

## Gene Note

## Genetic engineering of hexosamine with L-glutamine D-fructose-6-phosphate amidotransferase genes in plants

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**Abstract** In animals, yeasts, bacteria, and viruses, L-glutamine D-fructose-6-phosphate amidotransferase (GFAT) is well characterized, but not in plants. This study identified an *Arabidopsis* GFAT gene by detecting the enzymatic activity of the recombinant protein produced in a wheat germ *in vitro* translation system. The *Arabidopsis* gene and a chlorella virus GFAT gene under the CaMV 35S promoter were introduced into tobacco BY-2 cells. Transgenic cells with the viral gene had higher levels of hexosamines than wild type, but no such hexosamine production was observed in transgenic cells with the *Arabidopsis* gene. These results clarify metabolic engineering of hexosamines in plants.

**Key words:** BY-2 cells, L-Glutamine D-fructose-6-phosphate amidotransferase, hexosamine.

A major hexosamine, N-acetyl-D-glucosamine (GlcNAc), exists in polymeric form in chitin, a component of fungal cell walls and the exoskeletons of crustaceans, in heteropolysaccharides of mammalian proteoglycan, and in the peptidoglycan of the bacterial cell walls (Kent et al. 1953). Derived from GlcNAc, the nucleotide sugar uridine 5'-diphospho-N-acetyl-D-glucosamine (UDP-GlcNAc) is prerequisite for biosyntheses of GlcNAc-containing polymers and glycoproteins, which have important cellular functions. Although higher plants are devoid of GlcNAc in polymeric form, GlcNAc is activated into UDP-GlcNAc; then it is transferred into carbohydrate moieties of glycoproteins in plant cells, as it is as in mammalian cells. L-Glutamine D-fructose-6-phosphate amidotransferase (GFAT) catalyzes the following reaction irreversibly: D-fructose-6P+L-Glutamine→D-glucosamine-6P+L-glutamate. This is the first step in the hexosamine biosynthetic pathway, and a key step leading to formation of UDP-GlcNAc. The mammalian, yeast, and bacterial enzymes and genes encoding the enzymes have been well characterized (Milewski 2002). The presence of GFAT in higher plants was demonstrated (Hassid et al. 1959) and partially purified enzyme fractions were characterized (Vessal and Hassid 1972). Although the genes which were putatively assigned as GFAT genes are found in the *Arabidopsis* genome sequence (Kaneko et al. 2000), the catalytic activity of the proteins encoded by the genes remains unclear. We describe the identification of an *Arabidopsis* GFAT gene

by measuring the enzymatic activity of the protein encoded by the gene, and metabolic engineering of hexosamines using a viral GFAT gene in plant cells.

To express the protein encoded by the *Arabidopsis* gene (accession no. AT3g24090), a cDNA clone was isolated from 1-week-old seedlings of *Arabidopsis thaliana*. Total RNA was isolated from *Arabidopsis thaliana* using an RNeasy Plant Mini Kit (Qiagen Inc., Hilden, Germany). A single stranded cDNA was

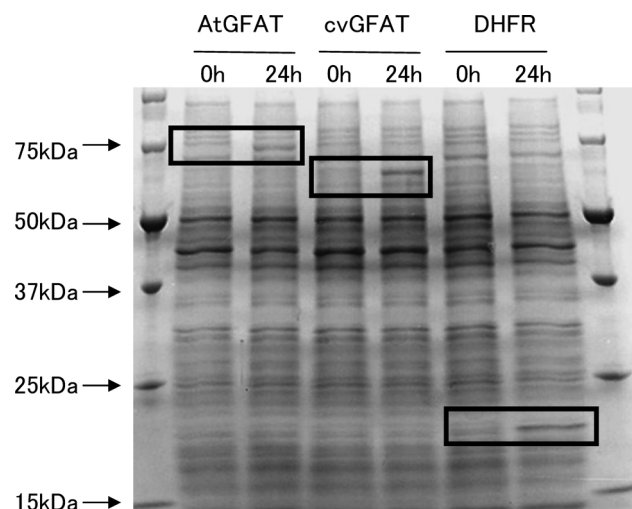


Figure 1. Recombinant GFAT proteins produced in a wheat germ *in vitro* translation system. SDS-PAGE analysis of the synthesized proteins were performed by staining with Coomassie blue. DHFR, Dihydrofolate reductase

Table 1. Hexosamine contents in transgenic BY-2 cells<sup>a</sup>

Cell lines	Hexosamine conc. ( $\mu\text{mol g}^{-1}$ fresh weight) <sup>b</sup>
AtGFAT	nd
cvGFAT	$0.78 \pm 0.34$
WT	nd

<sup>a</sup> Log-phase cells were harvested.

<sup>b</sup> nd, Not detected. Each value is shown as mean  $\pm$  SE ( $n=3$ ).

synthesized from 1  $\mu\text{g}$  of total RNA using random hexamer primer (Toyobo Co. Ltd., Osaka, Japan) and a Rever Tra Ace- $\alpha$  first strand cDNA synthesis kit (Toyobo Co. Ltd.). The cDNA for the *Arabidopsis* gene AT3g24090, which is assigned putatively as GFAT, was isolated from the single strand cDNA by PCR using the following primers: 5'-ATGTGTGGAATCTTCGCGT-ATCTGAATTTTCACGC-3' (forward) and 5'-GAGA-GTCGACCTATTGAGTAGTCACACTCTTTGCCAGAT-3' (reverse). The AtGFAT cDNA was cloned into pEU-NII (pEU-atGFAT).

In contrast, chlorella virus PBCV-1 encodes functional GFAT enzyme (Landstein et al. 1998). The predicted

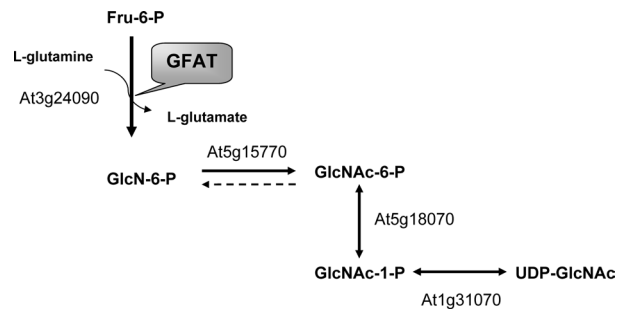


Figure 2. Aminosugar biosynthetic pathway in *Arabidopsis*. L-Glutamine D-fructose-6-phosphate amidotransferase (GFAT) is a key enzyme leading to formation of UDP-N-acetyl-D-glucosamine (UDP-GlcNAc); AT3g24090, L-Glutamine D-fructose-6-phosphate amidotransferase (GFAT); At5g15770, N-Acetyltransferase/glucosamine 6-phosphate N-acetyltransferase; At5g18070, Phosphoacetylglucosamine mutase; At1g31070, UDP-N-acetylglucosamine pyrophosphorylase; Fru-6-P, D-Fructose 6-phosphate; GlcN-6-P, D-Glucosamine 6-phosphate; GlcNAc-6-P, N-Acetyl-D-glucosamine 6-phosphate; GlcNAc-1-P, N-Acetyl-alpha-D-glucosamine 1-phosphate; UDP-GlcNAc, UDP-N-acetyl-D-glucosamine

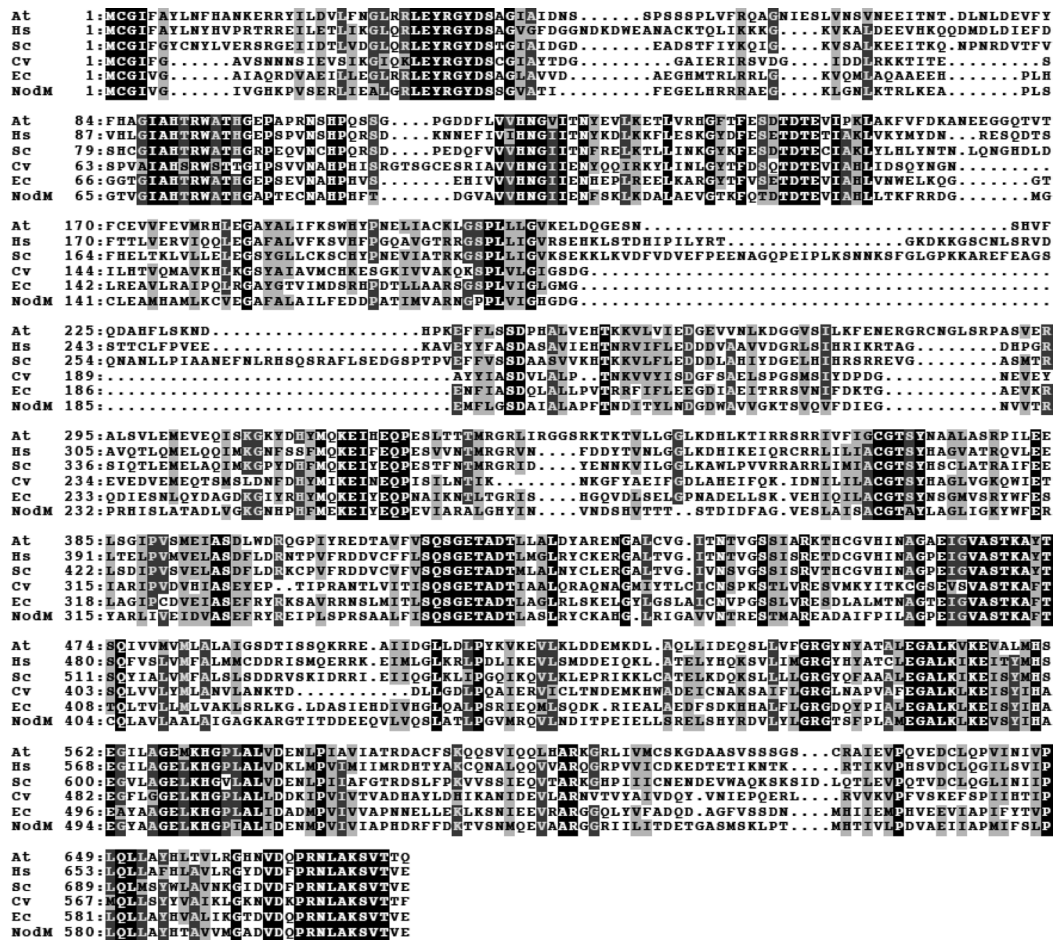


Figure 3. Multiple alignment of amino acid sequences of L-glutamine D-fructose-6-phosphate amidotransferase (GFAT). At, *Arabidopsis thaliana* GFAT (AT3g24090); Hs, Homo sapiens GFAT (accession number M90516); Sc, *Saccharomyces cerevisiae* GFAT (CAA50453); Cv, chlorella virus GFAT (U42580); Ec, *Escherichia coli glmS* GFAT (AAC76752); NodM, *Rhizobium* GFAT (CAK10404). The black-filled boxes and the gray-framed boxes indicate identical and similar residues, respectively.

amino-acid sequences of the chlorella virus GFAT (cvGFAT) enzyme is more similar to bacterial GFAT enzyme than to their eukaryotic GFAT enzyme (Landstein et al. 1998). The GFAT ORF in the chlorella virus Hirosaki chromosomal DNA (kindly provided from Dr. Yamada, Hiroshima University) was amplified by PCR using the following primers: 5'-ATGTGTGGCA-TCTTTGGAGCACTGTCAAACAAC-3' (forward) and 5'-AACTGCAGTAAAAGGTGGTCACGGATTTTGC-CAAGATTC-3' (reverse) complementary to the genomic DNA sequence of PBCV-1 (accession number U42580). The cvGFAT ORF was cloned into pEU-NII (pEU-cvGFAT). The GFAT exhibits 98% identity at the DNA level and 99% identity at the amino acid level to PBCV-1.

Then, AtGFAT and cvGFAT were synthesized using the bilayer method with PROTEIOS Wheat germ cell-free protein synthesis core kit (Toyobo Co. Ltd.) according to the supplier's protocol. Subsequent SDS-PAGE analysis confirmed the synthesis of AtGFAT and cvGFAT (Figure 1). The synthesized proteins were added to a GFAT reaction mixture containing 15 mM Fru-6P, 15 mM L-glutamine, 1 mM EDTA, 1 mM DTT, and 60 mM  $\text{KH}_2\text{PO}_4$  (pH 7.0), and incubated for 4 h at 37°C. Using a Morgan–Elson method (Reissig et al. 1955), GlcN-6P content was determined. In both AtGFAT (2.6 mU·mg<sup>-1</sup> protein) and cvGFAT (19.1 mU·mg<sup>-1</sup> protein), GFAT activities were confirmed.

The AtGFAT and cvGFAT were expressed in plant cells. To the *AtGFAT* ORF, *Xba*I and *Bam*HI sites, respectively, were added by PCR using pEU-atGFAT as template. The amplified fragment was digested with *Xba*I and *Bam*HI, and inserted into the *Xba*I-*Bam*HI site of pBI121 (Clontech, Palo Alto, CA, USA) between CaMV35S promoter and nopaline synthase terminator (nos-t) to construct AtGFAT expression plasmid pBI121-atGFAT. Similarly, cvGFAT was introduced into the pBI121 plasmid to create a pBI121-cvGFAT plasmid. The pBI121-atGFAT and pBI121-cvGFAT plasmids were electroporated respectively into *Agrobacterium tumefaciens* LBA4404 (Invitrogen Corp., Carlsbad, CA, USA). Tobacco BY-2 suspension-cultured cells (*Nicotiana tabacum* L. cv. Bright Yellow 2) were transformed according to the method described by An et al. (1985). Transformants of BY-2 cells were selected and maintained on modified Linsmaier and Skoog medium (Nagata et al. 1981) containing 30 g l<sup>-1</sup> of sucrose, 100 mg l<sup>-1</sup> of kanamycin and 100 mg l<sup>-1</sup> of carbenicillin. Suspension-cultured BY-2 cells were filtered and washed with distilled water. After addition of 1 ml of distilled water and 1 g of glassbeads (1 mm) per g fr. wt., cells were homogenized using a homogenizer BS-12 (Wakenyaku Co. Ltd., Kyoto, Japan). The cell debris was removed by centrifuging at 20,000×g for 10 min. The supernatant was used as a crude cell extract for

sugar analysis.

Hexosamine contents of the transgenic cells were determined using the Morgan–Elson method (Table 1). Cell lines carrying the cvGFAT exhibited a high level of accumulation of hexosamines when compared to the wild type cells. Because the Morgan–Elson method does not distinguish among D-glucosamine-6P, N-acetyl-D-glucosamine-6P, D-glucosamine and N-acetyl-D-glucosamine, as presented in Figure 2, it remains unclear what kinds of hexosamines are accumulated in the cells. The results indicate that the viral GFAT is suitable for metabolic engineering of hexosamines, which will be applied to produce polymeric forms of GlcNAc-containing sugars. It is particularly interesting that the cells carrying the *Arabidopsis* gene showed no significant accumulation of metabolites. The multiple sequence alignment of GFAT reveals that the *Arabidopsis* enzyme has sequence similarity with representative eukaryotic enzymes (Figure 3). Because the feedback inhibition of the eukaryotic GFAT by UDP-GlcNAc, but not the viral one, was reported, if the *Arabidopsis* enzyme exhibits the inhibition similarly to the eukaryotic one, then the enzymatic differences might explain the lack of accumulation of hexosamine in the transgenic cells. Further analysis of hexosamine derivatives is necessary to examine that hypothesis.

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