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Genes of folate biosynthesis in wheat☆

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ABSTRACT

Micronutrient deficiency is preventing an estimated one-third of the world's population from reaching their physical and intellectual potential. This results in reduced education attainment and work productivity which negatively impacts on regional development and economies. Contributing to the aetiology of these deficiencies is the over-refining and over consumption of basic food groups (cereals and tubers) which provide limited amounts of micronutrients. Folate deficiencies result from unbalanced diets and are responsible for an estimated 250,000 birth defects annually. Engineering plants like cereals to deliver daily requirements of folates (biofortification) is an attractive approach to address malnutrition in developing countries. We have isolated key folate genes including: 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase/7,8-dihydropteroate synthase, 4-amino-4-deoxychorismate synthase and folypolyglutamate synthetase from wheat seeds. Furthermore, we have identified homologous genes in the rice genome. Characterisation of sequences identified key functional and targeting regions. Analysis of the temporal and spatial patterns of gene expression supports *de novo* synthesis of folates in the developing wheat seed and uniquely in the mature seed transcriptome. The seed appears to have a unique potential to replenish its own pool of required glutamated folates at all stages in its life cycle.

1. Introduction

Folate [tetrahydrofolate (H_4F)] and its glutamated derivatives are classified as water soluble B group vitamins. These compounds are essential cofactors for the transfer of C1 units in one-carbon metabolism, the synthesis of purine nucleotides, amino acids and pantothenic acid (Blakely and Benkovic, 1984; Cossins, 1987). Folate derived methyl groups are supplied in the form of *S*-adenosylmethionine to a broad spectrum of methylation reactions through the action of methyltransferases (Scott, 1999).

The maintenance of cellular activities in all organisms requires the continual availability of folates. An incomplete biosynthesis pathway in animals has resulted in a dependence on dietary folate sources. A folate deficiency in human nutrition manifests itself in a number of disease states and potentially fatal disorders (Bottiglieri, 1996; Mason and Levesque, 1996; Scott, 1997). Normal neural function and development appear to be most affected by folate deficiencies and extend from dementia and Alzheimer's disease to congenital deformities like spina bifida and anencephaly (Clarke et al., 1998; Daley et al., 1995; Seshadri et al., 2002).

A balanced human diet requires the delivery of adequate levels of folates through high folate foods (fruits and vegetables). Although this is achievable in developed nations, global deficiencies are prevalent, primarily in diets where cereals and tubers provide the staple energy source. To alleviate deficiencies in the general population, many western countries have instigated fortification programs; however, these are neither practical nor achievable in poorer developing nations – often where the need is greatest. Efforts to enhance folate levels through biofortification in food crops may have greater impact at a global level.

Folate is a tripartite molecule comprised of *p*-aminobenzoate (pABA) condensed with a pterin ring and glutamate moieties. Furthermore, biosynthesis in plants is uniquely divided into three subcellular compartments. Pteridines are supplied through the catalytic action of GTP cyclohydrolase 1 (GCH1, EC 3.5.4.16) in the cytosol and is the first committed step in folate synthesis (Hossain et al., 2004; Schoedon et al., 1992; Yoneyama et al., 2001). The pABA moiety is synthesised in plastids from chorismate in a two step process initiated by the enzyme aminodeoxychorismate synthase (ADCS, EC 6.3.5.8) (Basset et al., 2004a). The pABA and pteridine moieties are condensed in the mitochondria through the catalytic action of 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK, EC 2.7.6.3) and 7,8-dihydropteroate synthase (DHPS, EC 2.5.1.15) both of which are unique to plants and microorganisms, allowing them to synthesise folates *de novo* (Rébeillé et al., 1997).

Abbreviations: dpa, days post anthesis; GTP, guanosine triphosphate; QRT-PCR, quantitative reverse transcription PCR; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription PCR.

☆ The sequences reported in this paper have been deposited in the GenBank database (accession numbers. EF208803, EF208804, EF208805).

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Folypolyglutamate synthetase (FPGS, EC 6.3.2.17) sequentially adds glutamate residues to form polyglutamate tetrahydrofolate ($H_4F\text{-}Glu_n$) in the final biosynthetic step (Ravanel et al., 2001).

The first biofortification programs in *Arabidopsis* and tomatoes found over-expression of GCH1 mRNA delivered up to 4-fold gains in total folates (Hossain et al., 2004; Diaz de la Garza et al., 2004). However, a depleted pABA pool in these transgenic plants identified ADCS as a second target for engineering. Transgenic tomato lines over-expressing both GCH1 and ADCS significantly elevated the endogenous folate levels up to 25-fold in ripening tomatoes (Diaz de la Garza et al., 2007). The accumulation of pteridine and pABA in these transgenic lines identified constraints in the folate pathway possibly at the point where the two moieties are condensed. Furthermore, the level of polyglutamylation was decreased when compared to control plant folates. These results suggest that HPPK, DHPS and FPGS may also be targeted to alleviate constraints on the folate pathway allowing for greater elevation of folate level with a high degree of polyglutamylation.

The high level of global consumption, post-harvest stability and an endogenous folate pathway make cereals an attractive target for improved folate nutrition via biofortification. Our strategy to elevate folates in cereals firstly relies upon an active folate pathway in the cereal seed and secondly through the over-expression of key folate biosynthesis genes. We first investigated folate biosynthesis in cereals using developing wheat seeds as our model system and identified transcripts for GCH1, suggesting the presence of an active biosynthesis pathway (McIntosh et al., 2008). In this study we have provided further evidence for the existence of a complete, active folate biosynthesis pathway in wheat seeds and have isolated cDNAs encoding ADCS, HPPK, DHPS and FPGS during developmental stages post anthesis. Identification of the amino acid sequences contributing to catalytic activity and the temporal and spatial gene expression profiles will assist metabolic engineering programs in cereals.

2. Experimental

2.1. Plant material

The Australian spring wheat (*Triticum aestivum*) variety 'Banks' was grown in a controlled plant growth cabinet (Phoenix Biosystems, Australia). The conditions for growth were altered at 3 week intervals to mimic field conditions. Conditions of growth ranged from 10 to 16 h of daylight and temperatures from 15 °C (dark) to 23 °C (light). Plants were monitored daily during flowering to identify the point of anthesis. Seed material was collected from the middle region of each ear and from the same tiller of each plant at 8 days post anthesis (dpa), 14 dpa, 16 dpa, 20 dpa, 30 dpa and 40–50 dpa (mature/dry). Samples were derived from pools of between 6 and 10 plants. Seeds were dissected into three tissue fractions including the embryo, endosperm and maternal layers (bran and aleurone).

2.2. RNA extraction

Seeds were surface sterilized prior to RNA extraction by shaking in 10% NaOCl for 10 min followed by a 70% ethanol wash and finally rinsed four times in DNase/RNase free water. For each developmental time point, 400 mg of seed was extracted first with TRIzol® reagent (Invitrogen, Carlsbad, CA.) according to the manufacturer's instructions. Following TRIzol extraction and ethanol precipitation, total RNA was purified with the Qiagen RNeasy Midi Kit (cat. no. 75142) according to the manufacturer's instructions. Total RNA quantity and quality were assessed by gel electrophoresis and absorbance at 260 nm. DNase treatment of total RNA prior to cDNA synthesis was achieved by incubation of 1 U of DNase (Roche)/μg RNA in 1/10th volume of 10× DNase I reaction buffer (Invitrogen) for 30 min at 25 °C.

2.3. First strand cDNA synthesis

First strand cDNA was synthesised using the Transcriptor™ First Strand cDNA synthesis Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Reactions contained 0.5–1 μg of total RNA, 10 U of Transcriptor™ reverse transcriptase and 50 pmol anchored-oligo$(dT)_{18}$ primer. Subsequent PCR amplification used between 1 μl and 5 μl of the first strand cDNA per 50 μl reaction.

2.4. Reverse transcription-PCR

All RT-PCRs were performed using the BD Advantage™ 2 PCR Enzyme System (BD Biosciences, Palo Alto, CA) in accordance with the manufacturer's instructions. Typical reactions contained 10 μM each primer and 2 μl of cDNA per 25 μl reaction volume. Thermal cycling parameters were as follows: 94 °C, 3 min for 1 cycle; 94 °C, 30 s; 50–60 °C, 35 s; 72 °C, 60–180 s for 30 cycles. RT-PCR primer sets are presented in Table 1. cDNA synthesis in the absence of reverse transcriptase served as a control for genomic DNA contamination in cDNA preparations.

2.5. 5′ and 3′ RACE

RACE (rapid amplification of cDNA ends) was utilised to amplify the 3′ and 5′ ends of the mRNA using the BD SMART™ RACE cDNA Amplification Kit (BD Biosciences, Palo Alto, CA). Amplification of the 5′ and 3′ ends employed a universal primer (UPM; Universal Primer A Mix, BD Biosciences) and a gene specific primer in both the antisense and sense orientation for 5′ and 3′ RACE respectively. The following cycling parameters were used: 94 °C, 30 s; 72 °C 3 min for 5 cycles; 94 °C, 30 s, 70 °C, 30 s; 72 °C, 3 min for 5 cycles; 94 °C, 30 s; 68 °C, 30 s; 72 °C, 3 min for 25 cycles.

2.6. Quantitative RT-PCR

Templates for each of the replicate QRT-PCRs were derived from three independent RNA extractions and cDNA synthesis reactions. All cDNA was amplified from 1 μg of total RNA. Each PCR contained: 0.2 μM each primer, 12.5 μl of SYBR® GreenER™ (Invitrogen; Carlsbad, CA), 1.0 μl of cDNA and water to 25 μl. Cycle parameters were as follows: 50 °C, 2 min for 1 cycle; 95 °C, 10 min for 1 cycle; 94 °C, 15 s; 54 °C, 15 s; 72 °C, 30 s for 40 cycles. All QRT-PCR primers were designed with Beacon Designer 4.0 software (PREMIER Biosoft International Palo Alto, CA) using default SYBR Green parameters.

Table 1
PCR primer pairs used to amplify full length cDNAs

Target gene	Primer sequence 5′–3′
ADCS	Sense: AAGATGGCCGCGCTCCGCC
	Antisense: TTCTAACTTATAGTCGTCCGCAC
HPPK/DHPS	Sense: ATGCTCCATGCTAAGGAGACACT
	Antisense: GTGTACCTTTTCATTTGACCAT
FPGS	Sense: ACGCGGGGGGCCGCGCGCCG
	Antisense: GTTCCCAAAAAATGTCACTTCT
β-Actin[a]	Sense: ATGGAAGCTGCTGGAATCCAT
	antisense-CCTTGCTCATACGGTCAGCAATAC

[a] Primer pair amplifies truncated fragment.