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THE GENETIC CONTROLLED HYDROXYLATION PATTERN OF THE ANTHOCYANIN B-RING IN *SILENE DIOICA* IS NOT DETERMINED AT THE *p*-COUMARIC ACID STAGE

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INTRODUCTION

The basic C-15 skeleton of flavonoids is formed by the head-to-tail condensation of three malonyl-CoA units to one molecule of hydroxycinnamoyl-CoA [1–4]. The A-ring and part of the heterocyclic ring are derived from the acetate units, the B-ring from the hydroxycinnamoyl ester. The basic C-15 flavonoid is then further modified to yield the various flavonoid classes [5]. In this biosynthetic pathway, the hydroxylation pattern of the B-ring can either be determined at the C-9 level, by starting with the appropriate hydroxycinnamoyl-CoA ester [6], or at the C-15 level, by hydroxylation of one of the C-15 intermediates [7].

In *Silene dioica* the hydroxylation pattern of the B-ring of the anthocyanidin molecule, and of the acyl group bound to the terminal sugar at the 3-position, is controlled by gene *P*, whereas the binding of the acyl group is governed by gene *Ac*. The hydroxylation pattern of the acyl group, however, corresponds with that of the B-ring of the anthocyanidin molecule. Thus in *p/p Ac/Ac* plants, in which only pelargonidin glycosides are found, the acyl group is *p*-coumaric acid. In *P/P Ac/Ac* plants the anthocyanidin is cyanidin, and the acyl group is caffeic acid [8, 9]. This suggests that homozygous recessive *p/p* plants are unable to synthesize caffeic acid, which is used both as a precursor in the biosynthesis of the anthocyanidin molecule and for acylation.

In this paper, we have investigated whether gene *P* is involved in the conversion of *p*-coumaric to caffeic acid.

RESULTS AND DISCUSSION

The enzyme catalysing the hydroxylation of *p*-coumaric acid to caffeic acid was present in petals and leaves of *Silene dioica*. Most of the activity is lost by polyclar AT (PVP) chromatography or by gel filtration. Therefore the supernatant of the crude homogenate was used to study the properties of the enzyme. The amount of caffeic acid formed was proportional to time, for periods

up to 10 min. Maximal synthesis took place at pH 6.5. The divalent metal ions Mn^{2+}, Mg^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+} and Hg^{2+} did not stimulate the reaction rate, neither did EDTA. β-Mercaptoethanol was inhibitory. The presence of NADPH is required; when NADH was used instead of NADPH only 30% of the activity remained. The formation of caffeic acid was independent of the action of gene *P*. In the green parts of *P/P* plants a V_{max} of 147 pmol/min/mg protein was found, in *p/p* plants this value was 158 pmol/min/mg protein. The apparent K_m for *p*-coumaric acid was 1.8 mM.

In contrast to all the other enzymes involved in anthocyanin biosynthesis which have been shown to be present in the petals of *S. dioica* (Kamsteeg *et al.*, unpublished results), the hydroxylation of *p*-coumaric acid was hardly detectable in the petals. V_{max} values of 5 and 4 pmol/min/mg protein, respectively, were found in *P/P* and *p/p* plants. This rate of activity is two orders of magnitude lower than the rate of other enzymes involved in anthocyanin biosynthesis, e.g. UDP-glucose: cyanidin 3-*O*-glucosyltransferase [10]. This suggests that this hydroxylating activity is not involved in anthocyanin biosynthesis in the petals. The finding that this hydroxylating activity is independent of the action of gene *P*, since the activity is present both in leaves and petals of *P/P* and *p/p* plants, also points in this direction. So either gene *P* is pleiotropic in its action, i.e. it is both able to govern the hydroxylation of a C-15 anthocyanidin precursor or pelargonidin and the hydroxylation of the acyl group bound to the 3-*O*-terminal sugar of the anthocyanin molecule, or it acts on another C-9 precursor in flavonoid biosynthesis, *p*-coumaroyl-CoA, which is both used as substrate for the anthocyanidin biosynthesis and for acylation.

EXPERIMENTAL

Synthesis of ^{14}C-*labelled p-coumaric acid.* *p*-Coumaric acid was synthesized according the Knoevenagel reaction. The specific activity of the [2-^{14}C]-malonic acid was adjusted with carrier malonic acid to 1.25 Ci/mol. *p*-Hydroxybenzaldehyde (0.2 mmol) was added, and the mixture was dissolved in 50 μl pyridine. Piperidine (1.5 μl) was added for catalysis. This mixture was incubated for 50 hr at 70°. The ^{14}C-labelled *p*-coumaric acid formed was isolated from the reaction mixture by PC in 2% HCO_2H. The yield was *ca* 20%.

Enzyme preparation. Petals and green parts were homogenized in an all glass Potter–Elvehjem homogenizer, in 50 mM phosphate, pH 6.5. The homogenate was centrifuged for 10 min at 36000 *g*. The supernatant was used for the enzyme assays.

Enzyme assay. The reaction mixture contained in a total vol. of 50 μl: 2 mM NADP, 10 mM glucose-6-phosphate, 1.4 units glucose-6-phosphate dehydrogenase, 1 mM [2-^{14}C]-*p*-coumaric acid and 25 μl enzyme. After 10 min incubation at 30°, the reaction was stopped with 50 μl 12% trichloroacetic acid. The reaction mixture was applied as a spot together with carrier caffeic acid on Whatman No. 3, and developed two-dimensionally in 2% formic acid and toluene–HOAc–H_2O (10:7:3, upper phase), respectively. The caffeic acid spot, visible as a blue spot in UV light, was cut out, placed into a scintillation vial with toluene liquifluor and counted in a liquid scintillation spectrometer.

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