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A flavonoid from *Brassica rapa* flower as the UV-absorbing nectar guide

Katsunori Sasaki[a], Takashi Takahashi[b,*]

[a]*Research Institute of Seed Production Co., Ltd., 6-6-3 Minamiyoshinari, Aoba-ku, Sendai, 989-3204, Japan*
[b]*Watanabe Seed Co., Ltd., 109 Machiyashiki, Kogota-cho, Miyagi 987-8607, Japan*

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Abstract

The corolla of *Brassica rapa* has an UV-absorbing zone in its center, known as the nectar guide for attracting pollinating insects. The pigment which plays the role of the nectar guide was isolated from the petals and identified to be isorhamnetin 3,7-*O*-di-β-D-glucopyranoside on the basis of MS and NMR spectroscopic data. The D-, L-configurations of the sugar moieties were determined by the fluorometric HPLC method. In plants raised in open field, there was a 13-fold higher content of the compound in the basal parts of the petals compared with the apical parts. This difference in flavonoid content is presumed to contribute to the visual attractiveness of *B. rapa* flowers to insect pollinators.

Keywords: *Brassica rapa*; Cruciferae; Corolla; UV absorption; UV-B; Nectar guide; Pollination; Flavonoids; Isorhamnetin 3,7-*O*-di-β-D-glucopyranoside

1. Introduction

The group *Brassica rapa* includes many significant crops, such as Chinese cabbage, which is important in China, Korea, Japan and other Asian countries. Its recent popularity has resulted in a considerable increase in its production in Europe and in the USA. Additionally, the turnip has been known to the Greeks and Romans since the pre-Christian era, and has since spread worldwide. Other local leafy vegetables such as komatsuna and pak-choi are widely distributed and consumed in many regions. Furthermore, seed extracts have been used as both edible and/or industrial oils since the ancient times.

The most important commercial cultivars of *B. rapa* in Japan are the F_1 hybrids possessing a self-incompatibility system (Bateman, 1955; Takasaki et al., 2000; Sakamoto and Nishio, 2001), which prevents self-fertilization. The hybrids are produced by the activities of insect pollinators such as honey bees, which recognize the flowers by some guiding factors (Wyatt, 1983) such as scent (Omura et al., 1999), patterns and markings in the UV or in the visible range (Thompson et al., 1972; Horovitz and Cohen, 1972; Utech and Kawano, 1975; Tanaka, 1982; Kandori and Ohsaki, 1998; Gronquist et al., 2001). To the human eye, the corolla of *B. rapa* appears uniformly bright yellow, but harbors an UV-absorbing zone in its center giving contrast against the other areas (Omura et al., 1999). The areas located near the nectary, known as the nectar guide (Thompson et al., 1972; Horovitz and Cohen, 1972; Utech and Kawano, 1975; Tanaka, 1982; Omura et al., 1999; Gronquist et al., 2001), are only visible and of orientation value to pollinating insects that land on the flower in search for their food. The visiting frequency of the pollinators influences the amount of hybrid seed production. Although much is known about the floral pigments responsible for the visible colors of flowers, in the case of *B. rapa* the chemical basis of UV absorption in the nectar guide has remained unexplained. In the present study we report the characterization of a compound as the UV nectar guide in *B. rapa*.

2. Results and discussion

The UV photographs of the petals of *B. rapa* reveal conspicuous UV nectar guides in the basal parts of the

* Corresponding author. Tel.: +81-229-31-1683; fax: +81-229-31-1683.
E-mail address: takaws@orion.ocn.ne.jp (T. Takahashi).

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corollas (Fig. 1). The left portion of Fig. 1 shows the flowers raised in the glasshouse for a forced breeding of new cultivars, and the other in the open field for F_1 seed production. The absorption spectra of the basal and apical extracts by MeOH are shown in Fig. 2A. Only the basal extract absorbed UV ray near 360 nm, an established attractant wavelength for many insects, particularly bees (Utech and Kawano, 1975), while the absorptions due to carotenoids, absorbing visible rays in the range of 400–500 nm with three characteristic maxima, were measured equally in both extracts. In their difference spectrum, setting the apical extract as a reference, two absorption maxima emerged at 255 and 355 nm (Fig. 2B). These absorptions, which correspond with the UV photographs (Fig. 1), are typical for flavonoids. TLC of the basal extract showed one spot (R_f 0.47; *n*-BuOH–HOAc–H_2O 6:1:2), which was absent from the apical extract. It absorbed UV and turned pale yellow with NH_3 vapor. These results indicate the involvement of a flavonoid as the compound responsible for the UV nectar guide.

The concentrated methanolic extract from petals of *B. rapa* was dissolved in *n*-hexane and extracted with 80% MeOH/H_2O three times. The residue of the aqueous methanolic fraction was washed with water, and then extracted with MeOH. Subsequently, compound **1** (Fig. 3) was isolated as yellow powder by reversed-phase HPLC.

By comprehensive interpretation of HR–FABMS, ^{1}H NMR, ^{13}C NMR, DQF–COSY, HSQC, HMBC spectra, and the fluorometric HPLC analysis to determine the absolute configurations of sugars, compound **1** was identified as isorhamnetin 3,7-*O*-di-β-D-glucopyranoside (Fig. 3).

The HR–FABMS showed a molecular ion $[M+H]^+$ at *m*/*z* 641.1719, corresponding to a molecular formula $C_{28}H_{33}O_{17}$. The ^{1}H and ^{13}C NMR spectral data for **1** are shown in Table 1. The connectivities around the quaternary carbons were established by the HMBC data. Although no correlations to C3 and to C4 were observed, they were assigned by default as the two remaining unassigned carbons; the oxygenated enolic carbon and the conjugated carbonyl carbon, respectively. The positions of both a methoxy group and two glycosidic residues were deduced from cross peaks due to $_3J_{CH}$ between C3′/OMe3′, C3/H1″ and C7/H1‴ in the HMBC spectra. Although the cross peaks of H2″/H3″ and H2‴/H3‴ were highly overlapped in the DQF–COSY spectra, the connectivities of the two sugars were individually assignable due to the separate cross peaks of C1″/H2″ and C1″/H3″ in the HMBC spectra.

The coupling constants (Table 1) of each oxymethine proton of the sugars were consistent with axial configurations involving H2″ (9 and 7 Hz), H3″ (10 and 9 Hz) and H2‴ (9 and 8 Hz). Particularly in the complicated region, proton decoupling difference experiments were carried out, although the anisotropy effects of the flavonoid skeleton, which made these proton signals separate relatively. The data from these experiments concluded the two sugars to be glucoses. The coupling constants of the anomeric protons (H1″ and H1‴) were observed at 7 and 8 Hz, respectively, indicating β-orientations of their glucosidic bonds. Thus two sugar moieties were identified only by the NMR spectroscopic data without the hydrolysis. The absolute configurations of the two glucoses were identified to both be D-isomers by HPLC analysis using a chiral reagent, (+)-2-methyl-2-β-naphthyl-1, 3-benzodioxole-4-carboxylic acid [(+)-MNB carboxylic acid] (Bai et al., 1997). This method was successfully applied to a very small amount of **1**, 33 μg (52 nmol).

According to the quantitative HPLC analytical data, the basal parts of the petals contained 4200±190 ppm (w/w) of **1**, whereas the content of **1** in the apical parts were 330±99 ppm (w/w), in the case of plants raised in the open field (Fig. 1, right).

Although **1** is a known flavonoid previously reported in the Crassulaceae (Wolbis and Krolikowska, 1988), the Zygophyllaceae (Al et al., 1988), the Balanitaceae (Maksoud et al., 1988), and the Cruciferae (Durkee and Harborne, 1972; Classen and Nozzolillo, 1980; Aguinagalde, 1988), in relation to chemotaxonomy, this is the first report of **1** involvement as nectar guides of *B. rapa* flowers. The UV profile of **1** showing two absorption

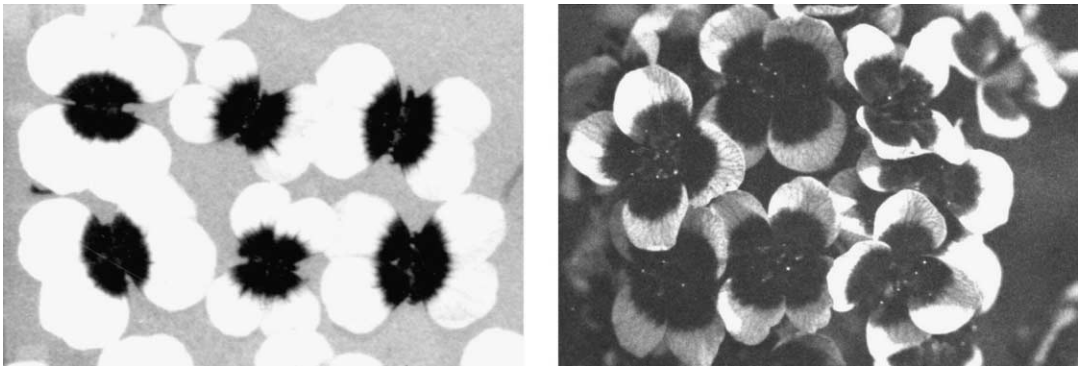

Fig. 1. UV photographs of *Brassica rapa*. Left: corollas raised in a glasshouse. Right: those raised in an open field.

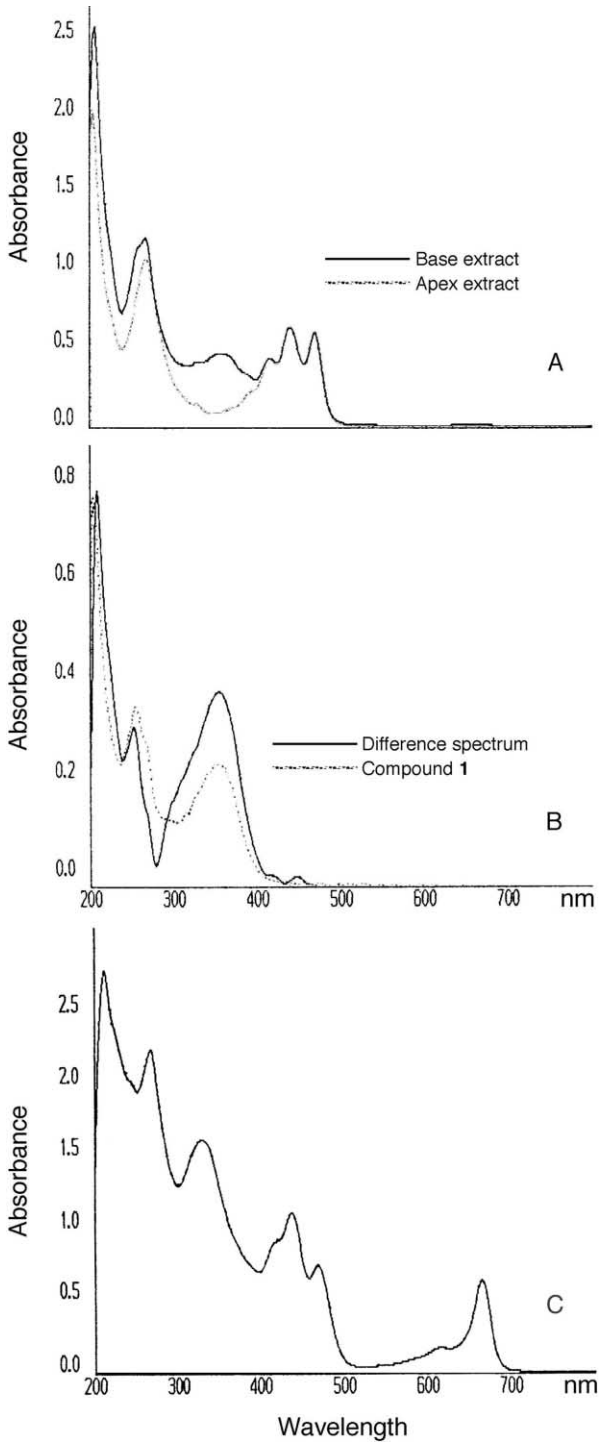

Fig. 2. UV and visible absorption spectra: (A) methanol extracts of petal bases and apices; (B) difference spectrum and compound **1**; (C) methanol extracts of leaves.

maxima, at 255 and 355 nm (Fig. 2B), similar to the pattern observed in the difference spectrum between the basal and apical petals (Fig. 2B), whereas that of the leaves (Fig. 2C) was very different (Fig. 2A, B). In addition, the HPLC analysis exhibiting the peaks due to the flavonoids from the methanolic extracts of the leaves was complicated where **1** was detected as a minor compound.

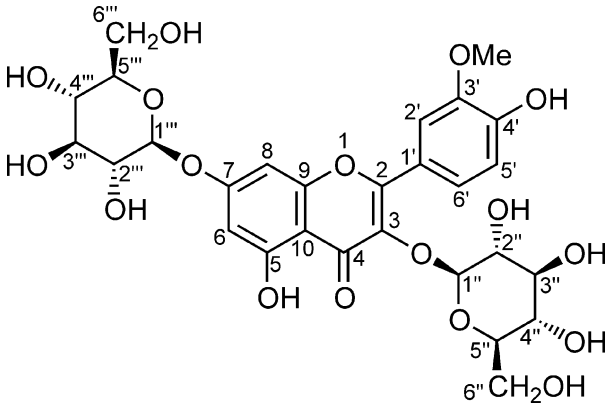

Fig. 3. Compound **1** from nectar guide of *Brassica rapa*.

This result shows that in the center of the corolla, a visual attractant composed of **1**, would be contributing to attract pollinating insects toward *B. rapa* flowers.

In a previous investigation by Thompson et al. (1972), three flavonols were identified as the UV nectar guides of *Rudbeckia hirta*. Additionally a recent investigation (Gronguist et al., 2001) reported UV pigments from several flowers, and identified various flavonoids and dearomatized isoprenylated phoproglucinols.

It is established that the biosynthesis of flavonoids is enhanced by UV-B exposure (Koes et al., 1994; Konishi et al., 1996). In the case of *B. rapa* flower, where the pigment playing the role as UV nectar guide is a flavonoid, the nectar guide pattern, the area and content of **1** was highly influenced by UV exposure. Indeed, in the left hand picture of Fig. 1, representing the corollas raised in the glasshouse with no UV exposure, the area of nectar guide was smaller than those raised in an open field (right). In the petals shown in Fig. 1 (left), the contents of **1** at the basal parts analyzed by HPLC were 1400 ± 70 ppm (w/w), whereas that of **1** in the apical parts were 73 ± 8.2 ppm (w/w). Both values were less than those of flowers raised in the open, which where 4200 ± 190 ppm (w/w) (basal) and 330 ± 99 ppm (w/w) (apical), attributable to the difference in UV-B exposure. In the open fields, where the production of F_1 hybrid seeds were carried out, for insect pollinators both wide and dense UV nectar guides in the corollas as shown the right of Fig. 1 seemed to confer greater visual attractiveness, which influenced their visiting frequency.

3. Experimental

3.1. General

All reagents were analytical grade unless otherwise stated. UV photographs were taken with a Nikon F-801S camera using a UV transmitting filter (Nikon UV-Nikkor, 105 mm, f/4.5), which has a transmission band centered on 330 nm, and transmits UV rays at

Table 1
NMR spectroscopic data for compound **1**[a]

Position	δ_H	δ_C	HMBC correlations[b]
2		160.2	
3		136.2	
4		180.3	
5		163.6	
6	6.48 *d* (2)	101.6	5, 7, 8, 10
7		165.5	
8	6.77 *d* (2)	96.5	6, 7, 9, 10
9		158.8	
10		108.4	
1′		123.0	
2′	7.93 *d* (2)	115.1	2, 1′, 3′, 4′, 6′
3′		149.5	
3′-OMe	3.93	57.5	3′
4′		153.0	
5′	6.88 *d* (8)	117.1	1′, 3′, 4′
6′	7.62 *dd* (8, 2)	124.9	2, 2′, 4′
1″	5.45 *d* (7)	104.1	3
2″	3.47 *dd* (9, 7)	78.8	1″
3″	3.49 *dd* (10, 9)	75.5	1″, 2″, 4″
4″	3.39 *dd* (10, 10)	72.0	2″, 5″
5″	3.53 *ddd* (10, 6, 2)	79.2	6″
6″	3.69 *dd* (12, 6)	63.3	
	3.91 *dd* (12, 2)		
1‴	5.06 *d* (8)	102.4	7
2‴	3.48 *dd* (9, 8)	78.6	1‴
3‴	3.44 *dd* (9, 9)	76.7	2‴, 4‴
4‴	3.30 *dd* (10, 9)	72.3	2‴
5‴	3.23 *ddd* (10, 5, 2)	79.4	4‴
6‴	3.55 *dd* (12, 5)	63.2	4‴, 5‴
	3.73 *dd* (12, 2)		

[a] Spectra were measured in CD_3OD at 600 MHz for ^1H and at 150 MHz for 13C; $CD_2\underline{H}OD$ (3.31 ppm in ^1H NMR) and $\underline{C}D_3OD$ (50.0 ppm in ^{13}C NMR) were used as internal standards. Chemical shifts are expressed in ppm; coupling constants in parentheses are in Hz.
[b] Protons correlated with carbons on position number.

220–420 nm, to eliminate visible rays. TLC was carried out on precoated silica gel 60 F_{254} plates (MERCK, Germany). UV and visible spectra were recorded on an Amersham Pharmacia Biotech Ultrospec 3000. The HPLC system consisted of a Hitachi L-7100 pump, a Hitachi L-7420 UV–visible detector, a Hitachi F-1000 fluorescence spectrophotometer and a Hitachi L-7300 column oven (Tokyo). The separation column used was either a Cosmosil 5C18AR (0.46×25 cm, Nacalai Tesque) or a Develosil 60-3 (0.46×25 cm, Nomura Chemical). HR–FABMS spectrum was measured on a Jeol JMS-700 (70 eV), and NMR spectra were on a Varian UNITY INOVA-600 at 20 °C in CD_3OD. (+)-MNB carboxylic acid was a gift from Dr. Ohrui and Dr. Akasaka of Division of Life Science, Graduate School of Agricultural Science, Tohoku University.

3.2. Plant material

Petals were obtained from *B. rapa* (spp. *pekinensis*) which had been raised in a greenhouse, utilizing an UV transmitting (above 360 nm) polyethylene terephthalate film (Shikusuraito®, Mitsubishi chemical MKV) in the Kogota district (Miyagi prefecture, Japan). Petals were not collected from plants raised in open fields since those were being used for F_1 seed production.

3.3. Extraction and isolation

Fresh petals (30.1 g) were extracted with MeOH. The concentrated extract was re-dissolved in *n*-hexane and extracted with MeOH–H_2O (4:1) three times. After evaporation of the aqueous methanolic fraction, in vacuo, brownish deposits were removed from the residue by washing with water. The residue was then re-extracted with MeOH three times. The methanolic extract was diluted by a fourth volume of water without concentration to avoid precipitation and its subdivision was subjected to purification by HPLC employing an ODS column (Cosmosil 5C18AR) at a flow rate of 1 ml/min using a gradient H_2O–MeCN 9:1 to 7:3 (v/v) in 40 min. The column temperature was set at 60 °C and the monitoring wavelength was 350 nm. The peak corresponding to compound **1** eluted at ca. 12 min to give 9.2 mg of the desired compound.

3.4. Isorhamnetin 3,7-O-di-β-D-glucopyranoside (*1*)

R_f TLC: 0.47 (*n*-BuOH–HOAc–H_2O 6:1:2, v/v/v); UV λ_{max}^{MeOH} nm (log ε): 255 (4.51), 270 sh (4.36), 355 (4.39); ^1H NMR, ^{13}C NMR and HMBC spectral data are shown in Table 1; HR–FABMS $[M+H]^+$ *m/z* 641.1719 (calc. for $[C_{28}H_{33}O_{17}]^+$ *m/z* 641.1718).

3.5. Absolute configuration of sugar moieties of *1*

The D-, L-configurations of sugar moieties of **1** were identified by an HPLC method using the chiral derivatizing reagent, (+)-MNB carboxylic acid (MNB–COOH) according to the literature (Bai et al., 1997). To a mixture of **1** (33 μg, 52 nmol) and 50% NaOH aq (10 μl) in DMSO (100 μl), CH_3I (100 μl) was added, with the mixture stirred at room temperature for 0.5 h. To the mixture was added $CHCl_3$ (500 μl), and the organic layer was washed with water (250 μl×3) and dried over $MgSO_4$. After filtration and evaporation under a dry nitrogen stream, the residue was reacted with a mixture of acetyl chloride (100 μl) and 12 N HCl (10 μl) at 50 °C for 2 h. After evaporation, the resulting residue was reacted with MNB–COO^- Cs^+ (100 μl, 10 mM in DMF) at 60 °C for 1 h. To the reaction mixture, Et_2O (1 ml) was added; the organic layer was washed with water (500 μl), satd. aq. $NaHCO_3$ (500 μl×3), and water (500 μl). After evaporation, the resulting ester was diluted with 500 μl of the eluant mentioned later, from which 5 μl was injected into the normal-phase HPLC column (Develosil 60-3) using a mixture of *n*-hexane–EtOAc–THF

(53:10:7, v/v/v, 1 ml/min) as eluant at 22 °C. The excitation and emission wavelengths were set at 310 and 370 nm, respectively. The MNB-derivatized D-glucopyranose eluted at 14.6 min, and was distinguishable from the L-isomer eluting at 13.5 min. The peak due to the MNB derivative from **1** was ≈2× the area compared with that from the authentic standard, isorhamnetin 3-β-D-glucopyranoside (Funakoshi), establishing the two glucoses of **1** to be D-isomers.

3.6. Analytical HPLC of *1*

To determine the quantities of **1** in both the basal and apical parts of petals or whole leaves, each methanolic extract was quantified by HPLC; fresh samples (both basal and apical parts of three petals or ca. 50 mg of leaves) were extracted with 2 ml of MeOH. After a centrifugation at 1800 rpm for 3 min, a 500 μl portion of the supernatant was evaporated under a dry nitrogen stream. The residue was re-dissolved in 1 ml of *n*-hexane and extracted with MeOH–H_2O (4:1, 1 ml) using a centrifuge as mentioned above. No carotenoids were detected in the MeOH–H_2O (4:1) phase as evidenced by the UV and visible spectrometric analysis, and no flavonoid absorbances were detected in the *n*-hexane phase either. A 5 μl portion of the aqueous MeOH fraction was diluted with 10% MeCN–H_2O, with 50 μl solutions subsequently being injected onto the HPLC system as above. Compound **1** eluted at ca. 12 min as a dominant peak although a few minor peaks were also detected. Each analysis was carried out in quintuplicate.

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References

Aguinagalde, I., 1988. Flavonoids in *Brassica nigra* (L.) Koch, *B. oleracea* L., *B. campestris* L. and their natural amphidiploids. Bot. Mag. Tokyo 101, 55–60.

Al, W.S.A.M., El, G.E., Saleh, N.A.M., 1988. Differences in flavonoid constituents among the species of *Fagonia-sinaica* complex Zygophyllaceae. Plant Syst. Evol. 161, 87–90.

Bai, C., Ohrui, H., Nishida, Y., Meguro, H., 1997. A fluorometric determination method for D,L configurations of per-O-methylated monosaccharides by anomeric 2-methyl-2-β-naphthyl-1,3-benzodioxole-4-carboxylation and high-performance liquid chromatography. Anal. Biochem. 246, 246–252.

Bateman, A.J., 1955. Self-incompatibility systems in angiosperms. III. Cruciferae. Heredity 9, 53–68.

Classen, D., Nozzolillo, C., 1981. Developmental aspects of flavonoid patterns in *Brassica campestris* var. *oleifera*: a preliminary study. Can. J. Bot. 59, 1382–1385.

Durkee, A.B., Harborne, J.B., 1973. Flavonol glycosides in *Brassica* and *Sinapis*. Phytochemistry 12, 1085–1089.

Gronquist, M., Bezzerides, A., Attygalle, A., Meinwald, J., Eisner, M., Eisner, T., 2001. Attractive and defensive functions of the ultraviolet pigments of flower (*Hypericum calycinum*). Proc. Natl. Acad. Sci. USA 98, 13745–13750.

Horovitz, A., Cohen, Y., 1972. Ultraviolet reflectance characteristics in flowers of crucifers. Am. J. Bot. 59, 706–713.

Kandori, I., Ohsaki, N., 1998. Effect of experience on foraging behavior towards artificial nectar guide in the cabbage butterfly, *Pieris rapae crucivora* (Lepidoptera: Pieridae). Appl. Entomol. Zool. 33, 35–42.

Koes, R.E., Quattrocchino, F., Mol, J.N.M., 1994. The flavonoid biosynthetic pathway in plants: function and evolution. BioEssays 16, 123–132.

Konishi, T., Kamoi, T., Matsuno, R., Sasaki, Y., 1996. Induction of cytosolic acetyl-coenzyme A carboxylase in pea leaves by ultraviolet-B irradiation. Plant cell Physiol. 37, 1197–1200.

Maksoud, S.A., El, H.M.N., 1988. The flavonoids of *Balanites-aegyptiaca* Balanitaceae from Egypt. Plant Syst. Evol. 160, 153–158.

Omura, H., Honda, K., Hayashi, N., 1999. Chemical and chromatic bases for preferential visiting by the cabbage butterfly, *Pieris rapae*, to rape flowers. J. Chem. Ecol. 25, 1895–1906.

Sakamoto, K., Nishio, T., 2001. Distribution of S haplotypes in commercial cultivars of *Brassica rapa*. Plant Breed. 120, 155–161.

Takasaki, T., Hatakeyama, K., Suzuki, G., Watanabe, M., Isogai, A., Hinata, K., 2000. The *S* receptor kinase determines self-incompatibility in *Brassica* stigma. Nature 403, 913–916.

Tanaka, H., 1982. Relationship between ultraviolet and visual spectral guidemarks of 93 species of flowers and the pollinators. J. Jpn. Bot. 57, 146–159.

Thompson, W.R., Meinwald, J., Aneshansley, D., Eisner, T., 1972. Flavonols: pigments responsible for ultraviolet absorption in nectar guide of flower. Science 177, 528–530.

Utech, F.H., Kawano, S., 1975. Spectral polymorphisms in angiosperm flowers determined by differential ultraviolet reflectance. Bot. Mag. Tokyo 88, 9–30.

Wolbis, M., Krolikowska, M., 1988. Flavonol glycosides from *Sedum acre*. Phytochemistry 27, 3941–3943.

Wyatt, R., 1983. Pollinator-plant interactions and the evolution of breeding systems. In: Real, L. (Ed.), Pollination Biology. Academic Press, New York, pp. 51–95.