Relationship between Flower Development, Anthocyanin Accumulation and Activity of Enzymes Involved in Flavonoid Biosynthesis in *Matthiola incana* R. Br.

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Matthiola incana, Anthocyanin Biosynthesis, Anthocyanin Content, Enzyme Activities, Flower Development

The activity of five enzymes concerning anthocyanin biosynthesis as well as the anthocyanin accumulation were studied during the development of buds and flowers of *Matthiola incana*. The investigations included the first three enzymes in the anthocyanin pathway, chalcone synthase, chalcone isomerase and flavanone 3-hydroxylase, and the flavonoid-modifying enzymes, flavonoid 3′-hydroxylase and flavonoid 3-O-glucosyltransferase. The bud and flower development was subdivided into eight stages with respect to morphological criteria. On a fresh weight basis, a substantial correlation between anthocyanin content and the activities of all of the five enzymes were found in the various developmental stages. Furthermore, the anthocyanins formed are obviously not or only less subject to degradation. Although all maxima of activity proved to be in buds, clear differences were observed between the five enzymes with regard to increase and stage of maximum activity. The isolation of other enzymes involved in flavonoid biosynthesis is likely to be most successful in the bud stages.

Introduction

In the last decade substantial progress has been achieved in elucidating enzymology and regulation of flavonoid biosynthesis. Plant cell suspension cultures have played a major role in these developments [1]. But recent work has shown that flowers or ornamental plants are also a good source for flavonoid elaborating enzymes. This allowed now successful enzymological studies on a genetically defined plant material [1, 2].

Among ornamental plants, flowers of *Matthiola incana* are one of the best investigated sources for the demonstration of enzymes involved in flavonoid biosynthesis. Up to now, however, the question remained, in which stage of flower development the known enzymes exhibit their maximum activity and whether there exists a relationship between enzyme activity and flavonoid accumulation. Furthermore, the knowledge of the time course of enzyme activity during bud and flower development is very important in context with our efforts to demonstrate still unknown enzymes of flavonoid biosynthesis [2], and to study gene action and regulation on a molecular level [3, 4].

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In the present paper, the activity of five enzymes concerning flavonoid biosynthesis, chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, flavonoid 3′-hydroxylase and flavonoid 3-O-glucosyltransferase (Fig. 1), were measured in relation to the developmental stages of buds and flowers of *M. incana*.

Materials and Methods

Plant material

The investigations were performed with flowers of an inbred line of *Matthiola incana* (line 04), which contains acylated cyanidin triglycosides in the petals. The plant material was cultivated in a greenhouse of the institute.

Stages of flower development

To classify the stage of the flowers (Fig. 2), morphological criteria were used.

Stage 1: Closed bud. The visible top of the petals amounts to about 1/4 of the whole bud length.

Stage 2: Closed bud. The visible top of the petals amounts to about 1/3 of the whole bud length.

Stage 3: The petals are twice as long as the sepals. The top of the bud is still closed.

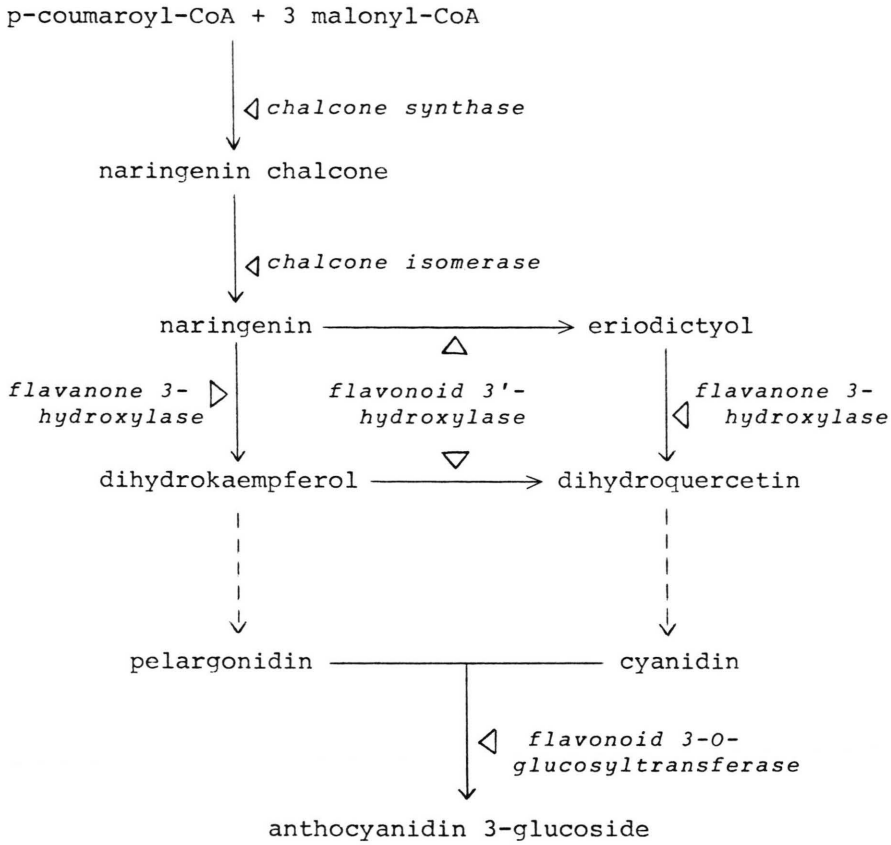

Fig. 1. Biosynthetic pathway of anthocyanins and the position of the five enzymes investigated.

Stage 4: The petals are unrolled at the top of the bud, but not yet unfolded.

Stage 5: The petals are opening to build a 90° angle.

Stage 6: The petals are completely unfolded. The pistil is not yet visible.

Stage 7: The petals are completely unfolded. The pistil appears between the petals.

Stage 8: Old and flaccid flower.

Enzyme preparations and assays

Buds or flowers, respectively, of the eight developmental stages were harvested at the same time to ensure comparable conditions. In all cases, only the less (buds) or more (flowers) coloured upper parts of the petals were used for enzyme preparation. Each stage was subdivided in five samples, because the five enzymes investigated need different work-up.

Standard procedures were used for preparing the extracts and for measuring the enzyme activities of chalcone synthase [5], chalcone isomerase [6], flavanone 3- and flavonoid 3′-hydroxylase [7] and flavonoid 3-O-glucosyltransferase [8].

Incubations were carried out immediately after preparation of a crude extract or microsomal fraction. In all cases, the rate of product formation was linear with time and protein concentration.

The given data are mean values from two series of independent preparations. All tests were run in duplicate. The enzyme activities were referred to gramme fresh weight of the petals and to 1 min of incubation time.

Analytical methods

Protein was determined by the method of Bradford [9]. The anthocyanin content of the petals was estimated as described by Forkmann and Seyffert [10].

Results

Since the velocity of the flower development of *Matthiola incana* is essentially dependent on environmental factors like temperature, illumination and nutrition, morphological criteria were used to divide the developmental process into eight significantly different stages (Fig. 2). This kind of subdivision proved to be sensitive enough to produce characteristic activity curves and to yield good agreement within the values of separate preparations.

The formation of anthocyanins during bud and flower development is shown in Fig. 2. In the less coloured buds of stage 1 the anthocyanin content of the petals is very low. In the following three developmental stages the anthocyanin content increases rapidly up to a maximum value which remains constant in the further flower stages, including the last stage with old and flaccid flowers.

The data for enzyme activity are calculated on a fresh weight basis, since the protein content of the enzyme preparations decreases rapidly and continuously with the growth of the buds and flowers. Regardless of the kind of enzyme preparations used, crude extracts of stage 1 were found to contain about ten times more protein (approx. 1.5 to 3.5 mg/ml) than those of stage 8 (approx. 0.15–0.5 mg/ml). In case of the chalcone synthase enzyme, however, immuno-precipitation experiments showed that in the first five stages of bud and flower development no visible differences in the amount of this enzyme exist (S. Rall, personal communication). Thus, the

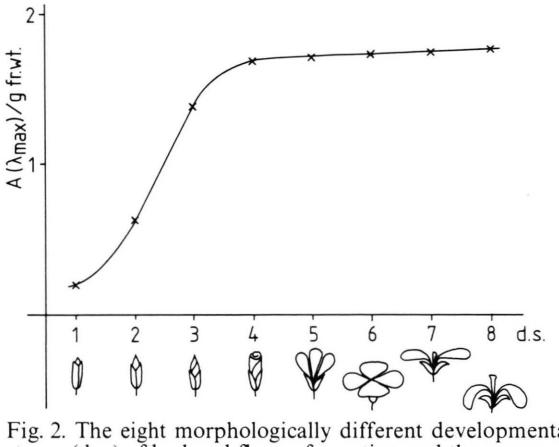

Fig. 2. The eight morphologically different developmental stages (d.s.) of bud and flower formation and the course of anthocyanin accumulation.

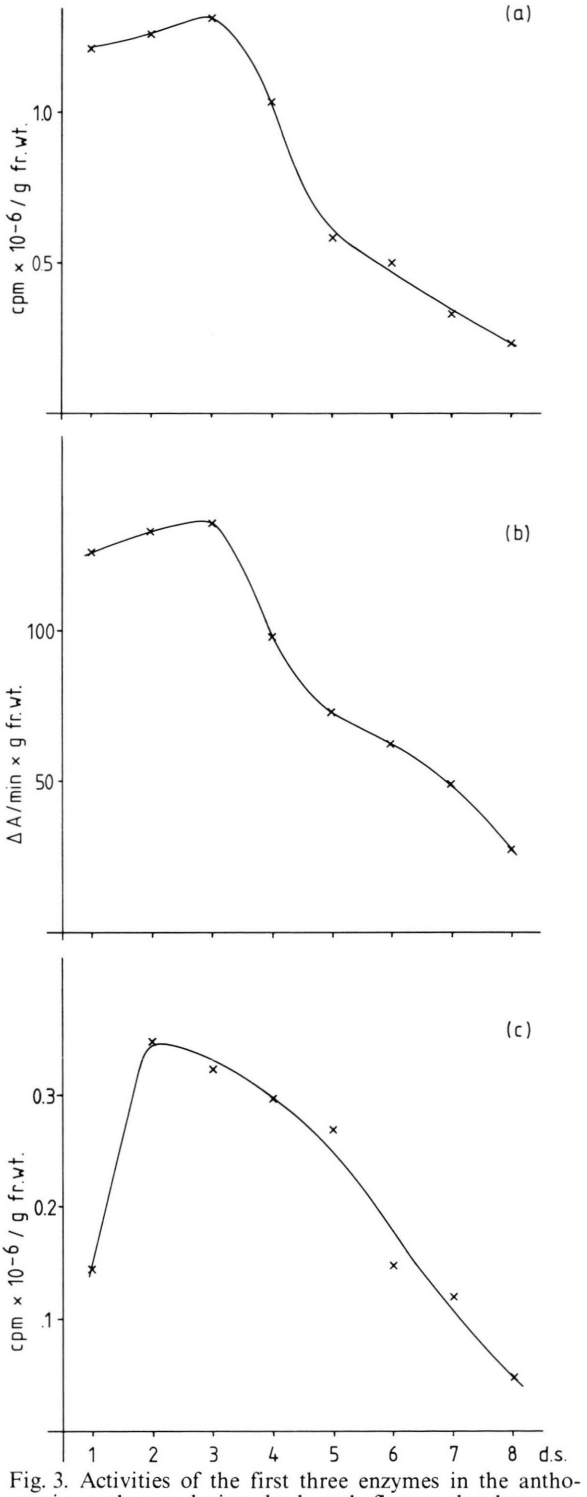

Fig. 3. Activities of the first three enzymes in the anthocyanin pathway during bud and flower development. (a) chalcone synthase; (b) chalcone isomerase; (c) flavanone 3-hydroxylase.

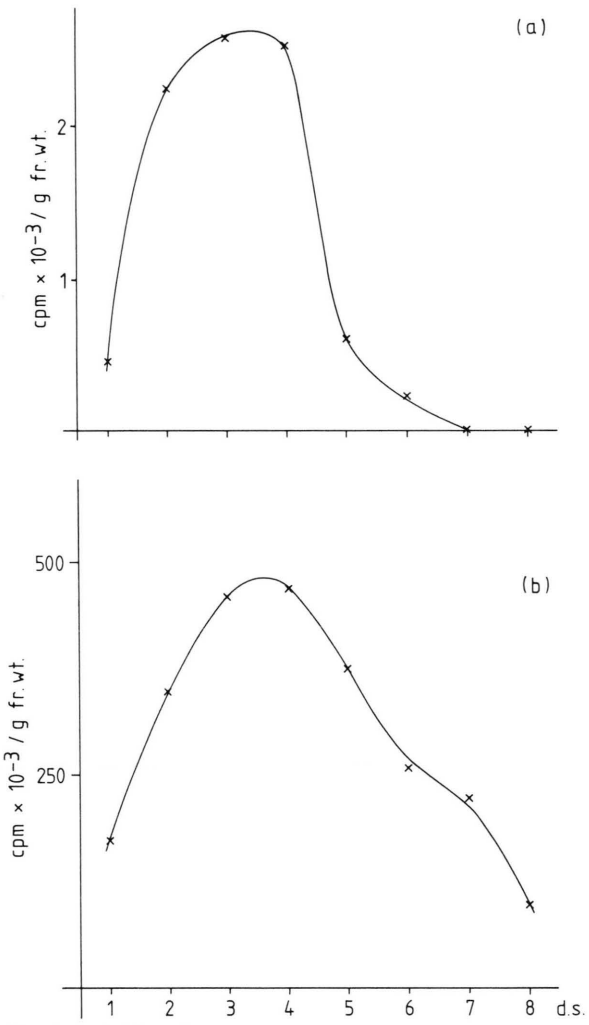

Fig. 4. Activities of the two modifying enzymes during bud and flower development. (a) flavonoid 3′-hydroxylase; (b) flavonoid 3-O-glucosyltransferase.

strong decrease of the protein content during flower development rather reflects the change of protein amount involved in the growth and differentiation process than that of the enzymes investigated.

On a fresh weight basis, the five enzymes exhibit distinct changes of enzyme activity during the development of buds and flowers. The course of activity of the first two enzymes of the flavonoid pathway, chalcone synthase and chalcone isomerase, is quite similar. Both enzymes show the highest enzyme activity already in the developmental stages 1–3. A significant decline in the activities is observed in extracts from later stages (Fig. 3a, b). The activity of the third enzyme in the biosynthetic pathway, flavanone 3-hydroxylase, rises from a considerably lower level in stage 1 rapidly to a maximum in stage 2. In enzyme preparations of the following stages the activity decreases at first slowly, than progressively (Fig. 3c).

The two flavonoid-modifying enzymes, flavonoid 3′-hydroxylase and flavonoid 3-O-glucosyltransferase, exhibit a rather low activity when extracted from the smallest bud stage. After a drastic increase they reach their highest activity in stage 3 and 4. Thereafter, flavonoid 3′-hydroxylase activity drops rapidly to zero in enzyme preparations from stage 7 and 8, whereas flavonoid 3-O-glucosyltransferase activity decreases much more slowly (Fig. 4a, b).

Discussion

The relationship between the activity of flavonoid elaborating enzymes, organ development and flavonoid content was first investigated in young parsley plants. Flavonoid content as well as enzyme activity were found to be maximal already in the youngest cotyledons and leaves investigated, and decreased to a rather low level in progressively older cotyledon and leaf tissue [11].

In contrast, a very low amount of anthocyanin was found to be present in very young flower buds of *Matthiola incana* and highest anthocyanin concentration was only observed in essentially older stages of flower development. Correspondingly, all of the five enzymes investigated in our work exhibited a clear maximum of activity during organ development. Comparison of the various enzyme activities with the amount of anthocyanin in the buds and flowers at the different developmental stages not only reveals a substantial correlation, but also provides evidence for a differential gene activity during flower development. Thus, the enzymes controlling the first steps of anthocyanin formation, chalcone synthase and chalcone isomerase, already show high activity in the earliest stages, where for the third enzyme in the pathway, flavanone 3-hydroxylase, and especially for the two modifying enzymes, flavonoid 3′-hydroxylase and flavonoid 3-O-glucosyltransferase, still a rapid increase of activity from a considerably lower level is observed. In this context, it should be noted that the developmental stages differ from each other not by a few hours, but rather by at least one day. Therefore, the differences observed be-

tween the five enzyme activities with regard to increase and maximum stage seem to be significant.

In opposition to the situation in parsley cell cultures, where a coordinate induction of phenylpropanoid enzyme activities (group I) and flavonoid enzyme activities (group II), respectively, was found after onset of irradiation [12], the expression of the flavonoid synthesizing enzymes of *M. incana* is not only influenced by light, but is also associated with a specific developmental stage of flower formation. The successive expression, however, is obviously not due to the presence of certain flavonoids used as substrates by the following enzymes, since in flower extracts of line 18, which largely lacks flavonoids by a complete deficiency of chalcone synthase activity [5], the other four enzymes investigated in this work exhibit high activities [7, 8].

Although all enzyme activities were found to be more or less drastically diminished in the older developmental stages, the anthocyanin content of the flowers remains constant over a long time, including the oldest flowers. This result suggests that the anthocyanins formed in the flowers are quite stable. If any anthocyanin degradation occurs, it is obviously low.

In all cases, the maximum enzyme activity was found in the bud stages. Therefore, chances for a successful demonstration of unknown enzymes involved in this pathway seem to be most promising in enzyme preparations of the developmental stages 2, 3 and 4; the most suitable source for the isolation of specific mRNAs for enzymes of the flavonoid biosynthesis should be stage 1 or still earlier. On the basis of this knowledge, it was already possible to demonstrate an enzyme activity in *M. incana* which catalyzes the conversion of dihydroflavonols to flavonols. A similar enzyme activity was recently found in cell cultures of parsley [13].

Acknowledgements

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