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Determination of Folate Concentrations in Diverse Potato Germplasm Using a Trienzyme Extraction and a Microbiological Assay

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Folate deficiency is a leading cause of birth defects and is implicated in several other diseases. We are interested in how much folate concentrations vary among potato germplasm. We determined total folate concentrations of potato tubers from 67 cultivars, advanced breeding lines, or wild species. Folates were extracted by a tri-enzyme treatment and analyzed by using a *Lactobacillus rhamnosus* microbiological assay. Folate concentrations varied from 521 ± 96 to 1373 ± 230 ng/g dry weight and were genotype and location dependent. The highest folate concentrations were mostly found in color-fleshed potatoes. Variations of folate concentrations within either color- or white-fleshed tubers were similar (~2-fold). Skin contained ~30% higher folate concentrations than flesh. Storage of tubers for 7 months generally led to an increase in folate contents. Semiquantitative RT-PCR analyses showed that higher folate contents were correlated with lower mRNA expression of some folate genes.

KEYWORDS: Folate; potato; *Solanum tuberosum*; wild species

INTRODUCTION

Tetrahydrofolate (THF) and its one carbon (1C) unit derivatives commonly named folates (vitamin B9) are essential micronutrients in the human diet. Indeed, while plants and microorganisms can synthesize folates, humans lack this ability and require a dietary supply. A deficiency of folates in the diet is associated with the increased risk of neural tube defects, cardiovascular diseases, anemia, and some cancers (*1*−*3*). Unfortunately, current folate intake is suboptimal in most of the world's populations, even in developed countries (*4*). Folic acid supplements and food fortification have been used to correct dietary folate deficiency in human populations. Observational studies and intervention trials showed that supplements of folic acid as multivitamins tablets led to a dramatic decrease in neural tube defects (*5*, *6*). Controlled studies also showed that folic acid fortification of grain products has led to a significant reduction in the incidence of neural tube defects (*7*−*9*), some childhood cancers (*10*), and stroke (*11*). Although these approaches proved to be efficient, they are expensive and hard to implement in developing countries.

All of the enzymes necessary for folate biosynthesis are present in potato tubers, and tuber mitochondria were shown to contain ~200 μM folate (*12*). Tubers are metabolically active organs, so not surprisingly they are rich in mitochondria. Thus, potato both has the potential and is a logical target to be developed as a significant source of dietary folate, especially because it is also the fourth most consumed staple food in the world and the most consumed vegetable in the West. Potatoes were found to be the third most important source of folate in the Dutch diet (*13*), provided 9−12% of the total folate in a Norwegian study (*14*), 10% in Finland (*15*), and are an important source in several other European studies (*16*, *17*). Several folate studies used store-bought potatoes of unspecified genotype, and the reported folate concentrations can vary substantially (*13*). The USDA nutrient database (http://www.nal.usda.gov/fnic/foodcomp/search; SR19) reports a small portion of a russet baked potato contains about 6% of the RDA (400 μg/day for an adult).

Besides transgenic approaches, one way to increase potato folate levels would be to identify germplasm with elevated amounts. Potato has tremendous genetic diversity, among which might be high-folate lines. Any such high-folate germplasm could be useful as is, or as a source of genes to introgress into domesticated cultivars. Currently, very little is known about how much folate concentrations vary among potato lines, because only very few genotypes have ever been analyzed for folate content (*18*, *19*). There are also few such studies for other crops. One study evaluated 13 strawberry cultivars, and another evaluated the effect of cultivar and location on folate in pulses (*20*, *21*).

Other vitamins and phytochemicals are known to vary substantially among different potato lines. Even among two

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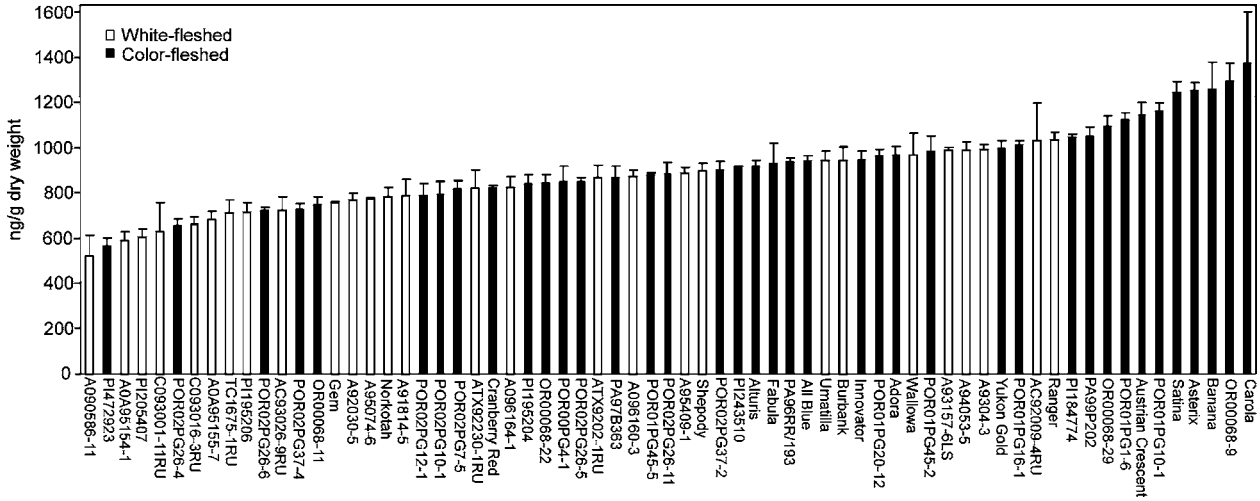

Figure 1. Total folate concentrations measured in 67 potato cultivars. Values for each cultivar are based on one harvest, except Burbank, Ranger, Norkotah, and Yukon Gold, which are means of two to four harvests. Folate extractions were made from three independent tubers or pools of tubers, and duplicate determinations were made on each extract. Data are means ± SE of all determinations.

mainstream white-fleshed varieties, substantial differences in many compounds were found (*22*), and over 30-fold variation for some compounds was found when increasingly diverse germplasm was screened (Shakya and Navarre, unpublished data). Other groups reported almost 2-fold variation of ascorbic acid content among potato germplasm (*23*). These data indicate that there is potential to use the genetic diversity of potato to increase contents of specific phytochemicals.

In this paper, we investigated variation of folate concentrations among 67 potato lines and analyzed the expression of four genes involved in folate metabolism.

MATERIALS AND METHODS

Potato Material. Tubers from commercial varieties and advanced breeding lines (see **Figure 1** for names) were obtained from fields at Hermiston, OR, Patterson, WA, Pullman, WA, and Othello, WA, during the years 2002, 2003, 2004, 2005, and 2006. Wild species (*Solanum kurtzianum*, PI472923; *S. spegazzinii*, PI205407; *S. tarijense*, PI195206; *S. stenotomum*, PI195204; *S. bulbocastanum*, PI243510; *S. pinnatisectum*, PI184774) were obtained from NSRP-6 (http://www.ars-grin.gov/) in Sturgeon Bay, WI. Within a few days after harvesting tubers, three slices (one upper third, one middle, one lower third, obtained longitudinally, and containing flesh and skin) were ground in liquid nitrogen, lyophilized, and stored at −80 °C. Tubers stored for 7 months were in cold storage at 4 °C and not treated with sprout inhibitors. Given the small size of tubers for wild species, whole tubers were used to prepare lyophilized samples. All folate measurements for a given genotype are from at least three different tubers.

Bacteria. Lyophilized cultures of *Lactobacillus rhamnosus* (ATCC 7469) were obtained from the American Type Culture Collection (Manassas, VA). Glycerol-cryoprotected cells were prepared as described previously (*24*).

Chemicals and Reagents. Folates (5-formyl-THF, 5-methyl-THF, 5,10-methenyl-THF, folic acid), pteroyltri-γ-L-glutamic acid ($PteGlu_3$), and pteroic acid ($PteGlu_1$) were from Schircks Laboratories (Jona, Switzerland). Purity was as specified by the manufacturer and ranged from 95% to 99.5%. α-Amylase (no. A6211) and protease (no. P5147) were obtained from Sigma Chemical. Rat plasma conjugase was from Pel-Freez (Rogers, AR; no. 36141-2) and was dialyzed before use as described (*25*). Difco folic acid casei medium was from Becton, Dickinson and Co. (Sparks, MD).

Folate Extraction. All folate procedures were done under low light. Folates were extracted by a tri-enzyme treatment according to the procedure in ref *25* with the following modifications. Two hundred milligrams of dry tissue was homogenized in 15-mL Eppendorf tubes containing 10 mL of extraction buffer (50 mM HEPES/50 mM CHES, pH 7.85, containing 2% (w/v) sodium ascorbate and 10 mM 2-mercaptoethanol, deoxygenated by flushing with nitrogen), boiled for 10 min, and cooled immediately in ice. The homogenate was treated with protease (1 mL at 4 mg/mL; 4.5 units per mg) for 2 h at 37 °C, boiled for 5 min, and cooled immediately in ice. The sample was then treated with α-amylase (1 mL at 20 mg/mL; 43 units per mg) and rat plasma conjugase in large excess (0.5 mL; activities from different batches were determined between 2.0 and 35.8 nmol min^{-1} mL^{-1} $PteGlu_1$ formed from $PteGlu_3$) for 4 h at 37 °C, boiled for 5 min, and cooled immediately in ice. The pH of 7.85 used is within the range of pH for optimal activities of these three enzymes. After centrifugation for 10 min at 3000*g*, the supernatant was transferred to a new tube. The residue was resuspended in 5 mL of extraction buffer and recentrifuged for 10 min. The combined supernatants were adjusted to a 20-mL final volume with extraction buffer, flushed with nitrogen, frozen in liquid nitrogen, and stored at −80 °C until analysis. Recoveries were estimated by adding a mixture of standards (5-formyl-THF, folic acid, 5-methyl-THF, 5,10-methenyl-THF, each in equal amounts) during the homogenization in Hepes/Ches buffer (300 ng of standards mixture was added per gram of dry sample). Between 98% and 106% of folate standards was recovered in extracts at the end of the assay.

Microbiological Assay. *Lactobacillus rhamnosus* (ATCC 7469) was used to determine total folate content of tri-enzyme-treated samples following the procedures described by Horne and Patterson (*24*) with the following modifications. The *L. rhamnosus* inoculum was prepared by mixing 1 mL of cryoprotected cells with 4 mL of 9 g/L NaCl, followed by centrifugation for 10 min at 3000*g*, and resuspension of the bacterial pellet in 5 mL of 9 g/L NaCl. The folic acid casei medium (double strength) was prepared as recommended by the manufacturer, with the addition of 8 g/L sodium ascorbate, and sterilized by 0.2 μm filtration. Wells of a 96-well plate (Falcon Microtiter Plates) contained 150 μL of double-strength folic acid casei medium containing either 5-formyl-THF standard (from 5 to 60 fmol of the (6*S*)-isomer in a maximum volume of 11 μL of extraction buffer) or 5 μL of potato extract at various dilutions. Sterile water was added to adjust the total volume to 300 μL. Ten microliters of bacteria was added to each well. The plate was incubated at 37 °C for ∼18 h. Bacterial growth was measured at 630 nm on a BioTek Instrument EL 311 SX microplate autoreader (BioTek Instrument, Winooski, VT) and analyzed with the KCJr EIA application software. Results were calculated by reference to a standard curve using 5-formyl-THF and were expressed as nanograms of 5-formyl-THF per gram of sample. Folate values were corrected for the endogenous folate contents in rat plasma conjugase, α-amylase, and protease.

RNA Extraction. Total RNAs were extracted from lyophilized tissues of potato tubers. Thirty-two milligrams of powdered tissue was homogenized in 0.4 mL of extraction buffer (100 mM LiCl,

Table 1. Primer Sequences Used for Semiquantitative RT-PCR, Size of Amplicons, and GenBank Accession Numbers and Organism of Sequences Used To Design Primers

primer name	primer sequence 5′→3′	amplicon size (bp)	EST or cDNA (potato cultivar or organism)
tubulin fwd	CCTACCGGACGTTACAAAGG	227	Z33382 (Record)
tubulin fev	ACCTTTCGCCCAGTTATTCC		
GCHI fwd	AAGGTGTTGTGCACATTGGT	330	CV499508 (Shepody)
GCHI rev	TCATCTTCCTGCAGAACAAG		AY069920 (tomato)
ADCS fwd	GGCTATCAAACCCATTCACA	433	CK252944 (Kennebec)
ADCS rev	TCTCAAGATCCTCATGATATC		CV503132 (Shepody)
GGH1 fwd	ATCAGATTCTTCCGCCGAG	428	CK257105 (Kennebec)
GGH1 rev	CGCTGCACTGAATTCCTCA		BI406650 (Kuras)
			BF187581 (Bintje)
GGH2 fwd	CCAATTGATGGCTACTCATGT	477	CV470185 (Shepody)
GGH2 rev	GCTTGATGCGATGCACTGAA		BG595634 (Kennebec)
			BI176848 (Bintje)

100 mM Tris-HCl pH 8.5, 10 mM EDTA, 1% SDS, 15 mM dithiothreitol) and 0.4 mL of phenol pH 4.3, and heated to 65 °C for 5 min. Chloroform:isoamylalcohol (24:1) was added, vortexed for 30 s, and centrifuged at 7500*g* for 15 min at 4 °C. The aqueous phase was transferred to a new tube and further clarified by addition of 0.4 mL of chloroform:isoamylalcohol (24:1) as previously. RNAs were precipitated by adding an equal volume of 4 M LiCl to the aqueous phase and kept overnight on ice. Total RNA was pelleted by centrifugation at 16 000*g* for 25 min at 4 °C, washed with 1 mL 70% ethanol, air-dried, and resuspended in 100 μL of DEPC-treated water.

Semiquantitative RT-PCR. First-strand cDNA was synthesized from 0.5 μg of total RNA using M-MuLV Reverse Transcriptase (New England BioLabs) and following the manufacturer's recommendations. Reaction products were stored at −20 °C. Sequences of primers used to amplify β-tubulin, GTP-cyclohydrolase I (GCHI), aminodeoxychorismate synthase (ADCS), and γ-glutamylhydrolases (GGH) are described in **Table 1**. Primers were designed to hybridize in conserved DNA regions as determined by alignment of DNA sequences from different potato cultivars available in databases (cultivars Kennebec, Bintje, Shepody, and Kuras; see **Table 1** for GenBank accession numbers). PCR reactions were carried out in a 10 μL total volume containing 25 ng of first-strand cDNAs. Conditions of amplification were as follows: 94 °C for 1 min followed by cycles at 94 °C for 10 s, 56 °C for 20 s, and 72 °C for 30−45 s. The cycle numbers were chosen so that amplifications were within the linear range.

Statistical Analyses. Statistical significances were assessed by Student *t* test for independent samples using the VassarsStats website (http://faculty.vassar.edu/lowry/VassarStats.html).

RESULTS AND DISCUSSION

Folate Concentrations in Diverse Potato Germplasm. Little is known about the extent to which folate levels vary in potato or any crop. To determine the extent that folate concentrations vary among potato germplasm, 67 different genotypes representing a wide range of germplasm and including several wild species were analyzed. These genotypes included white-, purple-, red-, blue-, and yellow-fleshed potatoes.

Folate concentrations ranged from 521 ± 96 to 1373 ± 230 ng/g dry weight (**Figure 1**), which is a 2.6-fold difference between the lowest and the highest folate concentrations. This range is considerably smaller than that found for tuber phenolic compounds (*26*, Shakya and Navarre, unpublished data), but is slightly greater than the 1.9-fold range found for folate among 13 strawberry cultivars (*20*) or ascorbate found among 33 potato lines (*23*). Among the most commonly grown white-fleshed varieties, cultivar Ranger Russet had the highest folate amounts at 1037 ± 34 ng/g dry weight. Of the five genotypes with the highest folate levels, all were color-fleshed, with four being yellow-fleshed. It is not clear why the highest folate genotypes are yellow varieties, as there is no obvious connection between carotenoid and folate biosynthesis. Furthermore, some yellow genotypes were on the low end of the concentration range, so this was not a universal trait of yellow potatoes. We also measured folate concentrations in a single accession of six wild potato species (**Figure 1**; names beginning with a PI prefix). Variation was almost as large in this small selection of wild species (1.85-fold) as it was in the much larger cultivar pool. This suggested wild species might have higher natural variation of folate than domesticated cultivars. Among the most commonly grown white-fleshed cultivars, differences (∼1.3-fold) were not as large as in diverse advanced breeding lines or the wild species.

Table 2. Folate Contents in Skin and Flesh of Two Potato Cultivars[a]

genotype	folate concentrations (ng/g DW) flesh	skin
Russett Burbank	1064 ± 35	1512 ± 104*
A93157-6LS	1026 ± 0	1430 ± 62**

[a] Asterisks indicate significant difference between flesh and skin folate concentrations as determined by Student *t* test (*, $P < 0.05$; **, $P < 0.01$).

The distribution of folates between tuber flesh and skin was determined in two varieties, and the skin had a ∼30% higher concentration (**Table 2**). However, as the periderm comprises a relatively small percentage of total tuber weight, most of a tuber's folate would be found in the flesh. The advanced breeding line OR00068-9, which had one of the highest folate concentrations among the germplasm screened, also had 1704 ± 50 ng/g dry weight folate in the skin.

Based on our results, a 175 g raw potato serving (∼6 ounces) would provide from 4.5% to 12% of the American RDA (400 μg). Because potatoes are eaten cooked, folate concentrations in potato tuber after cooking would need to be determined. Current data about the effect of cooking on folate concentrations in potatoes are inconsistent. Some studies report a nonsignificant loss after boiling (*18*), while others indicate that folate concentrations could either increase or decrease after cooking (USDA National Nutrient Database; http://www.nal.usda.gov/fnic/foodcomp/search). One study showed that potato suffered less loss of folate during boiling than leafy green vegetables (*19*). If we convert folate concentrations reported in previous studies to ng/g dry weight, assuming an average of 80% moisture content in potato tubers, our values are in general agreement with folate concentrations in raw potato reported in the literature of 600−2250 ng/g dry weight (*13*, *27*−*29*), but not with McKillop et al. (*18*) who reported a particularly elevated concentration (6250 ng/g dry weight; 175 g serving would provide 55% of the RDA). It should be noted that this latter paper also reported relatively higher folate concentrations in other vegetables such as broccoli and spinach as compared to other studies (*13*, *27*, *28*, *30*).

Environmental Effects on Folate Concentrations. One cannot preclude growing location, environmental, or harvest-related factors influencing folate concentrations in a given genotype, and this is a caveat when comparing a genotype from one location with one grown in a different location. To assess how much influence external factors might have on folate levels, four cultivars, Norkotah, Yukon Gold, Ranger, and Burbank, were harvested from different locations or years, and folate

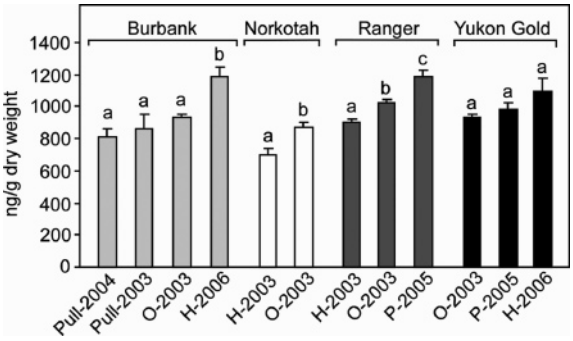

Figure 2. Total folate concentrations from four cultivars harvested at several locations and/or over several years. Folate extractions were made from three independent tubers or pools of tubers, and duplicate determinations were made on each extract. Data are means ± SE of all six determinations. Pull, Pullman, WA; O, Othello, WA; H, Hermiston, OR; P, Patterson, WA. Identical letters indicate that there was no significant difference ($P > 0.05$) as determined by Student t test.

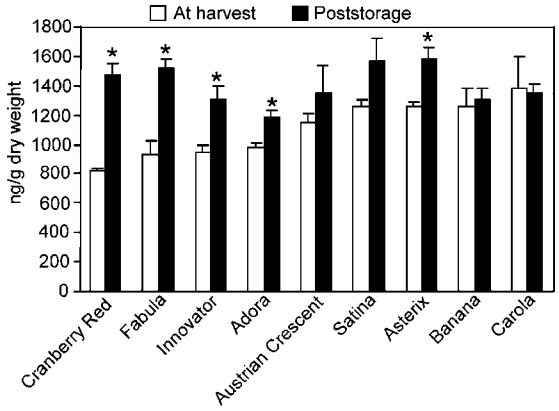

Figure 3. Effect of postharvest storage on folate concentrations. Tubers folate concentrations were determined either at harvest or after 7 months in cold storage room. Folate extractions were made from three independent tubers or pools of tubers, and duplicate determinations were made on each extract. Data are means ± SE of all six determinations. Asterisks indicate that samples were significantly different ($P < 0.05$).

levels were determined (**Figure 2**). The percentage difference between the high and low amounts found in a given cultivar planted at different locations was 20%, 15%, and 24% among Norkotah, Yukon Gold, and Ranger, respectively, while Russet Burbank had more substantial variation at 32%, ranging from 809 to 1187 ng/g DW. This suggests that environmental factors influence folate concentrations and that perhaps some varieties are more prone to variation than others. Nevertheless, these folate variations within one genotype were smaller than the variation seen among differing genotypes (521 ± 96 to 1373 ± 230 ng/g dry weight).

Postharvest Storage Affects Folate Concentrations. Potatoes are often cold stored for months after harvest. We measured folate concentrations in tubers that were stored for 7 months in cold-storage (**Figure 3**). All but one of nine cultivars analyzed had higher folate concentrations after storage as compared to concentrations at harvest. Differences between tubers measured at harvest or after storage ranged from 1.03- to 1.78-fold, and these differences were significant in five of the cultivars analyzed ($P < 0.05$). After storage, four cultivars reached folate levels substantially higher than that seen in any of the 67 genotypes in **Figure 1**. Cultivar Cranberry Red showed the biggest change, increasing from 825 ± 9 to 1473 ± 76 ng/g dry weight, an increase of 78.5%. Two cultivars (Banana and Carola) showed little or no difference. While Carola had the highest folate concentration in **Figure 1**, that concentration would only qualify as the sixth highest in **Figure 3**. This suggests that folate metabolism in varied potato genotypes during storage may be quite different. One possible explanation for these differences is that folate levels may increase as a tuber sprouts or nears sprouting, the timing of which can be quite cultivar specific. The growth taking place during sprouting may well involve increased folate synthesis. Folates are cofactors required for DNA synthesis, and folate content increases in actively dividing tissues such as root tips and germinating pea and rye (*31*, *32*). One interesting question is whether one of the higher folate genotypes shown in **Figure 1**, such as Carola, would increase if left longer in storage. For example, an increase equivalent to that seen in Cranberry Red would yield a potato with substantial amounts of folate. A detailed study of developmental and postharvest storage effects on folate content is underway.

Transcriptional Expression Levels of Some Folate Metabolism Genes. Folate is synthesized via an elaborate, well-defined pathway in plants, but it is still unknown as to why within a given plant species one variety might have more or less folates than another. Any effort to develop potatoes with higher folate concentrations would be facilitated by knowledge of the genetic basis of differing tuber folate levels and delineation of the key genes involved. Of the numerous genes involved in folate metabolism, we selected four for characterization and measured mRNA expression levels of two folate synthesis genes, GTP cyclohydrolase I (GCHI) and aminodeoxychorismate synthase (ADCS), and two γ-glutamylhydrolase (GGH) genes (*33*−*35*). Total RNA was extracted from three high-folate tubers (Asterix, Cranberry, and Fabula after 7 months in cold storage; folate concentrations of 1582, 1473, and 1515 ng/g dry weight, respectively) and three lower folate tubers (Norkotah, Russet Burbank, and Gem; 869, 804, and 759 ng/g dry weight, respectively). Semiquantitative RT-PCR analyses (**Figure 4**) showed that GCHI was expressed at the same level in all cultivars. ADCS expression was similar in all lower folate

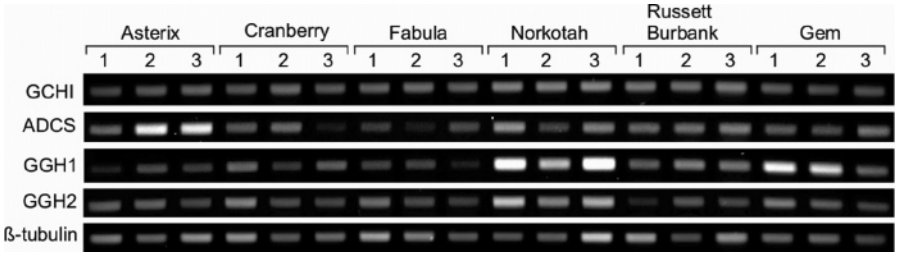

Figure 4. Analyses by semiquantitative RT-PCR of mRNA expression levels of some folate metabolism genes. For each cultivar, RNA extractions were made from three independent lyophilized potato tubers. The numbers of PCR cycles for GCHI, ADCS, GGH1, GGH2, and β-tubulin were 35, 34, 34, 36, and 31, respectively, and were in the linear range. Amplicons were run on a 1.2% agarose gel containing ethidium bromide and were visualized by UV illumination.

germplasm, but varied among "high folate" germplasm. Asterix had higher ADCS mRNA expression than all other cultivars. Interestingly, GGHs, especially GGH1, were more expressed in lower folate cultivars, suggesting that this enzyme may be one determinant of folate concentrations in potato cultivars.

One objective of this work was to determine how much folate varies among potato germplasm and whether high-folate germplasm could be identified that could be used as a source of potato genes useful to further increase folate in potatoes by breeding or transgenic methods. Identifying high and low folate varieties may prove useful toward understanding the molecular basis of folate variation in potatoes. A ~2.6-fold difference in folate concentration was found between the lowest and highest genotypes (**Figure 1**) and a ~3-fold difference if varieties that were stored for 7 months are included (**Figure 3**). Thus, potatoes and particularly the higher-folate varieties are likely a valuable source of folate in the diet, especially given the large quantities consumed per year, which approaches 140 lbs per person in the USA and is even higher in Europe. Further increasing folate in white-fleshed varieties, which are the most consumed form in the United States, could make potatoes dietary importance even more significant. This study showed that most of the commonly grown white varieties are in the mid-level range, and the difference between the highest white-fleshed variety (Ranger Russet) and highest overall (7 months Asterix; yellow flesh) was 32.8%. If the amounts found in the top varieties screened in this study represent or approach the ceiling of what potato can naturally contain, then perhaps only modest additional increases in the top white-fleshed cultivars may be obtainable through breeding. However, it is possible that crossing high-folate lines could be effective, as no breeding effort has previously been directed toward specifically increasing folate in potato. Alternatively, transgenic approaches might be a promising way to dramatically boost folate levels. Because there are many more cultivars extant than what we screened, we cannot preclude that the natural ceiling is substantially higher than what we observed. However, this study hints that there might be more variation in wild potato species and that this might be a fruitful direction to explore in a future study.

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