Anthocyanin Formation in the Petals of *Hibiscus mutabilis* L.

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Dedicated to Professor Achim Trebst on the occasion of his 60th birthday

Anthocyanin, L-α-Aminooxy-β-phenylpropionic Acid, Phenylalanine Ammonia-Lyase, *Hibiscus mutabilis*

When opening in the morning flowers of *Hibiscus mutabilis* L. appear white or ivory. The flower colour changes to red by late afternoon due to the accumulation of the anthocyanin cyanidin-3-sambubioside. At the onset, and during the rapid phase of pigment accumulation, phenylalanine ammonia-lyase (PAL) activity in the petals increases rapidly to seven times its initial level and then decreases while the flower senesces. In excised petals, the PAL inhibitor L-α-aminooxy-β-phenylpropionic acid (AOPP) suppresses pigment formation and causes the accumulation of phenylalanine. Anthocyanin synthesis depends, therefore, on the *de novo* production of cinnamic acid.

Introduction

Hibiscus mutabilis L., an ornamental shrub of tropical and subtropical regions, owes its name to the conspicuous change in flower colour which is observed during the shortlife time of its open flowers. When, after several weeks of development, the flowers finally open in the morning they appear white to ivory, some faint red coloration at the base of the petals being due to the presence of free cyanidin [1]. During the day, the petals produce and accumulate an anthocyanin, cyanidin-3-sambubioside (= 3-xylosylglucoside), which gives the red coloration to the flowers. In the following night, the flowers rapidly senesce and wilt. In addition to cyanidin-3-sambubioside, cyanidin-3-glucoside and flavonol glycosides have been identified in pink petals of *Hibiscus mutabilis* f. *versicolor* [2]. Two alternatives can be visualized for the rapid synthesis of cyanidin in the petals: 1) *de novo* synthesis *via* the shikimate and general phenylpropanoid pathways, and 2) synthesis from precursors, such as hydroxycinnamic acid conjugates or colourless flavonoids accumulated in the petals during flower development. The time course of phenylalanine ammonia-lyase (PAL) activity during pigment production, as well as the complete inhibition of pigment accumulation by the PAL inhibitor L-α-aminooxy-β-phenylpropionic acid (AOPP) [3–5] described in this communication clearly rule out the second alternative.

Materials and Methods

Seeds of *Hibiscus mutabilis* L. (Lot. No. 329) were obtained from the Botanical Gardens, University of Tokyo, Tokyo, Japan, in 1979 and plants were grown in a temperature-controlled greenhouse at 25 ± 2 °C under natural light conditions. AOPP was synthesized according to the procedure given in [6]; cinnamic acids and flavonoids were obtained from Roth, Karlsruhe, F.R.G. Homoeriodictyol was isolated from *Eriodictyon californicum* Greene [7] obtained from Interdrogas, Cologne, F.R.G. Anthocyanin extraction followed the procedure given in [8], and the A_{521} was used as a relative measure of the pigment content of the petals. Excised outer petals floating on 10 ml 0.01 M potassium phosphate buffer, pH 5.5, with additions as indicated in the text, were incubated at 25 °C and continuous illumination with fluorescent white light (Südlicht, 45 μmol m^{-2} s^{-1}). PAL was extracted from acetone powders of the petals and assayed spectrophotometrically as described previously [8, 9]. The methods for amino acid extraction and analysis are given in [5]. The protein concentration of the extracts was determined using the assay of Bradford [10] and bovine serum albumin as standard.

Abbreviations: AOPP, L-α-aminooxy-β-phenylpropionic acid; PAL, phenylalanine ammonia-lyase (EC 4.3.1.5).

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Results

Anthocyanin formation and PAL activity in petals in situ

Under the prevailing greenhouse conditions the shrubs of *H. mutabilis* showed healthy vegetative growth but flowered only sporadically. No systematic effort was made to determine the factors that induce flowering in this species; only in November 1981 the majority of the approximately 40 available plants burst into flowering and produced sufficient petal material to conduct the experiments described here. In the following five years, flowering was again erratic, and no further experiments could be carried out.

As described in the literature [1], flowers when fully open in the morning at 8:00 h appeared white to ivory (Fig. 1a); massive anthocyanin accumulation started around 11:00 h and began to level off around 17:00 h (Fig. 2), when all petals exhibited a deep pink to red coloration (Fig. 1b). At 20:00 h the petals had begun to curl up (Fig. 1c), and the flowers wilted in the following night. Specific PAL activity in the petals increased from a low activity of 25 pkat mg^{-1} between 7:00 and 8:00 h to over 170 pkat mg^{-1} at 14:00 h and then decreased somewhat less rapidly over the next 20 h (Fig. 2). This time-course of PAL activity strongly suggested the involvement of the enzyme in the rapid pigment production.

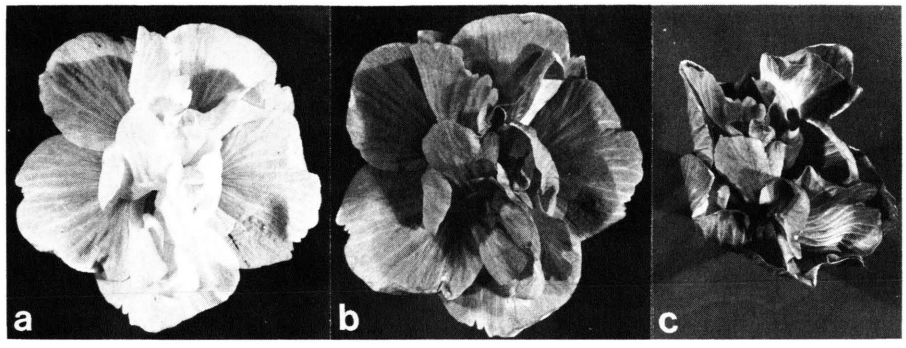

Fig. 1. Flowers of *Hibiscus mutabilis* at a) 8:00 h, b) 17:00 h, c) 20:00 h.

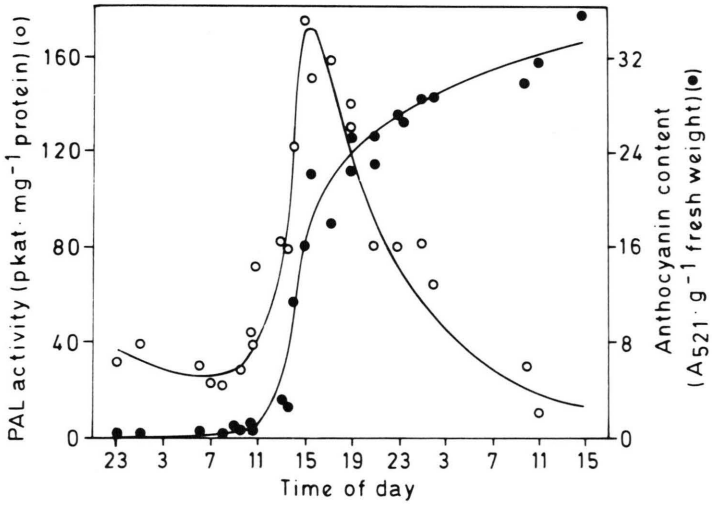

Fig. 2. Time course of anthocyanin accumulation (●) and PAL activity (○) in the petals of *Hibiscus mutabilis* flowers in the final phases of their development. Flowers were fully open at 8:00 h on the first day (see Fig. 1a), were curling up by 20:00 h of the same day (see Fig. 1c), and were withered the followed morning.

Effect of AOPP on anthocyanin formation and amino acid metabolism in excised petals

Outer petals excised at 8:00 h and subsequently floating on 0.01 M potassium phosphate buffer, pH 5.5, under continuous illumination accumulated anthocyanin just as under *in situ* conditions. Increasing concentrations of the PAL inhibitor AOPP progressively reduced the anthocyanin content of the petals. Complete inhibition was observed with a 1 mM concentration of the inhibitor (Fig. 3), while the I_{50}

Fig. 3. Outer petals of *Hibiscus mutabilis* flowers excised at 8:00 h (see Fig. 1a), after a 12 h incubation in the absence (left) or presence (right) of 1 mM AOPP.

value was determined to be 80 μM. Complementation experiments with putative precursors of the aglycon moiety, cyanidin, similar to those conducted previously with buckwheat hypocotyls [7], showed that only cinnamate, *p*-coumarate, naringenin and dihydroquercetin significantly reversed the inhibitory effect of AOPP (data not shown). These results are in agreement with the known pathway of cyanidin biosynthesis in other organisms (for discussion see [7]). Analyses of the soluble amino acids in the petals at the time of excision, and after 12 h incubation in the absence and presence of AOPP, revealed a substantial reduction in the concentrations of the acidic and polar neutral amino acids during incubation, irrespective of the presence of AOPP (with the exception of glycine) (Fig. 4). It was not investigated whether the decrease in the concentrations of these amino acids was due to metabolism or to their efflux from the petals. AOPP caused a specific 9-fold increase in the concentration of phenylalanine which can be attributed to the *in vivo* inhibition of PAL by AOPP. A far less pronounced effect on the concentration of tyrosine may be due to the inhibition of a tyrosine ammonia-lyase activity by AOPP. A similar effect on the concentration of glycine cannot be easily explained.

Discussion

To our knowledge, the striking colour changes in the flowers of *Hibiscus mutabilis* within a few hours after they have fully opened for a single day of beauty have not received the attention of plant physiologists so far. Phytochemical analyses [1, 2] revealed

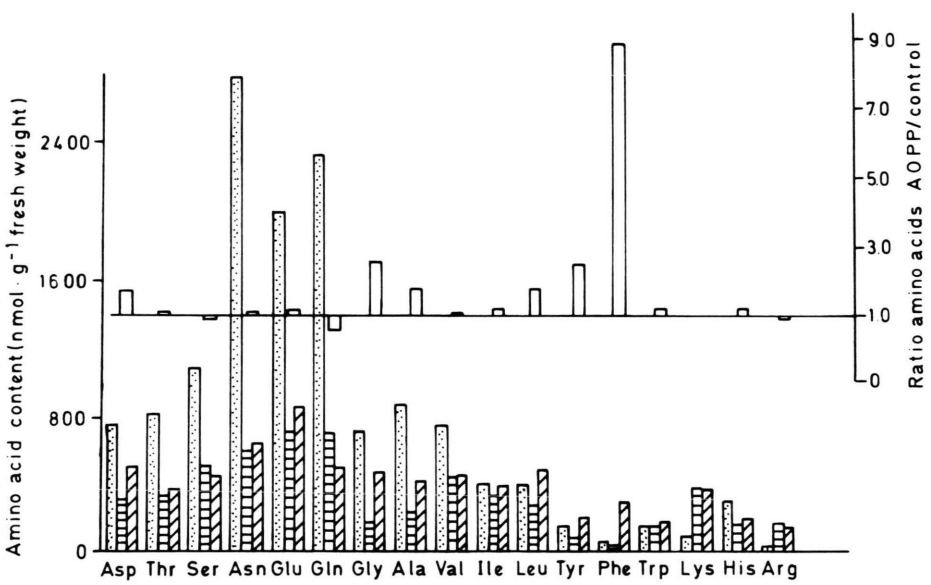

Fig. 4. Concentration of 80% ethanol-soluble amino acids in excised petals of *Hibiscus mutabilis*. Lower panel: the three bars for each individual amino acid in the lower panel represent from left to right: freshly excised petals (8:00 h); petals floated for 12 h on buffer only; petals floated for 12 h on buffer plus 1 mM AOPP. Upper panel: Ratio of amino acid concentrations in AOPP-treated *vs.* control samples.

that the pigmentation is due to cyanidin glycoside(s). The time course of anthocyanin formation in the flowers shows a rapid phase of pigment accumulation between 11:00 and 17:00 h, which coincides with a dramatic and transient increase in the activity of the key enzyme of phenylpropanoid metabolism, phenylalanine ammonia-lyase (Fig. 2). Inhibition of pigment formation in excised petals of *H. mutabilis* (Fig. 3) by the potent inhibitor of PAL, AOPP, clearly shows that PAL activity is necessary for anthocyanin synthesis. It is clear, therefore, that the cyanidin moiety of the pigment is synthesized *de novo* rather than from stored (hydroxy)cinnamate or flavonoid precursors.

While *H. mutabilis* flowers, as well as excised petals, are an attractive system to study the regulation of anthocyanin formation, the erratic flowering of the plants under our greenhouse conditions precluded any further systematic investigations.

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