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Bioaccessibility of Carotenoids from Transgenic Provitamin A Biofortified Sorghum

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Supporting Information

ABSTRACT: Biofortified sorghum (*Sorghum bicolor* (L.) Moench) lines are being developed to target vitamin A deficiency in Sub-Saharan Africa, but the delivery of provitamin A carotenoids from such diverse germplasms has not been evaluated. The purpose of this study was to screen vectors and independent transgenic events for the bioaccessibility of provitamin A carotenoids using an in vitro digestion model. The germplasm background and transgenic sorghum contained 1.0−1.5 and 3.3−14.0 μg/g β-carotene equivalents on a dry weight basis (DW), respectively. Test porridges made from milled transgenic sorghum contained up to 250 μg of β-carotene equivalents per 100 g of porridge on a fresh weight basis (FW). Micellarization efficiency of *all-trans*-β-carotene was lower ($p < 0.05$) from transgenic sorghum (1−5%) than from null/nontransgenic sorghum (6−11%) but not different between vector constructs. Carotenoid bioaccessibility was significantly improved ($p < 0.05$) by increasing the amount of coformulated lipid in test porridges from 5% w/w to 10% w/w. Transgenic sorghum event Homo188-A contained the greatest bioaccessible β-carotene content, with a 4−8-fold increase from null/nontransgenic sorghum. While the bioavailability and bioconversion of provitamin A carotenoids from these grains must be confirmed in vivo, these data support the notion that biofortification of sorghum can enhance total and bioaccessible provitamin A carotenoid levels.

KEYWORDS: *provitamin A, carotenoids, β-carotene, biofortification, bioaccessibility, sorghum, in vitro digestion, micellarization*

INTRODUCTION

Typical diets in Sub-Saharan Africa are based on significant portions of cereal grains including sorghum (*Sorghum bicolor* (L.) Moench) and millet, with limited intake of fruits, vegetables, and animal products.[1] The contribution of sorghum as a commodity to the food supply in Sudan, Chad, Niger, Mali, and Burkina Faso is 122−237 g per capita per day, providing 410−800 kcal/capita/day energy intake primarily as carbohydrates.[2] This dietary pattern is characterized by low levels of lysine, iron, zinc, and vitamin A, resulting in high prevalence of nutritional deficiencies. Of these, vitamin A deficiency is reported to be the greatest cause of mortality in children under 5 years of age in Sub-Saharan Africa.[3] Sorghum, like many other cereal grains, is not a sufficient dietary source to provide the *Estimated Average Requirement* (EAR) of these micronutrients. In response to micronutrient deficiencies worldwide, both conventional (traditional) breeding[4] and transgenic[5] approaches to the biofortification of staple crops are being developed. The goal of the Africa Biofortified Sorghum (ABS) Project is to develop a sorghum grain with enhanced bioavailable content of provitamin A carotenoids, zinc, and iron, along with improved protein quality through transgenic biofortification.

For vitamin A, plant biofortification is accomplished by addition or enhancement of genes involved in carotenoid biosynthesis (Figure 1).[6] Carotenoids are a diverse class of lipid-soluble isoprenoids with health benefits including protection against chronic disease[5] and provitamin A activity from species including β-carotene, α-carotene, α-cryptoxanthin, and β-cryptoxanthin.[7,8] Isoprenoid biosynthesis is limited by the first committed step catalyzed by deoxyxylulose 5-phosphate synthase (DXS).[9] Phytoene synthase (PSY) catalyzes formation of phytoene from two molecules of geranylgeranyl pyrophosphate, bringing flux into the carotenoid biosynthetic pathway. Phytoene is converted to lycopene by a multifunctional carotene desaturase (CRT-I). Lycopene cyclases (β-LCY and ε-LCY) produce β-carotene (β,β-carotene) and α-carotene (β,ε-carotene). Carotene hydroxylases (CRT-RB) convert α- and β-carotene to α- and β-cryptoxanthin and then subsequently to nonprovitamin A species lutein and zeaxanthin. Carotenoids with an unsubstituted β-ionone ring and *all-trans-* configuration have the potential for conversion to retinol (provitamin A activity). As such, *all-trans*-β-carotene may have twice the potential provitamin A activity as *cis-* isomers of β-carotene, α-carotene, α-cryptoxanthin, and β-cryptoxanthin after bioavailability and bioconversion are considered.

Sorghum is a diploid cereal grain with high homology to maize (*Zea mays*)[10] that is typically a very poor source of

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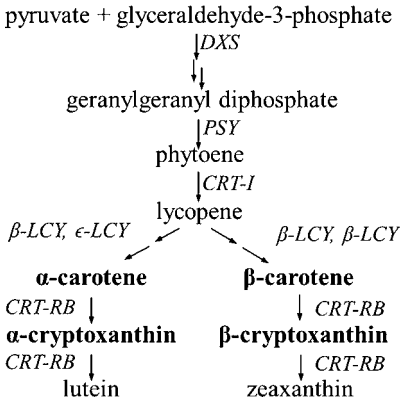

Figure 1. Overview of carotenoid biosynthesis. Provitamin A species are shown in bold. Isoprenoid synthesis is limited by the first step catalyzed by deoxyxylulose 5-phosphate synthase (DXS). Carotenoid synthesis is rate-limited by phytoene synthase (PSY), and subsequent conversion to lycopene is enhanced by multifunctional carotene desaturase (CRT-I). Two cyclizations by lycopene β-cyclase (β-LYC) form β-carotene, or one cyclization by each of β-LCY and lycopene ε-cyclase (ε-LCY) form α-carotene. From these species, carotene hydroxylase (CRT-RB) family enzymes generate α- and β-cryptoxanthin, and provitamin A activity is lost with hydroxylation of the remaining unsubstituted β-ionone ring to form lutein and zeaxanthin, respectively.

carotenoids, with <1 μg/g DW total carotenoids and <0.1 μg/g DW β-carotene.[11,12] The carotenoid content of maize is highly variable, but yellow maize contains about 15 μg/g FW total carotenoids and 0.7 μg/g FW β-carotene.[13] Provitamin biofortification of maize is possible by breeding due to natural genetic variation of genes pertaining to the carotenoid biosynthetic pathway. For instance, alleles with reduced CRT-RB expression can be selected to enhance β-carotene accumulation,[14] and reduction of ε-LCY in favor of β-LCY activity has reached 19 μg/g β-carotene in maize.[15] β-Carotene synthesis was promoted in Golden Rice 1 by insertion of phytoene synthase (PSY) from daffodil and reached as high as 37 μg/g in Golden Rice 2 with PSY from maize and CRT-I from *Erwinia uredovora*.[16] Cassava can contain as much as 2.5 μg/g DW β-carotene in nontransgenic roots and has been biofortified to 25 μg/g DW β-carotene with transgenic PSY and greater than 50 μg/g with transgenic PSY plus DXS constructs.[5,17]

While enhancement of provitamin A carotenoid content is promising, information on the bioefficacy and bioavailability of carotenoids from sorghum is limited, with no information currently available on biofortified sorghum. Bioconversion of provitamin A carotenoids to retinol is achieved by β,β-carotene-15,15′-monooxygenase (BCMO1) and is dependent upon several host factors such as vitamin A status, pathophysiological condition, and BCMO1 polymorphisms, as well as carotenoid bioavailability.[18] Carotenoid bioavailability is impacted by several factors including the food matrix, type and extent of processing, and the presence of lipid.[19] The purpose of this study was to screen the bioaccessibility of carotenoids from transgenic provitamin A biofortified sorghum samples in order to identify constructs and transgenic events with the greatest potential for bioaccessible provitamin A delivery. Bioaccessibility is the first step in overall bioavailability and is an indicator of absorption in vivo.[20] In this context, bioaccessibility is defined as the proportion of carotenoids released from the food matrix during digestion and partitioned into mixed micelles that are available for subsequent absorption by the intestine. It is critical that animal or human efficacy trials should proceed using only the most promising grain samples in order to manage cost and logistical hurdles, as well as provide the grain most likely to meet successful nutritional and public health end points. As such, in vitro digestion models have been developed and broadly applied to provide a rapid, cost-effective way of screening large sets of food products. The present study reports the application of an in vitro digestion model to comparatively screen the bioaccessibility of carotenoids from traditionally wet-cooked porridges formulated with biofortified and non-transgenic sorghum grains.

MATERIALS AND METHODS

Materials. Authentic *all-trans* standards of lutein, β-cryptoxanthin, β-carotene, lycopene, β-apo-8′-carotenal (Sigma-Aldrich, St. Louis, MO), α-cryptoxanthin, α-carotene (CaroteNature, Lupsingen, Switzerland), and zeaxanthin (IndoFine, Hillsborough, NJ) were used for carotenoid identification and calibration by HPLC. Ammonium acetate, butylatedhydroxytoluene, porcine pepsin, pancreatin, lipase, and bile extract were obtained from Sigma-Aldrich. All solvents were HPLC grade including acetone, methanol, ethyl acetate (JT Baker, Phillipsburg, NJ), petroleum ether, hexanes (Mallinckrodt, St. Louis, MO), and methyl *tert*-butyl ether (Sigma-Aldrich).

Transgenic Sorghum. *Agrobacterium*-mediated sorghum transformation was conducted using immature embryo isolated from sorghum TX430 following the protocol described by Zhao et al.[21,22] The transgenic grains used in this study were generated with vectors ABS168, ABS188, and ABS203. Vector ABS168 contained the same genes used in Golden Rice 2[16] but with different promoters: maize PSY-1 with sorghum α-kafirin promoter[23] and *Erwinia uredovora* CRT-I with sorghum β-kafirin promoter[23] in addition to the phosphomannose isomerase (PMI) gene from *Escherichia coli*[24] as the transformation selection marker. Vector ABS188 contained the genes used in ABS168, as well as the low phytic acid 1 (LPA-1) gene[25] to improve zinc and iron bioavailability from the sorghum grains. Vector ABS203 contained the genes used in ABS168, as well as DXS from *Arabidopsis*[9] with γ-zein promoter,[26] to enhance isoprenoid precursor synthesis and homogentisate geranylgeranyl transferase (HGGT) from Barley (*Hordeum vulgare* cv. Morex)[27] with α-kafirin promoter to enhance production of tocochromanols. Transgenic samples derived from the same vector represent different independent transgenic events (see Table 1). Vectors insert transgenes at random locations in the sorghum genome, such that the phenotype of independent transgenic events may vary. The transgenic samples were either homozygous transgenic grains or hemizygous transgenic grains that were derived from a self-pollinated hemizygous transgenic plant. These grains were mixed transgenic and nontransgenic grains. Nulls were nontransgenic grains produced from the segregated non-transgenic plants alongside ABS188. Nontransgenic TX430 was the germplasm background used for development of transgenic sorghum samples.

Sorghum Porridge Preparation. ABS 188 sorghum samples were received as coarsely milled meal. ABS 203 and 168 were received as whole kernels and coarsely milled with a Bühler-Miag MLI-204 grinder. Coarse meal was ground to <0.5 mm with a Foss Cyclotek 1093 mill and then stored at −80 °C under nitrogen until analysis or porridge preparation. Test porridge preparation reflected a traditional Tô preparation in Burkina Faso, following the neutral version described by Da, et al.[28] Briefly, a slurry of 10 g of fine sorghum meal and 20 mL of distilled water were added to 20 mL of boiling distilled water, stirred by hand with a spatula for 5 min, covered with foil, rested at 30 min at ambient temperature, and then stored at −80 °C until analysis or simulated digestion. This formulation contained 20% dry sorghum matter, which is very similar to 22.9% reported by the INSTAPA Project.[29]

Carotenoid Bioaccessibility. Porridges (8 g wet weight) were mixed with MUFA-rich canola oil (0.4 g, ∼5% w/w) to facilitate

Table 1. Sorghum Sample Information

event	vector	generation	transgene zygosity	transgenes
NonTG-A			nontransgenic	*a*
NonTG-B			nontransgenic	*a*
Null-A	ABS188	T2	null	
Null-B	ABS188	T2	null	
Hemi168	ABS168	T2	hemizygous	PSY-1, CRT-I, PMI
Homo168-A	ABS168	T2	homozygous	PSY-1, CRT-I, PMI
Homo168-B	ABS168	T2	homozygous	PSY-1, CRT-I, PMI
Homo168-C	ABS168	T2	homozygous	PSY-1, CRT-I, PMI
Homo188-A	ABS188	T2	homozygous	PSY-1, CRT-I, PMI, LPA-1
Homo188-B	ABS188	T2	homozygous	PSY-1, CRT-I, PMI, LPA-1
Homo188-C	ABS188	T2	homozygous	PSY-1, CRT-I, PMI, LPA-1
Homo188-D	ABS188	T2	homozygous	PSY-1, CRT-I, PMI, LPA-1
Hemi203-A	ABS203	T1	hemizygous	PSY-1, CRT-I, PMI, DXS, HGGT
Hemi203-B	ABS203	T1	hemizygous	PSY-1, CRT-I, PMI, DXS, HGGT
Hemi203-C	ABS203	T1	hemizygous	PSY-1, CRT-I, PMI, DXS, HGGT
Hemi203-D	ABS203	T1	hemizygous	PSY-1, CRT-I, PMI, DXS, HGGT
Hemi203-E	ABS203	T1	hemizygous	PSY-1, CRT-I, PMI, DXS, HGGT

[a]Nontransgenic: TX430 was the germplasm background for transgenic events.

micellarization of carotenes, which is minimal in the absence of coformulated/codigested lipid.[30] A control of carotenoid-rich vegetables was simultaneously digested during each set to confirm repeatability between assays. The vegetable mixture was used previously by our group[31] to assess carotenoid bioavailability in humans: spinach, Chinese wolfberry, tomato, carrot, and iceberg lettuce to supply major dietary carotenoids along with 5% w/w canola oil. A puree was prepared, aliquoted, and stored at −20 °C. To match the median concentration of β-carotene from sorghum in the intestinal digesta, 0.5 g of puree (in comparison to 8g sorghum porridge) was subject to in vitro digestion simultaneously with each set. Digestion was simulated by the oral, gastric, and intestinal conditions defined previously.[12] Briefly, 6 mL of oral phase base solution including α-amylase (3000 units) were added, vortexed for 1 min, and then incubated under nitrogen at 37 °C for 10 min in a shaking water bath. For the gastric phase, pepsin (final concentration 0.5 g/L) was added, the pH adjusted to 2.5 with 1 M HCl, the volume brought to 40 mL with saline (0.9% NaCl), and the mixture incubated under nitrogen at 37 °C for 60 min. For the intestinal phase, pancreatin (final concentration 0.8 g/L), lipase (0.4 g/L), and bile extract (1.8 g/L) were added, the pH was adjusted to 6.5 with 1 M $NaHCO_3$, and the mixture was incubated under nitrogen at 37 °C for 120 min. Aliquots of intestinal digesta were collected and frozen at −20 °C before analysis, and the remaining intestinal digesta was centrifuged at 10000*g* for 60 min and filtered through 0.22 μm cellulose acetate to isolate the aqueous micellar fraction. Micellarization efficiency (relative bioaccessibility) was calculated by the following: (concentration in aqueous micellar fraction/concentration in intestinal digesta) × 100%. Bioaccessible content was calculated as the following: (porridge carotenoid content μg/g DW) × (micellarization efficiency/100%).

Carotenoid Analysis. All extraction procedures were completed under yellow light to minimize the potential for photo-oxidative reactions. Carotenoids were analyzed by HPLC with diode array detection using a YMC C30 3 μm 2.0 mm × 150 mm column as previously described.[32] A typical separation from transgenic sorghum meal (Hemi203-A) is shown in Figure 2. *Sorghum meal and porridge*: Carotenoid extraction was based on a method previously reported for

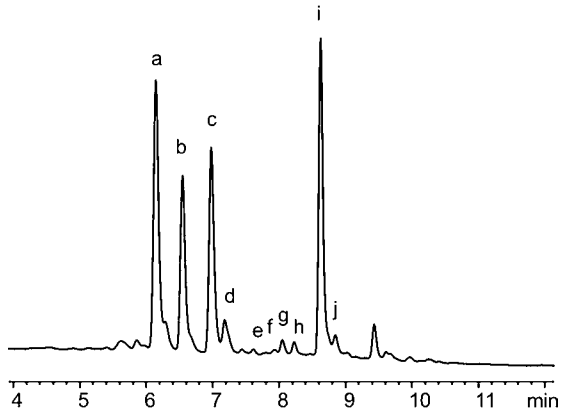

Figure 2. Chromatogram of major carotenoid species in transgenic sorghum meal (Hemi203-A) analyzed by HPLC with diode-array detection at 450 nm. Peak identification: lutein (a), zeaxanthin (b), β-apo-8′-carotenal internal standard (c), α-cryptoxanthin (d), β-cryptoxanthin (e), 15-*cis*-β-carotene (f), 13-*cis*-β-carotene (g), α-carotene (h), *all-trans*-β-carotene (i), 9-*cis*-β-carotene (j).

Golden Rice.[16] Briefly, sorghum meal (0.5 g) or porridge (2 g) was spiked with 100 μL internal standard (150 or 30 μM β-apo-8′-carotenal in ethanol). Sorghum meal was hydrated with 1 mL of distilled water on ice for 10 min, and carotenoids were extracted twice with 5 mL of cold acetone and once with 2 mL of methyl *tert*-butyl ether. The extract was dried under a stream of nitrogen, resolubilized in 1:1 methanol:ethyl acetate, and then analyzed by HPLC. Extraction recovery (mean ± standard deviation) of β-apo-8′-carotenal was 94.4 ± 4.5% and 91.4 ± 6.2% from sorghum meal and porridge, respectively. *Digestive fractions*: Intestinal digesta and aqueous micellar fractions were extracted three times with 1:3 acetone:petroleum ether with 0.1% w/v butylated hydroxytoluene, dried under nitrogen, and then resolubilized in 1:1 methanol:ethyl acetate. Extraction recovery of β-apo-8′-carotenal was 89.3% and 85.2% and for digesta and aqueous fractions, respectively.

Alternate Porridge Processing. To facilitate direct comparison with a previous study assessing carotenoid bioaccessibility from nontransgenic sorghum,[12] sorghum samples from vector ABS188 were also prepared with an alternate formulation. The two different test porridge formulations are shown in Table 2. As compared with the

Table 2. Nominal Test Porridge Formulations[a]

component	Da et al. (1982)[28] (%)	Kean et al. (2011)[12] (%)
lipid (canola oil)	5	10
sorghum meal (wet weight)	19	15
doubly distilled water	76	75

[a]Standard 100 g batches were made for each sorghum sample.

method used for all samples described by Da et al.,[28] the alternate method from Kean et al.[12] contained more lipid (10% instead of 5%) added during cooking instead of during digestion, was prepared from a coarser meal, and was slightly thinner (16.7% w/w instead of 20% w/w sorghum meal).

Data Analysis. All data are expressed as mean ± standard error of mean (SEM) with a minimum of triplicate sample extraction or in vitro digestion. *cis*-β-Carotene was reported as the sum of 9-*cis*, 13-*cis*, and 15-*cis* isomers. Provitamin A carotenoid concentration was determined in β-carotene equivalents, which is equal to *all-trans*-β-carotene + $^1/_2$(α-cryptoxanthin + β-cryptoxanthin + α-carotene + *cis*-β-carotene isomers). Statistical analysis was completed using SAS 9.2 (SAS Institute, Cary, NC) using the Tukey−Kramer method for pairwise comparison. Letters with different superscripts represent significant differences ($p < 0.05$) between samples within the carotenoid species.

Table 3. Carotenoid Content of Sorghum Meal (μg/g Dry Weight)[a,b]

sample	LUT	ZEA	α-CRP	β-CRP	α-CAR	Z-β-CAR	E-β-CAR	provitamin A[c]	total	% β-CAR
NonTG-A	3.7 ± 0.2 h	2.1 ± 0.1i	0.2 ± 0.1e	0.05 ± 0.01f	0.04 ± 0.00c	0.3 ± 0.1g	0.7 ± 0.1h	1.0 ± 0.1h	7.1 ± 0.2g	14 ± 2
NonTG-B	2.9 ± 0.1 h	2.9 ± 0.1h	0.6 ± 0.1de	0.10 ± 0.01def	0.04 ± 0.00c	0.3 ± 0.1g	0.8 ± 0.1h	1.3 ± 0.1h	7.6 ± 0.2g	14 ± 2
Null-A	3.1 ± 0.1 h	3.8 ± 0.1fg	0.7 ± 0.1de	0.10 ± 0.01def	0.04 ± 0.01c	0.3 ± 0.1fg	0.9 ± 0.1h	1.5 ± 0.1h	9.0 ± 0.2g	14 ± 2
Null-B	2.2 ± 0.1 h	3.5 ± 0.1gh	0.7 ± 0.1de	0.08 ± 0.00def	nd[d]	0.3 ± 0.1g	0.7 ± 0.1h	1.2 ± 0.1h	7.4 ± 0.2g	12 ± 2
Hemi168	7.6 ± 0.4 fg	4.3 ± 0.2def	0.7 ± 0.1de	0.09 ± 0.01def	0.18 ± 0.05bc	0.6 ± 0.1ef	2.6 ± 0.1g	3.3 ± 0.1g	16.0 ± 0.4f	20 ± 2
Homo168-A	10.7 ± 0.5 cd	5.4 ± 0.3bc	1.3 ± 0.1cd	0.30 ± 0.02b	0.34 ± 0.16bc	1.9 ± 0.1a	10.0 ± 0.5a	11.9 ± 0.6b	29.9 ± 0.8b	40 ± 8
Homo168-B	11.4 ± 0.6 bc	5.0 ± 0.2bcd	1.0 ± 0.3d	0.19 ± 0.02de	0.34 ± 0.05bc	1.4 ± 0.1bc	7.2 ± 0.3b	8.6 ± 0.5cd	26.6 ± 0.7bc	32 ± 6
Homo168-C	9.6 ± 0.3 cde	4.6 ± 0.2de	1.0 ± 0.1d	0.15 ± 0.02de	0.28 ± 0.02bc	1.1 ± 0.1bcd	5.5 ± 0.2cd	6.8 ± 0.3ef	22.3 ± 0.4cd	30 ± 6
Homo188-A	10.4 ± 0.2 cd	6.9 ± 0.1a	3.1 ± 0.2a	0.45 ± 0.02a	0.75 ± 0.03a	2.2 ± 0.1a	10.7 ± 0.3a	14.0 ± 0.3a	34.5 ± 0.5a	37 ± 6
Homo188-B	8.1 ± 0.3 efg	6.8 ± 0.1a	2.3 ± 0.2b	0.23 ± 0.01cd	0.26 ± 0.02bc	1.4 ± 0.1b	6.1 ± 0.1c	8.3 ± 0.2d	25.3 ± 0.4c	30 ± 2
Homo188-C	7.0 ± 0.1 g	4.8 ± 0.1cd	1.9 ± 0.2bc	0.13 ± 0.02def	0.11 ± 0.01c	0.5 ± 0.1efg	2.4 ± 0.1g	3.7 ± 0.2g	16.9 ± 0.3ef	17 ± 3
Homo188-D	7.4 ± 0.2 g	5.5 ± 0.1bc	2.4 ± 0.2ab	0.22 ± 0.01cd	0.15 ± 0.00bc	1.3 ± 0.1bcd	4.1 ± 0.1ef	6.1 ± 0.2f	21.0 ± 0.4de	26 ± 3
Hemi203-A	13.1 ± 0.4 b	5.6 ± 0.1b	1.2 ± 0.1cd	0.32 ± 0.04ba	0.47 ± 0.07ab	2.1 ± 0.1a	8.0 ± 0.2b	10.0 ± 0.2c	30.8 ± 0.5ab	33 ± 4
Hemi203-B	10.2 ± 0.3 cd	4.3 ± 0.1def	0.9 ± 0.1de	0.08 ± 0.03f	0.18 ± 0.03c	1.1 ± 0.1d	3.1 ± 0.1fg	4.2 ± 0.1g	19.8 ± 0.4def	21 ± 4
Hemi203-C	9.4 ± 0.3 def	3.9 ± 0.1efg	0.7 ± 0.1de	0.10 ± 0.02def	0.15 ± 0.03bc	1.1 ± 0.2cd	3.1 ± 0.1fg	4.1 ± 0.2g	18.5 ± 0.4def	23 ± 4
Hemi203-D	15.1 ± 0.6 a	5.1 ± 0.2bcd	2.2 ± 0.1b	0.32 ± 0.02b	0.35 ± 0.15bc	2.1 ± 0.1a	5.1 ± 0.2de	7.6 ± 0.3de	30.3 ± 0.7ab	24 ± 4
Hemi203-E	9.3 ± 0.3 def	3.9 ± 0.1fg	1.0 ± 0.1d	0.07 ± 0.02ef	0.17 ± 0.01bc	0.8 ± 0.1e	2.1 ± 0.1g	3.1 ± 0.1g	17.3 ± 0.4ef	17 ± 4
control[e]	64.0 ± 3.3	5.3 ± 0.4	2.5 ± 0.5	5.7 ± 0.8	20.6 ± 4.2	41 ± 3	66 ± 8	101 ± 10	205 ± 13	51 ± 3

[a]Abbreviations: lutein (LUT), zeaxanthin (ZEA), α-cryptoxanthin (α-CRP), β-cryptoxanthin (β-CRP), sum of *cis*-β-carotene isomers (Z-β-CAR), *all-trans*-β-carotene (E-β-CAR), total β-carotene as percent of total carotenoids (%β-CAR). [b]Letters with different superscripts represent significant differences ($p < 0.05$) between sorghum samples within carotenoid species using Tukey's test. [c]Provitamin A content in β-carotene equivalents = *all-trans*-β-carotene + $^1/_2$(α-cryptoxanthin + β-cryptoxanthin + α-carotene + *cis*-β-carotene). [d]Not detected. [e]Vegetable puree containing 5% w/w lipid.

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Table 4. Comparison of Transgene Vector Constructs and Nontransgenic/Null Sorghum for Delivery of Provitamin A Carotenoids (β-Carotene Equivalents) through Stages of Processing and in Vitro Digestion[a,b]

	mean (range) of events from vector or germplasm background			
stage of delivery	nontransgenic/null	ABS168	ABS188	ABS203
provitamin A content (μg/g DW)	1.2 (0.9−1.5)d	7.7 (3.3−11.9)b	8.3 (3.7−14.0)a	5.8 (3.1−10.0)c
test porridge provitamin A[b] content (μg/100 g FW)	25 (16−31)c	95 (47−164)ab	144 (71−250)a	89 (44−151)b
all-trans-β-carotene micellarization efficiency (%)	8.2 (6.3−11.3)a	2.0 (0.9−4.7)b	3.3 (1.9−4.9)b	2.9 (1.8−3.7)b
bioaccessible provitamin A content (μg/g DW)	0.14 (0.08−0.16)b	0.09 (0.05−0.13)b	0.33 (0.14−0.67)a	0.14 (0.09−0.16)b

[a]Letters with different superscripts represent significant differences ($p < 0.05$) between vectors within the stage of delivery using Tukey's test. [b]Provitamin A content in β-carotene equivalents = *all-trans*-β-carotene + $^1/_2$(α-cryptoxanthin + β-cryptoxanthin + α-carotene + *cis*-β-carotene).

RESULTS

Carotenoid Content. Transgenic sorghum grain events contained 16.0−34.5 μg/g total carotenoids DW, in comparison to 7.1−9.0 μg/g in null/nontransgenic sorghum (Table 3). Provitamin A carotenoid content was 0.9−1.5 μg/g DW β-carotene equivalents in nontransgenic samples and 3−14 μg/g in transgenic samples. The relative proportion of *all-trans*-β-carotene in relation to total carotenoids was 12−14% in nontransgenic sorghum and 17−40% in transgenic events. Carotenoid content varied between independent transgenic events within vector constructs. Homo188-A contained the greatest ($p < 0.05$) concentration of provitamin A carotenoids across all samples at 14.0 ± 0.3 ug/g DW. Comparisons between vector constructs are shown in Table 4. Only a hemizygous T1 generation was produced at the time of this study from vector ABS 203 (which contained DXS and HGGT in addition to PSY-1 and CRT-I), while one homozygous and three homozygous were available for ABS168, and all homozygous T2 events were assessed for ABS188. Therefore, the comparisons are between the events available and not the vector constructs in and of themselves. This being considered, the mean provitamin A content was greater from the ABS188 vector than other constructs.

Test Porridges. Provitamin A content of test porridges from vector constructs and nontransgenic sorghum are represented as μg/100 g fresh wet (FW) in Table 4 to facilitate estimation and comparison of the carotenoid content of prepared food products. Provitamin A content of test porridges ranged from 16 to 31 μg/100 g FW β-carotene equivalents in nontransgenic samples, and 40−250 μg/100 FW in transgenic biofortified samples. Retention of *all-trans*-β-carotene after cooking averaged 77 ± 3% and ranged from 48% to 100%. Detailed carotenoid content for each transgenic event is given in Supporting Information Table 1.

Micellarization Efficiency. The relative bioaccessibility of carotenoids from sorghum was determined by in vitro digestion and subsequent isolation of the aqueous micellar fraction. Carotenoids released from the cereal matrix and transferred to mixed bile salt−lipid micelles are considered bioaccessible and available to the intestinal enterocyte for subsequent absorption. *All-trans*-β-Carotene micellarization efficiency was greater ($p < 0.05$) from null/nontransgenic porridges (6.3−11.3%) than from transgenic sorghum porridges (0.9−4.9%), as summarized in Table 4. No difference was observed between the mean *all-trans*-β-carotene bioaccessibility of transgenic constructs. Micellarization efficiencies of all carotenoids from each individual sorghum samples are shown in Supporting Information Table 2. Micellarization efficiencies of carotenoids were higher from carotenoid-rich vegetable puree controls (33 ± 13% for *all-trans*-β-carotene) than from sorghum samples. This suggests that enhanced carotenoid levels in biofortified sorghum porridges did not result in saturation of micelles, as β-carotene levels in the digesta of controls were matched to the median level of transgenic sorghum.

Bioaccessible Carotenoid Content. Bioaccessible content represents the total amount of carotenoids released from the test porridge and solubilized during digestion that are available for subsequent intestinal uptake. Because bioaccessible content is a composite of both carotenoid content of the sorghum porridge and micellarization efficiency, it is the best measure to compare transgenic events/constructs and assess improvement from nontransgenic sorghum. Bioaccessible provitamin A content of digested test porridges ranged from 0.08 to 0.16 μg/g DW from nontransgenic samples and 0.05−0.67 μg/g DW from transgenic events (Table 4). ABS188 events contained on average a greater ($p < 0.05$) bioaccessible content than ABS168 events, heterozygous ABS203 events, and nontransgenic/null samples. Transgenic event Homo188-A had a greater ($p < 0.05$) bioaccessible content of provitamin A carotenoids, *all-trans*-β-carotene, lutein, zeaxanthin, and total carotenoids than all other sorghum samples (see Supporting Information Table 3).

Alternate Porridge Processing. Carotenoid micellarization efficiency was assessed from test porridges prepared by an alternate procedure to facilitate comparison to our previous assessments of nontransgenic sorghum and maize.[12,32] The alternative method contained 10% lipid added during cooking as opposed to 5% lipid added before digestion but was similar in other regards (Table 2). The micellarization efficiency of *all-trans*-β-carotene ranged from 6% to 22% in ABS188 transgenic samples and 25−27% in corresponding null/nontransgenic samples (Table 5). *All-trans*-β-Carotene micellarization efficiency was more bioaccessible ($p < 0.05$) from porridges

Table 5. Micellarization Efficiency (%) of *all-trans*-β-Carotene from ABS188 Samples with Alternative Processing Methods[a,b]

sample	5% lipid (Da et al. 1982[28])	10% lipid (Kean et al. 2011[12])
NonTG-B	8.3 ± 2.8ab	27.2 ± 7.0a
Null-A	6.3 ± 1.1ab	27.2 ± 13.4a
Null-B	11.3 ± 4.0a	25.5 ± 9.1ab
Homo188-A	4.9 ± 0.5ab	8.1 ± 2.9ab
Homo188-B	2.9 ± 0.4b	6.1 ± 0.8b
Homo188-C	3.6 ± 0.5ab	9.0 ± 5.0ab
Homo188-D	1.9 ± 0.1b	21.6 ± 6.2ab

[a]Letters with different superscripts represent significant differences ($p < 0.05$) between samples within the processing method. [b]*all-trans*-β-Carotene micellarization efficiency was significantly greater by processing test porridges with 10% lipid than with 5% lipid by overall *F*-test ($P < 0.001$).

prepared using 10% lipid than from 5% lipid for all samples except Homo188-C. The rank of carotenoid bioaccessibility between sorghum samples was similar in both methods with the exception of Homo188-D.

DISCUSSION

The purpose of this study was to screen independent transgenic events and three different vector constructs for the provitamin A biofortification of sorghum. Transgenic event Homo188-A was identified as the candidate with the greatest bioaccessible provitamin A carotenoid content. Provitamin A content of this transgenic event was 9−15 times greater than the germplasm background in the raw sorghum meal and 4−8 times greater after in vitro digestion. As for genetic constructs, ABS188 events delivered more bioaccessible provitamin A content on average than ABS168 or hemizygous ABS203 events. ABS188 events contained the most provitamin A in the sorghum meal, but the mean micellarization efficiency of β-carotene did not differ between the constructs. This suggests addition of LPA-1 or DXS and HGGT to the ABS168 construct does not affect carotenoid bioaccessibility. ABS188 contained LPA-1 to reduce phytate for mineral bioavailability, so it is not clear why this trait enhanced carotenoid accumulation or if this is a reflection of the particular transgenic events that were isolated and selected for this study. All ABS203 events were only hemizygous, so the potential of constructs containing DXS and HGGT to enhance provitamin A delivery should not be dismissed. Hemi203-A, with 10 μg/g DW provitamin A, seems to be a promising event to carry forward to the T2 generation.

Carotenoids were less bioaccessible from transgenic sorghum events than from the nontransgenic background. The bioaccessible provitamin A content of many transgenic samples was not improved in relation to the nontransgenic background despite enhanced provitamin A content in the raw meal. Failla, et al.[17] similarly reported the micellarization efficiency of β-carotene was significantly lower from transgenic provitamin A biofortified cassava than from nontransgenic cassava, although all transgenic cassava samples resulted in enhanced bioaccessible provitamin A content. Transgenic modifications to sorghum may affect the bioaccessibility of carotenoids through carotenoid localization and sequestration. Enhanced PSY expression in both nontransgenic *Arabidopsis*[33] and transgenic citrus[34] led to enhanced β-carotene crystallization in comparison to the corresponding wild-type species, and crystalline carotenoids are poorly bioaccessible due to poor aqueous solubility.[35] Further investigations on the localization and crystallinity of carotenoids in transgenic sorghum grains may be warranted to improve provitamin A bioaccessibility.

The bioaccessibility of carotenoids from sorghum seems to be poor in relation to other biofortified cereals, although the comparison of food matrixes across different studies is subject to confounding factors. β-Carotene micellarization efficiency was lower from transgenic sorghum in this study (1−5%) than from maize (17%)[13] and orange-flesh sweet potato (10%)[36] using similar in vitro methodologies. This may be related to carotenoid−protein binding and the digestibility of the sorghum porridge food matrix. The digestibility of proteins in sorghum after cooking is poor in relation to maize,[37] and carotenoids are likely bound to kafirin proteins in sorghum as they are to the analogous zein proteins in maize.[38,39] Localization of carotenoid synthesis in the ABS constructs was targeted to the endosperm by utilizing promoters of sorghum endosperm proteins α-kafirin and β-kafirin for PSY-1 and CRT-I, respectively, so it is likely that carotenoids were colocalized with kafirins. Improved protein digestibility is another target trait that has been developed as part of the ABS project,[40] so it may be insightful to assess the effect of protein digestibility on carotenoid bioaccessibility when this trait has been stacked with high provitamin A content.

In agreement with previous observations, comparison of porridge preparation methods suggests that additional bulk lipid enhances carotenoid bioaccessibility. Co-formulated lipid enhances bioaccessibility and bioavailability of provitamin A carotenoids in vitro and in vivo by enhancing solubilization, micelle formation, and chylomicrometer secretion.[7,19,31] Provitamin A bioaccessibility was enhanced 3−5-fold by processing the sorghum test porridges with 10% lipid instead of adding 5% lipid after processing. While the rank of one sample changed between the two methods, interactions between bulk lipid and the food matrix may differ between samples during wet-cooking and digestion processes. Because micellarization efficiency enhancement multiplies with the enhancement of provitamin A content of the starting grain to determine bioaccessible content, the preparation of biofortified sorghum foods should be considered as a complementary approach in raising vitamin A status. Implementation of provitamin A fortified sorghum foods should include education and access to plant-derived lipid sources in order to optimize provitamin A bioavailability. Future work may consider lipid sources common to Sub-Saharan Africa such as palm oil, sunflower oil, and peanut oil.

Nonprovitamin A carotenoids accounted for a significant proportion of total carotenoids in sorghum samples. The profile of β-carotene in relation to other carotenoids was enhanced 3-fold in comparison to the germplasm background to as high as 37−40% in biofortified sorghum meal. While this enrichment of β-carotene profile relative to the germplasm background is promising, potential exists to drive more carotenoids toward β-carotene. β-Carotene accounts for as much as 85−90% of carotenoids in cassava[5] and Golden Rice[16] and 46−66% in maize.[15] Within the most promising sorghum event Homo188-A, lutein and zeaxanthin accounted for 41% of carotenoids in the ground meal and over 71% of bioaccessible carotenoids. While carotenoid species may compete for micellarization and intestinal absorption, high amounts of lutein and zeaxanthin minimally affected in vitro bioaccessibility[13] and did not reduce in vivo bioefficacy[41] of provitamin A carotenoids from maize. Lutein and zeaxanthin accumulate in the macula of the retina, where they may be involved with retinal development and protection from light-induced damage.[42] Therefore, delivery of these nonprovitamin A carotenoids from biofortified sorghum may also be beneficial for the Sub-Saharan population.[43]

Biofortified sorghum is a potentially significant dietary source of provitamin A carotenoids. The *Estimated Average Requirement* of vitamin A intake for children age 4−8 years old is 275 μg of retinol activity equivalents (RAE) per day. The β-carotene to retinol conversion ratio is dependent upon many factors including bioavailability from the particular food matrix, which is estimated to be 12:1 by weight by the Institute of Medicine.[44] However, more favorable conversion rates have been recently reported for biofortified crops: 6:1 from maize,[45] 4.2:1 from cassava,[46] and 3.8:1 from Golden Rice.[47] A meal containing 200 g of test porridge from sorghum sample Homo188-A in this study would contain 500 μg of β-carotene equivalents in comparison to 2000 μg of β-carotene from 100 g of prepared cassava[46] or 1530 μg from 200 g of cooked Golden

Rice.[47] However, 200 g of this sorghum porridge would provide 30−48% of the vitamin A EAR for children 4−8 years old if the bioconversion rate were between 6:1 and 3.8:1 by weight. Further in vivo studies are required to determine the retinol equivalency of provitamin A from biofortified sorghum foods and provide evidence for bioefficacy in target populations.

In conclusion, this study is the first to have evaluated the delivery of provitamin A carotenoids from transgenic biofortified sorghum. Transgenic provitamin A biofortified sorghum developed by DuPont Pioneer contained 3−14 μg/g DW β-carotene at this stage of the ABS project. Sorghum sample Homo188-A was identified from in vitro screening as the event with the greatest concentration of bioaccessible provitamin A carotenoids. Carotenoid micellarization efficiency varied between transgenic events and was lower from transgenic samples than from nontransgenic samples. Provitamin A carotenoid accumulation was different between vector constructs, but micellarization efficiency was not. Additional research is warranted to better understand how these transgenic grains can be optimally processed and prepared to deliver highly bioaccessible carotenoids to the target population in Sub-Saharan Africa. New events and constructs are becoming available through the DuPont Pioneer ABS pipeline that may be able to address these challenges. These results suggest that biofortified sorghum can serve as a source of bioaccessible provitamin A carotenoids, but ultimately, the efficacy toward improving vitamin A status in target populations must be confirmed in vivo.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Carotenoid content of sorghum test porridges; carotenoid micellarization efficiency (%) from sorghum porridges digested with 5% lipid; bioaccessible carotenoid content of sorghum porridges digested with 5% lipid. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

DW, dry weight basis; FW, fresh weight basis; ABS, African biofortified sorghum; DXS, deoxyxylulose 5-phosphate synthase; PSY, phytoene synthase; CRT-I, carotene desaturase; LCY, lycopene cyclase; CRT-RB, β-carotene desaturases; BCMO1, β,β-carotene 15,15′-monooxygenase; HGGT, homogentisate geranylgeranyl transferase; HPLC, high-pressure liquid chromatography; SEM, standard error of the mean; EAR, *Estimated Average Requirement*; RAE, retinol activity equivalents; provitamin A, β-carotene equivalents

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