

Plant–Environment Interactions: Accumulation of Hypericin in Dark Glands of *Hypericum perforatum*

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• **Background and Aims** *Hypericum perforatum* is a perennial herbaceous plant and an extract from this plant has a significant antidepressant effect when administered to humans. The plant is characterized by its secretory glands, also known as dark glands, which are mainly visible on leaves and flowers. The current study evaluates the influence of several environmental factors and developmental stages of the plant on the accumulation and synthesis of hypericin and pseudohypericin (Hy-G), the major bioactive constituents, in *H. perforatum* plants.

• **Methods** The appearance of dark glands on different parts of the plant, under several environmental conditions, was monitored by microscopy. Hy-G concentrations were quantified by high-performance liquid chromatography.

• **Key Results** A significant presence of dark glands accompanying the highest concentrations of Hy-G was observed in the stamen tissues more than in any other organ of *H. perforatum*. A linear relationship between the number of dark glands and net photosynthetic rate of the leaf and Hy-G concentration in the leaf tissue was also established. A very high concentration of Hy-G was measured in the dark-gland tissues, but in the tissues without any dark glands it was almost absent. The presence of emodin, a precursor of Hy-G, at a high concentration in the dark-gland tissues, and its absence in the surrounding tissues was also observed, suggesting that the site of biosynthesis of Hy-G is in the dark-gland cells. A significantly low concentration of Hy-G (occasionally non-detectable) was measured in the xylem sap of the stem tissues. The dark-gland tissues collected from leaves, stems or flowers contained similar concentrations of Hy-G.

• **Conclusions** The concentration of Hy-G in various organs of *H. perforatum* plants is dependent on the number of dark glands, their size or area, not on the location of the dark glands on the plant. The study provides the first experimental evidence that Hy-G is synthesized and accumulates in dark glands.

Key words: Emodin, dark gland, net photosynthesis, hypericin accumulation, hypericin synthesis.

INTRODUCTION

Many plants synthesize a broad repertoire of complex organic molecules and are an important source of fine chemicals that have found extensive use in the pharmaceutical industry (Brodellius and Pedersen, 1993). *Hypericum perforatum*, commonly known as St John's-wort, produces one of the best-selling plant-based medicines, which is used mainly as an antidepressant. The major bioactive compound of this plant is the hypericin group (hypericin and pseudohypericin; called Hy-G hereafter), a red naphthodianthrone. Morphologically, *H. perforatum* plants are characterized by the presence of several types of secretory structures, including translucent glands, dark glands and secretory canals (Curtis and Lersten, 1990; Fornasiero *et al.*, 1998; Bottega *et al.*, 1999). Dark glands, probably first named by Green (1884), are also known as 'nodules' (Curtis and Lersten, 1990) or 'black nodules' (Onelli *et al.*, 2002; Maggi *et al.*, 2004). This gland is the most important secretory structure in *H. perforatum* plants. However, there is no experimental evidence that Hy-G or their precursors are synthesized or accumulated in dark glands, or that they are transported from dark glands to

different organs or vice versa, nor is there any information on the mechanisms of transport. In contrast, extensive research has been conducted on the anatomical and histological characterization of the dark glands on leaves and petals, starting in the 19th Century (Green, 1884, and references therein; Onelli *et al.*, 2002; Maggi *et al.*, 2004). In several studies, dark glands are proposed as the site where biologically active substances accumulate (McKey, 1979; Curtis and Lersten, 1990; Fornasiero *et al.*, 1998), although no direct evidence of chemical analysis has been presented.

The current study was designed to evaluate the interaction of some major aerial environmental factors (CO₂ and irradiance) and plant development stages in relation to Hy-G accumulation and synthesis in the dark glands located in different organs. The numbers of dark glands and Hy-G concentration were quantified in different parts of the plant under several environmental conditions. The aim was to establish whether there is a correlation between the number of dark glands developed on the leaf surface and net leaf photosynthetic rates and Hy-G concentrations. The xylem sap of the entire xylem pathway of the main stem was also measured to evaluate the possible transportation of Hy-G through the xylem network of *H. perforatum* plants. Accumulation of emodin,

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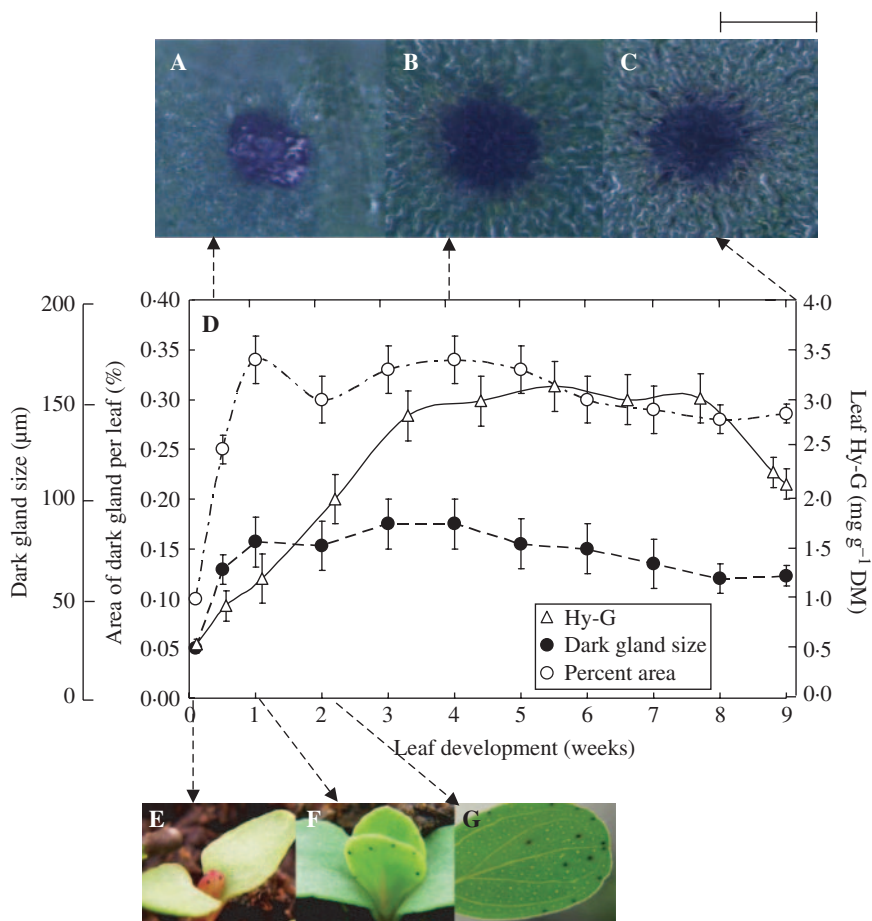


FIG. 1. (A–C) Dark glands of (A) 3-d, (B) 4-week and (C) 9-week-old leaves. Scale bar = 150 µm. (D) Dark gland size, percentage area of dark glands (cm⁻²) and Hy-G concentration in the leaf tissues during different stages of leaf development of *Hypericum perforatum* plants. Each symbol represents the mean ± s.e. of ten replicates. (E–G) The development of dark glands on seedlings (E, 2 d old, F, 1 week old; G, 2 weeks old). Scale bar = 6 mm.

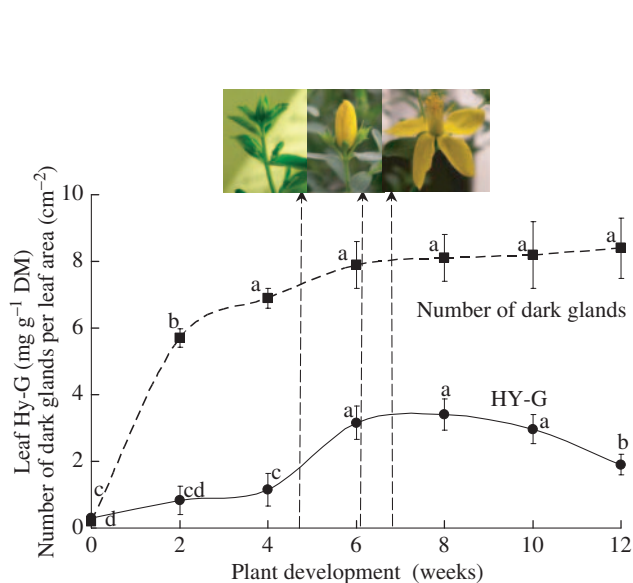


FIG. 2. Hy-G concentration and number of dark glands during the different stages of plant development of *Hypericum perforatum* plants. Each symbol represents the mean ± s.e. of ten replicates.

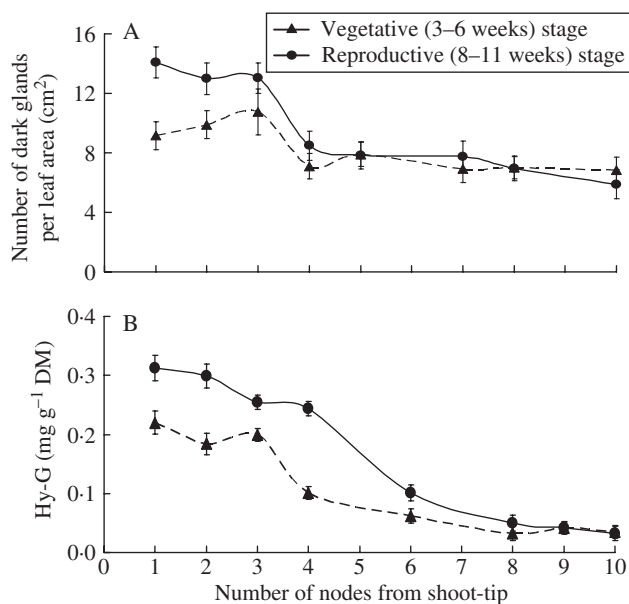


FIG. 3. (A) Number of dark glands on the leaves and (B) Hy-G concentration in the stem tissues of different nodes of *Hypericum perforatum* plants. Each bar represents the mean ± s.e. of 20 replicates.

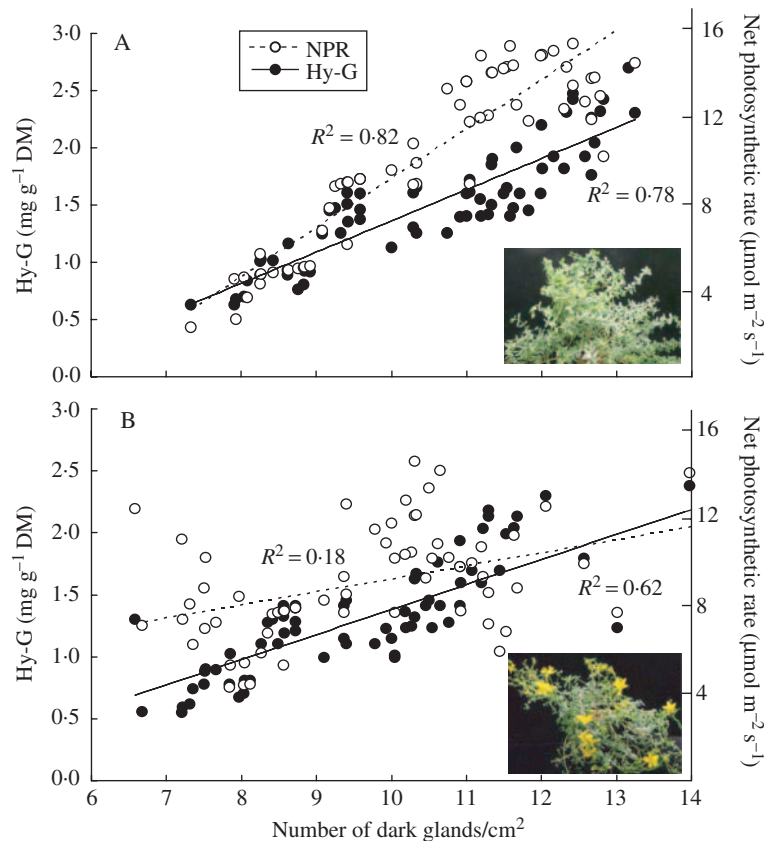


FIG. 4. Relationship between number of dark glands and Hy-G concentration and leaf net photosynthetic rates of *Hypericum perforatum* plants during (A) vegetative (3–6 weeks) and (B) reproductive stage (8–11 weeks); plants were grown with a PPFD of $300 \mu\text{mol m}^{-2} \text{s}^{-1}$, a CO_2 concentration of $1200 \mu\text{mol mol}^{-1}$ and an air temperature of $25/23^\circ\text{C}$ (photo- and dark period). Each symbol represents the mean of three samples from the same leaf tissue.

a precursor of hypericin, was also quantified in the dark glands of *H. perforatum* plants, and a hypothetical pathway is presented for the synthesis of Hy-G in the dark glands.

MATERIALS AND METHODS

Germination and plant establishment

Hypericum perforatum ‘Topas’ (St John’s-wort) seeds were obtained from Richters The Herb Specialists, Canada. The seeds were washed and placed on top of moist potting soil in a multicell tray covered with plastic wrap. Germination was conducted over a 10-d period at 27°C with a 16-h photoperiod, a light intensity (photosynthetic photon flux density; PPFD) of about $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ and periodic watering with tap water. Following germination, plants (about 1.0 cm high) were transferred to pots (9 cm diameter, one plant per pot) containing 200 g of soil mixture (Yanmar Agricultural Equipment Co., Ltd, Japan). The plants were grown under controlled-environment conditions and watered with a nutrient solution (Hyponex, N : P : K; 6 : 6 : 5; Hyponex Corp. Ltd, Osaka, Japan) every other day and with distilled water in between.

For the first experiment, plants were grown under artificial light (PPFD of $300 \mu\text{mol m}^{-2} \text{s}^{-1}$) with a 16-h

photoperiod and 60 % relative humidity, $1200 \mu\text{mol mol}^{-1}$ CO_2 concentration and $25/23^\circ\text{C}$ air temperature during the photo-/dark period. In the first set of plants, the number of dark glands, area and size (diameter) and Hy-G concentration were studied on selected leaves in relation to leaf development and expansion, i.e. at different leaf ages as well as at similar leaf ages (2 weeks old) but at different stages of plant development. In the second set of plants, the number of leaf dark glands and Hy-G concentrations in the stem tissues in different nodal zones (9- to 12-mm stem sections) during the vegetative (weeks 3–6) and reproductive stages of plant development (weeks 8–11) were evaluated. In the third set of plants, the leaves with various numbers of dark glands were selected, the dark glands were counted and the net photosynthetic rates were measured. Finally, in the fourth set of plants, xylem sap of the stem was isolated and analysed to quantify the hypericin and pseudohypericin concentrations.

In the second experiment, plants were grown under the following environmental conditions: (a) a range of light intensities (PPFDs of 100, 200, 300, 400 and $500 \mu\text{mol m}^{-2} \text{s}^{-1}$) each with a CO_2 concentration of $1200 \mu\text{mol mol}^{-1}$; and (b) a range of CO_2 concentrations (400, 800, 1200, 1600 and $2000 \mu\text{mol mol}^{-1}$) under a PPFD of $300 \mu\text{mol m}^{-2} \text{s}^{-1}$. The plants were tested in terms of number of

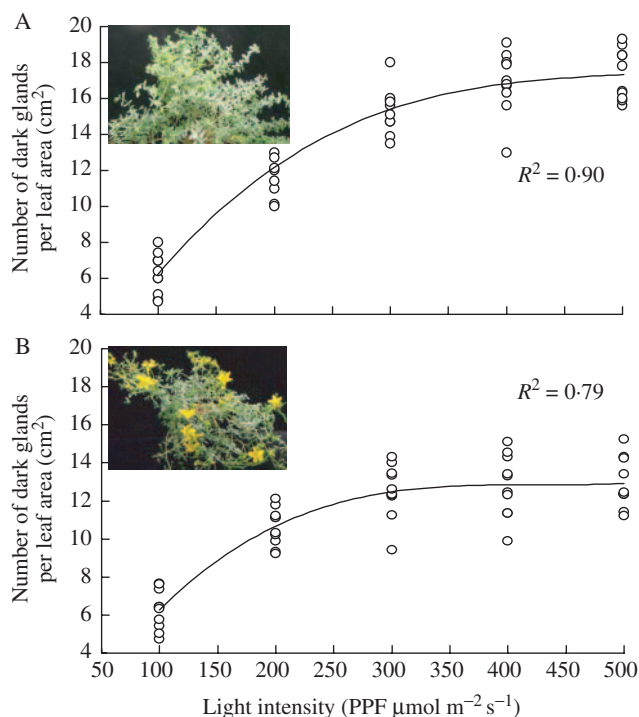


FIG. 5. Effect of different photosynthetic photon flux (PPFD) on the number of dark glands of *Hypericum perforatum* plants during (A) vegetative (3–6 weeks) and (B) reproductive stages (8–11 weeks) of plant development. Each symbol represents the mean of three samples from the same leaf tissue. CO_2 concentration = $1200 \mu\text{mol mol}^{-1}$.

dark glands per unit leaf area during the vegetative (weeks 3–6) and reproductive (weeks 8–11) stages of plant development.

In the third experiment, plants were grown under the same environmental conditions as for the first experiment, and the dark gland size, number and area, hypericin and pseudohypericin concentrations, dark canals, etc. were studied in the leaf, stem and different organs of flowers, and with different stages of flower development. For measuring the surface area of the stamen, anthers were considered as spherical.

In a separate set of plants, Hy-G and emodin concentrations in the dark-gland tissues and tissues without dark glands of leaf blades, petals and stems during the vegetative (weeks 3–6) and reproductive (weeks 8–11) stages were analysed. Dark glands were carefully separated under a dissecting microscope (each gland with about 10–20% green leaf or yellow petal tissue).

Morphological study and measurement of net photosynthetic rate

The morphological study was performed on fresh material observed under a precalibrated digital microscope (Keyence Corp., Tokyo, Japan). The net photosynthetic rate (P_n) per leaf area of the fourth to seventh uppermost, fully expanded leaves was measured using a portable

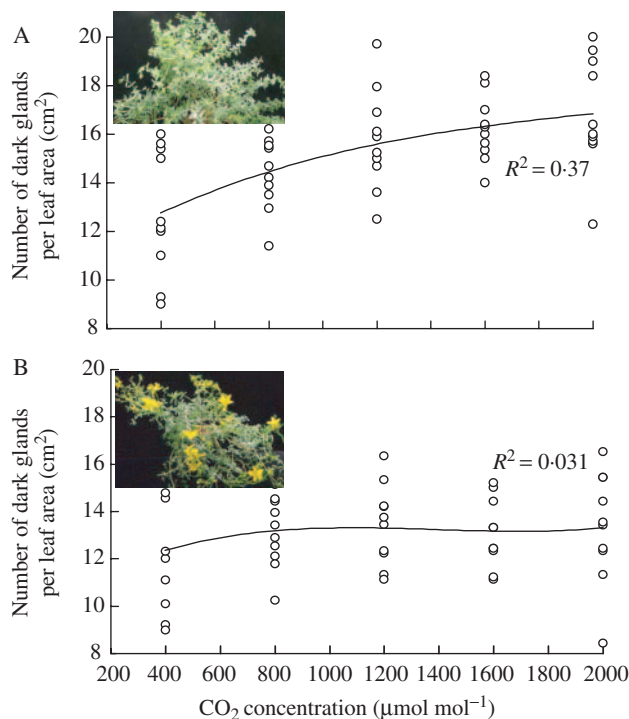


FIG. 6. Effect of different CO_2 concentration on the number of dark glands of *Hypericum perforatum* plants during (A) vegetative (3–6 weeks) and (B) reproductive stages (8–11 weeks) of plant development. Each symbol represents the mean of three samples from the same leaf tissue. Light intensity (PPF) = $300 \mu\text{mol mol}^{-2} \text{s}^{-1}$.

TABLE 1. *Hy-G concentrations in the xylem sap of the stem of Hypericum perforatum plants*

Stem position (distance from the shoot tip)	Concentration ($\mu\text{g mL}^{-1}$)	
	Vegetative stage	Reproductive stage
2nd–4th node	5.1 ± 0.3	ND*
5th–7th node	12.3 ± 1.1	16.7 ± 1.8
8th–10th node	ND	ND

Each value represents the mean \pm s.e. of seven replicates.

*Non-detectable.

photosynthesis system (LICOR-6400[®], LI-COR Inc., USA) with a 6400-15 arabidopsis chamber.

Xylem sap collection

Xylem sap collection was carried out immediately after harvesting the plants. In a stem portion (2 cm), a few millimetres of bark was removed from the higher cut end of the stem to avoid phloem contamination. The stem portion was immediately placed in a pressure chamber so that the section of stem with bark removed protruded. The pressure in the chamber was slowly increased to a maximum of 0.15 MPa, and the xylem sap which exuded was collected

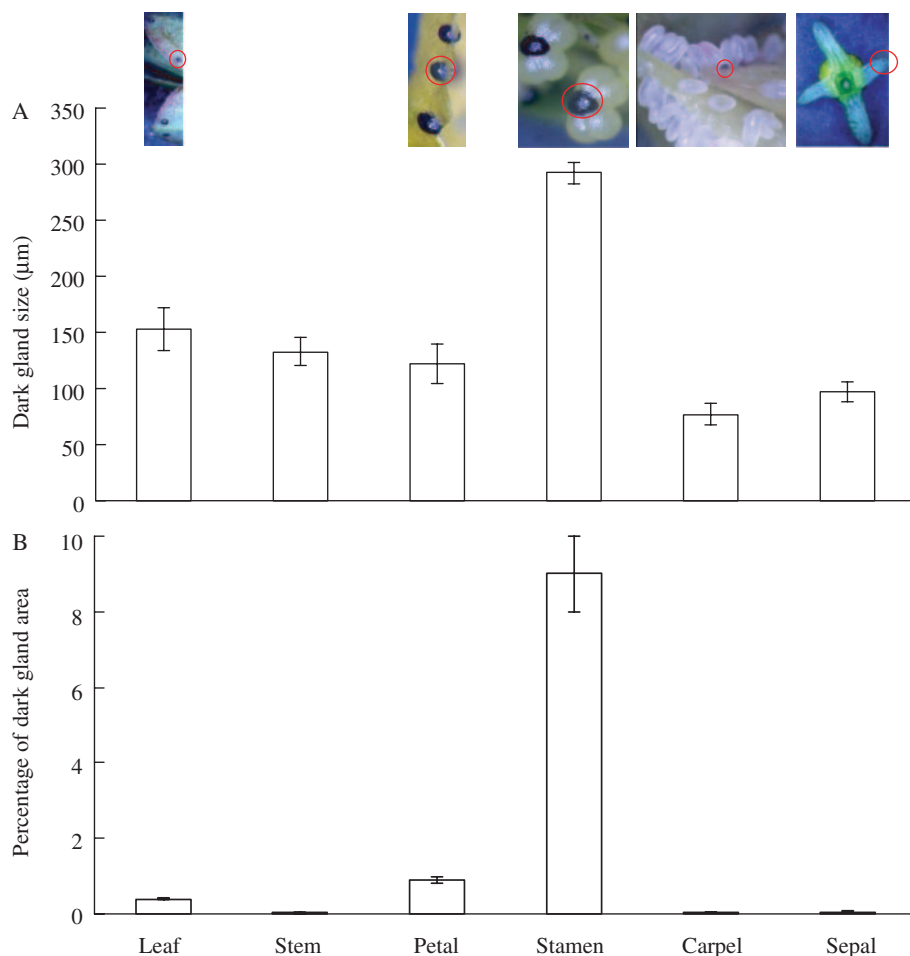


FIG. 7. (A) Size and (B) percentage area of dark glands developed in leaves, stems, flower petals, stamens, carpels and sepals tissues of *Hypericum perforatum*. Each bar represents the mean \pm s.e. of 30 replicates.

and separated with micro-capillary tubes for biochemical analysis. Initial tests indicated that pressures up to 0.15 MPa did not cause any contamination by cellular components.

Determination of hypericin, pseudohypericin and emodin concentrations

For the chemical analysis of hypericin, pseudohypericin and emodin concentrations, leaf or flower tissues were collected and flash frozen in liquid nitrogen and stored at -80°C until the analysis was conducted. The extraction, isolation and chemical analysis methods for hypericin and pseudohypericin were as described by Zobayed *et al.* (2005). Hy-G concentration was calculated by summing the hypericin and pseudohypericin concentrations.

For extraction of emodin, 1.0 mL of 2% (v/v) dimethyl sulfoxide in methanol was added to each sample, pulverized at 30 Hz for 6 min (MM 200; Retsch GmbH and Co., Haan, Germany) and centrifuged at 4000 rpm (300 g) at 4°C for 15 min (Kubota®; Kubota Co., Ltd, Tokyo, Japan). Extracts were filtered through a 0.2- μm syringe filter (Dismic-13HP, Advantec; Toyo Roshi Kaisha Ltd, Tokyo, Japan) and a 20- μL sample of the

filtrate was injected into a Phenomenex Hypersil C18 column (3.0 μm , 4.6 mm \times 100 mm) in a high-performance liquid chromatography (HPLC) system consisting of an SCL-10A system controller, SIL-10A auto-injector and an CTO-10A column oven (Shimadzu Corp., Kyoto, Japan). The analytes were separated isocratically using the mobile phase of 0.1 mol L^{-1} triethyl ammonium acetate and acetonitrile (25 : 75, v/v) at a flow rate of 0.5 ml min^{-1} , and emodin was quantified at 252 nm in the SPD-M10AV photodiode array detector. The standard curves were obtained by plotting the standard concentration (0.5, 2.5, 5, 10 and 50 $\mu\text{g mL}^{-1}$) as a function of peak area in HPLC chromatograms. High linearity ($r^2 = 0.99$) was obtained for each calibration curve.

All the concentrations of hypericin, pseudohypericin, Hy-G and emodin were expressed as milligrams per gram dry mass (DM) or fresh mass (FM) of the tissues.

Statistical analysis

The experiments were conducted twice. When necessary, statistical significance was determined by one-way analysis of variance (ANOVA) using the Sigma Stat program (SigmaStat™ for Windows® V2.03, SPSS Inc.).

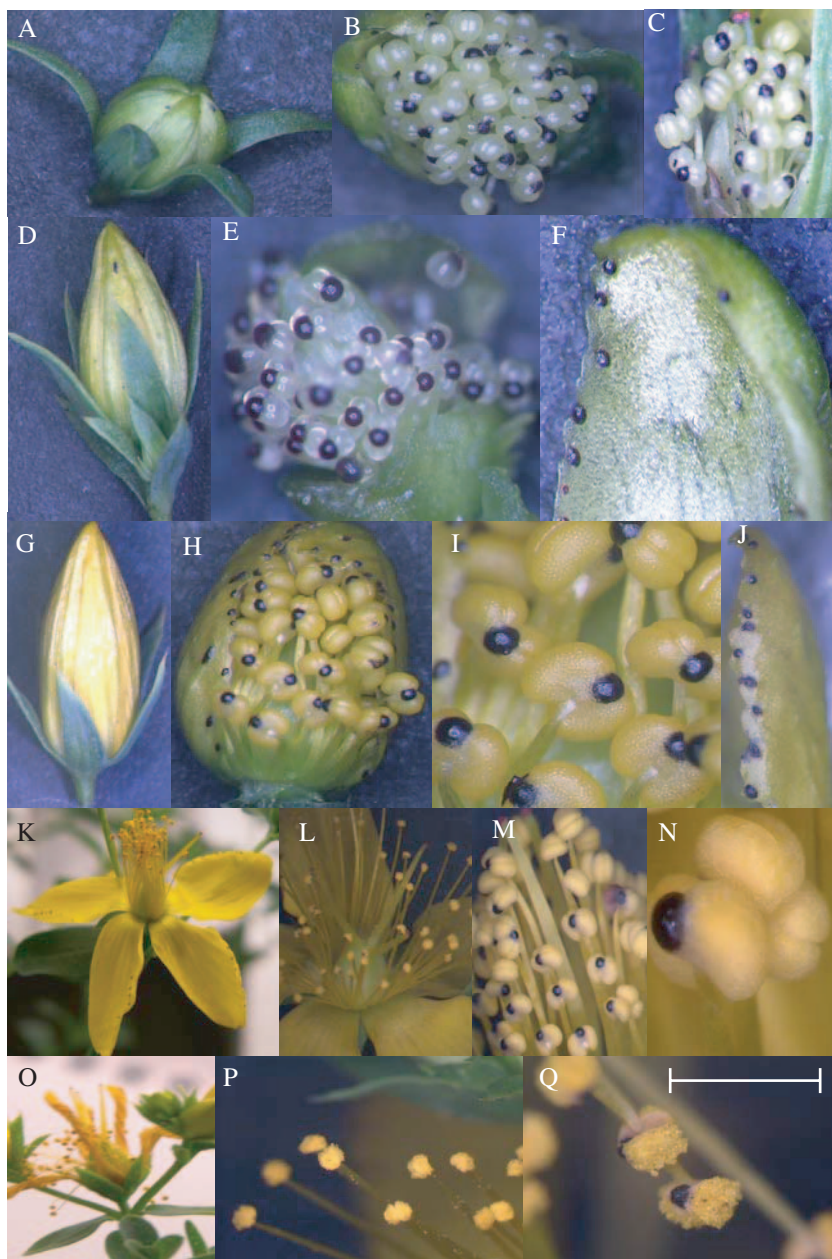


FIG. 8. Dark glands located in different types of flower tissues of *Hypericum perforatum*. (A–C) stage 1 of flower development: (A) an intact flower bud; (B and C) forcibly opened flower bud to show the dark glands located on the stamens. (D–F) Stage 2 of flower development: (D) an intact flower bud; (E) a forcibly opened flower bud to show the dark glands located on the stamens; and (F) on a petal. (G–J) Stage 3 of flower development: (G) an intact flower bud; (H) a forcibly opened flower bud to show the dark glands located on the stamens; (I) a close-up view of the stamens with dark glands; (J) dark glands located on a petal. (K–N) Stage 4 of flower development: (K) a fully opened flower; (L) a close-up view of flower; (M) stamens with dark glands; (N) a close-up view of an anther with dark gland. (O–Q) Stage 5 of flower development: (O) a mature flower; (P) stamens with dark glands; (Q) a close-up view of an anther with dark gland. Scale bar = 7 mm for K and O; 5 mm for A, D, G and L; 3 mm for P; 2.5 mm for C–E, H, J and M; 2 mm for Q; 1.5 mm for F; 1.0 mm for N).

Differences between means were assessed with the Student–Newman–Keuls test.

RESULTS

The number of dark glands on leaf tissues was studied in relation to leaf development and expansion (Fig. 1). The

dark glands were visible in few-day-old first leaves (Fig. 1A, E) but were absent in cotyledons (Fig. 1E). Generally a dark gland gained its full size only within 7–10 d of leaf development (Fig. 1D, F). In a young leaf (5–7 d old), the dark glands were smaller in size and slightly dome-shaped (Fig. 1A, B). Microscopy also revealed that during the vegetative stage of plant development, the cells in the dark glands in young leaves

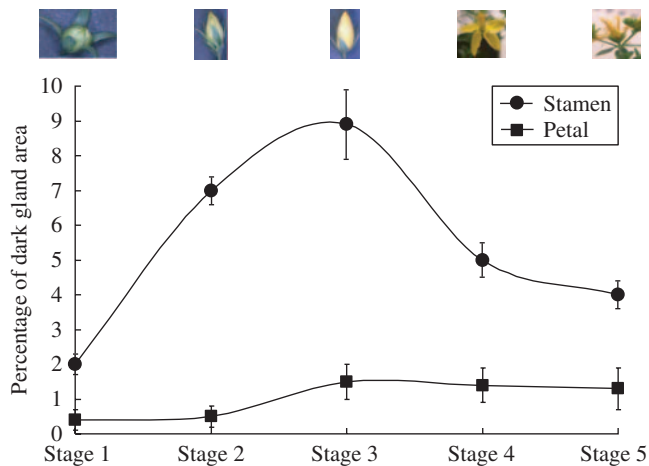


FIG. 9. Percentage of the area of dark glands on the stamen and petal tissues at different stages of flower development of *Hypericum perforatum*. Each symbol represents the mean \pm s.e. of 30 replicates.

mostly enlarged by expansion rather than through cell division. At the end of the reproductive stage, especially in the old leaves, most of the dark glands appeared to be shrinking and their dome-shape was no longer visible (Fig. 1C). Hy-G concentration also increased with plant development and the maximum concentration was measured only after 3 weeks of leaf development (Fig. 1D); the concentration of Hy-G was slightly lower in the older leaves (Fig. 1D).

The number of dark glands and Hy-G concentration in 2-week-old fully expanded leaves were studied throughout the vegetative and reproductive stages. The average number of dark glands studied on the second or third fully expanded leaves from the top generally increased with plant development and reached a maximum number of 8.2 cm^{-2} at the sixth week of plant development (Fig. 2). The Hy-G concentration in the second or third leaf tissue also increased with time and reached the maximum concentration only after 4 weeks of plant development (Fig. 2). The average concentration of Hy-G declined during the reproductive stage of plant development (especially in weeks 8–11; Fig. 2).

When leaves from different locations of a plant were studied, the number of dark glands was higher in the leaves of the upper part than the lower parts (Fig. 3A). In a 3- to 6-week-old plant, the number of dark glands was highest on the leaves of the third node from the shoot tip (Fig. 3A). In contrast, on an 8- to 11-week-old plant (after flower development), the highest number of dark glands was observed on the leaves of the first or second node from the top. Interestingly, the leaves on the upper part (nodes 1–3) have more glands in the 8- to 11-week-old plants than in the 3- to 6-week-old plants (Fig. 3A).

The Hy-G concentration was highest in the upper part of the stem and dramatically reduced in the lower parts, especially below the fourth node from the top (Fig. 3B). When the xylem sap was extracted and analysed, the concentration of Hy-G was similar in all parts of the stem

but was very low compared with that of any other tissue (Table 1). The value was about $5\text{--}16 \mu\text{g mL}^{-1}$ in the xylem sap (Table 1), $60 \mu\text{g mL}^{-1}$ in the leaf tissues (3 mg g^{-1} DM; Fig. 1D) and $6000 \mu\text{g mL}^{-1}$ in the stamen tissues (120 mg g^{-1} DM; Fig. 12).

An attempt was also made to see if there is a relationship between the number of dark glands and Hy-G concentration, as well as net photosynthetic rates during the vegetative (plants 3–6 weeks old) and reproductive (plants 8–11 weeks old) stages of plant development. Results revealed that in a 3- to 6-week-old plant, Hy-G concentration was linearly related to the leaf net photosynthetic rate (Fig. 4A, $R^2 = 0.82$). Also, the number of dark glands was linearly related to Hy-G concentration ($R^2 = 0.75$). When the net photosynthetic rates and Hy-G concentration were measured on an 8- to 11-week-old plant, the relationship with the number of dark glands in the leaves was not strong (Fig. 4B).

The number of dark glands on the leaves was also recorded under different light intensities (PPFD) and generally an increase in PPFD increased the number of dark glands, especially during the vegetative stage (Fig. 5A). The number was strongly related to PPFD and could be expressed as second-order functions of the number of dark glands with an R^2 value of 0.90. The number of dark glands was lower during the reproductive stage ($R^2 = 0.79$) than the vegetative stage, especially under higher PPFD (Fig. 5B). When number of dark glands was counted on the leaves of plants grown under different concentrations of CO_2 , increase in CO_2 concentration slightly increased the number of dark glands during the vegetative stage ($R^2 = 0.37$) as well as the reproductive stage ($R^2 = 0.31$) (Fig. 6).

Dark glands were also studied in different parts of flower tissues and in different stages of flower development. Dark glands in the stamen were generally largest in size and about twice the size of dark glands in the petal or leaf (Fig. 7A). The smallest size of dark glands was noticed in the carpel and sepal (Fig. 7A). The percentage of the dark-gland area over leaf area was also studied. The dark glands in the stamen occupied about 10% of the leaf area (Fig. 7B); in contrast, in the leaf and flower petal, the dark glands occupied only 0.7% and 1.6% of the leaf area (Fig. 7B). The dark glands in the stamens and petals were also studied at the different stages of flower development (Figs 8 and 9): stage 1, the premature flower bud, green in colour; stage 2, fully developed flower bud, greenish yellow; stage 3, flower buds just before they opened; stage 4, fully opened flower; stage 5, dried flower. The dark-gland area was largest in the stamen tissues at stage 3 (Figs 8 and 9), followed by the flower buds just before unfolding, while the lowest area of dark glands was observed in a premature flower bud, i.e. stage 1, and also in the dried flower, i.e. stage 5 (Fig. 9). In the petals, the dark-gland area reached the highest concentration during stage 3 (Fig. 9).

The secretory canals or the dark canals were also studied in different organs in the flower and the elongated canals were mainly observed in the petals (Figs 10A, B and 11), sepals (Figs 10C and 11) and filaments of stamens

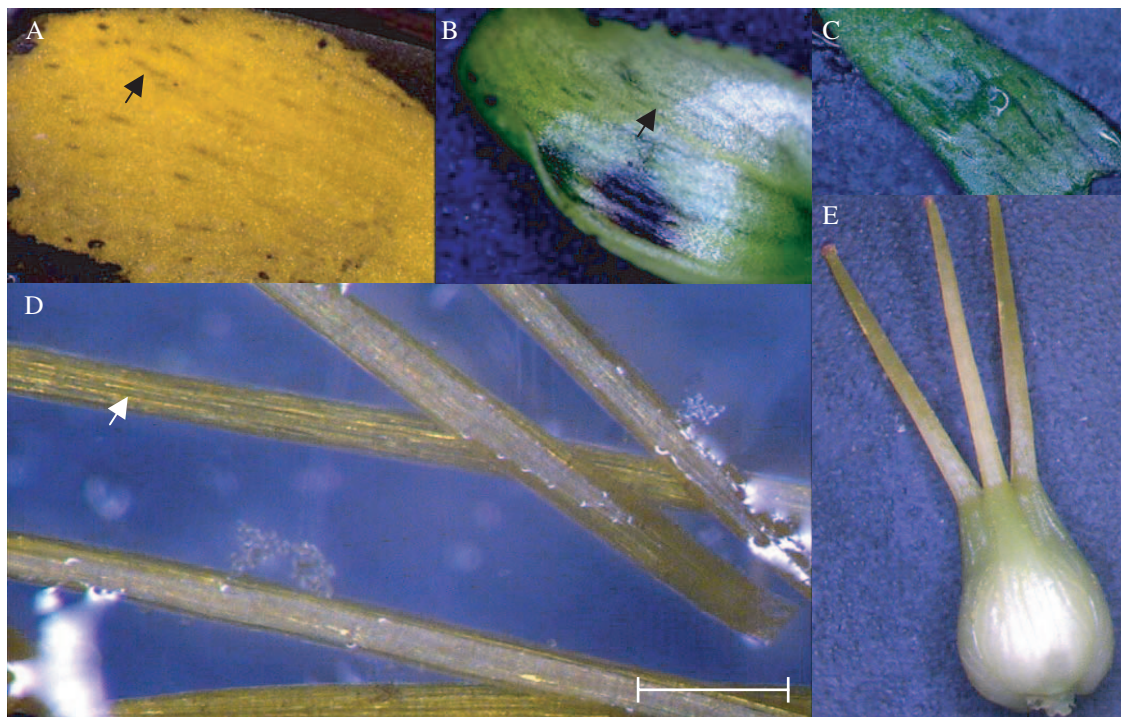


FIG. 10. Dark canals in (A) petal of a fully opened flower; (B) petal of a young flower bud; (C) sepal, (D) filaments of a stamen and (E) carpel of *Hypericum perforatum*. Scale bar = 4 mm for A–C; 0.5 mm for D; 2 mm for E.

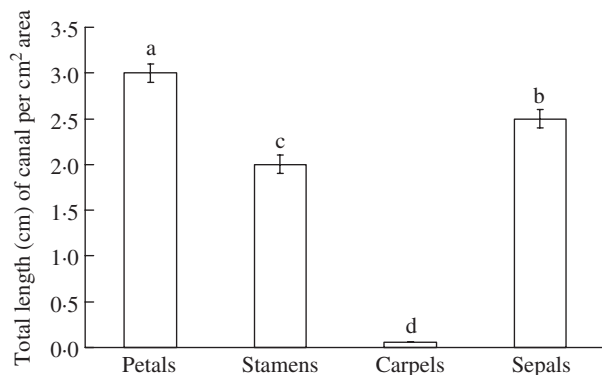


FIG. 11. Total length of dark canal on petal, stamen (filaments), carpel and sepal of a fully opened flower of *Hypericum perforatum*. Each bar represents the mean \pm s.e. of ten replicates.

(Figs 10D and 11). Small, narrow canals were also occasionally located in the carpel. The Hy-G concentration was about three to six times higher in the stamen tissues than in the petal tissues and was 15–20 times that in the carpel or sepal tissues at any stage of flower development (Fig. 12). Among the different developmental stages, the tissues of stages 2 and 3 (flower bud) contained the highest concentration of Hy-G (Fig. 12). When hypericin and pseudohypericin concentrations of the whole flower bud (or flower) tissue were measured, the concentrations again were highest in stages 2 and 3 of flower development (Fig. 13). The pseudohypericin concentration was highest in the tissues of stage 2 followed by stage 3, whereas the

hypericin concentrations were similar in the flower tissues of stages 2 and 3 (Fig. 13).

When Hy-G concentration was measured in the isolated dark glands, no significant difference was observed in the leaf, stem or flower tissues (Fig. 14A). Even the dark glands isolated from the leaves of 3-week-old (vegetative stage with fully expanded leaves) or 8-week-old plants (reproductive stage with visible flower buds and fully expanded leaves) had a Hy-G concentration which was not significantly different (Fig. 14A). When the dark glands were removed from the leaf, stem or flower tissues no significant difference in Hy-G concentration was noticed among the different parts of the tissues, except the petals (Fig. 14A). Generally, the concentration of Hy-G in tissues without dark glands was very low (Fig. 14A).

When the dark glands isolated from leaves and flower petals of 3- and 8-week-old plants were analysed for emodin, no significant difference was noted among the different types of tissues (Fig. 14B). The dark gland-free tissues of leaves, stems or petals generally showed a low concentration of emodin (Fig. 14B).

DISCUSSION

Among the numerous bioactive compounds synthesized in *H. perforatum* tissues, hypericin and pseudohypericin are the two major ones (Miller, 1998). Their synthesis is strictly regulated by the plant and its environment (Zobayed *et al.*, 2003, 2005; Zobayed and Saxena, 2004; Mosaleeyanon *et al.*, 2005). Major aspects of this

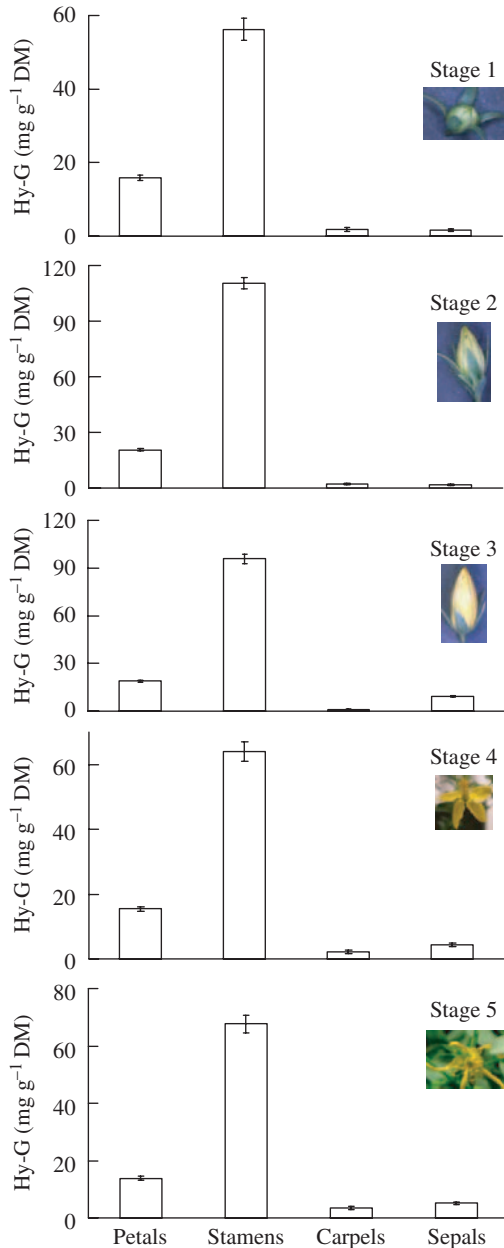


FIG. 12. Hy-G concentration in petal, stamen, carpel, and sepal tissues at different stages of flower development *Hypericum perforatum*. Each bar represents the mean \pm s.e. of ten replicates.

regulation may involve transport, including intracellular shuffling of intermediates and final products, as well as accumulation in the dark glands located in different organs of the plant, including leaf, stem and flower tissues. Flower tissues are already known to have the highest concentration of Hy-G which is five to eight times higher than the leaf tissues (Zobayed *et al.*, 2005). As far as is known, no report is available on the quantification of hypericin or pseudohypericin in the dark glands, although it has been speculated that the dark glands may be the site of accumulation of hypericin or pseudohypericin in *H. perforatum* plants

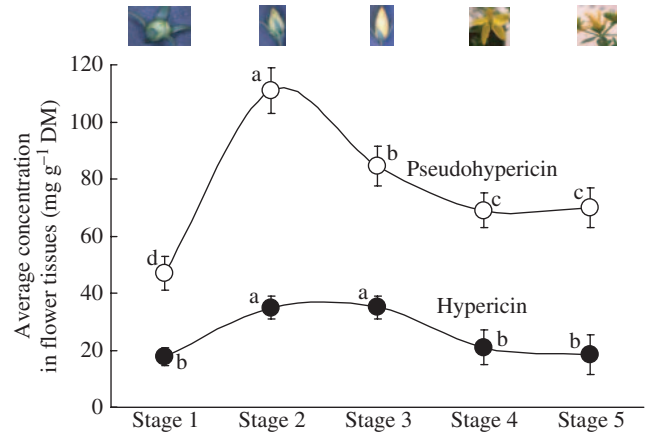


FIG. 13. Average concentrations of hypericin and pseudohypericin in flower tissues (whole flower) at different stages of flower development. Each symbol represents the mean \pm s.e. of ten replicates.

(McKey, 1979; Curtis and Lersten, 1990; Fornasiero *et al.*, 1998). Moreover, until now, the presence of large numbers of dark glands has been reported only in the petals and leaves (Curtis and Lersten 1990; Fornasiero *et al.*, 1998). In the current study, a significant presence of dark glands (Figs 7 and 8), accompanied by the highest concentrations of Hy-G, has been shown (Fig. 12) in the stamen tissues more than in any other organs of *H. perforatum* plants. This explains clearly why high concentrations of hypericin and pseudohypericin are generally reported in the flower tissues.

An attempt was also made to establish a relationship between the number of dark glands and Hy-G concentration in the leaf tissue, as well as net photosynthetic rates during the vegetative (weeks 3–6) and reproductive (weeks 8–11) stages. During the vegetative stage, linear relationships between the number of dark glands and net photosynthetic rates of the leaf and with Hy-G concentration in the leaf tissue were established (Fig. 4A). This relationship indicates that the leaves, capable of assimilating high levels of CO₂, can have a high number of dark glands, resulting in high Hy-G concentrations during vegetative growth (Fig. 4A). During the reproductive stage, these correlations were limited (Fig. 4B; $R^2 = 0.17$ and 0.62 for the net photosynthetic rates and Hy-G concentration, respectively). In particular, the leaves in the upper parts of shoots (newly developed) have comparatively high net photosynthetic rates but still have a low number of dark glands (Fig. 4B). The study also illustrated that during the vegetative stage, the location of accumulated Hy-G could be in the dark glands in leaf tissues only; however, during the reproductive stage, the dark glands located in the flower tissues, especially in the stamen, act as the major site for Hy-G accumulation. During this period of development, the leaf Hy-G concentration remained high (Figs 1 and 2) and the plant had already produced substantial leaf area for photosynthesis and vegetative growth was minimal.

Hy-G biosynthesis may lie on the polyketide pathway, presumably through emodin (Mazur *et al.*, 1992), an

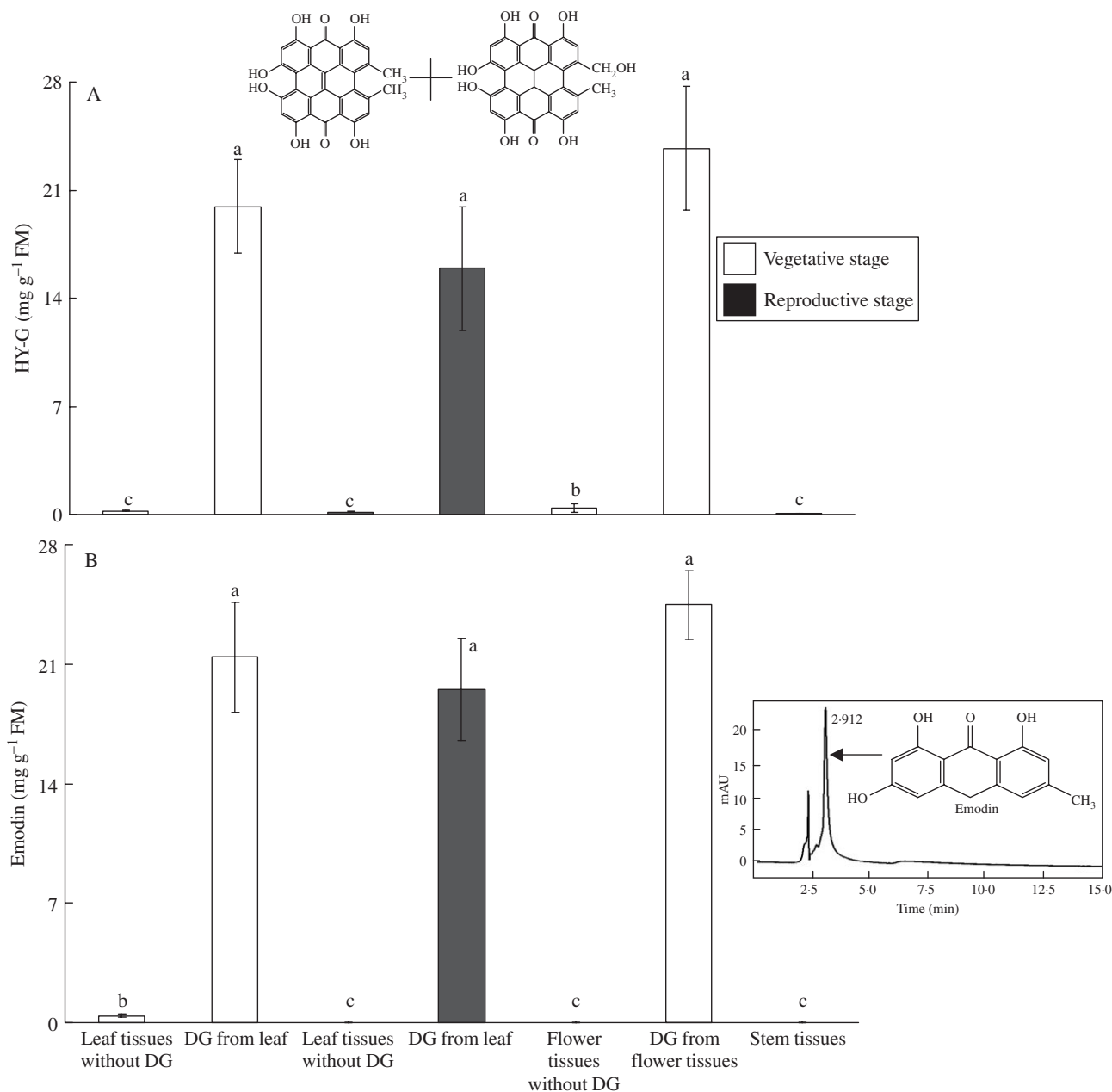


FIG. 14. Hy-G (A) and emodin (B) concentrations in the isolated dark glands (DG) and dark gland-free tissues from leaf, flower and stem tissues; each bar represents the mean \pm s.e. of ten replicates. Inset: HPLC peaks of emodin (standard; RT = 2.91 min) detected at a wavelength of 252 nm.

anthraquinone, and with proto-hypericin as the penultimate precursor (Zobayed *et al.*, 2006), and through the successive condensation of small carboxylic acids (Katz and Donadio, 1993) such as acetate and malonate. Acetate is generated as a product of glycolysis, as the result of fatty acid metabolism by β -oxidation, or by other pathways, and malonyl CoA can be derived from acetyl CoA by the addition of CO₂ as catalysed by acetyl CoA carboxylase (Fig. 15). Additionally, malonate is derived from oxaloacetate from the tricarboxylic acid cycle. A pool of acetyl CoA is generally present in the plastids (Ohlrogge and

Browse, 1995), and plastidal pyruvate dehydrogenase may act on pyruvate derived from glycolysis or from a side reaction of ribulose bisphosphate carboxylase to form acetyl CoA. Chloroplasts are known to contain an extremely active acetyl CoA synthetase, and Ohlrogge and Browse (1995) proposed that the synthesis of acetyl CoA in the mitochondria was followed by transport into the chloroplast. However, the absence of chloroplasts and the presence of only a few mitochondria in the dark gland cells (Onelli *et al.*, 2002) make it unlikely that these steps can take place in the dark glands.

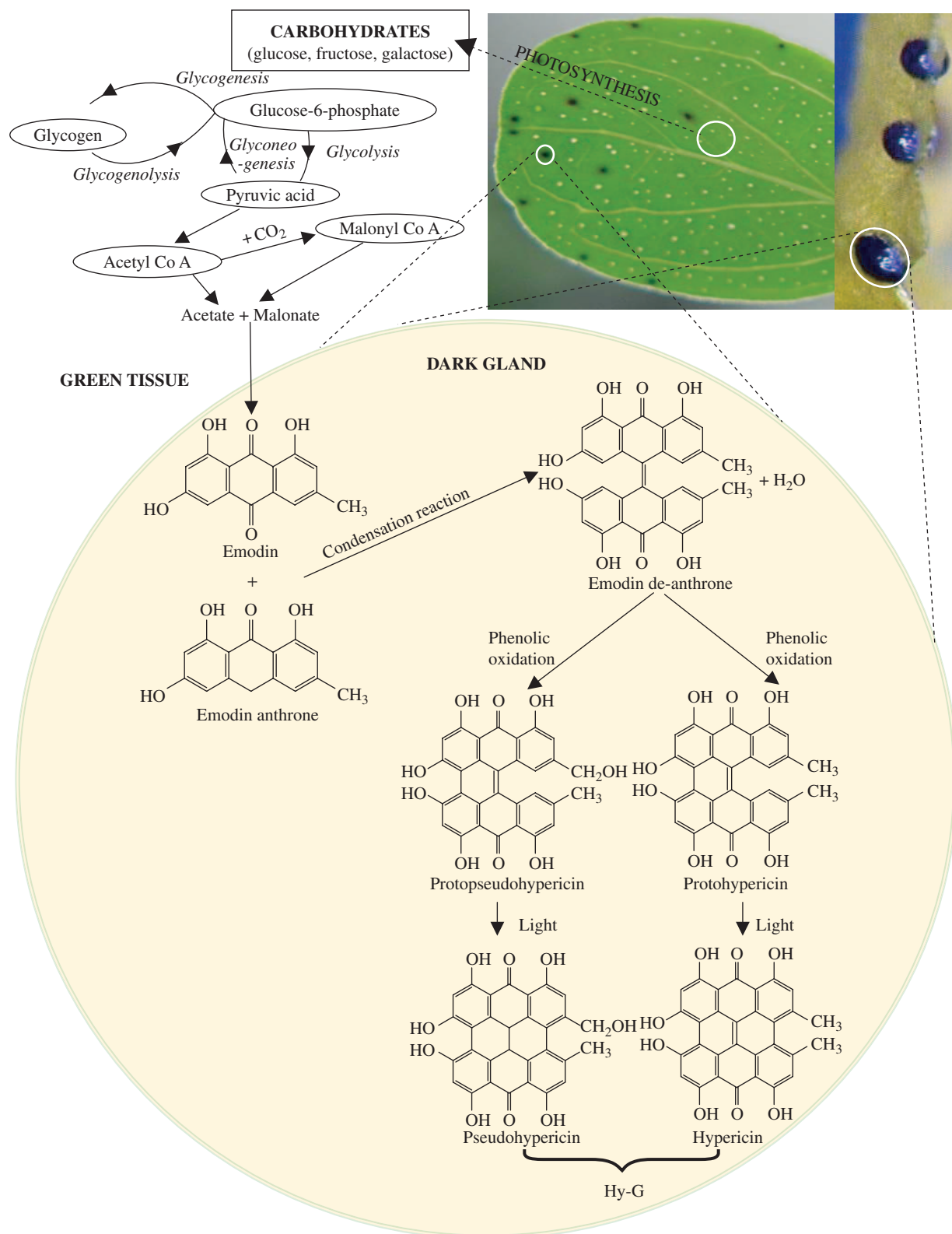


FIG. 15. Pathway for the synthesis of Hy-G from emodin in the cells of dark glands. Drawn from the data presented by Katz and Donadio (1993), Birch (1967), Zobayed *et al.* (2006) and the current study.

As the site of the production of photosynthates (green leaf) differs from the site of Hy-G accumulation in dark glands in flower and leaf tissues (Fig. 14A), it is concluded that long-distance transport of photosynthates via the phloem, xylem or apoplastic space occurs. Dark glands are already known to be connected to the minor veins and vein endings, and are surrounded by an irregular vascular reticulum, which typically includes contact with one or more vein endings (Curtis and Lersten, 1990). Moreover, the dark canals located in different organs (Figs 10 and 11), also known to be associated with veins (Curtis and Lersten, 1990), could be a route of transport as proposed by Williams (1954). Multi-vesicular structures in outer flat cells of developed dark glands were investigated by Onelli *et al.* (2002), who showed that the content is similar to the cytoplasm, with ribosome-like structures. The presence of ribosomes may indicate the production of cytoplasmic proteins such as enzymes for active transport of metabolites. Onelli *et al.* (2002) also showed the presence of numerous plasmodesmata in the outer flat cells of dark glands, suggesting an intense symplastic transport of photosynthates into the glandular cells.

For the conversion of emodin to Hy-G (Fig. 15), an initial condensation reaction between emodin and emodin anthrone, followed by dehydration to form emodin dianthrone takes place, which may undergo phenolic oxidation to form protohypericin and finally hypericin (Fig. 15). Conversion of protohypericin to hypericin generally takes place in the presence of light through a simple biochemical process. The presence of emodin at a high concentration in the dark glands and its absence in other tissues (Fig. 14B) suggests that all of the above-mentioned biochemical pathways for the synthesis of hypericins may take place in the dark-gland cells. The data presented in Table 1, showing the presence of a very low concentration of Hy-G (occasionally non-detectable) in the xylem sap, indicates the limited possibility of the transportation of Hy-G through the xylem network.

Hy-G is thought to function as defensive allelochemicals in *H. perforatum* and it has been proposed that these chemicals are localized in dark glands present primarily on the margins of leaves and petals (Curtis and Lersten, 1990; Fornasiero *et al.*, 1998). The data presented in Fig. 14A established that Hy-G is indeed mainly located in the dark glands. If the dark glands are removed, the Hy-G concentration remains similar in the leaf, flower or stem tissues (Fig. 14A). Moreover, the dark gland itself contained similar concentrations of Hy-G, irrespective of its location in the plants. Hence, the concentration of Hy-G in various organs depends on the number of dark glands, their size or area, not on the location of the dark glands in the plant. Transport of photosynthates from the green leafy cells into the dark gland cells of leaf or flower could be in the form of malonate and/or acetate (precursors of emodin)

in the polyketide pathway. Further investigations are in progress to establish the location of the metabolite and the correlation with the differences between dark-gland cells and the green cells of leaves.

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