Note

Plant Biotechnology, 21 (1), 79-83 (2004)

Homoglutathione Synthesis in Transgenic Tobacco Plants Expressing Soybean Homoglutathione Synthetase

Akifumi SUGIYAMA, Jun-ichi NISHIMURA, Yasuhiro MOCHIZUKI, Keisuke INAGAKI and Jiro SEKIYA*

Plant Nutrition Laboratory, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
**Corresponding author* E-mail address: jiro@kais.kyoto-u.ac.jp

Received 16 September 2003; accepted 7 October 2003

Abstract

Homoglutathione (hGSH) is present in some leguminous plants including soybean and bean. However, hGSH synthesis in non-leguminous plants has not been reported so far. We constructed transgenic tobacco plants expressing soybean hGSH synthetase (hGSHS). Although the transgenic tobacco plants exhibited higher hGSHS activity than glutathione (GSH) synthetase activity, hGSH content was much lower than GSH content. Precursor feeding experiments revealed that hGSH synthesis was limited by the availability of both γ-L-glutamyl-L-cysteine and β-alanine. The result also suggests that the major site of GSH synthesis in tobacco plants is not cytosol.

Key words: glutathione, homoglutathione, homoglutathione synthetase, leguminous plant, transgenic tobacco plant.

Abbreviations

BA, 6-benzyladenine; γ-GluCys, γ-L-glutamyl-L-cysteine; γ-ECS, γ-L-glutamyl-L-cysteine synthetase; GSH, glutathione; GSHS, glutathione synthetase; hGSH, homoglutathione; *hgshs*, homoglutathione synthetase; NAA, 1-naphthaleneacetic acid; ORF, open reading frame; PCR, polymerase chain reaction.

hGSH (γ-L-glutamyl-L-cysteinyl-β-alanine) is a major non-protein thiol compound found in leguminous plants, instead of or in addition to GSH (γ-L-glutamyl-L-cysteinylglycine) (Price, 1957; Carnegie, 1963; Klapheck, 1988). Synthesis of hGSH proceeds via two reaction steps catalyzed by γ-ECS and hGSHS, the former being common to GSH synthesis. Complementary DNAs of hGSHS were isolated from *Medicago truncatula* (Frendo *et al.*, 1999), pea (*Pisum sativum*) (Moran *et al.*, 2000), bean (*Phaseolus vulgaris*) (Moran *et al.*, 2000), and soybean (*Glycine max*) (accession no. AJ272035). Heterologous expression of cDNAs revealed that hGSHS has higher substrate specificity for β-alanine than glycine (Frendo *et al.*, 2001; Iturbe-Ormaetxe *et al.*, 2002). Although hGSH is assumed to be functionally interchangeable with GSH (Klapheck, 1988), hGSHS has not been expressed in non-leguminous plants before. We report herein the characterization of hGSH synthesis in transgenic tobacco plants expressing soybean *hgshs*.

Partial cDNA (462 bp) of soybean *hgshs* was isolated from soybean leaf cDNA library (GIBCO/BRL) by PCR using the following primers, 5′-AA(G/A)AT(T/C)CA(G/A)CA(G/A)GA(G/A)CT(G/T)GC-3′ (forward) and 5′-AG(G/C)(T/C/A)(G/A)(C/A)AAC(G/T/A/C)CC(G/A)CC(T/C)TCAT-3′ (reverse). Full-length cDNA of *hgshs* was isolated from soybean leaf cDNA library using an alkaline phosphatase labeled probe prepared from the 462 bp fragment. The *hgshs* cDNA obtained was ligated into pGEM-T Easy vector (pGEM-hgshs) and sequenced. Sequence analysis revealed that pGEM-hgshs contained an ORF identical to that of the previously reported soybean *hgshs* (AJ272035). Although the putative signal peptide is present in bean *hgshs* (AF258320), *M. truncatula hgshs* (AF075700), and *A. thaliana gshs* (U22359), it is absent in soybean *hgshs*, which is consistent with a previous report that hGSHS activity was found only in the cytosol of soybean leaves (Moran *et al.*, 2000).

*Bam*HI restriction sites were added to both ends of soybean *hgshs* ORF by PCR, using pGEM-hgshs as a template. The primers used were 5′-ACGGATCCATGTCTCAACCTTTGACCAC-3′

(forward) and 5′-ACGGATCCGCTCCATCAAG TTAGGTATAC-3′ (reverse). The amplified fragment was gel-purified and ligated into pGEM-T Easy vector. The resulting plasmid was digested with *Bam*HI and the fragment was subcloned into the *Bam*HI site of the modified pBI 121 vector (Koizumi *et al.*, 1999) to form pBI-hgshs. The integrity of the insert was verified by sequencing. The binary vector pBI-hgshs was introduced into *Agrobacterium tumefaciens* LBA4404 according to the method of Hofgen and Willmitzer (1988).

The hypocotyls of tobacco (*Nicotiana tabacum* cv. Bright Yellow-2) grown on an MS medium (Murashige and Skoog, 1962) were transformed with *A. tumefaciens* LBA4404 carrying the pBI-hgshs using the method of Horsh *et al.* (1985). Infected hypocotyls were incubated on MS medium supplemented with 3% sucrose, 2.1 mg l^{-1} NAA, and 0.02 mg l^{-1} BA for 2 days, followed by incubation in MS liquid medium supplemented with 3% sucrose, 2.1 mg l^{-1} NAA, 0.02 mg l^{-1} BA, and 500 mg l^{-1} carbenicillin for 2 day. Transformants were then selected on MS medium supplemented with 3% sucrose, 0.105 mg l^{-1} NAA, 0.5 mg l^{-1} BA, 100 mg l^{-1} carbenicillin, and 50 mg l^{-1} kanamycin. Regenerated shoots were excised from the calli and grown on half-strength MS medium supplemented with 1.5% sucrose, 0.02 mg l^{-1} 3-indolebutyric acid, 100 mg l^{-1} carbenicillin, and 50 mg l^{-1} kanamycin, and solidified with 0.2% gellan gum. Plants with good root systems were transferred to soil and allowed to grow to maturity in a greenhouse set at 25℃ with 16 h/8 h photoperiod. MS media were adjusted to pH 6.0 and solidified with 0.8% agar unless otherwise stated.

Thiol compounds were analyzed according to the methods of Newton *et al.* (1981) and Klapheck (1988) with some modifications. Plant tissues (ca. 100 mg) were ground in 2.5 ml of 0.1 N HCl containing 1 mM DTT with a mortar and pestle. The homogenate was centrifuged at 10,000*g* for 10 min at 4℃. The supernatant (200 μl) was added to a mixture of 300 μl of 200 mM Tris-HCl, pH 9.2, and 50 μl of 5 mM DTT, and incubated for 1 h at room temperature. A portion (130 μl) of the solution was added to 20 μl of 15 mM monobromobimane. After incubation for 15 min in the dark, the derivatization reaction was terminated by addition of 550 μl of 10% acetic acid. HPLC analysis of monobromobimane derivatives of thiol compounds was performed as described by Okumura *et al.* (2003).

All procedures for crude enzyme preparation were carried out at 4℃ unless otherwise stated. Plant tissues (ca. 1 g) were homogenized with 5 ml of 50 mM Tris-HCl, pH 8.0, containing 10 mM 2-mercaptoethanol, 5 mM EDTA, and 0.1% ascorbate, and 100 mg of insoluble polyvinylpyrrolidone using a mortar and pestle. The homogenate was centrifuged at 10,000*g* for 10 min. The supernatant obtained was brought to 80% saturation with ammonium sulfate. The precipitate was collected by the centrifugation and dissolved in a small volume of 50 mM Tris-HCl, pH 8.0, and then desalted with Sephadex G-25. The resulting solution was used as the crude enzyme.

GSHS and hGSHS activities were essentially measured according to the method of Klapheck *et al.* (1988). The standard reaction mixture contained 20 μl of crude enzyme solution, 40 mM Tris-HCl, pH 8.0, 3 mM ATP, 0.15 mM DTT, 40 mM $MgCl_2$, 3 mM phosphoenolpyruvate, 1 unit of pyruvate kinase, 0.75 mM γ-GluCys, and 7.5 mM glycine (for GSHS) or β-alanine (for hGSHS) in a total volume of 100 μl. After incubation at 30℃ for 30 min, the reaction was terminated by addition of 25 μl of 20% trichloroacetic acid. Thiol compounds formed were derivatized with monobromobimane and analyzed by HPLC as described by Okumura *et al.* (2003).

Protein concentration was measured according to the method of Bradford (1976) using bovine serum albumin as a standard. Free amino acids were extracted with 10 mg ml^{-1} citric acid solution from freeze-dried plant tissues. Amino acid contents were measured by the post-column method using *o*-phthalaldehyde.

Eight different lines of transgenic tobacco plants (T_0) expressing soybean *hgshs* were regenerated. The T_0 plants were allowed to self-fertilize and the seeds were collected. Studies were undertaken with T_1 plants (S-1 and S-2). Transgenic tobacco plants grew normally, similar to the wild-type. As shown in Table 1, hGSHS activity was detected in both lines of transgenic tobacco plants but not in wild-type tobacco plants. Transgenic tobacco plants exhibited higher hGSHS activity than GSHS activity, probably due to the CaMV 35S promoter. Foliar thiol content was measured (**Table 1**). hGSH was detected in the leaves of transgenic tobacco plants, whereas cysteine, γ-GluCys, and GSH were detected in both transgenic and wild-type tobacco plants at almost the same concentrations. hGSH content in transgenic tobacco plants was much lower than GSH content despite the higher hGSHS activity. The other substrate of hGSH, β-alanine, was not detected in tobacco leaves under the experimental conditions used here (**Table 2**), although it is known that β-alanine is a precursor of pantothenic acid and coenzyme A in all plants. β-

Table 1 Enzyme activities of GSHS and hGSHS and thiol content in leaves of wild-type and transgenic tobacco plants (T_1).

Line	Enzyme activity(pkat mg^{-1} protein min^{-1})		Thiol content (nmol g^{-1} FW)			
	GSHS	hGSHS	Cys	γ-GluCys	GSH	hGSH
WT	13.2 ± 1.6[1)]	nd[2)]	42 ± 4	18 ± 5	467 ± 46	nd
S-1	20.1 ± 3.0	48.5 ± 2.7	41 ± 5	30 ± 4	383 ± 20	49 ± 10
S-2	24.5 ± 5.7	110.5 ± 9.3	37 ± 6	16 ± 5	344 ± 9	47 ± 11

[1)] Values represent means ± standard error (n = 3-5).

[2)] nd, not detected.

Table 2 Foliar contents of β-alanine, GSH and hGSH in tobacco, broad bean, soybean and bean plants.

Plant	Content (nmol g^{-1} FW)		
	β-Alanine	GSH	hGSH
Tobacco	nd[1)]	467 ± 46 [2)]	nd
Broad bean	nd	430 ± 69	nd
Soybean	148 ± 8.1	14 ± 2.9	630 ± 52
Bean	42 ± 3.5	nd	363 ± 50

[1)] nd, not detected.

[2)] Values represent means ± standard error (n = 3-5).

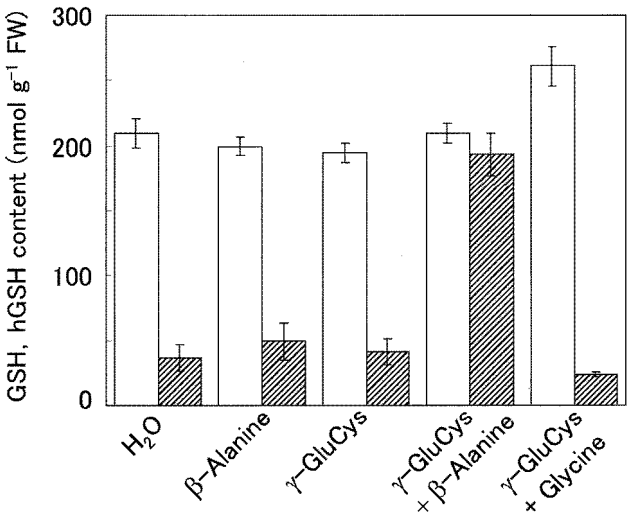

Fig. 1 Effects of exogenous γ-GluCys, β-alanine and glycine on GSH and hGSH contents in leaf discs of transgenic tobacco plants. Leaf discs of transgenic tobacco plants (S-2 line) were vacuum-infiltrated with γ-GluCys (10 mM), β-alanine (10 mM), and glycine (10 mM), either alone or in combination, as indicated. After incubation at 25℃ in the dark for 1 h, GSH and hGSH contents were measured. Open bars indicate GSH content and hatched bars, hGSH content. Data represent means ± standard error of four replicates.

Alanine was detected in soybean and bean that synthesize hGSH exclusively, but not in broad bean (*Vicia faba*) that synthesizes only GSH (**Table 2**). These results imply that the endogenous level of β-alanine may be correlated with hGSH synthesis in leguminous plants.

As β-alanine content was much higher in hGSH-synthesizing leguminous plants than in tobacco plants, we presumed that the β-alanine content in cells is a limiting factor for hGSH synthesis in transgenic tobacco plant. γ-GluCys has also been reported to be a limiting factor for GSH synthesis (Hell and Bergmann, 1990; Strohm *et al.*, 1995). In order to confirm whether the *in vivo* synthesis of hGSH in transgenic tobacco plants was limited by the availability of β-alanine or γ-GluCys, leaf discs of transgenic tobacco plants were vacuum-infiltrated with either β-alanine or γ-GluCys, or both. GSH and hGSH contents were measured after incubation for 1 h. hGSH content was not increased significantly by addition of β-alanine alone; however, it was increased 5.3-fold by addition of β-alanine and γ-GluCys (**Fig. 1**). Essentially the same results were obtained when leaf discs of a different line were used (data not shown). hGSH was not detected when leaf discs of wild-type tobacco plants were vacuum-infiltrated with β-alanine and γ-GluCys (data not shown). These results indicate that the synthesis of hGSH in trans-

genic tobacco plants is limited by the availability of γ-GluCys as well as β-alanine.

It has been reported that GSHS is present in cytosol and chloroplast at nearly the same level, and that GSH is synthesized in both compartments (Noctor *et al.*, 2002). If this is also the case in tobacco, γ-GluCys in the cytosol for GSH synthesis will also be used for hGSH synthesis in transgenic tobacco plants when β-alanine is exogenously supplied, and more hGSH will be synthesized than GSH in the cytosol due to the higher hGSHS activity. However, the result shown in **Fig. 1** is not consistent with this idea; therefore, it appears that the major site of GSH synthesis in tobacco is the chloroplast, and relatively low levels of GSH are synthesized in the cytosol. Strohm *et al.* (1995) reported that the incubation of leaf discs of poplar with γ-GluCys and glycine for 60 min resulted in a more than two-fold increase in GSH. However, we did not find as much increase in GSH as observed in poplar when leaf discs of transgenic tobacco were fed with either γ-GluCys or both γ-GluCys and glycine (**Fig. 1**). These contradictory results obtained with tobacco and poplar may be due to the difference in the level of GSHS in the cytosol. In other words, as we suggested above, GSH may be synthesized nearly equally in the cytosol and the chloroplast in poplar and mainly in the chloroplast in tobacco. This also implies that γ-GluCys and/or glycine are not spontaneously transported across the chloroplast membrane.

hGSH is detected only in leguminous plants. It has been surmised that the presence of hGSH in leguminous plants is due to the expression of legume-specific *hgshs* (Frendo *et al.*, 1999) and that the absence of hGSH in non-leguminous plants is due to the lack of *hgshs*. Our results, however, demonstrated that not only the expression of *hgshs* but also an adequate supply of β-alanine and γ-GluCys to the site of hGSH synthesis is necessary for some leguminous plants to synthesize detectable hGSH. Such questions as why high level of β-alanine is supplied in hGSH-synthesizing leguminous plants and how γ-GluCys and β-alanine are adequately supplied remain to be answered.

Acknowledgement

The authors thank Drs. Masanori Kaido and Kentaro Ifuku at Kyoto University for technical support and advice.

References

Bradford, M. M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., **72**: 248-254.

Carnegie, P. R., 1963. Structure and properties of a homologue of glutathione. Biochem. J., **89**: 471-478.

Frendo, P., Gallesi, D., Turnbull, R., Van de Sype, G., Hérouart, D., Puppo, A., 1999. Localization of glutathione and homoglutathione in *Medicago truncatula* is correlated to a differential expression of genes involved in their synthesis. Plant J., **17**: 215-219.

Frendo, P., Jimenez, M. J., Mathieu, C., Duret, L., Gallesi, D., Van de Sype, G., Herouart, D., Puppo, A., 2001. A *Medicago truncatula* homoglutathione synthetase is derived from glutathione synthetase by gene duplication. Plant Physiol., **126**: 1706-15.

Hell, R., Bergmann, L., 1990. γ-Glutamylcysteine synthetase in higher plants: catalytic properties and subcellular localization. Planta, **180**: 603-612.

Hofgen, R., Willmitzer, L., 1988. Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids Res., **16**: 9877.

Horsch, R. B., Fry, J. E., Hoffman, N. L., Eichholtz, D., Rogers, S. G., Fraley, R. T., 1985. A simple and general method for transferring genes into plants. Science, **227**: 1229-1231.

Iturbe-Ormaetxe, I., Heras, B., Matamoros, M. A., Ramos, J., Moran, J. F., Becana, M., 2002. Cloning and functional characterization of a homoglutathione synthetase from pea nodules. Physiol. Plant., **115**: 69-73.

Klapheck, S., 1988. Homoglutathione: isolation, quantification and occurrence in legumes. Physiol. Plant., **74**: 727-732.

Klapheck, S., Zopes, H., Levels, H. G., Bergmann, L., 1988. Properties and localization of the homoglutathione synthetase from *Phaseolus coccineus* leaves. Physiol. Plant., **74**: 733-739.

Koizumi, N., Ujino, T., Sano, H., Chrispeels, M. J., 1999. Overexpression of a gene that encodes the first enzyme in the biosynthesis of asparagine-linked glycans makes plants resistant to tunicamycin and obviates the tunicamycin-induced unfolded protein response. Plant Physiol., **121**: 353-361.

Moran, J. F., Iturbe-Ormaetxe, I., Matamoros, M. A., Rubio, M. C., Clemente, M. R., Brewin, N. J., Becana, M., 2000. Glutathione and homoglutathione synthetases of legume nodules: cloning, expression, and subcellular localization. Plant Physiol., **124**: 1381-1392.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., **15**: 473-497.

Newton, G. L., Dorian, R., Fahey, R. C., 1981. Analysis of biological thiols: derivatization with monobromobimane and separation by reverse-phase high performance liquid chromatography. Anal. Biochem., **114**: 383-387.

Noctor, G., Gomez, L., Vanacker, H., Foyer, C. H., 2002. Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. J. Exp. Bot., **53**: 1283-1304.

Okumura, R., Koizumi, Y., Sekiya, J., 2003. Synthesis of hydroxymethylglutathione from glutathione and L-ser-

ine catalyzed by carboxypeptidase Y. Biosci. Biotechnol. Biochem., **67**: 434－437

Price, C., 1957. A new thiol in legumes. Nature, **180**: 148－149.

Strohm, M., Jouanin, L., Kunert, K. J., Pruvost, C., Polle, A., Foyer, C. H., Rennenberg, H., 1995. Regulation of glutathione synthesis in leaves of transgenic poplar (*Populus tremula* × *P. alba*) overexpressing glutathione synthetase. Plant J., **7**: 141－145.