Anthranilate synthase forms in plants and cultured cells of *Nicotiana tabacum* L.

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Abstract. Tobacco (*N. tabacum* cv. Xanthi) cell lines contained two forms of anthranilate synthase (AS; EC 4.1.3.27) which could be partially separated by gel-filtration chromatography. One form was resistant to feedback inhibition by 10 μM tryptophan (trp) while the other form was almost completely inhibited by trp at the same concentration. Cell lines selected as resistant to 5-methyltryptophan (5MT) had more of the trp-resistant AS form. Only the trp-sensitive form was detected in plants regenerated from both normal and 5MT-resistant cell lines. Overexpression of the trp-resistant form in 5MT-resistant tobacco cells disappeared during plant regeneration but reappeared when callus was initiated from the leaves of these plants. The trp-sensitive form was localized in the particulate fraction and the trp-resistant form in the cytosol of tobacco cultured cell protoplasts. The trp-resistant form of AS from tobacco had an estimated MW of 200000, determined by Sephacryl S-200 chromatography, compared to an estimated MW of 150000 for the trp-sensitive form. The estimated molecular weights of AS from carrot and corn were 160000 and 150000, respectively. Analysis of AS activity from the diploid *Nicotiana* species *Nicotiana otophora* (chromosome number 2n = 24) by high-performance liquid chromatography showed two activity peaks identical in elution time and trp inhibition characteristics to the activity from *N. tabacum* (chromosome No. 48). Thus the two enzyme forms found in tobacco did not appear to have originated individually from the progenitor species genomes which combined to make up the tobacco genome.


Introduction

One objective of plant cell culture research is to select cells with altered properties and to regenerate plants which express the same altered properties. Many selection and plant regeneration studies have been carried out and in some cases the desired property has been found to be expressed in the regenerated plant while in other cases it has not (for reviews, see Widholm 1983; Chaleff 1983). In this laboratory, *Nicotiana tabacum* cells resistant to 5-methyltryptophan (5MT) have been selected and these, like many other 5MT-selected cell lines, contain a feedback-altered anthranilate synthase (AS) which causes an increase in free tryptophan (trp) (Widholm 1972). The enzyme AS converts the branch-point compound chorismic acid to anthranilic acid in the first committed step of the trp branch of the biosynthetic pathway for aromatic amino acids and is the site of regulation via feedback inhibition by trp (Widholm 1973). However, plants regenerated from these 5MT-resistant cells did not express the altered enzyme but suspension cultures initiated from leaves of these plants again expressed the 5MT-resistant traits of altered AS and increased free trp (Widholm 1980). These findings were published in Widholm (1980) but no quantitative data were presented. The work described here was undertaken to study further the expression of AS in cultured *N. tabacum* cells and in regenerated plants.
Materials and methods

Biological materials and culture methods. The Nicotiana tabacum L. cv. Xanthi cell lines used were TX2-4, a clone from 5MT-resistant cells (Widholm 1980); TX3, a 5MT-resistant line (Widholm 1977); TX6, a newly initiated wild-type line; and Xan 6-1, a clone from azetidine-2-carboxylic acid-resistant cells (Hauptmann and Widholm 1982). A 5MT-resistant Nicotiana tabacum ssp. tabacum cell line was C1, wild type (Widholm 1973), and C123, 5MT-resistant (Hauptmann and Widholm 1982).

Cells were cultured in 50 ml of Murashige and Skoog (1962) basal liquid medium with 0.4 mg l−1 2,4-dichlorophenoxyacetic acid (2,4-D) as the only growth regulator (denoted MX medium) in 125-ml Erlenmeyer flasks on reciprocating shakers at 80 rpm at 27-28°C with low continuous light. The cultures were maintained by inoculating 5 ml of cell suspensions at 7-d intervals.

Shoot development was promoted by transferring a small piece of callus or 0.5 ml of a 5-7-d suspension culture onto agar-solidified (0.8%) regeneration medium containing 1.0 mg l−1 indole-3-acetic acid and 0.64 mg l−1 kinetin (N4-furfurylamino purine) (denoted MS medium). Shoots were rooted on the same agar-solidified medium containing no hormones.

Corn seedlings (Zea mays L.; Golden Cross Bantam; Maxim Seed House, Champaign, Ill., USA) were grown on wet paper towels at room temperature in continuous light from fluorescent lamps (Econ-o-watt; cool-white; Westinghouse Corp., New York, N.Y., USA) at 25 pmol.m−2.s−1.

Subcellular fractionation and cell and tissue extract preparation. Protoplasts from TX2-4 cultured cells were prepared as described in Hauptmann and Widholm (1982) except incubation in the protoplast enzyme solution was for 3 h and the protoplasts were collected by centrifugation at 160 g. The protoplasts were disrupted (complete breakage seen under microscope) when forced with a 50-ml syringe through a 10-μm-mesh nylon screen in a 25-mm Swinnex filter holder. The debris and large organelles were collected by centrifugation at 160 g for 5 min. The supernatant was further centrifuged at 27000 g for 15 min. A 67% ammonium-sulfate precipitate was prepared from the supernatant by adding two volumes of a saturated ammonium sulfate solution (room temperature in water) and centrifuging at 27000 g for 10 min. The precipitate was resuspended in a minimum volume of the extraction buffer described below. The large organelles were washed two times with the buffer used for cell disruption (centrifugation each time at 160 g) and were then suspended in 10 ml extraction buffer and disrupted with a nitrogen pressure cell as described below. A 67% ammonium-sulfate precipitate of this extract was prepared.

Cells or tissues were dispersed by homogenization in a glass-glass homogenizer with one volume of extraction buffer (200 mM 3-amino-2-(hydroxymethyl)-1,3-propanediol [Tris]-HCl, pH 7.5, 0.2 mM ethylenediamine tetracetic acid [EDTA], 10 mM magnesium chloride, 10 mM glutamine, 60% glycerol), and 2 mg dithiothreitol and polyvinylpolypyrrolidone per 1 g (FW) of cells. The suspended cells were then passed three times through a Yeda (Rehovot, Israel) nitrogen pressure cell at 35 kg cm−2 and 5°C. After removal of cell debris by centrifugation for 10 min at 27000 g (5°C), one volume of supernatant was mixed with two volumes of ammonium sulfate, saturated at room temperature in water, and centrifuged as before. The resulting pellet was resuspended in a small volume of extraction buffer and used in the AS assay.

Anthranilate-synthase assay. Anthranilate synthase (AS; EC 4.1.3.27) activity was measured by continuous fluorometric detection of anthranilate production (modification of method used by De Moss, 1965, with bacterial extracts) at 30°C using an Amino-Bowan (SLM-Aminco, Urbana, Ill., USA) spectrophotofluorometer equipped with an Amino-Bowan Model 10-280 blank subtract microphotometer and a strip-chart recorder. Anthranilate was excited at 340 nm and fluorescence measured at 400 nm. The resuspended ammonium-sulfate pellet, 0.1 ml, was combined with 1.5 ml of buffer (50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [Hepes], pH 7.5, 0.05 mM EDTA, 2.0 mM magnesium chloride, 10 mM glutamine, 2.0 mM dithiothreitol, 5% glycerol), and 0.2 ml of substrate solution (80 mM chorismate, 15 mM magnesium chloride, 80 mM glutamine, pH 6.0). Stepwise additions of trp were made from 0.1 mM, 1.0 mM and 10 mM stocks, allowing the reaction to stabilize between each addition. The total volume of trp additions was less than 3% of the total assay volume except when AS was very resistant to trp inhibition in which case the final trp volume added was 10% of the total assay volume.

Chromatography. For Sephacryl S-200 chromatography, 2.0 ml of the ammonium-sulfate fraction were combined with 3.0 μl of glucose-6-phosphate dehydrogenase (internal standard; ammonium-sulfate suspension from Leuconostoc mesenteroides) and applied to the top of a 1.8 cm diameter, 98 cm long column. The flow rate was approx. 0.05 ml.min−1 (100 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5.0 mM magnesium chloride, 5.0 mM glutamine, 2.0 mM dithiothreitol, 50% glycerol) and 2-ml fractions were collected. The AS activity was assayed as described above except that 1.0 ml of a column fraction was combined with 0.6 ml buffer and 0.2 ml substrate solution. The activity of glucose-6-phosphate dehydrogenase was assayed by combining 0.1 ml of a column fraction with 0.1 ml of 60 mM NAD, 0.1 ml of 100 mM glucose-6-phosphate, and 2.8 ml of 100 mM potassium phosphate, pH 7.5. The increase in NADH was measured at 340 nm. The column was standardized using: horse-spleen ferritin (MW=440000), bovine liver catalase (210000), rabbit-muscle aldolase (158000), bovine heart lactate dehydrogenase (142000), horse-liver alcohol dehydrogenase (80000), and hen-egg ovalbumin (45000).

High-performance liquid chromatography (HPLC) of AS was performed as described in Brotherton and Widholm (1985).

Chemicals. Aldolase, ovalbumin and Sephacryl S-200 superfine were obtained from Pharmacia Fine Chemicals (Upssala, Sweden). All other enzymes used in the standardization of the Sephacryl S-200 column were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Chorismate was prepared as described by Gibson (1970). All other chemicals were reagent grade and obtained from commercial sources. Nylon screen (10 μm mesh) was obtained from Tefko (Elmsford, N.Y., USA) and disc filter holders were Swinnex, 25 mm, from Millipore Corp. (Bedford, Mass., USA).

Results

Direct fluorometric AS assays. In previous work from this laboratory (Widholm 1973), AS activity was assayed by measuring ethyl-acetate-extract-