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Tissue Culture Studies of Anthranilate Synthase the Tryptophan Biosynthetic Control Enzyme

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Abstract

Experiments initiated 30 years ago to obtain selectable markers have led to a series of studies of Trp biosynthesis and anthranilate synthase (AS) the control enzyme using largely plant tissue cultures since they have experimental properties that can be readily exploited. Enzymological and compound feeding studies provided evidence that AS is the control point in the Trp biosynthesis branch and that altering the AS feedback control by the selection of mutants resistant to the Trp analog 5-methyltryptophan (5MT) can lead to the overproduction of this important amino acid. Plants regenerated from these Trp overproducing lines of most species also had high free Trp levels but *Nicotiana tabacum* (tobacco) plants expressed the feedback altered AS only in cultured cells and not in the regenerated plants. Further tests by transient and stable expression of the cloned promoter for the naturally occurring tobacco feedback-insensitive AS, denoted ASA2, confirmed the tissue culture specific nature of the expression control. The $5MT^r$ caused by the expression of a feedback-insensitive AS from tobacco has been used to select protoplast fusion hybrids with several species since the resistance is expressed dominantly. Recently the ASA2 gene has been used successfully as a selectable marker to select transformed *Astragalus sinicus* and *Glycine max* hairy roots induced by *Agrobacterium rhizogenes*. These results show that the ASA2ÿ-subunit can interact with the ÿ-subunit of another species to form active feedback-insensitive enzyme that may be useful for selecting transformed cells. Plastid DNA transformation of tobacco has also effectively expressed ASA2 in the compartment in which Trp biosynthesis is localized in the cell.

Introduction

The results presented here come from continuous studies that were begun about 30 years ago using plant suspension cultures as experimental materials. The studies were initiated since I was interested in having resistance markers to use in genetic manipulation experiments. Even though the timeframe has been long, we have been able to use the tryptophan (Trp) analog 5-methyltryptophan (5MT) as a selection agent in protoplast fusion and genetic transformation experiments. The overall studies have also provided information about the control of Trp biosynthesis and the promoter that controls expression of anthranilate synthase (AS) in tobacco. Most of the results discussed are from work done in this laboratory even though other laboratories have carried out important experiments especially on mutant selection and recently on AS gene molecular analysis.

Trp is an "essential" amino acid since it is not synthesized by nonruminant animals including man. The amino acid is usually deficient in cereal grains so the manipulation of its levels could also be of importance. Trp or intermediates in the pathway are also precursors of many secondary compounds including indoleacetic acid, phytoalexins and in-

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secticidal compounds.

Trp is synthesized by a five enzyme branch from the branch point compound chorismate the last compound made by the shikimate pathway (Haslam, 1993). Chorismate also is used by chorismate mutase to form the other aromatic amino acids phenylalanine and tyrosine. Work with bacteria has shown that the Trp branch was controlled by repression of enzyme synthesis by Trp and also by feedback inhibition of the branchpoint enzyme AS.

AS usually is a tetrameric enzyme consisting of two ÿ-subunits and two ÿ-subunits (Crawford, 1989). The ÿ-subunit alone can convert chorismate to anthranilate in the presence of high ammonium levels. This subunit also contains the Trp feedback inhibition binding site. When the ÿ and ÿ-subunits are combined the natural substrate glutamine can act as the amino donor.

The first experiments that were done in this lab involved the selection of suspension cultured *Nicotiana tabacum* (tobacco) and *Daucus carota* (carrot) suspension cultured cells using growth inhibitory concentrations of 5MT (Widholm, 1972a, b). The selected resistant cultures were very resistant to 5MT and the resistance was stable in the absence of the selection agent. The resistant cells contained AS enzyme activity that was more feedback-insensitive to Trp and 5MT than the activity found in the wild-type cells which resulted in higher than normal free Trp levels.

These initial results led to the rest of the studies summarized here.

Enzymology and physiology of Trp biosynthesis

Development of the enzyme assays was assisted by the earlier work with plants by Delmer and Mills (1968) and Belser et al. (1971). Much effort had to be expended, however, to stabilize the AS activity in cell extracts since this was necessary for accurate activity and kinetic measurements.

In order to determine if the repression of enzyme synthesis occurred, as normally seen with bacteria, tobacco, rice, carrot, tomato and soybean suspension cultures were grown in the presence of high Trp levels (Widholm, 1971). The exogenous Trp increased the cellular Trp concentrations by 12 to 2500-fold but did not decrease the AS or Trp synthase enzyme activities. Addition of 50 ÿ M Trp to the reaction mixture completely inhibited the AS activity while 1 mM Trp decreased the Trp synthase activity by only 10-20% in extracts from all the cell lines. This shows that AS is much more sensitive to feedback inhibition than the last enzyme, Trp synthase.

To determine if indeed eight Trp analogs that were inhibitory to tobacco and carrot suspension cultures were toxic because they acted as Trp analogs, reversal studies using anthranilic acid, indole or Trp were carried out (Widholm, 1972c). The analogs were also used to inhibit the enzyme activity of AS and Trp synthase. The growth inhibition caused by the 4 and 5-methyl, the 5 and 6-fluoro, 5-hydroxy and 7-aza derivatives of Trp was reversed by the additives that all result in Trp accumulation in the cells. These analogs were also potent inhibitors of AS but not Trp synthase activity indicating that these six did act as toxic Trp analogs.

Both tobacco and carrot suspension cultures that were 5MT-resistant ($5MT^r$) and overproduced free Trp were at least partially resistant to the six Trp analogs listed above as well as to 4-fluoro, 5-methoxy and 4 and 7-methyl Trp analogs (Widholm, 1981). It was also demonstrated in this paper that Trp synthase could convert many indole analogs into the corresponding toxic Trp analogs. Thus this system might be useful for selecting Trp synthase auxotrophs since only lines with Trp synthase activity could make the toxic Trp analogs.

Each of the five enzyme activities was individually measured in carrot suspension culture crude extracts and some Trp was formed from added chorismate (Widholm, 1973). The AS activity was clearly lower than three of the other four enzymes in the extracts supporting its role as the control point. When the five enzyme activities were measured in wheat seedling extracts, AS had the lowest activity and was the only one inhibited by low Trp concentrations (Singh and Widholm, 1974).

When tobacco and carrot suspension cultures were grown in media containing shikimate, anthranilate, indole or Trp, all the compounds except shikimate caused a large increase in the free Trp pool in the cells (Widholm, 1974a). These results indicate that shikimate, which requires three enzymatic steps to form chorismate, does not freely flow past AS to make Trp, while anthranilate and indole which occur after AS in the pathway can flow freely to synthesize Trp. The results of these feeding studies and many of those presented above support the hypothesis that AS is the control point in Trp biosynthesis.

The AS enzyme activity in extracts from suspension cultured cells of tobacco, carrot, soybean and rice was found to be completely inhibited by from 5 to 50 ÿM Trp (Widholm, 1974c). The cellular levels in all lines were higher than this so compartmentalization would apparently be implicated in allowing the AS to be active to synthesize Trp. Studies with wild-type and a $5MT^r$ carrot line that over-

produces free Trp, due to the presence of feedback-insensitive AS, shows that the respective enzymes would be about 8 and 11% active during growth to produce the total Trp found in the cells. The levels of the cofactor Mg^{++} and the substrate glutamine were near optimal for AS activity in both carrot and tobacco cells but the chorismate levels were well below optimal.

Auxin-autotrophy was found with five of 10 carrot suspension cultures that were $5MT^r$ and overproduced Trp due to a feedback-insensitive AS (Widholm, 1977). A potato line with high Trp was also auxin-autotrophic while wild-type carrot, potato and tobacco and Trp overproducing tobacco lines were not auxin-autotrophic. It was possible to select auxin-autotrophic carrot and potato lines from wild-type cells incubated on auxin-free medium but these lines were not $5MT^r$. The additional of Trp or indole to the medium partially alleviated the auxin requirement of wild-type cell lines.

Selection of $5MT^r$ tissue cultures

Many cell lines have been selected as resistant to 5MT, including the tobacco and carrot lines already mentioned, and a carrot line from which plants were regenerated that carried the resistance trait when tissue cultures were reinitiated (Widholm, 1974b). This carrot line was 5MT-resistant due to decreased uptake and not due to a feedback-insensitive form of AS as found in all other cases.

It was also possible to select a carrot suspension cultured cell line sequentially for resistance to toxic analogs of phenylalanine, methionine, lysine and Trp (5MT) and this line contained 7, 6, 5 and 32-times the wild-type levels of the respective free amino acids (Widholm, 1978). The resistance was increased at least 100-fold for each analog.

Datura innoxia cell lines resistant to 5MT were selected by Ranch et al. (1983) and mutagens were found to increase the frequency. The resistant cell lines contained feedback-insensitive AS and increased free Trp. Leaves of regenerated plants and cultures initiated from the regenerated plants also showed 5MT-resistance, feedback-insensitive AS and increased free Trp. A later report (Brotherton et al., 1996) showed that the 5MT-resistance was retained by the *D. innoxia* plants propagated vegetatively for over 10 years. The trait was also inherited by progeny as a single, nuclear, dominant gene. The plants and cultures initiated from them retained 5MT-resistance, feedback altered AS and increased free Trp.

Rice callus initiated from mature seeds was selected for $5MT^r$ and plants were regenerated from the resistant callus (Wakasa and Widholm, 1987). Both the $5MT^r$ callus and regenerated plants contained increased levels of free Trp. The resistance, which could be assayed by seedling growth in 5MT, was inherited as a dominant trait that segregated 1:1 when heterozygous plants were selfed. Repeated selfing of $5MT^r$ plants did not produce homozygous plants.

Asparagus suspension cultured cells were selected for resistance to 5MT and these lines contained feedback-insensitive AS and increased free Trp (Curtiss et al., 1987). The lines also were resistant to 4-methyltryptophan and 6-fluorotryptophan.

Zea mays (maize) callus was also selected with 5MT and plants were regenerated (Miao et al., 1988). Both the $5MT^r$ callus and plants regenerated from this callus had very high levels of free Trp. Unfortunately all the regenerated plants were male and female sterile. Interestingly this same experiment was carried out to obtain fertile maize plants and the Trp overproduction trait due to feedback-insensitive AS was the first plant trait patented (Hibberd et al., 1987).

A potato suspension culture selected as 5MT-resistant contained increased free Trp due to a very high level of feedback-insensitivity of the AS activity (Carlson and Widholm, 1978). Preparative polyacrylamide gel electrophoresis of both wild-type and $5MT^r$ cell extracts separated feedback-insensitive and feedback-sensitive AS forms. The wild-type had predominantly the feedback-sensitive form while the $5MT^r$ line contained mainly the feedback-insensitive form. Thus potato appears to express two AS forms and selection for $5MT^r$ selected cells that overexpress the feedback-insensitive form.

We found that $5MT^r$ cell lines could be selected from newly initiated tobacco suspension cultures that exhibited the usual characteristics, feedback-altered AS and higher free Trp (Brotherton et al., 1986). However, the plants regenerated from these cultures did not show these characteristics but new cultures initiated from these plants did. High-performance liquid chromatography with a post-column reactor and fluorescence detector (Brotherton and Widholm, 1985) and conventional gel filtration chromatography showed that there were both feedback-sensitive and insensitive AS forms in both wild-type and $5MT^r$ cells. When regenerated plants were examined only the feedback-sensitive form was seen even in the case of the $5MT^r$ cell regenerants. Thus the feedback-insensitive AS form is expressed only in cultured cells.

Molecular studies of AS and its promoter

We have recently initiated studies of the ex-

pression of the feedback-insensitive AS form in tobacco at the molecular level. The cDNA for a naturally occurring feedback-insensitive form of AS was cloned from tobacco using PCR (Song et al., 1998). The cDNA was found to have 72% amino acid sequence identity to the AS ÿ2 of *Ruta graveolens* so the AS gene was denoted ASA2. The cDNA contains an apparent 61 amino acid transit sequence that would apparently target the mature protein to the plastids as found in other species. The expression pattern for this cDNA was similar to that found for the feedback-insensitive AS form observed above in the tobacco 5MT^r cultures and regenerated plant studies (Brotherton et al., 1986); the mRNA was very abundant only in the 5MT^r suspension cultures of tobacco and Nicotiana sylvestris but not in 5MT-sensitive cultures or in leaves, roots, stems or seeds of plants regenerated from 5MT^r cultures.

When the ASA2 cDNA was expressed in *E. coli trp*E5972 nonsense mutants, the cells were able to grow without added Trp and were also resistant to 5MT. When the site-directed mutant AS form with Phe-107, Arg-108 changed to Ser-107, Gln-108, the latter being the amino acids found in feedback-sensitive AS forms in other plant species, was expressed in the *E. coli* Trp nonsense mutant, the cells could grow without Trp but were no longer 5MT^r. The partially purified expressed ASA2 was active enzymatically *in vitro* and was more Trp feedback-insensitive than an expressed *Arabidopsis* ASA1 cDNA.

When the AS DNA fragment between +181 and + 392 that contains the Phe-107 and Arg-108 residues was PCR amplified, cloned and sequenced from genomic DNA from 5MT^r and 5MT^s *N. tabacum* and the putative progenitor species *N. sylvestris* and *N. tometosiformis*, a 115 bp intron was found in all cases. All the sequences were identical except there were eight base changes in *N. sylvestris* but these changes resulted in no amino acid changes. Southern hybridization with genomic DNA using ASA2 as probe indicates that tobacco and the two progenitor species contain two or more genes with homology to ASA2.

Thus it appears that ASA2 is a naturally occurring gene in tobacco and its progenitors that encodes a feedback-insensitive form of AS. This gene is expressed at high levels in 5MT^r cell lines but not in plants regenerated from these cell lines or in wild-type cultures. The 5MT selection thus apparently selects for cells that are overexpressing ASA2.

To attempt to learn more about the control of ASA2 expression, the 2252 bp fragment upstream of the coding sequence was cloned by inverse PCR and sequenced (Song et al., 1998). Deleted fragments were used to drive GUS expression in transient expression assays following particle bombardment. All the deleted fragments used, 370, 606, 1356 and 1252 bp, drove GUS expression at levels equal to or higher than the control CaMV35S in 5MT^r suspension cultured tobacco cells. The 370 and 606 bp fragments drove good expression in tobacco leaves while the 1356 and 2252 fragments had very low expression. These results support the concept that the ASA2 full-length promoter is tissue culture-specific with very low expression in plant tissue. This is also supported by stable transformation experiments where we find very low histochemical GUS expression in tobacco and *Astragalus sinicus* plant tissue when the 2252 bp promoter was used to drive GUS (Kim J, Cho HJ, Brotherton JE and Widholm JM, unpublished). Some of the callus tissue initiated from the tobacco plants transformed with the full-length promoter does show GUS expression.

The above results indicate that there may be transcriptional factors expressed in the 5MT^r *Nicotiana* sp. cultured cells that are not found in 5MT^s cells and regenerated plants. DNA binding gel-shift assays using fragments of the ASA2 promoter and nuclear extracts from suspension cultures do indeed show that the 5MT-resistant cultures have a protein that binds (Song HS, Brotherton JE, Widholm JM, unpublished). Further work is needed to clone and express this trans-acting factor or factors to elucidate the mechanisms.

Use of 5MT-resistance as a selectable marker

The 5MT^r trait of a number of cell lines has been used in the selection of protoplast fusion hybrids. One such study involved the fusion of protoplasts from a carrot cell line resistant to 5MT and another resistant to the lysine analog S(2-aminoethyl)-L-cysteine (AEC). Selection with toxic levels of both analogs produced a large number of hybrids while no colonies formed with unfused protoplasts (Harms et al., 1981). The hybrids usually contained the additive number of chromosomes and maintained the resistance with or without selection pressure.

Protoplasts from a 5MT and azetidine-2-carboxylate (A2C, proline analog)-resistant carrot cell line incapable of plant regeneration were fused with protoplasts from suspension cultured *Daucus capillifolius* cells and 5MT^r colonies were then selected (Kameya et al., 1981). The growing colonies were then selected for green pigmentation and embryo formation. Hybrid shoots then formed that contained the additive number of chromosomes and isozyme patterns. Callus initiated from these shoots showed resistance to both 5MT and A2C and feedback-in-

sensitive AS activity.

Protoplasts made from a 5MT^r tobacco cell line that was incapable of plant regeneration due to a long culture period were fused with *Nicotiana glutinosa* leaf mesophyll protoplasts (Horn et al., 1983). The fused protoplasts were selected for 5MT^r and subsequently plant regeneration. The hybrids were confirmed by isozyme and fraction 1 protein analysis.

A 5MT^r and a glyphosate-resistant carrot cell line were used in protoplast fusion experiments where the hybrids were selected using toxic levels of both inhibitors (Kothari et al., 1986). Restriction endonuclease analysis of the mitochondrial DNA from some of the hybrids showed that the mitochondrial DNA had undergone recombination and was different from either parent.

Protoplasts from a carrot suspension cultured line resistant to both 5MT and A2C were fused with protoplasts from a glyphosate-resistant carrot line (Hauptmann et al., 1988). The glyphosate-resistance was due to amplification of the glyphosate target gene, enolpyruvylshikimate-3-phosphate synthase (EPSPS), that results in overexpression of the enzyme encoded by this gene. The hybrids were selected with both glyphosate and A2C. Most of the hybrids were also 5MT^r even though this was not selected for and contained the amplified EPSPS genes as shown by Southern analysis and high levels of the EPSPS enzyme protein as shown by Western blots.

We have recently used the ASA2 gene driven by the CaMV 35S promoter in transformation experiments with hairy roots of both *A. sinicus* and soybean. In the first experiments with *A. sinicus*, the hairy roots induced by *Agrobacterium rhizogenes* were selected for kanamycin resistance (*Npt*II) that was also carried on the binary vector with ASA2 (Cho et al., 2000). The hairy roots did express ASA2 as shown by Northern blots, feedback resistance of AS activity and up to 6-fold increased free Trp. When the hairy roots were placed on 5MT-containing medium the controls were completely inhibited by 15 ÿM while the ASA2 expressing lines could grow with 100 M. Other experiments with *A. sinicus* and soybean hairy roots show that roots transformed with ASA2 can be selected directly in normally inhibitory levels of 5MT (Cho HJ, Brotherton JE and Widholm JM, unpublished).

The use of feedback-insensitive AS ÿ-subunit genes as selectable markers with 5MT as the selection agent has been reported with maize (Anderson et al., 1997) and rice (Wakasa et al., 1999), both in patents. In both cases mutant AS forms from the same respective species were used. In our work with *A. sinicus* and soybean, the ÿ-subunit, which does contain the feedback inhibition binding site, is from a different species (tobacco). This means that the feedback-insensitive ASA2 ÿ-subunit must be forming an active enzyme complex with the native ÿ-subunit, which has never been demonstrated before.

The ASA2 enzyme is more feedback-insensitive than the form from maize (Anderson et al., 1997) and the feedback-inhibition kinetics of the rice enzyme have never been published (Wakasa and Widholm, 1987; Wakasa et al., 1999). Thus we feel that ASA2 may be a good selectable marker that could be effective with many species.

We recently have transformed the plastid DNA of tobacco with the ASA2 gene without the transit sequence using the techniques described by Svab et al. (1990). AS is normally localized in the plastids where Trp biosynthesis occurs even though the genes are nuclear encoded. We found very high gene expression in transformed tobacco seedlings by Northern hybridization and did measure feedback insensitive AS activity (Zhang XH, Brotherton JE, Widholm JM, Portis AR, unpublished). The plants had very high free Trp concentrations also probably due to the high enzyme expression. The copy number of the ASA2 gene would be very high since plant cells contain many plastid chromosome copies which probably explains the high expression levels.

I have attempted to review the work mostly done in my laboratory using plant suspension cultures to study many aspects of Trp biosynthesis, selectable marker and promoter analysis. The studies were expedited since it is relatively easy to select mutants resistant to 5MT using suspension cultures. These cultures are also readily used in growth inhibition and compound feeding studies and enzyme extractions. In many cases plants can be regenerated. Thus I feel that tissue cultures can offer one ideal materials for biochemical, genetic and molecular biology studies of plants.

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