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A transgenic cereal crop with enhanced folate: rice expressing wheat HPPK/DHPS (Presentation to the 58th Australian Cereal Chemistry Conference)

Susan A. Gillies
Southern Cross University

Shane R. McIntosh
Southern Cross University

Robert J. Henry
Southern Cross University

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A TRANSGENIC CEREAL CROP WITH ENHANCED FOLATE: RICE EXPRESSING WHEAT HPPPK/DHPS

S.A. Gillies[1], S.R. McIntosh[2] and R.J. Henry[1]

[1]Grain Foods CRC, Centre for Plant Conservation Genetics, Southern Cross University, Lismore, NSW, 2480, Australia
[2] NSW Department of Primary Industries, Wollongbar Agricultural Institute, Wollongbar, 2477, NSW Australia

INTRODUCTION

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 folate intake causes megaloblastic anaemia and is linked to cardiovascular disease, various cancers and cognitive decline. Low prenatal folate levels can lead to low birth weight and premature infants, as well as catastrophic neural tube defects including spina bifida and anencephaly (MRC Vitamin Study Research Group 1991, Snowden et al 2000, McCullough et al 2004). Vertebrates are unable to synthesize folate de novo, accordingly plant foods are the primary source. Cereals, which provide 80% of dietry intake to over half the worlds population, are 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 to use metabolic engineering to create a cereal crop plant producing high levels of folate.

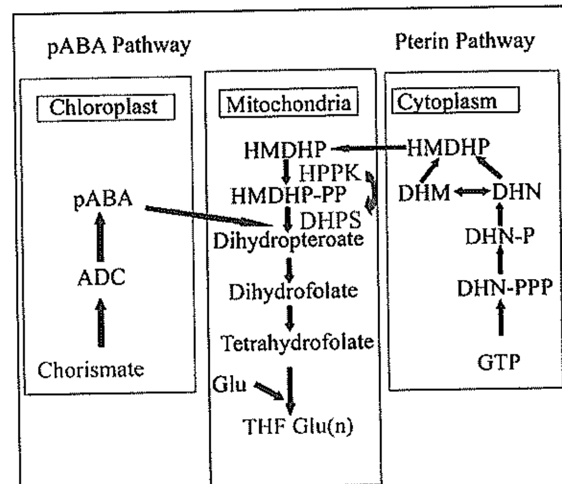

Figure 1. The folate production pathway.

Folate synthesis is a multi-step process involving 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 occurs in three separate subcellular compartments; pABA is synthesised from chorismate in the chloroplast and pterin is synthesised in the cytoplasm from GTP. The two are then condensed in the mitochondria where glutamate residues are added (Basset et al 2002, Basset et al 2004) (Figue 1). Our previous work has demonstrated that the key genes of the folate pathway are expressed in the

major tissues of the wheat plant, including the seed, providing evidence that de novo folate synthesis occurs throughout the plant (McIntosh et al 2007, McIntosh et al 2008). Additionally, we have confirmed these genes to be highly conserved among cereals plants, which allows for the introduction of wheat folate biosynthesis genes into rice plants to enhance folate production in transgenic rice. We selected 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase/7,8-dihydropteroate synthase (HPPK/DHPS), a bi-functional enzyme which operates at a central point of the folate biosynthesis pathway, to increase the levels of folate without a concomitant increase of any intermediates. This strategy obviates any need to adjust the levels of proteins required to transport components of the folate synthesis pathway 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. This work shows that expression of the wheat HPPK/DHPS gene in rice plants leads to enhanced folate levels in transgenic rice.

MATERIALS AND METHODS

Vector Construction

Wheat HPPK/DHPS coding sequence was isolated as previously described (McIntosh et al 2008). It was cloned into SmaI-SacI 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 sequence of the construct was 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) consisted of 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. 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

Caryopses were surface sterilized, 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 (Sanford et al 1993) 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 6 cm below the stopping plate and bombarded once at 1100 psi as described (Abedinia et al 1997).

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

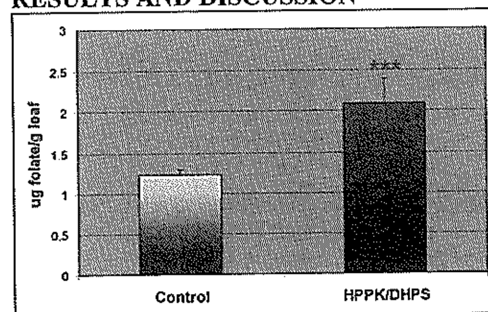

Figure 2. Total folate in transgenic plants.

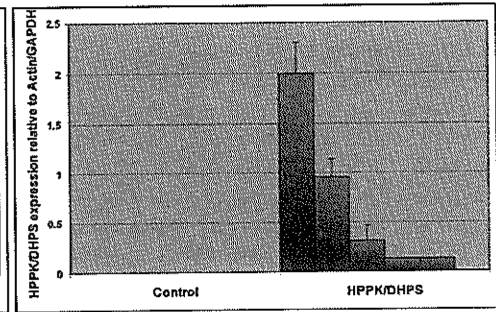

Figure3. Relative expression of HPPK/DHPS transgene

The wheat HPPK/DHPS gene was stably introduced via particle bombardment into Jarrah, an Australian variety of *Oryza sativa*, under the control of the maize ubiquitin promoter. Phenotypic changes from wild-type plants were not detected in transgenics throughout development and non-primary transgenic plants had similar ability to set seed. All plant lines which tested positive for the HPPK/DHPS gene, assessed through growth on hygromycin media and PCR screening, expressed higher folate levels than control plants. Analysis of total folate in leaves from T0 transgenic plants demonstrated, on average, almost double the folate levels of control plants (1.2 +/- 0.0 μg/g, and 2.1 +/- 0.32 μg/g respectively) (Figure 2). These increases were in the range of 1.2- to 2-fold (n =9). Additionally all transgenic lines showed significant expression of the transgene as detected by QRT PCR (Figure 3).

CONCLUSIONS

Inadequate folate consumption is a major global health concern and as such, efforts to alleviate this problem are being made from many different directions. One valid approach is the creation of genetically engineered cereal crops designed to produce higher folate levels. 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. We show that the stable introduction of the wheat HPPK/DHPS gene into rice results in an approximate doubling in the level of folate in the leaf tissue of primary transgenics. These results suggest the introduction of a single gene from a closely related species may be able to elevate folate levels in order to provide the recommended dietry levels of folate. Additionally this approach may be easily transferable to other food staples with more difficult transformation protocols, such as wheat.

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