Analysis of the transgenic tobacco plants expressing Panicum miliaceum aspartate aminotransferase genes

Abstract Expression of Panicum miliaceum L. (proso millet) mitochondrial and cytosolic aspartate aminotransferase (mAspAT and cAspAT, respectively) genes in transgenic tobacco plants (Nicotiana tabacum) and their influences on protein synthesis were examined. The mAspAT- or cAspAT-transformed plants had about threefold or 3.5-fold higher AspAT activity in the leaf than non-transformed plants, respectively. Interestingly, the leaves of both transformed plants had increased levels of phosphoenolpyruvate carboxylase (PEPC) and transformed plants with cAspAT also had increased levels of mAspAT in the leaf. These results suggest that the increased expression of Panicum cAspAT in transgenic tobacco enhances the expression of its endogenous mAspAT and PEPC, and the increased expression of Panicum mAspAT enhances the expression of its endogenous PEPC.

Key words Aspartate aminotransferase • Phosphoenolpyruvate carboxylase • Isoenzyme • Transgenic tobacco

Introduction

Aspartate aminotransferase (EC 2.6.1.1.; AspAT) catalyzes the reversible transfer of the amino group of aspartate to α-ketoglutarate to form oxaloacetate and glutamate. In plants AspAT has been proposed to play several metabolic roles including: (1) recycling of carbon skeletons during ammonia assimilation in roots (Ryan and Fottrell 1974), (2) providing amide precursors for biosynthesis of major nitrogen transport molecules such as asparagine and ureides (Rawsthorne et al. 1980), (3) recruiting asparagine nitrogen during seed filling (Murray 1978), (4) participating in intracellular carbon shuttles in C₄ plants (Hatch and Mau 1973), (5) providing precursors for the biosynthesis of the aspartate family of amino acids (Bryan 1980), and (6) participating in the malate/aspartate shuttle which moves reducing equivalents across the organellar membrane (Heber and Heldt 1981).

In cells of higher plants, multiple isozymes of AspAT have been detected in plastids, mitochondria, peroxisomes, and cytosol. Plant AspAT cDNAs have only recently been cloned from alfalfa (Udvardi et al. 1991, Gantt et al. 1992), carrot (Turano et al. 1992), Panicum (Taniguchi et al. 1992), lupin (Reynolds et al. 1992, Winefield et al. 1994), soybean (Wadsworth et al. 1993) and Arabidopsis (Schults and Coruzzi 1995, Wilkie et al. 1995). Genetic manipulation to decrease AspAT activity by the antisense method has been reported (Mett et al. 1996). However, no genetic manipulation has been reported to test the effects of increased AspAT activities on flux and diversion of amino acids through a particular pathway. Furthermore, although the targeting of the transgene product is powerful approaches to understand the metabolic role(s) of a specific enzyme and its regulatory properties on metabolism of related enzymes, in the case of mitochondria, only targeting of French bean glutamine synthetase to mitochondria of transgenic tobacco has been reported (Hemon et al. 1990).
In this report, genes for two isozymes of *Panicum* AspAT were expressed in tobacco plants under the control of CaMV (cauliflower mosaic virus) 35S promoter. The effects of the expression of these genes on amino acid metabolism and organelle-specific activity of the mitochondrial isozyme in transgenic tobacco were also evaluated.

### Materials and methods

Plasmid constructions and transformation of tobacco

The construction of the plasmid pmAAT-H1 is shown in Fig. 1A. The coding region of *Panicum* mAspAT cDNA, including the transit peptide, was isolated from the plasmid pmAAT1 (Taniguchi et al. 1992) by SacI/BamHI (partial digestion), and this fragment was inserted into the SacI/BamHI site of pBI221 (Jefferson et al. 1987) such that the gene was positioned between the CaMV 35S promoter (Odell et al. 1985) and nopaline synthase (Nos) terminator (Bevan et al. 1983). This plasmid was digested with HindIII/SacI, and the 35S-mAspAT fragment was inserted into the HindIII/SacI site of the binary vector pBI-H1 (kindly supplied by Dr. K. Nakamura, Nagoya University). The resulting plasmid, pmAAT-H1, was transferred to *Agrobacterium tumefaciens* LBA4404.

The construction of the plasmid pcAAT-H1 is shown in Fig. 1B. The coding region of *Panicum* cAspAT cDNA was isolated from the plasmid pcAAT2 (Taniguchi et al. 1992) by NcoI (partial digestion), and blunt-ended with T4 DNA polymerase. This fragment was inserted into the *Smal* site of pUC118. This plasmid was digested with XbaI/SacI, and the cAspAT fragment was inserted into the XbaI/SacI site of pBI221 such that the gene was positioned between the CaMV 35S promoter and the Nos terminator. This plasmid was digested with *Hind*III (partial)/SacI, and the 35S-cAspAT fragment was inserted into the HindIII/SacI site of the binary vector pBI-H1. The resulting plasmid pcAAT-H1 was transferred to *A. tumefaciens* LBA4404.

Leaf discs of tobacco, *Nicotiana tabacum* cv. Petit Havana SR-1 were infected with *A. tumefaciens* LBA4404 harboring binary vector plasmids pmAAT-H1 or pcAAT-H1 and cultured in MS-NB medium [MS medium (Murashige and Skoog 1962) containing 0.1 mg/l NAA (1-naphthylacetic acid) and 1 mg/l BAP (benzylationoinpurine)] supplemented with 500 mg/l Clororan and 0.8% (v/v) agar. The selection was carried out using 150 mg/l kanamycin sulfate and 50 mg/l hygromycin B during shoot formation. For root initiation, half concentrations of both reagents were used. After 3 weeks, green shoots regenerated from leaf disks were excised and placed onto MS-2 medium [50 mm HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-KOH (pH 7.5), 1 mm EDTA, 1 mm MgCl2, 2 mm DTT, 0.1% BSA (bovine serum albumin), pH 7.8]. After filtration through a 65 μm metal mesh, the homogenate was centrifuged for 2.5 min at 5000 g. The supernatant was centrifuged at 20 000 g for 4.5 min. The pellet of ‘crude’ mitochondria was resuspended in a suspension buffer [0.3 m sucrose, 10 mm MOPS, 1 mm EDTA, 0.1% BSA pH 7.2], and loaded onto a Percoll gradient. The gradient was centrifuged for 40 min at 11 400 g. The mitochondria were collected as the pale band on top of the 60% cushion. The purified mitochondria were washed once in a washing solution [0.3 m sucrose, 25 mm MOPS, 5 mm MgCl2, pH 7.2].

Western blot analysis

Leaf tissues harvested in the middle of the day or callus were homogenized in a grinding medium [100 mm HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-KOH (pH 7.5), 1 mm EDTA, 10 mm MgCl2, 1 mm MnCl2, 5 mm DTT (dithiothreitol)] with quartz sand in a 1.5 ml microtube. Following centrifugation (12 000 × g, 10 min), supernatant (soluble protein) was separated by SDS-PAGE [12% (w/v) polyacrylamide gel and 0.1% SDS (sodium dodecyl sulfate), Laemmli 1970]. After 5 μg of soluble total protein or 0.5 μg of mitochondrial fraction had been electrophoresed, the polypeptides were transferred from gel to an Immobilon-P membrane (Millipore, Japan). The membranes were first treated with mAspAT or cAspAT antibody raised in rabbit against *Eleusine coracana* mAspAT or P. maximum cAspAT, respectively, or phosphoenolpyruvate carboxylase (PEPC) antibody raised in rabbit against maize (*Zea mays*) C-form PEPC, and then with a second antibody raised in goat against rabbit IgG and conjugated with horseradish peroxidase.

Enzyme extraction and assay

About 0.5 g of leaf tissues was ground in a small mortar with 50 mg of insoluble polyvinylpyrrolidone and 2 ml of grinding medium [50 mm HEPES-KOH (pH 7.5), 1 mm EDTA, 1 mm MgCl2, 1 mm MnCl2, 5 mm DTT]. The supernatant obtained by centrifugation (12 000 g for 5 min) was used for the enzyme assay and western blot analysis. AspAT was assayed spectrophotometrically in 0.5 ml reaction mixture at 30 °C, as reported in Uchino et al. (1995). One unit of AspAT activity corresponds to the oxidation of 1 μmol of NADH min⁻¹ by the coupling enzyme.