Increasing Tryptophan Synthesis in a Forage Legume *Astragalus sinicus* by Expressing the Tobacco Feedback-Insensitive Anthranilate Synthase (ASA2) Gene[1]

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A cDNA clone that encodes a feedback-insensitive anthranilate synthase (AS), *ASA2*, isolated from a 5-methyl-tryptophan (Trp) (5MT)-resistant tobacco cell line under the control of the constitutive cauliflower mosaic virus 35S promoter, was introduced into the forage legume *Astragalus sinicus* by *Agrobacterium rhizogenes* with kanamycin selection. The 35S-*ASA2* gene was expressed constitutively as demonstrated by northern-blot hybridization analyses and the presence of feedback-insensitive AS. Hairy root lines transformed with 35S-*ASA2* grew in concentrations of up to 100 μM 5MT, whereas the controls were completely inhibited by 15 μM 5MT. Expression of the feedback-insensitive ASA2 resulted in a 1.3- to 5.5-fold increase in free Trp. Kinetic studies of the AS activity demonstrate the Trp feedback alterations and indicate that the ASA2 α-subunit can interact with the native *A. sinicus* β-subunit to form an active enzyme. The *ASA2* transcript and high free Trp were also detected in the leaves, stems, and roots of plants regenerated from the transformed hairy roots. Thus, we show for the first time that *ASA2* can be used to transform plants of a different species to increase the levels of the essential amino acid Trp and impart 5MT resistance.

Trp is an essential amino acid since it is not synthesized by animals and must be obtained in the diet of non-ruminants such as swine, poultry, and humans. Anthranilate synthase (AS) catalyzes the first reaction in the multi-step Trp biosynthesis branch by converting chorismate to anthranilate (Fig. 1). AS is feedback inhibited by the end product Trp, which binds to an allosteric site on the AS catalytic α-subunit. That AS is the control point in the Trp branch in plant cells is indicated by pathway intermediate-feeding studies (Widholm, 1974), enzyme activity levels (Singh and Widholm, 1974), feedback inhibition of the respective enzyme activities (Singh and Widholm, 1974), and 5-methyl-Trp (5MT) resistance selection (Widholm, 1972). Also, transformation with a feedback-altered *AS* gene has produced maize (Anderson et al., 1997) and rice (Wakasa et al., 1999) lines with altered feedback-inhibited AS and higher free Trp.

Plant cell culture systems have been useful for studying the regulatory mechanisms of amino acid biosynthesis since selection for resistance to toxic analogs such as 5MT can produce lines with feedback-insensitive AS enzyme activity, which result in increased amounts of the end product, Trp. For cellular selection to be useful for plant breeding, plants that express the trait and pass the selected trait to their progeny must be regenerated. In the case of 5MT resistance, tobacco plants regenerated from resistant cells did not express the altered form of AS (Brotherton et al., 1986) whereas *Datura innoxia* plants did (Ranch et al., 1983; Brotherton et al., 1996). In the case of rice the 5MT resistance of the regenerated plants (Wakasa and Widholm, 1987) was a nuclear, dominant trait but homozygosity was not attained even after several successive self-pollinations of resistant plants (Wakasa and Widholm, 1991). The use of genetic transformation technology with the Trp feedback-insensitive *ASA2* cDNA might allow more controlled expression.

Recent studies have shown that the plant AS consists of nonidentical large (α, component I) and small (β, component II) subunits similar to bacteria (Yanofsky and Crawford, 1987; Crawford, 1989). *AS* genes encoding an α-subunit (Niyogi and Fink, 1992; Bohlmann et al., 1995) and a β-subunit (Niyogi et al., 1993) have been cloned from Arabidopsis and *Ruta graveolens*. The two *AS* genes encoding the α-subunit of the enzyme cloned from Arabidopsis and *R. graveolens* have been designated *ASA1*/*ASA2* and *AS*α*1*/*AS*α*2*, respectively (Niyogi and Fink, 1992; Bohlmann et al., 1995). The expression of the *ASA1* and *AS*α*1* genes is induced by wounding and/or elicitor treatment and results in secondary compound production.

Maize and rice plants have been transformed with feedback-altered mutant *AS* genes isolated from the same respective monocot plants, maize and rice, as reported in the patents (Anderson et al., 1997; Wakasa et al., 1999). We describe here the transfor-

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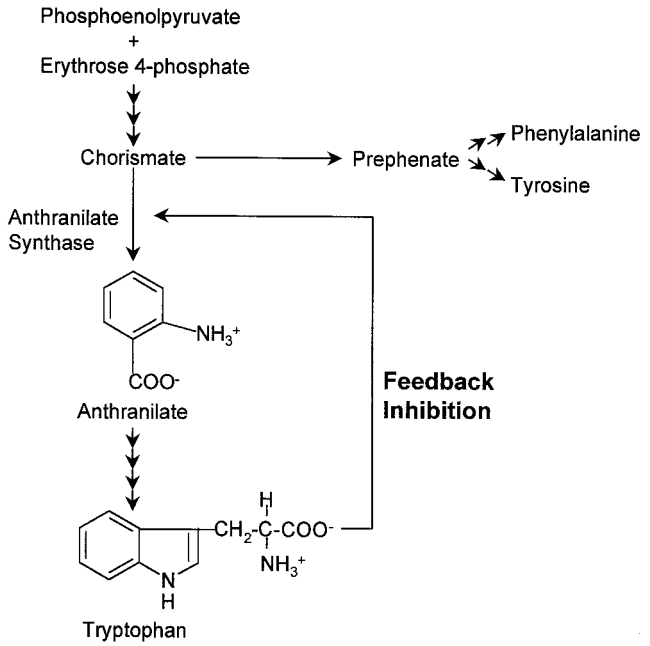

Figure 1. A diagram of the Trp biosynthetic pathway showing some key enzymes and their products. There are actually seven enzymes before chorismate.

mation of a model forage legume, *Astragalus sinicus*, with a cDNA designated *ASA2* that encodes a naturally occurring feedback-insensitive AS α-subunit cloned from an unselected but 5MT-resistant ($5MT^r$) tobacco suspension-cultured cell line (AB15-12-1; Song et al., 1998). These experiments were carried out to determine if expression of the feedback-insensitive ASA2 α-subunit of tobacco in a legume, *A. sinicus*, produces a feedback-altered AS that affects Trp biosynthesis and imparts tolerance to the Trp analog 5MT.

RESULTS AND DISCUSSION

Formation and Molecular Analysis of Transgenic *A. sinicus* Hairy Roots

Large numbers of kanamycin-resistant root lines were produced from *A. sinicus* seedlings transformed with *Agrobacterium rhizogenes* strain DC-AR2 containing pBIN-*ASA2*. The pBIN-*ASA2* plasmid contains the 2.2-kb coding and 3′-downstream regions of the *ASA2* cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter and also the kanamycin resistance gene, *nptII*, as a selectable marker (Fig. 2A). All 34 individual kanamycin-resistant hairy roots tested contained *ASA2* cDNA as determined by PCR analysis of genomic DNA using *ASA2* cDNA primers to produce a 1,107-bp fragment (data not shown). No band was produced with the control DNA samples from lines transformed by DC-AR2 without the binary vector.

Southern-blot analyses with hairy root genomic DNA corroborated the PCR screening and further demonstrated the stable incorporation of the 35S-*ASA2* gene into the *A. sinicus* genome (Fig. 2, B and C). The hybridization signal band corresponding to the 35S-*ASA2* gene was detected in all DNAs isolated from the hairy roots transformed with the pBIN-*ASA2* binary vector, but not from those transformed by DC-AR2 without the binary vector. Since *Bam*HI and *Eco*RI are unique sites in the T region of the binary vector, the presence of one to five fragments of variable size in the genomic DNA indicates the insertion of about one to five copies of the T-DNA with *ASA2* into the plant genome.

Total RNA isolated from 10 transformed and one control hairy root lines was hybridized with labeled *ASA2* cDNA as the probe, and a single band of approximately 2.2 kb was detected in RNA from all hairy root lines transformed with the 35S-*ASA2* gene and from the $5MT^r$ *Nicotiana sylvestris* control (Fig. 3A). No hybridization signal was detectable in RNA from the control hairy root line or the $5MT^s$ *N. sylvestris* suspension-cultured cell line. Some hairy root lines, A-7, A-8, and A-20, had very strong hybridization signals showing that the *ASA2* expression driven by the CaMV 35S promoter can be very high. The severalfold variation in expression observed among individual *ASA2* transformants is similar to that reported for other genes driven by the CaMV 35S promoter (Lagrimini et al., 1990) and can be attributed primarily to position effects (Weising et al., 1998) rather than to differences in gene copy number.

Leaf, stem, and root tissues from plants regenerated from the transformed hairy root line A-20 also showed strong expression of the 2.2-kb *ASA2* transcript, whereas the control did not (Fig. 3B). These results differ from those found with 5MT-selected tobacco suspension-cultured cells where high levels of the *ASA2* transcript were detected in RNA from $5MT^r$ cells, but not from wild-type suspension-cultured cells or leaves, roots, stems, or seeds of plants regenerated from the resistant cells (Song et al., 1998). Thus, *ASA2* cDNA was constitutively expressed in most tissues of *A. sinicus* when under the control of the 35S promoter.

AS Enzyme Activity

The kinetics of Trp feedback inhibition of AS activity in hairy root extracts were first measured using NH_4Cl instead of Gln as the second substrate since free ASA2 α-subunits can utilize ammonium to produce anthranilate from chorismate. In all four *ASA2* transformed lines tested, AS activity was less sensitive to Trp inhibition than was the activity from the two control lines (Fig. 4A). The apparent K_i values for Trp, estimated from the Trp concentration causing 50% inhibition, were 4, 5, 16, and 30 μM for the *ASA2* transformed lines, and 2 and 3 μM for the controls.

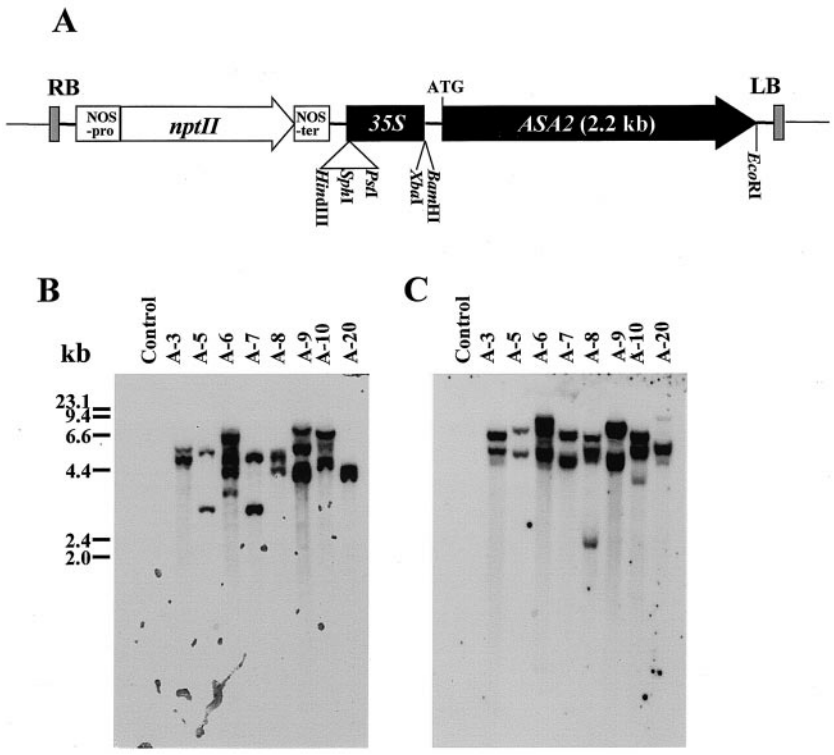

Figure 2. A, T-DNA region of pBIN-*ASA2*. Arrows indicate the direction of transcription. RB, Right border; LB, left border; NOS-pro, nopaline synthase promoter; NOS-ter, nopaline synthase terminator; *nptII*, neomycin transferase gene II; 35S, 35S promoter of CaMV; *ASA2*, the 2.2-kb coding and 3′-downstream regions of the AS gene. B and C, Southern-blot hybridization analyses. All DNAs were digested with *Bam*HI (B) and *Eco*RI (C). DNA from control hairy root transformed with *A. rhizogenes* strain DC-AR2 and independent hairy roots (A-3, A-5, A-6, A-7, A-8, A-9, A-10, and A-20) transformed with *A. rhizogenes* strain DC-AR2 harboring pBIN-*ASA2*.

These differences are smaller than those found with AS activity in extracts of wild-type and 5MT-resistant tobacco suspension-cultured cells where the apparent K_i values were 2 and 300 μM, respectively, and for ASA2 expressed in *Escherichia coli*, 100 μM (Song et al., 1998). These differences may be due to the level of expression, and differences in the amounts of the different β-subunits involved. Though the changes in K_i are modest compared with those observed in these other cell extracts, AS activity is still present at higher Trp concentrations, especially in transformed lines with increased total AS activity (*A. sinicus* hairy root lines A-20 and A-45). This apparently results in the higher free Trp observed in these lines.

When the AS activity was also measured using either 100 mM NH_4Cl or 10 mM Gln as the second substrate, the AS activity in the ASA2 transformed line, A-45, was more Trp-insensitive than the control β-glucuronidase-4 (GUS-4) with both substrates (Fig. 4B). This suggests that the ASA2 α-subunit product has complexed with a native β-subunit or subunits to form a holoenzyme capable of catalyzing the Gln-dependent reaction. The ratio of NH_4Cl-dependent activity to Gln-dependent activity was also higher in the *ASA2* transformed line than in the control, 0.76 and 0.63, respectively. A similar change in this ratio is observed in 5MT-selected tobacco suspension-cultured cells where ASA2 is overexpressed (data not shown). These results suggest that some free ASA2 α-subunits are present in the transformed line extract and are detected only when NH_4Cl is the substrate. Alternatively, this may represent an inherent kinetic difference between the *ASA2* gene product and other α-subunits. The hairy root free Trp level and AS feedback-insensitivity (K_i) are correlated up to K_i values of about 10 μM Trp above which higher Trp is not found (Fig. 4C). Additional studies are being carried out to see if this relationship always occurs, and thus represents the inherent limit in free Trp increase as some other step becomes limiting or Trp degradation or secretion occurs.

Kinetic studies with the maize AS (Anderson et al., 1997) show that this enzyme is not as feedback insensitive as the tobacco ASA2 enzyme (Song et al., 1998). No enzyme kinetic data has been presented for the rice feedback-insensitive enzyme (Wakasa and Widholm, 1987, 1991; Wakasa et al., 1999).

Free Trp Levels

The five control lines tested contained an average free Trp level of 57 nmol g^{-1} fresh weight, whereas one control line, GUS-4, consistently contained higher levels (91 nmol g^{-1} fresh weight; Table I) for some unknown reason. The higher free Trp in the GUS-4 line is clearly not due to altered feedback control of AS since, as shown in Figure 4A, the enzyme activity from the GUS-4 roots was more sensitive to inhibition by Trp than was the activity from another control line, GUS-75. Of the 22 independent *ASA2* transformed lines tested, 20 contained more free Trp than the control average and these ranged from 73 to 316 nmol g^{-1} fresh weight, a 1.3- to 5.5-fold increase. Similar free Trp increases are found

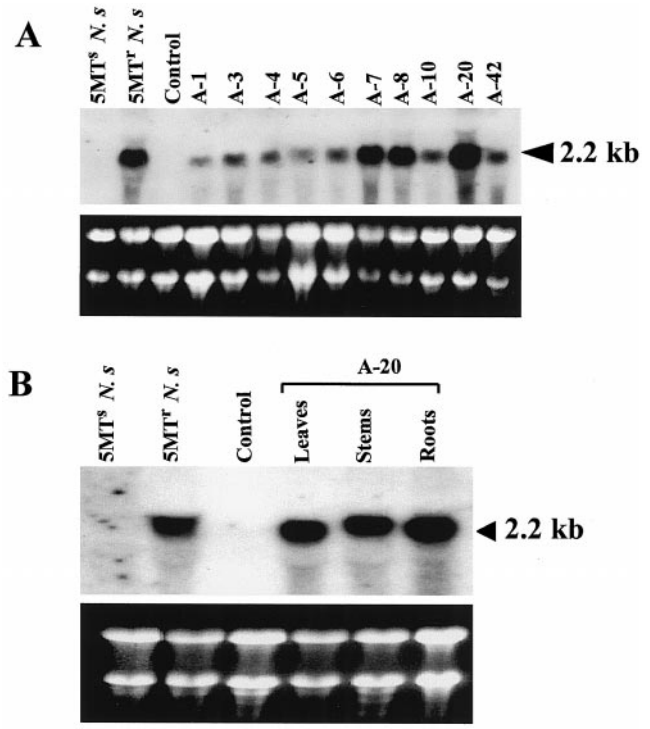

Figure 3. A and B, Northern-blot analyses. Total RNA isolated from 1-week-old 5MT^s and 5MT^r *N. sylvestris* suspension-cultured cell line, control hairy root transformed with *A. rhizogenes* strain DC-AR2, and independent hairy roots (A-1, A-3, A-4, A- 5, A-6, A-7, A-8, A-10, A-20, and A-42) transformed with *A. rhizogenes* strain DC-AR2 harboring pBIN-*ASA2* (A). One-week-old 5MT^s and 5MT^r *N. sylvestris* suspension-cultured cell line, plantlets of *A. sinicus* seedlings and leaves, stems, and roots from regenerants of the hairy root line A-20, respectively (B). In both A and B, the bottom panel shows the amount of rRNA stained with ethidium bromide.

in Trp analog resistant lines such as 5MT^r tobacco suspension-cultured cells (Song et al., 1998) and αmt^r Arabidopsis mutant (*amt-1*) plants (Kreps and Town, 1992), where feedback-insensitive AS is expressed. Transgenic maize transformed with 35S/ASA2C28 contained elevated levels of Trp relative to controls (Anderson et al., 1997). These levels ranged from 290 to 500 nmol Trp g^{-1} fresh weight in two of the nine transgenic cell lines, whereas the other lines contained levels in the range of 97 to 145 nmol Trp g^{-1} fresh weight. The Trp levels in the controls were 30 to 53 nmol g^{-1} fresh weight. Transgenic rice plants transformed with a rice mutant AS α-subunit gene contained from 143 to 1,522 nmol Trp g^{-1} fresh weight, whereas the level of the control was 33 nmol Trp g^{-1} fresh weight (Wakasa et al., 1999).

When the free Trp levels were measured in the leaves, stems, and roots of *A. sinicus* whole plants regenerated from controls (GUS-75 and GUS-76) and 35S-*ASA2* transformed hairy roots (A-3 and A-20), increases were found in those transformed with *ASA2*. The free Trp levels (nanomoles per gram fresh weight) in the leaves, stems, and roots of control transgenic plants, were 210, 184, and 105 (GUS-75)

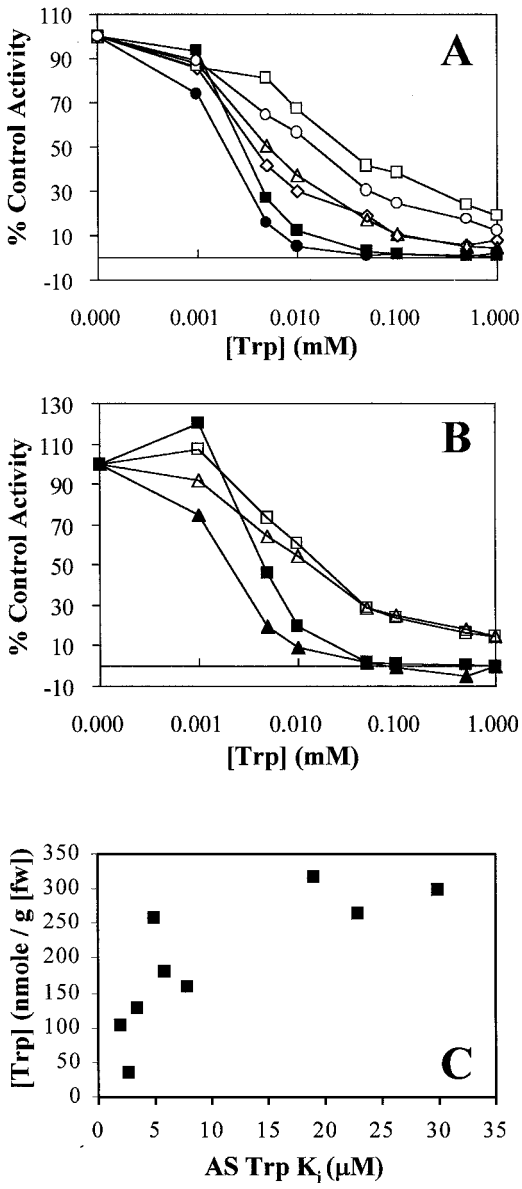

Figure 4. A, Trp inhibition of AS activity in extracts of *A. sinicus* hairy root lines. Control lines are GUS-75 (■), GUS-4 (●), and *ASA2* transformed lines are A-6 (◇), A-7, (Δ), A-20 (□), and A-45 (○). AS activity in the presence of Trp was measured as described in "Materials and Methods" with 100 mM NH_4Cl and 100 μM chorismate as substrates. Relative AS activity is the percentage of the activity observed when no Trp was added. The specific activity with no Trp added for each line was 24, 24, 17, 19, 39, and 36 pmol min^{-1} mg^{-1} protein, respectively. B, AS activity measured using either 100 mM NH_4Cl (A-45, Δ; GUS-4, ▲) or 10 mM Gln (A-45, □; GUS-4, ■) as the second substrate for AS. C, Trp insensitivity of AS activity versus free Trp levels found in the roots. The Trp concentration that resulted in 50% AS inhibition with NH_4Cl as the second substrate was extrapolated from the data in Figure 4A for six *A. sinicus* hairy root lines and from data (not shown) obtained with three other lines similarly tested. The Trp values are from Table I.

Table I. *Trp amounts in transformed hairy roots of 35S-ASA2 and controls*

Line	Trp Concentration[a]	Fold (Control Average: 57)
	nmol g^{-1} fresh wt	
Control		
GUS-75	34	
GUS-76	42	
GFP-77	56	
GUS-90	61	
GUS-4	91	
Transgenic		
A-2	42	0.7
A-44	43	0.7
A-11	73	1.3
A-19	79	1.4
A-10	81	1.4
A-1	92	1.6
A-18	92	1.6
A-5	127	2.2
A-22	127	2.2
A-6	128	2.2
A-12	144	2.5
A-25	149	2.6
A-4	180	3.2
A-16	198	3.5
A-9	202	3.5
A-42	215	3.8
A-3	247	4.3
A-7	256	4.5
A-8	264	4.6
A-20	297	5.2
A-27	313	5.5
A-45	316	5.5

[a] Values are mean of at least three replicates.

and 485, 176, and 161 (GUS-76) and the respective Trp levels in 35S-*ASA2* transformed plants were 4,103, 894, and 437 (A-3) and 3,176, 494, and 692 (A-20). In all plants the free Trp levels were highest in the leaves, which could be due to the higher number of plastids (chloroplasts) found there and the plastid localization of the Trp biosynthetic pathway (Schulze-Siebert and Schultz, 1989; Zhao and Last, 1995). Like all plant *AS* genes described to date, the tobacco *ASA2* cDNA sequence encodes a putative plastid transit peptide at the amino terminus, indicating plastid localization.

Measurement of all free amino acids in the shoots and roots of several transformed lines showed no alterations due to the increased Trp levels (data not shown). Similar results were obtained by Li and Last (1996) with an Arabidopsis feedback-insensitive AS mutant that contained 3-fold higher Trp. Brotherton et al. (1996) found higher Phe and Tyr in some 5MT-selected *D. innoxia* cells and plants that contained feedback-insensitive AS and higher free Trp. No change in other amino acids might be expected since the Trp branch pathway utilizes only a tiny fraction of the large, total shikimate pathway flux, which can be as much as 20% of the total fixed carbon in plants by some estimates and most of which is finally deposited as lignin (Haslam, 1993). The increase in Trp biosynthesis seen here would not cause a significant change in shikimate intermediates available for biosynthesis of other pathway products. This contrasts with situations where Trp is removed from the pool by expression of Trp decarboxylase, which results in a larger flux through this pathway (Yao et al., 1995).

The *A. sinicus* hairy root regenerants show the Ri plasmid syndrome including reduced plant height and plagiotropic roots as reported previously (Cho et al., 1998), so the altered morphology should not be due to the overexpression of ASA2. Mutant plants of a variety of species with elevated levels of Trp due to feedback-altered AS were normal in morphology and fertility (Ranch et al., 1983; Lee and Kameya, 1991; Kreps and Town, 1992).

5MT Resistance

Since expression of the feedback-insensitive *ASA2* cDNA gene should cause resistance to the Trp analog 5MT, as shown previously with *E. coli* (Song et al., 1998), a quantitative root growth test was used to assess the extent of $5MT^r$ of the transformed hairy roots. Control hairy root growth was inhibited by 10 μM 5MT and almost complete inhibition occurred at 15 μM or higher 5MT concentrations (Figs. 5 and 6). The growth of hairy root lines A-7, A-8, A-9, A-10, and A-20 was inhibited somewhat by 5MT, but growth did occur even in concentrations of up to 100 μM, the maximum concentration tested. Similar results were obtained when root growth was measured in liquid medium where the controls died in 20 μM, whereas lines A-7, A-8, A-9, A-10, and A-20 grew in concentrations of up to 100 μM 5MT. Thus, the transformed lines are clearly more $5MT^r$ than the control hairy root lines in both liquid and solid medium. This resistance occurs in lines A-7, A-8, A-9, and A-20, which have some of the higher free Trp levels (Table I), and also in A-10, where the increase in Trp is not very dramatic. Maize cell lines transformed with 35S/ASA2C28, which differed from the wild-type sequence by a single nucleotide that changed the codon at position 377 from Met (ATG) to Lys (AAG), were transferred to medium supplemented with 100 or 200 μM 5MT (Anderson et al., 1997). Nine transformed lines grew well, one showed reduced growth, and another eight lines exhibited little or no growth on the 5MT medium. The untransformed controls exhibited little or no growth on 100 μM 5MT medium. Wakasa et al. (1999) showed that hygromycin-resistant rice callus transformed with pUb-OSASA1D or pUb-OSASAW1 containing *hpt* and rice AS α-subunit genes could grow on 300 μM 5MT.

Although maize and rice plants have been transformed with mutated feedback-altered *AS* genes isolated from the same species (Anderson et al., 1997; Wakasa et al., 1999), we have used a naturally occur-

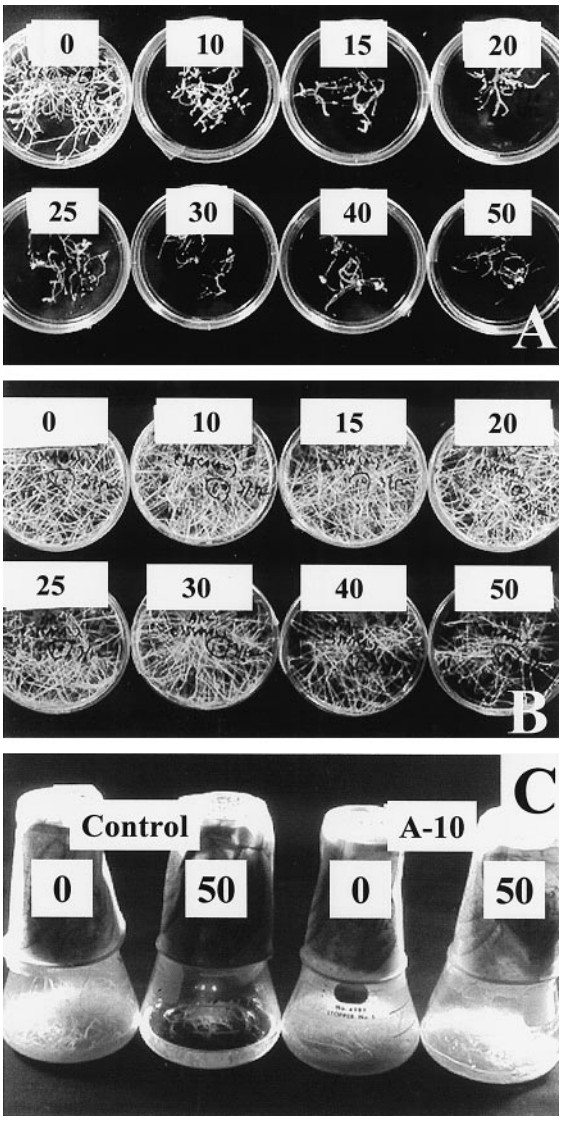

Figure 5. A through C, Effects of 5MT on the growth of *A. sinicus* hairy roots. A, Control *A. sinicus* hairy root line, GUS-75. Top, 0, 10, 15, and 20 μM 5MT. Bottom, 25, 30, 40, and 50 μM 5MT. B, Transformed *A. sinicus* hairy root line, A-10 on the same 5MT concentrations as in A. C, *A. sinicus* hairy root growth in liquid medium. Control hairy root line, GUS-75 in 0 and 50 μM 5MT. Transformed hairy root line, A-10 in 0 and 50 μM 5MT. Plates are 9 cm in diameter and the flasks are about 6 cm in diameter at the base. In all cases, about 200 mg of hairy roots were grown for 6 weeks. At least three independent experiments were analyzed.

ring feedback-insensitive AS α-subunit from a species different from that encoding the AS β-subunit. The results provide novel information about subunit interactions from the two species, in this case *Nicotiana tabacum* and *A. sinicus*. The results presented here show that one can use the feedback-altered form of AS as a new tool for studying the regulation of Trp biosynthesis in plants and for manipulating the free Trp levels to increase the amount of this essential amino acid in crops. Finally, the observation that the hairy roots expressing ASA2 are resistant to the toxic Trp analog, 5MT, suggests that this may be a useful selectable marker for plant transformation experiments even when used with a different species, since the feedback-insensitive α-subunit can interact with the native β-subunit to form an active enzyme. Further studies of these subunit interactions are needed.

MATERIALS AND METHODS

Construction of Chimeric 35S Promoter-ASA2 Vector

Two oligonucleotides, 5′-CGA TTG GAT CC**A TG**C AGT CGT TAC CTA-3′, and 5′-CAG CCG GAA TTC CCA AAT TGC TGA TGG CAT 3′ containing *Bam*HI and *Eco*RI overhangs, respectively (underlined), were synthesized and used for PCR amplification of full-length *ASA2* cDNA (Song et al., 1998) with *Pfu* DNA polymerase (Stratagene, La Jolla, CA). PCR amplification was performed for 30 cycles (95°C, 1 min; 50°C, 40 s; 72°C, 2 min). The amplified fragment was digested with *Bam*HI and *Eco*RI and this fragment used to replace the *gfp4-nos* terminator of binary vector pBIN-*gfp4* (Haseloff et al., 1997) to create pBIN-*ASA2*. The chimeric construct was transformed into *Escherichia coli* DH5α using $CaCl_2$ transformation (Sambrook et al., 1989), was purified by a plasmid kit (Qiagen, Valencia, CA), and was electroporated into *Agrobacterium rhizogenes* strain DC-AR2 (Cho et al., 1998).

Plant Materials and Transformation

$5MT^r$ (N300 green) and $5MT^s$ (NS-MX) suspension cultures of *Nicotiana sylvestris* (Song et al., 1998) were maintained by weekly transfers into 50 mL of liquid MX medium (Murashige-Skoog basal medium [Murashige and Skoog, 1962] with 1.8 μM 2,4-D) with and without 300 μM 5MT, respectively. *A. rhizogenes*-mediated *Astragalus sinicus* transformation was carried out as described previously with some modifications (Cho et al., 1998). Three days after

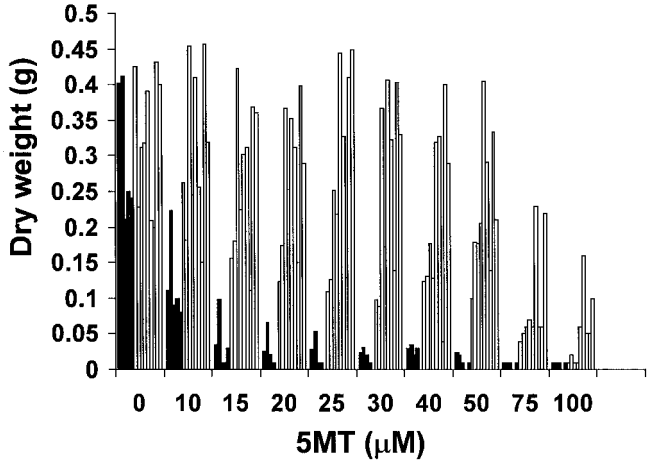

Figure 6. Quantitative data on the effect of 5MT on the growth of hairy roots. Five control lines (■) and nine *ASA2* transformed lines (□). About 200 mg of hairy roots were grown for 6 weeks. At least three independent experiments were carried out and the data combined.

cocultivation plants were transferred to Murashige-Skoog medium solidified with 3 g L^{-1} Gelrite (Greif Bros., Spotswood, NJ) containing 500 mg L^{-1} carbenicillin disodium and 75 mg/L^{-1} kanamycin. Kanamycin-resistant root tips were transferred and were freed from *A. rhizogenes* by two to three passages at weekly intervals on the same medium. The established root cultures were then transferred every 4 to 5 weeks on antibiotic-free medium and shoots were regenerated as described previously (Cho et al., 1998).

PCR Screening

DNA was extracted from hairy root tissues (150–300 mg) according to the methods of Dellaporta (1994). The primers used for amplification of a 1,107-bp fragment of the *ASA2* cDNA gene were 5′-CTG CAG CAA TTC ATG CAG TCG TTA CCT ATC-3′-and 5′-CTT CCC TCT TCT GCT TGT CCC-3′. The PCR reaction mixture consisted of 5 μL (100–200 ng) of plant DNA, 2.5 μL of 10× *Taq* buffer (Gibco/BRL, Cleveland), 1.25 μL of 50 mM $MgCl_2$, 0.25 μL of *Taq* DNA polymerase (5 U L^{-1}, Gibco/BRL), 0.5 μL of 10 mM dNTPs, 0.5 μL each of 10 μM primers, and 15 μL of sterile distilled water. Samples were heated to 95°C for 5 min, followed by 29 cycles of 95°C for 60 s, 57°C for 40 s, 72°C for 90 s, and 72°C for 10 min.

Nucleic Acid Analysis

Genomic DNA was isolated from 1-week-old suspension-cultured cells and 1-month-old hairy root cultures as described (Cho et al., 1998). Total RNA was prepared using a phenol extraction method (Wang et al., 1994) from 1-week-old suspension-cultured cells and 1-month-old hairy root cultures. DNA and RNA gels were blotted onto a nylon membrane (Hybond-N^+, Amersham, Buckinghamshire, UK) following a general capillary transfer method and cross-linked to the membrane by UV using Stratalinker (1,200 μJoules × 100, Stratagene). The full-length *ASA2* cDNA fragment was used as a probe following labeling with a Megaprime DNA labeling system (RPN1605, Amersham) with [α-^{32}P]dCTP (3,000 Ci $mmol^{-1}$) and the hybridization was conducted according to the protocol of the manual (Hybond-N^+, Amersham).

AS Enzyme Activity

Extracts were prepared using a Tenbroeck tissue grinder (Kontes Glass, Vineland, NJ) and the *ASA1* extraction buffer (2 mL g^{-1} tissue) described by Bernasconi et al. (1994). After removal of cellular debris by centrifugation (10 min at 35,000*g* and 4°C), 1 volume of the supernatant was combined with 2 volumes of room temperature saturated $(NH_4)_2SO_4$, and then centrifuged as before. The resulting pellet was resuspended in extraction buffer (1 mL g^{-1} tissue) and used immediately. When Gln was used as the second substrate for the AS-catalyzed reaction, the resuspended enzyme solution was desalted using Sephadex G25 to remove residual $(NH_4)_2SO_4$. AS activity was measured as described in Song et al. (1998) except the assay buffer was that described by Bernasconi et al. (1994) without NH_4Cl in the buffer. Either 100 mM NH_4Cl or 10 mM Gln was added to the assay mixture to determine α-subunit activity or total AS activity, respectively. Protein concentration was determined using a protein dye-binding assay kit (Bio-Rad Laboratories, Hercules, CA).

Free Trp Analysis

Tissue samples were frozen in liquid nitrogen and stored at −70°C until analyzed. Samples were ground frozen into a coarse powder and approximately 100 mg of tissue was homogenized with 0.1 N HCl (2 mL g^{-1} tissue) in a microfuge tube using a plastic pellet pestle (Kontes Glass). The sample was then frozen in liquid nitrogen, thawed, and microfuged to sediment debris. A portion of the supernatant was deproteinated using an UltraFree-MC (10,000) filter unit (Millipore, Bedford, MA) according to the manufacturer's directions. The filtrate was further diluted with 0.1 N HCl as necessary (1:10 for most samples) and 10 μL was analyzed by HPLC by methods similar to that of Berardino et al. (1990), using a 250- × 4.6-mm Adsorbosil C_{18} column (Alltech Associates, Deerfield, IL), an isocratic buffer system (85% [v/v]: 140 mM sodium acetate, 17 mM triethylamine, adjusted to pH 5.05 using phosphoric acid, and 15%: 60% [v/v] acetonitrile in water at 1 mL min^{-1}), and fluorescence detection (Kratos FS970; excitation, 215 nm; emission, band pass filter > 375 nm). All free amino acids were measured as described in Brotherton et al. (1996).

Measurement of Growth

About 200 mg fresh weight of hairy root cultures were subcultured to the medium containing various concentrations of 5MT, were weighed on foil of known weight, and were dried for 12 h at 75°C before dry weight determination.

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