

# Phytohormone-Mediated Induction of Red Colour in the Flower Florets of a Cultivar of Dyer's Saffron (*Carthamus tinctorius*)

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Z. Naturforsch. **53c**, 828–832 (1998); received August 14, 1997/April 16, 1998

Mogami-Benibana, Dyer's Saffron (*C. tinctorius*) Cultivar, Red Colour Appearance, Flower Floret, Phytohormone Mediated Induction

Gibberellic acid was fed to a homogenate of Mogami-Benibana, a cultivar of dyer's saffron (*Carthamus tinctorius*), at a 100  $\mu\text{M}$  level. After incubation for 60 min at 25 or 37 °C, the effect of externally supplied gibberellic acid on enzyme activities and the glucose level were investigated. Gibberellic acid enhances the activity of glucose oxidase by about 52%. It induces also a weak (9%) increase of glucosidase activity. Gibberellic acid increases the glucose content by about 6% during the initial 30-min incubation. The results relate to the red colour appearance in Mogami-Benibana at the later stage of blooming.

## Introduction

Mogami-Benibana is a cultivar of dyer's saffron (*C. tinctorius*) which is known to shift the flower colour conspicuously. This cultivar specific colour shift appears usually at the later phase of the flowering stage, changing slowly its original bright-yellow colour to rusty-red. This colour change is triggered directly by the pollination process, at which pistils elongate rapidly projecting over the capitula through a strangulated tubular region of the florets, inside of which many thick stamens are grown (Fukushima *et al.*, 1997). For the shift of the flower colour, various enzyme reactions are involved. Glucose oxidase (EC 1.1.3.4) has been shown to play a leading role in the red colour shift of Mogami-Benibana (Saito, 1992; 1993a). Galactose oxidase (EC 1.1.3.9) (unpublished results), alcohol oxidase (EC 1.1.3.13) (Saito and Utsumi, 1995a), glycolate oxidase (EC 1.1.3.1) (Saito and Utsumi, 1995b) and amino acid oxidase (EC 1.4.3.2) (Saito and Utsumi, 1995c) are also cooperative to the red shift reaction. For the completion of the enzyme catalyses, above oxidases generate hydrogen peroxide as an obligatory by-product, which reacts

with precarthamin to form carthamin in the floral tissues (Saito and Takahashi, 1985; Saito, 1993b). After pollination, pollen grains germinate on the stigma surface of pistils and insert their pollen tubes elongating in styles towards ovules. During these processes, various changes are introduced, namely secretion of hormones, induction of hydrolytic enzymes, abnormal relaxation of stylar tissues and so on. At this stage, a yellow precarthamin is entangled in the metabolic turnover systems, where it is oxidized to a red carthamin dye and served as a direct colour shift inducer. Once formed carthamin is fixed accumulating in floral tissues and thus the cultivar specific red colour shift is intensified gradually. To clarify the mechanism of the red colour shift, it seemed necessary to investigate hormonal effects on the colour modification process. However, only one preliminary work is found in the literature (Fukushima *et al.*, 1997).

The current communication deals with administering  $\text{GA}_3$  to a floral homogenate of Mogami-Benibana, demonstrating that  $\text{GA}_3$  is an inducer of the activities of glucosidase and glucose oxidase.

## Materials and Methods

### Chemicals

$\text{GA}_3$  and glucose were supplied by Kanto Kagaku (Tokyo, Japan). 4-Nitrophenyl- $\beta$ -D-glucopyranoside, *o*-dianisidine, citric acid, sodium borate,

**Abbreviations:**  $\text{GA}_3$ , gibberellic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MG, methyl- $\beta$ -D-glucoside; UV, ultraviolet light; VIS, visible light.

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borax, pyridine, acetic anhydride and acetone were obtained from Wako Pure Chemical (Osaka, Japan). HEPES was purchased from Dojin (Tokyo, Japan). ATP and NADP<sup>+</sup> were purchased from Oriental Kobo (Tokyo, Japan). Hexose kits were obtained from Gasukuro Kogyo (Tokyo, Japan). Hexokinase (EC 2.7.1.1) from yeast (specific act. 450 units/mg) was supplied by Boehringer Mannheim (Mannheim, Germany).

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from *Bacillus stearothermophilus* (specific activity 100 units/mg) was obtained from Seikagaku Kogyo (Tokyo, Japan). Avicel cellulose was a product of Asahi Kasei Kogyo (Tokyo, Japan). Amberlite IR-118 (H<sup>+</sup>-form) was furnished by Organo Kogyo (Tokyo, Japan). Other chemicals and reagents used were obtained from several commercial sources.

#### *Plant material*

The seeds of Mogami-Benibana were purchased from a market (Yamagata, Japan) and sown in our experimental field on April 21, 1993. After about three months of cultivation, the plants grew up to 70–80 cm height with spiny globular flowering heads. The plant materials were collected from the pre-opened immature heads and placed immediately in a vacuum desiccator. The desiccator was evacuated several times by a high-vacuum oil pump and stored in a freezer at  $-40^{\circ}\text{C}$  in the dark just before applying for the experiments.

#### *Administration of GA<sub>3</sub> to flower homogenates*

Young florets (0.2 g fresh wt. each), whose ovaries were cut off, were put in Thunberg tubes, in which 5 ml 100  $\mu\text{M}$  GA<sub>3</sub> had previously been supplied. Evacuation was carried out from five to seven times with a high-vacuum rotary pump to infiltrate GA<sub>3</sub> and incubated, if not mentioned otherwise, for 30 min at  $30 \pm 1^{\circ}\text{C}$  by gentle agitating at 80 strokes/min in a personal shaker. After incubation, the flowers were transferred to a mortar containing liquid nitrogen and crushed with a pestle to fine powders. The frozen powders were thawed in 5–6 ml 50 mM citrate/phosphate buffer, pH 7.0 and transferred to centrifuge teflon tubes. Centrifugation was performed at  $22,000 \times g$  for 10 min and the supernatant (4–5 ml) was passed through a column (4  $\times$  70 mm) of active carbon at

$5 \pm 1^{\circ}\text{C}$ . The decolourized flower liquid (3 ml) was placed immediately in a Thunberg tube filled with nitrogen gas and kept in the dark at  $5 \pm 1^{\circ}\text{C}$  just before using for the processes of enzyme activity determination or other assays.

#### *Assay of enzyme activities*

1. *Glucosidase*. Incubation mixtures were composed of 2 mM 4-nitrophenyl- $\beta$ -D-glucopyranoside and 100 mM phosphate buffer, pH 7.0 in a total volume of 1.2 ml and adjusted to  $37 \pm 1^{\circ}\text{C}$ . The enzyme reaction was started just after addition of 0.5 ml decolourized flower liquid (protein 50–100  $\mu\text{g/ml}$ ) at  $37 \pm 1^{\circ}\text{C}$ . After incubation for 15 min, the enzyme reaction was stopped by adding 20 mM sodium carbonate (2 ml) into the incubation mixture. Absorbance changes at 400 nm were monitored with a Hitachi UV/VIS spectrophotometer, model U-1100. One unit of glucosidase was defined as the amount that generated p-nitrophenol per min ( $\mu\text{mol/mg protein} \times \text{min}$ ). The experimental data were obtained from six to seven repetitions.

2. *Glucose oxidase*. The incubation mixture contained the following components. 0.2 mM *o*-dianisidine (O<sub>2</sub> gas was charged for 5 min), 560 mM glucose and 100 mM phosphate buffer, pH 7.0 in a total volume of 3.0 ml and adjusted to  $25 \pm 1^{\circ}\text{C}$ . The enzyme reaction was started after addition of 0.5 ml decolourized flower liquid (protein 50–100  $\mu\text{g/ml}$ ) for 5 min at  $25 \pm 1^{\circ}\text{C}$ . The absorbance changes at 436 nm were monitored with a Hitachi UV/VIS double-beam spectrophotometer, model U-1100. One unit of glucose oxidase was defined as the amount that produced D-glucono- $\delta$ -lactone per minute ( $\mu\text{mol/mg protein} \times \text{min}$ ). The data were obtained from six to seven repetitions.

3. *Measurement of protein*. Protein was determined after Lowry *et al.* (1951).

#### *Assay of glucose content*

Glucose contents in the test flower liquid were determined after Bergmeyer *et al.* (1974).

#### *Gas chromatography*

Young florets (0.32 g each), whose ovaries were cut off, were placed in a mortar, to which 8 ml 80% (v/v) methanol had been added and ground

for 10 min. The slurries were transferred to teflon tubes. Centrifugation was carried out at  $4,000 \times g$  for 10 min. The supernatant was evaporated to dryness at less than  $35^\circ\text{C}$ . The dark brown residue was suspended in distilled water (24 ml) and extracted with chloroform (4 ml). After removing chloroform, the residue was mixed with an internal standard solution (5 ml, 10 mg MG is contained) and sodium borate solution (3 ml, containing 40 mg sodium borate). The mixtures were left for 60 min at  $25 \pm 1^\circ\text{C}$ . Excess sodium borate was removed by passing through a column ( $1.0 \times 55$  cm, bed vol. 40 ml) of a cation exchanger, Amberlite IR-118 ( $\text{H}^+$ -form) and evaporated to dryness. To remove boric acid, evaporation was repeated further three times with each fresh glass-distilled methanol (5 ml each). For acetylation, the sugar extracts were mixed with acetic anhydride/pyridine (1:1, v/v) (2 ml each) for 10 h at  $30 \pm 1^\circ\text{C}$ . The resulting acetylated sugars ( $\mu\text{l}$ ) were used for gas chromatography. A Hitachi, model 263-50 (Hitachi, Tokyo, Japan) was used equipped with a glass column ( $3 \times 1000$  mm) and a column packing, 3% ECNSS-M Uniport HP 60-80 mesh (GL Science, Tokyo, Japan).

## Results and Discussion

Flower colour shift is a characteristic property of Mogami-Benibana for manifesting it at the later phase of the flowering stage. It shows up first in a constricted region of the bright-yellow tubular flowers closely associated with the pollination process. In our previous study, we have carried out feeding experiments using IAA and  $\text{GA}_3$  at 1–1000  $\mu\text{M}$  levels. Externally supplied 100  $\mu\text{M}$   $\text{GA}_3$  enhances the induction of the red colour most prominently, while IAA shows no such effect (Fukushima *et al.*, 1997). To establish the collaboration of phytohormones with the flower colour shift, feeding tests with  $\text{GA}_3$  have been performed once again at a 100  $\mu\text{M}$  dosage. Figure 1 illustrates the changes in three monosaccharides of immature flower florets collected from pre-opened flowering heads of three different sizes (group 1,2,3). This preliminary result shows that, before blooming, the monosaccharide content changes considerably. Mannose increases gradually during flower head elongation (relative ratios of group 1, group 2 and group 3, 1:2.5:6.6). Galactose, on the other hand, is reduced (1:0.7:0.5). A marked increase of glucose is observed (1:2.2:564). Table I lists data from a gas chromatographic analysis. It supports the re-

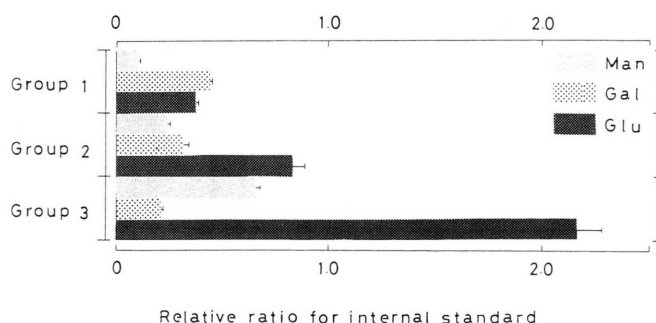


Fig. 1. Changes in the contents of monosaccharides in pre-mature florets of Mogami-Benibana of various lengths. Pre-mature florets were collected from flowering heads which are at pre-blooming stage and divided into three groups by their lengths (mm): group 1 = 5–7, group 2 = 10–12, group 3 = 15–17. After removing their ovaries, each 0.32 g floret was treated to prepare the starting materials for gas chromatography. For details of the experimental conditions, see Materials and Methods. Relative ratio shows the value calculated in proportion to that of an internal standard. Man: mannose, Gal: galactose, Glu: glucose.

Table I. Changes in the content of monosaccharides in pre-mature floret extracts of Mogami-Benibana.

Group*	Mannose		Galactose		Glucose	
		Ratio		Ratio		Ratio
1	0.050	1.0	0.241	1.0	0.196	1.0
2	0.257	5.1	0.131	0.54	0.909	6.5
3	0.684	13.7	0.069	0.29	114.5	589.2

\* Lengths of florets used were (mm): group 1 = 5–7, group 2 = 10–12, group 3 = 15–17. The value of each monosaccharide shows in proportion to that of an internal standard.

sults of Fig. 1, showing the same tendency for the quantitative changes in monosaccharides. The relationship between changes in glucose contents and incubation times is illustrated in Fig. 2. GA<sub>3</sub> fed at a 100 µM level, elevates glucose content a little (about 6%) at the initial 60 min incubation. The externally supplied GA<sub>3</sub> promotes the activity of glucosidase by about 9% after incubation for 60 min (Fig. 3). A striking increase in the activity of glucose oxidase is induced by GA<sub>3</sub> at 100 µM dosage (see Fig. 3). Incubation for 60 min, the oxidase activity is enhanced by about 52%. These results indicate that GA<sub>3</sub> is an efficient inducer for both enzyme activities. No check for the activity of enzymes not related to abolism has been done in the present study.

The internal glucose released by a certain phytohormones including GA<sub>3</sub> can be served as an en-

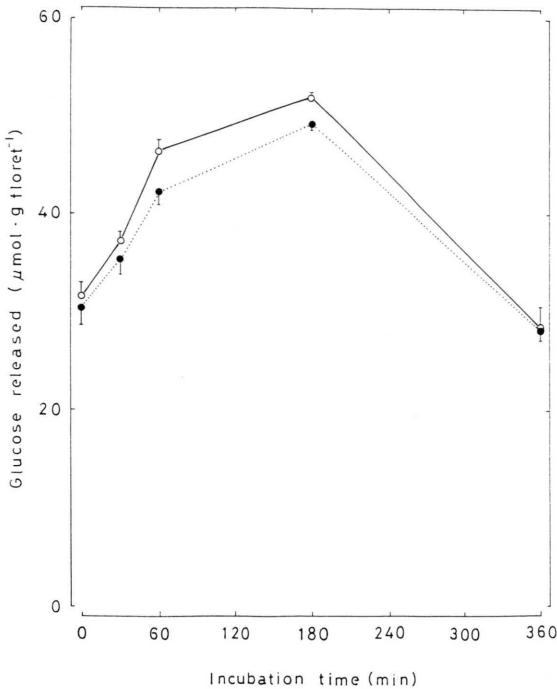


Fig. 2. Effect of GA<sub>3</sub> on glucose contents. Young florets (0.2 g fresh wt. each), whose ovaries were cut off, were used to prepare floret extracts. For details of the experimental conditions, see Materials and Methods. ○—○: GA<sub>3</sub> treated sample, ●—●: control sample.

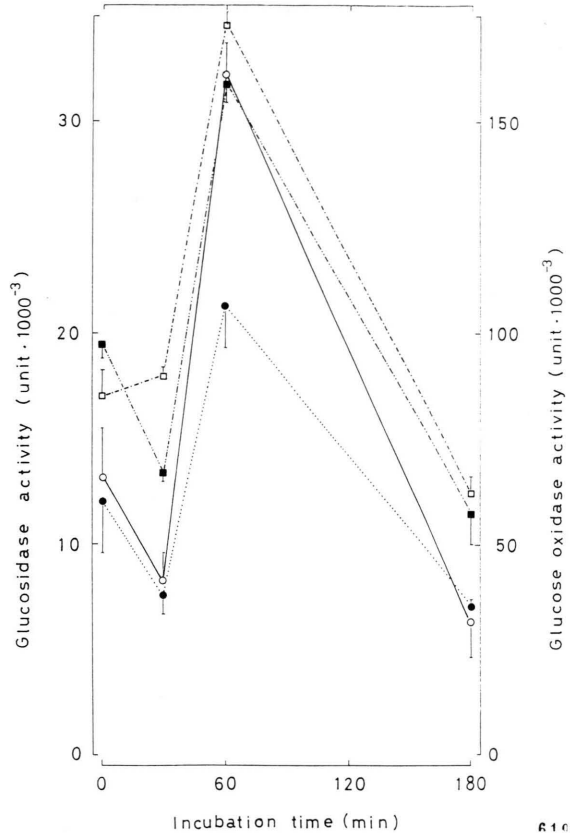


Fig. 3. Effect of GA<sub>3</sub> on the changes in activities of glucosidase and glucose oxidase. Young florets (0.2 g fresh wt. each), whose ovaries were cut off, were put into Thunberg tubes containing 5 ml 100 µM GA<sub>3</sub>, then the florets were treated as shown in Materials and Methods to prepare floret extracts for assaying enzyme activities. □—□: glucosidase activity, ■—■: control, ○—○: glucose oxidase activity, ●—●: control.

zyme substrate of glucose oxidase. Under aerobic conditions, glucose oxidase generates D-glucono-δ-lactone and hydrogen peroxide. Hydrogen peroxide reacts with a yellow precarthamin and accumulates a red carthamin dye, through the process of which red colour shift is visualized in the floral tissues (Saito, 1993a; b).

This is the first evidence that GA<sub>3</sub> is an efficient inducer for the red colour shift of flowers in *Mogami-Benibana*. More detailed studies on hormonal control of the flower colour shift is in progress in our laboratory.

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