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Maize provitamin A carotenoids, current resources, and future metabolic engineering challenges

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Vitamin A deficiency is a serious global health problem that can be alleviated by improved nutrition. Development of cereal crops with increased provitamin A carotenoids can provide a sustainable solution to eliminating vitamin A deficiency worldwide. Maize is a model for cereals and a major staple carbohydrate source. Here, we discuss maize carotenogenesis with regard to pathway regulation, available resources, and current knowledge for improving carotenoid content and levels of provitamin A carotenoids in edible maize endosperm. This knowledge will be applied to improve the nutritional composition of related *Poaceae* crops. We discuss opportunities and challenges for optimizing provitamin A carotenoid biofortification of cereal food crops.

Keywords: carotenoids, maize, vitamin A, metabolic engineering, endosperm, Poaceae, beta-carotene

VITAMIN A DEFICIENCY

The FAO estimates that 800 million people are chronically hungry, a problem that is most severe in sub-Saharan Africa, but stretches across the globe to include regions of Asia, the Pacific, Latin America, the Caribbean, and North Africa (FAO, 2000). Vitamin A deficiency in these nations is responsible for a number of disorders that range from impaired iron mobilization, growth retardation, and blindness to a depressed immune response, increased susceptibility to infectious disease and increased childhood mortality (Sommer and Davidson, 2002; WHO, 2009).

MAIZE, A MAJOR FOOD CROP AND MODEL FOR NUTRITIONAL IMPROVEMENT

The starchy endosperm tissue of cereal crops contributes a major portion of energy intake from the human diet but is typically of low provitamin A nutritional value. Provitamin A carotenoid biofortification of cereal crops would have a global impact on human health. Rice (*Oryza sativa*) is the most significant worldwide carbohydrate source, but does not accumulate any seed carotenoids. Rice biofortification could only be accomplished by transgenic approaches (Ye et al., 2000; Paine et al., 2005). Sorghum (*Sorghum bicolor*) is a major staple crop grown in semiarid regions due to its drought tolerance, which makes it a good candidate for biofortification. Yellow endosperm varieties contain provitamin A carotenoids and diverse collections of sorghum landraces have been analyzed to quantify pigment diversity (Fernandez et al., 2009). Wheat (*Triticum aestivum*) endosperm color is an important agronomic trait, however there is limited natural variation in this tissue (Howitt et al., 2009). Maize (corn, *Zea mays*) is the predominant food staple in much of sub-Saharan Africa and Latin America, regions that are also plagued by vitamin A deficiency (Sommer and Davidson, 2002). The significant variation in carotenoid content and composition of maize suggests that maize diversity may hold clues as to the target genes that could be manipulated by breeding or transgenics for improvement of cereal crop provitamin A content (Harjes et al., 2008).

Maize is an essential staple cereal crop that naturally accumulates carotenoids in the edible seed endosperm, and is thus an obvious target for biofortification projects. Maize is also a valuable model for other grass species due to historical collections of carotenoid mutants, genome sequence, and other molecular resources. Maize germplasm resources exhibit wide genetic diversity (Liu et al., 2003) with corresponding variation in carotenoid profiles (Harjes et al., 2008), features that are useful for investigating pathway regulation and generating breeding alleles. The close evolutionary relationship between maize and other food crops in the *Poaceae* provides an opportunity for using genome synteny to identify new maize targets for provitamin A improvement to gene homologs in other grass species.

LOCALIZATION OF CAROTENOID BIOSYNTHESIS

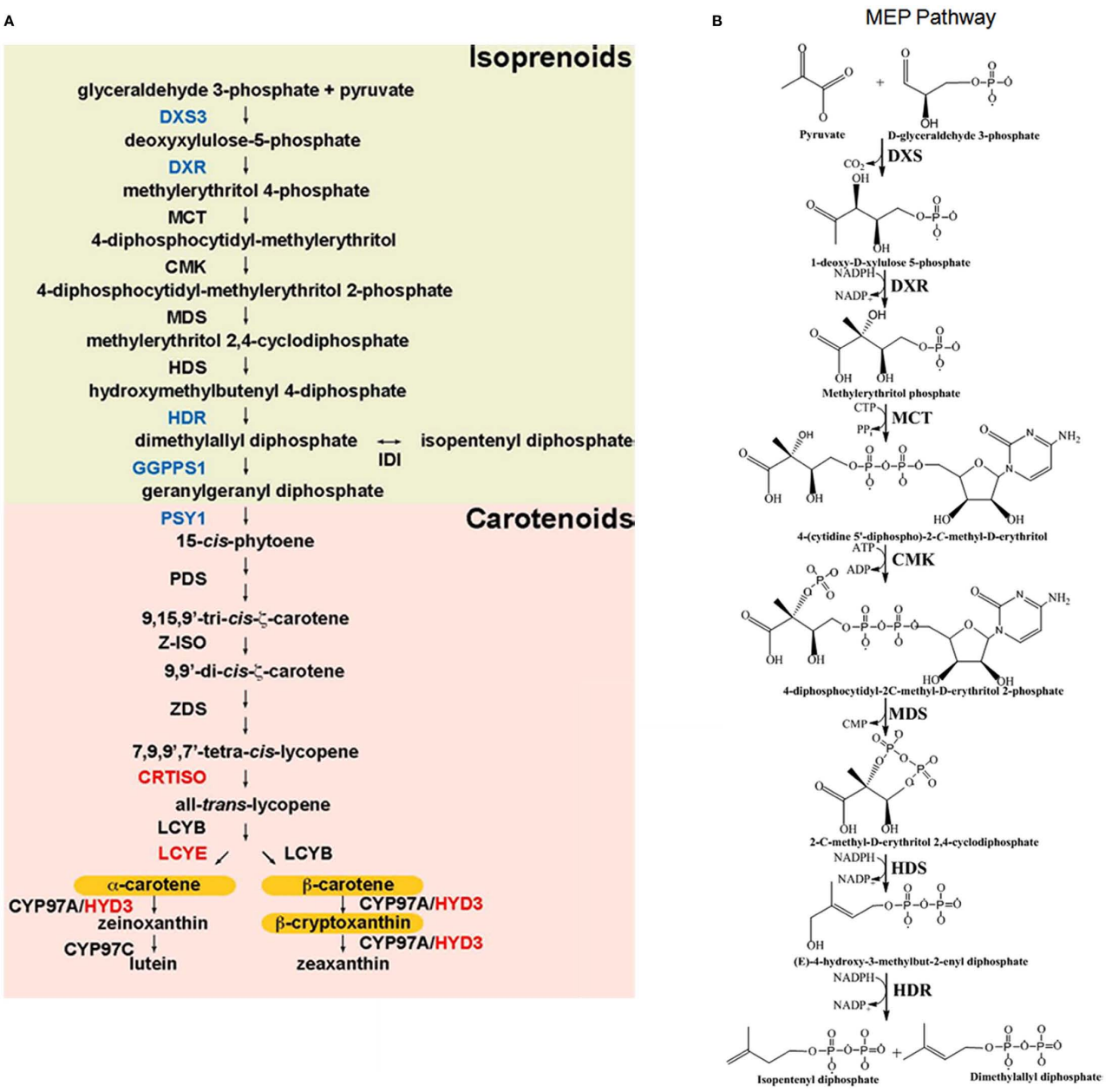
Carotenoid pigments are hydrophobic C40 isoprenoids that are synthesized in plant plastids, where they undergo a series of enzymatic modifications that impart different spectral properties and thus colors. Carotenoid biosynthetic enzymes are encoded by nuclear genes, and the proteins must be imported into plastids. Carotenoids that accumulate in cereal endosperm tissue are synthesized in amyloplasts, plastids that are specialized for storage of starch granules (Kirk and Tiliney-Bassett, 1978).

At least part of the biosynthetic pathway is associated with plastid membranes in maize endosperm (Li et al., 2008b). Proteomic analysis in plastids of other plants revealed sites of some but not all carotenoid enzymes in chloroplasts and chromoplasts

(Ytterberg et al., 2006; Joyard et al., 2009). However, metabolon organization is not well described and is further complicated by differences in plastid membrane organization. Chloroplasts possess a double envelope membrane and internal thylakoid membrane system whereas endosperm amyloplasts have only the double envelope membrane. Given that the endosperm and leaf plastids are architecturally distinct, further research on pathway localization in plastids is a prerequisite for optimizing carotenoid metabolic engineering efforts. An understanding of the biosynthetic pathway enzymes and associated genes will facilitate engineering of enhanced levels of the pathway intermediates that are provitamin A.

THE CAROTENOID BIOSYNTHETIC PATHWAY

The plant carotenoid biosynthetic pathway has been well characterized after decades of molecular genetic analyses (Cuttriss et al., 2011). In maize seed endosperm, the primary carotenoids that accumulate in diverse cultivars are either lutein or zeaxanthin or a combination of both (see **Figure 1**). Provitamin A compounds are biosynthetic pathway intermediates and therefore usually not the predominant carotenoids in endosperm, the target of provitamin A biofortification. Vitamin A (all-*trans*-retinol) is a C_{20} enzymatic cleavage product made in humans from plant carotenoids containing an unmodified β-ring (Von Lintig, 2010). α-carotene and β-cryptoxanthin have provitamin A potential, due to their single



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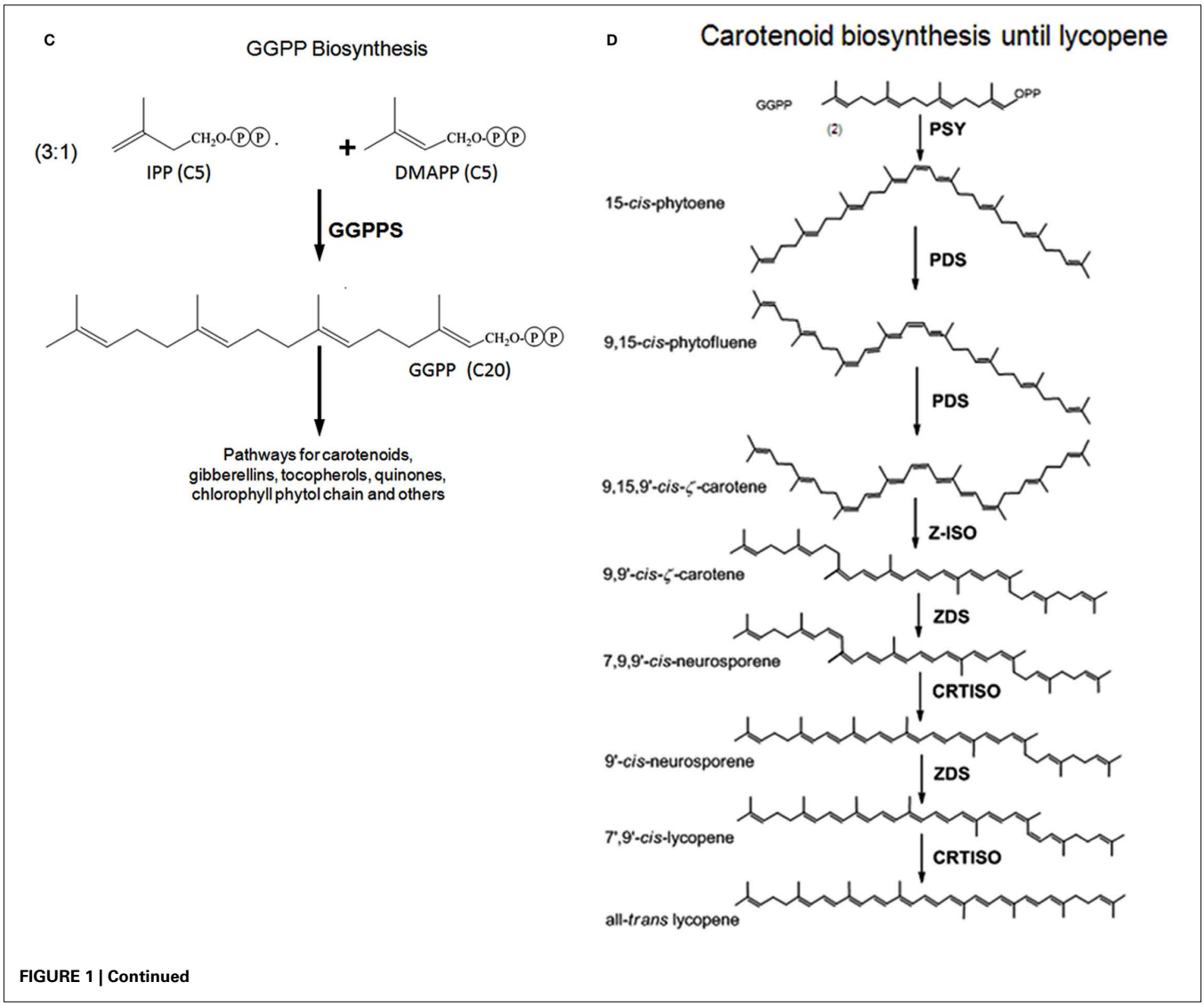

FIGURE 1 | Continued

unmodified β-ring, but β-carotene is the most efficient source, as two retinol molecules may be derived from each β-carotene molecule.

The plastid-localized methylerythritol 4-phosphate (MEP) pathway (Rodriguez-Concepcion, 2010) supplies isoprenoid precursors for carotenoids; glyceraldehyde-3-phosphate and pyruvate are combined to form deoxy-D-xylulose 5-phosphate (DXP), a reaction catalyzed by DXP synthase (DXS), and a number of steps are then required to form geranylgeranyl diphosphate (GGPP), the precursor to carotenoid biosynthesis (Lichtenthaler, 1999) as well as to other biosynthetic pathways. The first carotenoid, phytoene, is produced by the condensation of two GGPP molecules, a reaction that is catalyzed by phytoene synthase (PSY). Two desaturases (PDS, phytoene desaturase; ZDS, ζ-carotene desaturase) and two isomerases (Z-ISO, ζ-carotene isomerase; CRTISO, carotenoid isomerase) introduce a series of double bonds and alter the isomer state of each biosynthetic intermediate to produce all-*trans*-lycopene. At this point the main biosynthetic pathway branches, depending on cyclization activity. Asymmetric cyclization of lycopene by both ε- and β-lycopene cyclases (LCYE and LCYB, respectively) produces α-carotene with one ε- and one β-ionone ring (Cunningham and Gantt, 2001). Symmetric cyclization by LCYB yields β-carotene, with two unmodified β-ionone rings. Hydroxylation of the carotene β-ionone ring by one of two classes of structural distinct carotene hydroxylase enzymes eliminates provitamin A potential. The hydroxylated carotenes include the non-provitamin A xanthophylls, lutein, and zeaxanthin, which may be further modified to other xanthophylls, some of which are cleaved to form ABA (North et al., 2007).

MAIZE GENETICS OF CAROTENOGENESIS

Early maize mutant analyses were responsible for much of our understanding of the biosynthesis of carotenoids and the relationship of these pigments with the biogenesis of plastid ultrastructure (Robertson et al., 1966; Treharne et al., 1966; Robertson, 1975; Neill et al., 1986) (**Table 1**). Carotenoid biosynthesis occurs during seed development (Li et al., 2008b) and the accumulation of

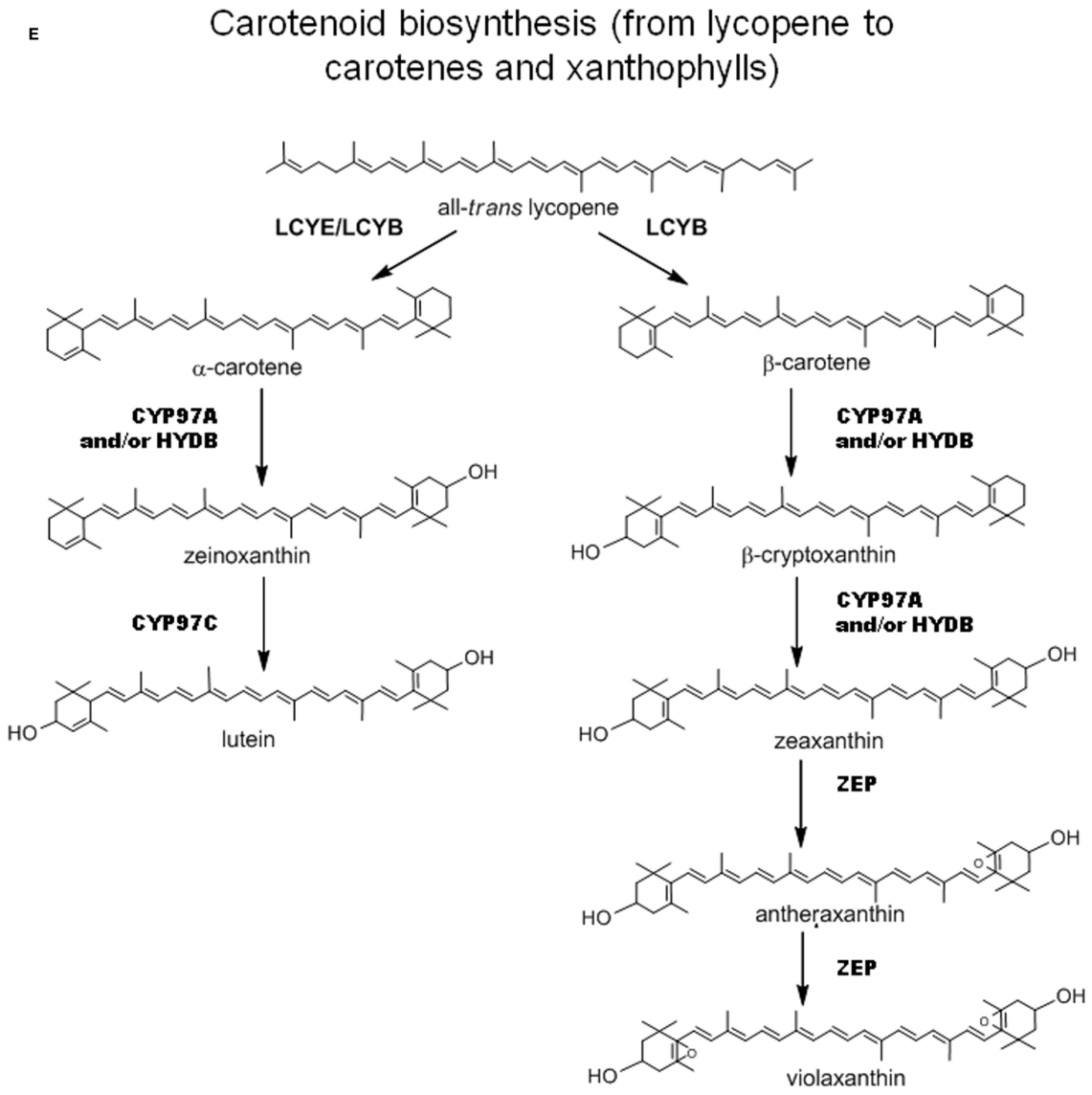

FIGURE 1 | The biosynthetic pathway and challenges for provitamin A metabolic engineering. (A) Regulatory points; **(B–E)** Structures of carotenoids and their precursors. Pro-vitamin A carotenoids are highlighted in orange. Enzymes with transcript abundance and/or allelic polymorphisms that positively associate with provitamin A carotenoid content are indicated in blue, and those with negative correlation in red (based on Harjes et al., 2008; Li et al., 2008b; Vallabhaneni and Wurtzel, 2009; Vallabhaneni et al., 2009, 2010; Yan et al., 2010). Not shown are CCD1 and ZEP for which transcripts levels are inversely associated with carotenoid content. CMK, 4-diphosphocytidyl-methylerythritol kinase; CRTISO, carotenoid isomerase; CYP97A/HYD3, β-hydroxylase; CYP97C, ε-hydroxylase; DXR, deoxyxylulose 5-phosphate reductoisomerase; DXS3, deoxyxylulose 5-phosphate synthase 3; GGPPS1, geranylgeranyl diphosphate synthase 1; HDR, hydroxymethylbutenyl 4-diphosphate reductase; HDS, hydroxymethylbutenyl 4-diphosphate synthase; IDI, isopentenyl diphosphate isomerase; LCYB, β-cyclase; LCYE, ε-cyclase; MCT, methylerythritol 4-phosphate cytidylyltransferase; MDS, methylerythritol 2,4-cyclodiphosphate synthase; PDS, phytoene desaturase; PSY1, phytoene synthase 1; ZDS, ζ-carotene desaturase; Z-ISO, 15-cis-ζ-carotene isomerase; CCD1, carotene cleavage dioxygenase 1; ZEP, zeaxanthin epoxidase.

carotenoids imparts a yellow–orange color to the endosperm, an easily scored phenotype. Genetic loci controlling carotenogenesis include alleles with recessive and dominant phenotypes. There are also duplicate factors and "modifier genes" (for which the underlying genes are largely unknown) that function together to control carotenoid phenotypes in the seed and seedling. In general, mutations blocking function of the pathway structural genes are recessive and lethal as the absence of carotenoids in seedlings leads to aberrant chloroplast development. "Mutant" seeds have white endosperm in comparison to the "normal" yellow endosperm. The mutant seeds may also exhibit a viviparous phenotype due to precocious germination in the absence of the apocarotenoid abscisic acid (ABA). If the pathway is blocked midway, biosynthetic intermediates accumulate throughout the plant.

As early as 1940, the *yellow1* (*y1*) locus was found to have a gene dosage effect on seed carotenoid content (Randolph and

Table 1 | Maize carotenoid candidate genes, quantitative trait loci, and phenotypic loci.

Enzyme	Genetic locus	Map	Reference
HYD6		1.01	Vallabhaneni et al. (2009)
	qtl^{bct}	1.01–1.03	Wong et al. (2004)
PDS	*vp5*	1.02	Matthews et al. (2003)
	qtl^{lt} *(umc1403–fad83)*	1.03	Chander et al. (2008)
	qtl^{c} *(bnlg2086–umc1988)*	1.04–1.06	Chander et al. (2008)
	wlu7	1.05	MaizeDatabase (www.maizegdb.org)
NCED1		1.08	Vallabhaneni et al. (2010)
	qtl^{bczlt}	1.05–1.09	Wong et al. (2004)
	wlu5	1.07	MaizeDatabase (www.maizegdb.org)
	lw1	1.1	MaizeDatabase (www.maizegdb.org)
HDR	*umc1082*	1.09	Vallabhaneni and Wurtzel (2009)
	qtl^{f} *(umc2047–phi30870)*	1.09–1.10	Chander et al. (2008)
	qtl^{f} *(umc2047–ols1)*	1.09	Chander et al. (2008)
	qtl^{zt} *(phi30870–umc1553)*	1.10–1.11	Chander et al. (2008)
	y3–al1	2.00–2.01	MaizeDatabase (www.maizegdb.org)
HYD4		2.03	Vallabhaneni et al. (2009)
CCD7		2.03	Vallabhaneni et al. (2010)
	se1	2.01	MaizeDatabase (www.maizegdb.org)
ZEP1	*gpm594*	2.04	Vallabhaneni and Wurtzel (2009)
	w3 (y11)	2.06	MaizeDatabase (www.maizegdb.org)
GGPPS1		2.08	Vallabhaneni and Wurtzel (2009)
CrtISO2		2.09	Vallabhaneni and Wurtzel (2009)
NCED3b		2.09	Vallabhaneni et al. (2010)
	qtl^{bclt}	3.01	Wong et al. (2004)
	qtl^{bczlt}	3.03–3.05	Wong et al. (2004)
	cl1	3.04	MaizeDatabase (www.maizegdb.org)
DXR		3.04	MaizeDatabase (www.maizegdb.org)
NCED2		3.04	Vallabhaneni et al. (2010)
CYP97C	343.75 cM	3.05	Vallabhaneni et al. (2009)
	qtl^{a} *(umc2408–bnlg197)*	3.06	Chander et al. (2008)
	qtl^{a} *(umc1399–phi046)*	3.07–3.08	Chander et al. (2008)
	y10	3.07	MaizeDatabase (www.maizegdb.org)
CCD8		3.07	Vallabhaneni et al. (2010)
	wlu1	3.07–3.08	MaizeDatabase (www.maizegdb.org)
	qtl^{czlt}	4.02–4.05	Wong et al. (2004)
CYP97A	392.43 cM	4	Vallabhaneni et al. (2009)
	lw4	4.06	MaizeDatabase (www.maizegdb.org)
DXS3		4.06	Vallabhaneni and Wurtzel, 2009
CCD4a		4.06	Vallabhaneni et al. (2010)
	qtl^{clt}	4.07–4.09	Wong et al. (2004)
CrtISO1	*csu704*	4.08	Vallabhaneni and Wurtzel, 2009
	qtl^{bczlt}	5.01–5.04	Wong et al. (2004)
HDS	umc1048	5.03	Vallabhaneni and Wurtzel, 2009
NCED9		5.03	Vallabhaneni et al. (2010)
	qtl^{bt} *(umc1692–umc2373)*	5.03–5.04	Chander et al. (2008)
	qtl^{l} *(umc1447–umc1692–umc2373)*	5.03–5.04	Chander et al. (2008)
	qtl^{t} *(umc2115–umc1447)*	5.02–5.03	Chander et al. (2008)
	vp2	5.04	Matthews et al. (2003)
LCYB	*vp7*	5.04	Singh et al. (2003)
	lw2/vp12	5.05	MaizeDatabase (www.maizegdb.org)
	lw3	5.06	MaizeDatabase (www.maizegdb.org)

(Continued)

Table 1 | Continued

Enzyme	Genetic locus	Map	Reference
CCD4b		5.06	Vallabhaneni et al. (2010)
	qtl^{bczlt}	5.06–5.07	Wong et al. (2004)
	qtl^{bczlt}	6.01	Wong et al. (2004)
PSY1	*y1*	6.01	Buckner et al. (1990)
	qtl^{abzlt} *(Y1ssr–umc1595)*	6.01–6.03	Chander et al. (2008)
	qtl^{lct} *(umc2313–Y1ssr)*	6.01–6.01	Chander et al. (2008)
DXS1		6.05	Vallabhaneni and Wurtzel (2009)
	qtl^{bzl}	6.05	Wong et al. (2004)
IPPI3		6.05	Vallabhaneni and Wurtzel (2009)
	qtl^{a} (*umc21–phi29985*)	6.06–6.07	Chander et al. (2008)
	y8 (lty2)	7.01	MaizeDatabase (www.maizegdb.org)
	qtl^{bczlt}	7.02	Wong et al. (2004)
GGPPS2		7.04	Vallabhaneni and Wurtzel (2009)
ZDS	*vp9*	7.02	Matthews et al. (2003)
	qtl^{bt} *(bnlg1792–atf2)*	7.02–7.03	Chander et al. (2008)
	qtl^{l} *(phi091–atf2)*	7.03	Chander et al. (2008)
	qtl^{lt} *(atf2–umc2332)*	7.03–7.04	Chander et al. (2008)
	wlu2	7.02–7.06	MaizeDatabase (www.maizegdb.org)
PSY3		7.03	Li et al. (2008a)
IPPI1		7.04	Vallabhaneni and Wurtzel (2009)
	clm1	8.00–8.09	MaizeDatabase (www.maizegdb.org)
GGPPS3		8.01	Vallabhaneni and Wurtzel (2009)
IPPI2		8.03	Vallabhaneni and Wurtzel (2009)
	qtl^{bclt}	8.02–8.03	Wong et al. (2004)
	qtl^{cz} *(umc1562–umc1141)*	8.05–8.06	Chander et al. (2008)
	qtl^{z} *(phi115–umc1735)*	8.03	Chander et al. (2008)
	wlu3	8.04–8.09	MaizeDatabase (www.maizegdb.org)
LCYE		8.05	Harjes et al. (2008)
	qtl^{bczlt}	8.05–8.06	Wong et al. (2004)
PSY2		8.07	Gallagher et al. (2004)
	qtl^{czlt}	9.02–9.04	Wong et al. (2004)
HYD5		9.07	Vallabhaneni et al. (2009)
	wlu4	9.03–9.08	MaizeDatabase (www.maizegdb.org)
DXS2		9.03	Vallabhaneni and Wurtzel (2009)
	qtl^{bczlt}	9.06–9.07	Wong et al. (2004)
CCD1	*wc1*	9.07–9.08	Vallabhaneni et al. (2010)
	qtl^{b}	10.01–10.02	Wong et al. (2004)
	qtl^{a} *(umc2016–bnlg1655)*	10.03	Chander et al. (2008)
	qtl^{c} *(phi050–umc1367–umc2016)*	10.03	Chander et al. (2008)
Z-ISO	*y9 (y12)*	10.03	Chen et al. (2010)
	qtl^{cl}	10.04–10.05	Wong et al. (2004)
ZEP2		10.04	Vallabhaneni and Wurtzel (2009)
HYD3		10.05	Vallabhaneni et al. (2009)
	qtl^{bzt} *(umc1506–bnlg1028)*	10.04–10.06	Chander et al. (2008)

Chromosome locations (Map) are indicated for each carotenoid locus representing a biosynthetic gene, quantitative trait locus (QTL), or known genetic (phenotypic) locus. Where the biosynthetic gene is known for a genetic locus, the enzyme is listed on the same line as the genetic locus. Gene family members are indicated numerically (i.e., DXS1, DXS2, DXS3). If there is no known corresponding carotenoid gene for a genetic locus or QTL, no corresponding enzyme is indicated. Mapping references are cited for the structural gene loci or in order of the gene and genetic locus, where applicable. QTL, quantitative trait locus for enhanced: a, α-carotene; b, β-carotene; c, β-cryptoxanthin; z, zeaxanthin; l, lutein; or t, total carotenoids in the seed endosperm.

Hand, 1940) and thus control of pathway flux; three copies of the dominant *Y1* allele in the triploid endosperm conditioned the most yellow seeds (endosperm) in contrast to homozygous *y1* seeds. White *y1* endosperm is an ancestral trait shared with teosinte, the wild progenitor of maize. Cloning and subsequent sequence analyses identified the *Y1* gene as encoding PSY1, the

first enzyme of the biosynthetic pathway (Buckner et al., 1996). Curiously, the yellow *Y1* seed accumulates carotenoids because of a gain of function mutation, which came at the loss of photoregulation in green tissue (Li et al., 2009). In contrast, the white endosperm *y1* allele has maintained photoregulation in green tissue. This alteration in PSY1 regulation across tissues, depending upon *y1* allele, highlights the potential ramifications of allele selection for breeding when we do not fully understand the role of gene family members with regard to carotenogenesis in different tissues. More detailed understanding of carotenoid gene families and their respective alleles will strengthen future predictive engineering strategies.

Recently, it was discovered that the maize *y9* locus encodes ζ-carotene isomerase (Z-ISO), a previously unknown pathway enzyme that is necessary for carotenogenesis in all plants (Chen et al., 2010). Without Z-ISO function, provitamin A carotenoids cannot be produced in the endosperm, the target tissue for biofortification. The importance of Z-ISO for endosperm carotenogenesis might explain a cluster of Quantitative Trait Loci (QTL) for seed carotenoids (**Table 1**), five of which were found within 15 cM of the *y9* locus (Chander et al., 2008). Another locus affecting seed carotenogenesis, *white cap1* (*wc1*), for which dominant alleles deplete endosperm carotenoids, was recently shown to be the map location (**Table 1**) for a carotenoid cleavage enzyme CCD1 (Vallabhaneni et al., 2010).

A detailed study of recombinant inbred lines identified more than 30 Quantitative Trait Loci (QTL; **Table 1**) for carotenoid content and composition (Wong et al., 2004; Chander et al., 2008), some of which are closely linked to biosynthetic pathway structural genes, such as *y1* or *y9* (Li et al., 2007). The number of QTL outnumber pathway structural genes and represent unknown genes that influence seed carotenoid content and composition. These QTL could lead to novel regulatory genes and other targets for future breeding efforts.

Gene duplication is a common feature in maize and for the maize carotenoid biosynthetic pathway. Carotenoid gene paralogs have been mapped in maize (**Table 1**), though the role of many duplicates is yet to be determined, which adds to the complexity of pathway regulation in different tissues. Genes encoding carotenoid enzymes are also duplicated in rice, wheat and sorghum (**Table 2**). As structural genes have been isolated over the years, they have been mapped to chromosome location to determine which known genetic loci might correspond to these structural genes. There are still a number of loci for which alleles affect levels of carotenoids and these loci do not correspond to known structural genes. Such loci represent additional opportunities for gene discovery for controlling seed carotenogenesis.

REGULATION OF SEED CAROTENOID ACCUMULATION

To improve the provitamin A potential of cereal seeds, the challenges are to increase total carotenoid content, optimize provitamin A composition, limit degradation of beneficial carotenoids, and control carotenoid sequestration. Approaches that may overcome these challenges are outlined below. These efforts have focused on elucidating factors that contribute to provitamin A accumulation in the endosperm through analysis of pathway regulatory points and timing of gene expression for key enzymes. This research has led to new tools and knowledge to help breed higher provitamin A cereal crops.

QUANTITATIVE CONTROL OF CAROTENOID CONTENT THROUGH MAXIMIZING PATHWAY FLUX

The first step in determining provitamin-A potential is to maximize biosynthetic flux and thus total carotenoid synthesis. *PSY* catalyzes the first committed biosynthetic step, and is encoded by three genes in maize and in other cereal crops such as rice and wheat (Palaisa et al., 2003; Li et al., 2008b; Welsch et al., 2008; Howitt et al., 2009; Chaudhary et al., 2010). PSY1 is responsible for endosperm carotenogenesis but also plays a role in carotenogenesis in other tissues (Gallagher et al., 2004; Li et al., 2008a,b). The various PSY paralogs respond differently to abiotic stimuli and have unique tissue specificities though their function remains redundant. In addition to its role in endosperm, PSY1 is needed for carotenogenesis related to thermotolerance in photosynthetic tissue (Li et al., 2008b). Salt and drought induced *PSY3* transcript abundance in maize roots, and correlated with increased carotenoid flux and ABA in maize roots (Li et al., 2008a). Similar responses were observed for the rice *PSY* homologs (Welsch et al., 2008), confirming that maize findings can be applied to other cereal biofortification projects and that each member of a conserved gene family has a specific role. QTL analysis determined that *PSY1* was responsible for 6.6–27.2% of phenotypic variation in seed carotenoid content (Chander et al., 2008), suggesting the contribution of additional unknown gene targets.

While PSY is clearly a significant flux determinant, other steps in the pathway are likely to have an effect on the size of the carotenoid pool. To this end, a maize diversity collection was analyzed to reveal those regulatory points and to determine the correlation between carotenoid content and biosynthetic gene transcript levels. A core germplasm collection representing extremes of seed carotenoid composition was created based on carotenoid profiles of 150 maize lines spanning 80% of maize genetic diversity. Statistical analysis of transcript levels was used to identify specific gene family members that influence carotenoid content and composition and the time during endosperm development when this effect was seen (Li et al., 2008b; Vallabhaneni and Wurtzel, 2009; Vallabhaneni et al., 2009). PSY1 was used to validate the approach. The comprehensive analysis of the maize carotenoid pathway gene families led to discovery of a number of new targets for endosperm carotenoid biofortification.

Rate-limiting steps were identified in both the biosynthesis of carotenoids and the supply of precursor isoprenoids, thus identifying potential metabolic engineering targets (Vallabhaneni and Wurtzel, 2009). Multiple control points both within the carotenoid pathway and MEP precursor pathway were identified in maize, and the timing of gene expression was found to be critical in determining carotenoid content. Transcript levels of CrtISO and ZEP1 and ZEP2 inversely correlated with seed carotenoid content, whereas several genes required to produce isoprenoid precursors showed positive correlation, including DXS3, DXR, HDR, and GGPPS1 (Vallabhaneni and Wurtzel, 2009). The genes identified in this study offer new opportunities for breeding and transgenic fortification. The timing of gene expression is critical in determining carotenoid composition, as expression

Table 2 | Map positions of candidate genes encoding carotenoid biosynthetic and degradative enzymes in *Zea mays, Oryza sativa, Triticum aestivum,* and *Sorghum bicolor*.

Enzyme	Maize (*Zea mays*)		Rice (*Oryza sativa*)		Wheat (*Triticum aestivum*)		Sorghum (*Sorghum bicolor*)	
	Chr. bin	Reference/ GenBank	Chr. bin	Reference/ GenBank	Chr. bin	Reference/ GenBank	Chr. Bin and (qtl)	Reference/ phytozome
DXS1	6.05	AY110050	5	AK064944			9	Sb09g020140
DXS2	9.03	AY946270	7	AK100909			2	Sb02g005380
DXS3	4.06	AY104478	6	AK121920			10	Sb10g002960
DXR	3.04	BG840684	1	AK099702			3	Sb03g008650
MCT		DR818082	1	AP003379			3	Sb03g042160
CMK	3.06	BG354410	1	AK067589			3	Sb03g037310
MDS		CD974184	2	AK060238			4	04g031830
HDS	5.03	AC198323	2	AK120769	6D	BE585702	4	Sb04g025290
HDR	1.09	DR789385	3	AK062113			1	Sb01g009140
IPPI1	7.04	AF330034	7	AK065871	1A	CD452500	2	Sb02g035700
IPPI2	8.03	AC203067	5	AK060336			9	Sb09g020370
IPPI3	6.05	AC206783						
GGPPS1	2.08	EE417573	7	AK121529			2	Sb02g037510
GGPPS2	7.04	EF417574	1	AP002865				
GGPPS3	8.01	AC211188					3	Sb03g009380
PSY1	6.01	AY324431	6	AY445521	7AL, 7BL, 7A	Yellow pigment qtl	10b, 0.0 cM, qtl (color; $p \leq 0.0001$)	Sb10g031020
PSY2	8.07	AY325302	12	AY773943	5A, 5B	Either PSY2 or PSY3	8	Sb08g022310
PSY3	7.03	DQ372936	9	FJ214953			2, 165.9 cM	Sb02g032370
PDS	1.02	L39266	3	AF049356	4A, 4B		6, 16.9 cM	Sb06g030030
Z-ISO	10.03	BT036679	12	BAF29644			8	Sb08g014610
ZDS	7.02	AF047490	7	AK065213	2A, 2B		2, 37.1 cM	Sb02g006100
CrtISO1	4.08	CC749876, AC218991	11	AC108871			5	Sb05g022240
CrtISO2^{pseudo}	2.09	EE045563/ AC183901						
LCYB	5.04	AY206862		AP008208			4	Sb04g006120
LCYE	8.05	BV727273		AK072015			3, 123.1 cM	Sb03g026020
HYD1^{pseudo}		EU638325						
HYD2^{pseudo}		EU638326						
HYD3	10.05	AY844958						
HYD4	2.03	AC196442	4	AL606624			6	Sb06g026190
HYD5	9.07	AC149033	3	AC119747			1	Sb01g048860
HYD6	1.01	AC155364	10	AE016959				
CYP97C2	3.05	BE552887	10	AK065689	6AL, 6BL, 6DL	USDA	1, 146.3 cM	Sb01g030050
CYP97A4	4	AY112171	2	AK068163			4	Sb04g037300
ZEP1	2.04	AC194845	4	AL606448			6	Sb06g018220
ZEP2	10.04	AC206194	NA	NA				
VDE	2.05	EU956472	1, 4	Os01g0716400, Os04g0379700			6	Sb06g012950
NXS			1	Os01g03750			3	Sb03g007320
CCD1	9.07	AY106323	12	Os12g44310			1, 48.5 cM qtl (color; $p \leq 0.05$)	Sb01g047540
NCED1	1.08	AC201886					1, 210.3 cM	
NCED2	3.04	AC199036	12	Os12g42280			1	Sbi_0.36552
NCED3a		AC212820	7	Os07g05940			2	Sb02g003230
NCED3b	2.09	AC205109						

(Continued)

Table 2 | Continued

Enzyme	Maize (*Zea mays*)		Rice (*Oryza sativa*)		Wheat (*Triticum aestivum*)		Sorghum (*Sorghum bicolor*)	
	Chr. bin	Reference/ GenBank	Chr. bin	Reference/ GenBank	Chr. bin	Reference/ GenBank	Chr. Bin and (qtl)	Reference/ phytozome
CCD4a	4.06	AC190588	2	Os02g47510			4	Sb04g030640
CCD 4b	5.06	AC194862	12	Os12g24800				
CCD7	2.03	AC211432	4	Os04g46470			6	Sb06g024560
CCD8a	3.07	AC185113	1	Os01g54270			5	Sb05g009950
CCD8b	7.02	AC198395	9	Os09g15240			2	Sb02g021490;
CCD8c			1	Os01g38580			10	Sb10g004360
							10 (8c-like)	Sb10g004370
CCD8d			8	Os08g28240			2	Sb02g021490
							7 (8d-like)	Sb07g024250
NCED9	5.03	AC190614	3	Os03g44380			1	Sb01g013520

Pseudo indicates the gene is not functional in the maize B73 inbred. The strength of the association between a QTL and a metabolite are indicated by a p value. The bin positions and QTL data are derived from the following references (Matthews et al., 2003; Wong et al., 2004; Chander et al., 2008; Fernandez et al., 2008; Chen et al., 2010; Vallabhaneni et al., 2010; MaizeDatabase, www.maizegdb.org).

of genes controlling steps that supply isoprenoid precursors to the carotenoid pathway correlated with carotenoid content at 25 days after pollination, whereas the carotenoid pathway genes (PSY1, CrtISO, and ZEP) showed earlier correlation (20 days after pollination).

QUANTITATIVE CONTROL OF CAROTENOID CONTENT THROUGH LIMITING DEGRADATION

Carotenoid degradation is important in determining total carotenoid accumulation as well as composition. A family of carotenoid cleavage enzymes is required to process violaxanthin and neoxanthin into ABA and other pathway intermediates into an array of apocarotenoids. All of the maize cleavage genes have now been identified, mapped to chromosomes (**Table 1**), and compared with homologs from related species (**Table 2**; Vallabhaneni et al., 2010). Carotenoid cleavage can deplete the carotenoid pool, as was observed in Arabidopsis seeds and chrysanthemum flowers (Auldridge et al., 2006; Ohmiya et al., 2006). One maize catabolic gene, *ZmCCD1*, was cloned and found to effectively cleave carotenoids at the 9, 10 position (Sun et al., 2008; Vogel et al., 2008). The position of *ZmCCD1*, chromosome 9.07, is linked to the dominant *white cap1* (*wc1*) locus (Vallabhaneni et al., 2010). Dominant *wc1* alleles exhibit reduced endosperm carotenoids, and there is evidence for a gene dosage effect as *ZmCCD1* transcript abundance negatively correlated with carotenoid content and an inbred line with high *ZmCCD1* copy number had concomitantly low endosperm carotenoid content (Vallabhaneni et al., 2010). Thus it is important to take advantage of the rich genetic resources to identify favorable CCD alleles that encourage retention of endosperm carotenoids.

QUALITATIVE CONTROL OF CAROTENOID COMPOSITION

Carotenoid composition is another important consideration as only carotenoids with unmodified β-ionone rings are converted to vitamin A. β-Carotene has the greatest provitamin A potential and it is therefore the preferred endosperm carotenoid for nutritional purposes. Optimizing β-carotene accumulation requires enhanced flux to the β-branch of the pathway in combination with limiting hydroxylation of β-carotene to downstream xanthophyll compounds that no longer have provitamin A activity.

The relative activities of the ε- and β-cyclases alter flux to either branch of the pathway; LCYE activity leads to α-carotene and lutein production at the expense of β-carotenoids. A diverse maize panel was subjected to association analysis, linkage mapping, and expression analyses showing that variation at the *LCYE* locus altered flux partitioning. Four polymorphisms were identified that controlled 58% of the variation between α- and β-branch accumulation, thus enabling selection of alleles that confer high provitamin A status for improved maize varieties (Harjes et al., 2008). This was a significant step in provitamin A enhancement, but still required the discovery of loci that were responsible for conversion of provitamin A β-carotene by hydroxylation to non-provitamin A products.

Breeding with select *LCYE* alleles to control increased flux through the β-branch of the pathway is only effective for biofortification if β-carotene remains unmodified by downstream hydroxylase enzymes. To identify target genes for blocking carotene hydroxylation, maize genes encoding carotene hydroxylases were investigated. Two structurally distinct classes of enzymes were found to be encoded by a total of eight genes in maize (Vallabhaneni et al., 2009). The gene families are similarly complex in other grasses. Using the maize diversity core collection produced by "metabolite sorting," it was possible to pinpoint the one carotene hydroxylase encoded by the *Hydroxylase3* (*HYD3*) locus, whose transcript levels negatively correlated with high β-carotene levels and positively correlated with zeaxanthin levels. *HYD3* was mapped close to a known QTL for β-carotene composition (see **Table 1**). PCR genotyping of 51 maize lines showed that the *HYD3* locus could explain 36% variation and fourfold difference in β-carotene levels (Vallabhaneni et al., 2009). Association and linkage population studies in maize confirmed that *HYD3* was indeed responsible for this particular QTL associated

with β-carotene accumulation (Yan et al., 2010). The most favorable alleles were found in temperate varieties and will be bred into tropical maize germplasm to help alleviate vitamin A deficiency in developing countries. Thus two significant gene targets to control β-carotene composition (*LCYE* and *HYD3*) have been identified, and the concomitant control of both of these steps promises to have a significant impact on provitamin A enhancement.

OPEN QUESTIONS ABOUT PATHWAY REGULATION

Carotenoid levels throughout the plant are regulated by developmental cues and various biotic and abiotic stresses (Cuttriss et al., 2011) In general, increases in carotenoid accumulation coincide with increased transcript abundance of some key (but not all) steps in the pathway; however no regulatory genes controlling carotenogenesis have been identified in maize or any other grass. As we tease apart the genetic diversity of maize carotenogenesis, it is hoped that we will identify transcription factors and feedback mechanisms that modulate carotenoid biosynthesis. Furthermore, the precise localization of carotenoid biosynthetic enzymes and their post-transcriptional interactions remain open questions along with unknown mechanisms of carotenoid sequestration in endosperm amyloplasts. Understanding protein import and interactions within the plastid will enable targeted manipulation of biosynthesis and more effective breeding strategies.

LOOKING FORWARD TO OPTIMIZE β-CAROTENE LEVELS IN MAIZE

Control points that determine carotenoid accumulation have produced promising molecular breeding tools, and while PSY has proven to be a major determinant, other biosynthetic and regulatory steps will have a significant impact on carotenogenesis. Understanding of allelic differences and new points of control can also be targeted in breeding projects. Certainly a molecular screen for a combination of preferable *LCYE* and *HYD3* alleles will have a large impact on β-carotene synthesis and retention.

An additional promising approach that remains to be tested is the endosperm specific upregulation of isoprenoid precursor synthesis. Transcript abundance of several isoprenoid genes (DXS3, DXR, HDR, and GGPPS1) was found to positively correlate with endosperm carotenoid content (Vallabhaneni and Wurtzel, 2009) and thus those genes are potential biofortification targets. Manipulation of *DXS3* is a particularly promising target, as *DXS* overexpression in Arabidopsis produced increased isoprenoids, including carotenoids (Estevez et al., 2001). Transgenic manipulations may offer the most expedient approach to control these additional targets given the absence of known regulatory factors to control multiple steps. Transgenic maize plants have been engineered to accumulate a wide variety of carotenoid intermediates and unusual keto-carotenoids and seeds ranged from white and yellow to dark-red, despite the white-endosperm genetic background (Aluru et al., 2008; Zhu et al., 2008). This achievement demonstrates remarkable plasticity in carotenoid accumulation and indicates that the targets identified by metabolite sorting and transcript profiling can be successfully manipulated.

Cereal breeding programs stand to be revolutionized with the increased availability of next generation and single-molecule sequencing technologies (Morozova and Marra, 2008; Edwards and Batley, 2010). The application of systems biology tools will enable us to expand analyses from candidate pathway genes to all genes in the genome, and to rapidly characterize germplasm diversity collections for crops that do not have the same wealth of genetic resources as maize. Proteomic and protein interaction analyses will precisely pinpoint where in the plastid carotenoid biosynthesis occurs. Combining these tools in an effective way will make for significant advances in metabolic engineering and biofortification of provitamin A carotenoids in cereal food crops. Improving the nutritional composition of such staples could have a positive impact on the health of millions of people worldwide.

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