Elevated vitamin E content improves all-*trans* β-carotene accumulation and stability in biofortified sorghum

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Edited by Ronald L. Phillips, University of Minnesota, St. Paul, MN, and approved August 12, 2016 (received for review May 4, 2016)

Micronutrient deficiencies are common in locales where people must rely upon sorghum as their staple diet. Sorghum grain is seriously deficient in provitamin A (β-carotene) and in the bioavailability of iron and zinc. Biofortification is a process to improve crops for one or more micronutrient deficiencies. We have developed sorghum with increased β-carotene accumulation that will alleviate vitamin A deficiency among people who rely on sorghum as their dietary staple. However, subsequent β-carotene instability during storage negatively affects the full utilization of this essential micronutrient. We determined that oxidation is the main factor causing β-carotene degradation under ambient conditions. We further demonstrated that coexpression of homogentisate geranylgeranyl transferase (*HGGT*), stacked with carotenoid biosynthesis genes, can mitigate β-carotene oxidative degradation, resulting in increased β-carotene accumulation and stability. A kinetic study of β-carotene degradation showed that the half-life of β-carotene is extended from less than 4 wk to 10 wk on average with *HGGT* coexpression.

β-carotene accumulation | β-carotene stability | vitamin E | HGGT | biofortified sorghum

The importance of vitamin A for human health has been widely addressed (1–6). A 2009 Global Report (7) summarized vitamin A as being "vital for survival and sight; to boost the immune system, vitamin A is a critical micronutrient for survival and physical health of children exposed to disease." In Africa, malnutrition is a serious challenge, but micronutrient deficiency also plays a dominant role in the overall food security of that continent. Based on this global report, the five countries having the highest proportions of preschool age children with vitamin A deficiency were all located in Africa: 95.6% in Sao Tome and Principe, 84.4% in Kenya, 75.8% in Ghana, 74.8% in Sierra Leone, and 68.8% in Mozambique.

Sorghum (*Sorghum bicolor* L.) is one of the most important staple foods for an estimated 500 million people, primarily those living in arid and semiarid areas. In Africa, it is the second most important cereal; about 300 million people rely on it as their daily staple food. Although sorghum is gluten-free and could be an attractive replacement for wheat-allergy sufferers, it is considered a nutrient-poor crop (8, 9) with very low amounts of β-carotene (10). The improvement of micronutrients in food crops has attracted considerable attention, and significant advances have been made in a range of major crops (11–22). Nutritional improvement in sorghum was undertaken a decade ago (23, 24); however, progress has lagged behind the progress in other crops. One reason was the recalcitrance of sorghum to genetic modification via transformation. Recent improvements in sorghum transformation have largely overcome this barrier and offer an alternative approach to genetic improvements in sorghum (25).

One of our objectives is to develop sorghum lines with enhanced and stabilized provitamin A (β-carotene). In Africa sorghum grain is commonly stored in ambient conditions for several months between harvest and consumption. The stability of β-carotene during storage is important for maintaining its nutritional value. During this study, a challenging issue was ensuring the stability during storage of the β-carotenes that were enhanced in our transgenic sorghum grain. A series of experiments revealed that oxidation was the major factor causing β-carotene degradation in sorghum grain. We found that coexpressing barley homogentisate geranylgeranyltransferase (*HGGT*) (26, 27) with genes responsible for enhancing β-carotene levels significantly improved β-carotene accumulation and stability in sorghum grain.

Results

Enhancing All-*Trans* β-Carotene Accumulation in Sorghum Endosperm. Sorghum contains very low levels of β-carotene. Approximately 45 d after pollination (DAP), mature WT TX430 inbred seeds accumulate 0.5 μg/g dry weight (DW) all-*trans* β-carotene and moderate levels of lutein (2.04 μg/g) and zeaxanthin (2.57 μg/g) (Fig. S1 and Table S1), which could correlate with the light yellow color of TX430 seeds (10). Because all-*trans* β-carotene was the predominant provitamin A carotenoid in both transgenic and WT sorghum grains (Fig. S1 and Table S1), we focus only on all-*trans* β-carotene in this report. To increase β-carotene accumulation in sorghum endosperm, we first tested the genes described by the Golden Rice-2 (GR2) research team (28) in sorghum and constructed vector–*ABS168* containing the *Zea mays* phytoene synthase 1 (PSY1) and the *Pantoea ananatis* carotene desaturase (CRTI) genes driven with different sorghum endosperm-specific promoters as illustrated in Fig. 1*A*. However,

Significance

Studies on the importance of vitamin A for human health continue to draw significant worldwide attention. However, the instability of provitamin A in crops resulted in a significant reduction of the potential nutrition values of these food crops. Our work demonstrates that provitamin A can be stabilized in sorghum by the coexpression of vitamin E through ectopic expression of homogentisate geranylgeranyltransferase (*HGGT*) and that vitamin E can enhance the stability of provitamin A *in planta*. This research has the potential to impact directly the lives of the millions of people who suffer from vitamin A deficiency, and we believe that these results will be applicable to enhancing provitamin A stability in many food crops.

Author contributions: P.C., Z.-Y.Z., K.G., D.F.G., and M.C.A. designed research; P.C., Z.-Y.Z., K.G., D.D., T.X.H., and M.C.A. performed research; D.D. and T.X.H. contributed new reagents/analytic tools; P.C., Z.-Y.Z., K.G., D.D., T.X.H., T.J.J., S.O., F.W., and M.C.A. analyzed data; and P.C., Z.-Y.Z., K.G., T.J.J., S.O., F.W., and M.C.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1605689113/-/DCSupplemental.

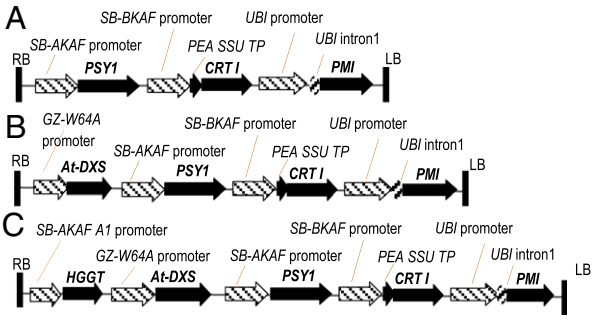


Fig. 1. Schematic representation of the molecular components in the transfer DNA (T-DNA) regions of the binary vectors used in sorghum transformations (see *SI Materials and Methods* for details). (*A*) Vector–*ABS168*. (*B*) Vector–*ABS198*. (*C*) Vector–*ABS203*.

implementing the technology developed for GR2 in sorghum achieved limited improvement in all-*trans* β-carotene accumulation, perhaps because of the different crop species and/or different promoters that drive PSY1 and CRTI genes. All transgenic *ABS168* quality events (see *Materials and Methods* for the definition of a "quality event"), such as event ABS168-1, accumulated less than 2 μg/g DW all-*trans* β-carotene in the mature seeds, without a visible change in seed color compared with WT (Fig. 2*A*). The events bearing multiple copies of the genes accumulated higher levels of total carotenoids in mature seeds, as previously reported (29). To investigate carotenoid accumulation during seed development, we further examined changes in seed color at different stages of seed developmental using a multiple-copy event ABS168-A. A light yellow color developed in the immature ABS168-A seeds around 30 DAP (Fig. 2*B*). This observation suggested that higher levels of carotenoids were produced in an early stage of seed development and that the decoloration occurred during seed maturation, further suggesting that the final carotenoid content in the seeds could reflect the balance between their biosynthesis vs. *in planta* degradation. This observation is consistent with a previous study demonstrating that carotenoid content increased at an early stage of kernel development and decreased sharply as kernels approached maturity in nontransgenic sorghum seeds (10).

DXS (1-deoxyxylulose 5-phosphate synthase) controls the rate-limiting step of the methylerythritol phosphate pathway that provides the precursor for carotenoid biosynthesis. It has been reported that overexpression of *Arabidopsis At-DXS* facilitates carotenoid biosynthesis *in planta* (30, 31). To improve carotenoid synthesis in sorghum further, vector–*ABS198* was designed in which *At-DXS* was coexpressed with *PSY1* and *CRTI* (Fig.1*B*). The top five *ABS198* quality events accumulated higher levels of total carotenoids (Table S2), and the all-*trans* β-carotene levels ranged from 2.5 to 9.1 μg/g DW in the mature seeds (Table 1). The endosperm of the best-performing *ABS198* quality event, ABS198-1, accumulated all-*trans* β-carotene up to 9.1 μg/g DW and showed a slightly visible orange color in the mature seeds (Fig. 2*A*).

Characterizing the Factors That Affect All-*Trans* β-Carotene Stability During Seed Storage. β-Carotene is a highly unsaturated molecule composed of many conjugated double bonds. It is very susceptible to oxidation (32). Many factors affect β-carotene stability, including temperature (thermal degradation), light intensity (photo-oxidative degradation), oxygen level (oxidative degradation), and enzymes (enzymatic oxidative degradation) (33–35).

To study all-*trans* β-carotene stability and to dissect the causes of degradation during ambient seed storage, we designed and conducted a series of experiments using the multiple-copy ABS168-A event. As shown in Table 2, soaking seeds in water for 30 min at room temperature (21 °C) had a minor effect (4.1% degradation) on the stability of all-*trans* β-carotene. In contrast, boiling seed for 30 min caused 22.9% degradation, consistent with previous reports (15, 36).

To assay the effect of light on all-*trans* β-carotene degradation in sorghum grain, ABS168-A grain was stored at room temperature either in the dark or under constant light (at an intensity of μmol $m^{-2}s^{-1}$) for 4 wk, and degradation data were compared. As shown in Table 2, about 51.9% of all-*trans* β-carotene was degraded under dark conditions. The degradation increased to 62.7% under light conditions. The slightly increased degradation (10.8%) represented photo-oxidative degradation of all-*trans* β-carotene over the 4-wk course.

Mature dry sorghum seeds contain about 10% moisture that might maintain limited enzyme activity causing enzymatic degradation of all-*trans* β-carotene. To test that possibility, ABS168-A seeds were boiled for 30 min to denature all enzymes and then were lyophilized overnight before being stored for 4 wk at room temperature in the dark. Given that boiling degraded all-*trans* β-carotene by about 22.9%, the actual all-*trans* β-carotene level before storage was 77.1% of the initial level. Considering that 33.4% of total all-*trans* β-carotene was retained after storage, the actual degradation caused by storage alone after protein denaturation was 56.7% [(77.1–33.4%)/77.1%] (Table 2), not significantly lower than the 51.9% degradation of all-*trans* β-carotene caused by storage without boiling treatment. Based on that observation, we concluded that the potential enzymatic degradation in the dry seeds could be ignored.

To determine if oxygen-induced oxidation is the main factor causing degradation, ABS168-A seeds were sealed in a container that was purged with pure oxygen (~100% O_2) to remove the air from the container and were stored for 4 wk at room temperature in the dark. The degradation of all-*trans* β-carotene increased to 70.4% in this oxygen-enriched environment (Table 2) but was significantly reduced in an oxygen-deprived environment (Fig. 3*A*).

These results suggested that oxygen-induced oxidative degradation was the main factor contributing to all-*trans* β-carotene degradation during ambient seed storage.

Improving All-*Trans* β-Carotene Stability by Ectopic Expression of *HGGT*. Vitamin E is a strong antioxidant (37) and is widely used along with β-carotene in the food industry as an additive to increase the shelf-life of β-carotene in foods (38). The biochemical effects of barley HGGT on tocotrienol and tocopherol biosynthesis have been well characterized *in planta* (26, 27). To prevent the oxidative degradation of all-*trans* β-carotene in sorghum grain and to increase its stability during seed storage, we introduced the HGGT gene from barley driven with an endosperm-specific promoter along with the carotenoid synthesis genes described for vector–*ABS198* and thereby created vector–*ABS203* (Fig. 1*C*). All the top five transgenic *ABS203* quality events displayed an orange color (Fig. 2 *A* and *C*) in the mature seed endosperms and enhanced all-*trans* β-carotene accumulation in the range of 7.3–12.3 μg/g DW. ABS203-4 was identified as the best-performing event (Table 1). Other carotenoids, such as lutein,

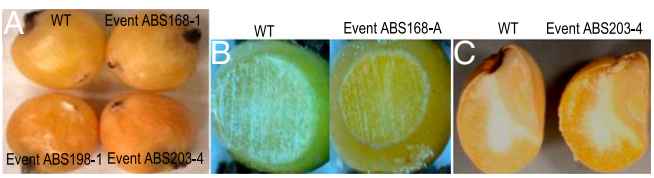


Fig. 2. Comparison of seed color in WT and transgenic sorghum. (*A*) Color in mature WT, single-copy event ABS168-1, ABS198-1, and ABS203-4 seeds. (*B*) Color in the endosperm of immature WT and event ABS168-A (multiple-copy event) seeds at 30 DAP. (*C*) Color of the endosperm of mature WT and ABS203-4 seeds.

Table 1. All-*trans* β-carotene $t_{1/2}$ with and without *HGGT* coexpression upon seed storage

Event with HGGT	All-*trans* β-carotene, μg/g	α-Tocotrienol, μg/g	α-Tocopherol, μg/g	γ-Tocopherol, μg/g	All-*trans* β-carotene $t_{1/2}$, wk	Event without HGGT	All-*trans* β-carotene, μg/g	All-*trans* β-carotene $t_{1/2}$, wk
ABS203-1	7.31 ± 0.65	1.09 ± 0.34	2.15 ± 0.54	7.96 ± 2.24	10.0 ± 0.9	ABS198-1	9.06 ± 1.31	3.5 ± 0.4
ABS203-2	10.49 ± 0.85	1.80 ± 0.72	3.07 ± 1.04	9.34 ± 1.63	9.4 ± 0.8	ABS198-2	5.87 ± 0.37	4.4 ± 01.3
ABS203-3	8.51 ± 0.92	1.27 ± 0.21	1.83 ± 0.65	8.27 ± 1.04	9.4 ± 0.4	ABS198-3	2.51 ± 0.34	2.4 ± 0.2
ABS203-4	12.30 ± 1.43	2.20 ± 0.62	3.79 ± 1.14	9.55 ± 1.01	11.0 ± 1.6	ABS198-4	3.95 ± 0.46	4.2 ± 0.7
ABS203-5	7.91 ± 0.51	1.85 ± 1.44	2.12 ± 0.81	8.70 ± 2.91	10.2 ± 0.6	ABS198-5	4.75 ± 0.85	5.5 ± 0.4
ABS203 average	9.31	1.64	2.95	8.76	10.0	ABS198 average	5.23	3.9
Fold change*	18.62	27.33	1.76	1.68	NA	Fold change*	10.46	NA
WT	0.50 ± 0.2	0.06 ± 0.03	1.67 ± 0.25	5.21 ± 0.98	NA	WT	0.50 ± 0.2	NA

Data are presented as the average ± SD of three biological replications.
*Compared with WT.

zeathanthin, α-carotene, 13-*cis* β-carotene, and 9-*cis* β-carotene, also were increased significantly (Fig. S1 and Tables S1 and S2), with all-*trans* β-carotene showing the greatest increase both in fold change (24.6-fold) and absolute accumulation (12.3 μg/g DW) (Fig. S1 and Table S1). In addition, both tocotrienols and tocopherols were significantly elevated in these ABS203 events compared with WT (Table 1). The three major vitamin E tocochromanols, α-tocotrienol, α-tocopherol, and γ-tocopherol, were increased 27.33-, 1.76-, and 1.68-fold, respectively. Although α-tocotrienol (1.64 μg/g DW) had the greatest fold-change increase, γ-tocopherol (8.76 μg/g DW) was the most abundant form of vitamin E accumulated in these ABS203 events.

To investigate the antioxidant effect of vitamin E on all-*trans* β-carotene stability, we compared the stability of all-*trans* β-carotene in the mature seeds of events ABS198-1 and ABS203-4 treated with different concentrations of oxygen for 4 wk at room temperature in the dark. As demonstrated in Fig. 3*A*, all-*trans* β-carotene degradation was increased with the increase of oxygen levels in both ABS198-1 and ABS203-4 events, as was consistent with the observation for event ABS168-A (Table 2). However, the degradation of all-*trans* β-carotene was much less in event ABS203-4 (with *HGGT*) than in event ABS198-1 (without *HGGT*), especially under higher oxygen concentrations. These results further support the hypothesis that oxygen-induced oxidation was the main source of all-*trans* β-carotene degradation. The antioxidant effect of vitamin E is important in enhancing the stability of all-*trans* β-carotene under ambient storage conditions.

We studied the kinetics of all-*trans* β-carotene degradation in sorghum grain during storage at room temperature by determining the relationship between storage time and all-*trans* β-carotene content at different storage intervals (see *Materials and Methods* for details). Using the ABS198-1 and ABS203-4 events as examples, the curves of all-*trans* β-carotene content vs. time represented the time course of all-*trans* β-carotene degradation in real time (Fig. 3*C*). The curves were converted into straight lines after the data were replotted with natural-log (ln) all-*trans* β-carotene content vs. time, indicating that all-*trans* β-carotene degradation in sorghum grain followed a first-order kinetic model (Fig. 3*B*) (39–41). The first-order rate constant (k) of β-carotene degradation was determined by the slope of each line, 0.2001/wk for event ABS198-1 and 0.0628/wk for event ABS203-4 (Fig. 3*B*). Accordingly, the $t_{1/2}$ of all-*trans* β-carotene was calculated as 3.5 ± 0.4 wk for event ABS198-1 and 11 ± 1.6 wk for event ABS203-4. As shown in Table 1, the average $t_{1/2}$ of all-*trans* β-carotene in the five ABS203 events (10 wk; range, 9.4–11 wk) was significantly longer than in the five ABS198 events (3.9 wk; range, 2.4–5.5 wk). These results showed that the stability of the all-*trans* β-carotene improved 2.6-fold when *HGGT* was coexpressed with β-carotene biosynthetic genes in sorghum grain.

To support further the notion that *HGGT* coexpression can mitigate all-*trans* β-carotene degradation under different storage temperatures, we measured the degradation of all-*trans* β-carotene at 30 °C and 37 °C. As shown in Fig. 3*D*, the higher temperature increased all-*trans* β-carotene degradation for both ABS198-1 and ABS203-4 events. At both temperatures, however, the degradation of all-*trans* β-carotene was significantly lower in event ABS203-4 than in event ABS198-1 (either $P < 0.03$ or $P < 0.002$).

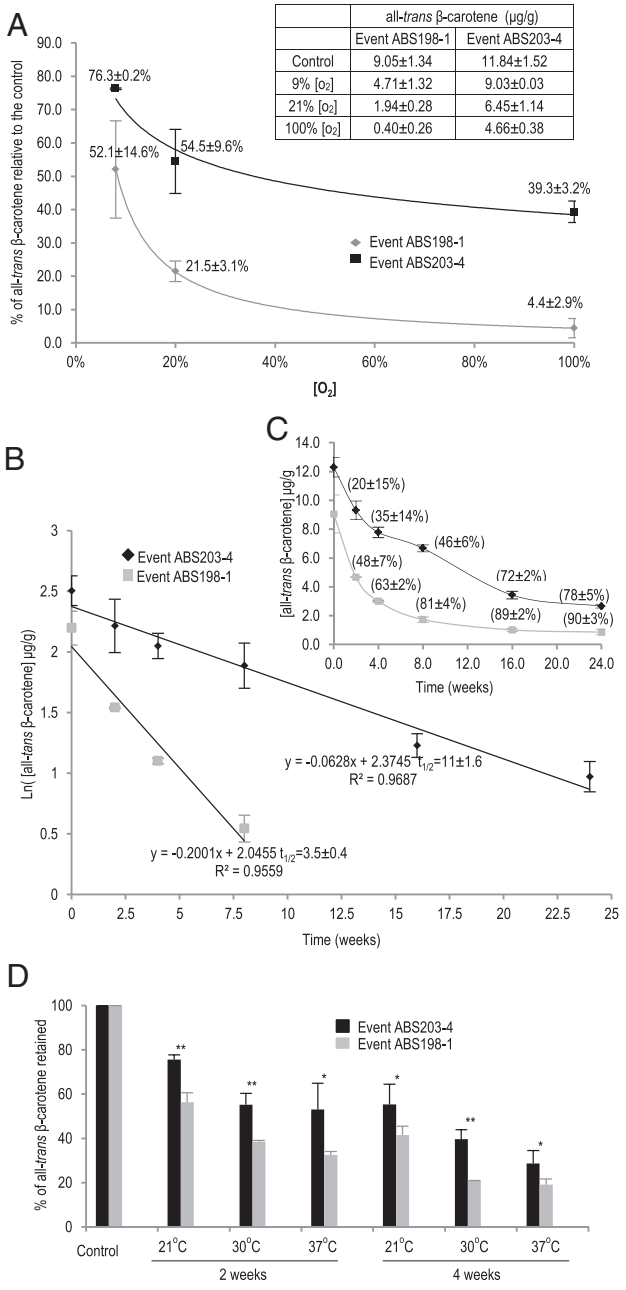
Collectively, the evidence described above supports the role of *HGGT* coexpression in increasing the stabilization of all-*trans* β-carotene during seed storage.

Table 2. All-*trans* β-carotene stability of event ABS168-A under different treatments

Treatment	All-*trans* β-carotene level after treatment	All-*trans* β-carotene of the control ± SD after treatments, %	Impact on all-*trans* β-carotene degradation
Seeds stored at −80 °C as control	9.67 ± 1.04	100	
Seeds soaked in water at room temperature in the dark for 30 min and then lyophilized	9.27 ± 0.69	95.9 ± 17.7	4.1% degradation with soaking
Seeds boiled for 30 min and then lyophilized	7.46 ± 1.53	77.1 ± 24.3	22.9% degradation with boiling
Seeds stored at room temperature in the dark for 4 wk	4.65 ± 0.45	48.1 ± 9.9	51.9% degradation with storage at room temperature
Seeds stored at room temperature under constant light (μmol $m^{-2}s^{-1}$) for 4 wk	3.61 ± 0.96	37.3 ± 14.0	10.8% photo-degradation
Seeds boiled for 30 min, lyophilized, and then stored at room temperature in dark for 4 wk	3.23 ± 0.92	33.4 ± 13.2	No detectable enzymatic degradation
Seeds sealed in a container purged with pure oxygen at room temperature in the dark for 4 wk	2.86 ± 0.16	29.6 ± 4.9	Degradation increased from 51.9 to 70.4%

Data are presented as the average ± SD of three biological replications.

Obtaining Higher All-*Trans* β-Carotene Accumulation in ABS203 Events. The decolorization of yellow endosperm during seed maturation in event ABS168-A indicated carotenoid degradation and suggested that the final carotenoid content in the mature sorghum seed could be the result of carotenoid biosynthesis vs. degradation.



	all-*trans* β-carotene (μg/g)	
	Event ABS198-1	Event ABS203-4
Control	9.05±1.34	11.84±1.52
9% [o_2]	4.71±1.32	9.03±0.03
21% [o_2]	1.94±0.28	6.45±1.14
100% [o_2]	0.40±0.26	4.66±0.38

Fig. 3. The effect of *HGGT* coexpression on increasing all-*trans* β-carotene stability during storage. (*A*) The impact of oxygen concentration on all-*trans* β-carotene stability. (*Inset*) The all-*trans* β-carotene level for each treatment is listed in the table. The control samples were stored at −80 °C. (*B*) Kinetics of all-*trans* β-carotene degradation upon ambient storage (see *SI Materials and Methods* for details). The all-*trans* β-carotene levels of event ABS198-1 at 16- and 24-wk storage times were too low to be included in the kinetics study. (*C*) Time course of all-*trans* β-carotene degradation during ambient seed storage. The percentile numbers represent the percentage of all-*trans* β-carotene degradation relative to the control seeds stored at −80 °C. (*D*) The effect of *HGGT* coexpression on all-*trans* β-carotene stability at different temperatures. $*P < 0.03$; $**P < 0.002$, *t* test. In all figures, error bars indicate ± SD from three replications.

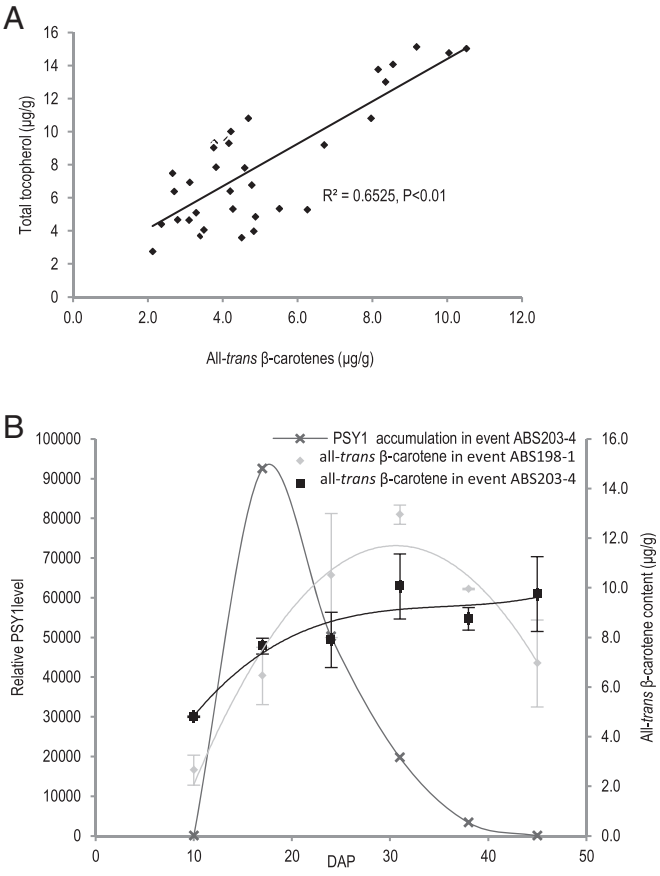


Fig. 4. The effect of *HGGT* coexpression on increasing all-*trans* β-carotene accumulation during seed development. (*A*) The positive correlation between the accumulation of all-*trans* β-carotene and total tocopherols in mature event *ABS203* seeds harvested from 35 independent plants. (*B*) The relationship between the accumulation of all-*trans* β-carotene and PSY1 protein during seed development in event ABS198-1 and ABS203-4. A similar pattern of PSY1 protein accumulation was observed in two other ABS203 events (Fig. S2). Error bars indicate ± SD from three replications.

Both vector–*ABS198* and vector–*ABS203* constructs contained exactly the same carotenoid biosynthesis genes (*PSY1*, *CRTI*, and *At-DXS*) regulated with the same promoters. The only difference between these two vectors was that vector–*ABS203* contained *HGGT*. The five best-performing ABS203 quality events contained significantly higher levels of all-*trans* β-carotene (9.3 μg/g DW by average) in the mature seeds than did the five best-performing ABS198 quality events (5.4 μg/g DW by average) (Table 1). Considering the potential for all-*trans* β-carotene degradation during seed maturation as indicated by the color change in event ABS168-A, we hypothesized that coexpressing *HGGT* could mitigate all-*trans* β-carotene degradation during seed maturation.

To test this hypothesis, we investigated the interrelationship between carotenoids and vitamin E content in mature seeds collected from 35 independent transgenic ABS203 plants. As shown in Fig. 4*A*, we observed a positive correlation ($R^2 = 0.6525$, $P < 0.01$) between all-*trans* β-carotene and total tocopherol (α- and γ-tocopherol) content in these 35 independent transgenic plants. This observation indicated that the antioxidant function of vitamin E could increase the stability of all-*trans* β-carotene through seed maturation, potentially leading to a higher accumulation of all-*trans* β-carotene in the mature seeds.

To gain insight into all-*trans* β-carotene accumulation throughout seed development, we determined the accumulation of PSY1

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protein (the enzyme that controls the rate-limiting step of carotenoid biosynthesis) by LC-MS/MS at six stages of seed development (10, 17, 24, 30, 38, and 45 DAP) in event ABS203-4. We found that PSY1 accumulated to the highest levels at the milk-stage (around 17 DAP) and declined sharply to an undetected level at maturity (around 45 DAP) (Fig. 4*B*), indicating that the capacity for carotenoid biosynthesis was higher at the early stages and was lower at the later stages of seed development. Because *PSY1* expression was driven by the same endosperm-specific promoter in ABS198 events and ABS203 events, it was reasonable to speculate that PSY1 accumulation followed a similar pattern in ABS198 and ABS203 events during seed development. To investigate the correlation between PSY1 accumulation and the all-*trans* β-carotene levels during the seed development, we further measured the accumulation of all-*trans* β-carotene at these six stages and found the highest level of all-*trans* β-carotene accumulation around 31 DAP for both event ABS203-4 and ABS198-1 (Fig. 4*B*). Afterward, the all-*trans* β-carotene levels declined sharply in event ABS198-1, similar to the previous report (10), but not in event ABS203-4. The sharp decline of all-*trans* β-carotene accumulation in event ABS198-1 correlated with a reduction in PSY1 accumulation, suggesting that all-*trans* β-carotene degradation surpassed the rate of biosynthesis at around 31 DAP in event ABS198-1. On the other hand, all-*trans* β-carotene accumulation remained constant through seed maturity in event ABS203-4, indicating that *HGGT* coexpression during seed development can mitigate all-*trans* β-carotene degradation in ABS203 events.

Discussion

Many attempts have been made to alter or to enhance the carotenoid biosynthetic pathways in various plant tissues (4, 12–15, 22), but little attention has been paid to carotenoid stability, especially all-*trans* β-carotene stability, in biofortified crops. The success of GR2 researchers in manipulating de novo carotenoid biosynthesis resulted in higher levels of β-carotene in rice endosperm (28), but the carotenoids degraded when Golden Rice was stored at ambient temperatures (42). Similarly, ectopic expression of the enzymes involved in carotenoid biosynthesis in sorghum endosperm resulted in increased all-*trans* β-carotene levels but with a $t_{1/2}$ of less than 4 wk (Figs. 2*A* and 4*B* and Table 1). Because sorghum grain must be stored and consumed over several months before newly harvested grain is available in most sub-Saharan Africa countries, developing a way to make all-*trans* β-carotene more stable during storage in ambient conditions is important for the full utilization of biofortified sorghum to deliver essential micronutrients. The focus of this research, therefore, was to develop technologies that not only increase all-*trans* β-carotene accumulation in sorghum grain but also make all-*trans* β-carotene more stable during seed storage.

The mechanisms of carotenoid metabolism have been well studied (43, 44). Factors identified as influencing carotenoid degradation in foods are oxygen, temperature, light, pH, ionic strength, moisture, and food matrix (33–35). In biofortified sorghum, oxygen-induced degradation is the greatest factor in all-*trans* β-carotene degradation during ambient seed storage (Fig. 3*A* and Table 2). Although vacuum sealing β-carotene–enriched grain potentially could slow β-carotene degradation, as demonstrated in Fig. 3*A*, this technique of storing biofortified sorghum is not practical for smallholder farmers in African countries. In the food industry, vitamin E has been shown to be the most effective antioxidant for preventing β-carotene degradation in dehydrated vegetables and fruits (45). Coexpression of *HGGT* to increase vitamin E biosynthesis, together with all-*trans* β-carotene biosynthesis, can mitigate the oxidative degradation of all-*trans* β-carotene (Fig. 3 *B*–*D*). The $t_{1/2}$ of all-*trans* β-carotene was extended significantly, from less than 4 wk to 10 wk on average, with coexpression of *HGGT* (Table 1). We observed the degradation of all three forms of vitamin E (α-tocotrienol, α-tocopherol, and γ-tocopherol) during seed storage, as shown in Table S3. This was correlated with an attenuated degradation of β-carotene during seed storage, suggesting that vitamin E may be protecting β-carotene from oxidative degradation. Although degradation was not completely prevented, we believe that the stability of all-*trans* β-carotene can be improved further by increasing vitamin E accumulation through the use of stronger endosperm-specific or even whole-seed promoters for ectopic expression of *HGGT*. In addition to preventing the degradation of all-*trans* β-carotene during mature seed storage, *HGGT* coexpression can prevent such degradation during seed development, resulting in higher all-*trans* β-carotene accumulation in freshly harvested grain (Fig. 4*B*).

Understanding how β-carotene biosynthesis (Fig. S3) and accumulation are regulated during seed development has helped in the design of new approaches to achieve higher β-carotene accumulation in mature sorghum grain. The correlation between all-*trans* β-carotene and PSY1 enzyme accumulation during seed development in event ABS198 (Fig. 4*B*) further supports the notion that PSY1 controls the rate-limiting step of carotenoid biosynthesis and provides insight into ways of further increasing β-carotene biosynthesis. If PSY1 gene expression and protein accumulation can be kept consistently at relatively higher levels throughout the later stages of seed development (e.g., by the use of promoters that achieve higher *PSY1* expression at all seed developmental stages, monocot codon optimization, or the selection of more stable and efficient versions of PSY), biofortified sorghum grain will accumulate more β-carotene in mature seeds. These methods combined with the recent discovery that carotenoid biosynthesis is controlled via posttranscriptional regulation of PSY by ORANGE could further improve β-carotene biosynthesis in sorghum grain (46).

A further role of α-tocopherol in promoting the central cleavage of the β-carotene molecule to form vitamin A rather than β-apo carotenoids was demonstrated using rat intestinal postmitochondrial fractions incubated with β-carotene (47). The multiple functions of vitamin E not only increase β-carotene stability by preventing oxidation during storage but also potentially increase the efficiency of vitamin A conversion by promoting central cleavage of the β-carotene molecule during consumption. Combining vitamin E biosynthesis with enhanced all-*trans* β-carotene biosynthesis will enable the development of biofortified sorghum that can eliminate vitamin A deficiency in people who consume sorghum as their staple diet. Based on the β-carotene bioconversion rate (4.3 μg β-carotene to 1 μg retinol) determined for event ABS203-4 using Mongolian gerbils as an animal model (48) and based on the stability of β-carotene determined in this research, we estimated that freshly harvested β-carotene–biofortified sorghum from this event could provide 90% of the estimated average requirement (EAR) of vitamin A for children under age 3 y and still would provide 20% of EAR after 6 mo of seed storage. On the other hand, event ABS198-1 would provide only 8% of EAR after 6 mo of seed storage.

Materials and Methods

Vector construction, transgenic events, treatments of sorghum seeds, kinetic studies of all-*trans* β-carotene degradation, HPLC and LC-MS/MS assays, and statistical analysis are described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Edgar B. Cahoon (University of Nebraska) for vitamin E validation; Heather Christenson, Tracy Asmus, David Draize, and Weiwei Zhu (DuPont Pioneer) for sorghum transformation; Brian Lenderts and Nancy Leysens (DuPont Pioneer) for quantitative PCR analysis; Mark Perkins (DuPont Pioneer) for HPLC analysis; Shifu Zhen (DuPont Pioneer) for greenhouse management; and the Donald Danforth Plant Science Center for certain funding administration. This research was supported by Bill and Melinda Gates Foundation Grand Challenges in Global Health Grant ID-37877 and by The Howard G. Buffett Foundation funding of the Africa Biofortified Sorghum Project. DuPont Pioneer provided funding and in-kind donations.

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