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ARTICLE

Synergy Effect of Sodium Acetate and Glycosidically Bound Volatiles on the Release of Volatile Compounds from the Unscented Cut Flower (*Delphinium elatum* L. "Blue Bird")

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Many modern floricultural varieties have lost their scent during traditional breeding programs. The factors that result in the nonscent emission of some cut flowers remain unclear. The objective of this study was to investigate whether the nonscent emission is due to one of the factors, the lack of suitable substrates (or precursors of scent compounds). Using solid-phase microextraction and dynamic headspace volatile sampling techniques, the supplement of nonvolatile compounds such as 2-coumaric acid glucoside to the unscented cut flower such as *Delphinium elatum* L. "Blue Bird" enhanced the emission of scent from the flower, which was sufficient for detection by the human olfaction. Interestingly, compared with feeding with each compound, the combination of sodium acetate and 2-coumaric acid glucoside showed the synergy effect on enhancement of coumarin, a cherry leaf-like scent emission from the flower, which is due to one of factors that sodium acetate enhanced the activity of β-glucosidase being involved in the formation of the scent compound. These results suggest that some enzymes responsible for the formation of floral scents indeed occur in the unscented flowers such as *Delphinium elatum* L. "Blue Bird", and the non- or low-scent emission of the flowers are due to the lack of suitable substrates.

KEYWORDS: ***Delphinium elatum* L. "Blue Bird"; sodium acetate; 2-coumaric acid glucoside; 2-phenylethyl glucoside; headspace; scent**

INTRODUCTION

In nature, floral scent plays an important role in attracting pollinators, thereby ensuring plant reproductive and evolutionary success (*1*). Also, humans have always been fascinated by floral scent because of their aesthetic, emotional, and economic values (*2*). Unfortunately, floral scent always has been lost during traditional breeding programs for the cut flower market and ornamental plants because of a negative correlation between flower longevity and fragrance (*1*, *3*).

Recently, some metabolic engineering has been made to improve scent quality of flowers. The most often used attempt to engineer scent, for example, the genetic engineering approach, has mainly concentrated on the introduction of the "scent genes" responsible for the final steps of the formation of volatiles, which lead to the successful and not-so-successful changes in floral scent bouquet (*4*). However, little attention has been given to the role of substrates (or precursors of volatiles) in regulating the scent formation. To investigate whether the non- or low-scent emission of some cut flowers are due to the lack of suitable substrates, in the present study, several precursors of volatile compounds were supplied to the unscented cut flowers, and the flower that achieves in the olfactory detectable enhancement was screened out. This study also focused on the effect of the precursors of volatile compounds supplied to the flower on the floral scent emission and the related floral scent emission mechanism. In addition, sodium acetate may enhance availability of acetyl coenzyme A (acetyl-CoA) that is an important precursor of secondary metabolites formed from acetate pathway (*5*). Therefore, sodium acetate was used for clarifying its effect on the scent emission. The information obtained from this study would advance our understanding of production and emission of secondary volatile compounds and also contribute to the metabolic engineering to modify floral scent.

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MATERIALS AND METHODS

Experimental Design. This research mostly focused on the precursors of phenylpropanoid/benzenoid volatiles, such as shikimic acid (purchased from Sigma Chemical Co. USA), L-phenylalanine, *trans*-cinnamic acid (purchased from Wako Pure Chemical Industries, Ltd., Japan), 2-phenylethyl α,β-D-glucoside (2PEG) (a gift from T. Hasegawa Co., Ltd., Japan), and 2-coumaric acid glucoside (chemically synthesized by the authors).

The unscented cut flowers (*Rosa* "Rote Rose", *Gerbera jamesonii* "Jaguar Deep Rose", *Dianthus caryophyllus* "Barbara", and *Delphinium elatum* L. "Blue Bird") and the flowers with unpleasant odors (*Eustoma grandiflorum* "Forever Blue", *Gypsophila elegans* "Covent Garden", and

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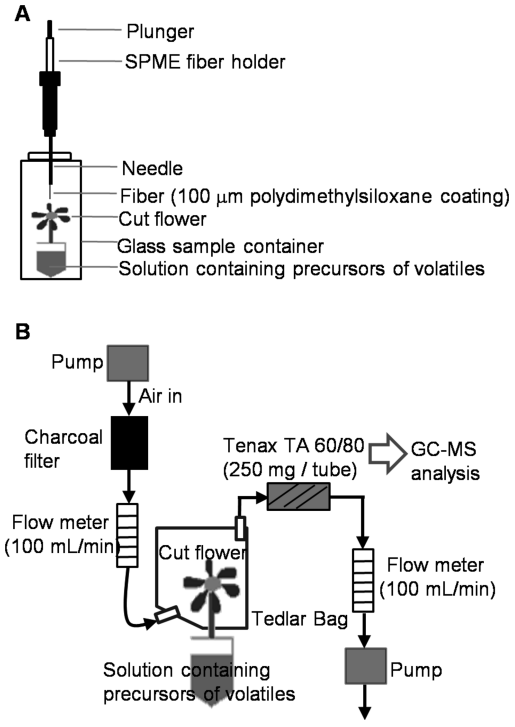

Figure 1. Solid phase microextraction (SPME) technique (A) and dynamic headspace volatile sampling technique (B).

Tulipa "Greenland") were used in this study. These cut flowers almost at the midopen stages were purchased from the flower shops in Shizuoka, Japan, and grown in the incubator under the conditions of 12 h light/12 h dark photoperiod, 70% humidity, and 20−22 °C. The flowers were kept for 2−3 days in tap water prior to use. Afterward, the flowers were independently placed in the following solutions: (1) water (as control), (2) 15 mM precursors of volatile compounds in water, (3) 10 mM sodium acetate in water, and (4) 15 mM precursors of volatile compounds and 10 mM sodium acetate in water. After 20 h treatments, the headspace volatiles were collected using two different techniques, solid phase microextraction (SPME) (**Figure 1A**, 8 h/sampling for 1 flower) and dynamic headspace volatile sampling (**Figure 1B**, 12 h/sampling for 1 flower). The sensory evaluation was carried out by three persons every 6 h in nighttime and every 2−4 h in daytime. The significant olfactory effect of 2-coumaric acid glucoside on *Delphinium elatum* L. "Blue Bird" was confirmed by the three persons who joined the sensory evaluation and another five persons who were invited.

Synthesis of 2-Coumaric Acid Glucoside. 2-Coumaric acid glucoside was synthesized from 2-hydroxycinnamic acid according to the literature (*6*) with modifications (**Figure 2**). 2-Hydroxycinnamic acid (2 g, 12.1 mmol) was dissolved in 2 mL of MeOH, and TMSCl (2.3 mL, 18.1 mmol) was dropped in the solution during 5 min at 0 °C. After 4−6 h stirring under ice-cooling, 1 mL of MeOH was added and stirred overnight at room temperature. Afterward, the solution was evaporated to dryness to obtain compound **2** (2.15 g, 12.0 mmol, 99.5%). Compound **2** (100 mg, 0.56 mmol), 2,3,4,6-tetra-*O*-acetylglucopylanosyl bromide (692 mg, 1.68 mmol), and Cs_2CO_3 (182 mg, 0.56 mmol) were dissolved in dry MeCN (10 mL) and stirred for 72 h at room temperature. The solution was diluted to 50 mL with CH_2Cl_2, washed with brine (2 × 50 mL), dried over Na_2SO_4, and concentrated. The residue was purified using Wakogel C-200 chromatography with 0→40% ethyl acetate in hexane to obtain compound **3** (187 mg, 0.37 mmol, 65.7%). To a solution of **3** (172 mg, 0.34 mmol) in 5 mL of THF, NaOH (2 M, 5 mL) was added and stirred for 2.5 h at room temperature. Afterward, the solution was acidified with HCl (2 M, 4.5 mL) and evaporated to dryness. The residue was purified by a cartridge C18 column (50 mm id×100 mm, Merck) to obtain 2-coumaric acid glucoside (64 mg, 0.19 mmol, 57.8%). ^{1}H NMR (270 MHz, DMSO): δ 3.16−3.41 (m, H-2″, H-3″, H-4″, H-5″), 3.46 (1H, dd, J = 11.5 and 5.6 Hz, H-6″b), 3.68 (1H, dd, J = 11.5 and 1.9 Hz, H-6″a), 4.97 (1H, d, J = 7.2 Hz, H-1″), 6.51 (1H, d, J = 16.1 Hz, H-2′), 7.02 (1H, t, J = 7.9 and 7.9 Hz, H-5), 7.18 (1H, d, J = 7.9 Hz, H-3), 7.35 (1H, ddd, J = 1.6 and 7.9 Hz. H-4), 7.65 (1H, dd, J = 1.6 and 7.9 Hz. H-6), 7.9 (1H, d, J = 16.1 Hz. H-3′).

Determination of Volatile Compounds by Gas Chromatography−Mass Spectrometry (GC-MS). Analyses of volatile compounds, 2-phenylethanol and coumarin, were performed using a GC-MS QP5050 (Shimadzu), which was controlled by a Class-5000 workstation. For the determination of volatile compounds absorbed in Tenax TA, a TurboMatrix Automated Thermal Desorber (PerkinElmer instruments) was connected to GC. Optimized operating conditions were: desorption temperature 250 °C, desorption time 10 min, 0.5−10% to the GC column. The GC-MS condition was described in our previous study (*7*), with a modification. The GC was equipped with a capillary TC-5 column (GL Sciences Inc., Japan), 30 m × 0.25 mm I.D., and 0.25 μm film thickness. Helium was used as a carrier gas at a flow rate of 1.5 mL/min. The injector temperature was 230 °C. The GC oven was maintained at 60 °C for 3 min. The temperature of the oven was programmed at 15 °C/min to 110 °C and then at 40 °C/min to 290 °C and kept at this temperature for 3 min. The mass spectrometer was operated by the full scan mode (mass range m/z 60−200) or by the selected ion monitoring (SIM) mode for quantitative analysis (m/z 122, 91 for 2-phenylethanol; m/z 146, 118 for coumarin).

To determine the internal pool sizes of volatile compound (coumarin) in the flowers, tissue (0.3 g fresh weight) was ground in liquid nitrogen and extracted in 2.5 mL of an azeotropic mixture of pentane−dichloromethane (2:1 v/v) for 16 h under dark and afterward filtered through a short plug of anhydrous sodium sulfate. One μL of the filtrate was obtained and subjected to GC-MS analyses. The GC-MS conditions were described as above. [5,6,7,8-$^2H_4$]-Coumarin was used as an internal standard.

Preparation of Cell-Free Extracts and β-Glucosidase Assay. Cell-free extracts were prepared as described previously (*8*) with modifications. Delphinium flower powder (300 mg) ground by liquid nitrogen was homogenized for 5 min at 0 °C in a mixture of 10 mL of buffer A (0.1 M potassium phosphate at pH 7.5, containing 0.5% 3-[(3-cholamidopropyl)-dimethylamino]-1-propanesulfonate (CHAPS), 2 mM dithiothreitol, 5% glycerol, and 1 mM ethylenediaminetetraacetic acid (EDTA)), 50 μL of 0.1 M 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, and 600 mg of polyvinyl polypyrrolidone. After centrifuging at 4000*g* for 20 min at 4 °C, the supernatant was desalted on a PD-10 column that had been equilibrated with buffer B (0.01 M potassium phosphate at pH 7.5, containing 0.05% CHAPS, 5% glycerol, and 1 mM EDTA) to give crude β-glucosidase solution.

The β-glucosidase activity was determined in a standard assay. The reaction mixture consisting 150 μL of enzyme solution, 150 μL of buffer B, 150 μL of 10 mM *p*-nitrophenyl β-glucoside, and 300 μL of 50 mM citric acid buffer (pH 6.0) was incubated for 15 min at 30 °C. The reaction was quenched by adding 750 μL of 1 mM Na_2CO_3. The amount of *p*-nitrophenyl released was measured by the absorbance at 405 nm. One unit of β-glucosidase was defined as the amount of enzyme releasing 0.01 μmol of *p*-nitrophenyl per min under the above conditions.

Statistical Analysis. Data are expressed as mean ± standard error. Student's t test was used to estimate significance for comparisons. A probability level of 5% ($p < 0.05$) was considered significant.

RESULTS AND DISCUSSION

Olfactory Effects of the Precursors Feeding on the Floral Scent Emission. To know whether newly introduced "precursors" could find appropriate scent enzymes and the intended products could be produced and emitted at levels that could be detected by humans, several precursors of volatile compounds were supplied to the different flowers. Because the first committed step in the pathways of some volatile phenylpropanoid and benzenoid compounds derived from shikimic acid is the conversion of L-phenylalanine to *trans*-cinnamic acid (*2*), this allowed us to use the three compounds, i.e., shikimic acid, L-phenylalanine, and *trans*-cinnamic acid as candidates for the feeding experiment. Also, the important precursors of volatiles such as glycosidically bound volatile compounds were applied to the feeding experiment. The olfactory detectable enhancement of volatiles emitted from the flowers was only achieved in the *Delphinium elatum* L.

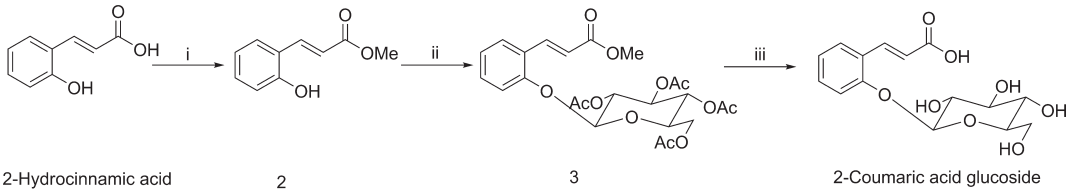

Figure 2. Synthesis of 2-coumaric acid glucoside.

Table 1. Sensory Evaluation of the Flowers Supplied with the Precursors of Volatile Compounds[a]

	Rosa "Rote Rose"	*Eustoma grandiflorum* "Forever Blue"	*Gerbera jamesonii* "Jaguar Deep Rose"	*Dianthus caryophyllus* "Barbara"	*Gypsophila elegans* "Covent Garden"	*Tulipa* "Greenland"	*Delphinium elatum* "Blue Bird"
2PEG	+	−	−	−	↓	−	−
2-coumaric acid glucoside	+	−	−	+	↓	+	++
trans-cinnamic acid	−	−	−	−	−	−	−
L-phenylalanine	+	−	+	+	−	−	−
shikimic acid	−	−	−	−	−	−	−

[a] −, No olfactory effect; ↓, Slight decrease in unpleasant odor; +, Very slight effect; ++, Significant olfactory effect. The precursors (10−30 mM) and sodium acetate (10 mM) were supplied to the flowers for 1 to 3 days.

"Blue Bird" supplied with 2-coumaric acid glucoside, while other attempts to modify the scent bouquet were less successful (**Table 1**). These could be due to several factors, for example, the absence of suitable enzymes for the introduced reaction, substrate specificity of scent enzymes, modification of the scent compound into a nonvolatile form, insufficient levels of emitted volatiles for olfactory detection by humans (or comparatively high olfactory threshold of produced volatiles), or masking of introduced compounds by other volatiles (*4*).

To evaluate whether 2-coumaric acid glucoside also could influence scent emission of other cultivars of delphinium flowers, *Delphinium grandiflorum* "Blue Butterfly" and *Delphinium grandiflorum* "Summer Morning" were used. Although amount of coumarin emitted from the flowers slightly increased after treatment with 2-coumaric acid glucoside, it had no significant olfactory effect on the both delphinium flowers.

On the basis of the olfactory detectable effect, in this work, the *Delphinium elatum* L. "Blue Bird" was used as a model to further study the emission mechanism of volatile compounds from the flowers supplied with the precursors.

Effects of 2PEG and 2-Coumaric Acid Glucoside Feeding on the Release of Volatile Compounds from *Delphinium elatum* L. "Blue Bird". In rose flowers, glycosides like 2-phenylethyl β-D-glucoside are stored inside the petals and can act as primary source of rhythmically emitted volatiles such as 2-phenylethanol, being released by the action of the β-glucosidase throughout the photoperiod (*9*). In the present work, using SPME technique (**Figure 1A**), 2-coumaric acid glucoside feeding resulted in the formation of coumarin in the *Delphinium elatum* L. "Blue Bird" (**Figure 3A**,**B**). Furthermore, 2-coumaric acid glucoside and 2PEG feeding enhanced the rhythmic emission of coumarin and 2-phenylethanol (**Figure 4A**,**B**) by the dynamic headspace volatile sampling analysis (**Figure 1B**). This indicates that some "scent enzymes" occur in some unscented flowers such as *Delphinium elatum* L. "Blue Bird", and the non- or low-scent emission of the flowers may be due to the lack of suitable substrates (or precursors of volatiles). However, in contrast to the produced coumarin, the formed 2-phenylethanol

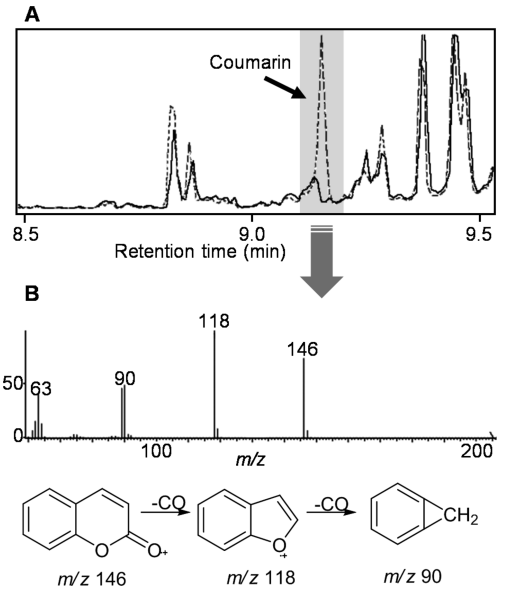

Figure 3. GC chromatogram (A) and mass spectrum (B) of coumarin released from the *Delphinium elatum* L. "Blue Bird" flowers placed in water (control, real line) and the mixed solution of 15 mM 2-coumaric acid glucoside and 10 mM sodium acetate (dashed line), respectively. After 20 h of treatment, a solid phase microextraction (SPME) technique was employed to collect the volatiles emitted from the flower for 8 h.

had no effect on the olfactory properties of the delphinium flower. Compared with olfactory threshold of coumarin (2.3×10^{-8} ppm in the air) (*10*), 2-phenyethanol shows comparatively high olfactory threshold (10 ppm in the air) (*11*). Indeed, a successful modification of the floral scent bouquet is not only to increase or change scent production. The reaction of human and insect olfactory system to the produced scents also greatly contributes to target selection. At present, success or nonsuccess in metabolic engineering of floral scent are always assessed on the basis of sensory evaluations by humans, whose odor

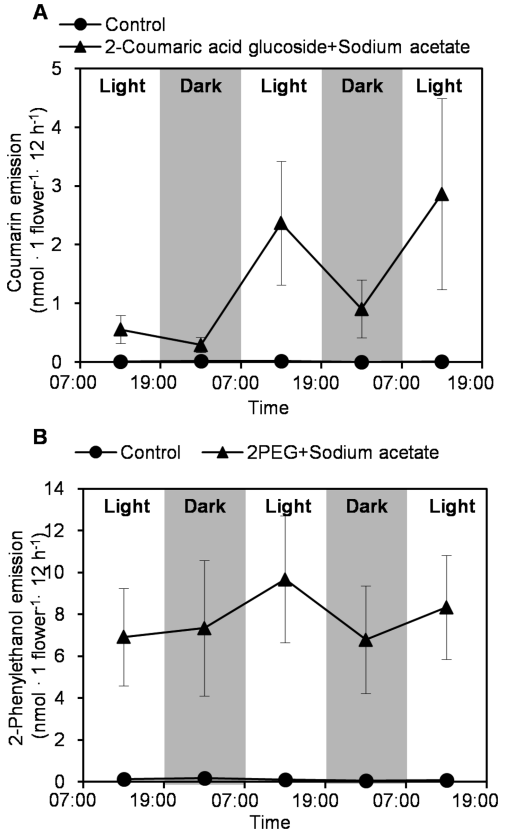

Figure 4. Effects of 2-coumaric acid glucoside (A) and 2PEG (B) on the emission of coumarin and 2-phenylethanol from the *Delphinium elatum* L. "Blue Bird" flowers, respectively. The flowers were placed in water (control), the mixture of 15 mM 2-coumaric acid glucoside or 2PEG, and 10 mM sodium acetate aqueous solution, respectively. Data are expressed as mean ± standard error (n = 3). After 20 h of treatment, a dynamic headspace volatile sampling technique was used to collect the volatiles emitted from the flower.

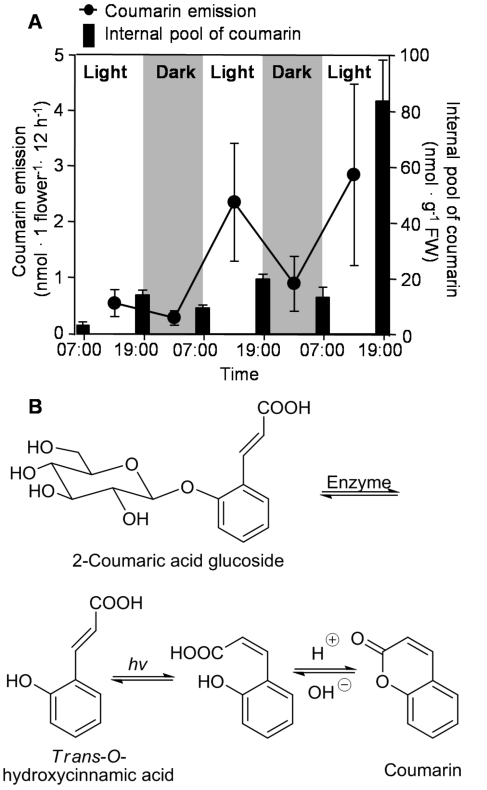

Figure 5. Rhythmic emission and internal pool of coumarin in the *Delphinium elatum* L. "Blue Bird" flowers supplied with 2-coumaric acid glucoside (A) and proposed pathway of coumarin formation in the plants (B, refer to ref *5*). (A) The flowers were placed in the mixture of 15 mM 2-coumaric acid glucoside and 10 mM sodium acetate aqueous solution. Data are expressed as mean ± standard error (n = 3). After 20 h of treatment, the flowers collected at 07:00 and 19:00 were used to the determination of internal pool.

threshold perception is much lower than that of most animals and insects (*2*, *12*). However, little is known about the impact of changes in the scent bouquet on insect and animal attraction (*2*).

Because coumarin was found to induce liver toxicity (*13*), the European Union Scientific Committee for Food set the maximum limits for coumarin of 2 mg/kg in foodstuffs and beverages with specific exceptions of 10 mg/kg in "certain" caramel confectionery and in alcoholic beverages (*14*). In the assessment of the safety of coumarin for human health, as discussed by Lake (*15*), the maximum daily human exposure to coumarin for a 60 kg consumer has been estimated to be 3524 μg coumarin/day (or 58.73 μg/kg body weight/day). Additionally, no adverse effects of coumarin have been reported in susceptible species in response to doses that are more than 100 times the maximum human daily intake (*15*). In this work, after chemical treatment, coumarin emitted from one flower ranges from 0.082 to 0.42 μg within 12 h (**Figure 4A**). It suggests that exposure to coumarin from a reasonable amount of the flowers depicted in this paper poses no health risk to humans.

Regulation of Emission of Scent Compound from the Delphinium Flower by the 2-Coumaric Acid Glucoside Feeding. In the *Delphinium elatum* L. "Blue Bird" flower without any treatment (control), the internal pool of coumarin varied from 0.35 to 0.86 nmol/g fresh weight during the 12 h light/dark photoperiod. After treatment with 2-coumaric acid glucoside, the internal pool of coumarin was significantly increased (3.4−83.4 nmol/g fresh weight) (**Figure 5A**). Moreover, it shows increases in the daytime and decreases in the nighttime, suggesting that the synthesis of coumarin is regulated by light. In the metabolic pathway leading from 2-coumaric acid glucoside to coumarin (**Figure 5B**, ref *5*), light can facilitate the isomerization of *trans*-*O*-hydroxycinnamic acid and thereby formation of coumarin. In addition, the coumarin exhibits rhythmic emission with a peak during the light period, which is in accordance with the change in internal pool of synthesized coumarin (**Figure 5A**). In nature, nocturnally pollinated flowers generally tend to have a maximum of scent emission during the dark period, which is controlled in most case by the endogenous circadian clock. In contrast, for diurnal pollinate flowers, the situation is reversed, which is controlled in most case directly by light (*16*, *17*). Recent studies also provide evidence that, in many cases, the scent emission regulation either by endogenous circadian clock or by light is controlled at the level of gene expression responsible for the scent formation (*18*). However, in some cases, the availability of substrates for the final step of scent formation in flowers determines the efficiency of their emission (*19*). For example, the rhythmic emission of geranyl acetate in hybrid rose flowers is regulated at the level of its substrate geraniol that can be controlled by light (*18*).

Our results demonstrate that changes in emission of coumarin from the flower coincided with the synthesized coumarin. However, because we know very little about scent emission, it remains to be determined whether coumarin is immediately emitted to the atmosphere instead of accumulating in the cells once the compound is synthesized, or when a certain threshold level of the compound is reached, flowers start a self-protection mechanism

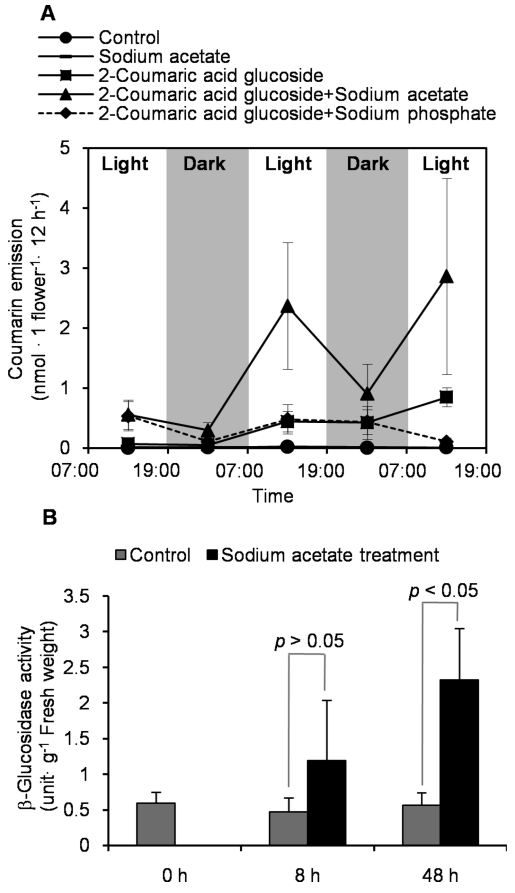

Figure 6. Effects of 2-coumaric acid glucoside individual feeding and synergy feeding with sodium acetate or sodium phosphate on the emission of coumarin from the *Delphinium elatum* L. "Blue Bird" flowers (A) and effect of sodium acetate on the activity of β-glucosidase of the delphinium flowers (B) . (A) The flowers were placed in water (control), 10 mM sodium acetate aqueous solution, 15 mM 2-coumaric acid glucoside aqueous solution, the mixture (pH 4.4) of 15 mM 2-coumaric acid glucoside and 10 mM sodium acetate aqueous solution, and the mixture (pH 4.4) of 15 mM 2-coumaric acid glucoside and a certain amount of sodium phosphate (NaH_2PO_4 for the pH adjustment) aqueous solution, respectively. Data are expressed as mean±standard error (n=3). After 20 h of treatment, a dynamic headspace sampling technique was used to collect the volatiles emitted from the flower. (B) The delphinium flowers were treated with water (control) and 10 mM sodium acetate at 22 °C under dark condition for 0, 8, and 48 h, respectively. Means are significantly different by Student's t test ($p < 0.05$). Data represent the mean ± standard error (n = 5).

to emit it in case that this much accumulation can be toxic to plant cells.

Synergy Effects of 2-Coumaric Acid Glucoside and Sodium Acetate on the Release of Coumarin from the Delphinium Flower. In the present study, the mixed feedings of 2-coumaric acid glucoside and sodium acetate (or sodium phosphate) were used from the delphinium flower. Interestingly, compared with the individual feeding, the combination of 2-coumaric acid glucoside with sodium acetate showed the synergy effect on enhancement of coumarin release from the flower, while the combination of 2-coumaric acid glucoside with sodium phosphate showed no significant effect (**Figure 6A**). To elucidate the contribution of sodium acetate to this synergy effect, the effect of sodium acetate on the activity of β-glucosidase of the delphinium flowers was examined in this study. As shown in **Figure 6B**, the activity of β-glucosidase of the flower was significantly increased after the 48 h treatment with sodium acetate. β-Glucosidase was suggested to be partly responsible for controlling the diurnal emission of 2-phenylethanol in the *Rosa damascena* (*20*) and play an important role in the emission of the floral scent compounds (*8*), although attempts to correlate glucosidase activity with rhythmic cycles in volatile emission from rose flowers were unsuccessful (*21*). It will be interesting to determine how sodium acetate influenced the activity of β-glucosidase. In addition, it remains to be determined whether this synergy effect of sodium acetate could be due to other possibilities. Acetate is the most linking between primary metabolism and secondary metabolism and occupies a central position in relation to the general metabolism of plants (*22*). Two entirely separate secondary biosynthetic routes, isoprenoids (i.e., terpenes, steroids, and carotenoids) and acetogenins (i.e., polyacetylenes, fatty acids, and polyketides) originate with acetate (*5*). Also, acetate condensation occurs in many possible routes, which give rise to variety of aromatic compounds. For example, isocoumarins, which are structurally related to the coumarins but with an inverted lactone ring, can derive from the acetate pathway (*23*). However, in the present work, feeding of sodium acetate itself did not significantly promote scent emission from the flower. It may be that sodium acetate or the activated intermediate acetyl-CoA become precursors of intermediates of carbohydrates, resulting in the activation of carbohydrate metabolism, thereby glycosidase and/or glycosyl transferase were activated to hydrolyze coumaric acid glucoside.

The loss of scent emission in flowers that can be due to the mutation or loss of the relevant biosynthesis genes was recently confirmed in flowers of petunia (*Petunia axillaris* subsp. *parodii*) (*24*). Isoeugenol synthase gene in *Petunia axillaris* subsp. *Parodii* contains a frame-shift mutation that renders it inactive, which leads to a decrease in isoeugenol biosynthesis. In addition, when isoeugenol synthase activity is reduced in *Petunia* hybrid, the coniferyl acetate substrate that would have been used by isoeugenol synthase is instead used by eugenol synthase, thereby the flowers synthesize higher levels of eugenol.

Our data shows that the supplement of nonvolatile compounds such as 2-coumaric acid glucoside to *Delphinium elatum* L. "Blue Bird" enhanced the emission of scent from the flower, indicating that the non- or low-scent of the flower also can be caused by the lack of suitable substrates. This result helps us with the better understanding of the mechanism formation in unscented flowers. Moreover, it suggests that metabolic engineering of the floral scent spectrum requires a more rational design based on the correct choice of species and complete understanding of metabolic pathways. In this work, it is interesting that the addition of sodium acetate has a synergetic effect on the release of coumarin from 2-coumaric acid glucoside, which is due to one of the factors that sodium acetate influenced the glucosidase activity. Some undesirable results obtained during the efforts to modify plant volatile profiles demonstrate that the complexity of the biosynthetic networks for plant volatiles. Therefore some combined strategies, such as enhancement of scent precursors and influence on the involved "scent enzyme", will help us to achieve desirable scent emission.

ABBREVIATIONS USED

CHAPS, 3-[(3-cholamidopropyl)-dimethylamino]-1-propanesulfonate; EDTA, ethylenediaminetetraacetic acid; GC-MS, gas chromatography−mass spectrometry; 2PEG, 2-phenylethyl α,β-D-glucoside; SPME, solid phase microextraction.

LITERATURE CITED

(1) Dudareva, N.; Negre, F. Practical applications of research into the regulation of plant volatile emission. *Curr. Opin. Plant Biol.* **2005**, *8* (1), 113–118.

(2) Pichersky, E.; Dudareva, N. Scent engineering: toward the goal of controlling how flowers smell. *Trends Biotechnol.* **2007**, *25* (3), 105–110.
(3) Vainstein, A.; Lewinsohn, E.; Pichersky, E.; Weiss, D. Floral fragrance. New inroads into an old commodity. *Plant Physiol.* **2001**, *127* (4), 1383–1389.
(4) Dudareva, N.; Pichersky, E. Metabolic engineering of plant volatiles. *Curr. Opin. Biotechnol.* **2008**, *19* (2), 1–9.
(5) Dewich, P. M., Ed.; Medicinal Natural Products: A Biosynthetic Approach, 2nd ed.; John Wiley & Sons Ltd: London, 2002; pp 8−12, pp 142−144.
(6) Yamazoe, A.; Hayashi, K.; Kuboki, A.; Ohira, S.; Nozaki, H. The isolation, structural determination, and total synthesis of terfestatin A, a novel auxin signaling inhibitor from *Streptomyces* sp. *Tetrahedron Lett.* **2004**, *45* (45), 8359–8362.
(7) Yang, Z. Y.; Kinoshita, T.; Tanida, A.; Sayama, H.; Morita, A.; Watanabe, N. Analysis of coumarin and its glycosidically bound precursor in Japanese green tea having sweet-herbaceous odour. *Food Chem.* **2009**, *114* (1), 289–294.
(8) Sakai, M.; Tomita, S.; Hirata, H.; Asai, T.; Dohra, H.; Hara, M.; Watanabe, N. Purification and characterization of β-glucosidase involved in the emission of 2-phenylethanol from rose flowers. *Biosci. Biotechnol. Biochem.* **2008**, *72* (1), 219–221.
(9) Picone, J. M.; Clery, R. A.; Watanabe, N.; MacTavish, H. S.; Turnbull, C. G. N. Rhythmic emission of floral volatiles from *Rosa damascena semperflorens* cv. "Quatre Saisons". *Planta* **2004**, *219* (3), 468–478.
(10) Frérot, E.; Bagnoud, A.; Vuilleumier, C. Menthofurolactone: a new *p*-methane lactone in *Mentha piperita* L.: analysis, synthesis and olfactory properties. *Flavour Fragrance J.* **2002**, *17* (3), 218–226.
(11) Sayama, H. Chemical synthesis of ^{13}C-shikimic acid and its metabolism in rose petals (*in Japanese*). *Master Dissertation*, Shizuoka University, Japan, **2008**.
(12) Stockhorst, U.; Pietrowsky, R. Olfactory perception, communication, and the nose-to-brain pathway. *Physiol. Behav.* **2004**, *83* (1), 3–11.
(13) Hazleton, L. W.; Tusing, T. W.; Zeitlin, B. R.; Thiessen, R., Jr.; Murer, H. K. Toxicity of coumarin. *J. Pharmacol. Exp. Ther.* **1956**, *118*, 348–358.
(14) European Communtiy Council Directive. A constituent of natural favouring source materials limited by Annex II of Flavourings Directive 88/388/EEC. **1988**. http://ec.europa.eu/food/fs/sfp/addit_flavor/flav09_en.pdf (accessed June 1, 2009).
(15) Lake, B. G. Coumarin metabolism, toxicity and carcinogenicity: relevance for human risk assessment. *Food Chem. Toxicol.* **1999**, *37* (4), 423–453.
(16) Matile, P.; Altenburger, R. Rhythms of fragrance emission in flowers. *Planta* **1988**, *174* (2), 242–247.
(17) Loughrin, J. H.; Hamilton-Kemp, T. R.; Andersen, R. A.; Hildebrand, D. F. Volatiles from flowers of *Nicotiana sylvestris, N. otophora* and *Malus domestica:* headspace components and day/night changes in their relative concentrations. *Phytochemistry* **1990**, *29* (8), 2473–2477.
(18) Hendel-Rahmanim, K.; Masci, T.; Vainstein, A.; Weiss, D. Diurnal regulation of scent emission in rose flowers. *Planta* **2007**, *226* (6), 1491–1499.
(19) Dudareva, N.; Pichersky, E. Floral scent metabolic pathways: their regulation and evolution. In *Biology of Floral Scent, Section II: Biochemistry and Molecular Biology of Floral Scent*; Dudareva, N., Pichersky, E., Eds.; CRC Press Taylor & Francis Group: Boca Raton, FL, 2006; pp 55−78.
(20) Hayashi, S.; Yagi, K.; Ishikawa, T.; Kawasaki, M.; Asai, T.; Picone, J.; Turnbull, C.; Hiratake, J.; Sakata, K.; Takada, M.; Ogawa, K.; Watanabe, N. Emission of 2-phenylethanol from its β-D-glucopyranoside and the biogenesis of these compounds from [$^2H_8$] L-phenylalanine in rose flowers. *Tetrahedron* **2004**, *60* (33), 7005–7013.
(21) Baldermann, S.; Yang, Z. Y.; Sakai, M.; Fleischmann, P.; Watanabe, N. Volatile constituents in the scent of roses. In *Floriculture and Ornamental Biotechnology: Special Issue 1: Roses*; Teixeira da Silva, J. A., Ed., Zlesak, D. C., guest editor; Global Science Books: London, 2009; pp 89−97.
(22) Seigler, D. S., Ed.; Plant secondary metabolism. Kluwer Academic Publishers: Norwell, MA, 1995.
(23) Hill, R. A. Naturally occurring isocoumarins. *Fortschr. Chem. Org. Naturst.* **1986**, *49*, 1–78.
(24) Koeduka, T.; Orlova, I.; Baiga, T. J.; Noel, J. P.; Dudareva, N.; Pichersky, E. The lack of floral synthesis and emission of isoeugenol in *Petunia axillaris* subsp. *parodii* is due to a mutation in the isoeugenol synthase gene. *Plant J.* **2009**, doi: 10.1111/j.1365−313X.2009.03834.x.

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