Daminozide reduces red color intensity in herbaceous peony (*Paeonia lactiflora* Pall.) flowers by inhibiting the expression of flavonoid biosynthetic genes

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Abstract

Daminozide is a plant growth retardant that inhibits 2-oxoglutarate-dependent dioxygenase activity in the gibberellin biosynthesis pathway, but few studies have focused on its effect on the flavonoid biosynthesis pathway involved in flower color. In the present study, the effect of daminozide on the regulation of flower color was investigated. An herbaceous peony (*Paeonia lactiflora* Pall.) cultivar ‘Fenzhu Pan’ was used as the material and treated with daminozide. Color parameters of petals were measured; flavonoid composition and content as well as flavonoid biosynthetic gene expression were monitored. The results showed that daminozide treatment caused less red coloration in herbaceous peony. The content of total anthocyanins was decreased by 23%, and the content of total anthoxanthins was increased by 8%; thus, it appeared that less red coloration in daminozide-treated flowers was associated with restricted anthocyanin accumulation. Furthermore, the gene expression patterns of flavonoid 3'-hydroxylase, dihydroflavonol 4-reductase and anthocyanidin synthase were lower after daminozide treatment, which could decrease anthocyanin accumulation and lead to less red coloration in flowers. These findings would improve our understanding of daminozide regulation of flower color in herbaceous peony.

Keywords Herbaceous peony · Daminozide · Flavonoids · Gene expression

Introduction

Herbaceous peony (*Paeonia lactiflora* Pall.) is a traditional ornamental plant in China with high ornamental value. The plant is popular due to its bright color and graceful appearance of flowers (Qing 2004), and it is widely used in various theme gardens and parks, such as peony gardens. For many ornamental plants, double-petaled cultivars exhibit higher ornamental value than single-petaled cultivars. In the market, double-petaled flowers have a greater appeal to consumers. Approximately, 300–400 herbaceous peony cultivars are distributed throughout China, and most contain some degree of red coloration, including those categorized as pink, red and purple, whereas few white cultivars are available (Wang and Zhang 2005; Wu 2006; Zhu et al. 2009). In addition,

studies have shown that white flowers can effectively reduce feelings of anxiety, anger and fatigue (Li et al. 2012). Based on these qualities, further study would be useful to regulate herbaceous peony flower color to provide the market with more white double-petaled cultivars.

In herbaceous peony, flower color is determined primarily by flavonoids, including anthocyanins and anthoxanthins (Zhao et al. 2014, 2016; Wu et al. 2016). Anthocyanin accumulation is related to red color intensity in herbaceous peony flowers (Zhong et al. 2012; Zhao et al. 2012a, 2016), and this accumulation is connected with flavonoid biosynthetic gene expression (Nakatsuka et al. 2005). Previously, Zhao et al. (2012a) predicted and summarized the putative flavonoid biosynthesis pathway in herbaceous peony, including the following genes: phenylalanine ammonialyase gene (*PAL*), chalcone synthase gene (*CHS*), chalcone isomerase gene (*CHI*), flavanone 3-hydroxylase gene (*F3H*), flavonoid 3'-hydroxylase gene (*F3'H*), dihydroflavonol 4-reductase gene (*DFR*), anthocyanidin synthase (*ANS*), *UDP*-glucoside gene (*UGT*) and methyl transferase gene (*MT*). However, until now, few studies have focused on the regulation of herbaceous peony flower color.

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Daminozide is a well-known chemical plant growth inhibitor that affects 2-oxoglutarate-dependent dioxygenase (2-ODD) activity in the gibberellin biosynthesis pathway, as it is structurally similar to 2-ODD (Rademacher 2000). By this characteristic, daminozide can affect plant growth and metabolites (Schmiderer et al. 2010; Karimi et al. 2015). Apart from this, daminozide would produce a side effect on flower color (Menhenett 1984; Reiss and Lewis 1984; Roepke et al. 2013). For example, daminozide can cause red color loss in the ray florets of two bronze *Chrysanthemum morifolium* cultivars. The researchers have postulated that this phenomenon is due to the inhibition of 2-oxoglutarate-dependent ANS activity by daminozide treatment, which is involved in the flavonoid biosynthesis pathway (Roepke et al. 2013). However, few studies have focused on the effects of daminozide on the flavonoid biosynthesis pathway and how daminozide regulates herbaceous peony flower color is still unclear. To explore the effect of daminozide on the flavonoid biosynthesis pathway in herbaceous peony, we first selected a natural herbaceous peony cultivar ‘Fenzhu Pan’ and treated it with daminozide. Then, color parameters of petals were measured; qualitative and quantitative analyses of flavonoids in petals were performed. Finally, the expression patterns of relevant flavonoid biosynthetic genes (*PIPAL*, *PICHS*, *PICHI*, *PIF3H*, *PIF3'H*, *PIFLS*, *PIDFR* and *PIANS*) in petals were examined. These results would contribute to our understanding of daminozide regulation of red color reduction in herbaceous peony flowers.

Materials and methods

Plant materials and treatments

A pink herbaceous peony cultivar ‘Fenzhu Pan’ was selected as the experimental material. The cultivar was transplanted into eight rows (three plants per row) in the germplasm repository of Horticulture and Plant Protection College, Yangzhou University, China (32°23'31"N, 119°24'50"E) in 2009 and grown in a natural state since then.

Four rows of plants were treated with solutions of 5 g/L daminozide (85%; Shanghai Yuanye Bio-Technology Co., Ltd, China). The other four rows of plants received distilled water. Both groups were foliar sprayed once the above-ground parts began to leaf on March 30th. They were treated once a week at dusk for a total of five treatments. The petals at three flower-developmental stages (stage 1, S1, flower-bud stage; stage 2, S2, initiating bloom stage; stage 3, S3, bloom stage; S1, S2 and S3 in Fig. 1) were collected as samples from May 8th to May 20th. Color parameters of the samples were measured immediately after each collection, and then the samples were frozen in liquid nitrogen and stored at − 80 °C for further study.

Color parameter measurements

The color parameters of samples were measured by a TC-P2A colorimeter (Beijing Optical Instrument Factory, China) in the laboratory at room temperature. The middle part of the inner part of ten random petals from the control

Fig. 1 Flowers of herbaceous peony in the control and daminozide-treated groups during flower development. S1 (Stage 1) = flower-bud stage, S2 (Stage 2) = initiating bloom stage, S3 (Stage 3) = bloom stage

and daminozide-treated groups at each stage was measured to reduce errors. After measuring, the color parameter values (L^* , a^* , b^*) indicated the color differences between samples.

Qualitative and quantitative analyses of flavonoids

The methods used for qualitative and quantitative analyses of flavonoids were the same as those reported by Zhao et al. (2014) with some modifications. In brief, fresh petals from each sample (approximate 1 g) were ground to powder in liquid nitrogen in the laboratory. Then, the samples were extracted with 6 mL of acidic methanol solution (70: 0.1: 29.9; v/v/v, CH₃OH: HCl: H₂O) at 4 °C for 24 h. The supernatant was passed through a 0.22 µm nylon membrane filter (Shanghai ANPEL Scientific Instrument Co., Ltd., China) and transferred to a vial (Agilent Technology, USA) prior to analysis. The analysis was performed by high-performance liquid chromatography–electrospray ionization–mass spectrometry (HPLC–ESI–MSⁿ, LCQ Deca XP MAX, Thermo Company, USA) coupled with a photodiode array and mass spectrometry detectors (HPLC–PDA–MS, Thermo Company, USA) with a three-dimensional quadrupole ion trap mass spectrometer. The HPLC column was TSK gel ODS-80Ts QA (4.6 mm × 250 mm) (Tosoh, Japan). Any absorbance value for both anthocyanins and anthoxanthins detected at 525 nm and 350 nm was noted. Cyanidin-3-*O*-glucoside (Cy3G) and rutin were used as reference standards for quantitative analysis to calculate the relative content in fresh weight (FW) units. Additionally, the total content of flavonoids was the sum of anthocyanins and anthoxanthins. Three replicate extractions were made for each biological sample.

Gene expression analysis

The relative expression levels of genes were analyzed using real-time quantitative polymerase chain reaction (qRT-PCR) with a BIO-RAD CFX Connect™ Real-Time System (Bio-Rad, USA). The samples were the same as those used for flavonoid analysis. Total RNA was separately extracted from

each sample using a Trizol Regent Kit (TaKaRa, Japan) according to the manufacturer's instructions with some modifications. All RNA samples were purified with a DNase I kit (TaKaRa, Japan) to remove co-extracted genomic DNA. Purity and integrity were analyzed with a nanodrop spectrophotometer (Thermo Fisher Scientific, USA). The cDNA, transcribed from the RNA for which the absorbance ratios were within 1.8–2.2 (A260/A280 nm) and 2.0–2.5 (A260/A230 nm), was prepared for qRT-PCR. Finally, qRT-PCR was performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems, USA) with three replicates using the following thermal cycle conditions: 95 °C for 30 s, followed by 40 cycles of denaturation for 5 s at 95 °C, annealing for 30 s at the annealing temperature of each primer pair and extension for 30 s at 72 °C. All gene-specific primers in this study are shown in Table 1. The qRT-PCR efficiency of each gene was obtained by analyzing the standard curve of a cDNA serial dilution. Relative transcript level was calculated with the $2^{-\Delta\Delta CT}$ method. Herbaceous peony *Actin* (JN105299) was used for template normalization (Zhao et al. 2012b). Primers were designed using the Primer 5.0 program.

Statistical analyses

All data were average values of at least three replicates with standard deviations. The results were analyzed for variance using the SAS/STAT statistical analysis package (version 9.4, SAS Institute, Cary, NC, USA).

Results

Color parameters

In our study, after daminozide treatment, the color intensity of flowers was reduced compared to the control at each stage (Fig. 1). To verify the naked eye observation, the petal color parameters were measured by a chroma meter. As shown in Fig. 2, the value of color parameter

Table 1 Gene-specific primers used in qRT-PCR analysis

Gene	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
<i>PAL</i>	TGAGTCAAGTTGCCAAGAG	GCATTAAAGGGTAAGTAGCG
<i>CHS</i>	ATGACTGGAACCTCGATACTCT	CTCACTCAAAAACATGCCTAG
<i>CHI</i>	CAACTTTCCGAGGCTGTG	CTCCTTTGACCTTATCCATC
<i>F3H</i>	AAGGAAGCACTCACAACG	GAAGACCACCCACCTGAT
<i>F3'H</i>	GGCGAGAAACCAACATT	CCATCCGTAGCCCTAAACT
<i>FLS</i>	GCTATCTCATCCGCACAA	CTTCAAACCTTTCCTCTACCAG
<i>DFR</i>	GCACTTTCTCCAATCACAG	CAAATGTAGCGACCCTCT
<i>ANS</i>	AGATTAGAACAAGAAGTCGGTG	GGAGGATGAAAGTGAGGG
<i>Actin</i>	GTTGCCCTTGATTACGAG	CAGCTTCCATTCCGATTA

Fig. 2 Color parameters of herbaceous peony in the control and daminozide-treated petals during flower development. S1 (Stage 1) = flower-bud stage, S2 (Stage 2) = initiating bloom stage, S3 (Stage 3) = bloom stage. The values represented mean $\pm$ SE, and different letters marked significant differences ($p < 0.05$)

L^* presented an increasing trend during flower development. In contrast, the values of color parameter a^* and b^* demonstrated a decreasing trend in both control and daminozide-treated groups. The values of color parameters L^* and b^* were higher in the daminozide-treated groups at each stage compared to the control, whereas the value of color parameter a^* was lower. Specifically, the value of color parameter L^* had increased to on average more than 20% higher than the control after daminozide treatment during flower development. Additionally, the value of color parameter a^* was positive rather than negative after daminozide treatment at each stage, whereas the value of color parameter b^* was negative instead of positive, except in the case of the control at S1. The differences in these

values between daminozide-treated groups and the control were all statistically significant.

Identification of flavonoid composition

Flavonoids, including anthocyanins and anthoxanthins, each have a different ultraviolet absorption wavelength. By detecting two ultraviolet–visible absorption bands at 525 and 350 nm, anthocyanin and anthoxanthin peaks are distinguished, respectively. The results revealed the presence of similar compounds in both control and daminozide-treated groups, although their peak areas varied in both anthocyanin and anthoxanthin chromatograms (Fig. 3). At 525 nm, the areas under each peak were all smaller than the control after

Fig. 3 HPLC chromatograms of herbaceous peony in the control and daminozide-treated petals in S1 (Stage 1 = flower-bud stage). **a** Anthocyanins, detected at 525 nm; **b** Anthoxanthins, detected at 350 nm; a1–a3 indicate identified anthocyanins; f1–f7 indicate identified anthoxanthins

daminozide treatment. In contrast, at 350 nm, the area of peaks varied in different ways after daminozide treatment. Additionally, most areas of the low absorption peaks were less than the control, whereas the high absorption peaks were greater than the control. Based on retention time, ultraviolet–visible spectral properties, mass spectrometric data and main fragmentations, ten main flavonoids, including three anthocyanins and seven anthoxanthins, were separated and characterized (Table 2). At 525 nm, the maximum absorption wavelengths ($\lambda_{\max}$) of the obvious peak a2 were at 275 and 515 nm, the $[M + H]^+$ was at m/z 625.38, and the fragment ions were at m/z 463.06 and 301.24. Fragments with an m/z of 301 at 525 nm were tentatively assigned as peonidin derivatives. Two fragment ions with 162 mass units indicated two glucuronides. Comparing the HPLC retention time with spectral and mass spectra data for anthocyanins reported in the literature, the compound was identified as peonidin-3,5-di-*O*-glucoside (Pn3G5G; Fan et al. 2012). At 350 nm, the obvious peak f2, with a retention time of 25.20 min, $\lambda_{\max}$ at 270 and 335 nm, $[M + H]^+$ at m/z 611.16 and fragment ions at m/z 449.32 and 287.45 was identified as a kaempferol derivative. The loss of two 162 mass unit fragment ions from m/z 611 indicated that the compound was a kaempferol derivative with two glucoses. Comparing the HPLC retention time with spectral and mass spectrum data for anthoxanthins reported in the literature, the compound was identified as kaempferol-3,7-di-*O*-glucoside (Km3G7G; Zhong et al. 2012). Similarly, peaks a1 ($\lambda_{\max}$ 275 and 515 nm, $[M + H]^+$ (m/z) 611.23, MS^2 (m/z) 448.99 and 287.15), a3 ($\lambda_{\max}$ 275 and 515 nm, $[M + H]^+$ (m/z) 711.09, MS^2 (m/z) 549.45, 463.24 and 301.11), f1 ($\lambda_{\max}$ 265 and 355 nm, $[M + H]^+$ (m/z) 627.11, MS^2 (m/z) 464.93 and 303.24), f3 ($\lambda_{\max}$ 265 and 355 nm, $[M + H]^+$ (m/z) 617.12, MS^2 (m/z) 465.22 and 302.93), f4 ($\lambda_{\max}$ 265 and 355 nm, $[M + H]^+$ (m/z) 465.12, MS^2 (m/z) 303.21), f5 ($\lambda_{\max}$ 270 and 350 nm, $[M + H]^+$ (m/z) 601.11, MS^2 (m/z) 448.66 and 287.24), f6 ($\lambda_{\max}$ 265 and 365 nm, $[M + H]^+$ (m/z)

449.32, MS^2 (m/z) 287.25) and f7 ($\lambda_{\max}$ 270 and 345 nm, $[M + H]^+$ (m/z) 449.24, MS^2 (m/z) 287.26) were characterized as cyanidin-3,5-di-*O*-glucoside (Cy3G5G), peonidin-3-*O*-malonylglucoside-5-*O*-glucoside (Pn3MloG5G), quercetin-3,7-di-*O*-glucoside (Qu3G7G), quercetin-3-*O*-galloylglucoside (Qu3GloG), quercetin-3-*O*-glucoside (Qu3G), kaempferol-3-*O*-galloylglucoside (Km3GloG), kaempferol-7-*O*-glucoside (Km7G) and kaempferol-3-*O*-glucoside (Km3G), respectively (Li et al. 2009; Fan et al. 2012; Zhong et al. 2012).

Quantitative analysis of flavonoids

The relative content of each flavonoid was obtained using Cy3G and rutin as standards. The relative content of the ten main components is shown in Fig. 4. The abundance of all ten peaks dropped gradually during flower development in both control and daminozide-treated groups, except for the content of Qu3GloG at S2. With daminozide treatment, the contents of Cy3G5G, Pn3G5G, Pn3MloG5G, Qu3G7G, Qu3GloG and Qu3G were lower at each stage compared to the control, whereas the contents of Km3G7G, Km3GloG, Km7G and Km3G were higher. For anthocyanins, the content of Pn3G5G, which on average accounted for more than 80% of the total anthocyanin, was dramatically higher, by an average of 19%, compared to the control during flower development. Regarding anthoxanthins, on the whole, the contents of quercetin derivatives (f1, f3 and f4) were significantly lower, at an average of 80.60%, compared to the control during flower development. In contrast, the contents of kaempferol derivatives (f2, f5, f6 and f7) were higher, especially Km3G7G and Km7G, which were consistently 1.23 and 1.43 times higher compared to the control during flower development. Km3G7G and Km3G were the two major anthoxanthins, which on average accounted for 25.18 and 63.96% of total anthoxanthins during flower development, respectively. The total contents of anthocyanin,

Table 2 Retention time, ultraviolet–visible spectral properties, mass spectrometric data and tentative identification of the compounds detected in the control and daminozide-treated petals

Peak	Retention time (min)	Mass spectrometry information			Tentative identification
		$\lambda_{\max}$ (nm)	$[M + H]^+$ (m/z)	MS^2 (m/z)	
a1	18.01	275; 515	611.23	448.99; 287.15	Cyanidin-3,5-di- <i>O</i> -glucoside
a2	25.61	275; 515	625.38	463.06; 301.24	Peonidin-3,5-di- <i>O</i> -glucoside
a3	36.61	275; 515	711.09	549.45; 463.24; 301.11	Peonidin-3- <i>O</i> -malonylglucoside-5- <i>O</i> -glucoside
f1	19.27	265; 355	627.11	464.93; 303.24	Quercetin-3,7-di- <i>O</i> -glucoside
f2	25.20	270; 335	611.16	449.32; 287.45	Kaempferol-3,7-di- <i>O</i> -glucoside
f3	37.44	265; 355	617.12	465.22; 302.93	Quercetin-3- <i>O</i> -galloylglucoside
f4	45.87	265; 355	465.12	303.21	Quercetin-3- <i>O</i> -glucoside
f5	53.77	270; 350	601.11	448.66; 287.24	Kaempferol-3- <i>O</i> -galloylglucoside
f6	55.01	265; 365	449.32	287.25	Kaempferol-7- <i>O</i> -glucoside
f7	60.47	270; 345	449.24	287.26	Kaempferol-3- <i>O</i> -glucoside

Fig. 4 Simplified schematic overview of the major flavonoid biosynthetic pathway in herbaceous peony petals and the contents of tentative flavonoid compounds in the control and daminozide-treated pet-

als during flower development. The values represented mean $\pm$ SE, and different letters marked significant differences ($p < 0.05$)

anthoxanthin and flavonoid are shown in Fig. 5, which demonstrate a downward trend during flower development in both control and daminozide-treated groups. The level of total anthocyanin content was lower in the daminozide-treated groups compared to the control, whereas the level of

total anthoxanthin content was higher. Considering that the content of total anthoxanthin was on average accounted for nearly 99% of total flavonoid during flower development, the trend in total flavonoid was consistent with that of total anthoxanthin.

Fig. 5 Total anthocyanin, anthoxanthin and flavonoid content of herbaceous peony in the control and daminozide-treated petals during flower development. S1 (Stage 1) = flower-bud stage, S2 (Stage 2) = initiating bloom stage, S3 (Stage 3) = bloom stage. The values represented mean $\pm$ SE, and different letters marked significant differences ($p < 0.05$)

Expression analysis of flavonoid biosynthetic genes

As shown in Fig. 6, from the overall level, the expression level of the upstream gene *PICHS* was the highest, whereas the downstream gene *PIFLS* was the lowest. From the overall trend, the expression patterns of *PICHS*, *PICHI*, *PIF3H*, *PIF3'H*, *PIDFR* and *PIANS* exhibited a similar downward trend during flower development in both control and daminozide-treated groups, but the expression pattern of *PIFLS* was the opposite. Unlike these genes, the expression pattern of *PIPAL* first showed a rise from S1 to S2 followed by a

decline from S2 to S3, reaching a minimum at S3. With daminozide treatment, there were no significant differences in the expression patterns of *PIPAL*, *PICHS*, *PICHI* and *PIF3H* at each stage compared to the control. However, the expression levels of *PIF3'H*, *PIDFR* and *PIANS* were lower with daminozide treatment than the control, except for *PIDFR* at S3, which remained about the same as the control. The differences between control and daminozide-treated groups at S1 and S2 were statistically significant. *PIFLS* was expressed at a higher level in daminozide-treated groups compared to the control.

Fig. 6 Flavonoid biosynthetic gene expression analysis of herbaceous peony in the control and daminozide-treated petals during flower development. S1 (Stage 1) = flower-bud stage, S2 (Stage 2) = initiating bloom stage, S3 (Stage 3) = bloom stage. The values represented mean $\pm$ SE, and different letters marked significant differences ($p < 0.05$)

Discussion

In this study, less red coloration occurred in herbaceous peony cultivar ‘Fenzhu Pan’ flowers with daminozide treatment compared to untreated flowers, which were in consistent with previous results in bronze, pink and purple *Chrysanthemum morifolium* cultivars (Menhenett 1984; Reiss and Lewis 1984; Roepke et al. 2013). To characterize the differences in flower color more scientifically and accurately, the CIE1976LAB system was used. This system is an international standard of color measurement wherein positive values for the color parameter a^* indicate red, whereas negative values along this parameter indicate green. Positive values for the color parameter b^* correspond to yellow, whereas negative values on this axis correspond to blue. Color lightness is expressed by the percentage of the color parameter L^* . The value of this color parameter ranges from 0 to 100, indicating dark to bright. Measured by a colorimeter, the difference of color parameter a^* value indicated that the flower color was a greenish coloration rather than reddish with daminozide treatment compared to the control, whereas the difference of color parameter b^* value indicated that the flower color was a yellowish coloration for the daminozide-treated groups instead of bluish (as seen with controls) except for the control at S1. This verified the observation of the naked eye, which had indicated that the color intensity of flowers was reduced by daminozide treatment. Furthermore, the difference in the value of color parameter a^* between the control and daminozide-treated groups was, on average, 3.8 times greater than the value of color parameter b^* during flower development, suggesting that the difference of flower color was particularly the intensity of red coloration. In addition, the values of color parameter L^* , a^* and b^* demonstrated that the intensity of flower color was gradually reduced during flower development. This was consistent with results from other herbaceous peony cultivars (Zhao et al. 2014, 2016; Wu et al. 2016).

In ornamental plants, the flower color is relevant to the tissue structure of the petal and to the type and amount of pigments in petal cells. Among these factors, pigments have a significant impact on the flower color (Zhao and Tao 2015). Through chemical structures, cellular location and biochemical synthesis pathways, pigments can be separated into three categories: carotenoids, flavonoids and alkaloids. In herbaceous peony, flavonoids, including anthocyanins and anthoxanthins, are the crucial pigments contributing to flower color. Zhong et al. (2012) found that in herbaceous peony, an increase in the brightness of flower color along with a decline in red and chroma could be related to a decrease in total anthocyanin content and increase in total anthoxanthin content during flower

development. Zhao et al. (2014) observed that total anthocyanin and anthoxanthin content varied in the red outer petals and the yellow inner petals of the herbaceous peony cultivar ‘Jinhui’. A similar result was reported in the herbaceous peony cultivar ‘Qiaoling’, which has white outer petals and yellow inner petals (Wu et al. 2016). In the present study, the contents of flavonoid were indeed different after daminozide treatment compared to the control. The content of total anthocyanin was an average of 23% lower, and the content of total anthoxanthin was an average of 8% higher with daminozide treatment compared to the control during flower development. A similar result was reported in two bronze *Chrysanthemum morifolium* cultivars ‘Baton rouge’ and ‘Pelee’ by Roepke et al. (2013). This previous study showed a 75% reduction in anthocyanins in ‘Baton rouge’, a 98% decline in anthocyanins and a 22–68% rise in flavones and flavonols content in ‘Pelee’. Interestingly, in the present study, unlike ‘Pelee’, though the content of total anthoxanthin was higher, the content of flavonols was differently affected. Among the flavonols, the content of the kaempferol derivatives was higher with daminozide treatment, whereas the content of the quercetin derivatives showed the opposite effect. Furthermore, anthoxanthin was responsible for flower colors from white to dark-yellow, whereas anthocyanin was responsible for a wide range of flower colors from pink to blue–purple (Zhao and Tao 2015). In rose (*Rosa hybrid*), Schmitzer et al. (2012) observed a positive correlation between the content of anthocyanin and the value of color parameter a^* . Additionally, Pn3G5G was the major anthocyanin in herbaceous peony petals and was mainly responsible for red coloration in flowers (Jia et al. 2008). In herbaceous peony, Zhao et al. (2016) found that the content of total anthocyanidin, with Pn3G5G being the main component, accumulate to higher levels in red flowers and lower levels in white flowers. In the present study, Pn3G5G was also identified as the major anthocyanin. Hence, less anthocyanin accumulation with daminozide treatment would be associated with the reduction of red color intensity in herbaceous peony flowers, whereas the increase in anthoxanthin might only play a minor role.

The accumulation of flavonoids was controlled by a series of genes in the flavonoid biosynthesis pathway (Nakatsuka et al. 2005). In herbaceous peony, Zhao et al. (2012a) proposed a putative flavonoid biosynthesis pathway, including *PAL*, *CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, *ANS*, *F3GT*, *F5GT* and *MT*. Among these genes, the expression patterns of *DFR* and *ANS* played a major role in anthocyanin biosynthesis, which was consistent with petals exhibiting red. In peony (*Paeonia suffruticosa*), low expression of *DFR* and *ANS* in white petals compared to red petals led to a significant decrease in the anthocyanin accumulation (Zhao et al. 2015). A similar result was found in herbaceous peony; the expressions of

DFR and *ANS* basically had the highest levels in red cultivars and the lowest levels in white cultivars (Zhao et al. 2016). Daminozide is a plant growth inhibitor. The inhibitory effect of daminozide was a result of competition with the structurally similar natural co-substrate 2-oxoglutaric acid (Rademacher 2000). A similar plant growth inhibitor, prohexadione-calcium (Pro-Ca), was reported to inhibit several 2-ODDs activities involved in the flavonoid biosynthesis pathway in some plants. For example, Kavalier et al. (2014) showed that *F3H* was inhibited by Pro-Ca in the hop (*Humulus lupulus* L.) cones. In 'Braeburn' apple (*Malus pumila*), Bizjak et al. (2013) found that the expression of *F3H* and *ANS* was reduced in response to autumn application of Pro-Ca. This reduction resulted in dramatic changes to the flavonoid composition in apple peels, associated with a loss of red coloration in the skin of the apple. For daminozide, inhibition of *F3H* was unlikely because it displayed negligible inhibition of the catalytic activity of *F3H* (Halbwirth et al. 2006). In bronze *Chrysanthemum morifolium*, Roepke et al. (2013) proposed that the red color reduction with daminozide treatment was associated with inhibition of 2-ODD *ANS*, which was involved in the flavonoid biosynthesis pathway. In the present study, the expression levels of *PIPAL*, *PICHS*, *PICHI* and *PIF3H* remained the same after daminozide treatment, which confirmed the results of Halbwirth et al. (2006). The expression levels of *PIF3'H*, *PIDFR* and *PIANS* were lower, suggesting that the catalytic activities of *F3'H*, *DFR* and *ANS* might be inhibited after daminozide treatment. Inhibition of *ANS* would to some extent validate the previous study in bronze *Chrysanthemum morifolium* (Roepke et al. 2013). However, the expression level of *PIFLS* was higher after daminozide treatment. This might have resulted in increases in metabolic precursors upstream of *FLS*. Due to the lower expression of upstream *PIF3'H* and downstream *PIDFR* and *PIANS*, a small amount of anthocyanin was produced. Based on the inhibition, more metabolic precursors upstream of *F3'H* and *DFR* had accumulated. With normal expression of the upstream genes *PIPAL*, *PICHS*, *PICHI* and *PIF3H*, as well as higher expression of *PIFLS* combined with more metabolic precursors, slightly more anthoxanthin was produced (Fig. 4). In addition, little accumulation of quercetin derivatives might be caused by the lower expression level of *PIF3'H*.

In conclusion, the reduction of red color intensity in herbaceous peony flowers occurred in response to daminozide treatment. After daminozide treatment, the content of total anthocyanin was, on average, 23% lower than the control during flower development. Additionally, Pn3G5G was identified as the major anthocyanin responsible for red coloration. The significant decrease in anthocyanin accumulation was directly related to the reduction of red color intensity in flowers. The total anthoxanthin content was, on average, 8% higher, despite the lower quercetin derivatives content

during flower development. The lower expression of *PIF3'H*, *PIDFR* and *PIANS* after daminozide treatment might result in a decrease in anthocyanin accumulation and eventually lead to the reduction of red color intensity in flowers. Besides, the inhibition would give rise to more anthoxanthin accumulation, whereas inhibition of *F3'H* would be associated with a decrease in quercetin derivative accumulation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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