Metabolic changes in carrot cells in response to simultaneous treatment with ultraviolet light and a fungal elicitor

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Abstract. Ultraviolet light induces anthocyanin biosynthesis in cell cultures of an Afghan cultivar of Daucus carota (Daucus carota L. ssp. sativus). Simultaneous treatment with a fungal elicitor from Pythium aphanidermatum results in an inhibition of the catalytic activity of chalcone synthase (CHS), which in turn correlates with an inhibition of anthocyanin biosynthesis. On immunoblots, one isoenzyme (40 kDa) of CHS disappears upon elicitor treatment. On an mRNA level, only the mRNA for the 40-kDa-CHS is active after treatment with ultraviolet light. After inhibition of anthocyanin biosynthesis by the elicitor the enzyme protein disappears and the CHS mRNA is strongly diminished. This inhibition depends on the concentration of the elicitor. In addition, elicitor treatment leads to an induction of the general phenylpropanoid pathway as well as to the accumulation of 4-hydroxybenzoic acid which is covalently bound to wall polysaccharides of the carrot cells. The possible function of phenylalanine ammonia-lyase in providing precursors for 4-hydroxybenzoic acid is discussed.

Key words: Anthocyanin – Cell culture – Chalcone synthase – Daucus – Elicitor (Pythium) – 4-Hydroxybenzoic acid – Phenylalanine ammonia-lyase

Introduction

Plant cells respond actively to different exogeneously applied stimuli by synthesizing secondary metabolites. In cell cultures of parsley the accumulation of flavonoids is mediated by ultraviolet (UV) light. Their rate of synthesis depends on the de novo synthesis of enzymes which are part of the biosynthetic pathway and is regulated by temporal gene activation leading to transient maxima in their catalytic activity (for a review see Hahlbrock and Scheel 1989). These flavonoids are thought to be filter substances that protect tissues from damage by excessive light irradiation (Möhle et al. 1985).

On the other hand, microbial attack leads to the synthesis of phytoalexins belonging to different classes of compounds (for a review see Ebel 1986). In parsley, furanocoumarins are synthesized upon fungal infection (Jahnen and Hahlbrock 1988). The responses of parsley are also demonstrated in model systems such as cell cultures and protoplasts, and in intact plants as well (Dangl et al. 1987). In legumes, isoflavonoid-derived phytoalexins are synthesized in response to infection. The differential activation of a gene family encoding for chalcone synthase as a key enzyme in flavonoid biosynthesis has been described in Phaseolus vulgaris (Ryder et al. 1987). In soybean, glyceollin biosynthesis is also regulated by differential transcription of one of the chalcone-synthase (CHS) genes (Wingender et al. 1989).

Cell cultures of an anthocyanin-containing carrot (Daucus carota L. ssp. sativus) are also well suited for observing rapid responses to UV-light (Seitz and Gleitz 1988) and to a fungal elicitor from the oomycete Pythium aphanidermatum (Schnitzler and Seitz 1989). After UV irradiation the synthesis of anthocyanins is induced. Chalcone synthase is thought to be a key element in anthocyanin biosynthesis. In carrot this homodimeric enzyme is present in two isoforms exhibiting different subunit molecular weights (40 kDa and 43 kDa). The synthesis of anthocyanins is linked to a transient induction of phenylalanine ammonia-lyase (PAL) and the 40-kDa CHS (Seitz and Gleitz 1988).

After treatment with an elicitor from Pythium aphanidermatum, PAL is rapidly synthesized de novo whereas the catalytic activity of CHS is inhibited, as is anthocyanin biosynthesis. On Western blots the 40-kDa CHS disappears but the second isoform with a molecular weight of 43 kDa per subunit is still present. In addition to the activation of PAL, elicitor treatment leads to metabolic changes resulting in an accumulation of 4-hydroxybenzoic acid in a wall-bound form (Schnittzler and Seitz 1989).
were propagated as previously described.

In the present report we compare the responses of cultured anthocyanin-containing carrot cells to UV light and a fungal elicitor. The metabolic alternative is depicted in Fig. 1. The hierarchy in the regulation is discussed.

Materials and methods

Cell cultures. The experiments were carried out with an anthocyanin-containing cell line of Daucus carota L. ssp. sativus. Callus cultures (Seitz and Richter 1970) and cell cultures (Noe et al. 1980) were propagated as previously described.

Irradiation conditions. Dark-grown cell cultures were irradiated with UV light (315–420 nm) 10d after the onset of cultivation, as described earlier (Gleitz and Seitz 1989).

Elicitor preparation. The elicitor from Pythium aphanidermatum was prepared as described previously (Schnitzler and Seitz 1989).

Protein preparation. Four grams of cells (fresh weight) and 1 g Dowex 1 × 2, equilibrated in 100 mM potassium phosphate buffer (KPi) pH 8, were resuspended in 2 ml of 200 mM KPi, 50 mM sodium hydrosulfite, pH 7. The cells were homogenized by sonication (30 s, 70 W) with a micro-tip sonifier (Branson, Danbury, Conn., USA). The supernatant of a 27 000 × g centrifugation (10 min) was frozen in liquid nitrogen and stored at −20°C prior to enzyme-activity measurements.

Enzyme assays. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activity was determined according to Koukol and Conn (1961).

Chalcone-synthase activity was determined according to Gleitz and Seitz (1989).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), Western blotting, and immunostaining. All details are as described earlier by Gleitz and Seitz (1989). The antisera to PAL and CHS were gifts from K. Hahlbrock.

Isolation of total RNA. Total RNA was isolated from cell cultures of carrot. The cells were frozen in liquid nitrogen and ground with pestle and mortar. The powder was allowed to thaw in one volume of freshly prepared phenol (Maniatis 1982) and stirred for 10 min at room temperature. After the addition of equal volumes of extraction buffer (50 mM Tris-HCl pH 7.6, 5 mM MgCl₂) and chloroform/isoamyl alcohol (24:1, v/v) the mixture was stirred for another 10 min. After a centrifugation at 20 000 × g the aqueous phase was extracted twice with an equal volume of chloroform/isoamyl alcohol. Nucleic acids were precipitated overnight at −20°C in two volumes of ethanol containing 0.1 volume of 3 M sodium acetate at pH 5.5. The pellet of a 20 000 × g centrifugation was dissolved in 0.5 ml DNase buffer (40 mM Tris-HCl pH 7.5, 6 mM MgCl₂) and incubated with 20 units of DNase I (Pharmacia LKB, Freiburg i. Br., FRG) for 10 min at 37°C. Subsequent to a chloroform/isoamyl alcohol extraction and ethanol precipitation (see above) the resulting pellet was washed twice with 70% ethanol and then dissolved in sterile water. The purity of RNA was checked by non-denaturing agarose gel electrophoresis (Slater 1986). The RNA concentration was determined photometrically at 260 nm. The A₂₆₀/A₂₈₀ ratio varied from 1.79 to 1.9.

In-vitro translation. An mRNA-dependent rabbit reticulocyte lysate (Boehringer, Mannheim, FRG) was used according to the supplier’s instructions. The lysate was diluted in the reaction mixture to final concentrations of 0.25 mg · ml⁻¹ of total RNA, 1.5 mM magnesium acetate, 50 mM potassium acetate and 18.5 kBq · μl⁻¹ of L-[³⁵S]methionine. The incorporation of methionine into total protein was measured as described by Lawton et al. (1983). For immunoprecipitation of the translation products, 20 μl of the sample were diluted (1:10) by addition of the precipitation buffer (0.1 M NaCl; 1 mM EDTA; 1% ethylphenyleneglycol (Nonidet P-40, Fluka, Neu-Ulm, FRG); 10 mM Tris-HCl pH 7.5). The anti-CHS serum (1.5 μl) was added and then the mixture was incubated at room temperature for 3 h. Then 25 μl of a suspension (100 mg · ml⁻¹) of protein A-Sepharose were added and incubated at room temperature for 1 h. The resulting immunocomplex was collected by centrifugation and washed with precipitation buffer. The procedure was repeated twice. During the last washing step, Nonidet P-40 was omitted. The resulting pellet was heated to 100°C in the presence of 10 μl sample buffer (80 mM Tris-HCl pH 6.8; 5% mercaptopethanol (v/v); 20% glycerol (w/v); 0.01% bromphenol blue; 2% sodium dodecyl sulphate (w/v). The proteins were separated on 10% (w/v) polyacrylamide gels with a 5% stacking gel according to Laemmli (1970). The gels were dried on a gel dryer and autoradiographed with Kodak XAR-5 at room temperature. The relative mRNA activity was determined by scanning and integration of the autoradiograms with a scanner (Elscript 400; Hirschmann, Unterhaching, FRG).

Determination of 4-hydroxybenzoic acid. The 4-hydroxybenzoic acid content was determined by slightly modifying a procedure already described by Schnitzler and Seitz (1989). The butanic extract from cell walls was dried in a stream of filtered air and the resulting residue was redissolved in methanol/H₂O (1:1) and then used for high-performance liquid chromatography analysis by (HPLC). The chromatographic conditions were the same as already described, the sole exception being that a steeper gradient was applied ranging from 5 to 30% methanol.

Protein determination. The protein concentration was determined by Bradford’s method using bovine serum albumin (BSA) as a standard (Bradford 1976).