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Genetic Enhancement of Grain Quality-Related Traits in Maize

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1. Introduction

Maize (*Zea mays*) 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 & Johnson, 2003). Currently more than 70% of maize production is used for food and feed; therefore, knowledge of genes involved in grain structure and chemical 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. However, important advances were made by breeders in this area as well, resulting in maize with a wide range of compositions. In fact, by exploiting genetic variation, the composition of the kernel was altered for both the quantity and quality (structure and chemical diversity) of starch, protein, and oil throughout kernel development. Furthermore, the ability of plant scientists to use existing genetic variation and to identify and manipulate commercially important genes will open new avenues 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.

This chapter focuses on gene discovery, exploitation, and genetic variation known to affect the development and chemical composition of maize kernel. Throughout the chapter we have attempted 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 enhance kernel quality-related traits. Finally, we provide a brief outlook on future developments in this field and the resultant opportunities and application of conventional and molecular breeding for the development of new maize products better suited to its various end uses.

2. 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).

As a typical angiosperm, the maize kernel comprise two zygotic tissues, namely the embryo (germ) and the endosperm, that are embedded in the testa (or seed coat) and the pericarp (or fruit wall), which fuse into a thin protective envelope. The endosperm is the main storage site of starches and proteins, whereas the embryo reserves mainly lipids. However, the economical and nutritional value of the kernel is mostly derived from the endosperm, a starch-rich tissue, that supports the embryo at germination.

In maize, endosperm makes up the majority of kernel dry matter (70-90%) and is the predominant sink of photosynthates and other assimilates during reproductive growth; therefore, factors that mediate endosperm development to a large extent also determine grain yield. Furthermore, the endosperm of seed can serve as a valuable system to address fundamental questions related to the improvement of seed size in crops.

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. Recently, large databases of maize gene expressed sequence tags (ESTs) have been made 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 to systematically characterize them, has made possible to analyze gene expression in developing maize endosperm more thoroughly to identify tissue-specific genes involved in endosperm 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 5000 different genes could be expressed during development. However, about 35% of them 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). Furthermore, Mèchin 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, defence, 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. Although, mRNAs are the primary products of gene expression and their levels are often weakly correlated to corresponding protein levels (Gygi et al., 1999), the analyses of the changes in the transcription profiles of endosperm mutants may allow to formulate predictions regarding the biological role of these loci in endosperm development and metabolism. This information is useful for identifying distinctive, previously uncharacterised, endosperm-specific genes; in addition, it provides both further research material for academic laboratories, and material for plant breeders and food processors to include in their respective research or product pipelines.

3. Accumulation of storage products

The structure and biochemical properties of seed storage compounds have been widely investigated over the past 30 years 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. For more detailed reviews describing the nature and biochemistry of maize endosperm and embryo storage products, we refer the reader to a number of recent reviews (i.e. Hannah, 2007; Holding & Larkins, 2009; Motto et al., 2009; Val et al. 2009).

3.1 Storage protein

The primary storage proteins in the maize grain are prolamines called "zeins". Specifically, the zeins are the most abundant protein storage component (>60%) in developing endosperm tissues and 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 & Larkins, 2005). 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).

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 & 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. According to these workers once genes are copied, expression of donor genes is reduced relative to new copies. In particular, epigenetic processes that modify the information content of the genome without changing the DNA sequence, seems to contribute to silencing older copies: some of them can be reactivated when endosperm is maintained as cultured cells, indicating that copy number variation might contribute to a reserve of gene copies.

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 as the signal peptide is processed. After being secreted into the RER, the β- and γ-zeins form a matrix, which is penetrated by the α- and δ-zeins, enlarging the PB and making it a spherical structure of 1-2 μm (Lending & Larkins 1989). 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). Recently, maize storage protein mutants created through RNAi showed that γ- zein RNA interference (RNAi) maize mutant lines exhibited slightly altered PB body formation and that a more drastic effect was observed in the β-γ- combined mutant, where protein bodies showed an irregular shape, particularly in their periphery (Wu & Messing, 2010). 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. To gain a deeper insight into the relationship between RNA and protein

localization in plants, 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.

3.1.1 Endosperm mutants altering storage protein synthesis

As highlighted before, endosperm growth and development is a complex phenomenon that may be driven by the coordinate expression of numerous genes. Strategies using spontaneous and induced mutants allow the characterization of the complex underlying gene expression system integrating carbohydrate, amino acid, and storage protein metabolisms and operating during endosperm growth and development. In this respect several endosperm mutants altering the timing and the rate of zein synthesis have been described (reviewed by Motto et al., 2009). The mutants altering the rate of zein synthesis 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, as zein synthesis is reduced, the overall lysine content is elevated, giving potential for use in the development of "high-lysine" maize.

Genotype	**Inheritance**	**Effect on zein accumulation**	**Molecular bases**
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 reduction, reduction γ-zein	
Opaque-2 modifiers	Semidominant	27-kDa overproduction	
Floury-1 (fl1)	Semidominant	General reduction	Transmembrane protein
Floury-2 (fl2)	Semidominant	General reduction	Defect 22-kDa zein
Floury-3 (fl3)	Semidominant	General reduction	
*Defective-endospermB30 (De*B30)*	Dominant	General reduction	Defect 20-kDa zein
Mucronate (Mc1)	Dominant	General reduction	Abnormal 16-kDa γ-zein
Zpr10(22)	Recessive	10-kDa reduction	

Table 1. Some features of maize mutans affecting zein accumulation.

Genetics has played an important role in discovering a series of opaque endosperm mutants and demonstrating their effects on genes mediating zein deposition (Motto et al., 2009). For

example, the recessive mutation *opaque-2* (*o2*) induce a specific decrease in the accumulation of 22-kDa α-zeins, while the *opaque-15* (*o15*) mutation exerts its effect primarily on the 27-kDa γ-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. Similarly, Myers et al. (2011) have found that the *opaque5* (*o5*) mutant phenotype is caused by a reduction in the galactolipid content of the maize endosperm, with no change in zein proteins. Furthermore, these workers reported that *O5* locus encodes the *monogalactosyldiacylglycerol synthase (MGD1)* and specifically affects galactolipids necessary for amyloplast and chloroplast function. A further interesting maize opaque endosperm mutant, termed *mto140*, which also shows retarded vegetative growth has been studied by Holding et al., (2010). The seeds showed a general reduction in zein storage protein accumulation and an elevated lysine phenotype typical of other opaque endosperm mutants; however, it is distinct from the 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. Because the opaque phenotype co-segregated with a Mutator transposon insertion in an *arogenate dehydrogenase* gene (*zmAroDH-1*), this has led the previous authors the characterization of the four-member family of maize *arogenate dehydrogenase* genes (*zmAroDH-1–4*) which share highly similar sequences. Their differential expression patterns, as well as 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 (reviewed in Motto et al., 2009). These studies showed that *O2* activates the expression of 22-kDa α-zein and 15-kDa β-zein genes by interacting with the TC-CACGT(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). Moreover, these last workers via sequence analysis indicated that the *O7* gene showed similarities with members of the larger family of *acyl-CoA synthetase*-like genes (*ACS*), although its exact enzymatic activity is 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).

3.1.2 Regulation of storage protein synthesis

The expression of zein genes is regulated coordinately and zein mRNAs accumulate at high concentrations during early stages of endosperm development (reviewed in Motto et al., 2009). From these studies it was also noted that the coordinate expression of zein genes in maize is controlled primarily at the level of transcription according to specific spatial/temporal patterns. Therefore, attention has turned to understanding the regulatory mechanisms responsible for zein gene expression. Highly conserved *cis*-regulatory sequences have been identified in the promoter of prolamine genes and corresponding *trans*-activity factors (cf Motto et al., 2009). Zein gene expression can also be affected by other regulatory mechanisms, such as methylation, aminoacid supply and phosphorylation. In this context, Locatelli et al. (2009) have provided evidence that O2-mediated transcriptional activation occurs in two-phases, first a potentiated and second a transcriptional activated phase, both characterized by a specific profile of chromatin modifications. The dependency on O2 activity in the establishment of these chromatin states was different for distinct sub-sets of *O2* targets, indicating a gene-specific interaction of *O2* with chromatin modifying mechanisms in driving transcription.

3.1.3 Practical applications and perspectives

Despite efforts to develop opaque mutations that are commercially useful, its inherent phenotypic deficiencies, such as soft endosperm texture, lower yield, increased seed susceptibility to pathogens and mechanical damages, have limited their use. To overcome these drawbacks Quality Protein Maize (QPM) strains were created by selecting genetic modifiers that convert the starchy endosperm of an o2 mutant to a hard, vitreous phenotype. 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 at a different chromosome 7 location, which causes decreased 27-kDa γ-zein expression suggesting that the amount of γ-zeins would become critical 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.

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 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.

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 *o2*, (M*o2*s). 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 nonvitreous 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.

3.2 Starch synthesis

Maize, like other cereals, accumulate starch in the seed endosperm as an energy reserve. Moreover, 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 that is accomplished by a multiplicity of conserved enzymatic activities (see Hannah & 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). Briefly starch synthesis has 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 relatively 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. Two DBE classes have been conserved separately in plants (Beatty et al., 1999). These are referred as pullulanase-type DBE (PUL) and isoamylase-type DBE (ISA), based on similarity to prokaryotic enzymes with particular substrate specificity. ISA functions in starch production are implied from genetic observations that mutations typically result in reduced starch content, abnormal amylopectin structure, altered granule morphology, and accumulation of abnormally highly branched polysaccharides similar to glycogen.

3.2.1 Genes affecting starch biosynthesis

Starch biosynthesis in seeds is dependent upon several environmental, physiological, and genetic factors (reviewed in Boyer and Hannah, 2001). Moreover, the maize kernel is a suitable system for studying the genetic control of starch biosynthesis. 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. As there seems little point in reviewing these data, we will simply summarize in Table 2 cloned maize genes and their gross phenotypes. Although, the effects shown in this table may not necessarily be the primary effect of a mutant, these are the ones presently known. More recently, Kubo et al. (2010) have described novel mutations of *sugary1 (su1*) and *isa2* loci, coding for isoamylase-type starch-DE enzyme (ISA) ISA1 and ISA2, respectively: Their data indicate that in maize endosperm these enzymes function to support starch synthesis either as a heteromeric multisubunit complex containing both ISA1 and the noncatalytic 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 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.

Genotype	Mayor biochemical changes[a]	Enzyme affected
Shrunken-1 (sh1)	↑ Sugars, ↓ Starch	↓ Sucrose synthase
Shrunken-2 (sh2)	↑ Sugars, ↓ Starch	↓ ADPG-pyrophosphorylase, ↑ Hexokinase
Brittle-1 (bt1)	↑ Sugars, ↓ Starch	↓ Starch granule-bound phospho-oligosaccharide synthase
Brittle-2 (bt2)	↑ Sugars, ↓ Starch	↓ ADPG-pyrophosphorylase
Shrunken-4 (sh4)	↑ Sugars, ↓ Starch	↓ Pyridoxal phosphate
Sugary-1 (su)	↑ Sugars, ↓ Starch	↑ Phytoglycogen branching enzyme, ↓ Phytoglycogen debranching enzyme
Waxy (wx)	↑ 100% Amylopectin	↓ Starch-bound starch syntase, ↑ Phytoglycogen branching enzyme
Amylose-extender(ae)	↑ Apparent amylose, ↑ Loosely branched polysaccharide	↓ Branching enzyme IIb
Dull-1 (du1)	↑ Apparent amylase	↓ Starch synthase II, ↓ Branching enzyme Iia,↑ Phytoglycogen branching enzyme

Table 2. Summary of mutant effects in maize where an associated enzyme lesion has been reported.[a] Changes relative to normal. ↑, ↓ = increase or decrease, respectively. Sugars = the alcohol-soluble sugars.

Many biochemical and molecular studies on starch synthesis have been also focused on identifying the rate limiting enzymes to control 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 heterotetramer of two small subunits encoded by *Brittle2* (*Bt*2) gene, and two large subunits, encoded by the *Shrunken2* (*Sh2*) gene. Transgenic approaches focused on allosteric regulation of AGPase, although studies of the kinetic mechanism of maize endosperm AGPase has uncovered complex regulatory properties (Kubo et al., 2010), increase starch content and caused an increased seed weight than lines expressing wild-types (Giroux et al., 1996; Wang et al., 2007). Additional research has been also devoted to the over-expression of the wide-type genes encoding maize AGPase. For example Li et al. (2011), have transferred the *Bt*2 and *Sh2* genes from maize, with an endosperm-specific promoter from 27-kDa zein or an endosperm-specific promoter from 22-kDa zein, into elite inbred lines, solely and in tandem, by *Agrobacterium tumefaciens*-mediated transformation. They found that developing transgenic maize kernels exhibited higher *Bt2* and *Sh2* gene expression, higher AGPase activity, higher seed weight, and 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.

3.2.2 Regulation of starch synthesis

In spite of the above mentioned studies as a complex metabolic pathway, the regulation of starch biosynthesis 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 interconversion. In this connection, it has been argued that glucose also serves as a signal molecule in regulating gene expression, 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 major grain-filling pathway is highly integrated in endosperm. Gene responses to sugars and C/N balance have been implicated. For example, Sousa et al. (2008) have recently identified in maize a gene for *Sorbitol dehydrogenase1* (*Sdh1).* They showed that this gene is highly expressed early in seed development throughout the endosperm, with greatest 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 analyse this large, complex phenotypic class. As far as transcription factors are concerned, Fu & Xue (2010) have recently identified in rice candidate regulators for starch biosynthesis by gene coexpression analysis. 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 approaches 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 fuond 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 have 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.

3.3 Lipids

While intensive agricultural and industrial uses of the maize kernel is widely due to its high starch content, the oil stored in the maize kernel also has considerable importance. Moreover, its oil is the most valuable co-product from industrial processing of maize grain through wet milling or dry milling and is high-quality oil for human.

Research in this field (for review see Val et al., 2009) indicate that i) the mature embryo is approximately 33% lipid in standard hybrids and contains about 80% of the kernel lipids; ii) 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; iii) maize oil is highly regarded for its low level of saturated fatty acids, on average 11% palmitic acid and 2% stearic acid, and its relatively high levels of polyunsaturated fatty acids such as linoleic acid (24%); and iv) maize oil is relatively stable, since it contains only small amounts of linolenic acid (0.7%) and high levels of natural antioxidants. Additionally, it was found that 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. However, 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).

3.3.1 Lipid biosynthetic pathway and genetic inheritance

The primary determinant of amount of lipids in maize kernels is the genetic makeup (Lambert, 2001). 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. High-oil varieties of maize were developed at the University of Illinois through successive cycles of recurrent selection (Dudley and Lambert, 1992). Although these lines have an improved energy content for animal feeding applications, the poor agronomic characteristics, including disease susceptibility and poor standability. These deficiencies precluded their commercial introduction on broad hectarage.

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. However, Zheng et al. (2008) have recently found that a oil QTL (*qHO6*) affecting

maize seed oil and oleic-acid content, encodes an acyl-CoA:diaglycerol acytransferase (DGAT1-2), which catalyze the final step of oil synthesis.

As far as the composition in concerned, maize oil is mainly composed of palmitic, stearic, oleic, linoleic, and linolenic fatty acids. Evidence has shown that genetic variation existed also for the fatty acid composition of the kernel (Lambert, 2001). 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 this plant 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 gene 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 powerful 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 (i.e. ZmLEC1),* that directly activate in Arabidopsis genes involved in TAG metabolism and storage, and to the transcription factor *WRINKLED1* (i.e.. *ZmWRI1a* and *ZmWRI1b)*, necessary to mediate the regulatory action of the master regulators towards late glycolytic and oil metabolism. In this crop, 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. Exhaustive analyses relying on ChIP experiments would allow determining whether these sets are identical. Interestingly, the DNA AW-box proposed to be bound by *AtWRI1* (Maeo et al., 2009) was also identified in promoter sequences of putative target genes of *ZmWRIa*, suggesting that even the *cis*-regulatory element recognized by *WRI1* seems have been conserved between dicots and monocots. Additionally this study has shown that transgenic *ZmWri1a-OE* kernels did not only induce a significant increase in saturated and unsaturated fatty acids with 16 to 18 C atoms but also cause a significant increase for 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 (citric acid, succinic acid). 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.

3.4 Carotenoid pigments

Along with their essential role in photosynthesis, carotenoids are of significant economic interest as natural pigments and food additives (reviewed in Botella-Pavía & 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 & Bramley 2004). 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 exhibits considerable natural variation for kernel carotenoids, with some lines accumulating as much as 66 µg/g (e.g. Harjes et al., 2008), with provitamin A activity (*β*-cryptoxanthin, *α*- and *β*-carotene is typically small (15% to 18% of the total carotenoids fraction) compared to lutein or zeaxanthin (45% and 35%, respectively; Kurlich & Juvik, 1999; Brenna and Berardo, 2004). Moreover, a moderate to high heritability estimates indicate that breeding for increased levels of both carotenes and xanthophylls should be feasible.

3.4.1 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 apocarotenoids (Matthews and Wurtzel, 2007). In maize 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 pro-vitamin 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 *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 & 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. Toledo-Ortiz et al. (2010) have recently identified in *Arabidopsis* seedlings that *phytochrome-interacting factor1 (PIF1*) and other transcription factors of the *phytochrome-interacting factor* (*PIF*) family down-regulate the accumulation of carotenoids by specifically repressing the gene encoding *PSY*, the main rate-determining enzyme of the pathway. Their results also suggest a role for *PIF1* and other *PIFs* in transducing light signals to regulate *PSY* gene expression and carotenoid accumulation during daily cycles of light and dark in mature plants. In this context, manipulating the levels of *PIF* transcription factors by transgenic or marker-assisted breeding approaches might help improve carotenoid accumulation in plants for the production of varieties with enhanced agronomical, industrial, or nutritional value.

4. 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. 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 seed-specific 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. 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 (Jung and Falco, 2000). 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 (Jung and Falco, 2000) compared to around 0.2% in wild-type maize. Similarly, Rascon-Cruz et al. (2004) have 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 *a*-lactalbumin in the endosperm. They noted that the lysine content of the lines examined was 29-47% greater in endosperm from transgene positive kernels. Furthermore, Wu et al. (2007) provided a novel approach to enrich the lysine content (up to 26%) in the maize grain by endosperm-specific expression of an *Arabidopsis* lysyl tRNA synthate. Combining these traits with seed-specific reduction of lysine catabolism offers an optimistic future for commercial application of high-lysine maize.

Single mutations in starch biosynthesis have been commercially used for the production of some 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. Another strategy is to reduce the energy requirements for the starch to ethanol conversion process. For example, gelatinization is the first step in bioethanol production from starch. It is conceivable that a modified starch with decreased gelatinization temperature might require less energy for the conversion process. Research showed that expression of a recombinant amylopullulanase in rice resulted in starch that when heated to 85°C was completely converted into soluble sugars (Chiang, 2005). 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). This could potentially be exploited within the high-fructose maize syrup market. Moreover, 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. The expression of that gene yielded fully functional GTF-D enzyme as shown by accumulation of novel soluble α-(1→6)-linked glucan at high levels in the mature maize kernels (up to 14% of their dry weight). These findings suggest that the introduction of greater diversity in linkages within α-glucan polymers will enable the generation of specialty glucans to replace modified starches used for several products (e.g. 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. 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. If no modification of kernel size was observed in these transgenic lines, other agronomic characteristics remain to be studied to evaluate the economic potential of such material. Another strategy to increase oil accumulation in the grain may consist in improving both 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). Accordingly, the oil content of the kernel was positively correlated with malic enzyme activities in maturing embryos (Doehlert & Lambert, 1991), which makes NADP-ME an attractive target for engineering high oil concentrations in embryos of maize. Furthermore, in oilseed species, numerous biotechnological approaches have been carried out that were aimed at maximizing the flow of C into oil by overexpression of enzymes of the TAG assembling network. For example in maize, several attempts have been made to over-express *diacylglycerol acyltransferases* (*DGAT*). *DGAT* catalyses the transfer of an acyl chain from the acyl-CoA pool to the *sn-3* position of a diacylglycerol molecule, resulting in the synthesis of TAG. The embryospecific over-expression of both maize *DGAT1-2* and of fungal *DGAT2 (*Zheng et al., 2008; Oakes et al., 2011) resulted in limited (1.25 fold) but statistically significant increases in kernel oil content. Whereas it has been shown that grain yield was not affected by expression of fungal *DGAT2*, data concerning the putative incidence of the over-expression of maize *DGAT1-2* on yield and other agronomic characteristics of the modified lines are missing. Nevertheless these works provide insights into the molecular basis of natural variation of oil and oleic-acid contents in plants and highlight *DAGT* as a promising target for increasing oil and oleic-acid content in other crops.

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 *ZmWRI1*, a WRI1-like gene of maize, enhanced oil accumulation in transgenic maize without detectable abnormalities. However, expression of *ZmLEC1* under similar conditions severely affected growth and development of the resulting transgenic maize plants (Shen, et al., 2010*).* Similar results

were obtained by constitutive overexpression of the *ZmWRI1* gene in the transgenic maize plants (Pouvreau et al., 2011*)*. It was also found that *ZmWri1* not only increases the fatty acid content of the mature maize grain but also the content of certain amino acids (Lys, Glu, Phe, Ala, Val) of several compounds involved in amino acid biosynthesis (pyro-Glu, aminoadipic acid, Orn, nor- Leu), and of two intermediates of the TCA cycle(citric acid, succinic acid) (Pouvreau et al., 2011). 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 (see above), over-expression of *ZmWRIa* in the starchy endosperm was not sufficient to trigger oil accumulation in this compartment (Shen et al., 2010). Since there is no evidence that WRI1 regulates TAG assembly, it is not surprising that over-expression of ZmWRI1a only proves to be efficient in tissues already accumulating oil, and thus already expressing the TAG biosynthetic machinery. 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, although question remains about the rate-controlling steps that limit the predictability of metabolic engineering 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 & 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. Moreover, this is a very important proof of concept for genetic manipulation of distinct metabolic pathways.

There is evidence indicating that tocophenols, in particular γ-tocophenol the predominant form of vitamine E in plant seeds, are indispensable for protection of the polyunsaturated fatty acid in addition to have benefits to the meet industry (Rocheford et al., 2002). The same authors have also shown that considerable variation is present among different maize inbreds from tocophenol levels, as well as different ratios of α-tocophenol to γ-tocophenol. This result suggested that breeders can use natural varieties, molecular marker assisted selection strategies and transgenic technologies to alter overall level of tocophenols and ratio of α- to γ-tocophenol. However, current nutritional research on the relative and unique benefits of α- to γ-tocophenol should be considered in developing breeding strategies to alter levels of these vitamin E compounds.

Another area in which transgenic approaches may help solve an important problem with maize as a feed grain is in the reduction of phytic acid levels. In maize, 80% of the total phosphorous (P) is found as phytic acid, and most of that is in the germ (O'Dell et al., 1972). Phytate P is very poorly digested by non-ruminant animals, therefore inorganic supplementation is necessary. Phytate is also a strong chelator that reduces the bioavailability of several other essential minerals such as Ca, Zn, Cu, Mn, and Fe. In addition, since the phytate in the diet is poorly digested, the excrement of monogastric animals (e.g. poultry and pigs), is rich in P and this contributes significantly to environmental pollution. *Low phytic acid* mutants (*lpa*) of maize are available; these have received considerable attention by breeders in order to develop commercially acceptable hybrids with reduced levels of phytic acid (Raboy, 2009).

In maize, several mutants with low levels of phytate have been isolated and mapped; this includes *lpa 1-1*, *lpa 2-1,* and *lpa 241*, (Raboy, 2009). The *lpa1* mutant does not accumulate *myo*-inositol monophosphate or polyphosphate intermediates. It has been proposed that *lpa1* is a mutation in *myo*-inositol supply, the first part of the phytic acid biosynthesis pathway (Raboy et al., 2000). The *lpa2* mutant has reduced phytic acid content in seeds and accumulates *myo*-inositol phosphate intermediates. Maize *lpa2* gene encodes a *myo*-inositol phosphate kinase that belongs to the Ins(1,3,4)P3 5/6-kinase gene family (Shi et al., 2003). The *lpa3* mutant seeds have reduced phytic acid content and accumulate *myo*-inositol, but not *myo*-inositol phosphate intermediates was found to encode *myo*-inositol kinase (Shi et al., 2005).

Despite efforts to elucidate and manipulate phytic acid biosynthesis, *low phytic acid* mutants have limited value to breeders because of adverse effects on agronomic traits such as low germination rates, reduced seed weight (*lpa1-1*), stunted vegetative growth and impaired

seed development (*lpa241*). However, Shi et al. (2007) have recently identified the gene disrupted in maize *lpa1* mutants as a multidrug resistance-associated protein (MRP) ATP-binding cassette (ABC) transporter. Silencing expression of this transporter using the embryo-specific globuline promoter produced low-phytic acid, high phosphate transgenic maize seeds that germinate normally and do not show any significant reduction in seed dry weight.

To increase the amount of bioavailable iron in maize, Drakakaki et al. (2005) have generated transgenic maize plants expressing aspergillus phytase and iron-binding protein ferritin. This strategy has proven effective for increasing iron availability and enhancing its absorption. However, much work is still to be done to transfer this technology to tropical and subtropical maize genotypes normally grown in the areas of greatest need for enhanced iron content maize.

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 & 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.

5. Conclusion and future perspectives

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.

Metabolism is one of the most important and best recognized networks within biological systems. However, advances in the understanding of metabolic regulation still suffer from insufficient research concerning the modular operation of such networks. Elucidation of metabolic regulation within the context of the entire system, including transcriptional, translational and posttranslational mechanisms, is rarely attempted (Sweetlove et al., 2008).

Instead, to date, studies on metabolic regulation have mostly been limited to regulatory interactions within the metabolic pathways themselves (Sweetlove & Fernie, 2005; Sweetlove et al., 2008). Strategies to detect intermediary metabolic fluxes can now be estimated by computer- aided modeling of the central metabolic network and by mapping the pattern of metabolic fluxes underlying, via the possibility of labeling data collected by NMR and GC-MS and the biomass composition. For example Alonso et al., (2011), to map the pattern of metabolic fluxes underlying this efficiency, have labeled maize embryos to isotopic steady state using a combination of labeled ^{13}C-substrates. The resultant flux map reveals that even though 36% of the entering C goes through the oxidative pentose-phosphate pathway; this does not fully meet the NADPH demands for fatty acid synthesis. Metabolic flux analysis and enzyme activities have highlighted the importance of plastidic NADP-dependent malic enzyme, which provides one-third of the C and NADPH required for fatty acid synthesis in developing maize embryos.

It should worth to be mentioned that metabolic engineering of maize has been relatively slow due to the difficulty of maize transformation. Maize transformation with *Agrobacterium* is now more efficient than currently used particle gun transformation (reviewed in Jones, 2009; Reyes et al., 2010). In addition, larger DNA fragments can be inserted with *Agrobacterium* than those previously reported by other methods. The ability to routinely insert metabolic pathway quantities of DNA into the maize genome will further speed up maize metabolic engineering. Furthermore, site-directed mutagenesis via gene targeting, based on homologous recombination such as the application of designed zinc finger nucleases that induce a double stranded break at their target locus, are promising tools for genetic applications (Shukla et al., 2009; Saika et al., 2011). The use of these technologies may lead to both targeted mutagenesis and targeted gene replacement at remarkably high frequencies and enable to modify useful information, acquired from structural- and computational-based protein engineering, to be applied directly to molecular breeding of crops, including metabolic engineering.

Advances in plant genetics and genomic technologies are also contributing to the acceleration of gene discovery for maize 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 map-based 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 ESTs databases (http://www.maizegdb.org) and, DNA microarray technology to investigate mRNA-level controls of complex pathways. 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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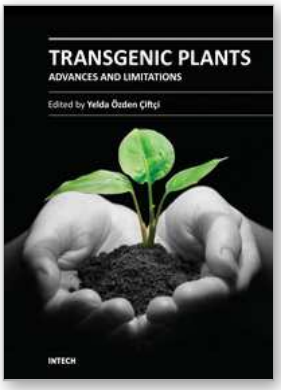

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Development of efficient transformation protocols is becoming a complementary strategy to conventional breeding techniques for the improvement of crops. Thus, Transgenic Plants - Advances and Limitations covers the recent advances carried on improvement of transformation methods together with assessment of the impact of genetically transformed crops on biosafety. Each chapter has been written by one or more experienced researchers in the field and then carefully edited to ensure throughness and consistency.

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