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Characterising the functional importance of folate polyglutamation in plants

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ABSTRACT

Folate cannot be synthesized by humans and plant food is the main source of this essential vitamin. Folate deficiency can have severe health consequences such as birth defects e.g. spina bifida, and marginal deficiency may impact on cardiovascular vascular diseases and certain cancers. Research suggests that folate intake needs to increase to improve human health. In the USA, cereal-based products are fortified with folic acid and there has been a recommendation for its introduction into the UK. There is some concern over fortification with folic acid since this is not the "natural form" of folate. This project aimed to establish the potential to harness natural variation to enhance folate content in wheat.

The potential for natural variation was demonstrated by a three fold range of folate levels within a population of the model plant *Arabidopsis*. The total folate levels were then determined in the seed from a range of elite wheat lines. In this case there was minimal natural variation with a range of folate of between 40 and 55μg.100g^{-1}. The term folate encompasses a wide range of related compounds. The project developed a novel technique to profile these folates. The method was able to differentiate and quantify around 20 individual folates in *Arabidopsis* leaf samples. When applied to folates from wheat seed, it proved possible to separate and quantify several mono-glutamated folates but there was no evidence for any folates with higher levels of glutamation as found in the leaf tissue. The effect of partial germination on the folate content of the wheat seed was also determined. Germination resulted in a significant increase in total folate, and in particular in the levels of 5-methyl tetrahydrofolate, but again only mono-glutamated forms were detected. An investigation into the physiological role of polyglutamation was thus carried out using *Arabidopsis.* Three isoforms of folylpolyglutamate synthase (FPGS) were identified - cytoplasmic (cFPGS), mitochondrial (mFPGS) or plastid (pFPGS). The expression of these within the plant and the effect of knocking out their activity on growth and folate metabolism was examined. Spatial expression, particularly for cFPGS and pFPGS was observed. There was minimal phenotypic response to knocking these enzymes out individually, although silencing of pFPGS appears to have the most significant impact on the accumulation of polyglutamated forms of folate.

INTRODUCTION

Folate, or vitamin B_9, cannot be synthesized by humans and animals; plant food is the main source of this essential vitamin (Scott *et al.,* 2000). Folate deficiency can have severe health consequences. There is a definite link to birth defects such as spina bifida (Brzozowska *et al.* 2004) and women are strongly recommended to take folate supplements prior to conception and during the early stages of pregnancy. However, marginal folate deficiency also results in an increased plasma homocysteine level and this is an emerging risk factor for cardiovascular vascular diseases such as heart attacks and strokes and certain cancers (Finglas *et al.,* 2003). The reference nutrient intake for folate has been set at 200μg/day in the UK but the recommended dietary allowance in the US is twice this value. The average daily intake of folate within the UK has been estimated to be between 200μg and 300μg/day dependent on age and gender but research suggests that this current intake needs to increase two to three-fold to significantly improve human health (Scott *et al.,* 2000).

There are essentially three different strategies to increase folate intake; supplementation, food fortification, and higher intake of folate-rich foods, in practice all have had limited success. In the USA, cereal-based products have been fortified with folic acid since 1998 (Gregory, 2004); nevertheless, this only increased daily intake to 350-450 μg, still below the 600μg thought to be optimal. Furthermore, the majority of the European countries (including the UK) have rejected this move although a recent draft report by the Scientific Advisory Committee on Nutrition has recommended its introduction into the UK (Mayor, 2005). There is still some concern over fortification with folic acid since this is not the "natural form" of folate and requires bioconversion following absorption. Biofortification of folate in food through genetic modification needs to be explored but is likely to meet with consumer opposition in Europe at the present time. The third strategy is to harness natural variation for enhanced folate content in crops of interest and then use conventional breeding approaches to improve current elite lines.

Folate is found in a wide variety of foods (Table 1). The national diet and nutritional survey carried out by the FSA between 2000/01showed that cereals and their

products account for around 33% of dietary folate with over half of that attributed to breakfast cereals fortified with folic acid. Vegetables account for 27% of total intake. Some vegetables, such as broccoli represent a rich source of dietary folates with levels of up to 177μg/100g (Rychlik, 2004). Other vegetables may have lower levels of folate but due to the much larger quantities consumed represent major sources of dietary folate. Thus, tomato and potato have around 20μg and 100μg /100g folate, respectively and potato provides about 10-12% of the normal dietary intake of folate in the UK.

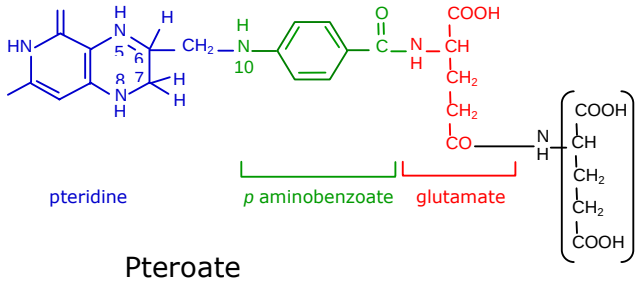
Table 1 Examples of different foods and their folate content (Holland *et al.*, 1991).

Food	**Amount (μg/100g)**
Cereals & cereal products	
Yeast extract	2620
Fortified breakfast cereals	167 - 350
Granary bread	88
Wheatgerm bread	46
Fruits	
Raspberries, raw	33
Satsumas	33
Oranges	31
Grapefruit	26
Orange juice	18
Avocado	13
Vegetables	
Asparagus, raw	175
Black-eye bean curry	140
Brussel sprouts, boiled	110
Spinach, boiled	81
Spinach, frozen, boiled	52
Cabbage, raw	75
Broccoli, boiled	64
Cauliflower, boiled	51
Peas, frozen, boiled	47
Meat & meat products	
Chicken livers, fried	1350
Lamb's kidney, fried	70
Beef mince, cooked	30
Beef sirloin steak, grilled	20

Genetic modification has already been employed to give a 25 fold increase in folate levels within tomato fruit (Diaz *et al.,* 2004) showing that the potential is present to enhance this nutrient in crops. The complex pathway leading to the synthesis of folates in plants has been elucidated over the last 10 years (Rebeille *et al.,* 2006a).

Much of the research to elucidate the pathway has been carried out in *Arabidopsis* and tomato (Rebeille *et al.,* 2006a) and this has led to the identification of many biosynthetic enzymes and their corresponding genes in these two organisms. However, important aspects remain unclear in particular the regulation of synthesis and homeostasis.

Folate is a generic term for a range of structurally related compounds that exhibit a common vitamin activity based on the parent structure of folic acid (pteroyl-L-monoglutamic acid). They basically consist of a pteroate head group linked to one or more glutamate residues through a PABA linkage (Figure 1). Variations occur in the substitutions of the pteroate head group and in the number of glutamic acid residues in the "tail". Figure 1 actually shows the tetra-reduced form of this molecule 5,6,7,8-tetrahydropteroyl-*γ*-glutamate (THF) which serves as a coenzyme in the body.



Pteroate

Figure 1 Structure of THF, showing the different moieties of this tripartite molecule (modified from Cossins & Chen, 1997).

The pteroate head group of folate can exist in a number of oxidative states and can be associated with either mono or polyglutamated chains. The polyglutamation may impact on the bioavailability of folate from the diet since folates require deglutamation to a mono glutamated form in the gut prior to uptake by the reduced folate carrier. However, the significance of this for bioavailability is unclear (Gregory *et al.,* 2005). Some studies have suggested that the bioavailability of heptaglutamated folate may be as little as 40 or 50% that of the monoglutamated equivalent (Melse-Boonstra *et al.,* 2004) whilst other studies have demonstrated no

effect (Boddie *et al.,* 2000). Thus it is important that any approach to enhance folate accumulation must also take into account the form in which the folate is present. This requires the technical ability to profile folates in plant tissues.

The *de novo* biosynthetic pathway for THF is found in organisms from different kingdoms, including fungi, prokaryota, and plantae. Due to the structural properties of this molecule, the general biosynthetic pathway is conserved among the different organisms. The biosynthesis of folate is complicated but basically occurs in the following order: first the pteridine and the *p*ABA moieties must be formed from GTP and chorismate, respectively. This is followed by their condensation, glutamylation, and reduction to produce 5,6,7,8-tetrahydropteroyl-*γ*-glutamate (THF). Addition of the polyglutamyl tail is the final step of this pathway, leading to THF-polyglutamate biosynthesis (Hanson & Gregory III, 2002).

Three compartments, namely cytoplasm, plastid and mitochondria are involved in the biosynthesis of folate in plants. Additionally, the folate salvage reactions incorporate a fourth compartment, the vacuole. In plants, five reactions are required to produce the polyglutamylated form of THF within the mitochondria. The reaction starts with the bifunctional enzyme hydroxymethyldihydropterin pyrophosphokinase/ Dihyropteroate synthase (HPPK/DHPS) phosphorylating the hydroxymethyldihydropterin, followed by its attachment to the pABA moiety, thus forming dihydropteroate (Rébeillé *et al.*, 2006b). The next step in the pathway is catalysed by the ATP-dependent dihydrofolate synthetase (DHFS) which leads to the attachment of one glutamate residue to the carboxyl moiety of pABA to form dihydrofolate ($H_2PteGlu_1$). This molecule is then reduced by the bifunctional enzyme dihydrofolate reductase/thymidylate synthase (DHFR/TS) to form THF. The monoglutamate THF molecule can then be further glutamylated through the action of folylpolyglutamate synthetase (FPGS) in the three compartments involved in THF synthesis (Rébeillé *et al.*, 2006b).

MATERIALS AND METHODS

Folate Extraction for *Arabidopsis* samples

Samples consisting of 200mg plant material were homogenised within a microfuge tube using a micro-pestle. Further homogenisation was conducted after the addition of 470μL ice-cold 95% methanol/extraction buffer [75mM KH_2PO_4, 0.4M ascorbic acid, 0.8% 2-mercaptoethanol, pH 6.0], samples were then used for either microbiological assay or folate profiling. For profiling 30μL of an internal standard mixture [0.1 mg/mL of each of methotrexate, triglutamic acid and hexaglutamic acid (1:1:1 v/v)]. The sample extracts were then centrifuged (12000g, 5 min, 4°C), and the supernatants were filtered through a 0.45 mm Whatman Vectaspin microfilter (12000g, 10 min, 4°C). They were then evaporated to dryness under nitrogen gas to approximately 20μL, and re-suspended in 200μL of extraction buffer [75mM KH_2PO_4, 52mM ascorbic acid, 0.1% 2-mercaptoethanol, pH 6.0] prior to analysis. Samples for analysis were maintained at 48°C in a HPLC autosampler before analysis by LC/MS/MS. The ability of the extraction procedure to maintain folate stability and enzyme inhibition was verified using a range of mono- and polyglutamated folate standards, both in the presence and absence of a plant-derived matrix.

Folate Extraction for wheat samples

Samples (5g) of wheat seed were homogenized using a ball mill (2 min @30f/s). Extraction buffer (EB) - 0.1M $NaPO_4$ pH 6.2- was brought to the boil and Na ascorbate (5g/L of extraction buffer) added. Then 80mL of the EB was added to the ground wheat sample and then incubated at 90°C for 10 min. This was then transferred to a 100mL volumetric flask and the volume made to 100mL by the addition of extraction buffer. Samples were then either assayed for folate content using the microbiological assay or treated as for *Arabidopsis* for folate profiling.

Microbiological assay for folate content

Preparation of media (once prepared media was autoclaved to sterilise and stored in the fridge):
Lactobacilli agar: 4.8g added to 100mL water and boiled for 2-3 min, 2mL aliquots were then dispensed into Bijou bottles and autoclaved.

Lactobacilli broth (LB): 7.6g was added to 200mL water and boiled for 2-3 min. Once cooled 10mL aliquots were dispensed into thick-walled glass test-tubes, capped with bacticaps and autoclaved.

Folic Acid Casei Medium (FACM): 23.5g was added to 500mL water and boiled for 1-2 mins, once cooled 0.5g ascorbic acid was added and the pH was adjusted to 6.2, with 1M acetic acid or NaOH. Aliquots of 10mL were placed in test-tubes with Bacticaps and 100ml aliquots were placed in 200mL screw top Duran bottles and autoclave for 5 min only at 121^{o}C.

Sub-culturing *Lactobacillus rhamnosus* NCIMB 6375: this strain belongs to ACDP Group 1, defined as 'a biological agent unlikely to cause human disease' but it should not be assumed that infection can never occur. Cultures are maintained as stabs in agar medium in the fridge and transferred to LB when required for assays and preparing fresh stabs (monthly).

Deconjugation:
Human plasma deconjugase was prepared by adding 20mL 1M L-cysteine (3.15g in 20mL water) to freeze-dried 5mL plasma (Sigma product no. P9523). Up to 9.6mL, but usually less than mL extract, is placed in a thick-walled screw top tube, 0. mL plasma solution is added and the volume made up to 10mL with fresh extraction buffer. A blank is prepared without sample. Incubate at 37^{o}C for 60 min. (maximum 2h). Heat at 90^{o}C for 20 min. After cooling transfer to a 2mL volumetric and make up to volume with fresh extraction buffer. Filter and store several aliquots in microtubes at -20^{o}C.

Assay procedure:
On the afternoon before the assay transfer bacteria from stab to LB broth and incubate overnight at 37°C. On the day of the assay stock folic acid was diluted as follows:
Solution A (200ng/mL) 100μL stock to 100mL with water
Solution B (4ng/mL) 2.0mL A to 100mL with water
Solution C (4ng/mL) 2.0mL A to 100mL with freshly prepared 1% ascorbic acid pH 6.0
Solution D, 1% ascorbic acid pH 6.0.

250μl solution B was added to a 10mL FACM tube (=I tube), 100μL LB was added and then incubated at 37°C for 6h.

A folic acid standard curve was prepared from 0 to 2000pg/mL using solution C and diluting with solution D.

Samples were diluted with solution D and 4x50μL standards and samples were transferred to a sterile microassay plate with a lid. This was done using sterile tips and in a laminar flow cabinet to keep the conditions as clean as possible.

0.5mL was transferred from I-tube to a 100ml FACM bottle, mixed and 200μL added to all wells using a multi-channel pipette with sterile tips and an autoclaved reservoir.

Plates were put into a plastic box with a sealable lid which had been lined with wetted absorbent paper. Plates were stacked on top of two unused plates to keep them off the damp paper lining the bottom of the box and a small dish of water was placed in the box to maintain humidity. The box was sealed and incubated overnight (18h).

After incubation, the plates were allowed to cool to room temperature to prevent condensation in the plate reader and were read at 630nm.

Folate Profiling

All solvents used were of HPLC grade. Mobile phases were filtered through either 0.45 mm nylon or cellulose nitrate Whatman disk filters (Maidstone, UK) prior to use. Folate standards PteGlu, H2PteGlu, H4PteGlu, 5,10-CH2-H4PteGlu, 5-CH3-H4PteGlu, 5-CHO-H4PteGlu, 10-CHO-H4PteGlu, 5,10-CH^{+}-H4PteGlu, PteGlu3, PteGlu4, PteGlu5, PteGlu6, PteGlu8, 7,8-dihydroneopterin, 6-hydroxymethyl-7,8-dihydropterin, and 7,8-dihydropteroic acid were used. The choice of standards reflects the existing knowledge of folates in plants. Examples of all monoglutamates and a selection of polyglutamates of different chain lengths were selected, which allowed the retention time of the non-standard folates to be estimated.

Standard and sample preparation

All folate standards were dissolved in methanol containing 0.1% ascorbic acid and 0.1% citric acid. Standards were then diluted in a 1:10 ratio with acetonitrile/0.1% ammonium acetate (3:1, v/v), to a final concentration of 0.1 mg/mL (pH 6.0) and were subdivided into aliquots and stored at ~80^{o}C until required.

Mass spectrometry (MS)

A Micromass Quattro Ultima triple quadrupole mass spectrometer (Waters, Manchester, UK) equipped with an ESI interface was used for all the MS experiments. The instrument parameters for negative ion ESI were optimised by direct infusion of the standards in methanol/water (22:78, v/v) containing 5mM dimethylhexylamine, at a flow rate of 200 mL/min, under conditions closely resembling the real chromatographic conditions. During optimisation the spectral peak width was set to 0.7Th FWHM for both Q1 and Q3 but increased to 1.0Th FWHM during LC/MS/MS quantitative experiments. The probe capillary was optimised at 3.2kV, and the desolvation and source temperatures were set to 500 and 125°C, respectively. The desolvation gas flow rate was set to 800 L/h, the cone gas flow rate to 160 L/h, and the nebuliser gas flow rate to maximum.

Argon was used for collisionally induced dissociation (CID) and the cell vacuum was 2.4 ~ 10~^{3} mbar. Precursor and product ions for multiple reaction monitoring (MRM), cone voltage and collision energy optimisations for each of the available standards were subsequently verified by flow injection analysis, using the QuanOptimise facility of the MassLynx operating software (Table 1). Where standards were not available, the optimal cone voltages and collision energies were predicted from those obtained from a closely related standard (Table 1). The product ion spectra for each of the standards were obtained at a higher resolution (0.7Th FWHM) for comparison with endogenous folate species. For each folate form, a second MRM was made, as identified from a previously obtained product ion spectrum, to confirm its identity.

Liquid chromatography

Liquid chromatography was performed using an Agilent 1100 series LC system, equipped with binary pump, auto-sampler, temperature control and vacuum degasser (Agilent Technologies, Waldbronn, Germany). A Luna C18(2) 150 ~ 2.0 mm column fitted with a C18 SecurityGuard guard column was used throughout (Phenomenex, Macclesfield, UK). The mobile phase consisted of (A) methanol/water (5:95, v/v) with 5mM dimethylhexylamine, pH 8.1, and (B) methanol with 5mM dimethylhexylamine, at a flow rate of 200 mL/min. A linear gradient from 22% B to 80% B over 20.5 min was followed by a 5min isocratic hold at 80% B. The column was then re-equilibrated for 12.5 min at 22% B. The injection volume was 20μL and the column was maintained at 20°C.

Calibration and validation

Eight-point calibration curves containing the following amounts of folate standards: 0, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 5.0 and 10ng, were prepared using the sample extraction procedure, for each of the standard analytes: PteGlu, H2PteGlu, H4PteGlu, 5,10-CH2-H4PteGlu, 5-CH3-H4PteGlu, 5-CHOH4PteGlu, 10-CHO-H4PteGlu, 5,10-CH^{þ}-H4PteGlu, PteGlu3, PteGlu4,

PteGlu5, PteGlu6, PteGlu8, 7,8-dihydroneopterin, 6-hydroxymethyl-7,8-dihydropterin, and 7,8-dihydropteroic acid. The ratios of the LC/MS peak areas of the analyte/internal standard were calculated and used to construct calibration lines of peak area ratio against analyte concentration using unweighted linear regression analysis. The linearity and reproducibility of calibration were assessed in eight replicate analyses. Analyte recovery was determined by comparing extracted standards with non-extracted standards at 400pg, 2.0ng and 20ng injected (n ¼ 8). Samples prepared at the same fixed concentrations were used to determine intra-day (five replicates analysis) and inter-day (20 replicates conducted over four separate runs) precision and accuracy. The lower limit of quantification for each analyte was determined as the lowest concentration of the analyte to give a relative standard deviation (RSD) of 20% or less for intra-day precision and accuracy.

Quantification

Folates for which authentic standards were available were quantified in plant extracts using an internal standard method with appropriate duplicate calibration standards for each analytical run. Three internal standards were used: methotrexate for monoglutamyl folates, triglutamic acid for folates with 2,4-conjugated glutamates, and hexaglutamic acid for folates with five or more conjugated glutamates. Folates for which authentic standards were unavailable could not be quantified directly. In this case a semi-quantitative approach was used, in which a peak area ratio for the folate was calculated using the most structurally appropriate internal standard. The concentration was then determined using the calibration line of the folate with the closest level of polyglutamation. The structural identities of folates extracted from plant material were confirmed by comparison with authentic standards where possible, using HPLC retention time, diagnostic precursor and productions, and full-scan mass spectra by ESI-MS.

Preparation of β-Glucuronidase (GUS) fusions

The promoter region from -2Kb relative to the initiation of the ATG codon was amplified from Col-0 genomic DNA via PCR. The restriction sites incorporated into the primers were used to digest the amplified promoter fragments and the Gateway entry vector pENTR11 (GATEWAY, Invitrogen; UK). The promoter was then ligated with the vector, and used to transform DH5α competent cells. Following confirmation of cloning through PCR analysis of 5 colonies with the pENTR11 forward, and reverse gene primers, one positive colony was used to set up an overnight LB and Kanamycin (50 μg/μl w/v) culture in a shaker incubator at 37°C. The plasmid DNA was isolated from cultures the following day, using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich Ltd., Dorset, UK). An LR reaction was set up to introduce the promoter into the expression vector pGWB3 following GATEWAY manual instructions (GATEWAY, Invitrogen; UK). This allows the promoter to be inserted upstream the uidA gene in this promoterless vector. The construct was used to transform Columbia plants. Homozygous single copy transgenic plants were characterized from the T_3 population by analysis of offspring segregation. Multiple plants homozygous for the transgene were used for GUS activity assays.

Transformation of *Arabidopsis*

The plant transformation was carried out using the floral dip method of Clough and Bent (Clough & Bent, 1998). This protocol requires the growth of 3 healthy *Arabidopsis* plants, grown in the greenhouse in 8cm diameter pots, until the immature flower bud clusters can be seen. To encourage proliferation of secondary buds, the initial bolted stems were clipped. For each construct, a starter culture was initiated by inoculating 5ml of LB, supplemented with the appropriate antibiotic(s) and 25 μg mL^{-1} (w/v) Rifampicin, with a single colony of *Agrobacterium*, and was grown for 24 hours at 25-28°C in a shaker incubator. 1 mL of this starter culture was used to inoculate 100mL of liquid LB culture containing the appropriate antibiotics. The culture was incubated (as above) until the OD_{600} was 0.8 to 1. *Agrobacterium* cells were then spun down for 20 min at 5500g in the centrifuge, followed by re-suspension in infiltration medium

comprising 100mL LB, 0.5% w/v Sucrose, and 0.05% v/v Silwet L77 (OSi Specialities, Inc., Danbury, CT, USA).

For floral dip, the inoculum was added to a beaker, and the plants were inverted into this suspension such that all above-ground tissues were submerged. Plants were then removed after 5-20 seconds of gentle agitation, and they were placed in plastic trays, protected by plastic covers (sleeves).

The plants were grown for 3-5 weeks, until the siliques became brown and dry. Seeds were then harvested, and used in the screening phase.

β-Glucuronidase staining and seedling clarification

Histological analysis of GUS enzyme activity in *Arabidopsis* tissue was carried out with modifications from Willemsen *et al*. (1998). The seedlings were incubated for 2-16 hours at 37°C in 0.5 mg ml^{-1} (w/v) X-gluc (Biosynth AG) dissolved in n-dimethyl-formamide, 0.1% Triton X-100, 0.5 mM $K_4Fe(CN)_6.H_2O$, 0.5 mM $K_3Fe(CN)_6$, and 50 mM sodium phosphate buffer, pH 7.2. Following staining, the seedlings were clarified as described by Malamy and Benfey (1997). The seedlings were placed in a petri dish containing acidified MeOH (0.24 N HCL in 20% methanol) for 20 mins at 55°C. This solution was replaced with 7% NaOH, in 60% ethanol for 15 mins at room temperature. The seedlings were rehydrated for 10 mins each in 40%, 20% and 10% ethanol. Seedlings were mounted in 50% glycerol on glass microscope slides.

RESULTS

Folate levels are likely to be subject to natural variation and to test this hypothesis folate was measured in a number of *Arabidopsis* recombinant inbred lines (RILs). These had been generated by crossing two landraces of *Arabidopsis* namely Lansberg (Ler) and Columbia (Col). The results are shown in Figure 2.

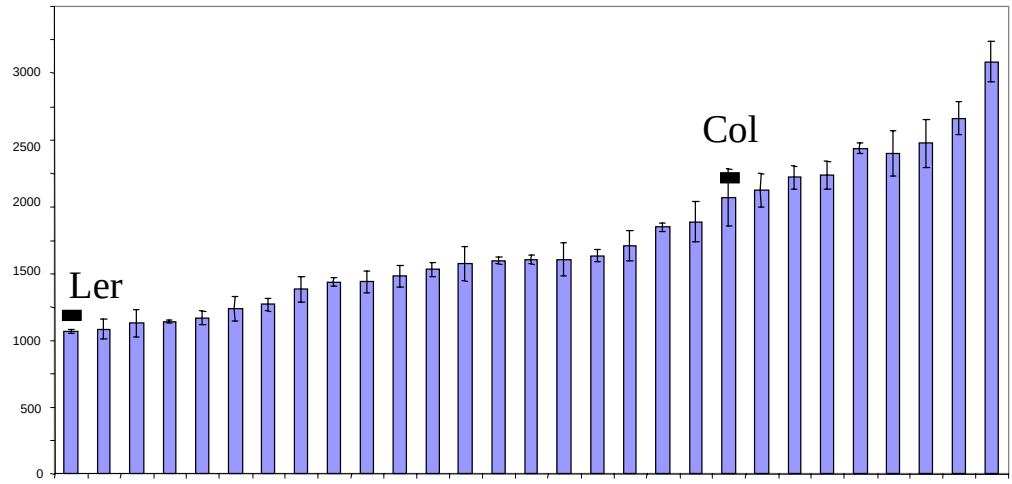


Figure 2 Folate levels in Arabidopsis recombinant inbred lines.

The Lansberg parent showed the lowest folate level whilst that for the Columbia was almost twice as high. As expected the progeny from the crosses showed a range of folate levels and exhibited a three fold variation from the lowest to highest accumulators. This demonstrates that natural variation in folate level can be quite significant and present a potential approach to manipulate the levels of this nutrient in crops.

In order to assess whether there was a similar natural variation in wheat seven elite lines were examined. Whole grains were collected from each of these lines, total folate was extracted and then measured using the microbiological assay. The results are shown in Figure 3. The folate in these seven lines ranged between 40 and 55µg.100g^{-1}. It can be seen that in this case there was minimal natural variation shown in the level of folate. Literature values for folate in whole grain wheat flour are limited but range from about 29 to 60µg.100g^{-1}. However, these have been estimated using a variety of techniques, which have been shown to introduce a two fold variation in detected levels. Taking the results here for the seven lines and the literature values it would seem that there may be limited value in trying to utilise natural variation for the enhancement of folate in wheat.

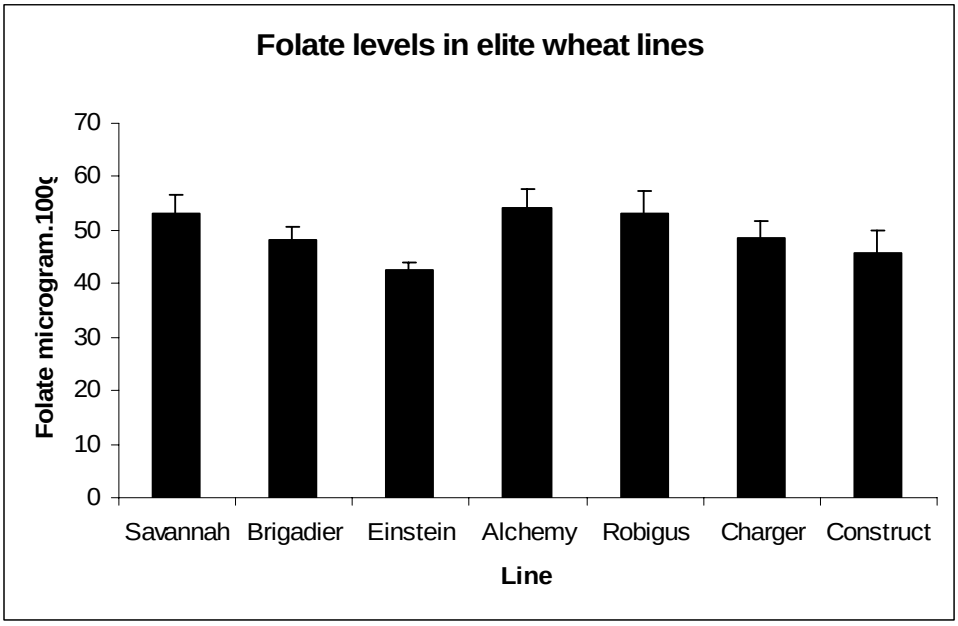


Figure 3 Folate levels in seven elite wheat lines.

Total folate is only one potential parameter of "quality" in terms of the nutritional value of a crop. Folate occurs in many forms most notably there is a variation in the number of residues associated with the polyglutamate chain. A part of this project was thus to develop an analytical technique to monitor the various folates present in wheat. The methodology involved the analysis of folates using LC-MS and was developed in the first instance using spinach as this contains very high levels of total folate. A typical result for folate distribution in spinach leaves is shown in Figure 3.

The columns in the table in Figure 3 represent the various pteroate head groups that can be found in folate. The rows represent the number of glutamine residues that are associated with that particular head group. The figures represent the percentage contribution of each of the folate moieties to the total. Thus the figure highlighted in red represents the major folate, this having a 5-methyl tetrahydro pteroate head group and a single glutamine residue and representing 36.8% of the total folate. The figures highlighted in orange represent the other "major" contributors to the folate pool. It can be seen from Figure 3 that this technique could detect a total of 32 different forms of folate but that only 10 of these contributed more than 1% to the total folate pool. The

graph in Figure 4 shows the distribution of folates in terms of the length of their polyglutamate chain. Single residues predominate but there was also a significant proportion of folates with 5,6 or 7 glutamate residues.

number of glutamates	formyl THF	5-methyl THF	5,10 methylene THF	5,10 methenyl THF	tetrahydrofolic acid	dihydrofolic acid	Folic acid	% of total folate pool
1	2.5	38.8	0.5			8.7	0.4	**50.9**
2	1.4		0.5			1.9		**3.7**
3	0.9	0.4	1.4	1.2		0.3		**4.1**
4	0.8	0.7		1.9			0.4	**3.8**
5	3.0	5.4	3.8					**12.1**
6		2.0	8.5		3.3	0.6	1.0	**15.4**
7		5.3	1.7					**7.0**
8	0.7	0.3	0.8	1.0			0.1	**2.9**
%	**9.3**	**52.8**	**17.1**	**4.1**	**3.3**	**11.5**	**1.9**	

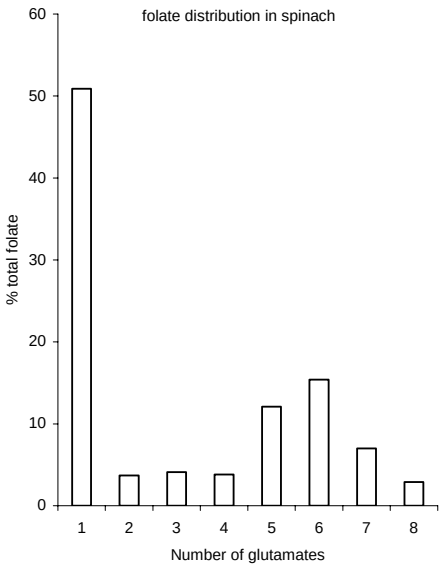


Figure 4 Folate profile from spinach leaves.

Preliminary studies with extracts from wheat grains only detected folate moieties containing a single glutamate residue. To test whether this was a complication brought about by the lower levels of folate recoverable from wheat, in comparison to spinach, folate profiles from *Arabidopsis* leaves was also carried out. The results are shown in Table 2.

Table 2 Distribution of folates in *Arabidopsis* leaves.

Glutamates	**1**	**2**	**5**	**6**	**7**	**8**
Tetra hydro folic acid	5.9			12.7		
5-methyl THF	16.6	0.7	2.7			
5-formyl THF	3.4	7.8		0.3		
5,10 methylene THF	1.2					0.4
5,10 methenyl THF	27.1					1.3
10 Formyl THF	2.1	7.6			0.5	

It can be seen from Table 2 that whilst monoglutamated folates predominated in the *Arabidopsis* profile it was none the less possible to detect a considerable amount of polyglutamated forms as well.

It was hypothesised that germination of the wheat seed would increase extractable folate levels and that this would lead to an alteration in the folate profile. To test this wheat seed were imbibed and allowed to germinate. Samples were collected before imbibing and at two stages of germination. Stage 1 was selected as the stage at radical emergence was first evident (early stages of germination) and stage 2 when the shoot became apparent (advanced stage of germination). These stages are illustrated in Figure 5.

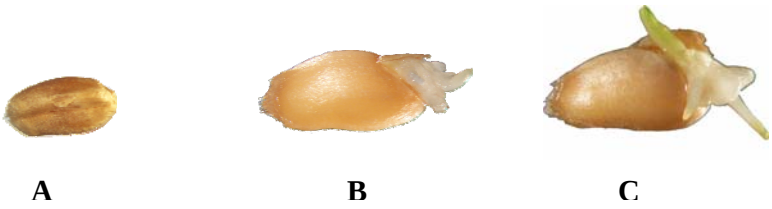


Figure 5 Stages of germination for analysis of foalte in wheat. (A) Unimbibed; (B) Stage 1 early germination: (C) Stage 2 advanced germination.

Wheat grains were selected at the three stages of germination as depicted in Figure 5, total folate was extracted and the profile determined using LC-MS. The results are shown in Figure 6.

From Figure 6 it was evident that as expected the total level of folate increased as the wheat seed germinated. It was also evident that the majority of this increase was associated with an increase in the level of the folate moiety containing a 5-methyl tetrahydrofolate head group. Also evident was the fact that as with the preliminary studies only folate moieties with a single glutamate residue could be detected. Only four of the possible pteroate head groups were detected in this analysis. The 5-methyl THF was predominant in germinating seedlings and this is consistent with the profiles from both spinach and *Arabidopsis*. The other three pteroate groups detected in wheat actually represent minor components in both spinach and *Arabidopsis* perhaps highlighting species differences in these profiles. Of particular interest was the relatively high proportion of folic acid detected in this case.

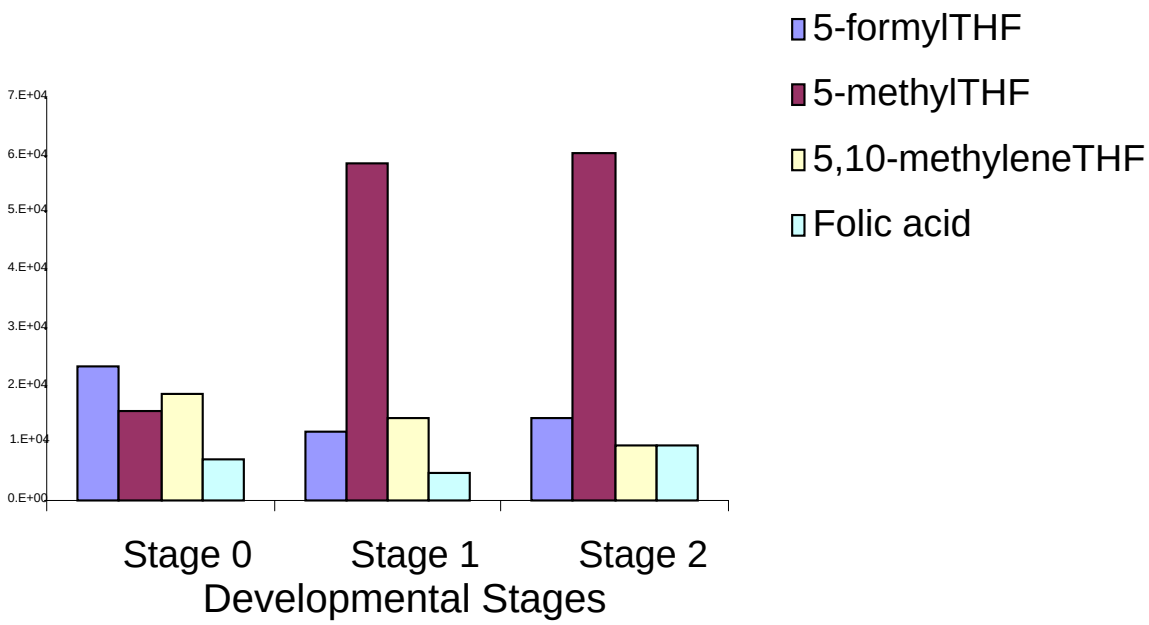


Figure 6 Folate profile from wheat grains at different stages of germination. All values are for monoglutamated forms.

The inability to detect folate moieties with more that one glutamate residue in the wheat grains may represent a technical difficulty associated with this tissue. The apparent lack, of polyglutamated forms of folate in wheat may impact on its nutritional value. The bioavailability of the folate may actually be enhanced by it being predominantly in the monoglutamated form as this may make absorption across the intestinal wall more efficient. However, it may also impact negatively on the stability of the folate during the storage and processing of the wheat grain. This possibility was illustrated by an experiment carried out to look at the stability of folates during cooking of broccoli. In this experiment the folate profile of broccoli florets was determined before and after boiling for 10 minutes. Retention of folate during boiling was found to depend on both the nature of the pteroate head group and the degree of polyglutamation. Folates with the 5-methyl THF head group and longer chain glutamates showed the greatest retention during processing.

Following on from these results the factors effecting polyglutamation in plant tissues was investigated. This employed the use of the model plat *Arabidopsis*

since this is amenable to rapid genetic modification and analysis. A key process influencing the degree of polyglutamation is the synthesis of polyglutaqmated folates - catalysed by folylpolyglutamate synthases (FPGS). This enzyme was selected for further investigation.

Whilst previous studies have identified three *A. thaliana* FPGS isoforms involved in the polyglutamylation process (Ravanel *et al.*, 2001), the unique tri-compartmentalised nature of this enzyme and its effects on the organellar, cellular and whole plant folate profiles remain largely unknown. The three isoforms encode cytoplasmic (cFPGS), mitochondrial (mFPGS) or chloroplastic, or plastid (pFPGS) forms of the enzyme. These were all isolated and their expression within the plant examined by the construction of three separate FPGS promoter:GUS fusion expressing lines. The expression of the FPGS can be easily visualised by the fact that tissues in which expression is occurring are blue when exposed to the substrate for the GUS enzyme. The results for the three FPGS isoforms are shown in Figures 7 to 9.

The results reveal meristematic pFPGS, and vegetative cFPGS expression whilst mFPGS isoform appears to be redundantly expressed in both tissues. Insertion knockout lines (KO) were isolated for each of the 3 FPGS isoforms. These are plants in which the relevant gene has been inactivated. The plants were grown in the glasshouse and it was found that the pFPGS KO mutant exhibited a conditional root growth defect, anthocyanin accumulation, and anchor root emergence. The effect of silencing these three FPGS enzymes on folate metabolism was investigated by carrying out a folate analysis on wild type and mutant plants. The results of the folate profile are shown in Figure 10.

This folate profiling revealed a range of polyglutamylation capacity in the FPGS KOs. cFPGS and mFPGS KOs showed reduced polyglutamylation, while pFPGS KO alleles lacked polyglutamylation of 5-CH_3-THF.

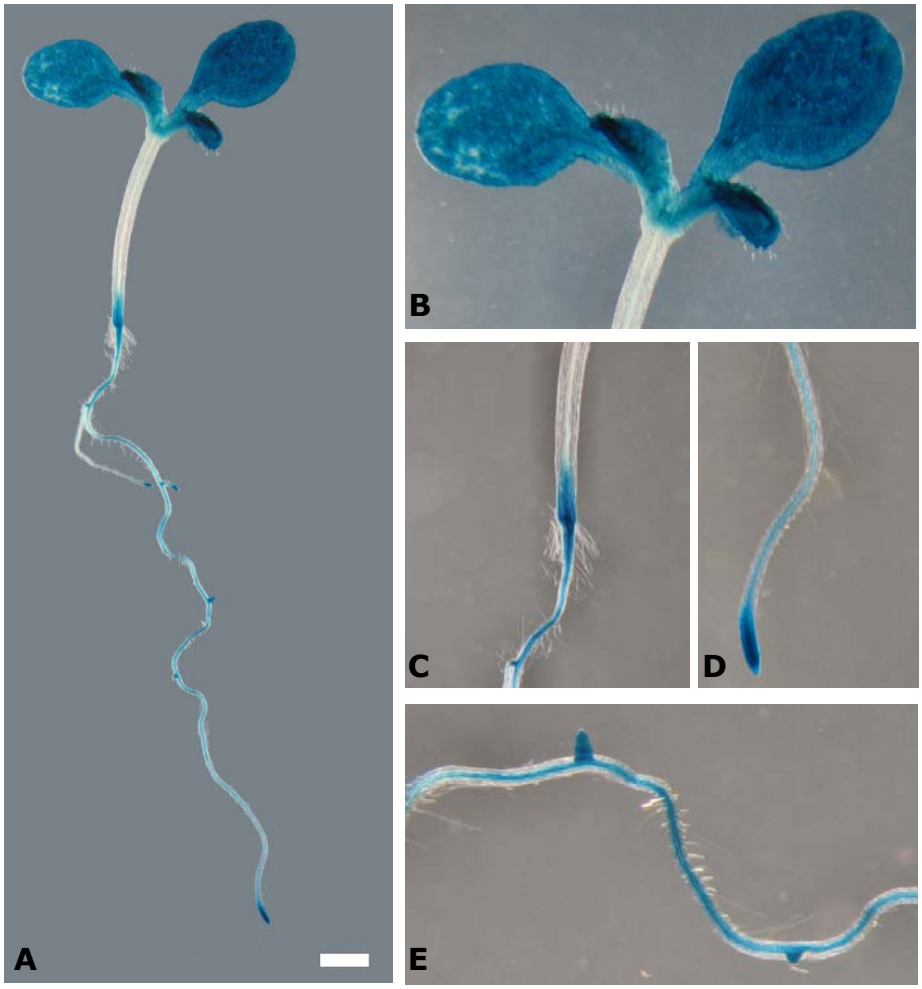


Figure 7 GUS staining of mFPGS promoter:GUS line. GUS expression in 8 days old seedling is seen in panel A following 6 hours of histochemical staining (scale: 1mm), with panel B-E showing sites of intense GUS staining.

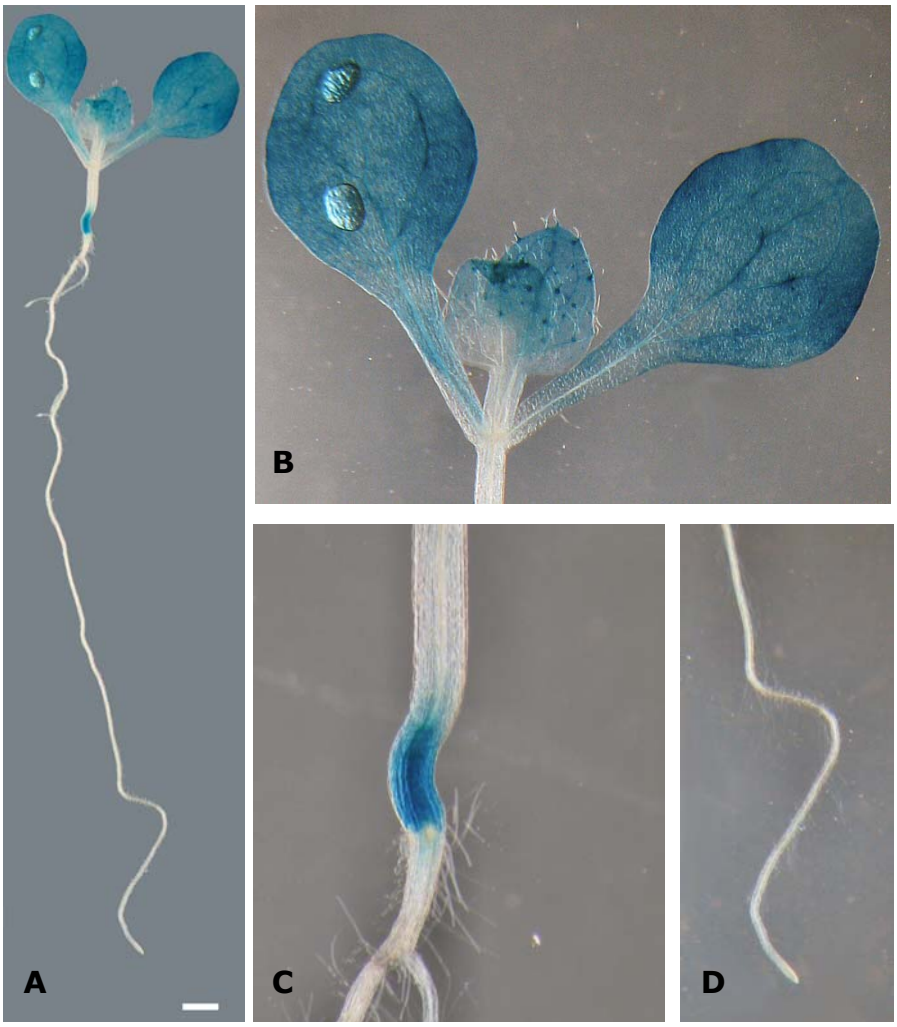


Figure 8 GUS staining of cFPGS promoter:GUS line. GUS expression in 8 days old seedling is seen in panel A following 6 hours of histochemical staining (scale: 1mm), with panel B and C showing sites of intense GUS staining. Panel D highlights the lack of GUS expression in the root.

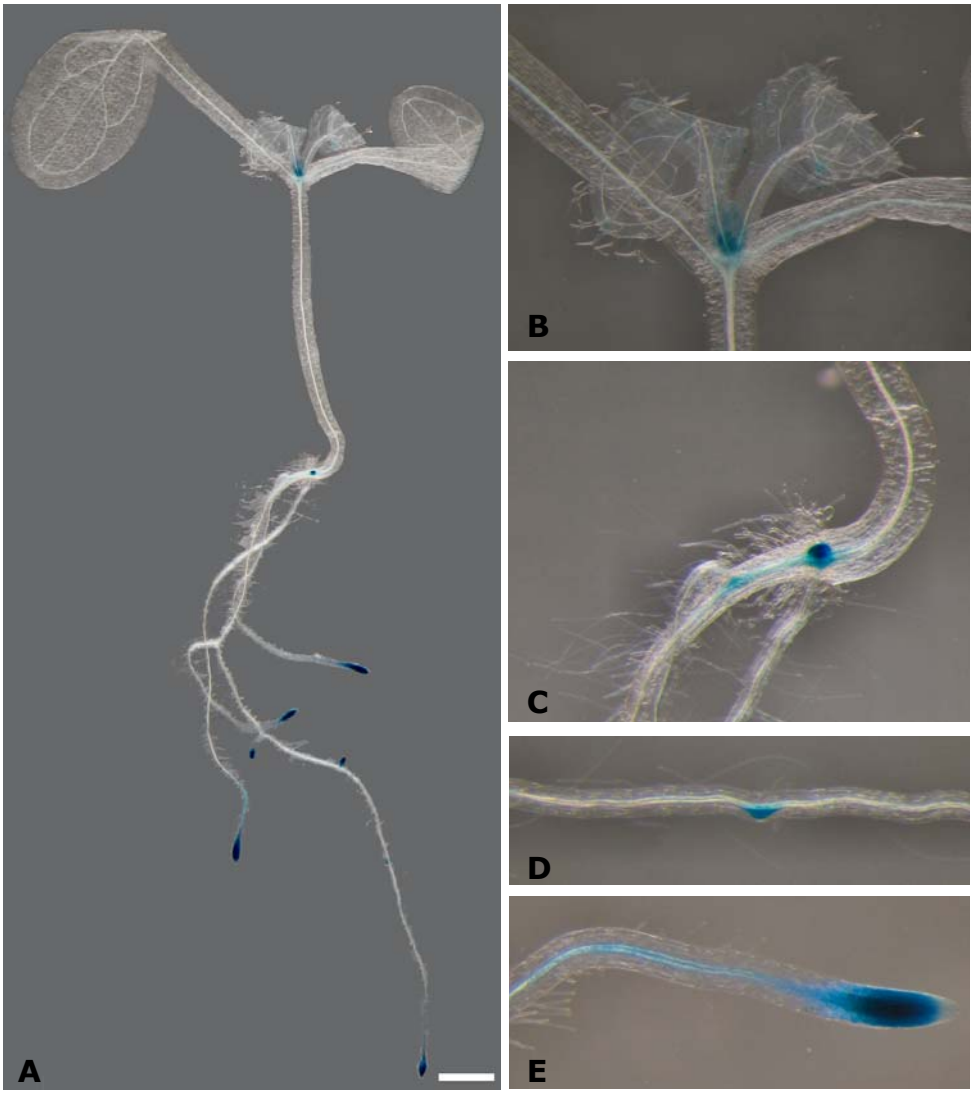


Figure 9 GUS staining of pFPGS promoter:GUS line. GUS expression in 8 days old seedling is seen in panel A following 22 hours of histochemical staining (scale: 1mm), with panel B-E showing sites of intense GUS staining.

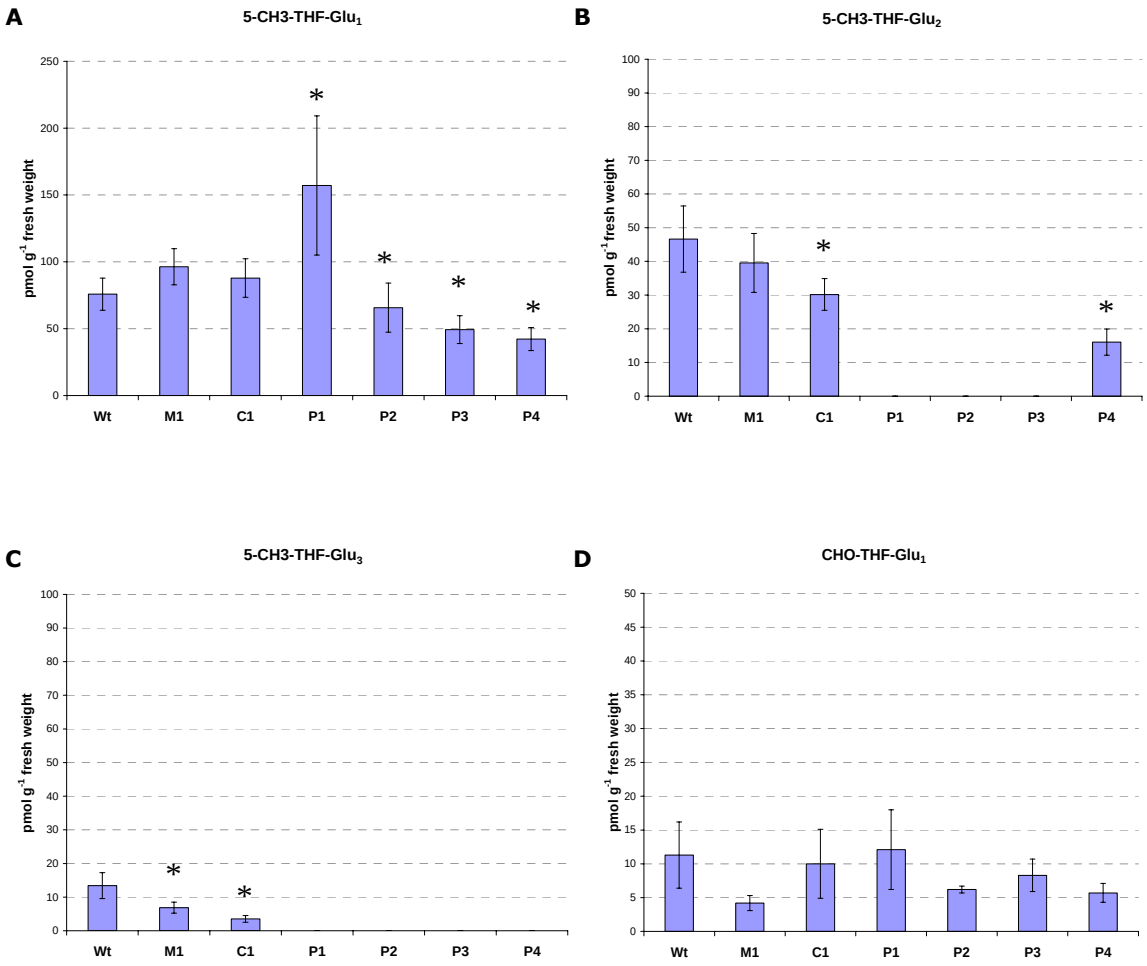


Figure 10 Folate profile of FPGS KO mutants and wild type (Col) rosette leaves using LC-MS/MS. 5-CH_3-THF and (5&10)-CHO-THF levels (pmol g^{-1} fresh weight) in rosette leaves of *Arabidopsis* wild type (Col-0), and FPGS KO mutants mFPGS (M1), cFPGS (C1), and pFPGS (P1, P2, P3, and P4) ±SEM. A, B, C mono, di, and tri glutamate 5-CH_3-THF levels. D Monoglutamate CHO-THF levels. *-Samples which have a significantly reduced concentration compare to wild type ($P<0.05$).

CONCLUSIONS

Natural variation in folate was detected within a population of Arabidopsis recombinant inbred lines. This could be interpreted to show that exploitation of natural variation may be a feasible approach to the enhancement of folate levels in crops. However, when the range of elite wheat lines was investigated there was much less variation in total folate. The levels measured were high when compared to those often quoted for wheat products and as such may reflect the

situation that breeding has in some way already selected for high folate level in these elite lines.

The effect of partial germination on the folate levels was examined and this was indeed shown to enhance the levels significantly. This may present a potential tool to further enhance the folate in these lines during processing.
Using methodology, developed in this project, to profile the folates from wheat seeds has shown that whilst a range of mono-glutamated folates can be detected there was little evidence for any polyglutamated forms as found in leaf tissue. This may reflect different physiological roles for the various folates in different tissues. This observation prompted an examination of the major enzyme responsible for polyglutamation – folylpolygltamate synthase (FPGS). It was found that the isoforms of this enzyme are indeed differentially expressed in tissues of *Arabidopsis* and that silencing of individual isoforms has little phenotypic impact. Interestingly, it seems that the chlorplastic or plastid localised enzyme has the greatest impact on polyglutamation.

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