Plant Physiol. (1993) 103: 1285–1290

Ectopic Overexpression of Asparagine Synthetase in Transgenic Tobacco[1]

Timothy Brears[2], Christopher Liu, Thomas J Knight, and Gloria M. Coruzzi*

Department of Biology, New York University, 1009 Main Building, Washington Square East, New York, New York 10003 (T.B., C.L., G.M.C.); and Department of Biology, University of Southern Maine, 96 Falmouth Street, Portland, Maine 04103 (T.J.K.)

Here, we monitor the effects of ectopic overexpression of genes for pea asparagine synthetase (AS1) in transgenic tobacco (*Nicotiana tabacum*). The AS genes of pea and tobacco are normally expressed only during the dark phase of the diurnal growth cycle and specifically in phloem cells. A hybrid gene was constructed in which a pea AS1 cDNA was fused to the cauliflower mosaic virus 35S promoter. The 35S-AS1 gene was therefore ectopically expressed in all cell types in transgenic tobacco and constitutively expressed at high levels in both the light and the dark. Northern analysis demonstrated that the 35S-AS1 transgene was constitutively expressed at high levels in leaves of several independent transformants. Furthermore, amino acid analysis revealed a 10- to 100-fold increase in free asparagine in leaves of transgenic 35S-AS1 plants (construct z127) compared with controls. Plant growth analyses showed increases (although statistically insignificant) in growth phenotype during the vegetative stage of growth in 35S-AS1 transgenic lines. The 35S-AS1 construct was further modified by deletion of the glutamine-binding domain of the enzyme (glnΔAS1; construct z167). By analogy to animal AS, we reasoned that inhibition of glutamine-dependent AS activity might enhance the ammonia-dependent AS activity. The 3- to 19-fold increase in asparagine levels in the transgenic plants expressing glnΔAS1 compared with wild type suggests that the novel AS holoenzyme present in the transgenic plants (glnΔAS1 homodimer) has enhanced ammonia-dependent activity. These data indicate that manipulation of AS expression in transgenic plants causes an increase in nitrogen assimilation into asparagine, which in turn produces effects on plant growth and asparagine biosynthesis.

In higher plants, AS (EC 6.3.5.4) catalyzes the formation of Asn and glutamate from Gln and aspartate. Thus, together with GS, which is responsible for the assimilation of nitrogen from ammonia, AS plays an important role in primary nitrogen metabolism (Miflin and Lea, 1976; Sieciechowicz et al., 1988). Asn and Gln represent the major long-distance nitrogen transport compounds in plants and are abundant in phloem sap. Typically, Gln is more abundant in phloem exudates of light-grown plants, whereas Asn, which has a higher N:C ratio, is more abundant in dark-grown plants where photosynthetic carbon is limited (Joy et al., 1983; Sieciechowicz et al., 1988). The cloning of plant AS genes has revealed that the AS genes of pea and tobacco (*Nicotiana tabacum*) are expressed exclusively in the dark (Tsai and Coruzzi, 1990, 1991). Light dramatically represses the expression of the AS1 gene in peas in both photosynthetic and nonphotosynthetic organs (Tsai and Coruzzi, 1991). The cell-specific expression of AS1 in the phloem (Tsai, 1991) is similar to that of cytosolic GS (Edwards et al., 1990; Brears et al., 1991) and suggests that the pea AS1 gene functions to generate Asn for long-distance nitrogen transport. These data indicate a metabolic preference for Asn as a nitrogen transport compound under nonphotosynthetic conditions.

In addition to elucidating the regulation of AS gene expression, the cloned plant AS genes (Tsai and Coruzzi, 1990) are valuable molecular tools that may be used to clarify whether the encoded plant AS enzyme is a Gln- and/or an ammonia-dependent enzyme. Plant AS has been shown to utilize Gln in generating Asn in vitro (Sieciechowicz et al., 1988). However, AS activity in maize roots can utilize ammonia directly, albeit at high ammonia concentrations (Stulen and Oaks, 1977; Oaks and Ross, 1984). Since such high levels of ammonia do not normally arise in plant tissues, the ammonia-dependent activity of plant AS seen in vitro may have no physiological significance in vivo. However, ammonia-dependent AS activities have been found in other organisms. *Escherichia coli* has two AS genes, *asnA* and *asnB*; the *asnA* gene encodes an ammonia-dependent AS, whereas the *asnB* gene encodes an enzyme that prefers Gln as a substrate (Felton et al., 1980; Humbert and Simoni, 1980). In mammalian cells, both Gln- and ammonia-dependent AS activities are located on the same AS polypeptide. Antibodies that specifically bind to the amino-terminal Gln binding site of mammalian AS inhibit Gln-dependent AS activity and actually enhance the ammonia-dependent AS activity (Pfeiffer et al., 1986, 1987). Thus, in animals, Gln- and ammonia-dependent AS activities are both encoded by the same gene,

[1] This work was supported by Department of Energy grant DE-FG02-92-ER20071 and a technology transfer grant from New York University. In addition, T.B. gratefully acknowledges the support of a long-term fellowship from the European Molecular Biology Organization. C.L. was awarded a Westinghouse semi-finalist prize for his part in this research.

[2] Present address: Ciba-Geigy Corporation, Agricultural Biotechnology Research Unit, 3054 Cornwallis Road, Research Triangle Park, NC 27709.

* Corresponding author; fax 1–212–995–4204.

Abbreviations: AS, asparagine synthetase; CaMV, cauliflower mosaic virus; GS, glutamine synthetase; OPA, *o*-phthaldialdehyde.

although the substrate-specificity domains exist as distinct domains on the AS polypeptide.

The studies reported herein concern the manipulation of AS gene expression in plants with the aim of increasing Asn production and testing the effects on plant growth. There are several features of Asn that make it preferable to Gln as a nitrogen transport/storage compound, and hence, the increased assimilation of nitrogen into Asn may be valuable in vivo. Asn is a long-distance nitrogen transport compound in peas and has a higher N:C ratio than Gln. Therefore, it could serve as a more economical compound for nitrogen transport. In addition, Asn is more stable than Gln and can accumulate to high levels in vacuoles (Lea and Fowden, 1975; Sieciechowicz et al., 1988). In developing pea leaves, Asn is actively metabolized, but in mature leaves that no longer need nitrogen for growth, Asn is not readily metabolized and is reexported (in the phloem) from the leaf to regions of active growth such as developing leaves and seeds (Ta et al., 1984; Sieciechowicz et al., 1988). Thus, we were interested in determining whether the ectopic overexpression of AS in all cell types would increase Asn production in a light-independent fashion. Furthermore, we tested whether the increased Asn production provides an advantage in the nitrogen use efficiency and growth phenotype of transgenic plants.

In addition to overexpressing wild-type AS, we ectopically expressed a modified form of the AS enzyme (glnΔAS1), which was missing the Gln-binding domain. We hypothesized that ectopic overexpression of a glnΔAS1 form of the enzyme might produce a novel plant AS enzyme with enhanced ammonia-dependent AS activity, or that such a mutation might have a dominant negative effect (Herskowitz, 1987) due to co-assembly with endogenous wild-type AS subunits to form a heterodimer (Rognes, 1975; Hongo and Sato, 1983). The analysis of the transgenic plants that ectopically express pea AS demonstrated an increased accumulation of Asn and an improved growth phenotype in the case of 35S-AS1, and an increased accumulation of Asn accompanied by a detrimental effect on growth phenotype in the case of 35S-glnΔAS1. These results indicate that it is possible to manipulate nitrogen metabolism and growth phenotype by ectopic overexpression of AS genes. Because nitrogen is often the rate-limiting element in plant growth and is typically applied to crops several times during the growing season, designing molecular technologies that improve nitrogen use efficiency in crop plants is of considerable interest to agriculture.

MATERIALS AND METHODS

Transfer of the AS1 cDNA to a Binary Expression Vector

The AS1 cDNA, previously cloned from pea (Tsai and Coruzzi, 1990), was transferred from pTZ18U to the *Eco*RI site of pBluescript KS− (Stratagene). A glnΔAS1 deletion mutant was constructed using "inside-outside" polymerase chain reaction (Innis et al., 1990). Coding sequences corresponding to amino acids 2 to 4 (CGI) were deleted from the amino terminus of the AS1 cDNA, leaving the initiating Met and the untranslated leader intact. This deletion corresponded to the presumed Gln-binding domain of the AS enzyme comprising amino acids MCGI, which have been defined for animal AS (Pfeiffer et al., 1986, 1987). cDNAs corresponding to wild-type AS1 and glnΔAS1 were then transferred from pBluescript to the binary expression vector pTEV5. This vector contains the CaMV 35S promoter (from −941 to +26), a multiple cloning site, and the nopaline synthase terminator (T. Brears, C. Liu, T. Knight, and G. Coruzzi, unpublished data). Figure 1 shows details of the binary vector constructions containing the AS1 cDNAs that were transformed into tobacco (*Nicotiana tabacum*).

Plant Transformations

Binary vector constructions were transferred into the disarmed *Agrobacterium* strain LBA4404 and subsequently into *N. tabacum* SR1 using standard procedures described elsewhere (Bevan, 1984; Horsch et al., 1985).

RNA Analysis of Transformants

RNA was isolated using "RNA matrix" from Bio101 and total RNA was electrophoresed as previously described (Thomas, 1983). Gels were capillary blotted onto Hybond-N nylon membrane (Amersham). cDNAs were labeled using the random primer plus extension reagent labeling system supplied by New England Nuclear. Hybridizations were done in aqueous solution and blots were washed in 0.1× SSPE/0.1× SDS. Northern blots were probed with the pea AS1 cDNA, pAS1 (Tsai and Coruzzi, 1990).

Extraction of Free Amino Acids

Tobacco leaf tissue samples were collected and immediately frozen in liquid nitrogen. The frozen tissue was then extracted in 10 mL of extraction media consisting of methanol:chloroform:water (12:5:3, v/v/v). The homogenate was centrifuged at 12,000*g* for 15 min. The pellet was extracted again and the supernatants were combined. Addition of 2.5 mL of chloroform and 3.8 mL of distilled water to the supernatant induced separation. The methanol:water phase was collected and dried under vacuum and redissolved in 1 mL of distilled water. The solution was filtered by passing it through a 0.45-μm nylon filter microcentrifuge tube filter system centrifuged at 12,000*g* for 2 min.

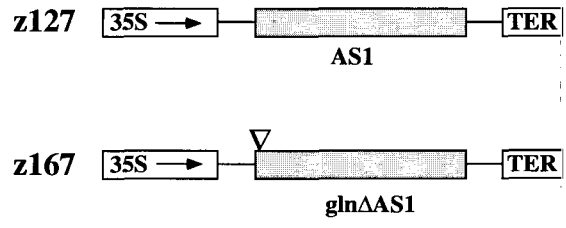

Figure 1. AS constructions transferred to transgenic plants. cDNAs for the AS1 gene (Tsai and Coruzzi, 1990) and the glnΔAS1 gene were fused to the 35S promoter and nopaline synthase transcriptional terminator for transfer to tobacco using the binary expression vector pTEV5.

HPLC Determination of Amino Acid Pools

The amino acids were determined as OPA derivatives on a Microsorb Type O AAAnalysis column (Rainin, Woburn, MA) using a DuPont HPLC instrument. Sample (100 μL) was derivatized with 100 μL of OPA working reagent. After 2 min of derivatization, 50 μL of the derivatized sample was injected. The gradient was produced using two eluents: A, 95% 0.1 M sodium acetate (pH 7.2) with 4.5% methanol and 0.5% tetrahydrofluoran; B, 100% methanol. Eluents were filtered and degassed with He before use. Detection of OPA-derivatized amino acids was accomplished with a UV spectrophotometer at 340 nm. Each determination was done twice and the values represent the average.

Plant Growth Conditions

Progenies of primary transformants characterized as expressing high levels of either the AS1 mRNA or the mutated glnΔAS1 mRNA were germinated on Murashige-Skoog medium containing 100 μg/mL kanamycin. After 14 d, kanamycin-resistant seedlings were transferred to 4-inch pots filled with white sand, which were covered with Saran Wrap for approximately 1 week to prevent excessive transpiration and to enable seedlings to become established. Pots were treated with an excess of 1× Hoagland solution containing 10 mM potassium nitrate as the only nitrogen source. Subsequently, between three and seven plants were sacrificed for fresh weight determination each week, continuing for a period of 4 weeks until shading of neighbors was apparent. Plants were grown under a light/dark cycle of 16/8 h with a temperature cycle of 24/18°C. Daytime light intensity was 1000 lux.

RESULTS

Construction of Transgenic Plants Expressing Pea AS1 and glnΔAS1

The pea AS1 cDNA (Tsai and Coruzzi, 1990) expressed from the 35S-CaMV promoter was transferred into transgenic tobacco (see Fig. 1 and "Materials and Methods") and five independent primary (T_1) transformants (z127, 1–5) were shown to express high levels of the AS1 mRNA (see below). Three independent transgenic lines (z167, 1–3) that contained the AS1 cDNA with a deletion in the Gln-binding domain (glnΔAS1) were also shown to contain high levels of transgene RNA (see below).

Identification of Transformants Expressing High Levels of AS1 and glnΔAS1 mRNA

Northern blot analysis of RNA extracted from transgenic plants was undertaken to identify plants in which the 35S-AS1 transgene was expressed at high levels (Fig. 2). As a positive control, RNA for AS was detected in leaves of pea plants grown in the dark (Fig. 2, lane PL). By contrast, no AS mRNA was detected in leaves of light-grown wild-type tobacco (Fig. 2, lane TL). Our previous studies have shown that tobacco AS mRNA is expressed exclusively in tissues of plants grown in the dark (Tsai and Coruzzi, 1991). The transformants that overexpress AS1 (z127–1, -3, -4, and -5) all contained high levels of AS1 mRNA, even though these plants were grown in the light (Fig. 2). Thus, the 35S CaMV promoter produces constitutive expression of pea AS1, whereas the endogenous AS mRNA is not expressed in tobacco leaves in the light. The glnΔAS1 transformants also showed constitutive high-level expression of mRNA (z167–2, -3, and -4), compared with tobacco controls (Fig. 2). Because the AS enzyme is notoriously unstable, the AS enzyme has never been purified to homogeneity and antibodies for plant AS were not available for AS protein analysis. In addition, we were unable to detect AS activity by in vitro assay due to enzyme instability (T. Knight, T. Brears, G. Liu, and G. Coruzzi, unpublished data).

Amino Acid Analysis of Transgenic Lines Expressing AS1 and glnΔAS1

Based on the northern results, two independent transgenic lines that showed high levels of AS1 mRNA (z127–1 and z127–4) were selected for further analysis. Similarly, lines z167–2 and z167–4 were selected as high-expressing representatives of the glnΔAS1 construction. The progeny of these primary T_1 transformants (e.g. the T_2 generation) were subjected to amino acid and growth analysis described below.

AS1-Overexpressing Lines

Both z127 lines selected (z127–1 and z127–4) showed increased levels of Asn (10- to 100-fold higher than wild-type controls) (Table I). The variation apparent among the individual T_2 sibling plants from each line possibly reflects the homozygosity or heterozygosity of individual progeny, and the approximate 2:1 ratio of intermediate:high Asn levels would substantiate this assertion. In some cases the large deviations from a 2:1 ratio could be explained if multiple

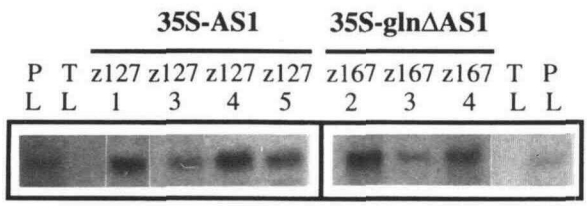

Figure 2. Northern analysis of transgenic plants expressing either AS1 or glnΔAS1. Ten micrograms of total RNA isolated from leaves of individual transformants was loaded in each lane. Blots were probed with the AS1 cDNA from pea (Tsai and Coruzzi, 1990). A positive control includes AS mRNA in dark-grown pea leaves (PL). A negative control includes AS mRNA in light-grown tobacco leaves (TL).

copies of T-DNA were segregating in the T_2 generation. In all cases, however, a considerable increase in Asn is seen extending up to nearly 100 times the control concentration. Interestingly, there is a corresponding reduction in Gln concentrations in these plants (although the z127–4 data are skewed by a single high value) and this most likely reflects the use of Gln as a substrate in the AS reaction; equally predictable is the reduction in concentration of the other AS substrate, aspartate. Somewhat unexpected is the reduced concentration of glutamate, a product of the AS reaction. This may be due to the high turnover rate of glutamate due to its use as a substrate in processes such as transamination or in the supply of Gln for plant growth. Since the amino acid data are not normally distributed, it is not possible to make assessments of stoichiometry of the amino acid substrates and products. However, we can identify "high" and "low" amino acid pool values that are consistent for each T_2 plant.

glnΔAS1-Overexpressing Lines

In the two lines selected that overexpress glnΔAS1, we assessed whether the deletion of the Gln-binding domain of AS would have a dominant negative effect on Asn biosynthesis. The data collected for these lines (z167–2 and z167–4) are somewhat difficult to interpret due to the variation of data points (Table I). However, in almost every case there is a substantial increase in Asn concentration, ranging from 3- to 19-fold compared with wild-type nontransgenic tobacco. These results suggest that the transgenic lines have the ability to accumulate Asn with little effect on aspartate, glutamate, or Gln pools. One possibility is that the glnΔAS1 enzyme is able to synthesize Asn directly from ammonia and aspartate.

Table I. *Amino acid analysis in transgenic lines overexpressing AS1 or glnΔAS1*

Amino acid concentrations are in nmol/gram fresh weight.

Plant ID	Asn	Glu	Gln	Asp
Control				
C	34	1399	309	1935
C	38	1452	405	1861
C	36	965	425	2015
C	47	1526	275	1720
Mean	39	1335	353	1883
AS1 wild type				
z127-1-A	553	228	14	182
z127-1-B	3399	808	60	922
z127-1-C	213	525	81	240
z127-1-D	487	537	17	264
z127-1-E	3159	983	43	796
Mean	1562	616	43	481
z127-4-A	1105	838	132	451
z127-4-B	902	2947	389	1092
z127-4-C	373	1606	17	678
z127-4-D	4109	691	923	1664
Mean	1622	1520	365	971
glnΔAS1				
z167-2-A	684	838	352	761
z167-2-B	1341	2947	944	3119
z167-2-C	173	1606	1224	1946
Mean	733	1797	840	1942
z167-4-A	47	691	75	948
z167-4-B	109	864	346	1491
z167-4-C	137	1313	714	1705
z167-4-D	165	1534	838	2069
Mean	114	1100	493	1553

Plant-Growth Experiments on Transformants Expressing AS1 and glnΔAS1

Growth analysis was undertaken using individual transgenic T_2 plants grown in white sand. These studies were aimed at assessing growth rate under conditions that minimized interference from neighboring plants. For this reason, fresh weight measurements were taken only during the vegetative stage of growth (up to 6 weeks postgermination) to ensure that this growth analysis occurred prior to the onset of bolting, when internal pools of nitrogen are remobilized. Plants were grown under an excess of inorganic nitrogenous fertilizer (10 mM nitrate) under growth conditions that reduced the photosynthetic interference of neighboring plants. The growth analysis was terminated when such interference became apparent. All plants analyzed were of the same age at each time point, and analysis started at between 0.1 and 0.3 g fresh weight/plant and continued until the plants were approximately 6 weeks old, when the interference of neighboring plants became apparent and bolting was imminent.

Table II shows the results of mean total fresh weight determinations for lines z127–1 and z127–4 (overexpressing wild-type AS1) and z167–2 and z167–4 (overexpressing glnΔAS1). Transgenic lines overexpressing wild-type AS outgrew the higher-yielding control by 33 and 10% (Table II), although in neither case was this statistically significant when analyzed by unpaired *t* test. Transgenic lines overexpressing the glnΔAS1 construction (z167) did not perform comparably. The z167–4 plants that survived until the 6th week were indistinguishable in growth from controls, and the z167–2 plants that survived were much smaller than controls ($P = 0.041$; significant at the 5% level) (Table II). Comparing the three different lines in the experiment, it was of interest that a greater proportion of kanamycin-resistant z167 plants died. Typically the z167 plants were slow to germinate and looked unhealthy when grown in pots. This was clearly reflected in the fresh weight data collected for z167–2, although less apparent for the z167–4 data, suggesting that the glnΔAS1 gene product did indeed have a dominant-negative effect on plant growth.

DISCUSSION

Here, we report studies in which AS is ectopically overexpressed in transgenic plants to test the effects of this manipulation on primary nitrogen assimilation and on plant growth. In particular, we have altered the cell-specific expression pattern of AS and have also modified its regulation with regard to light. In wild-type plants, AS is normally expressed

Table II. *Growth of transgenic lines overexpressing AS1 or glnΔAS1*

Total fresh weight means (g) for transgenic lines (z127 and z167) and controls (C-1, C-2) for weeks 3 to 6. The statistical analysis was done for the final week's measurement only (week 6) and control-1 was selected for the *t* test. df, Degrees of freedom; the probability of the populations being related was deemed to be significant (*) for $P < 0.05$; NS, not significant.

	C-1	C-2	z127-1	z127-4	z167-2	z167-4
Week 3	0.12	0.07	0.28	0.12	0.11	0.19
Week 4	0.60	0.41	1.30	0.51	0.31	0.57
Week 5	1.19	1.11	1.87	1.72	0.71	0.99
Week 6	6.49	5.83	8.63	7.16	3.83	6.13
Percent increase at week 6 compared with control 1	100	90	133	110	59	94
No. of plants	7	6	7	7	3	5
SE	0.60	1.07	1.15	0.88	0.92	0.85
SD	1.58	2.61	3.05	2.34	1.60	1.89
t for unpaired test to control-1 (df)			1.65 (12)	0.63 (12)	2.43 (8)	0.35 (10)
Probability			0.125	0.54	0.041	0.731
Significance			NS	NS	*	NS

only in the phloem (Tsai, 1991), and its expression is dramatically repressed by light in both photosynthetic and non-photosynthetic tissues (Tsai and Coruzzi, 1990, 1991). In this paper we have expressed wild-type AS1 of pea and a mutated form of AS1 (glnΔAS1) under the control of a constitutive promoter (35S-CaMV) in transgenic tobacco so that AS1 is expressed in all cell types in a light-independent fashion. The physiological significance of constitutively expressing AS1 in cells where it is not normally expressed may have considerable impact on plant nitrogen metabolism. For example, Asn is involved in photorespiratory nitrogen recycling (Ta et al., 1984; Givan et al., 1988), so the ectopic expression of AS in photosynthetic cells may have dramatic impact on photorespiration. Furthermore, the expression of an ammonia-dependent AS enzyme in this context may aid in the reassimilation of photorespiratory ammonia.

Four independent transgenic tobacco lines expressing 35S-AS1 have been shown to express a wild-type pea AS1 transgene constitutively. Two lines were analyzed further (z127–1 and z127–4) and it was shown that the expressed AS1 gene was functional since free Asn accumulated to high levels in transgenic leaf tissue; typically transgenic lines z127–1 and z127–4 accumulated between 10- and 100-fold more Asn than control untransformed tobacco lines. These increased Asn levels were predictably accompanied by a reduction in the AS substrates Gln and aspartate. However, the expected increase in glutamate (the other AS product) was not observed, presumably due to the reactivity of glutamate and its use as a substrate in several related processes. Due to the differential reactivity of the amino acids involved in the AS reaction and the possibility that their synthesis is dependent on feedback mechanisms, we are not surprised that the amino acid data do not completely correspond to the expected ratios predicted from the known biochemical stoichiometries involved in the Asn biosynthetic biochemical reaction. These results demonstrate that the availability of Gln is not limiting in the AS reaction. However, it may still be possible to channel more inorganic nitrogen into the nitrogen transport compound Asn by providing higher endogenous levels of Gln.

The plant growth experiment on the z127 transgenic plants was aimed at determining whether the accumulation of Asn in the AS1-overexpressing plants might have a positive effect on growth during the vegetative stage of plant development. The rapid leaf development that occurs during vegetative growth imposes a strong demand for nutrient availability, and nitrogen is typically the most critical nutrient at this time due to the synthesis of new proteins in expanding and enlarging tissues. Nitrogen assimilated and accumulated at this time is subsequently recycled in the plant and deposited in seed reserves; as well as being a major long-distance transport amino acid, Asn also plays an important role in the formation of seed reserves (Dilworth and Dure, 1978; Sieciechowicz et al., 1988). The two z127 lines were found to outgrow untransformed controls over a 6-week period up to the end of vegetative growth and conferred a 10 and 33% growth advantage. However, these figures are not statistically significant when a *t* test is performed. Thus, although the plants make 10- to 100-fold higher levels of Asn, it is possible that Gln levels are limiting relative to increases in growth. We have recently found that overexpressed GS in transgenic tobacco can confer a greater growth advantage that is statistically significant (T. Brears, C. Liu, T. Knight, and G. Coruzzi, unpublished data). Because Gln and Asn are pivotal amino acids in the primary assimilation of inorganic nitrogen, we anticipate that creating transgenic lines that express both GS and AS at high levels (by crossing AS and GS overexpressers) may be even more advantageous. In particular, our experiments have the advantage that assimilation in transgenic lines will not be restricted to a few cell types, enabling available nitrogen in all plant cells to be utilized. The ectopic overexpression of both GS and AS in a single plant may have the advantage of avoiding Gln accumulation; since Gln is an active metabolite the presence of high concentrations of Gln may upset cell metabolism. By contrast, Asn is a relatively inert compound able to store nitrogen economically. In ad-

dition, Asn is formed in a reaction that liberates a molecule of glutamate then available to accept a further unit of ammonia (Lea and Fowden, 1975).

In addition to the ectopic overexpression of wild-type AS, we also attempted to modify plant Gln-dependent AS to enhance its ammonia-dependent activity. In particular, it has been shown in animals that antibodies to the Gln-binding domain of AS inhibit Gln-dependent AS activity yet enhance the ammonia-dependent activity (Pfeiffer et al., 1986, 1987). By analogy, we created a site-specific mutant in a pea AS1 cDNA (Tsai and Coruzzi, 1990) that specifically deleted the three amino acids required for Gln binding (glnΔAS1). By introducing this glnΔAS1 into transgenic plants, we might expect to enhance the ammonia-dependent AS activity and/or inhibit the endogenous Gln-dependent activity through subunit poisoning and the formation of heterodimers of wild-type and mutant subunits. Two independent transgenic lines, z167–2 and z167–4, which overexpress the glnΔAS1 transgene, were found to be capable of accumulating Asn levels approximately 3 to 19 times greater than untransformed tobacco controls. The activity of the glnΔAS1 gene in Asn biosynthesis is suggestive of the modified enzyme having the capability of utilizing a nitrogen substrate other than Gln (e.g. ammonia). By analogy to the known ammonia-dependent AS activities of the *E. coli asnA* gene and mammalian AS, the high levels of Asn in the transgenic plants that express the mutated plant glnΔAS1 enzyme suggest that the glnΔAS1 enzyme in transgenic plants can assimilate ammonia directly into Asn and therefore bypass GS in primary nitrogen assimilation. If this suggestion is correct, it is also apparent that the glnΔAS1 gene is not as efficient in synthesizing Asn as the overexpressed wild-type AS1, based on the relative levels of Asn in these transgenic plants (z167 versus z127).

Transgenic lines expressing glnΔAS1 (z167–2 and z167–4) did not outgrow untransformed controls; indeed, they typically grew more poorly than untransformed plants as evidenced by the performance of z167–2 and the higher proportion of z167 plants to die before the end of the experiment. It is curious that these plants should accumulate 3- to 19-fold higher levels of Asn in their leaves yet grow more poorly. Plant AS is believed to assemble as a homodimer (Rognes, 1975). In leaf mesophyll tissue where wild-type AS is not normally expressed, the glnΔAS1 form is able to self-assemble into homodimers and form an enzyme capable of generating Asn. In phloem cells, however, glnΔAS1 subunits may co-assemble with wild-type AS subunits, thereby inactivating wild-type AS as a dominant-negative mutation (Herskowitz, 1987). In the glnΔAS1 plants, Asn synthesized in leaf mesophyll cells may be unable to be transported to and loaded into the phloem, and this could account for the poor growth phenotype of these transgenic lines. These observations highlight the specialization of cell-type function and cell-specific gene expression of nitrogen metabolic genes and their impact on plant nitrogen metabolism.

Received May 11, 1993; accepted August 10, 1993.

LITERATURE CITED

Bevan M (1984) *Agrobacterium* vectors for plant transformation. Nucleic Acids Res **12:** 8711–8721

Brears T, Walker EL, Coruzzi GM (1991) A promoter sequence involved in cell-specific expression of the pea glutamine synthetase GS3A gene in organs of transgenic tobacco and alfalfa. Plant J **1:** 235–244

Dilworth MF, Dure L (1978) Developmental biochemistry of cotton seed embryogenesis and germination. X. Nitrogen flow from arginine to asparagine in germination. Plant Physiol **61:** 698–702

Edwards JW, Walker EL, Coruzzi GM (1990) Cell-specific expression in transgenic plants reveals nonoverlapping roles for chloroplast and cytosolic glutamine synthetase. Proc Natl Acad Sci USA **87:** 3459–3463

Felton J, Michaelis S, Wright A (1980) Mutations in two unlinked genes are required to produce asparagine auxotrophy in *Escherichia coli*. J Bacteriol **142:** 221–228

Givan CV, Joy KW, Kleczkowski LA (1988) A decade of photorespiratory nitrogen recycling. Trends Biochem Sci **13:** 433–437

Herskowitz I (1987) Functional inactivation of genes by dominant negative mutations. Nature **329:** 219–222

Hongo S, Sato T (1983) Some molecular properties of asparagine synthetase from rat liver. Biochim Biophys Acta **742:** 484–489

Horsch RB, Fry JW, Hoffman NL, Eicholtz D, Rogers SG, Fraley RJ (1985) A simple and general method for transferring genes into plants. Science **227:** 1229–1231

Humbert R, Simoni RD (1980) Genetic and biochemical studies demonstrating a second gene coding for asparagine synthetase in *Escherichia coli*. J Bacteriol **142:** 212–220

Innis MA, Gelfand DH, Sninsky JJ, White TJ (1990) PCR Protocols: A Guide to Methods and Applications. Academic Press, New York

Joy KW, Ireland RJ, Lea PJ (1983) Asparagine synthesis in pea leaves, and the occurrence of an asparagine synthetase inhibitor. Plant Physiol **73:** 165–168

Lea PJ, Fowden L (1975) The purification and properties of glutamine-dependent asparagine synthetase isolated from *Lupinus albus*. Proc R Soc Lond **192:** 13–26

Miflin BJ, Lea PJ (1976) The pathway of nitrogen assimilation in plants. Phytochemistry **15:** 873–885

Oaks A, Ross DW (1984) Asparagine synthetase in *Zea mays*. Can J Bot **62:** 68–73

Pfeiffer NE, Mehlhaff PM, Wylie DE, Schuster SM (1986) Monoclonal antibodies specific for bovine pancreatic asparagine synthetase. J Biol Chem **261:** 1914–1919

Pfeiffer NE, Mehlhaff PM, Wylie DE, Schuster SM (1987) Topographical separation of the catalytic sites of asparagine synthetase explored with monoclonal antibodies. J Biol Chem **262:** 11565–11570

Rognes SE (1975) Glutamine-dependent asparagine synthetase from *Lupinus luteus*. Phytochemistry **14:** 1975–1982

Sieciechowicz KA, Joy KW, Ireland RJ (1988) The metabolism of asparagine in plants. Phytochemistry **27:** 663–671

Stulen I, Oaks A (1977) Asparagine synthetase in corn roots. Plant Physiol **60:** 680–683

Ta TC, Joy KW, Ireland RJ (1984) Amino acid metabolism in pea leaves. Plant Physiol **74:** 822–826

Thomas PS (1983) Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. Methods Enzymol **100:** 255–266

Tsai F-Y (1991) Molecular biology studies of the light-repressed and organ-specific expression of plant asparagine synthetase genes. PhD thesis. The Rockefeller University, New York

Tsai F-Y, Coruzzi GM (1990) Dark-induced and organ-specific expression of two asparagine synthetase genes in *Pisum sativum*. EMBO J **9:** 323–332

Tsai F-Y, Coruzzi GM (1991) Light represses the transcription of asparagine synthetase genes in photosynthetic and non-photosynthetic organs of plants. Mol Cell Biol **11:** 4966–4972