Provitamin-A Enrichment in Maize

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Introduction

Maize is an important cereal crop of the world and it is a staple food for 1.2 billion people of Sub-Saharan Africa and Latin America (www.iita.org). Some of the important maize-growing countries are the USA, China, Brazil, India, Philippines, South Africa, and Indonesia. While vitamin A deficiency (VAD) is prevalent in Sub-Saharan Africa and Southeast Asia (FAO, 2009), maize bio-fortification (i.e., increasing essential micronutrient levels) with high-levels of proVA carotenoids in the grain, is considered a promising solution to overcome this deficiency. HarvestPlus, through the International Maize and Wheat Improvement Center (CIMMYT) and other partners, has been breeding maize hybrids and open pollinated varieties with increased total proVA carotenoid concentrations since 2004 (Pfeiffer and McClafferty, 2007b).

Vitamin A is an essential micronutrient controlling several biological processes including vision, growth, and immunity. VAD affects growth; increases risk to several diseases including night blindness possibly leading to corneal blindness, and can cause reproductive disorders, as well as stunted growth among affected children (West, 1991; Ortiz-Monasterio et al., 2007; West and Darnton-Hill, 2008; www.harvestplus.org). Vitamin A deficiency related-health impacts are most severe among preschool-aged children and pregnant woman (Rice et al, 2004). Deficiency of vitamin A can compromise the immune system thereby increasing the risk of mortality from infectious diseases such as measles and malaria and other disorders such as diarrhea (Rice et al, 2004).

Several interventions to scale improved levels of vitamin A consumed in diets include supplementation, food fortification, and dietary diversification (FAO, 2009). However, because maize is a staple food in many parts of Africa, development of maize varieties that are bio-fortified with considerably high-concentrations of proVA carotenoids in the grain is a key approach for alleviating VAD in these regions. The minimum proVA content in maize grains needed to reduce VAD is 15 µg/g. Although there is no consensus about the exact ratio, recent reports indicate that bio-conversion of β-carotene from maize to vitamin A occurs at a ratio of about 2.8 mg:1 mg (Howe and Tanumihardjo, 2006) or 3.2 mg:1 mg (Muzhingi et al., 2011). Other carotenoids, lutein and zeaxanthin, are available in greater quantity in maize than β-carotene; however, although they have other health benefits, they do not have proVA (Ortiz-Monasterio et al., 2007). Furthermore, a recent study indicated that proVA could enhance the bio-availability of Fe. Deficiency of this essential micronutrient causes widespread health problems (Pixley et al., 2011).

Breeding for increased concentrations of proVA is promising because there is considerable genetic variation available in maize germplasm. Initial CIMMYT studies revealed that among 1,000 tropical maize genotypes, total proVA varied from 0.24 to 8.80µg/g, while the proportion of total proVA carotenoids ranged between 5 percent and 30 percent (Ortiz-Monasterio, 2007). The HarvestPlus project conducts extensive work on improving proVA levels in elite maize lines, hybrids and synthetic populations. Classical and molecular breeding methods have been implemented, including use of various temperate- and tropical-sources with high-concentrations of proVA, and marker assisted selection.

During the initial years of the HarvestPlus breeding program, measurements of carotenoid content in breeding materials were performed using the high-cost, high-performance liquid chromatography (HPLC),. Through extensive search and characterization, some genotypes that possessed more than 15 µg/g proVA were identified. Further, through genetic studies using three-germplasm panels containing genotypes with varied carotenoids content, two genes encoding two key enzymes in the carotenoid pathway, β-carotene hydroxylase-1 (*CrtRB1*) on chromosome 10 (Yan et al., 2010; Babu et al., 2012) and lycopene epsilon cyclase (*LcyE*) on chromosome 8 (Harjes et al. 2008), have been recently reported to affect proVA carotenoid concentrations in maize grain. A diversity of haplotypes related to these two genes has been found in the breeding materials.

During the development of breeding program, selection of homozygous genotypes at the *CrtRB1* locus was performed using a marker linked to the gene. This marker assisted selection (MAS) approach resulting in identification of several genotypes having higher β-carotene concentrations than those previously identified. Utilization of these genotypes as new parent lines for crosses, accelerates the progress of the

breeding program. Recently, in a number of improved inbred lines and populations, the concentration of proVA in the grain has reached 30 µg/g.

In terms of breeding products, three-way cross are preferred over single-cross hybrids in several maize consuming countries where VAD is prevalent, because seed production is less expensive while considerable uniformity of the hybrid plants can still be obtained. In all hybrid development programs, understanding general and specific combining ability of and between lines, and forming and exploiting meaningful heterotic groups is key to success. Egesel et al. (2003) evaluated a 10-parent diallel (45 maize hybrids) and found that variation for carotenoids was more attributable to general than to specific combining ability effects, indicating a major role for additive gene action.

ProVA maize varieties are not only expected to have a high-content of proVA, but also need to have other key characteristics desired by farmers, including high-yield, good agronomic performance, and good-eating quality. In addition, consumers need to be convinced that the bio-fortified grain can produce palatable foods, and proVA produced are bio-available. Breeding for bio-fortification requires an interdisciplinary approach, including the field of plant breeding, nutrition, biochemistry, and food technology.

Maize carotenoid profile

ProVA carotenoids are available only in orange and yellow maize (not white maize). Genetic diversity of maize carotenoid is quite high (Table 1). In general, yellow maize has a proVA carotenoid content of <2 µg/g. ProVA carotenoids which become a precursor of vitamin A are β-cryptoxanthin, α- and β-carotene. Lutein and zeaxanthin are available in greater quantity in maize than beta-carotene and serves to reduce the risk of cataracts, however they do not have proVA activity (Ortiz-Monasterio et al., 2007). The proportion of lutein and zeaxanthin ranged between 30-50 percent of total carotenoids in maize, while proVA carotenoids by 10-20 percent.

Table 1. Examples of ranges of carotenoid content in maize kernels (adapted from Pixley et al, 2013). Data are expressed in µg/g DW.

Carotenoids	Germplasm adaptation			
	Temperate		Tropical/ Subtropical	
	Example 1	Example 2	Example 1	Example 2
Lutein	0.0 – 27.5	0.0 – 31.0	1.3 – 32.3	0.4 - 19
Zeaxanthin	0.01 – 7.7	0.76 – 43.9	0.3 – 34.8	0.3 – 21.5
β-cryptoxanthin	0.07 – 2.4	0.16 – 10.8	0.0 – 6.13	0.3 – 4.6
β-carotene	0.07 – 7.6	0.07 – 13.6	0.7 – 5.8	0.3 – 4.3
Total proVA	0.1 – 8.8	0.15 – 19.0	0.7 – 8.8	0.45 – 6.6
References	Kurilich and Juvik (1999)	Harjes et al (2008)	Ortiz-Monasterio et al (2007)	Menkir et al (2008)

Tropical maize generally contains more β-cryptoxanthin and less β-carotene than temperate maize. Hence, in order to improve β-carotene content, several source genotypes for the HarvestPlus breeding program were selected from temperate regions. This became a challenge because although the improved varieties have many desirable traits from source genotypes, they may not suitable to the environments where distribution is targeted, because, for example, they may have low-grain production.

ProVA maize bio-fortification breeding programs require fast, accurate, and inexpensive screening techniques to determine proVA concentrations in the grain. A screening alternative includes the use of a color score and near-infrared reflectance spectroscopy (NIRS). The deep orange-colored maize is likely to have higher proVA content than the pale yellow maize, and therefore this indicator was commonly used by breeders as an early-selection criteria in the field. However, it should be noted that the correlation coefficient between color scores (shades of yellow) and proVA carotenoids concentration is relatively low (0.2-0.4) (Harjes et al, 2008). A color measurement method using HunterLab miniscan produces a higher-correlation coefficient (0.6) (Lozano-Alejo et al, 2007), yet the reliability of this method has not yet been proven for a wide range of germplasm. Researches on the use of NIRS to quantify carotenoids showed that this method can be used to estimate the lutein, zeaxanthin, and total carotenoids, but is not accurate for proVA carotenoids (Berardo et al, 2009; Zum Felde, pers. comm.). Thus, quantification of the proVA content in the laboratory is still required to determine an accurate value.

Analyses of carotenoid concentrations could occur from grain harvested from self-pollinated plants. Random samples of 20 to- 30 seeds should be refrigerated at -80°C and ground to a very fine powder

(Howe and Tanumihardjo, 2006). The laboratory procedure for carotenoid quantification includes saponification to remove lipids, extraction, separation, and quantification (Galicia et al, 2008). One widely-used extraction method involves the use of one or more organic solvents including hexanes, tetrahydrofuran, methanol, ethanol, or ethyl acetate.

Carotenoids quantification can be performed using a reverse-phase high-performance liquid chromatography (HPLC) coupled with a photodiode array detector, or an ultra-performance liquid chromatography (UPLC). The use of UPLC can increase the speed of output as much as six times the speed of HPLC, at a lower cost. Carotenoid compounds that can be measured include lutein, zeaxanthin, β-cryptoxanthin, and β-carotene (all-trans, 9-cis, and 13-cis isomers). Total proVA concentration can be calculated as all-trans β-carotene + 9-cis β-carotene + 13-cis β-carotene + 0.5(β-cryptoxanthin).

Carotenoids are easily degraded by interactions with other molecules, and therefore, to maximize the consistency of proVA carotenoids measurements results across genotypes breeders should: (1) use kernels with the same stadia of physiological maturity and same moisture content; (2) minimize storage time of samples; (3) utilize low-temperatures in the sample storage (-20 to -80 C); (4) minimize the time for extraction and analysis; and (5) protect the sample from oxygen and white light. Some other important measures include the use of checks, internal standards, and laboratory replicates.

Prospects and progress on proVA biofortification breeding

The existence of genetic diversity for carotenoid content in maize grains, is the ultimate requirement in conventional plant-breeding activities for carotenoid enhancement. Therefore, breeding activity begins by evaluating the carotenoid profile in several maize genotypes. Initial CIMMYT studies revealed that there is variation in total proVA among more than 1,000 tropical genotypes, ranging from 0.24 to 8.80 µg/g (Ortiz-Monasterio, 2007). Also, the study revealed that the proportion of total proVA in total carotenoids varies between 5 percent to 30 percent.

ProVA trait is controlled by relatively few genes, thus its inheritance is relatively simple (Pfeiffer and McClafferty, 2007a). In terms of combining ability, a diallel study of 45 corn hybrids indicated that variation for carotenoids was more attributable to general combining ability (GCA) than to selected combining ability (SCA) effects, indicating a major role for additive gene action (Egesel et al., 2003). Additionally, in our recent study on heterotic group formation in a proVA breeding program, we found no evidence of heterosis for carotenoid traits among line combinations from different putative heterotic groups, as indicated by non-significant comparison on between versus within heterotic groups (Suwarno et al, 2014; Table 2). This study also revealed that the repeatability for carotenoid traits is relatively high (67 percent to 89 percent), indicating that these traits are less-affected by environmental effects (Suwarno et al., 2014; Table 2).

The HarvestPlus' proVA maize breeding program conducts extensive work to improve proVA levels in elite maize lines, hybrids and synthetic populations. Beginning in 2004, early variety development crossed white elite strains from Zimbabwe with yellow proVA strains from temperate regions (received from T. Rocheford, University of Illinois, USA). Breeders backcrossed each F_1 to its' elite parent before the formation of inbred lines. A number of early generation (S_2) genotypes were crossed with two testers, and the resulting hybrids were evaluated in Stage 1 trials. A total of approximately 20 percent were selected and these lines were self-pollinated and subsequently crossed with three-testers, for further evaluation in Stage 2 trials. As many as 10 percent to 20 percent of the selected lines from Stage 2 trials were crossed with three, single-cross testers, and the resulting three-way cross hybrids were evaluated at six- to eight-locations. Five hybrids with proVA content of 6-9 µg/g were tested in Zambia's National Performance Trials (NPT) in 2010 to 2011. Currently, the target of 15 µg/g of proVA carotenoids has been achieved in some of the breeding lines and populations, and three outstanding hybrids with total proVA carotenoid concentration more than 7 µg/g have been officially released for commercialization in Zambia in 2012.

More recently, the HarvestPlus' proVA maize breeding program utilized some lines with considerably higher-proVA concentrations (compared to those available at the early stages of the program). The process in Figure 1 continues, repeatedly, resulting in a breeding pipeline with better products than before the onset of the program. Two important advancements in the program were: (1) discovery of allelic diversity for the LcyE; and CrtRB1 genes and development of MAS to select genotypes with favorable alleles of these genes; and (2) use of UPLC in place of HPLC, which increases efficiency of carotenoids phenotyping.

Table 2. Examples of grand mean, between and within putative heterotic groups mating means, and repeatability for carotenoid traits (adapted from Suwarno et al, 2014). Data are expressed in µg/g DW.

	Lutein	Zeaxanthin	β-cryptoxanthin	β-carotene	Total proVA
Grand mean	3.49	5.90	4.87	3.88	10.76
Between groups mean (B) ¶	3.39	5.74	4.84	3.88	10.78
Within groups mean (W) ¶	3.91	6.57	5.00	3.89	10.67
B-W difference#	-0.52^{+}	-0.83 NS	-0.16 NS	-0.01 NS	0.11 NS
Rpeatability	0.89	0.67	0.89	0.56	0.85

Probability of significance: ** P<0.01, * P<0.05, + P<0.10, NS=not significant

¶ Estimated across four environments (TL10, TL11, TLCA11, CL11) for grain yield and three environments (TL10, TL11, TLCA11) for carotenoid concentrations.

F-tests using models with the 'bw' and 'bw x env' factors aggregating between groups ('b') and within group ('w') hybrids, respectively.

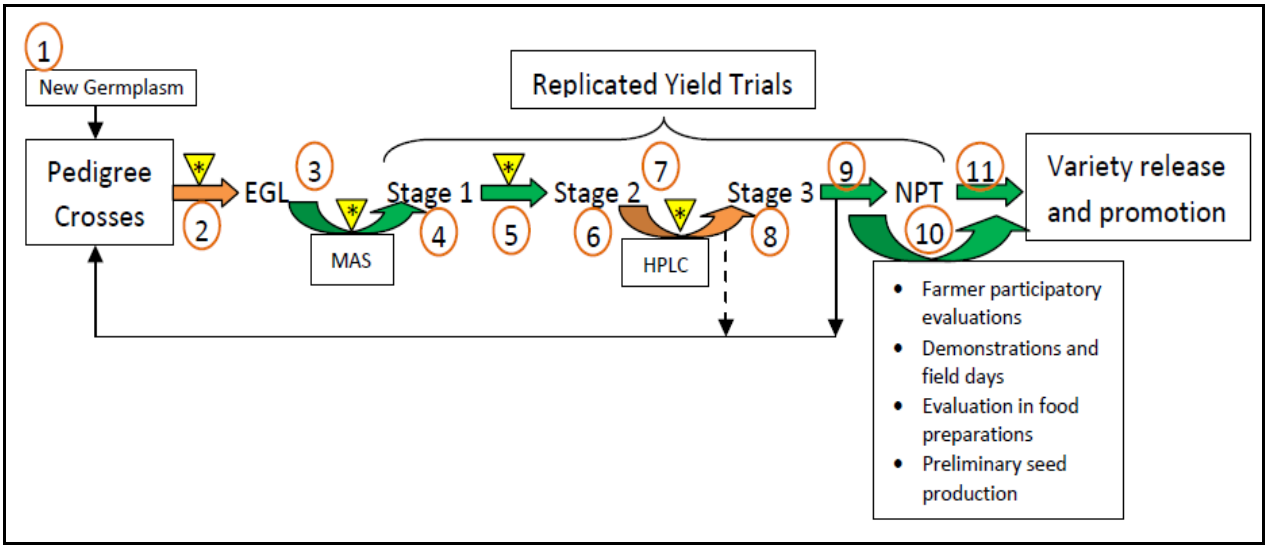


Figure 1. Generalized plant breeding or biofortified product development scheme (adapted from Pixley et al, 2013). Circled numbers refer to steps discussed in the text. EGL, early generation line; MAS, marker-assisted selection; HPLC, high performance liquid chromatography; NPT, national performance trial (Zambia). *Indicates major opportunities for agronomic selection, e.g. for disease resistance.

Molecular tools for facilitating efficient proVA breeding

Recent development of the HarvestPlus' proVA maize varieties involves the use of marker-assisted selection (MAS), this approach facilitates efficient breeding for high-levels of proVA carotenoids in maize (Prasanna et al., 2010). MAS is a method of selecting plants having desired alleles at one or more locus using DNA markers. The effectiveness of MAS associated with the complexity of the trait are currently being studied. A number of quantitative traits, including yield, are controlled by many minor genes and are highly-influenced by environmental factors, hence MAS is not effective for this case. A number of other quantitative traits, including proVA content in maize seeds, are controlled by a few major genes. For such a trait, selection using markers linked to relevant genes may result in a desired response. Additionally, a MAS method would be effective for plant breeding only if it has been successfully validated using a wide-range of germplasm.

Several studies validate that proVA content in maize grain is controlled by three major genes: phytoene synthase 1 (Y1 syn. Psy1); lycopene epsilon cyclase (LcyE) (Harjes et al., 2008); and β-carotene hydroxylase 1 (*CrtRB1*) (Yan et al., 2010; Babu et al., 2012) (Table 3). *Y1* activates the carotenoid pathway and is responsible for color variation of maize seeds from white to yellow, and other colors. *LcyE* and *CrtRB1* genes play a role in the accumulation of carotenoids in the endosperm. Natural mutant alleles of *LcyE* with reduced function can lead to the division of lycopene more in the direction toward the β- branch of the pathway, which results in more accumulation of proVA carotenoids (12). In addition, natural genetic variation for *CrtRB1* with reduced functionality plays a role in flux inhibition from β-carotene to β-

cryptoxanthin, resulting in an increased accumulation of β-carotene.

In practice, the MAS approach using markers associated with *CrtRB1* is proven to be efficient in improving selection for accumulated proVA concentrations. *CrtRB1* was found to explain a 15-fold change in the β-carotene to β-cryptoxanthin ratio (Yan et al., 2010), and therefore, it is a favorable allele for advancing the breeding process. Although not as significant as *CrtRB1*, *LcyE* was also found to have considerable influence on proVA carotenoid concentrations, with four-polymorphisms of this gene accounting for three-fold differences of β-carotene and β-crypoxanthin ratio (Harjes et al., 2008).

Conventional selection (without molecular aid) during the early stages of the HarvestPlus breeding program resulted in improved genotypes. These had higher proVA concentrations (8 µg/g). Recently, a number of genotypes were identified that possessed up to 26 µg/g β-carotene and 30 µg/g total proVA. These were obtained from *CrtRB1* MAS and individuals with homozygous favorable allele at the CrtRB1 locus were selected. Application of seed-based DNA extraction enabled breeders to only plant individuals having favorable alleles at target loci. This saves time and resources. Analysis using HPLC and UPLC are still required to quantify the actual amount of carotenoids, even though MAS is low-cost and effective.

While marker assisted selection for favorable allele(s) of *CrtRB1* has been very helpful during the development of outstanding high proVA maize cultivars, moving forward, the search for new genes and the use of innovation such as genomic prediction tools offer opportunities to improve efficiencies in the selection processes and to explain phenotypic variation. Association-mapping has been used extensively in the past, to identify genes that control important phenotypes in plants, such as flowering time in maize (Ducrocq et al., 2008; Buckler et al., 2009), arabidopsis (Brachi et al., 2010), barley (Stracke et al., 2009), and ryegrass (Skøt et al., 2005); yield and its components in rice (Agrama et al., 2007); quality traits in potato (D'hoop et al., 2007) and cotton (Abdurakhmonov et al., 2008).

Table 3. Carotenoid-associated genes in maize (adapted mainly from www.maizesequence.org)

Chromosome	Position	Gene name	Abbv.
1	17,660,941-17,667,054	Phytoene desaturase	Pds1
2	15,865,938-15,868,219	β-carotene hydroxylase	Hyd1
2	44,440,299-44,449,237	Zeaxanthin epoxidase	ZEP
6	55,671,246-55,674,458	Phytoene synthase	Psy
7	17,470,585-17,479,020	ξ-carotene desaturase	Zds1
8	138,882,594-138,889,812	Lycopene epsilon-cyclase	LcyE
8	168,273,042-168,276,092	Phytoene synthase 2	Psy2
9	153,692,212-153,694,576	β-carotene hydroxylase	Hyd5
10	136,057,214-136,060,219	β-carotene hydroxylase	CrtRB1
10	4,705,086-4,705,639	Phytoene synthase 3	Psy3

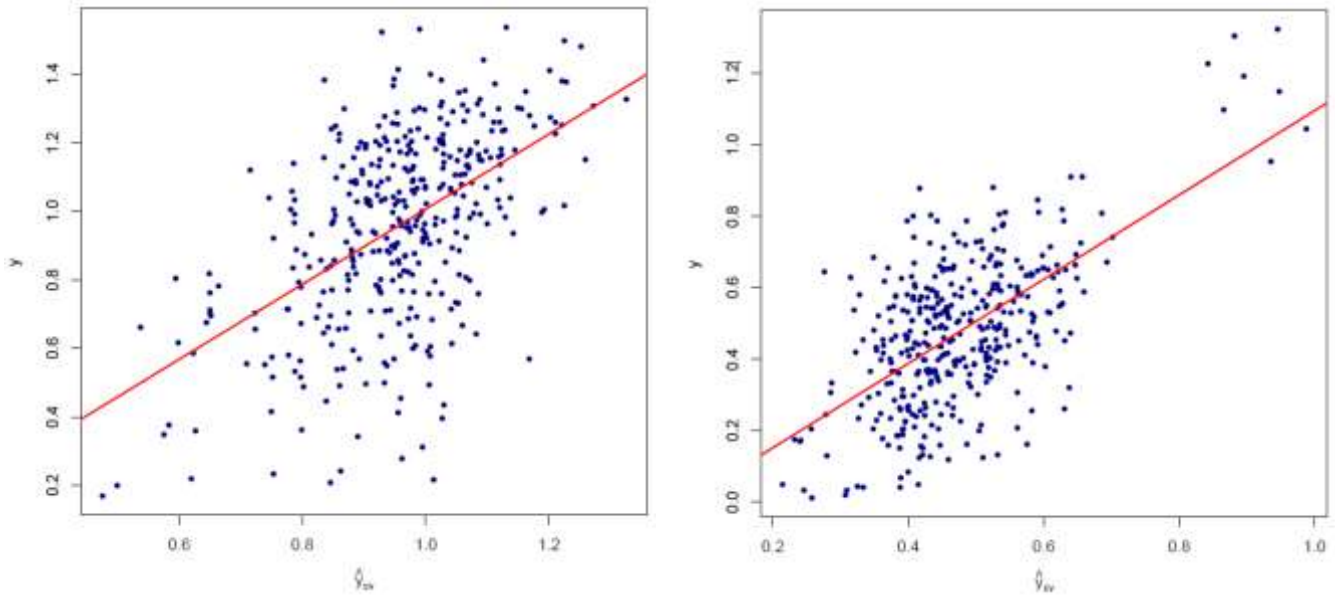
However, association-mapping has a few significant advantages compared to the linkage-mapping approach. It enables selection from wider variances of the trait of interest and more precise, finite, mapping results (reviewed in Yu and Buckler, 2006). Rather than utilizing a structured population (for example, F_2, backcross, or recombinant inbred lines [RIL]) in the linkage-mapping approach, association-mapping technique relies on historical recombination that have occurred over many years. Therefore, the association-mapping approach would be more applicable for cross-pollinated species rather than for self-pollinated ones because in the former, natural recombination occurred at a much higher frequency and developed greater polymorphism among germplasm accession at the loci of interest, enabling higher-mapping resolutions.

Because a diverse collection of inbred lines are used in association studies, population structure and genetic relationships among lines are two-major challenges that could cause spurious associations between markers and the trait of interest. Several advanced statistical methods have been used to obtain more precise results; one of the most recent approaches is using a mixed-linear model involving genotype (G) and population structure (Q) as fixed effects and familial relatedness (K) as a random effect to optimize type I and II error rate control (Yu et al., 2006). However, in some cases, a model with G and Q only is sufficient while adding the K term over-corrects the results. Correcting for population structure could be accomplished by Principal Components Analysis (PCA) or through Bayesian approaches such as STRUCTURE (Pritchard et al., 2000), while familial relatedness could be represented in the model by a pair-wise kinship matrix (Yu et al., 2006).

Other important considerations for association-mapping are marker density, population size, and genetic control of the trait of interest. Use of high-density of SNP marker panel in association-mapping is of a great importance to capture rare variations in the genome. Most recently, the genotype-by-sequencing (GBS) platform enables the possibility of SNP genotyping using more than a million SNPs. This platform offers an opportunity to obtain higher coverage of the genome but the call-rate is typically low. Moreover, population size would affect the power of identifying associated polymorphism. To generate linkage, mapping studies of 10 QTL using F_2 mapping population, power to detect QTL increases drastically from 0.1-0.4 to 0.5-0.9 using a population size of 100 and 500, respectively, depending on the heritability of the trait (reviewed in Bernardo, 2000).

While using a large, well-defined population (such as the nested-association-mapping (NAM) in maize) (McMullen et al., 2009) for general association studies, is ideal, developing a breeding program from a derived population, for example, using panels from CIMMYT's Carotenoid Association-mapping (CAM), Drought Tolerant Maize for Africa (DTMA), Improved Maize for African Soils (IMAS), and Nutritional Quality (NQ) would help identify marker associations with specific traits targeted in the respective breeding programs. The use of broad-genotypic variation for specific traits of interest is expected to increase the capacity to identify rare variants. Furthermore, association-mapping is more suitable for identifying qualitative traits (controlled by a few genes with large effects) such as endosperm color in maize. However, results for quantitative traits (many genes, small effects) are influenced by heritability of that trait and the magnitude of the gene's effect (Yu and Buckler, 2006).

Genomic predictions and selections open the opportunity to predict carotenoids with more accuracy, especially for direct prediction of total proVA concentrations. While recent SNP genotyping technologies offer faster mass analysis at a lower cost, genomic selection, especially for early-generation screening is useful. Although genomic selection is a fairly recent trend in plant breeding, not many applications have been found to date; however, a recent study in European maize revealed that genomic prediction using 960 SNPs across six-segregating populations, using random-regression-best-linear-unbiased-prediction, produced correlation between observed and predicted values of 0.81 for grain moisture and 0.36 for grain yield (Zhao et al., 2012). In building models for genomic predictions, co-linearity between SNP markers is a major challenge. However, several advanced statistical tools are available for SNP model development, including Ridge Regression, BayesA, BayesB, LASSO, and Stepwise selection. Preliminary results from a 10-fold genomic prediction exercise using 380 lines in the CAM panel and only 5,000 SNP markers, exhibited considerably larger prediction ability of zeaxanthin (r=0.53) and β-carotene (r=0.62) concentrations (Figure 2).



(a) (b)

Figure 2. Plot of actual phenotype value (y) versus genomic-estimated breeding value ($\hat{y}_{CV}$) for and zeaxanthin (a) and β-carotene (b) from a genomic prediction exercise using 380 maize lines and 5,000 SNPs.

Conclusions

Breeding for increased concentrations of proVA is promising since there is variation available in the germplasm. Classical and molecular breeding methods have been implemented, including use of various temperate and tropical sources with high concentrations of proVA as well as marker assisted selection for the favorable CrtRB1 allele. It is important to note that the improved varieties should not only have high-proVA content, but also should have high-yield, good-agronomic performance, tolerance to abiotic stress, resistance to pests and diseases, as well as good-food-quality and taste.

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