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Quality related traits of the maize (*Zea mays* L) grain: gene identification and exploitation

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Abstract

Developing maize plants with improved kernel quality traits involves the ability to use existing genetic variation and to identify and manipulate commercially important genes. This will open opportunities for designing novel variation in grain composition and will provide the basis for the development of the next generation of specialty maize. This paper provides an overview of current knowledge on the identification and exploitation of genes affecting the composition, development, and structure of the maize kernel with particular emphasis on pathways relevant to endosperm growth and development, differentiation of starch-filled cells, and biosynthesis of starches, storage proteins, lipids, and carotenoids. The potential that the new technologies of cell and molecular biology will provide for the creation of new variation in the future are also indicated and discussed.

Keywords: endosperm development, starch and protein synthesis, genetic variability, gene discovery

Introduction

Maize (*Zea mays* L) is a major food and animal feed worldwide and occupies a relevant place in the world economy and trade as an industrial grain crop (White and Johnson, 2003). Currently, more than 70% of maize production is used for food and feed. Therefore, knowledge of genes involved in grain structure and chemistry is important for improving the nutritional and food-making properties of maize. Although, plant breeding has been extremely successful at improving the yield of maize, quality has received less attention. Nevertheless, by exploiting genetic variation, important advances have been made also in this area, resulting in maize lines with a wide range of kernel variations in starch, protein, and oil composition. Furthermore, the ability of plant scientists to use existing genetic variability and to identify and manipulate commercially important genes will open new venues to design novel variation in grain composition. This will provide the basis for the development of the next generation of speciality in maize and of new products to meet future needs.

Throughout this manuscript, we will focus on gene discovery, exploitation, and genetic variation known to affect the development and chemical composition of the maize kernel, trying to summarize the current status in these areas with a particular reference to deposition of storage proteins, starches, lipids, and carotenoids, and research pertinent to enhancing kernel quality-related traits. Furthermore, we will provide a brief outlook on future developments and resultant opportunities deriving from the application of conventional and molecular breeding aimed at the development of new, better suited maize products.

Kernel growth and development

The great economical and nutritional value of the maize kernel is mainly due to its high starch content, as it represents approximately 75% of the mature seed weight. However, the protein complement (ca. 10% of the mature seed weight), mainly found in the form of zeins (storage proteins) is essential for human and animal nutrition. Yet the question remains of why the selection for higher starch level irremediably results in less protein content, as illustrated by the Illinois Long-Term Selection Experiment, which is spanning over more than 100 generations of classical breeding (Mooses et al, 2004). In maize, the endosperm is the main storage site, a starch-rich tissue that supports the embryo at germination, making up the majority of kernel dry matter (70-90%) and the predominant sink of photosynthates and other assimilates during reproductive growth. Conversely, the embryo reserves mainly lipids. Since the economical and nutritional value of the kernel is based on the utilization of its endosperm, factors that mediate endosperm development largely determine grain yield.

High-throughput genomics and post-genomics approaches are now providing new tools for a better understanding of the genetic and biochemical networks operating during kernel development. Large databases of maize expressed sequence tags (ESTs) are now available (i.e http://www.maizegdb.org), and transcriptome analyses aimed at identifying genes involved in endosperm development and metabolism have been published, along with computer software allowing a systematical characterization. Hence, it has become possible to analyze gene expression in developing maize endosperm more thoroughly and to identify tissue-specific genes involved in endosperm

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development and metabolism (Lai et al, 2004; Verza et al, 2005; Liu et al, 2008; Prioul et al, 2008). These studies have shown that, in maize, at least 5,000 distinct genes are expressed during kernel development. Unfortunately, approximately 35% of these are orphan genes, whose functions remain enigmatic, possibly corresponding to endosperm specific genes (Liu et al, 2008), as also observed in wheat (Wan et al, 2008). Méchin and co-workers (2004) have established a proteome reference map for the maize endosperm. They found that metabolic processes, protein destination and synthesis, cell rescue, defense, cell death and ageing were the most abundant functional categories detected in the maize endosperm. Collectively, the transcriptome and proteome maps constitute a powerful tool for physiological studies and are the first step for investigating maize endosperm development and metabolism.

Storage proteins

Over the past 30 years, the structure and biochemical properties of seed storage proteins have the focal point of many investigations due to their abundance, complexity, and impact on the overall nutritional value of the maize seed. A great deal is now known about the compounds that are made and stored in seeds, as well as how they are hydrolyzed and absorbed by the embryo (see Hannah, 2007; Holding and Larkins, 2009; Motto et al, 2009; Val et al, 2009 for a review).

The most abundant protein storage components (>60%) in developing endosperm tissues of the maize grain are prolamines called «zeins». These proteins are constituted by alcohol-soluble compounds with a characteristic amino acid composition, being rich in glutamine, proline, alanine, and leucine, and almost completely devoid of lysine and tryptophan (Gibbon and Larkins, 2005). Based on their evolutionary relationships, zeins are divided into four protein subfamily of α- (19- and 22-kDa), β- (15 kDa), γ- (16-, 27-, and 50-kDa), and δ-zeins (10- and 18-kDa), that are encoded by distinct classes of structural genes (Holding and Larkins, 2009). Miclaus et al (2011) have recently reported that α-zein genes have evolved from a common ancestral copy, located on the short arm of chromosome 1, to become a 41-member gene family in the reference maize genome, B73. From a nutritional point of view, the exceedingly large proportion of codons for hydrophobic amino acids in α-zeins is mostly responsible for the imbalance of maize protein reserves. Therefore, the reduction in α-zein protein accumulation with biased amino acid content could provide a correction to this imbalance. Zeins have also unique functional and biochemical properties that make them suitable for a variety of food, pharmaceutical, and manufactured goods (Lawton, 2002).

The proper deposition of zeins inside subcellular structures called protein bodies (PBs) confers the normal vitreous phenotype to the endosperm. PBs are specialized endosperm organelles that form as an extension of the membrane of the rough endoplasmic reticulum (RER), into which zeins are secreted once their signal peptide is processed. PBs first appear as small γ-zein accretions, and later, α- and δ-zeins penetrate and appear as inclusions with the γ-zein backgrounds. Mature protein bodies of 1 to 2 µm have an even, round shape, with α-zeins confined within the core surrounded by a shell of α- and β-zeins. This model, developed from immunogold transmission electron microscopy (TEM) of chemically fixed endosperm samples, does not distinguish between the locations of 19- and 22-kD α-zeins (Lending and Larkins, 1989). However, antibodies specific to 22- and 19-kD α-zeins revealed that these proteins have distinct patterns of accumulation. Whereas the 19-kD α-zein is found throughout the protein body core, the 22-kD α-zein is found only in a discrete ring at the interface between the 19-kD α-zein-rich core and the 27-kD γ-zein-rich peripheral region (Holding et al, 2007). Alterations in size, shape or number of PBs generally determine the opaque phenotype (Holding and Larkins, 2009), the sole exception being floury1 (fl1), an opaque mutant with no alterations in PB size or shape (Holding et al, 2008). Further studies reported by Llop-Touset et al (2010) have indicated that the N-terminal proline-rich domain of γ-zein plays an important role in PB formation, while Washida et al (2009) have identified that the cis-localization elements of the 10-kDa δ-zein are responsible for PB-ER targeting. Their results indicate that there is a close relationship between RNA and protein localization in plant cells and that RNA localization may be an important process in mediating the deposition of storage protein in the endomembrane system in plants.

Endosperm mutants altering storage protein synthesis

Endosperm growth and development is a complex phenomenon, driven by the coordinate expression of several genes. Strategies using spontaneous and induced endosperm gene mutations allow the unravelling of the complex underlying expression system operating during endosperm growth and development integrating carbohydrate, amino acid, and storage protein metabolisms. In this respect, several endosperm mutants with altered timing and zein synthesis rate have been described (reviewed by Motto et al, 2009). These mutants exhibit a more or less defective endosperm and have a lower than normal zein content at maturity. Many of these genes have been mapped to chromosomes and their effect on zein synthesis has been described (Table 1). All mutants confer an opaque phenotype to the endosperm, and, exhibit, concomitantly with zein synthesis reduction, an increased overall lysine content, allowing for their potential use in the development of «high-lysine» maize.

The recessive mutation *opaque-15* (*o15*) mutation exerts its effect primarily on the 27-kDa γ-zeins,

while the *opaque-2* (*o2*) induces a specific decrease in the accumulation of 22-kDa α-zeins. The *floury1* (*fl1*) mutation is somewhat different, since it does not affect the amount or composition of zein proteins but rather results in the abnormal placement of α-zeins within the PB: *Fl1* encodes a transmembrane protein that is located in the protein body ER membrane. The *opaque5* (*o5*) mutant phenotype is caused by a reduction in the galactolipid content of the maize endosperm, with no change in zein proteins (Myers et al. 2011). Furthermore, these workers reported that O5 locus encodes the monogalactosyldiacylglycerol synthase (MGD1) and specifically affects galactolipids necessary for amyloplast and chloroplast function. The *opaque-1* (*o1*) locus has recently been isolated by map-based cloning and was shown to encode a plant-specific myosin XI protein. Myosin XI proteins are involved in cytoplasmatic streaming and organelle motility and structure (Wang et al, 2012). This recessive mutant, exhibiting identical amounts of protein with respect to wild-type, with a nearly identical amino acid composition.

Holding et al (2010) have studied an interesting maize opaque endosperm mutant, termed *mto140*, which also shows retarded vegetative growth. Its seeds show a general reduction in storage protein accumulation and an elevated lysine phenotype typical of other opaque endosperm mutants. However, *mto140* is distinct from other opaque mutants because it does not result from quantitative or qualitative defects in the accumulation of specific zeins but rather from a disruption in amino acid biosynthesis.

A co-segregation of the opaque phenotype with a Mutator transposon insertion in an arogenate dehydrogenase gene (*zmAroDH-1*), led the previous authors towards the characterization of the four-member family of maize arogenate dehydrogenase genes (*zmAroDH-1–4*). The differential expression patterns of these genes,together with subtle mutant effects on the accumulation of tyrosine and phenylalanine in endosperm, embryo, and leaf tissues, suggested that the functional redundancy of this gene family provides metabolic plasticity for the synthesis of these important amino acids.

The *o2* mutation has been widely studied at the genetic, biochemical and molecular levels. *O2* encodes a basic leucine zipper (bZIP) transcriptional regulator that is specifically expressed in the endosperm (Hartings et al, 1989). These studies showed that O2 activates the expression of 22-kDa α-zein and 15-kDa β-zein genes by interacting with the TCCACGT(a/c)R(a/t) and GATGYRRTGG sequences of their promoters, therefore displaying a broad binding specificity and recognizing a variety of target sites in several distinct genes. *O2* also regulates directly or indirectly a number of other non-storage protein genes, including *b-32*, encoding a type I ribosome-inactivating protein, one of the two cytosolic isoforms of the *pyruvate orthophosphate dikinase* gene (*cyPPDK1*), and *b-70*, encoding a heat shock protein 70 analogue, possibly acting as a chaperonin during PB formation. *O2* also regulates the levels of lysine-ketoglutarate reductase (Brochetto-Braga et al, 1992) and aspartate kinase1 (Azevedo et al, 1997). These broad effects suggest that *O2* plays an important role in the developing grain as a coordinator of the expression of genes controlling storage protein, and nitrogen (N) and carbon (C) metabolism.

The *O7* gene was recently cloned by two different groups, using a combination of map based cloning and transposon tagging and confirmed by transgenic functional complementation (Miclaus et al, 2011; Wang et al, 2011). These last workers showed furthermore, via sequence analysis, that the *O7* gene exhibits similarities with members of the *acyl-CoA synthetase-like* (*ACS*) gene family, although its exact enzymatic activity remains uncertain. In particular, Miclaus et al (2011) have hypothesized a mechanism in which the O7 protein functions in post-translational modification of zein proteins, thus contributing to membrane biogenesis and stability of PBs and conferring the normal vitreous phenotype of the kernel. Alternatively, Wang et al (2011) have suggested, by analysis of amino acids and key metabolites, that O7 gene function might affect amino acid biosynthesis by affecting α-ketoglutaric acid and oxaloacetic acid phenotype, indicating a conserved biological function of *O7* in cereal crops. In this respect, Hartings et al (2011), in a study to clarify the role that *O2* and *O7* play in endosperm gene expression through transcriptomic analyses, indicated that the o2 and o7 mutants alter gene expression in a number of enzymatic steps in the tricarboxylic acid cycle (TCA) and glycolysis pathways that are of central importance for the amino acid metabolism in developing seeds. Although a systematic characterization of such enzymes will be necessary before any inferences are warranted, the cloning of *O7* revealed a novel regulatory mechanism for storage protein synthesis and highlighted an effective target for the genetic manipulation of storage protein contents in cereal seeds, maize included.

An alternative approach to understand the relationship between zein synthesis and the origin of the opaque endosperm phenotype is to perturb zein accumulation transgenically. In this respect, a number of laboratories have reported a reduction in 22-kDa (Segal et al, 2003) and 19-kDa α-zeins (Huang et al, 2004) by RNAi and by seed-specific expression of lysine rich protein (Rascon-Cruz et al, 2004; Yu et al, 2004).

Although maize endosperm storage protein genes have been studied for many years, many questions regarding their sequence relationships and expression levels have not been solved, such as structure, synthesis and assembly into protein bodies, and their genetic regulation (Holding and Larkins, 2009). The development of tools for genome-wide studies

Table 1 - Some features of maize mutants affecting zein accumulation.

Genotype	Inheritance	Effect on zein accumulation	Molecular bases
Opaque-1 (*O1*)	recessive	no reduction	myosin XI protein
Opaque-2 (*O2*)	recessive	22-kDa elimination, 20-kDa reduction	transcriptional activator
Opaque-5 (*O5*)	recessive	no reduction	MGD1
Opaque-6 (*O6*)	recessive	general reduction	
Opaque-7 (*O7*)	recessive	general reduction 20- and 22-kDa	ACS-like protein
Opaque-15 (*O15*)	recessive	27-kDa and γ-zein reduction	
Opaque-2 modifiers	semi-dominant	27-kDa overproduction	
Floury-1 (*fl1*)	semi-dominant	general reduction	transmembrane protein
Floury-2 (*fl2*)	semi-dominant	general reduction	defective 22-kDa zein
Floury-3 (*fl3*)	semi-dominant	general reduction	
Defective Endosperm B30 (*De*B30*)	dominant	general reduction	defective 20-kDa zein
Mucronate (*Mc1*)	dominant	general reduction	abnormal 16-kDa γ-zein
Zpr10(22)	recessive	10-kDa reduction	

of gene families makes a comprehensive analysis of storage protein gene expression in maize endosperm possible with the identification of novel seed proteins that were not described previously (Woo et al, 2001). For example, to advance our understanding of the nature of the mutations associated with an opaque phenotype, Hunter et al (2002) assayed the patterns of gene expression in a series of opaque endosperm mutants by profiling endosperm mRNA transcripts with an Affimetrix GeneChip containing approximately 1,400 selected maize gene sequences. Their results revealed distinct, as well as shared, gene expression patterns in these mutants. Similar research on the pattern of gene expression in *o2*, *o7*, and in the *o2o7* endosperm mutants was carried out by Hartings et al (2011) by profiling endosperm mRNA transcripts at 14 DAP. Their results, based on a unigene set composed of 7,250 ESTs, allowed to identify a series of mutant related up-regulated (17.1%) and down-regulated (3.2%) transcripts. In addition, the same authors identified several differentially expressed ESTs, homologous to gene encoding enzymes involved in amino acid synthesis, C metabolism (TCA cycle and glycolysis), storage protein and starch metabolism, gene transcription and translation processes, signal transduction, and in protein, fatty acid, and lipid synthesis. Those analyses demonstrate that the mutants investigated are pleiotropic and play a critical role in several endosperm metabolic processes. Although, by necessity, these data are descriptive and more work is required to define gene functions and dissect the complex regulation of gene expression, the genes isolated and characterized to date give us an intriguing insight into the mechanisms underlying amino acid metabolim in the endosperm.

Practical applications and perspectives

Inherent phenotypic deficiencies, such as soft endosperm texture, lower yield, increased seed susceptibility to pathogens, and mechanical damages, have limited efforts to develop opaque mutations that are commercially useful. Quality Protein Maize (QPM) strains, created by selecting genetic modifiers that convert the starchy endosperm of an *o2* mutant to a hard, vitreous phenotype were developed to overcome these drawbacks. Genetic studies have shown that there are multiple, unlinked o2 modifiers (*Opm*), (review in Gibbon and Larkins, 2005). Genetic analysis of *o2* modifiers identified several disperse quantitative trait loci (QTLs). Although their molecular identities have remained unknown, QTLs could be correlated with observed increases in 27-kDa γ-zein transcript and protein in QPM (Holding et al. 2008, and references therein). Two different QTLs, which are candidates for *o2* modifier genes, affect 27-kDa γ-zein gene expression. The first of these is associated with increased expression and the other is linked to *o15*, a mutation on chromosome 7, which causes decreased 27-kDa γ-zein expression, suggesting that the amount of γ-zeins might be critical in order to keep starch granules embedded in the vitreous area. To examine the role of γ-zeins in QPM, Wu et al, (2010) have used an RNAi construct, designed from the inverted coding sequences of the 27- kDa γ-zein gene, to knock down both 27- and 16-kDa γ-zeins by taking advantage of their DNA sequence conservation. Their findings reinforce the fact that different zeins have evolved to play distinct roles in the development of the endosperm.

A useful strategy to develop more quickly new QPM varieties has been proposed by Wu and Messing (2011). In fact, conversion of QPM into local germplasm is a lengthy process that discourages the spread of the benefits of QPM because breeders have to monitor a high lysine level, the recessive *o2* mutant allele, and the modifiers of *o2*, (*Mo2s*). Accordingly, to overcome this problem these last authors presented a simpler and accelerated QPM selection. Instead of using the recessive *o2* mutation, they used an RNAi

construct directed against both 22- and 19-kDa zeins, but linked to the visible green fluorescent protein (GFP) marker gene. Indeed, when such a green and non-vitreous phenotype was crossed with QPM lines, the Mo2s produced a vitreous green kernel, demonstrating that high lysine and kernel hardness can be selected in a dominant fashion.

High-throughput genomics and post-genomics approaches are now providing new tools for a better understanding of the genetic and biochemical networks operating during kernel development. Large databases of maize expressed sequence tags (ESTs) are now available (i.e http://www.maizegdb.org), and transcriptome analyses aimed at identifying genes involved in endosperm development and metabolism have been published, along with computer software allowing a systematical characterization. Hence, it has become possible to analyze gene expression in developing maize endosperm more thoroughly and to identify tissue-specific genes involved in endosperm

Carbohydrate synthesis

Maize, like other cereals, accumulates starch in the seed endosperm as an energy reserve. Its starch is one of the most important plant products and has various direct and indirect applications in food, feed, and industries. For this reason, attempts to increase starch accumulation have received a great deal of attention by plant breeders and plant scientists. Starch biosynthesis is a central function in plant metabolism and is accomplished by a multiplicity of conserved enzymatic activities (see Hannah and James, 2008, for a review). Roughly, three-quarters of the total starch is amylopectin, which consists of branched glucose chains that form insoluble, semi-crystalline granules. The remainder of the starch is amylose, which is composed of linear chains of glucose that adopt a helical configuration within the granule (Myers et al, 2000). Starch synthesis proceeds by means of two fundamental activities represented by starch synthase, which catalyzes the polymerization of glucosyl units into α(1/4)-linked «linear» chains, and starch-branching enzyme, which catalyzes the formation of α(1/6)-glycoside bond branches that join linear chains. Acting together, the starch synthases and starch-branching enzymes assemble the highly branched polymer amylopectin, with approximately 5% of the glucosyl residues participating in α(1/6)-bonds, and the lightly branched molecule amylose. A third activity necessary for normal starch biosynthesis is provided by starch-debranching enzyme (DBE), which hydrolyzes α(1/6)-linkages. According to Beatty et al (1999), two separate but conserved DBE classes are present in plants i) pullulanase-type DBE (PUL) and ii) isoamylase-type DBE (ISA), identified based on similarities with prokaryotic enzymes with particular substrate specificities. The ISA functions in starch production are deduced from mutation behavior, typically resulting in reduced starch content, an abnormal amylopectin structure, altered granule morphology, and the accumulation of abnormally highly branched polysaccharides similar to glycogen.

Genes affecting starch biosynthesis

Starch biosynthesis in seeds is dependent on several environmental, physiological, and genetic factors (reviewed in Boyer and Hannah, 2001). The maize kernel is a suitable system for studying the genetic control of starch biosynthesis, since a large number of mutations that cause defects in various steps in the pathway of starch biosynthesis in the kernel have been described. Their analysis has contributed greatly to the understanding of starch synthesis (reviewed in Boyer and Hannah, 2001). In addition, these mutations have facilitated the identification of many genes involved in starch biosynthetic production. The cloned maize starch biosynthesis genes and their gross phenotypes are summarized in Table 2. In addition, Kubo et al (2010) have recently described novel mutations of the *sugary1* (*su1*) and *isa2* loci. The involved genes encode isoamylase-type starch-DE enzymes (ISA1 and ISA2, respectively). Their data indicate that, in maize endosperm, these enzymes function to support starch synthesis either as a heteromeric multi-subunit complex containing both ISA1 and the non-catalytic protein ISA2 or as a homomeric complex containing only ISA1. In particular, it was found that i) homomeric ISA has specific functions that determine amylopectin structure and that are not provided by heteromeric ISA and ii) tissue-specific changes in relative levels of ISA1 and ISA2 transcripts, or functional changes in the ISA1 protein, could explain how maize endosperm acquired the homomeric enzyme.

Many biochemical and molecular studies on starch synthesis have been focused on identifying the rate limiting enzymes in order to be able to control the metabolism. In this context, ADP-glucose pyrophosphorylase (AGPase) plays a key role in regulating starch biosynthesis in cereal seeds. The AGPase in the maize endosperm is a hetero-tetramer of two small subunits encoded by *Brittle2* (*Bt2*) gene, and two large subunits, encoded by the *Shrunken2* (*Sh2*) gene. Studies of the kinetic mechanism of maize endosperm AGPase have uncovered complex regulatory properties (Kubo et al, 2010). Nevertheless, transgenic approaches focused on allosteric regulation of AGPase, determined an increase in starch content and caused an increased seed weight with respect to lines expressing wild-type alleles (Giroux et al, 1996; Wang et al, 2007). Additional research has been devoted to the over-expression of the wide-type genes encoding maize AGPase. For example, Li et al (2011), have transferred the *Bt2* and *Sh2* genes from maize, with either an endosperm-specific promoter from a 27-kDa zein or an endosperm-specific promoter from a 22-kDa zein, into elite inbred lines, solely and in tandem, by *Agrobacterium tumefaciens*-mediated transformation. They found that developing

Table 2 - Summary of mutant effects in maize where an associated enzyme lesion has been reported.

Genotype	Major biochemical changes[§]	Enzyme affected
Shrunken-1 (*sh1*)	↑ sugars ↓ starch	↓ sucrose synthase
Shrunken-2 (*sh2*)	↑ sugars ↓ starch	↑ hexokinase ↓ ADPG- pyrophosphatase
Brittle-1 (*bt1*)	↑ sugars ↓ starch	↓ starch granule-bound phospho-oligosaccharide synthase
Brittle-2 (*bt2*)	↑ sugars ↓ starch	↓ ADPG- pyrophosphatase
Shrunken-4 (*sh4*)	↑ sugars ↓ starch	↓ pyridoxal phosphate
Sugary-1 (*su1*)	↑ sugars ↓ starch	↑ phytoglycogen branching enzyme ↓ phytoglycogen debranching enzyme
Waxy (wx)	↑ 100% amylopectin	↑ phytoglycogen branching enzyme ↓ starch-bound starch synthase
Amylose-extender (*ae*)	↑ apparent amylose, loosely branched polysaccharide	↓ branching enzyme IIb
Dull-1 (*du1*)	↑ apparent amylose	↓ starch synthase II ↓ branching enzyme IIa ↑ phytoglycogen branching enzyme

[§]changes relative to normal, ↑ ↓ = increase or decrease, respectively, sugars = alcohol-soluble sugars

transgenic maize kernels exhibited higher *Bt2* and *Sh2* gene expression, higher AGPase activity, higher seed weight, and that the kernels accumulated more starch compared with non-transgenic plants. The over-expression of either gene enhanced AGPase activity, seed weight (+15%) and starch content compared with the wild type, but the amounts were lower than plants with over-expression of both *Bt2* and *Sh2*. Collectively, these results indicate that over-expression of those genes in transgenic maize plants could improve kernel traits and provide a feasible approach for enhancing starch content and seed weight in maize.

Regulation of starch synthesis

Despite the several above-mentioned studies shedding light on such a complex metabolic pathway as starch synthesis, its regulation is still poorly understood. This is surprising, considering the number and variety of starch mutations identified so far, which may indicate that nutrient flow is the key regulatory stimulus in carbohydrate inter-conversion. In this connection, it has been argued that glucose and in some cases, different sugars or sugar metabolites might act as the actual signal molecules (reviewed in Koch, 2004). There is evidence that regulation of the major grain-filling pathway is highly integrated in endosperm and gene responses to sugars and C/N balance are likely involved. For example, Sousa et al (2008) have recently identified a gene for *Sorbitol dehydrogenase1* (*Sdh1*) in maize. They showed that this gene is highly expressed throughout the endosperm during early seed development, with the greatest expression levels in the basal region, compatible with SDH involvement in the initial metabolic steps of carbohydrate metabolism. The same authors also presented genetic, kinetic, and transient expression evidence for regulation at the transcriptional level by sugars and hypoxia. Moreover, many pleiotropic defective kernel (*dek*) mutations that fail to initiative or complete grain filling have been identified, but not studied in detail. These are likely to include mutations in «housekeeping genes» as well as important developmental mutants or transcription factors. In this respect, a key challenge is to devise molecular and genetic strategies that can be used to effectively analyze this large, complex phenotypic class.

As far as transcription factors are concerned, Fu and Xue (2010) have recently identified candidate regulators for starch biosynthesis by gene co-expression analysis in rice. Among these genes, *Rice Starch Regulator1* (*RSR1*), an APETALA2/ethylene-responsive element binding protein family transcription factor, was found to negatively regulate the expression of type I starch synthesis genes. Moreover, *RSR1* deficiency results in the enhanced expression of starch synthesis genes in seeds. Collectively, these results demonstrate the potential of co-expression analysis for studying rice starch biosynthesis and the regulation of a complex metabolic pathway and provide informative clues, including the characterization of *RSR1*, to facilitate the improvement of seed quality and nutrition. It is expected that similar orthologous loci will be soon identified in maize; this will allow us to deeper our knowledge on regulatory mechanisms affecting starch biosynthesis in maize. Different ap-

proaches in this area are needed to identify direct interaction among starch biosynthetic enzymes, as well as modifying factors that regulate enzyme activity. In this respect, Wang et al (2007) described a study in which a bacterial *glgC16* gene, which encodes a catalytically active allosteric-insensitive enzyme, was introduced into maize. The results of this study showed that developing transgenic maize seeds exhibited higher AGPase activity (a rate limiting step in glycogenesis and starch synthesis), in the presence of an inhibitory level of Pi *in vitro*, compared with the untransformed control. More interestingly, the same authors found the seed weight of transgenic plants was increased significantly. Furthermore, tools for genome-based analyses of starch biosynthesis pathway are now available for maize and other cereals. This may eventually help to explain species differences in starch granule shape and size, and thus provide the potential for agricultural advances. Recently, Prioul et al (2008) have provided information on carbohydrate metabolism by comparing gene expression at three levels - transcripts, proteins and, enzyme activities - in relation to substrate or product in developing kernels from 10 to 40 DAP. Their study has identified two distinct patterns: during endosperm development invertases and hexoses are predominant at the beginning, whereas enzyme patterns in the starch pathway, at the three levels, anticipate and parallel starch accumulation, suggesting that, in most cases, transcriptional control is responsible for the regulation of starch biosynthesis.

Lipids

Plant oil is an important renewable resource for biodiesel production and for dietary consumption by humans and livestock. The mature maize embryo consists of approximately 33% lipid in standard hybrids and contains about 80% of the kernel lipids (Val et al, 2009). High-oil maize shows a greater feed efficiency than normal-oil maize in animal feed trials: the caloric content of oil is 2.25 times greater than that of starch on a weight basis and its fatty acid composition, mainly oleic and linoleic acids. Furthermore, maize oil is highly regarded for its low level of saturated C16:0 and C18:0 fatty acids (on average 11% palmitic acid and 2% stearic acid), and its relatively high levels of polyunsaturated fatty acids such as the essential omega-6 fatty acid, linoleic acid (24%). Maize oil contains only small amounts of the essential omega-3 fatty acid linolenic acid (0.7%) and high levels of natural antioxidants and is therefore relatively stable (Val et al, 2009). Oil and starch are accumulated in different compartments of the maize kernel: 85% of the oil is stored in the embryo, whereas 98% of the starch is located in the endosperm. Therefore, the relative amounts of oil and starch are correlated with the relative sizes of the embryo and endosperm and successful breeding for high oil content in the Illinois High Oil strains has mainly been achieved through an increase in embryo size (Moose et al, 2004). Whereas the embryo represents less than 10% of the kernel weight in normal or high-protein lines, it can contribute more than 20% in high-oil lines. Genetic components may also modulate oil content in the embryo, independently of its size, as shown by the cloning of a high-oil QTL in maize that is caused by an amino acid insertion in an acyl-CoA:diacylglycerol acyltransferase catalyzing the last step of oil biosynthesis (Zheng et al, 2008).

Lipid biosynthetic pathway and genetic inheritance

The primary determinant of amount of lipids in maize kernels is the genetic makeup (Lambert, 2001). In spite of a good understanding of the oil biosynthetic pathway in plants and of the many genes involved in oil pathway have been isolated, the molecular basis for oil QTL is largely unknown. In maize studies through genetic mapping of oil traits reported that multiple (>50) QTLs are involved in lipid accumulation (Laurie et al, 2004), making yield improvement through conventional breeding difficulty. However, evidence has shown that genetic variation exists also for the fatty acid composition of the kernel (Lambert, 2001). Zheng et al (2008) have recently found that an oil QTL (qHO6) affecting maize seed oil and oleic acid content, encodes an acyl-CoA:diaglycerol acytransferase (DGAT1-2), which catalyzes the final step of oil synthesis. A single gene *linoleic acid1* with a recessive allele, *ln1*, which conditions high linoleic acid levels, was identified in genetic studies involving Illinois High Oil strains. Single gene inheritance has also been identified in other reports (Poneleit and Alexander, 1965). Additionally, *oleic acid1* (*olc1*), which reduces further desaturation of oleic acid to linoleic acid was identified and mapped to chromosome 1 (Wright, 1995). Other studies, using monosomic lines, have identified genes controlling oleic and linoleic acid composition on chromosomes 1, 2, 4, and 5 (Windstrom and Jellum, 1984). High stearic acid and high oleic acid contents were reported to be under the control of one major gene (Wright, 1995). In essentially all studies, researchers suggested that major gene effects were being modulated by modifier genes for oil composition. Although it seems that sources of major genes for composition of maize oil can be utilized, other studies indicate that the inheritance of oleic, linoleic, palmitic, and stearic acid content when considered together is complex and under multigenic control (Sun et al, 1978). Molecular characterization of *fatty acid desaturase-2* (*fad2*) and *fatty acid desaturase-6* (fad6) in maize indicates that fad2 and fad6 clones are not associated with QTLs for the ratio of oleic/linoleic acid, suggesting that some of the QTLs for the oleic/linoleic acid ratio do not involved variants of *fad2* and *fad6*, but rather involved other genes that may influence flux via enzymes encoded by *fad2* or *fad6*. Additional studies are needed to more precisely identify the genes and enzymes involved in determining the composition of maize oil. Application of pow-

erful new technologies, such as transcription profiling, metabolic profiling, and flux analyses, should prove valuable to achieving this scope.

In addition, identification of transcription factors or other regulatory proteins that exert higher level control of oil biosynthesis or embryo development will be particularly attractive candidate for biotechnology approaches in the future. In maize, Pouvreau et al (2011) have recently identified orthologs related, respectively, to the master regulators LEAFY COTYLEDON1-2 (ie. *ZmLEC1*), that directly activate in Arabidopsis genes involved in TAG metabolism and storage, and to the transcription factor WRINKLED1 (ie. *ZmWRI1a* and *ZmWRI1b*), necessary to mediate the regulatory action of the master regulators towards late glycolytic and oil metabolism. In maize, both genes are preferentially expressed in the embryo and exhibit a peak of expression at the onset of kernel maturation. *ZmWRI1a* is induced by *ZmLEC1* (Shen et al, 2010). Additionally, transcriptomic analyses carried out on *ZmWRI1a* over-expressing lines have allowed to the previous workers to identify putative target genes of *ZmWRI1a* involved in late glycolysis, fatty acid or oil metabolism. Though not fully overlapping, the sets of *AtWRI1* and *ZmWRI1a* target genes are very resembling. Kernels from transgenic lines, overexpressing *ZmWri1a*, did induce a significant increase in saturated and unsaturated fatty acids with 16 to 18 C atomsas well as several free amino acids (Lys, Glu, Phe, Ala, Val), intermediates or cofactors of amino acid biosynthesis (pyro-Glu, aminoadipic acid, Orn, nor-Leu), and intermediates of the TCA cycle (Maeo et al, 2009). Since the transcriptome analysis suggests that *ZmWri1a* essentially activates genes coding for enzymes in late glycolysis, fatty acid, CoA, and TAG biosynthesis, and considering that no misregulated candidates participate in any additional pathways, the increase in amino acids and TCA intermediates probably reflects secondary adjustments of the C and N metabolism to the increased oil biosynthesis triggered by *ZmWri1a*. The three amino acids Phe, Ala, and Val are derived from PEP or pyruvate, and their increase may simply be a byproduct of a strongly increased C flux through glycolysis.

Carotenoid pigments

Carotenoids are a complex class of isoprenoid pigments providing nutritional value as provitamin A and non-provitamin A. Along with their essential role in photosynthesis, carotenoids are of significant economic interest as natural pigments and food additives (reviewed in Botella-Pavía and Rodríguez-Concepción, 2006). Their presence in the human diet provides health benefits as nontoxic precursors of vitamin A and antioxidants, including protection against cancer and other chronic diseases (review by Fraser and Bramley, 2004). Furthermore, their varied colours provide additional commercial value as colorants in foods and nutrient supplementation (reviewed in Matthews and Wurtzel, 2007). These motives have promoted scientists to explore ways to improve carotenoid content and composition in staple crops (reviewed in Sandmann et al, 2006; Zhu et al, 2009). Analyses of genotypes with yellow to dark orange kernels evinces considerable natural variation for kernel carotenoids, with some lines accumulating as much as 66 µg/g (e.g. Harjes et al, 2008). Provitamin A activity (β-cryptoxanthin, α- and β-carotene is typically small ranging from 15% to 18% of the total carotenoids fraction, compared to lutein or zeaxanthin with average contents of 45% and 35%, respectively (Kurlich and Juvik, 1999; Brenna and Berardo, 2004). A moderate to high heritability estimate indicates that breeding for increased levels of both carotenes and xanthophylls should be feasible.

Carotenoid biosynthesis and genetic control

Carotenoids are derived from the isoprenoid biosynthetic pathway and are precursors of the plant hormone abscisic acid (ABA) and of other apo-carotenoids (Matthews and Wurtzel, 2007). 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, therefore, 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).

The plant carotenoid biosynthetic pathway has been well characterized (Figure 1) 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. Provitamin A compounds are biosynthetic pathway intermediates and therefore usually not the predominant carotenoids in endosperm, the target of provitamin A biofortification. α-carotene and β-cryptoxanthin have provitamin A potential, due to their single 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). 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;

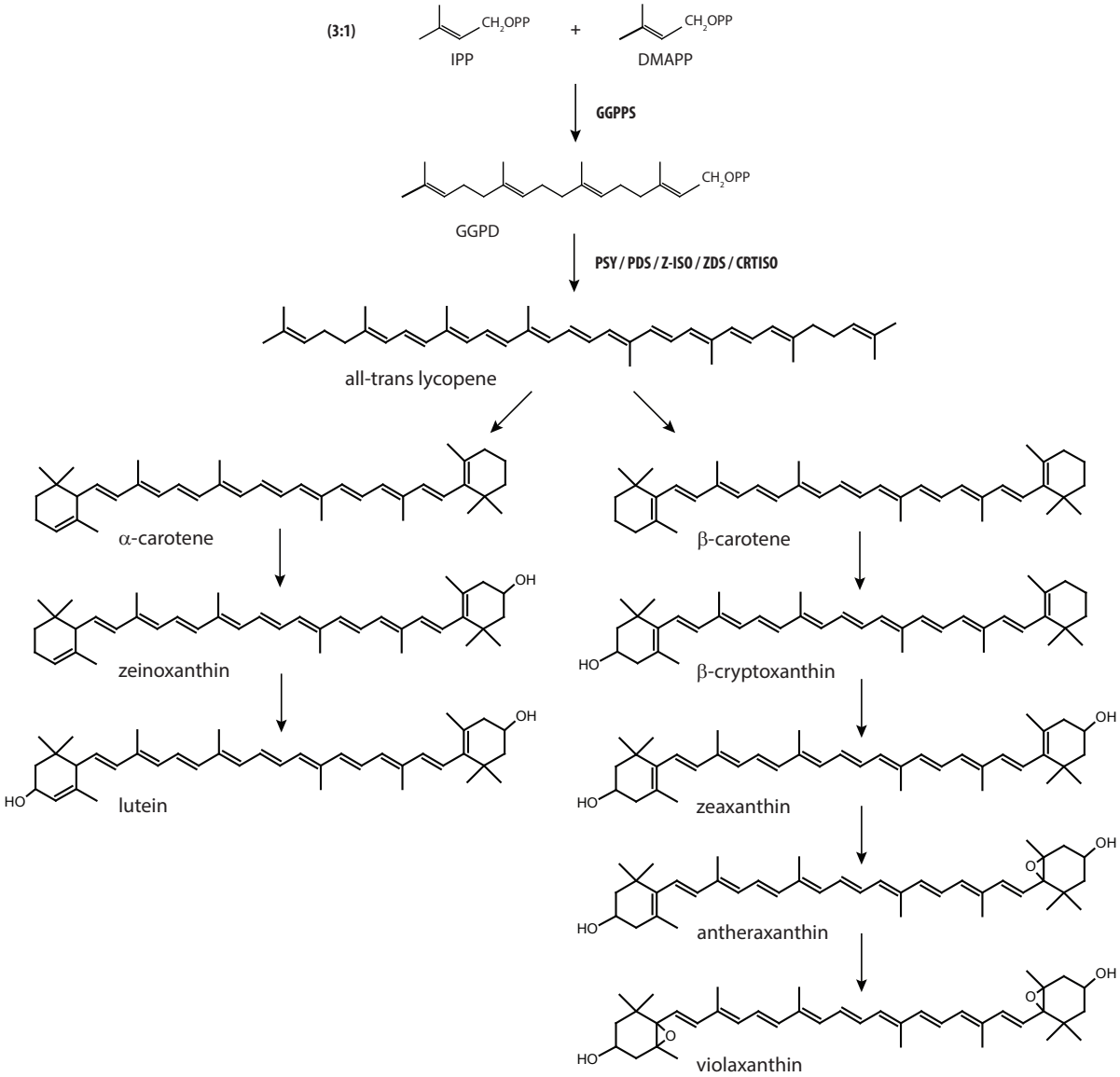

Figure 1 - Carotenoid synthesis in maize. IPP - isopentenyl diphosphate, DMAPP - dimethylallyl diphosphate, GGDP - geranylgeranyl diphosphate, GGPPS - geranylgeranyl diphosphate synthase, PSY - phytoene synthase, PDS - phytoene desaturase, Z-ISO - ζ-carotene isomerase, ZDS - ζ-carotene desaturase, CRTISO - carotenoid 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).

Characterization of the carotenoid biosynthetic pathway has been facilitated by the analysis of mutants associated with reduced levels of carotenoids. In fact, by using this approach in maize three genes controlling early steps in the carotenoid pathway have been cloned. The use of these cloned genes as probes on mapping populations will enable the candidate gene approach to be used for studying the genetic control of quantitative variation in carotenoids. Accordingly, Wurtzel et al (2004) detect major QTLs affecting accumulation of β-carotene and β-cryptoxanthin indicating that these QTLs could be selected to increase levels of provitamin A structures.

Chander et al (2007), using a RIL population found 31 QTL including 23 for individual and 8 for total carotenoid accumulations. Moreover, Harjes et al (2008), via association mapping, linkage mapping, expression analysis, and mutagenesis, showed that variation in the *lycopene epsil cyclase* (*lcyE*) locus alters flux down α-carotene versus β-carotene branches of the carotenoid pathway. Additional experimental evidence obtained by Yan et al (2010) have documented that also the gene encoding β-*carotene hydroxylase1* (*crtRB1*) underlies a principal QTL associated with β-carotene concentration and conversion in maize kernels. Moreover, the same workers noted that the *crtRB1* alleles associated with reduced transcript expression correlate with higher β-carotene concentrations. Genetic variation at *crtRB1* also affects hydroxylation efficiency among encoded allozymes, as observed by resultant carotenoid profiles in recombinant expression assays. Similarly, studies on natural maize genetic diversity carried out by Vallabhaneni et al (2009), have provided the identification of hydroxylation genes associated with reduced endosperm provitamin A content. In particular, transcript profiling led to discovery of the *hydroxylase3* locus that coincidently mapped to a carotene QTL, thereby prompting investigation of allelic variation in a broader collection. Vallabhaneni and Wurtzel (2009) have sampled a maize germplasm collection via statistical testing of the correlation between carotenoid content and candidate gene transcript levels. They observed multiple pathway bottlenecks for isoprenoid biosynthesis and carotenoid biosynthesis acting in specific temporal windows of endosperm development. Transcript levels of paralogs encoding isoprenoid isopentenyl diphosphate and geranylgeranyl diphosphate-producing enzymes, such as DXS3 (1-deoxy-D-xylulose-5-phosphate synthase3), DXR (DXP reductoisomerase), HDR (4-hydroxy-3-methylbut-2-enyl diphosphate reductase), and GGPPS1 (geranylgeranyl pyrophosphate synthase1), were found to positively correlate with endosperm carotenoid content.

New strategies for creating variation

The use of molecular biology to isolate, characterize, and modify individual genes followed by plant transformation and trait analysis will introduce new traits and more diversity into maize database. Metabolic engineering of maize has been relatively slow due to the difficulty of maize transformation. Maize transformation with Agrobacterium (reviewed in Jones, 2009) is now more efficient than currently used particle gun transformation. In addition, larger DNA fragments can be inserted with Agrobacterium than those previously reported by other methods. Furthermore, a strategy for targeted genome modification through the use of designed zinc finger nucleases that induce a double stranded break at their target locus has been recently developed (Shukla et al, 2009). The ability to routinely insert metabolic pathway quantities of DNA into the maize genome will further speed up maize metabolic engineering.

Maize-based diets (animals or human) require lysine and tryptophan supplementation for adequate protein synthesis. Tryptophan is also the precursor for the synthesis of some neurotransmitters and for niacin (Heine et al, 1995, Morris and Sands, 2006). The development of high-lysine maize for use in improved animal feeds illustrates the challenges that continually interlace metabolic engineering projects. For example, maize-based diets (animals or humans) require lysine and tryptophan supplementation for adequate protein synthesis. The development of high-lysine maize to use in improved animal feeds illustrates the challenges that continually interlace metabolic engineering projects. From a biochemical standpoint, the metabolic pathway for lysine biosynthesis in plants is very similar to that in many bacteria. The key enzymes in the biosynthetic pathway are aspartakinase (AK) and dihydrodipicolinic acid synthase (DHDPS), both of which are feedback inhibited by lysine (Galili, 2004). Falco et al (1995) isolated bacterial genes encoding lysine-insensitive forms of AK and DHDPS from *Escherichia coli* and Corynebacterium, respectively. A deregulated form of the plant DHDPS was created by site-specific mutagenesis (Shaver et al, 1996). The expression of the bacterial DHPS in maize seeds overproduced lysine, but they also contained higher level of lysine catabolic products then their wild-type parents (Mazur et al, 1999), despite the fact that lysine catabolism was suggested to be minimal in this tissue (Arruda et al, 2000). Likewise, a gene corresponding to a feedback-resistant form of the enzyme anthranilate synthase (AS) has been cloned from maize and re-introduced via transformation under the control of seedspecific promoters. This altered AS has reduced sensitivity to feedback inhibition by tryptophan; thus, tryptophan is overproduced and accumulates to higher than normal levels in the grain. This strategy has been successful in reaching commercially valuable levels of tryptophan in the grain (Anderson et al, 1997). More recently, Houmard et al (2007) reported the increase in maize grains by specific suppression of lysine catabolism via RNAi. An important observation from these studies was that the lysine content was increased in the transgenic lines by 15-20% to 54.8%. These experiments showed that transgenic approaches, in addition to investigating relationships between zein synthesis and opaque endosperm, could be useful to increase kernel lysine content. Similarly, Reyes et al (2008), using RNAi, have produced transgenic maize lines that had LKR/SDH suppressed in the embryo, endosperm or both. These authors noted a synergist increase in free lysine content in the mature kernel when LKR/SDH was suppressed in both embryo and endosperm; these results have also suggested new insights into how free lysine level is regulated and distributed in

developing grains. A different approach to enhance the level of a given amino acid in kernels is to improve the protein sink for this amino acid (Kriz, 2009). This goal can be achieved by transforming plants with genes encoding stable proteins that are rich in the desired amino acid(s) and that can accumulate to high levels. Following this approach, Jung and Falco (2000), deduced that, among a variety of natural, modified or synthetic genes that were tested, the most significant increases in seed lysine levels were obtained by expressing a genetically-engineered hordothionine (HT12) or a barley high-lysine protein 8 (BHL8), containing 28 and 24% lysine, respectively. These proteins accumulated in transgenic maize to 3-6% of total grain proteins and when introduced together with a bacterial DHPS, resulted in a very high elevation of a total lysine to over 0.7% of seed dry weight compared to around 0.2% in wild-type maize. Similarly, Rascon-Cruz et al (2004) found, that the introduction of a gene encoding amarantin protein from Amaranth plants, which is known to be balanced in its amino acid content, increases from 8 to 44% essential amino acid content. Bicar et al (2008) have developed transgenic maize lines that produce milk α-lactalbumin in the endosperm. They noted that the lysine content of the lines examined was 29-47% greater in endosperm from transgene positive kernels.

Single mutations in starch biosynthesis have been commercially used for the production of specialty maize. For example, specialty varieties such as waxy can result in 99% amylopectins, while the use of «amylomaize varieties» (amylose extender endosperm mutants) have kernels up to 20% amylopectin and 80% amylose. These varieties are of interest for commercial purposes in starch industry, such as food ingredients, sweeteners, adhesives, and for the development of thermoplastics and polyurhetanes. However, advances in understanding the starch biosynthetic pathway provide new ways to redesign starch for specific purposes, such for ethanol production. Alteration in starch structure can be achieved by modifying genes encoding the enzymes responsible for starch synthesis, many of which have more than one isoform (Boyer and Hannah, 2001). Transgenic lines with modified expression of specific starch synthases, starch branching enzymes or starch debranching enzymes are being generated in attempts to produce starch granules with increased or decreased crystallinity, and thus altered susceptibility to enzymatic digestion.

The expression of microbial genes in transgenic plants represents also an opportunity to produce renewable resources of fructans. Transgenic maize expressing the *Bacillus amyloliquefaciens SacB* gene accumulates high-molecular weight fructose in mature seed (Caimi et al, 1996) with a potential use within the high-fructose maize syrup market. Zhang et al (2007) have developed transgenic maize endosperm, via the introduction of a *Streptococcus mutans gtfD* gene that accumulates novel glucan (oligo- and polysaccharides composed solely of glucose molecules) polymers at levels relevant to commercial production allowing the possible generation of specialty glucans to replace modified starches used for several products (eg., thickening reagents, adhesives, textile modification, and papermaking polymers with economical and environmental benefits).

Efforts to increase oil content and composition in maize kernels through breeding have considerable success, but high oil lines have significant reduced yield (cf. Moose et al, 2004). Several and complementary approaches might be considered to try and enhance oil content in maize kernels. First, this goal may be achieved by increasing the relative proportion of the oil-rich embryonic tissue within the grain. It has been recently reported that embryo size and oil content could be increased in transgenic maize by ectopic expression of the wheat *Purindoline a* and *b* (*PINA* and *PINB*) genes (Zhang et al, 2010). While total oil content of the kernel was increased by 25% in these transgenic lines, the molecular mechanism responsible for the increase remains to be clarified. Another strategy to increase oil accumulation in the grain may consist in improving the oil content of embryonic tissues. A close examination of C metabolism in maize embryos suggested that the flux of C through NADP-ME may constitute a metabolic bottleneck (Alonso et al, 2010). The oil content of the kernel was positively correlated with malic enzyme activities in maturing embryos (Doehlert and Lambert, 1991), which makes NADP-ME an attractive target for engineering high oil concentrations in embryos of maize. The recent identification of transcriptional regulators of the oil biosynthetic network in maize has opened the way for designing and testing new original biotechnological strategies. A study has shown that seed-specific expression of *ZmWRI1*, a WRI1-like gene of maize, enhanced oil accumulation in transgenic maize without detectable abnormalities. Finally, a third approach to increase oil content in maize grains may consist in diverting C flux from starch to oil in the endosperm. Considering both the elevated amounts of ATP consumed in futile cycling processes and the rates of reductant production in endosperm tissues of maize kernels, Alonso et al (2010) have speculated that increasing biomass synthesis and redirecting part of the C flux toward fatty acid production by metabolic engineering could theoretically be obtained. This would require inhibiting futile cycling whilst overexpressing the whole set of enzymes involved in TAG production. To date, no successful attempt has been reported. If the use of *ZmWRI1* as a biotechnological tool for improving oil content in embryos of maize seems promising, overexpression of *ZmWRI* in the starchy endosperm was not sufficient to trigger oil accumulation in this compartment (Shen et al, 2010). What is more, the structure and size of maize kernels may impair large accumulation of oil in

the endosperm.

The cloning of carotenogenic genes in maize and in other organisms have opening up the possibility of modifying and engineering the carotenoid biosynthetic pathways in plants. Engineering high levels of specific carotenoid structures requires controlled enhancement of total carotenoid levels (enhancing pathway flux, minimizing degradation, and optimizing sequestration) plus controlled composition for specific pathway end products. While most of the nuclear genes for the plastid-localized pathway are available (Li et al, 2007) and/or can be identified, questions remain about the rate-controlling steps that limit the predictability of metabolic engineering in plants. Predictable manipulation of the seed carotenoid biosynthetic pathway in diverse maize genotypes necessitates the elucidation of biosynthetic step(s) that control carotenoid accumulation in endosperm tissue. Studies have implicated PSY, the first committed enzyme, as rate controlling for endosperm carotenoids (e.g. Pozniak et al, 2007; Li et al, 2008). However, upstream precursor pathways may also positively influence carotenoid accumulation (Matthews and Wurtzel, 2000; Mahmoud and Croteau, 2001), while downstream degradative pathways may deplete the carotenoid pool (Galpaz et al, 2008). Transgenic strategies can also be used as tools to complement breeding techniques in meeting the estimated levels of provitamin A. In this respect, Aluru et al (2008) reported that the overexpression of the bacterial genes *crtB* (for PS) and *crtI* (for the four desaturation steps of the carotenoid pathway catalyzed by PDS and β-carotene desaturase in plants), resulted in an increase of total carotenoids of up to 34-fold with a preferential accumulation of β-carotene in the maize endosperm. The levels attained approach those estimated to have a significant impact on the nutritional status of target populations in developing countries. Furthermore, the same authors, via gene expression analyses, suggested that increased accumulation of β-carotene is due to an up-regulation of the endogenous lycopene β-cylase. These experiments set the stage for the design of transgenic approaches to generate provitamin A-rich maize that will help alleviate vitamin A deficiency in developing countries. Similarly, Naqvi et al (2009) produced transgenic maize plants with significantly increased contents for β-carotene, ascorbate, and folate in the endosperm via that simultaneous modification of 3 separate metabolic pathways. The transgenic kernels contained 169-fold the normal amount of β-carotene, 6-fold, and 2-fold the normal amount of ascorbate and folate, respectively. This finding, which largely exceeds any realized thus far by conventional breeding alone, opens the way for the development of nutritional complete cereals to benefits the consumers in developing countries.

A relatively new area in plant biotechnology is the use of genetically-engineered maize to produce high-value end products such as vaccines, therapeutic proteins, industrial enzymes and specialty chemicals (see Hood and Howard, 2009 for a review). The long-term commercial expectations for this use of «plants as factories», often also called «molecular farming», are large. Transgenic maize seed has many attractive features for this purpose, including: i) well-suited for the production and storage of recombinant proteins; ii) ease of scale-up to essentially an infinite capacity; iii) well-established infrastructure for producing, harvesting, transporting, storing, and processing; iv) low cost of production; v) freedom from animal pathogenic contaminants; vi) relative ease of producing transgenic plants which express foreign proteins of interest. However, there is a need, apart the public issues related with the acceptance of genetically-engineered maize, for continued efforts in increasing expression in order to reduce cost effectiveness for products at protein accumulation levels in transgenic plants to broaden this new uses.

Conclusion and future perspectives

Currently more than 70% of maize production is used for food and feed; therefore, knowledge of genes involved in protein, starch, and lipids production is relevant for improving the nutritional and food-making properties of maize kernels. However, developing plants with improved grain quality traits involves overcoming a variety of technical challenges inherent in metabolic engineering programs. Advances in plant genetics and genomic technologies are contributing to the acceleration of gene discovery for product development. In the past few years, there has been much progress in the development of strategies to discover new plant genes. In large part, these developments derive from four experimental approaches: firstly, genetic and physical mapping in plants and the associated ability to use mapbased gene isolation strategies; secondly, transposon tagging which allows the direct isolation of a gene via forward and reverse genetic strategies as well as the development of the Targeting Induced Local Lesions IN Genomes (TILLING) technique; thirdly, protein-protein interaction cloning, that permits the isolation of multiple genes contributing to a single pathway or metabolic process. Finally, through bioinformatics/genomics, the development and use of large expressed sequence tags (ESTs) databases (http://www.maizegdb.org) and, DNA microarray technology to investigate mRNA-level controls of complex pathways.

Two prominent features of agriculture in the 20th century have been the use of breeding and genetics to boost crop productivity and the use of agricultural chemicals to protect crops and enhance plant growth. In the 21st century, crops must produce good yields while conserving land, water, and labor resources. At the same time, industries and consumers require plants with an improved and novel variation

in grain composition. We expect that genomics will bolster plant biochemistry as researchers seek to understand the metabolic pathways for the synthesis of these compounds. Identifying rate-limiting steps in synthesis could provide targets for genetically engineering biochemical pathways to produce augmented amounts of compounds and new compounds. Targeted expression will be used to channel metabolic flow into new pathways, while gene-silencing tools will reduce or eliminate undesirable compounds or traits. Therefore, developing plants with improved grain quality traits involves overcoming a variety of technical challenges inherent in metabolic engineering programs.

Moreover, new technologies and information continue to increase our understanding of maize; for instance, the complete DNA sequence of the maize genome, along with comprehensive transcriptome, proteome, metabolome, and epigenome information, is also a key resource for advancing fundamental knowledge of the biology of development seed quality-related traits to be applied in molecular breeding and biotechnology. These additional layers of information should help to further unravel the complexities of how genes and gene networks function to give plants including quality-traits. This knowledge will drive to improved predictions and capacities to assemble gene variation through molecular breeding as well as more optimal gene selection and regulation in the development of future biotechnology products. In conclusion, although, conventional breeding, molecular marker assisted breeding, and genetic engineering have already had, and will continue to have, important roles in maize improvement, the rapidly expanding information from genomics and genetics combined with improved genetic engineering technology offer a wide range of possibilities for the improvement of the maize grain.

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