'-*O*-[6-*O*-(malonyl)-glucoside] were detected for *c35662_gl_i1*, suggesting that
49 *c35662_gl_i1* has butein 4'-glucosyltransferase activity. Recombinant enzyme analysis
50 also supported butein 4'-glucosyltransferases activity of *c35662_gl_i1*. Therefore, our

results suggested that both *c34671_gl_il* and *c35662_gl_il* are 6'-deoxychalcone 4'-glucosyltransferases but with different substrate preference.

Key words: Butein, Flavonoid, Flower color, Glycosylation, Isoliquiritigenin, Yellow pigment

Abbreviations

4'CGT	chalcone 4'- <i>O</i> -glucosyltransferase
AKR	aldo-keto reductase
CH3H	chalcone 3-hydroxylase
CHS	chalcone synthase
GGT	glucoside glucosyltransferase
UGT	UDP-dependent glycosyltransferase

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Introduction

Dahlia (*Dahlia variabilis*) is a popular ornamental plant belonging to Asteraceae. *Dahlia* is autoallooctaploid ($2n = 8X = 64$) (Gatt et al., 1998), and it exhibits a variety of traits, one of which is abundant flower color, including black, red, white, and yellow. These flower colors are due to combination of flavonoids and related pigments (Ohno et al. 2013, 2021; Deguchi et al. 2016b), and many genes related to flavonoid biosynthesis in ray florets have been characterized (Suzuki et al. 2002; Ohno et al. 2011a, 2011b, 2013, 2018a, 2022; Deguchi et al. 2013). Pigment that plays an important role in yellow and red flowers of dahlias is 6'-deoxychalcone (isoliquiritigenin and butein). 6'-deoxychalcone is one of yellow flavonoid pigments, which contributes to yellow flower color by solo accumulation and orange to red flower color by co-accumulation with anthocyanins, and its derivatives have also been detected in the leaves (Ohno et al. 2011a, 2018b, 2021).

Yellow flower color in many species is derived from carotenoids and betaxanthins. Thus, most plant species that do not synthesize those pigments in flower lack bright yellow flower color. In addition to carotenoids and betaxanthins, flavonoids also exhibit yellow color. Chalcononaringenin and aurone are known as yellow flavonoids in flowers as well as 6'-deoxychalcone (Harborne 1966; Miyajima et al. 1991; Itoh et al. 2002; Ono et al. 2006; Saito et al. 2011; Deguchi et al. 2016a). Flavonoids can be synthesized in almost all plant species, thus, they are promising targets for molecular breeding of yellow flowers.

The presumed biosynthetic pathway of 6'-deoxychalcone in *dahlia* (Ohno et al. 2021) is shown in Fig. 1. First, isoliquiritigenin (2',4,4'-trihydroxychalcone) is synthesized by chalcone synthase (CHS) and aldo-keto reductase (AKR) from coumaroyl-CoA and malonyl-CoA as substrates (Welle and Grisebach, 1988; Ohno et al.

2022). Then, butein is synthesized from isoliquiritigenin by chalcone 3-hydroxylase (CH3H) (Schlangen et al. 2010). In coreopsis (*Coreopsis grandiflora*), butein is used as a substrate to form aurone, sulfuretin (Kaintz et al. 2014; Molitor et al. 2015). Flavonoids including 6'-deoxychalcones are generally glycosylated after synthesis *in vivo*. Glycosylation is one of the major modifications in flavonoids, enhancing solubility and facilitating transport into the vacuole (Jones and Vogt 2001; Bowles et al. 2005). Glycosylation is generally catalyzed by UDP-dependent glycosyltransferases (UGTs) superfamily enzymes (Ross et al. 2001), and in *Arabidopsis thaliana*, 120 UGTs have been reported (Li et al. 2001). Flavonoids are glycosylated in various positions depending on the substance by UGT (Hofer 2016). For example, aurone is glycosylated at 4'-position to form 4-hydroxyaurone (Ono et al. 2006). In snapdragon (*Antirrhinum majus*), 4-hydroxyaurone is synthesized by two enzymes; aureusidin synthase (AmAS1) and chalcone 4'-O-glucosyltransferase (Am4'CGT) (Nakayama et al. 2000; Ono et al. 2006). 6'-deoxychalcone in dahlia ray florets is also known to be glycosylated at the 4'-position, and the final product inferred from the structure is butein 4'-malonylsophoroside (Harborne et al. 1990; Ohno et al. 2021). Pigment analysis of dahlia 'Shukuhai' (SH) ray florets using fast-atom-bombardment mass spectrometry and nuclear magnetic resonance techniques identified four 4'-glycosides for butein (butein 4'-O-glucoside, butein 4'-O-[6-O-(malonyl)-glucoside], butein 4'-O-[2-O-(glucosyl)-glucoside] and butein 4'-O-[2-O-(glucosyl)-6-O-(malonyl)-glucoside]) and four 4'-glycosides for isoliquiritigenin (isoliquiritigenin 4'-O-glucoside, isoliquiritigenin 4'-O-[6-O-(malonyl)-glucoside], isoliquiritigenin 4'-O-[2-O-(glucosyl)-glucoside] and isoliquiritigenin 4'-O-[6-O-(rhamnosyl)-glucoside]) (Ohno et al. 2021). Structure of the pigments detected in 'SH' suggests the presence of chalcone 4'-glucosyltransferase (4'CGT). In addition to GT, the presence of glucoside glucosyltransferase (GGT) and rhamnosyltransferase as

glycosyltransferases and malonyltransferase as an acyltransferase was indicated in ray florets, and the modification pathway of 6'-deoxychalcone was inferred (Fig. 1). Stich et al. (1994) reported 6'-deoxychalcone 4'-glucosyltransferase activity from ray florets in dahlia and Halbwirth et al. (1997) reported 6'-deoxychalcone 4'-glucosyltransferase activity from ray florets in coreopsis, however, genes encoding 6'-deoxychalcone 4'-glucosyltransferase have not yet been identified.

In our previous study, we demonstrated that co-overexpression of *Am4'CGT* in combination with *CaMYBA*, which is a chili pepper (*Capsicum annuum*) anthocyanin biosynthesis MYB transcription factor, and *DvAKR1* resulted in successful accumulation of isoliquiritigenin 4'-glucoside in *Nicotiana benthamiana* leaves (Ohno et al. 2022). This result indicated that *Am4'CGT* is able to function as isoliquiritigenin 4'-glucosyltransferase. Furthermore, co-overexpression of *DvAKR1* and *CaMYBA* without glucosyltransferase did not induce accumulation of isoliquiritigenin, suggesting that 4'CGT is required for the accumulation of isoliquiritigenin in *N. benthamiana*.

In this study, with the aim of molecular breeding of yellow flowers by 6'-deoxychalcone glycosides in the future, we attempted to identify 6'-deoxychalcone 4'-glucosyltransferase genes in dahlia by exploration of genes those are expressed in ray florets and have high homology to *Am4'CGT*. We found two candidate genes, and function of the candidate genes was analyzed by transient overexpression in *N. benthamiana* leaves and recombinant enzymatic analysis.

Materials and methods

Plant materials

A red-white bicolor *Dahlia variabilis* ‘Shukuhai (SH)’ and a purple-white bicolor dahlia ‘Rinka (RK)’, which is bud mutation of ‘SH’, were used. ‘SH’ accumulates 6'-deoxychalcone, anthocyanins and flavones in its ray florets, while ‘Rinka’ accumulates only anthocyanins and flavones (Ohno et al. 2021; 2022). Ray florets for subsequent analyses were collected at different developmental stages from field- or greenhouse-grown plants in the experimental field at Kyoto University (Kyoto, Japan). *N. benthamiana* plants for agroinfiltration were grown in a growth chamber without environmental controls in Kyoto University.

Isolation of candidate genes

Since 6'-deoxychalcone accumulation was started from stage 2 in ray florets (Ohno et al. 2022), RNA-seq data of ‘SH’ stage 2 ray florets (DRR337625) and ‘RK’ stage 2 ray florets (DRR337626) were *de novo* assembled by Trinity (Grabherr et al. 2011) with default settings, and contigs that have high homology with other flavonoid glycosyltransferases related to flavonoids were selected for the analysis.

Genomic DNA was extracted from leaves or ray florets using MagExtractor Plant Genome (Toyobo, Osaka, Japan). Transcripts or genomic fragments were cloned into pTAC-1 vector (BioDynamics Laboratory Inc., Tokyo, Japan) for sequencing. Plasmids were extracted using Quick Gene Plasmid Kit S II (Kurabo, Osaka, Japan). cDNA sequence in ray florets and genomic fragment of *c34671_g1_i1* and *c35662_g1_i1* from ‘SH’ and ‘RK’ were sequenced 16 clones for each cultivar and were compared. Primers used in this analysis were shown in Table S1.

Phylogenetic analysis

A phylogenetic tree was constructed with the putative amino acid sequences of flavonoid glycosyltransferase using neighbor-joining method, and bootstrap consensus inferred with 1,000 replicates in MEGA11 (<https://www.megasoftware.net>) (Tamura et al. 2021; Saitou and Nei 1987). The accession numbers for amino acid sequences are listed in Table S2.

Gene expression analysis

Total RNA was extracted from ray florets of 'SH' at four different developmental stages using Sepasol RNA I Super G (Nacalai Tesque, Kyoto, Japan) and was purified with High-salt precipitation solution (Takara Bio, Ohtsu, Japan). The relative expression of *c34671_g1_i1* and *c35662_g1_i1* genes was investigated using qRT-PCR as described by Ohno et al. (2022). *DvActin* is used as an internal control. The primers used for qRT-PCR are shown in Table S3.

High-performance liquid chromatography (HPLC) analysis

For pigment analysis, samples were frozen and pulverized with liquid nitrogen, and the pigment was extracted with 5% acetic acid methanol (acetic acid: methanol: distilled water = 1: 10: 9, by vol.) to make the crude extract. For hydrolyzed samples, the crude extract was air-dried and replaced with 5% hydrochloric acid methanol (hydrochloric acid: methanol: distilled water = 1: 10: 9, by vol.) and boiled in water for two hours to

make the hydroxylated extract. HPLC analysis was performed as described by Ohno et al. (2022). Isoliquiritigenin and butein as standard chemicals were obtained from Tokyo Chemical Industries (Tokyo, Japan). For analysis of crude extract, HPLC chromatogram data of 'SH' (Ohno et al. 2021) ray floret extract was used as a standard, and prediction of each peak was conducted based on retention time, order of peaks and maximum absorption wavelength measured by the photodiode array attached to the HPLC. Quantification of 6'-deoxychalcones 4'-glucosides in ray florets were performed with three replications.

Agroinfiltration assay

Transient overexpression of 6'-deoxychalcone glucosyltransferase genes was examined in *N. benthamiana* to verify their functional activities. cDNA of *DvAKR1*, *chalcone reductase 5* in soybean (*Glycine max*) (*GmCHR5*) (Mameda et al. 2018), *c34671_g1_i1*, *c35662_g1_i1*, *Am4'CGT*, *DvCH3H* and *CaMYBA* were subcloned in pDONR221 vector (Invitrogen), and then recombined in pGWB2 Gateway binary vector, which overexpressed under the CaMV35S promoter (Nakagawa et al. 2007). *CaMYBA* was cloned from the purple flowers of *C. annuum* 'Peruvian Purple' (Ohno et al. 2020). All constructs were transformed into *A. tumefaciens* EHA105 strain. The *BETA-GLUCURONIDASE* (*GUS*) gene was used in control assays. The primers used for cloning are shown in Table S4.

Agrobacterium harboring pGWB2 transgene was shaken in liquid YEP medium with 50 ppm kanamycin at overnight. After centrifugation, the pellet was collected and redissolved in buffer (50 mM MES, 50 mM magnesium chloride (pH5.5)). The concentration of the solution was prepared to OD=0.7. For co-expression of *Am4'CGT* or

c34671_gl_i1 or *c35662_gl_i1*, *DvAKR1* or *GmCHR5*, *DvCH3H*, *CaMYBA* and *p19*, agrobacterium harboring transgene were equally mixed, and injected into 4–5-week-old *N. benthamiana* leaves. *p19* (pDGB3alpha2_35S:p19:Tnos, addgene) is a *Tomato bushy start virus*-derived RNA silencing repressor that was introduced to increase the success rate of infiltration. Leaves about 2 cm² were sampled between the fifth and the seventh day post infiltration, and divided into two parts and one is used for pigment extraction and the other is used for RT-PCR to validate transgene expression. Samples were obtained in three biological replicates consisting of at least three different leaves for each experiment. The *GUS* expression was confirmed by GUS staining as described by Ohno et al. (2022). Primers used for RT-PCR to validate transgene expression are shown in Table S5. Primers for *GmCHR5* were designed according to Mameda et al. (2018), and primers for *Am4'CGT* were designed according to Hoshino et al. (2019).

Recombinant enzyme assay

cDNA of *Am4'CGT*, *c34671_gl_i1* and *c35662_gl_i1* were cloned into pET6xHN-N vector (Takara Bio) for expression of recombinant protein in *Escherichia coli*. Plasmids were extracted using Quick Gene Plasmid Kit S II (Kurabo). Plasmid sequences were confirmed with the primers shown in Table S6. Plasmids were introduced into *E. coli* BL21 strain by electroporation. Transformed BL21 strains were shaken at 37°C in liquid LB medium containing 50 ppm ampicillin. The culture medium was diluted 20-fold into new LB liquid medium and incubated until OD₆₀₀ = 0.7. 1% volume of IPTG (final concentration 500 ppm) was added for induction of recombinant protein production. After centrifugation (3000 x g, 4°C, 10 min), pellet was resuspended by xTractor Buffer (Takara Bio USA, San Jose, CA), and crude protein extract was prepared. To detect

chalcone 4'-glucosyltransferase activity, 100 mM Tris-HCl buffer (pH8.0), 100 µL crude protein extract, 30 µL of 20 mM UDP-glucose (Cayman Chemical, Ann Arbor, MI), 50 µL of 1 mg/mL butein (Tokyo Chemical Industries) or isoliquiritigenin (Tokyo Chemical Industries) (total volume 1 mL) were gently mixed and kept overnight at 30°C. The mixture was analyzed by HPLC under the above-described conditions.

Results

***c34671_g1_i1* and *c35662_g1_i1* are candidate genes for 6'-deoxychalcone 4'-glucosyltransferase in dahlia**

De novo assembled RNA-seq data of 'SH' and 'RK' was analyzed to search for genes with high homology to other flavonoid related glycosyltransferases, and seven contigs (*c34671_g1_i1*, *c35662_g1_i1*, *c37087_g1_i1*, *c37958_g1_i1*, *c38610_g1_i2*, *c38674_g1_i1* and *c38795_g2_i3*) were identified. Then, a phylogenetic tree among these contigs and known flavonoid-related glycosyltransferases in other plant species was constructed (Fig. 2). Flavonoid-related glycosyltransferases were classified into eight clades: chalcone 4' and isoflavone 7 positions, chalcone 2 position, flavonoid C-glycosyltransferase, flavonoid position-3, flavonoid position-5, flavonoid position-7, UGT89B and GGT, respectively. The phylogenetic tree shows that *c34671_g1_i1* and *c35662_g1_i1* are most closely distributed to *Am4'CGT*. Other five contigs were assumed to belong other glycosyltransferase clades; *c38674_g1_i1*: chalcone 2 position, *c38610_g1_i2*: flavonoid C-glycosyltransferase, *c37087_g1_i1*: flavonoid 5 position,

c38795_g2_il: UGT89B, *c37958_g1_il*: GGT, respectively. Thus, *c34671_g1_il* and *c35662_g1_il* were selected as candidate genes for 6'-deoxychalcone 4'-glucosyltransferase in dahlia.

c34671_g1_il and *c35662_g1_il* encode 460 and 466 amino acids, respectively, and many variations in cDNA sequences existed in 'SH' and 'RK' (Table S7 and S8). In putative amino acid sequence, the identity of *c34671_g1_il* and *c35662_g1_il* to *Am4'CGT* was 47% and 43%, respectively, and the identity between *c34671_g1_il* and *c35662_g1_il* was 50% (Fig. S1). *c34671_g1_il* and *c35662_g1_il* belong to UGT88 group, which contains *Am4'CGT*, *Lv4'CGT* and soybean isoflavone 7-O-glucosyltransferase (*GmIF7GT*) (Ono et al. 2006; Noguchi et al. 2007). The 7-position of isoflavone is equivalent to the 4'-position of chalcone, thus, this result suggests that isoflavone and 6'-deoxychalcone glucosyltransferases are of single origin. *At3GT* (Q9LFJ8) and *At7GT* (AAR01231) have one intron and two exons, while *c34671_g1_il*, *c35662_g1_il*, *Am4'CGT* and *GmIF7GT* have no introns (Ono et al. 2006; Noguchi et al. 2007). Therefore, genomic structure also indicated these *c34671_g1_il*, *c35662_g1_il* and *Am4'CGT* are phylogenetically related.

Gene expression analysis of *c34671_g1_il* and *c35662_g1_il* at different developmental stages in 'SH' ray florets

To investigate the expression characteristics of *c34671_g1_il* and *c35662_g1_il*, we analyzed the gene expression levels of the seven identified glycosyltransferase genes at four developmental stages of 'SH' ray florets (Fig. 3a). For pigment analysis, a total of seven glucosides of 6'-deoxychalcone were detected in 'SH' ray florets, of these, butein-4'-[6-O-(malonyl)-glucoside] had the highest content. The content of butein-4'-[6-O-

(malonyl)-glucoside] began to increase rapidly from stage 2, followed by a gradual decrease (Fig. 3b). And the other six glucosides showed similar content trends (Fig. S2). *c34671_gl_i1* was highly expressed in stage 1 and stage 2 and its expression level eventually decreased after stage 3 (Fig. 3c). *c35662_gl_i1* showed a similar expression pattern to *c34671_gl_i1*, with high expression in stage 1 and stage 2, and a sharp decrease in expression from stage 3 (Fig. 3c). *c38610_gl_i2*, *c38674_gl_i1* and *c38795_g2_i1* showed similar pattern with *c34671_gl_i1* and *c35662_gl_i1*, while *c37087_gl_i1* has high expression in stage 1 and stage 2, and *c37958_gl_i1* has highest expression in stage 1 (Fig. 3c). *c34671_gl_i1* and *c35662_gl_i1* showed similar expression characteristics with increased expression levels prior to pigment accumulation, and another isoliquiritigenin biosynthetic gene, *DvAKR1* also showed the highest expression at stage 2 (Ohno et al. 2022), suggesting that *c34671_gl_i1* and *c35662_gl_i1* might be involved in the accumulation of 6'-deoxychalcones.

Transient co-overexpression of candidate glucosyltransferase genes with 6'-deoxychalcone biosynthesis-related genes induced accumulation of 6'-deoxychalcone 4'-glucosides in *N. benthamiana* leaves

In the previous study, transient co-overexpression of *DvAKR1*, *Am4'CGT* and *CaMYBA*, or *GmCHR5*, *Am4'CGT* and *CaMYBA* both induced accumulation of isoliquiritigenin 4'-glucosides in *N. benthamiana* leaves (Ohno et al. 2022). To verify whether *c34671_gl_i1* and *c35662_gl_i1* have 4' glucosylation activity to 6'-deoxychalcone, we performed agroinfiltration in *N. benthamiana* leaves. We at first used *DvAKR1*. The first transgene combinations are combination 1: *DvAKR1* x *CaMYBA* x *p19*, combination 2: *Am4'CGT* x *DvAKR1* x *CaMYBA* x *p19*, combination 3: *c34671_gl_i1* x *DvAKR1* x *CaMYBA* x *p19*

and combination 4: *c35662_g1_i1* x *DvAKR1* x *CaMYBA* x *p19*. Transgene expression was confirmed by RT-PCR (Fig. S3). As a result, for combination 1 (*DvAKR1* x *CaMYBA* x *p19*), two peaks (Fig. 4a peaks 1 and 2) were detected. Comparing our data with previously reported 'SH' ray florets extract profiles (Ohno et al. 2021), the detected peaks were presumed to isoliquiritigenin 4'-*O*-glucoside (peak 1) and isoliquiritigenin 4'-*O*-[6-*O*-(malonyl)-glucoside] (peak 2). In addition, the detected peak in hydrolyzed extract also corresponded to that of isoliquiritigenin standard (Fig. 4b). For combination 2 (*Am4'CGT* x *DvAKR1* x *CaMYBA* x *p19*) and combination 3 (*c34671_g1_i1* x *DvAKR1* x *CaMYBA* x *p19*), similar to combination 1, two major peaks (Fig. 4b peaks 1 and 2) were detected in crude extract. In hydrolyzed extract, peak of isoliquiritigenin were detected (Fig. 4b). For combination 4 (*c35662_g1_i1* x *DvAKR1* x *CaMYBA* x *p19*), two peaks (Fig. 4a peaks 1 and 2) in crude extract and peak of isoliquiritigenin in hydrolyzed extract was detected (Fig. 4b). However, peak areas detected in combination 4 were similar to combination 1 (Fig. 4c). We obtained similar results using *GmCHR5* instead of *DvAKR1* (Fig. 4a). Therefore, these results suggested that *Am4'CGT* and *c34671_g1_i1* have isoliquiritigenin 4'-glucosyltransferase activity, but *c35662_g1_i1* does not have isoliquiritigenin 4'-glucosyltransferase activity and detected peaks in combination 4 might be due to background *N. benthamiana* UGT activity.

To further investigate whether the candidate genes have 4'-glucosylation activity for butein, in addition to *DvAKR1* or *GmCHR5*, *CaMYBA* and candidate glucosyltransferase genes, we co-expressed *DvCH3H*, which is required for the conversion of isoliquiritigenin to butein. The transgene combinations are combination 5: *DvAKR1* x *CaMYBA* x *DvCH3H* x *p19*, combination 6: *Am4'CGT* x *DvAKR1* x *CaMYBA* x *DvCH3H* x *p19*, combination 7: *c34671_g1_i1* x *DvAKR1* x *CaMYBA* x *DvCH3H* x *p19*, and combination 8: *c35662_g1_i1* x *DvAKR1* x *CaMYBA* x *DvCH3H* x *p19*.

Transgene expression was confirmed by RT-PCR (Fig. S3). First, for combination 5 (*DvAKR1* x *CaMYBA* x *DvCH3H* x *p19*), in crude extract, three peaks were detected and two of them were presumed to be isoliquiritigenin 4'-*O*-glucoside (peak 1) and butein 4'-*O*-[6-*O*-(malonyl)-glucoside] (peak 5) (Fig. 5a). In hydrolyzed extract, no major peaks were detected (Fig. 5b). This was supposed to be due to the degradation of the pigments during the hydrolysis reaction. For combination 6 (*Am4'CGT* x *DvAKR1* x *CaMYBA* x *DvCH3H* x *p19*), in crude extract, several peaks were detected and four of them were predicted to glycosides of 6'-deoxychalcone: butein 4'-*O*-glucoside (peak 3), isoliquiritigenin 4'-*O*-glucoside (peak 1), butein 4'-*O*-[6-*O*-(malonyl)-glucoside] (peak 5) and isoliquiritigenin 4'-*O*-[6-*O*-(malonyl)-glucoside] (peak 2) (Fig. 5a). Although another major peak (peak 4) was detected in the crude extract, we could not identify the compounds. In hydrolyzed extract, in addition to isoliquiritigenin and butein, another peak was detected (black arrow in Fig. 5b), and it showed a maximum absorbance wavelength at 380 nm and continued to around 420 nm (Fig. 5c), suggesting this compound might be a chalcone-like compound, and peak 4 might be its derivative. For combination 7 (*c34671_g1_i1* x *DvAKR1* x *CaMYBA* x *DvCH3H* x *p19*), similar peak pattern to combination 6 was detected, but the highest peak of combination 6 was peak 4, while that of combination 7 was peak 1. Two 4'-butein glucosides (butein 4'-*O*-[6-*O*-(malonyl)-glucoside] and butein 4'-*O*-glucoside) were detected, indicating two possibilities that *c34671_g1_i1* functions as butein 4'-glycosyltransferase, or that *c34671_g1_i1* functions as isoliquiritigenin 4'-glycosyltransferase and then isoliquiritigenin glucosides were converted to butein glucosides by *DvCH3H*. The peak of unknown compound (black arrow in Fig. 5b) was also detected in the hydrolyzed extract. Finally, for combination 8 (*c35662_g1_i1* x *DvAKR1* x *CaMYBA* x *DvCH3H* x *p19*), in crude extract, two major peaks were detected, namely butein 4'-*O*-glucoside

(peak 3) and butein 4'-O-[6-O-(malonyl)-glucoside] (peak 5) (Fig. 5a). Isoliquiritigenin glucosides were similar level to combination 5 (Fig. 5d). Thus, it was suggested *c35662_g1_i1* functioned as butein 4'-glucosyltransferase. In hydrolyzed extract, the peak of unknown compound (black arrow in Fig. 5b) was also detected as well as butein. Overall, these results suggested that *c35662_g1_i1* have butein 4'-glucosyltransferase activity but not isoliquiritigenin 4'-glucosyltransferase activity.

Recombinant c35662_g1_i1 protein showed glucosylation activity against butein

To verify 4'-glucosyltransferase activity against isoliquiritigenin and butein, recombinant proteins of *Am4'CGT*, *c34671_g1_i1* and *c35662_g1_i1* were expressed by pET6xHN-N vector and crude extract was used for enzyme activity assay. Unfortunately, we could not confirm enzyme activity when isoliquiritigenin was used as a substrate (Fig. 6a). However, enzyme activity was detected when butein was used as a substrate. For *c35662_g1_i1*, a major peak of butein 4'-O-glucoside was detected (Fig. 6b). For *Am4'CGT* and *c34671_g1_i1*, one peak other than butein was detected (Fig. 6b). The peak showed maximum absorbance wavelength at about 400 nm and continued to around 420 nm. Thus, the peak was supposed to be not butein derivative but another chalcone-like compound. From these results, it was suggested that *c35662_g1_i1* has 4'-glycosylation activity against butein *in vivo*.

Discussion

In dahlia ‘SH’ ray florets, four 4'-glucosides for isoliquiritigenin and four 4'-glucosides for butein were identified (Ohno et al. 2021). We explored candidate genes for 6'-deoxychalcone 4'-glucosyltransferase from the transcriptome data of ray florets, and two candidate genes (*c34671_g1_i1* and *c35662_g1_i1*) were detected (Fig. 2). In this study, we conducted agro-infiltration and recombinant enzyme assay to verify function of the candidate genes. In the control experiment, co-overexpression of *DvAKR1* x *CaMYBA* x *p19* (combination 1) without glucosyltransferase induces accumulation of small amount of isoliquiritigenin 4'-O-glucoside (peak 1) and isoliquiritigenin 4'-O-[6-O-(malonyl)-glucoside] (peak 2) (Fig. 4a). Glycosylation is important modifications in flavonoids, enhancing solubility and facilitating transport into the vacuole (Jones and Vogt 2001; Bowles et al. 2005), and in *N. tabacum* and *N. benthamiana*, there are several flavonoid glucosyltransferases and acyltransferases with wide range of substrate preference (Taguchi et al. 2001; Liu et al. 2017). Thus, it was supposed that forced glycosylation of these glucosyltransferases in *N. benthamiana* resulted in detection of two isoliquiritigenin 4'-glucosides in combination 1. Co-overexpression of three genes (combination 2-4) in tobacco leaves induced isoliquiritigenin 4'-glucoside. However, peak area detected from combination 4 was similar level to combination 1 (Fig. 4c), suggesting *c35662_g1_i1* does not have isoliquiritigenin 4'-glucosyltransferase activity, and only *Am4'CGT* and *c34671_g1_i1* have isoliquiritigenin 4'-glucosyltransferase activity.

Co-overexpression of four genes (combination 6-8) induced butein 4'-glucosides in addition to isoliquiritigenin glucosides (Fig. 5). In both combination 7 (*c34671_g1_i1* x *DvAKR1* x *CaMYBA* x *DvCH3H* x *p19*) and combination 8 (*c35662_g1_i1* x *DvAKR1* x *CaMYBA* x *DvCH3H* x *p19*), the same 4'-glucosides of butein (butein 4'-O-glucoside (peak 3) and butein 4'-O-[6-O-(malonyl)-glucoside] (peak 5)) were detected. These results indicated both *c34671_g1_i1* and *c35662_g1_i1* are

involved in butein 4'-*O*-glucoside formation. However, for *c34671_gl_il*, we could not judge from these results whether *c34671_gl_il* glucosylated butein or *c34671_gl_il* glucosylated isoliquiritigenin at first and then isoliquiritigenin 4'-glucoside was converted to butein 4'-glucoside by DvCH3H. From recombinant enzyme assay, butein 4'-*O*-glucosyltransferase activity was not detected from *c34671_gl_il* (Fig. 6b), indicating the latter hypothesis is more likely. In combination 8, detected level of isoliquiritigenin 4'-*O*-glucoside (peak 1) and isoliquiritigenin 4'-*O*-[6-*O*-(malonyl)-glucoside] (peak 2) were again similar to the control experiment (combination 5) (Fig. 5d), indicating *c35662_gl_il* does not have isoliquiritigenin 4'-glucosyltransferase activity. Unfortunately, we could not confirm isoliquiritigenin 4'-*O*-glucosyltransferase activity by recombinant enzyme assay (Fig. 6a), however, clear butein 4'-*O*-glucosyltransferase activity was detected from *c35662_gl_il* (Fig. 6b). Therefore, it was suggested *c35662_gl_il* is mainly involved in butein 4'-glucosylation but not isoliquiritigenin 4'-glucosylation.

Am4'CGT is a chalcone 4'-*O*-glucosyltransferase involved in aurone formation in snapdragon and it showed 2', 4', 6', 4-tetrahydroxychalcone 4'-*O*-glucoside activity (Ono et al. 2006). Compared to *c34671_gl_il* and *c35662_gl_il*, *Am4'CGT* showed similar HPLC chromatogram pattern with *c34671_gl_il* (Figs 4 and 5), indicating *Am4'CGT* and *c34671_gl_il* have similar function as isoliquiritigenin 4'-glucosyltransferase and substrate preference. However, there was slight difference in combination 6 and combination 7 (peak 4 was the highest in combination 6, while peak 1 was the highest in combination 7: Fig 5a), indicating function of *Am4'CGT* and *c34671_gl_il* is not exactly the same.

In combination 6 and 7, one peak (peak 4) of unknown compound was detected from crude extract (Fig. 5a). In hydrolyzed extract, one peak (arrowed in Fig. 5b) was

detected, and photodiode array analysis indicated this compound is chalcone-like compound (Fig. 5c). This compound was not detected without *DvCH3H* (Fig. 4b), suggesting *DvCH3H* function is required for this chalcone-like compound formation. One possibility is that *DvCH3H* hydroxylated some moiety and produced this unidentified compound.

In conclusion, *c34671_gl_il* and *c35662_gl_il* were demonstrated to function as 6'-deoxychalcone 4'-glucosyltransferase but with different substrate preference. It was suggested that *c34671_gl_il* is a major isoliquiritigenin 4'-glucosyltransferase and *c35662_gl_il* is a major butein 4'-glucosyltransferase in dahlia. Based on these results, hereafter *c34671_gl_il* was designated as *Dv4'CGT* and *c35662_gl_il* was designated as *Dv4'BGT*, and the putative 6'-deoxychalcone glucosylation pathway in dahlia was inferred (Fig. 7). The difference in glucosylation properties of the two genes may be useful for molecular breeding of yellow flowers using 6'-deoxychalcone. In a previous study, it was reported that there is the best correlation between dahlia *b** values and butein 4'-malonylglucoside (Ohno et al. 2021). Therefore, it may be possible to create brighter yellow flowers more efficiently by using *c35662_gl_il/Dv4'BGT*. Further study is needed to clarify interactions among *c34671_gl_il /Dv4'CGT*, *c35662_gl_il/Dv4'BGT* and other glycosylation or acylation-related enzymes, that will be of great help in the molecular breeding of yellow flowers.

Supplementary data

Table S1 Primers used for sequencing

Table S2 Accession numbers for amino acid sequences used in the phylogenetic analysis

Table S3 Primers used for qRT-PCR

Table S4 Primers used for gateway cloning

Table S5 Primers used for validation of transgenic expression

Table S6 Primers used for recombinant enzyme assay

Table S7 SNPs of *c34671_g1_i1* cDNA in 'SH' and 'RK'

Table S8 SNPs of *c35662_g1_i1* cDNA in 'SH' and 'RK'

Fig. S1 Comparison of putative amino acid sequence among Am4'CGT, *c34671_g1_i1* (Dv4'CGT) and *c35662_g1_i1* (Dv4'BGT).

Fig. S2. Content of 6'-deoxychalcone 4'-glycosides at each developmental stage of 'SH' ray florets.

Fig. S3. Validation of transgene expression in infiltrated leaves by RT-PCR.

Conflict of interests

The authors declare no conflict of interests.

Data availability statement

Accession numbers: *c34671_g1_i1/Dv4'CGT* (LC775776), *c35662_g1_i1/Dv4'BGT* (LC775777), *c37087_g1_i1/flavonoid 3-O-glucosyltransferase-like* (LC775778), *c37958_g1_i1/glucosyl glycoside transferase-like* (LC775779), *c38610_g1_i2/flavonoid C-glucosyltransferase-like* (LC775780), *c38674_g1_i1/tetrahydroxychalcone 2'-*

glucosyltransferase-like (LC775781) and *c38795_g2_i3/UDP-glycosyltransferase* (LC775782).

Figure legends

Fig. 1 Presumed biosynthetic pathway for isoliquiritigenin and butein derivatives in dahlia proposed by Ohno et al. (2021). Bt, butein; Bt4'G, butein 4'-*O*-glucoside; Bt4'MG, butein 4'-*O*-[6-*O*-(malonyl)-glucoside]; Bt4'MS, butein 4'-*O*-[2-*O*-(glucosyl)-6-*O*-(malonyl)-glucoside]; Bt4'S, butein 4'-*O*-[2-*O*-(glucosyl)-glucoside]; Iso, isoliquiritigenin; Iso4'G, isoliquiritigenin 4'-*O*-glucoside; Iso4'MG, isoliquiritigenin 4'-*O*-[6-*O*-(malonyl)-glucoside]; Iso4'RG, isoliquiritigenin 4'-*O*-[6-*O*-(rhamnosyl)-glucoside]; Iso4'S, isoliquiritigenin 4'-*O*-[2-*O*-(glucosyl)-glucoside]; 4'CGT, chalcone 4'-glucosyltransferase; AKR, aldo-keto reductase; CH3H, chalcone 3-hydroxylase; CHS, chalcone synthase; C4'GGT, chalcone 4'-glucoside glucosyltransferase; C4'GMaT, chalcone 4'-glucoside malonyltransferase; C4'GRhT, chalcone 4'-glucoside rhamnosyltransferase.

Fig. 2 Phylogenetic tree for plant flavonoid glucosyltransferase based on amino acid sequence. Glucosyltransferase putative amino acid sequences were aligned using ClustalW, and the tree was constructed by the neighbor-joining method. Bootstrap values obtained with 1000 repetitions are indicated on each branch. Putative amino acid sequences used for the phylogenetic tree are shown in Table S2.

Fig. 3 Analysis of expression levels of candidate glycosyltransferase genes and pigment content at different developmental stages of ‘SH’ ray florets. **a** Inflorescence and ray florets at different developmental stages of ‘SH’. Bars indicate 1 cm. **b** Butein-4'-[6-O-(malonyl)-glucoside] content. **c** Relative expression levels of *c34671_g1_i1*, *c35662_g1_i1*, *c37087_g1_i1*, *c37958_g1_i1*, *c38610_g1_i2*, *c38674_g1_i1* and *c38795_g2_i3*. *DvActin* was used as an internal standard. Bars indicate $\pm$ SE ($n=3$).

Fig. 4 Transient over-expression analysis by agroinfiltration in *N. benthamiana* leaves to analyze glucosylation activity against isoliquiritigenin. **a** and **b** HPLC chromatograms of over-expressed leaves analyzed at 380 nm (**a**: crude pigments, **b**: hydrolyzed pigments). Number in chromatograms indicates 1: isoliquiritigenin 4'-O-glucoside, 2: isoliquiritigenin 4'-O-[6-O-(malonyl)-glucoside]. **c** Quantification of peak areas of peak 1 and peak 2 in combinations 1-4. Bars indicate $\pm$ SE ($n=4-9$).

Fig. 5 Transient over-expression analysis by agroinfiltration in *N. benthamiana* leaves to analyze glucosylation activity against butein. **a** and **b** HPLC chromatograms of over-expressed leaves analyzed at 380 nm (**a**: crude pigments, **b**: hydrolyzed pigments). Number in chromatograms indicates 1: isoliquiritigenin 4'-O-glucoside, 2: isoliquiritigenin 4'-O-[6-O-(malonyl)-glucoside], 3: butein 4'-O-glucoside, 4: not identified chalcone-like compound, 5: butein 4'-O-[6-O-(malonyl)-glucoside]. **c** Photodiode array analysis of an unknown compound showing a peak indicated by arrows in **b** of *DvAKR1* x *Am4'CGT* x *CaMYBA* x *DvCH3H* x *p19*. **d** Quantification of peak areas of peak 1, peak 2, peak 3, peak 4 and peak 5 in combinations 5-8. Bars indicate $\pm$ SE ($n=3-6$).

Fig. 6 Enzymatic activity of recombinant *Am4'CGT*, *c34671_gl_il* and *c35662_gl_il* proteins against isoliquiritigenin (**a**) and butein (**b**). Chromatograms show the absorption at 380 nm in each reaction mixture.

Fig. 7 Presumed major glycosylation pathway for isoliquiritigenin and butein derivatives in dahlia ray florets. C4'GGT, chalcone 4'-glucoside glucosyltransferase; C4'GMaT, chalcone 4'-glucoside malonyltransferase; CH3H, chalcone 3-hydroxylase.

References

- Bowles D, Isayenkova J, Lim EK, Poppenberger B (2005) Glycosyltransferases: managers of small molecules. *Curr Opin Plant Biol* 8:254–263. <https://doi.org/10.1016/j.pbi.2005.03.007>
- Dastmalchi M, Bernards MA, Dhaubhadel S (2016) Twin anchors of the soybean isoflavonoid metabolon: evidence for tethering of the complex to the endoplasmic reticulum by IFS and C4H. *Plant J* 85:689–706. <https://doi.org/10.1111/tpj.13137>
- Deguchi A, Ohno S, Hosokawa M, Tatsuzawa F, Doi M (2013) Endogenous post-transcriptional gene silencing of flavone synthase resulting in high accumulation of anthocyanins in black dahlia cultivars. *Planta* 237:1325–1335. <https://doi.org/10.1007/s00425-013-1848-6>
- Deguchi A, Ohno S, Tatsuzawa F, Doi M, Hosokawa M (2016a) Identification of the yellow pigment in *Saintpaulia* flowers. *Hort. Res. (Japan)* 15: 123–128. <https://doi.org/10.2503/hrj.15.123>

576 Deguchi A, Tatsuzawa F, Hosokawa M, Doi M, Ohno S (2016b) Quantitative evaluation
 577 of the contribution of four major anthocyanins to black flower coloring of dahlia ray
 578 florets. Hort J 85:340–350. <https://doi.org/10.2503/hortj.MI-121>
 579 Gatt M, Hong D, Keith H, Brian M (1998) Polyploidy and evolution in wild and cultivated
 580 dahlia species. Ann Bot 81:647–656. <https://doi.org/10.1006/anbo.1998.0614>
 581 Grabherr G, Haas J, Yassour M, Levin J, Thompson D, Amit I, Adiconis X, Fan L,
 582 Raychowdhury R, Zeng Q, Chen Z, Mauceli E, Hacohen N, Gnirke A, Rhind N,
 583 Palma F, Birren B, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A (2011) Full-
 584 length transcriptome assembly from RNA-Seq data without a reference genome. Nat
 585 Biotechnol 29:644–652. <https://doi.org/10.1038/nbt.1883>
 586 Halbwirth H, Wimmer G, Wurst F, Forkmann G, Stich K (1997) Enzymatic glucosylation
 587 of 4-deoxyaurones and 6'-deoxychalcones with enzyme extracts of *Coreopsis*
 588 *grandiflora*, Nutt. I. Plant Sci 122: 125-131. <https://doi.org/10.1016/S0168->
 589 9452(96)04553-0
 590 Harborne JB (1966) Comparative biochemistry of flavonoids-I.: Distribution of chalcone
 591 and aurone pigments in plants. Phytochemistry 5:110–115.
 592 [https://doi.org/10.1016/S0031-9422\(00\)85088-8](https://doi.org/10.1016/S0031-9422(00)85088-8)
 593 Harborne JB, Greenham J, Eagles J (1990) Malonylated chalcone glycoside in dahlia.
 594 Phytochemistry 29:2899–2900. [https://doi.org/10.1016/0031-9422\(90\)87101-Y](https://doi.org/10.1016/0031-9422(90)87101-Y)
 595 Hofer B (2016) Recent developments in the enzymatic O-glycosylation of flavonoids.
 596 Appl Microbiol Biotechnol 100:4269–4281. <https://doi.org/10.1007/s00253-016->
 597 7465-0
 598 Hoshino A, Mizuno T, Shimizu K, Mori S, Fukada-Tanaka S, Furukawa K, Ishiguro K,
 599 Tanaka Y, Iida S (2019) Generation of yellow flowers of the Japanese morning glory

600 by engineering its flavonoid biosynthetic pathway toward aurones. *Plant Cell*
601 *Physiol* 60:1871–1879. <https://doi.org/10.1093/pcp/pcz101>

602 Itoh Y, Higeta D, Suzuki A, Yoshida H, Ozeki Y (2002) Excision of transposable
603 elements from the chalcone isomerase and dihydroflavonol 4-reductase genes may
604 contribute to the variegation of the yellow-flowered carnation (*Dianthus*
605 *caryophyllus*). *Plant Cell Physiol* 43:578–585. <https://doi.org/10.1093/pcp/pcf065>

606 Jones P, Vogt T (2001) Glycosyltransferases in secondary plant metabolism: tranquilizers
607 and stimulant controllers. *Planta* 213:164–174.
608 <https://doi.org/10.1007/s004250000492>

609 Kaintz C, Molitor C, Thill J, Kampatsikas I, Michael C, Halbwirth H, Rompel A (2014)
610 Cloning and functional expression in *E. coli* of a polyphenol oxidase transcript from
611 *Coreopsis grandiflora* involved in aurone formation. *FEBS Lett* 588:3417–3426.
612 <https://doi.org/10.1016/j.febslet.2014.07.034>

613 Li Y, Baldauf S, Lim EK, Bowles DJ (2001) Phylogenetic analysis of the UDP-
614 glycosyltransferase multigene family of *Arabidopsis thaliana*. *J Biol Chem*
615 276:4338–4343. <https://doi.org/10.1074/jbc.M007447200>

616 Liu Y, Wang X, Mo T, Yan Y, Song Y, Zhao Y, Li J, Shi S, Liu X, Tu P (2017)
617 Identification and functional application of a new malonyltransferase NbMaT1
618 towards diverse aromatic glycosides from *Nicotiana benthamiana*. *RSC Adv*
619 7:21028–21035. <https://doi.org/10.1039/C7RA01940H>

620 Mameda R, Waki T, Kawai Y, Takahashi S, Nakayama T (2018) Involvement of chalcone
621 reductase in the soybean isoflavone metabolon: identification of GmCHR5, which
622 interacts with 2-hydroxyisoflavanone synthase. *Plant J* 96:56–74.
623 <https://doi.org/10.1111/tpj.14014>

624 Miyajima I, Maehara T, Kage T, Fujieda K (1991) Identification of the main agent
 625 causing yellow color of yellow-flowered cyclamen mutant. J Japan Soc Hort Sci
 626 60:409–414. <https://doi.org/10.2503/jjshs.60.409>
 627 Molitor C, Mauracher SG, Pargan S, Mayer RL, Halbwirth H, Rompel A (2015) Latent
 628 and active aurone synthase from ray florets of *C. grandiflora*: a polyphenol oxidase
 629 with unique characteristics. Planta 242:519–537. [https://doi.org/10.1007/s00425-](https://doi.org/10.1007/s00425-015-2261-0)
 630 015-2261-0
 631 Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka
 632 K, Jinbo T, Kimura T (2007) Development of series of gateway binary vectors,
 633 pGWBs, for realizing efficient construction of fusion genes for plant transformation.
 634 J Biosci Bioeng 104:34–41. <https://doi.org/10.1263/jbb.104.34>
 635 Nakayama T, Yonekura-Sakakibara K, Sato T, Kikuchi S, Fukui Y, Fukuchi-Mizutani M,
 636 Ueda T, Nakao M, Tanaka Y, Kusumi T, Nishio T (2000) Aureusidin synthase: a
 637 polyphenol oxidase homolog responsible for flower coloration. Science 290:1163–
 638 1166. <https://doi.org/10.1126/science.290.5494.1163>
 639 Noguchi A, Saito A, Homma Y, Nakao M, Sasaki N, Nishino T, Takahashi S, Nakayama
 640 T (2007) A UDP-glucose: Isoflavone 7-*O*-glucosyltransferase from the roots of
 641 soybean (*Glycine max*) seedlings: Purification, gene cloning, phylogenetics, and an
 642 implication for an alternative strategy of enzyme catalysis. J Biol Chem 282:23581–
 643 23590. <https://doi.org/10.1074/jbc.M702651200>
 644 Ohno S, Deguchi A, Hosokawa M, Tatsuzawa F, Doi M (2013) A basic helix-loop-helix
 645 transcription factor *DvIVS* determines flower color intensity in cyanic dahlia
 646 cultivars. Planta 238:331–343. <https://doi.org/10.1007/s00425-013-1897-x>
 647 Ohno S, Hori W, Hosokawa M, Tatsuzawa F, Doi M (2018a) Post-transcriptional
 648 silencing of chalcone synthase is involved in phenotypic lability in ray florets and

649 leaves of bicolor dahlia (*Dahlia variabilis*) 'Yuino.' Planta 247:413–428.
 650 <https://doi.org/10.1007/s00425-017-2796-3>

651 Ohno S, Hori W, Hosokawa M, Tatsuzawa F, Doi M (2018b) Identification of flavonoids
 652 in leaves of a labile bicolor flowering dahlia (*Dahlia variabilis*) 'Yuino'. Hort J
 653 87:140–148. <https://doi.org/10.2503/hortj.OKD-099>

654 Ohno S, Hosokawa M, Hoshino A, Kitamura Y, Morita Y, Park Y, Nakashima A,
 655 Deguchi A, Tatsuzawa F, Doi M, Iida S, Yazawa S (2011a) A bHLH transcription
 656 factor, *DvIVS*, is involved in regulation of anthocyanin synthesis in dahlia (*Dahlia*
 657 *variabilis*). J Exp Bot 62:5105–5116. <https://doi.org/10.1093/jxb/err216>

658 Ohno S, Hosokawa M, Kojima M, Kitamura Y, Hoshino A, Tatsuzawa F, Doi M, Yazawa
 659 S (2011b) Simultaneous post-transcriptional gene silencing of two different
 660 chalcone synthase genes resulting in pure white flowers in the octoploid dahlia.
 661 Planta 234:945–958. <https://doi.org/10.1007/s00425-011-1456-2>

662 Ohno S, Ueno M, Doi M (2020) Differences in the *CaMYBA* genome between
 663 anthocyanin-pigmented cultivars and non-pigmented cultivars in pepper (*Capsicum*
 664 *annuum*). Hort J 89:30–36. <https://doi.org/10.2503/hortj.UTD-097>

665 Ohno S, Yamada H, Maruyama K, Deguchi A, Kato Y, Yokota M, Tatsuzawa F, Doi M
 666 (2022) A novel aldo–keto reductase gene is involved in 6'-deoxychalcone
 667 biosynthesis in dahlia (*Dahlia variabilis*). Planta 256:47.
 668 <https://doi.org/10.1007/s00425-022-03958-4>

669 Ohno S, Yokota M, Yamada H, Tatsuzawa F, Doi M (2021) Identification of chalcones
 670 and their contribution to yellow coloration in dahlia (*Dahlia variabilis*) ray florets.
 671 Hort J 90:450–459. <https://doi.org/10.2503/hortj.UTD-305>

672 Ono E, Fukuchi-Mizutani M, Nakamura N, Fukui Y, Yonekura-Sakakibara K,
 673 Yamaguchi M, Nakayama T Tanaka T, Kusumi T, Tanaka Y (2006) Yellow flowers

674 generated by expression of the aurone biosynthetic pathway. Proc Natl Acad Sci
675 USA 103:11075–11080. <https://doi.org/10.1073/pnas.0604246103>

676 Ross J, Li Y, Lim EK, Bowles DJ (2001) Higher plant glycosyltransferases. Genome Biol
677 2:reviews3004.1. <https://doi.org/10.1186/gb-2001-2-2-reviews3004>

678 Saito N, Tatsuzawa F, Hoshino A, Abe Y, Ichimura M, Yokoi M, Toki K, Morita Y, Iida
679 S, Honda T (2011) Anthocyanin pigmentation controlled by *speckled* and *c-1*
680 mutations of Japanese morning glory. J Japan Soc Hort Sci 80:452–460.
681 <https://doi.org/10.2503/jjshs1.80.452>

682 Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing
683 phylogenetic trees. Mol Biol Evol 4:406–425.
684 <https://doi.org/10.1093/oxfordjournals.molbev.a040454>

685 Schlangen K, Miosic S, Thill J, Halbwirth H (2010) Cloning, functional expression, and
686 characterization of a chalcone 3-hydroxylase from *Cosmos sulphureus*. J Exp Bot
687 61:3451–3459. <https://doi.org/10.1093/jxb/erq169>

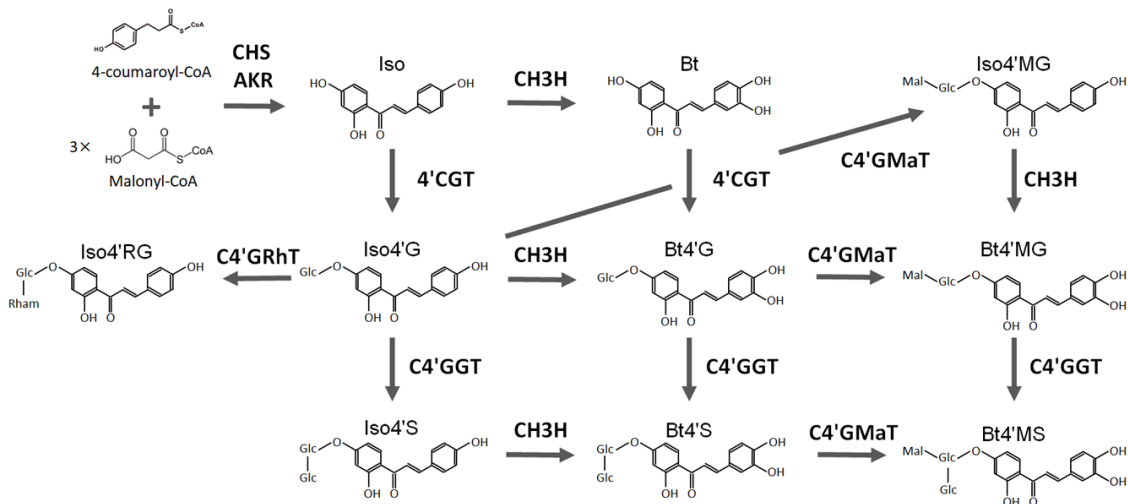
688 Stich K, Halbwirth H, Wurst F, Forkmann G (1994) Formation of 6'-deoxychalcone 4'-
689 glucosides by enzyme extracts from petals of *Dahlia variabilis*. Zeitschrift für
690 Naturforschung C 49:737–741. <https://doi.org/10.1515/znc-1994-11-1207>

691 Suzuki H, Nakayama T, Yonekura-Sakakibara K, Fukui Y, Nakamura N, Yamaguchi M,
692 Tanaka Y, Kusumi T, Nishino T (2002) cDNA cloning, heterologous expressions,
693 and functional characterization of malonyl-coenzyme A: anthocyanidin 3-*O*-
694 glucoside-6'-*O*-malonyltransferase from dahlia flowers. Plant Physiol 130:2142–
695 2151. <https://doi.org/10.1104/pp.010447>

696 Taguchi G, Yazawa T, Hayashida N, Okazaki M (2001) Molecular cloning and
697 heterologous expression of novel glucosyltransferases from tobacco cultured cells

698 that have broad substrate specificity and are induced by salicylic acid and auxin. Eur
699 J Biochem 268:4086–4094. <https://doi.org/10.1046/j.1432-1327.2001.02325.x>
700 Tamura K, Stecher G, Kumar S (2021) MEGA11: Molecular Evolutionary Genetics
701 Analysis Version 11. Mol Biol Evol 38:3022–3027.
702 <https://doi.org/10.1093/molbev/msab120>
703 Welle R, Grisebach H (1998) Isolation of a novel NADPH-dependent reductase
704 which coacts with chalcone synthase in the biosynthesis of 6'-deoxychalcone.
705 FEBS Lett 236:221–225. [https://doi.org/10.1016/0014-5793\(88\)80318-1](https://doi.org/10.1016/0014-5793(88)80318-1)
706

Fig. 1



712



Fig. 3

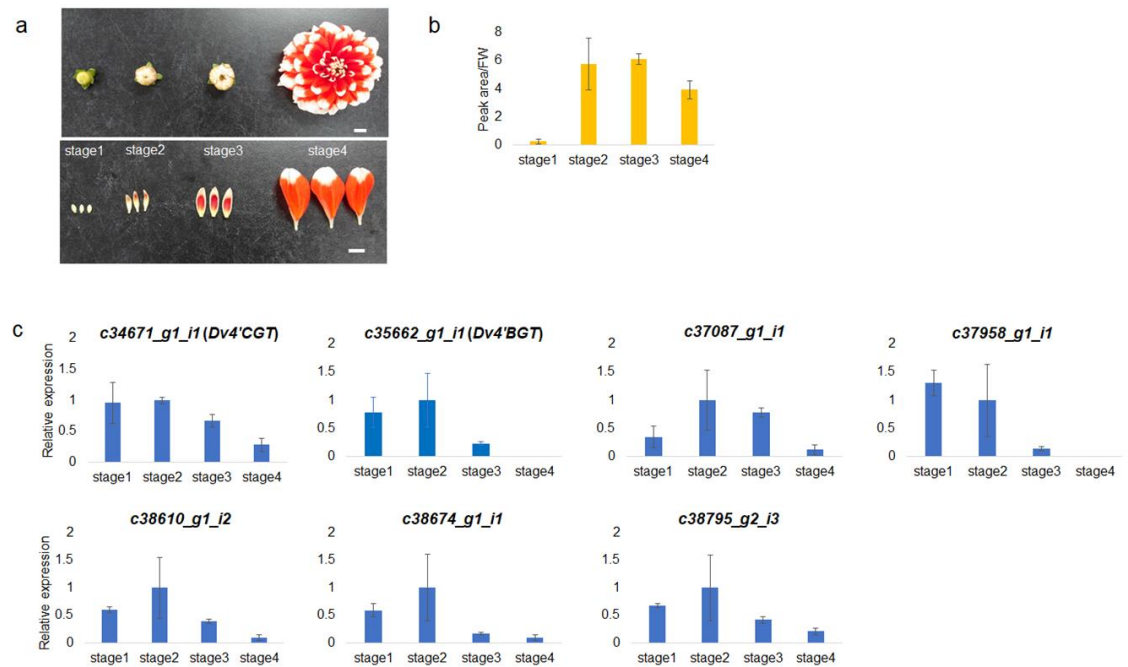


Fig. 4

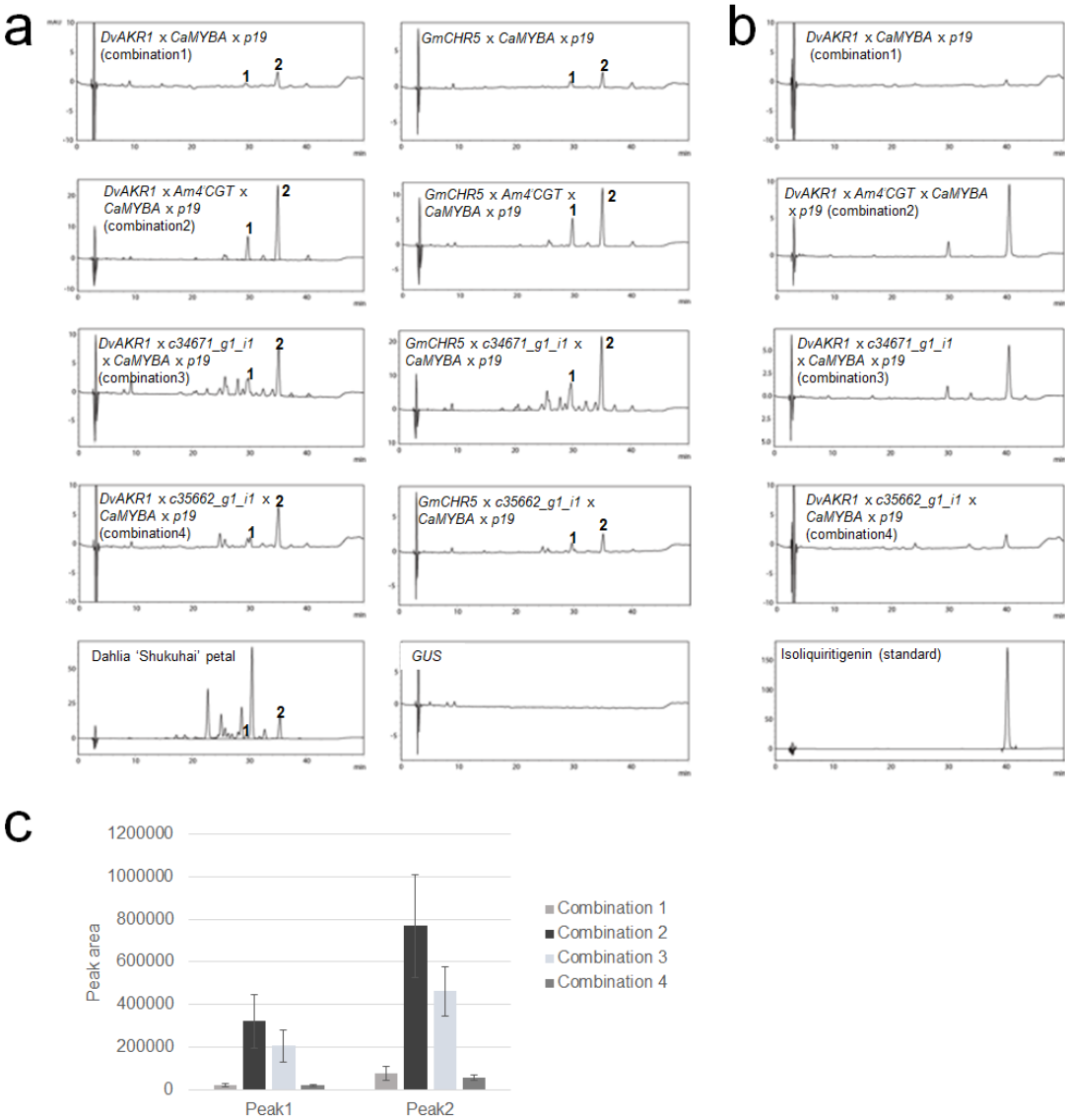


Fig. 5

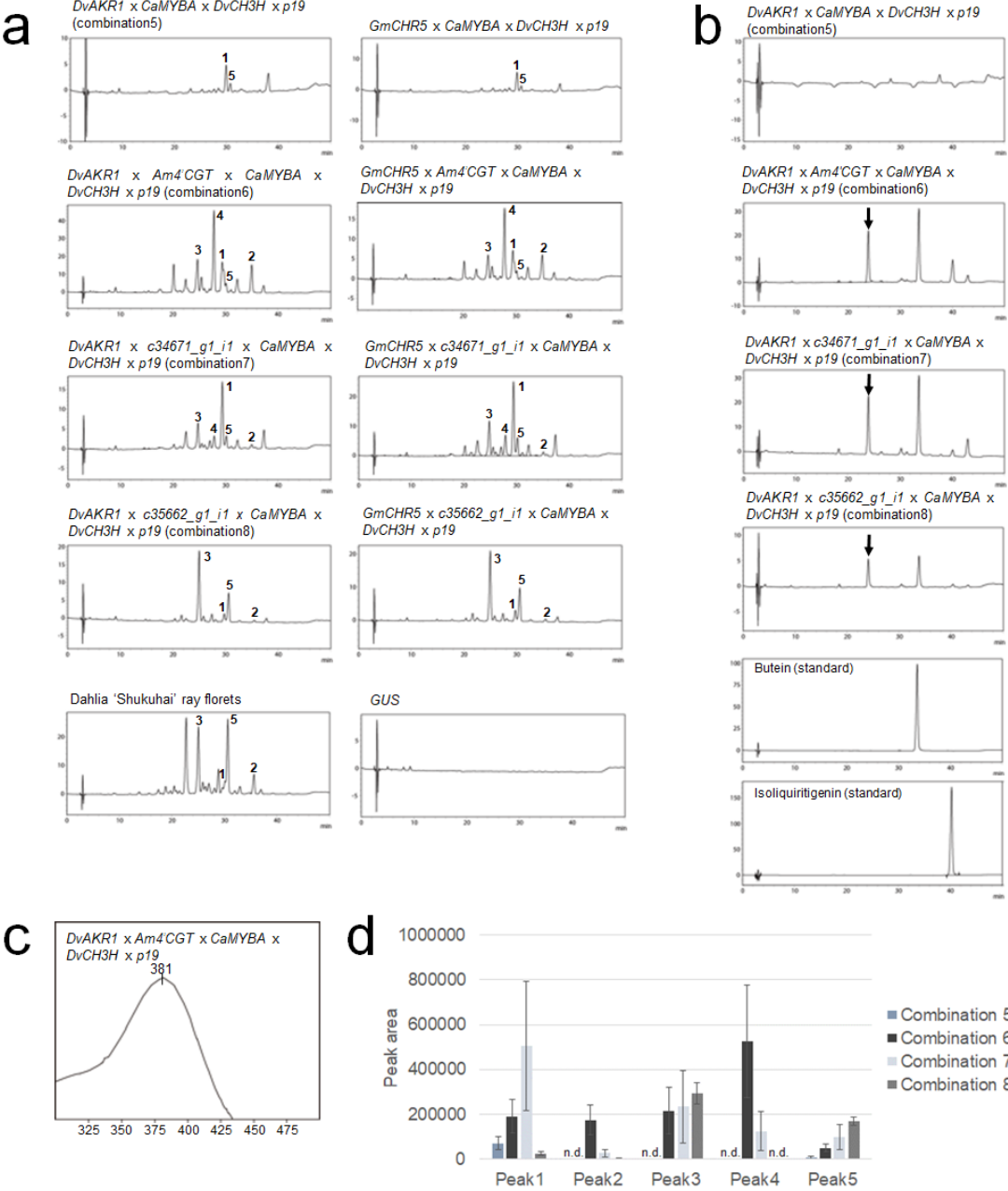


Fig. 6

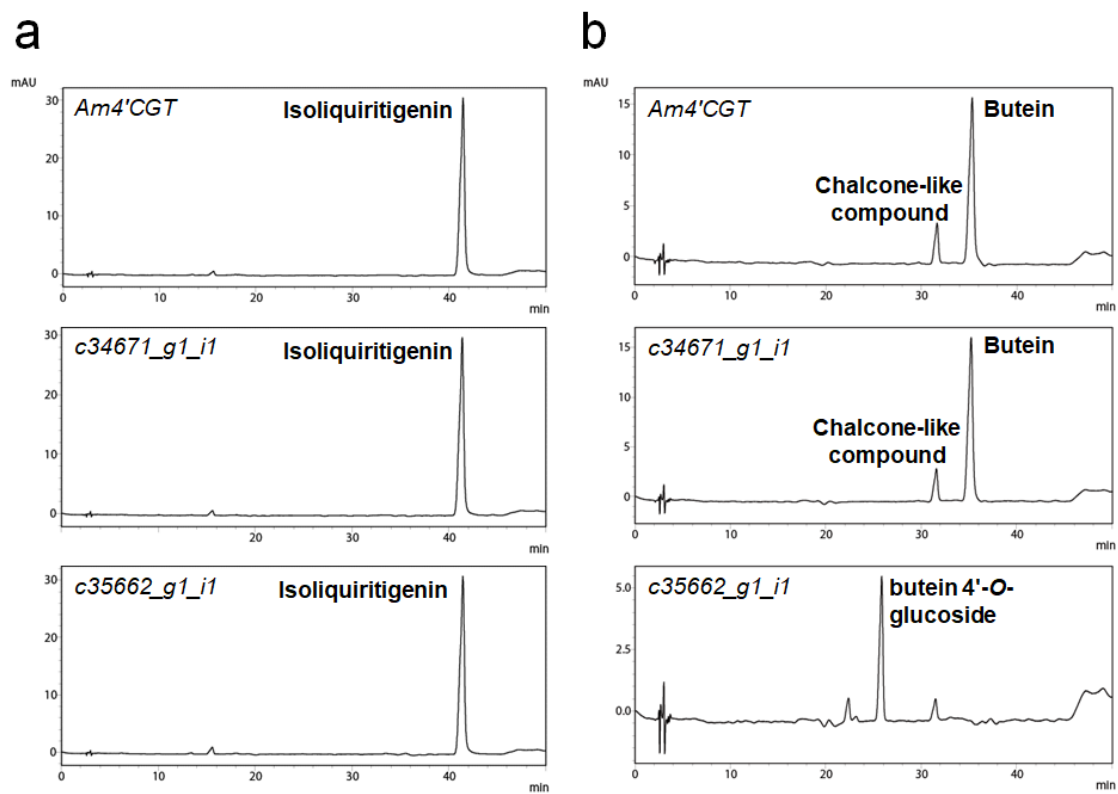


Fig. 7

