RESEARCH

Effects of S_1 Recurrent Selection for Provitamin A Carotenoid Content for Three Open-Pollinated Maize Cultivars

Thanda Dhliwayo, Natalia Palacios-Rojas, Jose Crossa, and Kevin V. Pixley*

ABSTRACT

Maize (*Zea mays* L.) cultivars with increased concentrations of provitamin A (proVA) carotenoids can improve the health of millions of consumers who are vitamin A deficient and rely on maize as a staple food. Three open-pollinated maize cultivars (OPC) were subjected to three cycles of S1 recurrent selection for increased proVA concentration. Agronomic performance of Cycles 0, 1, 2, and 3 for each OPC was evaluated using three replications at 10 locations, and changes in proVA concentration were assessed for hand-pollinated grain produced at two sites. Selection resulted in significant ($P < 0.01$ for 11, and $P < 0.05$ for 1 occurrence) linear increases of 25 to 67% per cycle for total proVA, 28 to 60% for β-carotene, 18 to 70% for β-cryptoxanthin, and 11 to 46% for zeaxanthin. These findings are especially significant because, in contrast to recent trends, they demonstrate the feasibility of developing proVA-enhanced maize while meeting nutritionists' recommendations not to sacrifice β-cryptoxanthin and zeaxanthin to increase β-carotene concentration in grain. Grain yield increased in one but decreased ($P < 0.01$) in two of the OPCs, and we hypothesize that linkage drag associated with proVA-enhancing genes from exotic donor lines may be responsible for the negative trends. We conclude that breeding proVA-enriched maize without sacrificing β-cryptoxanthin and zeaxanthin concentrations is feasible, but that (i) it remains unknown whether such approaches can achieve the high concentrations of proVA reported elsewhere by using marker-assisted selection for genes that favor β-carotene accumulation, and (ii) that grain yield and agronomic performance should be simultaneously selected if useful cultivars are desired.

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Abbreviations: CIMMYT, International Maize and Wheat Improvement Center; HPLC, high-performance liquid chromatography; LD, linkage disequilibrium; MAS, marker-assisted selection; OPC, open-pollinated cultivar; ProVA, provitamin A; QPM, quality protein maize; VAD, vitamin A deficiency.

Vitamin A deficiency (VAD) is associated with several health disorders including night blindness, an irreversible form of blindness called xerophthalmia, growth retardation, depressed immune response, and increased childhood mortality (West and Darnton-Hill, 2008; WHO, 2009; Muthayya et al., 2013). Despite several supplementation and food fortification programs, VAD affects about 190 million children and 19 million pregnant women and results in more than 4 million children suffering xerophthalmia and about 800,000 deaths annually, mostly in Africa and Southeast Asia (Rice et al., 2004; West, 2002; Muthayya et al., 2013). Vitamin A deficiency, together with other micronutrient deficiencies, is most prevalent where diets consist predominantly of cereal grains that are poor in micronutrient contents (Nuss and Tanumihardjo, 2010). Maize (*Zea mays* L.) is a staple food for hundreds of millions of consumers in Africa and Latin America, where in 11 countries, including Guatemala, Lesotho, Malawi, Zambia, and Zimbabwe, it provides more than 30% of both total dietary calories and protein (Atlin et al., 2011; Nuss and Tanumihardjo, 2010). Areas of high maize consumption overlap with regions of

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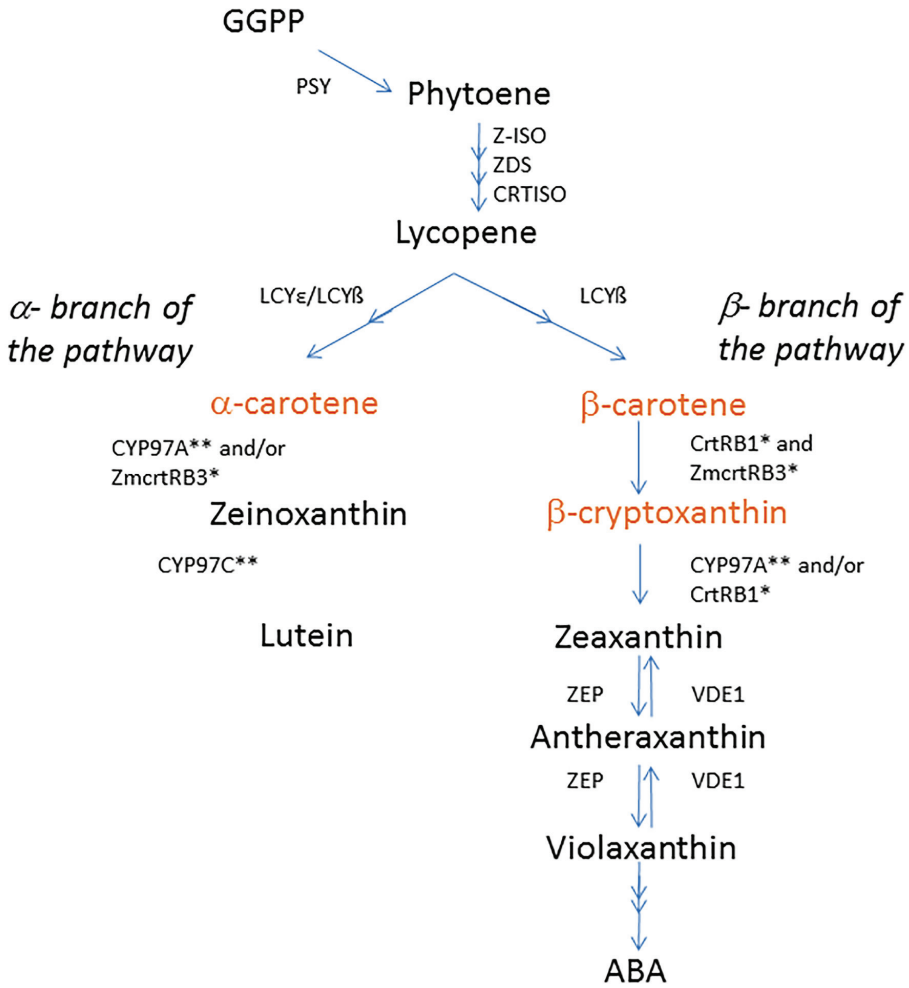

Figure 1. The carotenoid biosynthetic pathway, with provitamin A carotenoids highlighted in orange. Single asterisks indicate non-heme di-iron enzymes; double asterisks indicate cytochrome p-450 enzymes. *GGPP*, geranyl geranyl diphosphate; *PSY*, phytoene synthase; *Z-ISO*, 15-*cis* zeta carotene isomerase; *ZDS*, zeta carotene desaturase; *CRTISO*, carotenoid isomerase; *LCYβ*, lycopene β-cyclase; *LCYε*, lycopene ε-cyclase; *CRTRB1*, β-carotene hydroxylase 1; *ZEP*, zeaxanthin epoxidase; *VDE1*, violaxanthin de-epoxidase; ABA, abscisic acid. (Adapted from Babu et al., 2013a.)

VAD in large regions of sub-Saharan Africa and parts of South Asia and Latin America (West, 2002; WHO, 2009).

Biofortification is the breeding of staple food crops to increase micronutrient density (Bouis and Welch, 2010; Bouis et al., 2011; Pfeiffer and McClafferty, 2007; Pixley et al., 2013). Biofortification has been identified as one of the most practical and economically effective strategies to combat micronutrient deficiencies in developing countries (Nestel et al., 2006; Qaim et al., 2007). HarvestPlus (www.harvestplus.org, accessed 21 July 2014) is a special or "challenge" program of the Consultative Group on International Agricultural Research (CGIAR) dedicated to breeding crops for better nutrition, focusing on vitamin A, zinc, and iron enrichment of staple crops. Provitamin A (proVA) biofortified maize is the cornerstone of the HarvestPlus strategy to combat VAD in sub-Saharan African regions where maize is a major staple crop. Breeding of proVA-biofortified maize has been ongoing at the International Maize and Wheat Improvement Center (CIMMYT), International Institute of Tropical Agriculture (IITA), the Zambian Agricultural Research Institute (ZARI), and elsewhere for nearly 10 yr, and biofortified maize cultivars have been released in Nigeria and Zambia (Pixley et al., 2013).

The plant carotenoid biosynthetic pathway (Fig. 1) is well understood, and several genes involved in pathway regulation have been cloned and characterized (Vallabhaneni et al., 2009; Wurtzel et al., 2012; Cuttriss et al., 2011; Von Lintig, 2010). Three loci, phytoene synthase (*PSY*), lycopene epsilon cylase (*LCYε*), and β-carotene hydroxylase1 (*CRTRB1*), are known to regulate key steps in the accumulation of proVA carotenoids in the grain. The first gene, *PSY1*, is primarily a biosynthetic flux determinant and catalyzes the first committed step in the pathway by converting geranylgeranyl diphosphate to phytoene, resulting in the change from white to yellow in maize grain endosperm. *LCYε* and other associated genes catalyze the conversion of phytoene to α-carotene

via z-carotene. Mutant alleles of *LCYε* have been identified that preferentially increase flux of phytoene into the β branch of the carotenoid biosynthetic pathway and result in increased concentrations of proVA carotenoids, especially β-carotene (Harjes et al., 2008). However, selecting for the *LCYε* mutation to increase proVA concentration is most effective if β-carotene hydroxylation by downstream *CRTRB1* to β-cryptoxanthin, and then to zeaxanthin, is reduced. Allelic variation at the *CRTRB1* locus is associated with β-carotene levels, and selection for favorable alleles has resulted in four times higher proVA than the wild-type allele (Babu et al., 2013a; Vallabhaneni et al., 2009; Yan et al., 2010). The most favorable alleles were initially found in temperate germplasm, and these alleles are being introgressed into tropical germplasm. DNA markers linked to causal polymorphisms at the *LCYε* and *CRTRB1* loci have also been recently developed and validated (Babu et al., 2013b) and are already being routinely used along with phenotypic quantification of carotenoid concentrations by high performance or ultra-high-performance liquid chromatography (HPLC or UPLC) to select for proVA concentration in the CIMMYT–HarvestPlus breeding program (Galicia et al., 2012). Kandianis et al. (2013) have proposed other genes in the carotenoid biosynthetic pathway, in addition to *PSY*, *LCYε*, and *CRTRB1*, that appear promising for use in the improvement of total carotenoid and β-carotene in future breeding efforts.

Although there is increasing adoption of hybrids in many developing countries, including in sub-Saharan Africa, improved open-pollinated cultivars (OPCs) are still a viable and preferred option for many resource-poor farmers (Pixley, 2006). Consequently, CIMMYT's proVA maize biofortification project invests in developing OPCs and in enhancing proVA concentration for selected already-popular OPCs. Crosses or backcrosses of popular OPCs with proVA donor inbred lines are subsequently selected for increased proVA carotenoid concentrations and agronomic performance. Medium to high (0.55–0.90) heritability and a preponderance of additive over nonadditive effects determining proVA concentrations in maize (Egesel et al., 2003; Menkir et al., 2014; Suwarno et al., 2014) suggest that recurrent selection for proVA content should be effective (Coors, 1999; Hallauer and Miranda, 1988).

An important concern in trait-focused recurrent selection programs is the possibility of correlated effects, or unintended associated changes in other traits. Increasing the oil concentration in the long-term recurrent selection Illinois high oil population, for example, resulted in significantly decreased starch content, decreased kernel weight (yield), and increased germ (embryo) size as a percentage of the kernel (Dudley and Lambert, 2004). Similarly, recurrent selection for increased protein content not only decreased grain yield but also resulted in numerous secondary physiological changes, including greater efficiency in absorbing and translocating N, enhanced asparagine level, higher enzymatic activity in N metabolism, increased seed phytic acid, and decreased grain sugar (Below et al., 2004). Thus, while recurrent selection generally has proven effective for changing quantitative traits in maize, correlated effects are less predictable.

The objective of this study was to evaluate progress and scout for correlated effects from selection for increased concentrations of proVA carotenoids in three maize open-pollinated cultivars subjected to three cycles of S1 recurrent selection.

MATERIALS AND METHODS

Experimental Germplasm

Three open-pollinated maize cultivars, Obatanpa-SR, SAM4, and ZM305, were chosen because of their relevance to resource-poor farmers in southern Africa. 'Obatanpa' is a quality protein maize (QPM or modified *opaque-2*) cultivar originating from CIMMYT's population 63 (tropical, white, dent, QPM) that was first released in Ghana and subsequently in more than 10 African, Asian, and Latin-American countries (Atlin et al., 2011; Gunaratna et al., 2010; Krivanek et al., 2007). Obatanpa-SR is a modified version of Obatanpa that was improved at CIMMYT for resistance to maize streak virus disease, which is a serious disease of maize in many sub-Saharan African countries. Sintetico Amarelo de Milho 4 (SAM4) is a yellow-grained OPC that is popular in Angola, while ZM305 is an early maturing, white-grained OPC developed by CIMMYT breeders in southern Africa.

More than 400 plants of each OPC were planted, and at least 100 were used as females for crosses with proVA donor lines during the summer of 2003–2004 in Zimbabwe. The 12 proVA donor lines (KUI2007, A6, KUI3, KUI11, SC55, TZI18, A619, SC213, M162W, KUI43, CML124, and Pob. 445-57-2-1-B★3) were mostly temperate, but included tropical (Thailand origins) and two lines of mixed subtropical and temperate origin, identified by Torbert Rocheford (University of Illinois) as having the highest concentrations of proVA (5–8 μg g^{-1}) among lines evaluated to that date. The F_1 crosses were made between individual OPC female plants and individual male plants, making as many different crosses as possible for each OPC on the basis of synchronization of flowering; all males were planted on two dates to increase the probability of synchronization with each female. At harvest, the F_1 ears were bulked for each OPC, and a balanced bulk was used to form the first backcross generation (BC_1) using the respective OPC as the recurrent, female parent, during winter 2004 in Zimbabwe. The BC_1F_1 seed of each OPC was selected visually, and seeds with the most intense yellow or orange color were planted during winter 2004–2005 at Tlaltizapan, Mexico, to develop BC_1 S_1 ears. There were no white seeds or kernels among the BC_1S_1s or later generations of the OPCs.

The three OPCs were improved by S_1 recurrent selection for three cycles, during which 10% selection intensity was applied for total proVA concentration. The fewest number of S_1 lines evaluated for any OPC and cycle of selection was 120, and the most was 225, but for all cases we selected the 10% of lines with the largest concentration of proVA. Each cycle consisted of self-pollinating

Table 1. Experimental maize (*Zea mays* L.) genotypes and number of hand-pollinated full-sib ears produced for each of them at Agua Fria during winter 2007–2008 (AF08A) for use as seed for yield trials and at Tlatizapan and Agua Fria during winter 2008–2009 (TL09A and AF09A, respectively) for use in provitamin A laboratory analyses.

Open-pollinated cultivar	Selection method–cycle of selection	Pedigree	AF08A	TL09A	AF09A
			—— Number of full-sib ears harvested ——		
ZM305	S_1–Cycle 0	ZM305(ProA)BC_1c0	105	90	96
	S_1–Cycle 1	ZM305(ProA)BC_1c1	171	90	125
	S_1–Cycle 2	ZM305(ProA)BC_1c2	155	106	118
	S_1–Cycle 3	ZM305(ProA)BC_1c3	120	94	132
	FS/S_1–Cycle 1	ZM305(ProA)BC_1c2FS	206	104	115
Obatanpa-SR	S_1–Cycle 0	Obatanpa(ProA)BC_1c0	129	67	98
	S_1–Cycle 1	Obatanpa(ProA)BC_1c1	174	58	111
	S_1–Cycle 2	Obatanpa(ProA)BC_1c2	144	85	109
	S_1–Cycle 3	Obatanpa(ProA)BC_1c3	186	53	120
	FS/S_1–Cycle 1	Obatanpa(ProA)BC_1c2FS	171	91	112
SAM4	S_1–Cycle 0	SAM4(ProA)BC_1c0	124	81	129
	S_1–Cycle 1	SAM4(ProA)BC_1c1	168	95	129
	S_1–Cycle 2	SAM4(ProA)BC_1c2	163	67	111
	S_1–Cycle 3	SAM4(ProA)BC_1c3	168	69	109
	FS/S_1–Cycle 1	SAM4(ProA)BC_1c2FS	193	63	105

plants of the Syn_1 (first recombination of synthetic population, or F_1s among selected lines) of Cycle *n* to obtain S_1s. Seeds of each S_1 were then divided into a sample for analysis of proVA concentration, a sample for planting, and a reserve sample. S_1s were planted ear-to-row, and laboratory analyses were simultaneously initiated about 2 wk after being harvested. ProVA concentrations were available before flowering, and S_1 lines selected only on the basis of highest proVA concentration were recombined by making as many crosses as possible among individual plants of different S_1s (i.e., diallel mating scheme) to form the Syn_1 of Cycle *n* + 1. The crosses were harvested as half-sib families, that is, all crosses with the same female were bulked together, and each half-sib family was equally represented when planting the Cycle *n* + 1 for S_1 development. Exceptions to this process were: (i) during the first cycle (from zero to one), laboratory data were not available before flowering, so we developed S_2 ears that were subsequently used in the recombination to form Cycle 1, but the selection units were the S_1 lines; and (ii) for Obatanpa, we first evaluated protein quality and eliminated S_1s that did not meet the standards for QPM before evaluating and selecting those with most proVA concentration, but we still recombined about 10% of the original population size.

Four cycles were evaluated for each OPC: Cycles 0 (the initial BC_1 for each OPC), 1, 2, and 3 (Table 1). We included three genotypes in addition to the 12 key genotypes (four cycles of selection for each of three OPCs) for the evaluation of progress from S_1 recurrent selection described above (Table 1). Starting from Cycle 2 of each OPC, we conducted one cycle of full-sib (FS) selection for grain yield and agronomic performance (50% culling), combined with subsequent selection for proVA concentration among S_1s from selected FSs. This was done because of concerns that we should take the opportunity of this work to simultaneously improve yield potential and agronomic traits for the OPCs. Thus, three entries in the evaluation trial were the "BC_1c2FS" versions for each OPC. Seed of each genotype for use in yield trials was a bulk of 105 to 211 FS ears produced by hand pollination at Agua Fria, Mexico, during winter 2007–2008.

The same seed used to plant yield trials was also planted in single eight-row plots with about 200 plants each at two environments, Agua Fria and Tlaltizapan, Mexico, during winter 2008–2009, to produce grain for laboratory analyses of carotenoid concentrations. Previous reports indicating that genotype by environment interaction effects are small or not significant for proVA carotenoid concentrations (Egesel et al., 2003; Menkir and Maziya-Dixon, 2004; Pfeiffer and McClafferty, 2007; Menkir et al., 2014), as well as grain production and laboratory analysis cost considerations, were our justification for using only two environments for estimating carotenoid concentrations. Full-sib pollinations were made by hand within each plot, and 53 to 104 FS ears were kept at harvest for each plot at Tlatizapan, and 85 to 132 were kept at Agua Fria (Table 1). The ears were individually shelled and a balanced bulk was formed for each plot without exercising selection, except to avoid use of rotten kernels. Subsamples of 50 seeds were prepared for carotenoid analyses, and two such replications were sent to each of three laboratories, CIMMYT, the University of Wisconsin, and the International Potato Center (CIP) for analysis of carotenoid concentrations by HPLC. Each laboratory analyzed two samples from each of two environments for each genotype.

Field Experiments

The field experiments used a randomized incomplete block design in which each OPC (plus checks of similar maturity) was a main plot, and cycles of selection within OPC were the subplots. This design was necessary to block entries according to maturity, given that ZM305 is significantly earlier maturing than the other OPCs. Three replications of two-row plots were grown at each site, with row length typically 5 m, row spacing 0.75 m, and 53,000 to 90,000 plants per hectare, according to local recommendations. The trial was grown at 10 locations between 2008 and 2009 (Table 2).

Table 2. Sites where the agronomic evaluation trial was grown.

Site name	Country	Season	Latitude	Elevation	Important stresses
				masl†	
Tlaltizapan	Mexico	2008 summer	18.4 N	940	None
Celaya	Mexico	2008 summer	20.3 N	1750	Fusarium stalk rot
El Batan	Mexico	2008 summer	19.3 N	2250	Turcicum leaf blight
El Batan	Mexico	2008 summer	19.3 N	2250	Low soil nitrogen
Agua Fria	Mexico	2008–2009 winter	20.3 N	60	None
Agua Fria	Mexico	2008–2009 winter	20.3 N	60	Drought at flowering
ART Farm	Zimbabwe	2008–2009 summer	17.5 S	1500	None
Harare	Zimbabwe	2008–2009 summer	17.5 S	1500	Maize streak virus
Ikenne	Nigeria	2009	6.9 N	150	None
Tepalcingo	Mexico	2009 summer	18.6 N	1160	None

† Meters above mean sea level.

Laboratory Analysis of Carotenoids

As described above, carotenoid analysis was performed for hand-pollinated seed produced in two environments, Tlaltizapan and Agua Fria, during winter 2008–2009. The grain was promptly dried, shelled, counted, and transferred to cold storage (−80°C) to minimize carotenoid degradation while awaiting analysis. At the time of this study, carotenoid analysis capabilities at CIMMYT had just been established, so to validate our data, two 50-kernel samples of each entry were analyzed at three independent laboratories: the University of Wisconsin (Howe and Tanumihardjo, 2006a), the Food Quality Laboratory at the International Center of Potato (CIP) (Burgos et al., 2009), and at CIMMYT (Galicia et al., 2012).

At CIMMYT, analysis was performed as described in Babu et al. (2013a). Briefly, 50 kernels per entry were used for the carotenoid analysis using HPLC. Carotenoids were released from finely ground dried maize grain samples by adding ethanol. Samples were then saponified, followed by carotenoid extraction using hexane. Carotenoid separation and quantification were done using HPLC with a C30 column (YMC Carotenoid S-5 4.6 × 150 mm) attached to a C30 filter insert (YMC Carotenoid 5u, 4 × 2). A multiwavelength detector set at 450 nm was used, and data were collected and processed using Waters Millennium 2010 software (Waters Chromatography). Lutein, zeaxanthin, β-cryptoxanthin, and all-trans-β-carotene were identified through their characteristic spectra and comparison of their retention times with known standard solutions.

Total proVA content (μg g^{-1}) was calculated for each sample as the sum of β-carotene plus one-half of β-cryptoxanthin.

Statistical Analysis of Carotenoid Concentrations

Preliminary analysis showed strong laboratory effects but no laboratory by genotype interactions for all five carotenoid traits. This result indicated that the three laboratories ranked the genotypes relatively the same, and we, therefore, conducted combined analyses across all three labs. The general linear model (GLM) procedure of SAS (SAS Institute, 2008) was used to analyze carotenoid data across locations and laboratories for Cycles 0 to 3 for each of the OPCs. Locations and laboratories were fit as fixed effects, whereas genotypes, including cycles of selection (nested within OPC), and samples (50 g grain of each genotype) were considered random effects. To test for response to selection in any of the three OPCs, cycles of selection sum of squares (nested within each population) were further partitioned into linear and quadratic sums of squares. A second ANOVA with cycles of selection fit as fixed effects was then conducted individually for each OPC to test for response to selection for traits with significant response effects in the first ANOVA. Average response to selection per cycle was estimated for each OPC as the coefficient of regression from a linear regression model containing only the linear term using the REG procedure of SAS (SAS Institute, 2008). Percentage response to selection was estimated as the ratio of the linear regression coefficient (or slope) to the cycle zero intercept. Inferences about the statistical significance of linear or quadratic responses to selection were based on *F* tests of orthogonal polynomial contrasts using coefficients for equally spaced treatment levels as described in Steel and Torrie (1980, p. 363–372). Differences between specific entries or groups of entries in the experiment were estimated using CONTRAST and ESTIMATE statements in SAS (SAS Institute, 2008).

Repeatability of entry effects was estimated on a plot mean basis as the ratio of the genotypic variance to the total phenotypic variance.

Statistical Analysis of Agronomic Data

Managed drought, managed low nitrogen, and the highland location at El Batan in Mexico were analyzed individually because they were dissimilar according to Ward's minimum variance clustering method (Ward, 1963) for grain yield. The seven remaining environments clustered and were analyzed together as a nested randomized completed block with cycles of selection nested within each OPC. Open-pollinated cultivars were considered fixed, while cycles of selection (nested within OPC), environments, and replications were considered random. Analysis of variance was performed using the GLM procedure of SAS (SAS Institute, 2008). Cycles of selection (nested within OPC) sum of squares were also subpartitioned into linear and quadratic contrast sum of squares as described for carotenoid traits above. A second ANOVA was performed for each OPC for each trait with significant linear or quadratic response in the first analysis.

Linear and quadratic contrasts were used to test for trends in response to selection in each population, and *F* tests were performed using the genotype by environment interaction as

Table 3. Means across seven locations for grain yield (GY) number of days to anthesis (AD), and plant height (PH), and across two locations for lutein, zeaxanthin, β-cryptoxanthin (BCX), β-carotene (BC), and total provitamin A (ProVA) concentrations, as well as probabilities of significance for *F* tests of linear (β_1) and quadratic (β_2) contrasts.

Population	GY	AD	PH	Lutein	Zeaxanthin	BCX	BC	ProVA
	Mg ha^{-1}	d	cm			μg g^{-1}		
Obatanpa(ProA)BC$_1$c0	6.38	65.9	206.6	6.42	4.18	1.87	1.71	1.80
Obatanpa(ProA)BC$_1$c1	7.12	67.1	202.9	3.31	8.77	2.87	2.30	2.84
Obatanpa(ProA)BC$_1$c2	6.40	66.3	197.3	4.41	7.92	4.29	3.12	3.87
Obatanpa(ProA)BC$_1$c3	5.41	66.1	195.1	4.93	9.64	4.58	3.19	4.24
β_1 (linear)	**	ns[§]	*	ns	*	**	**	**
β_2 (quadratic)	**	**	ns	ns	ns	ns	ns	ns
ΔG cycle^{-1}[†]	−0.36 ±0.29	–	−4.0 ±0.4	–	1.55 ±0.73	0.95 ±0.16	0.53 ±0.11	0.84 ±0.12
ΔG cycle^{-1} (%)[‡]	−5.7	–	−2.0	–	37.1	50.8	31.0	46.7
SAM4(ProA)BC$_1$c0	7.80	67.7	204.1	4.34	9.81	3.52	2.00	2.84
SAM4(ProA)BC$_1$c1	6.83	67.5	190.5	5.42	12.23	4.39	2.37	3.59
SAM4(ProA)BC$_1$c2	6.80	67.3	193.1	5.93	12.56	4.79	3.00	4.19
SAM4(ProA)BC$_1$c3	6.15	67.7	183.7	5.14	13.36	5.49	3.65	4.97
β_1 (linear)	**	ns	**	ns	**	**	**	**
β_2 (quadratic)	Ns	ns	ns	**	ns	ns	ns	ns
ΔG cycle^{-1}[†]	−0.50 ±0.12	–	−5.9 ±2.1	–	1.10 ±0.31	0.63 ±0.06	0.56 ±0.05	0.70 ±0.02
ΔG cycle^{-1} (%)[‡]	−6.4	–	−2.9	–	11.22	17.89	28.03	24.65
ZM305(ProA)BC$_1$c0	5.13	61.0	183.5	4.12	4.75	2.48	1.10	1.78
ZM305(ProA)BC$_1$c1	6.55	62.2	175.2	3.62	8.22	3.87	1.94	3.04
ZM305(ProA)BC$_1$c2	5.65	63.0	170.1	3.29	6.42	5.36	2.24	3.74
ZM305(ProA)BC$_1$c3	6.23	64.2	175.0	4.25	12.65	7.74	3.18	5.49
β_1 (linear)	**	**	*	ns	**	**	**	**
β_2 (quadratic)	*	ns	ns	*	ns	ns	ns	ns
ΔG cycle^{-1}[†]	0.24 ±0.30	1.04 ±0.05	−3.1 ±2.1	–	2.19 ±1.04	1.73 ±0.17	0.66 ±0.09	1.18 ±0.14
ΔG cycle^{-1} (%)[‡]	4.7	1.70	−1.7	–	46.15	69.66	60.18	66.68
SEM	0.08	0.1	9.7	0.71	1.07	0.37	0.30	0.29
LSD (P = 0.05)	0.79	0.8	8.6	2.00	3.03	1.04	0.86	0.83
Repeatability	0.93	0.98	0.91	0.55	0.88	0.80	0.60	0.78

* Different from zero at $P \leq 0.05$.

** Different from zero at $P \leq 0.01$.

[†] Refers to the average genetic gain from recurrent selection, which was estimated as the coefficient of linear regression on cycle means. Inference on statistical significance of linear and quadratic responses to selection was made based on *F* tests of polynomial contrasts rather than regression coefficients because we had more power to detect trends with the contrasts.

[‡] Percentage of genetic gain per cycle for each open-pollinated cultivar was obtained by dividing the genetic gain per cycle by the cycle zero mean.

[§] ns, not significant.

the error term. Average and percentage response to selection per cycle and differences among specific entries were calculated as described for carotenoid traits above.

RESULTS AND DISCUSSION

Response to Selection for Provitamin A Concentration

The proVA carotenoids, β-cryptoxanthin and β-carotene, as well as total ProVA concentration increased linearly ($P < 0.01$) with cycles of selection for all three populations (Table 3 and Fig. 2). Lutein and zeaxanthin were not under direct selection, but the latter increased with cycles of selection while there was generally no change in the former; the linear model was not significant for lutein for all three populations, while the quadratic model was significant for SAM4 and ZM305 (Table 3).

Responses per cycle of selection for proVA carotenoids were large, ranging from 0.53 μg g^{-1} for β-carotene in Obatanpa to 1.73 μg g^{-1} for β-cryptoxanthin in ZM305 (Table 3). β-Cryptoxanthin was more abundant than β-carotene in all populations, and selection for increased total proVA concentration resulted in larger increases in β-cryptoxanthin (average of 1.10 μg g^{-1}) than β-carotene (average of 0.58 μg g^{-1}) concentration. Increases in proVA concentration were smallest for SAM4, which was not surprising because it was the only OPC that was already yellow and therefore contained some proVA before crossing with donors of large concentration of proVA. Being yellow, SAM4 already had the functional allele, whereas the white OPCs carried the null allele for the *PSY* gene, which encodes the phytoene synthase enzyme that catalyzes the first dedicated step in the carotenoid biosynthetic pathway and is responsible for the color difference

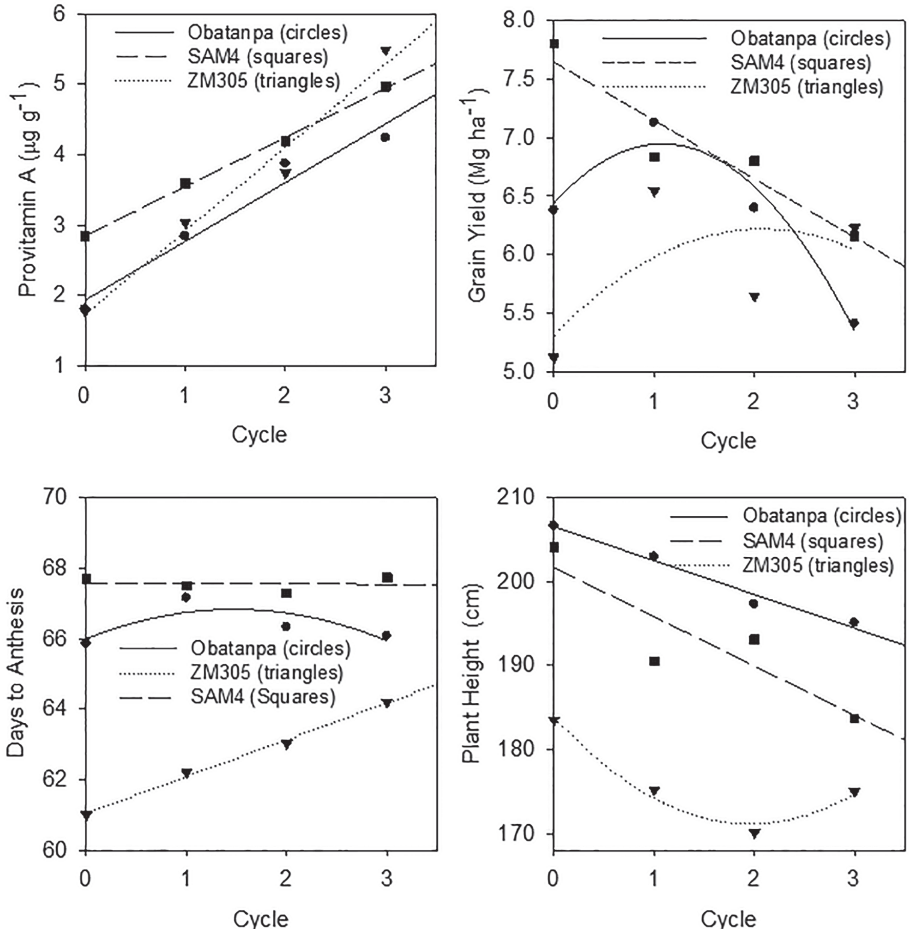

Figure 2. Response to three cycles of S_1 recurrent selection for provitamin A carotenoids and correlated responses for grain yield, plant height, and number of days from planting to anthesis in 'Obatanpa', 'SAM4', and 'ZM305' open-pollinated cultivars of maize (*Zea mays* L.).

between yellow and white in maize (Buckner et al., 1990; 1996). Thus the effect of the *PSY* allele from the proVA donor lines, which were yellow to orange in color, was expected to be large on Obatanpa and ZM305 and small or insignificant for SAM4. This is evidenced by the differences in Cycle 0 population means for total proVA content, which were 60% (1 µg g^{-1}) larger for SAM4 than for Obatanpa and ZM305 (Table 3).

Although direct comparison of the results reported here with other studies is difficult because of the differences in traits and selection methods, our responses are significantly larger than most results reported in the literature, even for other single-trait-focused selection studies (Dudley and Lambert, 2004). Our study was not designed to answer questions regarding the genetic basis of the observed large responses to selection; however, we believe that two main reasons may be responsible for this. First, the results are consistent with effects of a few major quantitative trait loci associated with proVA carotenoid concentration in Obatanpa, SAM4, and ZM305. Only a few major loci have been reported to be associated with proVA concentration in genetic mapping studies, including *PSY1* (Buckner et al., 1990; 1996), *LCYε* (Harjes et al., 2008), and *CRTRB1* (Yan et al., 2010). Second, we selected only for proVA concentration, and direct response to selection for a single trait is expected to be larger than response when selecting simultaneously for multiple traits (Hazel and Lush, 1942; Falconer and Mackay, 1996).

Genetic drift, or random changes in allele frequencies, can cause changes in population means, especially for small populations typical of recurrent selection experiments, such as this study (Falconer and Mackay, 1996). In recurrent selection studies, effects of drift are impossible to predict or quantify; however, random genetic drift would not be expected to cause large and consistent increases in proVA carotenoid concentrations, which we observed with selection across the three populations. Our results conclusively demonstrate that S_1 recurrent selection was highly effective at increasing proVA carotenoid concentration in the three OPCs.

Correlated Effects from Selection for Provitamin A

Differences for grain yield among the cycles of selection were highly significant ($P < 0.01$), with a heritability (repeatability) estimate across seven environments of 0.93 (Table 3). Differences among the cycles of selection were also statistically significant ($P < 0.01$) for number of

days to anthesis, number of days to silking, plant height, ear height, resistance to turcicum leaf blight (*Exserohilum turcicum*), and stalk lodging (data not shown). Grain yield, number of days to anthesis, number of days to silking, and plant height each showed significant linear responses associated with the three cycles of S_1 recurrent selection for increased proVA carotenoid concentrations in at least one of the three OPCs. Because number of days to anthesis and number of days to silking were highly correlated with each other ($r = 0.99$, $P < 0.01$), only data for number of days to anthesis are shown in Table 3.

Across the seven "target" environments, grain yield decreased (linear effect, $P < 0.01$) at an average rate of 0.36 Mg ha^{-1} cycle^{-1} and 0.50 Mg ha^{-1} cycle^{-1} for Obatanpa and SAM4, respectively, and increased (linear effect, $P < 0.01$) 0.24 Mg ha^{-1} cycle^{-1} for ZM305 (Fig. 2; Table 3). Grain yield under rainfed, well-fertilized conditions at the highland location (El Batan) declined on average by 9% ($P < 0.05$) for Obatanpa, 10% ($P < 0.01$) for SAM4, and 7% ($P < 0.05$) for ZM305 for each cycle of selection for increased proVA concentration (Table 4). These results at El Batan are consistent with those observed across the seven target environments, except for ZM305; however, at 2250 meters above sea level, El Batan is not a recommended environment for the OPCs evaluated in this study, and this result may reflect genotype by environment interaction. Grain yield of the different cycles of selection for the three OPCs did not differ under managed drought and low-N conditions, except a small negative trend ($P < 0.05$) for Obatanpa under drought (Table 4). Because we only had one location each for low-N, drought, and highland conditions, we cannot make strong conclusions about such environments from these data.

The number of days from planting to anthesis did not change with selection for increased proVA content for Obatanpa and SAM4 but increased 1.0 d cycle^{-1} ($P < 0.05$) for ZM305. Selection for increased proVA content significantly reduced plant height by an average 4.0, 5.9, and 3.1 cm cycle^{-1} for Obatanpa, SAM4, and ZM305, respectively (Table 3).

This study was not designed to answer questions regarding the genetic basis for the observed results, but we have a couple of hypotheses. First, given the limited opportunities for recombination after each synthetic was formed, we speculate that the levels of linkage disequilibrium (LD) in these populations were relatively high. Also, because the proVA donor inbred lines were derived mostly from temperate germplasm, they were presumably less elite for agronomic performance in tropical environments than the tropical OPCs. Linkage blocks contributed by the temperate donors of increased proVA concentration may have compromised grain yield under the target, tropical environments. With high LD, selection for increased proVA content would have favored selection for the proVA donor haplotypes relative to the generally more adapted OPC haplotypes, resulting in reduced grain yield.

Table 4. Correlated response of grain yield to three cycles of S_1 recurrent selection for provitamin A content under managed drought at Agua Fria, low nitrogen at El Batan, and normal highland conditions at El Batan (2250 m above sea level).

Population	Drought	Low N	Normal (highland)
	Mg ha^{-1}		
Obatanpa(ProA)BC$_1$c0	3.96	1.97	4.24
Obatanpa(ProA)BC$_1$c1	3.14	1.74	4.55
Obatanpa(ProA)BC$_1$c2	3.28	2.02	3.92
Obatanpa(ProA)BC$_1$c3	3.01	1.35	3.19
β_1 (linear)	*	ns[§]	*
β_2 (quadratic)	ns	ns	ns
ΔG cycle^{-1}[†]	−0.27 ±0.11	–	−0.38 ±0.14
ΔG cycle^{-1} (%)[‡]	−6.82	–	−8.96
SAM4(ProA)BC$_1$c0	3.19	1.81	4.14
SAM4(ProA)BC$_1$c1	3.57	2.06	4.26
SAM4(ProA)BC$_1$c2	3.02	1.63	3.51
SAM4(ProA)BC$_1$c3	3.16	2.14	2.95
β_1 (linear)	ns	ns	**
β_2 (quadratic)	ns	ns	ns
ΔG cycle^{-1}[†]	–	–	−0.43 ±0.11
ΔG cycle^{-1} (%)[‡]	–	–	−10.39
ZM305(ProA)BC$_1$c0	3.71	2.74	5.51
ZM305(ProA)BC$_1$c1	3.37	3.12	4.5
ZM305(ProA)BC$_1$c2	2.85	2.95	4.9
ZM305(ProA)BC$_1$c3	3.74	2.81	4.14
β_1 (linear)	ns	ns	*
β_2 (quadratic)	*	ns	ns
ΔG cycle^{-1}[†]	–	–	−0.37 ±0.13
ΔG cycle^{-1} (%)[‡]	–	–	−7.0
SEM	0.18	0.16	0.28
LSD ($P = 0.05$)	0.52	0.47	0.83

* Different from zero at $P \leq 0.05$.

** Different from zero at $P \leq 0.01$.

[†] Refers to the average genetic gain from recurrent selection, which was estimated as the coefficient of linear regression on cycle means. Inference on statistical significance of linear and quadratic responses to selection was made based on *F* tests of polynomial contrasts rather than regression coefficients because we had more power to detect trends with the contrasts.

[‡] Percentage of genetic gain per cycle for each open-pollinated cultivar was obtained by dividing the genetic gain per cycle by the cycle zero mean.

[§] ns, not significant.

A second possibility is that inbreeding depression contributed to the declines in plant height and grain yield associated with selection for increased proVA concentration. The recurrent selection was performed on closed populations and the scheme involved only one recombination of crosses among selected S_1 lines to form F_1s per cycle of selection. Although F_1s with the same female were bulked to form half-sib F_1 bulks that were planted separately in an effort to maximize parental representation when generating S_1s for each cycle of improvement, it is possible that there was little allelic variation for the relatively few genes (e.g., one or more of *PSY*, *LCYε*, and *CRTRB1*) determining the observed differences for proVA

Table 5. Contrasts of means for Cycle 3 of S_1 recurrent selection for total provitamin A concentration versus one cycle of full-sib selection for grain yield (50% selection) plus total provitamin A concentration conducted on the same initial Cycle 2 populations.

Contrast†	GY‡	AD‡	PH‡	Lutein	Zeaxanthin	BCX‡	BC‡	ProVA‡
	Mg ha^{-1}	d	cm			μg g^{-1}		
Obatanpa (C3-FS)	−1.59**	−1.98**	−10.12*	1.29	0.69	1.07*	1.01*	1.22**
SAM4 (C3-FS)	−0.89**	0.100	−4.00	−0.69	−1.29	0.29	0.67	0.68
ZM305 (C3-FS)	−0.41*	1.10*	−4.19	0.16	−0.68	3.11**	0.92*	1.99**

* Different from zero at $P \leq 0.05$.

** Different from zero at $P \leq 0.01$.

† Differences between means were estimated by subtracting the FS mean from the C3 mean using the ESTIMATE statement in SAS. Estimates were tested for statistical significance from zero using a *t* test.

‡ GY = grain yield, AD = anthesis date, PH = plant height, BCX = β-cryptoxanthin, BC = β-carotene, ProVA = total provitamin A.

concentration. This may have led to narrowing of genetic diversity (a genetic "bottleneck") and inbreeding depression, measurable as decreased plant height and grain yield. This hypothesis would also help explain the contrast with results reported by Menkir et al. (2014) and Suwarno et al. (2014) and found for numerous unpublished hybrid evaluation trials from our proVA biofortification breeding program, which consistently found no significant correlation between grain yield and proVA carotenoid concentrations.

Data in Table 5 indicate that 50% selection intensity for grain yield before selection for increased proVA concentration among FS families derived from Cycle 2 of S_1 selection for each population, resulted in significantly increased grain yield for all three populations compared with their respective third cycle of S_1 selection for proVA concentration alone. Incidentally, plant height also increased, although this was only significant for Obatanpa. Unfortunately, these gains in grain yield were at the expense of 0.68 to 1.99 μg g^{-1} of total proVA carotenoid concentration. It is interesting to note that Ceballos et al. (2013) recently reported analogous results for FS recurrent selection for increased total carotenoid concentration in cassava (*Manihot esculenta* Crantz); they achieved consistent gains for total carotenoid and β-carotene concentrations with an associated declining trend in tuber dry matter content (yield) and were able to select outstanding clones (families) with superior carotenoid and dry matter content values from each cycle of selection.

The total carotenoid concentration (lutein + zeaxanthin + β-cryptoxanthin + β-carotene) of the three populations increased by an average of 74% during the three cycles of selection for proVA concentration (data not shown), which may have resulted from selection for alleles (e.g., for PSY, phytoene desaturase, or zeta-carotene desaturase) that result in enhanced total production of lycopene and therefore increased flux into the carotenoid pathway (Fig. 1; see also Kandianis et al., 2013). Alternatively, alleles may have been selected that decrease the onward flux (e.g., of zeaxanthin toward abscisic acid) or catabolism (e.g., carotenoid cleavage dioxygenase 1) of carotenoids. Interestingly, S_1 recurrent selection for increased total proVA resulted in quite different changes in concentrations for the four carotenoids. As might be explained by selection for preferential flux into the β branch of the carotenoid pathway, as is the case with selection for proVA-enhancing allele(s) of *LCYε* (Harjes et al., 2008; Babu et al., 2013a), lutein concentration was unchanged for all populations, while zeaxanthin more than doubled, except for the yellow-grained population (SAM4) in which it also increased, but only by 36%. On the other hand, because β-cryptoxanthin and β-carotene increased in similar proportions within each population and across populations, it appears that no significant selection occurred (and perhaps no genetic variation existed) for alleles of *CRTRB1* that would reduce hydroxylation of β-carotene. The fact that zeaxanthin concentration increased for all populations also suggests that hydroxylation-reducing alleles of *CRTRB1* were not selected (Babu et al., 2013a).

Given recent experience and publication of results for 26 tropical maize populations (Babu et al., 2013a) demonstrating the effectiveness of marker-assisted selection (MAS) for alleles of *CRTRB1* that result in reduced hydroxylation of β-carotene to β-cryptoxanthin, proVA breeding programs are increasingly implementing this approach. At the same time, MAS for a favorable allele of *LCYε* first reported by Harjes et al. (2008) has become less common or is no longer conducted, because this allele has less effect than *CRTRB1* on total proVA concentration, and because the available markers are less efficient than those for *CRTRB1* (CIMMYT, R. Babu, personal communication, 2011; see Babu et al. [2013a] for general discussion). Our breeding program has developed maize lines with more than 20 μg g^{-1} of total proVA by applying this strategy; however, there is evidence suggesting that less aggressive selection for the favorable *CRTRB1* allele may be more effective in sustaining long-term gains from selection without significantly affecting the concentration of other essential carotenoids.

There is a growing body of literature examining nutritional aspects of proVA carotenoids and providing insights for efficacious biofortification strategies (see Tanumihardjo et al., 2010). Burri et al. (2011) demonstrated experimentally and reviewed literature to conclude that β-cryptoxanthin is generally more bioavailable than β-carotene from foods

typical of Western diets, while Schmaelzle et al. (2014) concluded the same using biofortified maize genotypes. Davis et al. (2008a,b), Howe and Tanumihardjo (2006b), and Schmaelzle et al. (2014) have reported that β-cryptoxanthin in maize has similar bioconversion and bioefficacy as β-carotene. This finding for bioefficacy is significant because current calculations, including herein, assume the theoretical bioconversion rates of two β-cryptoxanthin molecules versus only one β-carotene molecule to form one retinol (vitamin A) molecule. If the calculation of total proVA is adjusted according to these new findings, becoming the sum of β-carotene plus β-cryptoxanthin instead of β-carotene plus one-half of β-cryptoxanthin, the proVA-enhancing value of breeding for enhanced β-carotene at the expense of β-cryptoxanthin, as occurs when selecting for the partial-knockout allele of *CRTRB1*, disappears. Selecting for reduced hydroxylation of β-carotene would still have the apparent proVA-enhancing advantage of reducing the total "leakage" of carotenoid to non-proVA carotenoids, zeaxanthin and further downstream in the pathway (Fig. 1); however, this potential advantage is likely negated in many genetic backgrounds by feedback inhibition of total flux into the pathway in response to this partial blockage by *CRTRB1* (Babu et al., 2013a). In addition, Babu et al. (2013a) reported a potentially undesirable, greatly reduced concentration of zeaxanthin for some populations in which selection for the proVA favorable allele of *CRTRB1* had been applied. In view of this controversy—to breed or not to breed for more β-carotene at the expense of β-cryptoxanthin—our results provide important evidence that proVA biofortification of maize can be achieved without following the demonstrated "short-cut" route of selecting defective (partial knockout) alleles of *CRTRB1*.

The limits of selection for increased proVA concentration in maize are unknown, and it is also unknown whether the levels already achieved using MAS for alleles of *CRTRB1* that reduce hydroxylation of β-carotene can be achieved otherwise. A strategy toward enhancing proVA concentrations without reducing the action of *CRTRB1* would likely include MAS for proVA-enhancing alleles of *LCYε*, which preferentially increase flux to the proVA-carotenoid-rich β branch (away from the α branch), without affecting the ratio of β-carotene to β-cryptoxanthin and without decreasing zeaxanthin concentration. It is also likely that untapped allelic diversity exists for genes upstream of lycopene and for genes coding cleavage enzymes that catabolize carotenoids, some or all of which could be incorporated into new strategies to biofortify maize with increased concentrations of proVA carotenoids.

CONCLUSIONS

Our results demonstrate that proVA concentrations were increased during three cycles of S_1 recurrent selection in three diverse maize populations with no indications of declining rates of improvement. The breeding strategy did not select nor result in reduced hydroxylation of β-carotene, which is the cornerstone of many of the most effective proVA breeding programs today. The value of our findings is enhanced by recommendations from some nutritionists suggesting that it may be undesirable to sacrifice β-cryptoxanthin to increase β-carotene concentration in grain. The negative trend in grain yield observed in association with single-trait selection for enhanced proVA concentration for two of the three populations in this report contrasts with multiple previous findings of no correlation between grain yield and proVA concentration in maize. We hypothesize that linkage drag from the proVA donor parents or inbreeding depression resultant from specific circumstances of this project may be responsible for this undesirable association and conclude that breeding for enhanced proVA concentrations in maize should simultaneously consider grain yield.

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