A transgenic cereal crop with enhanced folate: rice expressing wheat HPPK/DHPS

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ABSTRACT

Folate is a B-group vitamin critical for normal cellular function and division. It acts in one-carbon transfer systems essential in nucleotide synthesis, methylation and gene expression. Insufficient intake causes megaloblastic anaemia and there are strong linkages to cardiovascular disease, various cancers and cognitive decline. Low levels prenatally can lead to low birth weight and premature infants and catastrophic neural tube defects including spina bifida and anencephaly (1-3).

Vertebrates are unable to synthesize folate *de novo*, accordingly plant foods are the primary source. Cereals, which provide over half the worlds population with 80% of their diets are unfortunately particularly poor in folate. Consequently the majority of developed nations have fortification programmes. In the developing world however, such programmes are logistically far more difficult. A practicable alternative is metabolic engineering, to create a cereal crop plant producing high levels of folate.

Folate is produced in a multi-step process from a pterin ring, p-aminobenzoate (pABA) and glutamate residues. Here we show rice plants transgenic for wheat 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase/7,8-dihydropteroate synthase (HPPK/DHPS) which operates at a central point in the production pathway, gives elevated folate levels. Consequently we have a cereal crop transgenic for a single cereal gene expressing enhanced folate levels.
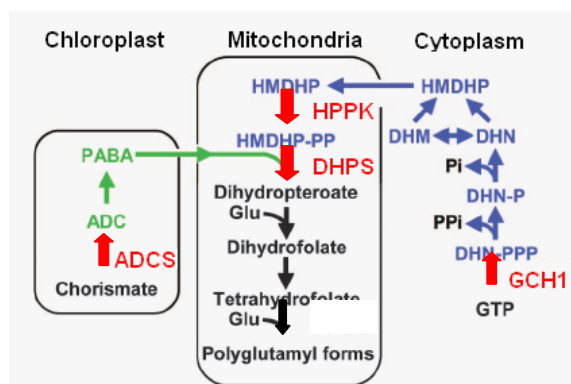
INTRODUCTION

Folate is produced from the condensation of a pterin ring with a p-aminobenzoate (pABA) moiety and the subsequent addition of a variable number of glutamate residues. In plants, folate production is divided into three separate subcellular compartments. PABA is synthesised from chorismate in the chloroplast, the first step of which is catalysed by 4-amino-4-deoxychorismate synthase (ADCS). Pterin is synthesised in the cytoplasm from GTP through the initial action of GCH1 (GTP cyclohydrolase-1). The two are condensed in the mitochondria where glutamate residues are added (4-5) (Fig 1).

Our previous work demonstrated the key genes of the folate pathway are expressed in the major tissues of the wheat plant including the seed, evidence *de novo* folate synthesis occurs throughout the plant (6-7). Additionally we confirmed these genes are highly conserved, validating our selected experimental strategy of introducing wheat genes into rice plants to enhance folate. We selected HPPK/DHPS rationalising use of a bi-functional enzyme which operates at a central point of the pathway would increase the levels of folate without the concomitant increase of any intermediates. This strategy additionally obviates any need to adjust the levels of proteins required to transport pathway components into the required compartment. Moreover the introduction of a single gene would make it more likely this technology could be replicated into other cereal staples such as sorghum.

We introduced the wheat HPPK/DHPS gene under the control of the maize ubiquitin promoter into the Australian rice variety Jarrah via particle bombardment. We showed an approximate doubling in the level of folate in the leaf tissue of primary transgenics, a similar level was transmitted to transgenic seeds. This work sheds light on control of the folate pathway and suggests the introduction of a single gene from a closely related species may be able to elevate folate to a level where the recommended dietary folate is provided per 100g serving. Additionally this approach should be easily transferable to other food staples with more difficult transformation protocols, such as wheat.

Further analysis of transgenic plants is ongoing.

Fig 1 The folate production pathway. PABA is synthesized from chorismate in the chloroplast, pterin is synthesized in the cytoplasm. These are transported into the mitochondria where the two are condensed and the product glutamated.

MATERIALS AND METHODS

Vector Construction

Wheat HPPK/DHPS cDNA was isolated as previously described (6). It was cloned into Sma1-Sac1 digested bombardment vector pUbi.gfp.nos (provided by A. Futardo) so as to replace the green fluorescent protein (gfp) fragment with HPPK/DHPS and drive its expression from the maize ubiquitin promoter. The construct was sequence verified by an ABI 3730 48 capillary DNA analyser (Applied Biosystems). Sequence analysis and alignments were undertaken using SEQUENCHER™ 4.5 software (Gene Codes Corporation, MI., USA).

Plant material and media

Embryogenic calli were induced from mature seed of the Australian rice cultivar Jarrah (Yanco Agricultural Institute, New South Wales Department of Agriculture) Basal media (CIM) was MS basal salts with B5 vitamins (Gamborg *et al*. 1968), 30g/l sucrose, 500mg/l proline, 500mg/l glutamine and 300mg/l casein enzymatic hydrolysate. 2 mg/l 2,4D was added for callus induction (CIM). 36.4g/l of both mannitol and sorbitol were added for osmotic medium (CIMO) Proliferation medium (CPM) was CIM with 1mg/l 2,4D. Regeneration medium (CRM) was CM with 30g/l sucrose, 3mg/l BAP and 0.5mg/l NAA. Plantlet/rooting medium (PM) consisted of half strength MS salts and vitamins with 10g/l sucrose and 0.05mg/l NAA. Hygromycin B resistant callus was selected on media supplemented with 30 or 50mg/l hygromycin B (H30 or H50) (PhytoTechnology Laboratories) All media was solidified using 3g/l Phytagel and adjusted to pH 5.8.

Culture conditions

After standard surface sterilizing procedures the caryopses were plated onto CIM and incubated in the dark at 27°C and 80% humidity. After 14-18 days scutellum derived embryogenic callus was excised and plated onto CIM and cultured as above for a further 2 weeks, and again for 9 days before plating onto CIMO for osmotic conditioning as described by Vain *et al.* (1993). After 4 hours calli were subject to particle bombardment and returned to the incubator for 20 hours before plating onto CIM with 30mg/l hygromycin B for initial selection. 10-14 days later healthy, pale yellow hygromycin B resistant calli were transferred to CPMH50. This step was repeated after a similar subculturing interval and after a further 10-14 days hygromycin resistant lines were plated onto CRMH50 and incubated at 23°C with a 16 hour photoperiod and 80% humidity for 10 days. The regenerating calli were then transferred to CRM without selection to enhance embryo development and subcultured every 10-14 days onto the same medium until plantlets had formed. Plantlets were transferred to PM for root production. For the final passage PMH15 or PMH30 (15 or 30mg/l hygromycin) was used to kill any non transformed escapes. Surviving, well rooted plants 10-15cm high were grown in the plant house. All healthy calli derived from a single bombarded callus piece were considered an independently transformed line and maintained separately.

Particle bombardment

Bombardment was carried out using the Biolistic PDC-1000/He system (BIO-RAD, Hercules, California). 1µm gold particles were used as microcarriers and prepared as reported (9) except 50mg of gold was used and resuspended in 1ml of sterile 50% glycerol before being dispensed in 50µl aliquots. Each aliquot was coated with 10µg of HPPK/DHPS plasmid and 5µg pGL2 and resuspended in 30µl 100% ethanol. 5 µl of this suspension was dispensed onto macro carrier discs per bombardment. The calli were placed 6cm below the stopping plate and bombarded once at 1100 psi as described (10).

Folate Measurement

Total folates were quantified using a microbial assay relying on the turbidimetric bacterial growth of *Lactobacillus rhamnosus* (Strain -ATCC27773). Total folates were liberated from the complex seed matrix by mechanical homogenisation and incubation at 121°C for 10 min; followed by a 120min tri-enzyme extraction at 37 °C using amylase, protease and pancreatic hydrolase (Davis et al., 1970). Growth of *L. rhamnosus* in test extracts is compared to growth in the presence of varying concentrations of folic acid with a typical detection limit of 40ng/g test sample. Folate analysis was performed by PathWest Laboratory Medicine (Royal Perth Hospital), an Australian NATA accredited laboratory (ISO/IEC 17025; 1999).

RESULTS AND DISCUSSION

Expression of transgene in rice lines

The HPPK/DHPS gene was stably introduced into Jarrah, an Australian variety of *Oryza Sativa*. There were no detectable phenotypic changes from wild-type throughout development and non-primary transgenic plants had similar ability to set seed. Transgenic plants were selected for by growth on hygromycin media, and further subject to PCR for the presence of the HPPK/DHPS gene.

All plant lines which tested positive for the HPPK/DHPS gene expressed higher folate levels than control plants. Analysis of total folate of leaves from T0 transgenic plants demonstrated on average almost a doubling from controls (1.2 +/- 0.0 μg/g, and 2.1 +/- 0.32 μg/g respectively), Fig 1. The increases were in the range of 1.2 to 2 fold (n =9). A limited number of seed lines from these primary transgenic plants showed increases from 1.2 to 1.5 fold from control (average = 1.4, n =3).

The highest level of folate seen in transgenic seed in our experiments was 60 μg folate/100g of rice seed, significantly below the recommended daily supplemental level of 400 μg. However it is possible that producing greater numbers of plant lines may result in the generation of seed with folate close to the recommended level per standard 100g serving.

the transgene and folate, and folate levels in subsequent generation transgenic plants.

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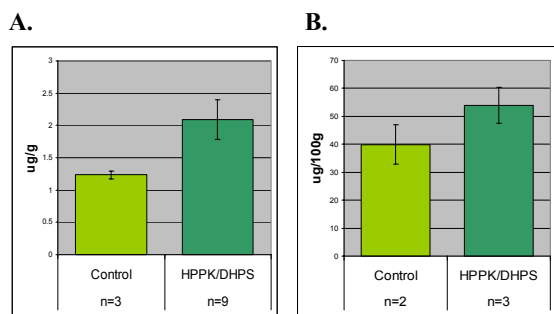
A.   **B.**

Fig 2. Total folate in transgenic plants. A. Leaf tissue from T0 plants. B. Seed from T0 plants.

CONCLUSIONS

Inadequate folate consumption is a major global health concern. Efforts are being made from many directions toward alleviating this problem and a valid approach is the creation of genetically engineered cereal crops. This work provides insight into the utility of the simplest strategy of transgenic approach, the introduction of a single gene from a closely related species. Work is ongoing to examine correlation of expression levels of