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ORIGINAL PAPER

Folate biofortification of lettuce by expression of a codon optimized chicken GTP cyclohydrolase I gene

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Abstract Folates are essential coenzymes involved in one-carbon metabolism. Folate deficiency is associated with a higher risk of newborns with neural tube defects, spina bifida, and anencephaly, and an increased risk of cardiovascular diseases, cancer, and impaired cognitive function in adults. In plants folates are synthesized in mitochondria from pterin precursors, which are synthesized from guanosine-5′-triphosphate (GTP) in the cytosol (pterin branch), and *p*-aminobenzoate (PABA), derived from chorismate in plastids (PABA branch). We generated transgenic lettuce lines expressing a synthetic codon-optimized GTP-cyclohydrolase I gene (*gchI*) based on native *Gallus gallus* gene. Immunoblotting analyses confirmed the presence of the *gchI* in transgenic lines. Twenty-nine transgenic lines were generated and 19 exhibited significant increase in the folate content, ranging from 2.1 to 8.5-fold higher when compared to non-transgenic lines. The folate content in enriched lettuce would provide 26% of the Dietary Reference Intakes for an adult, in a regular serving. Although the lettuce lines generated here exhibited high folate enhancement over the control, better folate enrichment could be further achieved by engineering simultaneously both PABA and pterin pathways.

Keywords Biofortification · Folate · GTP cyclohydrolase I · *Lactuca sativa* · Nutrigenomics · Transgenic lettuce

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Introduction

Folates are part of an extended family of polyglutamates (usually containing 5–7 glutamyl residues) of pteroic acid and related analogs, and are essential cofactors for the one-carbon transfer reactions needed for de novo biosynthesis of purines, pyrimidines, formylmethionyl-tRNA and thymidylate and in the metabolism of several amino acids, including methionine, serine and glycine (Cossins and Chen 1997; Storozhenko et al. 2005). In plants, pterin precursors are synthesized from GTP in the cytosol (pterin branch), whereas *p*-aminobenzoate (PABA) is derived from chorismate in plastids (PABA branch). The biosynthesis of pterins is regulated by GTP-cyclohydrolase I (*gchI*; EC 3.5.4.16), the first and rate limiting enzyme of this pathway. Both pterin precursors and PABA are imported into the mitochondria to participate in the condensation to folates (Cossins 2000).

Deficiency of folate in the diet is likely to result in a reduction in the capacity to synthesize DNA and maintain the usual rate of cell division. This most evidently results in the production of megaloblastic anemia from folate deficiency due to a reduction in the biosynthesis of cells in the bone marrow. Its deficiency has been associated with neural tube defects in infants (such as spina bifida and anencephaly), increased risk of vascular disease and some cancers (Eskes 2000; Krishnaswamy and Madhavan 2001; Lucock 2000; Molloy and Scott 2001). In addition, lower serum folate levels have been observed in patients with Alzheimer disease and vascular dementia (Gallucci et al. 2004). Supplements of folic acid as multivitamin tablets led to a dramatic decrease in neural tube defects (MRC Vitamin Study Research Group 1991). Controlled studies also showed that folic acid fortification of grain products has led to a significant reduction in the incidence of neural tube defects (Honein et al. 2001; Lopez-Camelo et al. 2005; Persad et al. 2002), some childhood cancers (French et al. 2003), and stroke (Goyer and Navarre 2007; Yang et al. 2006).

Although plants and microorganisms are able to synthesize folates, animals lack a complete folate synthesis pathway. Consequently, humans need to consume a certain proportion (a minimum of 400 μg/day) through the diet, mostly from plant sources (Scott et al. 2000; Konings et al. 2001). There is good evidence for folate deficiency in a considerable number of the population in both developing and developed countries, even where folate supplementation of foods is practiced, and for a level of intake in excess of the recommended dietary allowance (Scott et al. 2000; Morris and Tangney 2007).

Folate biofortification of food crops by means of genetic engineering is a cost-effective and sustainable complementary approach to the existing interventions (industrial fortification, folate pill distribution and diet diversification), particularly for rural populations in developing countries, where conventional approaches have significant recurrent costs and are difficult to implement. Recently, De La Garza et al. (2004, 2007) expressed a mammalian *gchI* gene in tomato fruits and raised its pteridine content by 3 to 140-fold and fruit folate content by an average of twofold relative to vector-alone controls. Storozhenko et al. (2007) reported folate biofortification of rice seeds (100 times above wild type) achieved by overexpressing two *Arabidopsis thaliana* genes [coding for GTP-cyclohydrolase I (*gchI*) and aminodeoxychorismate synthase (*adcs*)] of the pterin and PABA branches of the folate biosynthesis. Several dietary sources are rich in natural folate, such as fruits, dark green leafy vegetables, i.e., spinach, Brussel sprouts and broccoli, oranges, beans, yeast and liver. A considerable amount of folate, however, can be destroyed by cooking, processing and storage (Hall and Solehdin 1998; Eskes 2000). Lettuce (*Lactuca sativa*) is one of the most consumed vegetables in many countries, where it is typically eaten cold and raw, in salads, hamburgers, tacos, and many other dishes. Consequently, this is a very attractive crop as a vehicle for high temperature sensitive nutritional molecules. Since the activity of plant *gchI* is presumably subject to negative feedback regulation (Sohta et al. 1997; Yoneyama and Hatakeyama 1998) we constructed a vector to express an avian *gchI* by overexpressing a synthetic code-usage optimized gene in leaves of transgenic lettuce to increase total folate.

Materials and methods

Vector construct and lettuce transformation

GTP-cyclohydrolase I gene (*gchI*) was synthesized by Epoch Biolabs Inc (Sugar Land, TX, USA) according to the sequence from *Gallus gallus* (Genebank accession number Z49267), replacing 10 rare codons (TGT to TGC, CCA to CCG, GGT to GGC, AGA to CGT, GCA to GCG, AGA to AGG, ACA to ACG, CCT to CCC, GGT to GGC, CAT to CAC). The synthetic DNA was inserted between the *Nco*I/*Sac*I of the pBI426 vector (Datla et al. 1991), replacing the *gus:nptII* fusion gene. Then the expression cassette was transferred to pCAMBIA1300 using *Eco*RI and *Hin*dIII to generate the vector pCGCHI that was used to transfect *Agrobacterium tumefaciens* strain EHA105 by electroporation. The vector pCGCHI was used for lettuce (cv. Verônica) transformation according to Dias et al. (2006).

Screening of transgenic plants by PCR

DNA was isolated from leaf disks according to Doyle and Doyle (1987). PCR was carried out according to Bonfim et al. (2007). The primers CHMUT591C

(5′-ATGTGTAGCTTCGATCACCACTCC-3′) and CH MUT130 (5′-AGACCAAGAAGCGAGGAGGACA AC-3′) within the *gchI* gene were utilized to amplify a 461 bp sequence. The mixture was treated at 95°C (5 min) and subjected to 35 cycles of amplification (95°C for 1 min, 55°C for 1 min, and 72°C for 1 min), with a final elongation cycle of 7 min at 72°C. The reaction mixture was then loaded onto 1% agarose gel and visualized under UV light following blue green dye (LGC Biotecnologia, Brazil) staining.

Expression of *gchI* in *E. coli*

The *gchI* gene was expressed in *Escherichia coli* to be used as a positive control. The *gchI* coding sequence was inserted into pDEST17 *E. coli* expression vector (Invitrogen) generating the vector pDEST GCHI. LB medium (0.5 l) plus ampicillin (50 mg) was inoculated with an overnight cell culture of transformed BL21-Lys cells containing the plasmid pDESTGCHI. The cells were grown with shaking at 37°C to an $A_{600\ nm}$ of 0.7–0.9, with the expression of the *gchI* gene induced by 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and growth continued for 5 h at 37°C. The cells were harvested and stored at −80°C. Accumulation of recombinant protein was monitored by SDS–PAGE in whole cell soluble fractions. The His_6-tagged *gchI* was purified in a single step with HisTrap HP affinity columns (GE Healthcare, USA) following the manufacturer's protocol. The purity of the protein was analyzed by SDS–PAGE. The His_6-tagged *gchI* was used as a positive control.

Western blot analysis

Protein extraction was performed by mixing the leaf powder (0.1 g) and 30 µl sample buffer (125 mM Tris, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.04% bromophenol blue, pH 6.8). The mixture was boiled for 5 min. Total protein was quantified using the Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins were resolved in a 12% SDS–PAGE mini gel, electrotransferred to Immobilon-P PVDF membrane (Millipore) under 100 mA for 70 min, followed by 4°C overnight blocking in Tris-buffered saline solution (20 mM Tris base, 137 mM NaCl, pH 7.6) containing 5% dry milk and 0.1% tween 20. The membrane was incubated with mouse IgG polyclonal anti-GCHI partial recombinant protein (NP000152, Human GCHI epitope a.a. 84–173, Novus Biologicals Inc, Littleton, CO, USA) diluted 1:4,000 in blocking buffer, for 4 h at 25°C. Secondary antibody incubation was performed for 4 h at 25°C with goat anti-mouse IgG conjugated with alkaline phosphatase (Bio-Rad) in a dilution of 1:7,000 in blocking buffer. Blots were developed with chemioluminescent substrate CSPD (Applied Biosystems) according to the manufacturer's instructions using Kodak Standard films.

Folate content determination

The microbiological method was used for the determination of folate in plant leaves following the AACC Method 86-47 (DeVries et al. 2001; Hyun and Tamura 2005) with modifications. Five grams of fresh leaves were ground in liquid nitrogen and homogenized with 25 ml of 0.1 M phosphate buffer containing 114 mM ascorbic acid (pH 4.1). The homogenate was heated in a water bath at 100°C for 10 min, cooled, and stored at −80°C until used. Enzyme treatments were carried out according to Pandrangi and LaBorde (2004) except that a bovine conjugase, prepared according to Wilson and Horne (1982), was used. Glycerol-cryoprotected *Lactobacillus rhamnosus* (= *Lactobacillus casei* ATCC 7469, American Type Culture Collection, Manassas, VA, USA) was prepared according to Wilson and Horne (1982). Folate content in lettuce lines was determined using the ninety-six-well microplate procedure. Bacteria were cultivated in sterilized folic acid assay medium (Sigma). Pteroyl-L-glutamic acid (Sigma) was used as the folic acid standard. Optical density at 595 nm was used to measure microbial growth. Triplicate samples were assayed and analyses were repeated four times. Folate concentration data was normalized using the total protein and expressed as ng of folate per mg of total protein.

Statistical analysis

Analysis of variance was performed with the SAS System for Windows (version 8.02; SAS Institute, Cary, NC).

Results

Twenty-nine transgenic lines were generated and all contained the *gchI* transgene in PCR analyses (data not shown). Each line originated from an independent regenerating callus and could be considered an independent transformation event. All twenty-nine lines were advanced to the T_3 generation. To confirm that the *gchI* gene was expressed, protein from leaf tissue from the T_3 plants was extracted and analyzed by SDS–PAGE and immunoblotting. The transgene product *GCHI* was detected in transgenic lines (Fig. 1). No cross-reaction with lettuce GCHI was detectable and no bands were observed in the wild-type controls (Fig. 1). As expected, an immunoreactive band was observed, corresponding in size to the predicted molecular mass (30.1 kDa, including N- and C-terminal tail extensions) of the *gchI* gene product expressed in *E. coli*.

To determine whether the expression of *gchI* increased folates, the total folate levels of transgenic and non-transgenic lines were measured by microbial assay with *L. rhamnosus*. Results revealed that 19 lines exhibited significant increase ($P = 0.05$) in the folate content, ranging from 2.1 to 8.5-fold higher when compared to non-transgenic lines (Fig. 2). In addition, spinach (*Spinacea oleracea*), a plant rich in folates, was analyzed. Results revealed that some transgenic lines, such as AA16-C3-3, 47B5, 47B2, had 72.6, 67.4 and 51.5%, respectively of the total folate found in spinach.

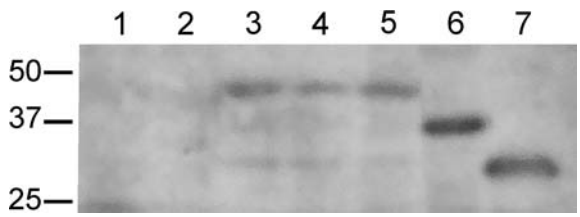

Fig. 1 Expression of GgGCHI in transgenic lettuce leaves. Western blot of GgGCHI expressed in transgenic plants, chicken and *E. coli* detected by using anti-hGCHI antibody. *Lanes 1–2* non-transgenic lettuce leaves; *Lanes 3–5* transgenic lettuce lines (60 μg of total was loaded in each lane); *Lane 6* 30 μg of total protein isolated from chicken liver; *Lane 7* recombinant GCHI-6His from *E. coli* cells transformed with plasmid pDESTGCHI. Molecular mass standards are indicated on *left* (in kDa)

Discussion

Despite the fact that plants are a primary source of dietary folates, the folate content of most consumed vegetables is quite low. Among the strategies to increase folate intake by population, i.e., supplementation by capsule intake, food fortification, and educational incentive for higher intake of folate-rich food, enhancement of folate contents in edible crops through "classical" and molecular breeding (biofortification) could offer a sustainable alternative, especially for developing countries, where other methods of folate fortification are hampered. In this work, we introduced the *gchI* gene encoding an unregulated GTP-cyclohydrolase I from *G. gallus* (chicken *gchI*) into lettuce to increase the folate content in leaves. We have chosen to engineer the folate pathway in lettuce because of its worldwide importance, low folate content, and because it is typically eaten raw.

Immunoblotting analyses with anti-GCHI raised against the epitope corresponding to aminoacids from position 84 to 173 of the human GCHI showed that the *G. gallus* codon-optimized gene was expressed in lettuce leaves. No signal was observed in the non-transgenic lines. Indeed, the GCHI from *G. gallus* is very divergent from plant GTP-cyclohydrolases, presenting an identity of 39% with the most related GCHI from *Solanum lycopersicum* (*E*-value = $1e^{-26}$) and *Arabidopsis thaliana* (*E*-value = $3e^{-26}$). In addition, the human GCHI epitope has high identity with GgGCHI (94%) and low identity with plant GCHI epitopes (<41%).

One major and one minor GCHI-immunoreactive band were observed in the transgenic lines. The GgGCHI protein detected in both transgenic lettuce leaves and chicken liver exhibited a higher molecular mass when compared to GgGCHI expressed in *E. coli*. This enzyme is a multimer composed of identical subunits, but its exact number of subunits has not yet been determined for most species. Differences in electrophoretic pattern, related to the in silico expected molecular mass, have been observed in both plant and animal GCHI (Yoneyama and Hatakeyama 1998; Cha et al. 1991). This difference in protein size could also be attributed to post-translational modifications, such as glycosylation and phosphorylation. Hesslinger et al. (1998) have shown that GCHI is subject to modification at

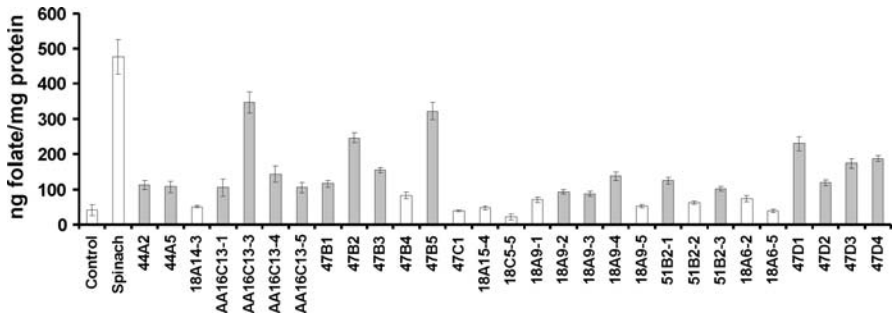

Fig. 2 Total folate levels in T_3 transgenic and non-transgenic (control) lettuce lines. Folate values of 19 transgenic lines (*gray bars*) were significantly different from the non-transgenic plants. *Bars* represent the mean ± SD (Student's *t*-test; $P < 0.05$ vs. controls). Spinach was also analyzed because it is considered to be rich in folates

the post-translational level. Additionally, an *in silico* analysis using the YinOYang 1.2 algorithm (Gupta and Brunak 2002) predicted five Ser/Thr residues in the GgGCHI with high potential (0.77–0.99; threshold = 0.5) for N-acetylglucosamine glycosylation as well as phosphorylation. In transgenic lettuce leaves, GgGCHI seems to be electrophoretically slightly higher than the protein found in chicken liver. Although plants possess an endomembrane system and a secretory pathway similar to that of animal cells, differences between animal and plant glycosylation patterns, such as in glycan side chains synthesis, have been observed in recombinant plant systems (Ma et al. 2003; Balen and Krsnik-Rasol 2007; Streatfield 2007). Further experiments should be carried out to confirm if this difference could influence enzymatic activity.

Plants expressing the *gchI* from chicken exhibited 8.5-fold more total folates in leaves than controls. A similar strategy, but using bacterial *gchI*, was similarly successful in enhancing pteridine and folate production in *A. thaliana* leaves (Hossain et al. 2004) and tomato fruits (De La Garza et al. 2004). Despite increasing folate content in tomato fruits by an average of twofold (threefold in the highest case), fruits that were harvested and ripened after detaching at breaker stage contained folate levels similar to control fruits that ripened on the plant (De La Garza et al. 2004). In *Arabidopsis*, the expression of the *E. coli* gene encoding *GCHI* resulted in a two to fourfold enhancement of folates. In all cases, expression of the *gchI* gene generated much higher pteridine content increase when compared to folate enhancement (Hossain et al. 2004; De La Garza et al. 2004). These results suggested the existence of a constraint on flux in the folate pathway, probably the PABA supply. Recently, De La Garza et al. (2007) crossed tomato lines overproducing both PABA and pteridines to produce plants accumulating up to 25-fold more folate in fruits. Although the lettuce lines generated here exhibited high folate enhancement over the control, better folate enrichment could be achieved by increasing the PABA biosynthesis.

The folate content achieved in leaves of lettuce was as high as 188.5 μg/100 g on a fresh weight basis (346.5 ng folate/mg protein), which is 5.4-fold more folate than observed in lettuce raw green leaves (var. crispa) (USDA National Nutrient Database for Standard Reference, http://www.nal.usda.gov/fnic/foodcomp/search/). The folate-enriched lettuce serving (56 g, according to USDA National Nutrient) provides about 26% of the Dietary Reference Intakes (DRI) (400 μg/day for an adult). Further research will be necessary to evaluate the bioavailability and bioefficacy of folate-fortified lettuce.

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