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Grain quality-related traits in maize: gene identification and exploitation

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Abstract

Maize grain is a relevant source of food, feed, and industrial row materials. Developing plants with improved grain structure and quality traits involves the ability to use existing genetic variation and to identify and manipulate economically important genes. This will open new avenues for designing novel variation in kernel size, structures, and 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 development, structure, and composition of the maize kernel with particular emphasis on pathways relevant to endosperm growth and development, and biosynthesis of storage proteins, starch, lipids, and carotenoids. The potential that the new technologies of cell and molecular biology will provide for the creation of new variation or novel compounds in the future are indicated and discussed.

Keywords: kernel development, endosperm mutants, storage products syntheses, genetic variability, breeding strategies

Introduction

Maize (*Zea mays*) is a relevant food and animal feed worldwide and occupies a dominant place in the world economy and trade as an industrial grain crop. (White and Johnson, 2003). Therefore, knowledge of genes involved in determining quality-related traits (structure and chemical diversity) of starch, proteins, oil, and other compounds is important for improving the industrial, nutritional and food-making properties of the grains.

Although plant breeding has been extremely successful at improving the yield of maize, quality has received less attention. However, advances were made by breeders in this area as well, resulting in maize kernels with a wide range of structures and compositions. By exploiting genetic variation, the composition of the kernel was altered for both the quantity and quality of starch, proteins, and oil throughout kernel development. Furthermore, the ability of maize breeders to use existing genetic variation and to identify and manipulate economically important genes will open new avenues for the design of novel variation in grain composition, thus providing the basis for the development of the next generation of speciality in maize and of new products to meet future needs.

The objective of this paper is to overview various aspects of current knowledge on gene discovery, exploitation, and genetic variation known to affect maize kernel development and chemical composition with special emphasis on storage proteins, starch, lipids, and carotenoids. Additionally, we provide a brief outlook on future developments in this field and the opportunities that they may provide for the development of new maize products better suited to its various end uses.

Kernel growth and development

The great economical and nutritional value of the maize kernel is mainly due to its high starch content that 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) and oil (ca. 4.6%) are essentials for human and animal nutrition. Yet the question remains of why the selection for higher starch or oil levels irremediably results in less protein or starch content, respectively, 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 comprises two tissues, the embryo (germ) and the endosperm, that are embedded in the testa (or seed coat) and the pericarp (or fruit wall), a thin protective envelope (see Consonni et al, 2005, for a review). The endosperm is the main storage site of starch and proteins, whereas the embryo reserves mainly lipids.

The entire process of kernel development can be roughly divided in five stages: cell division, cell enlargement, deposition of reserves, maturation and dessication. (Larkins and Vasil, 1997). In the following paragraphs we will focus on the endosperm because is important both for the breeders, as a major component of maize yield, and for the physiologists because it provides the foundation for seedling germination and initial growth. Those interested in the genetics

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of embryogenesis and germination are referred to comprehensive reviews covering these topics, (e.g. Dumas and Rogowsky, 2008).

Seed size controls

Although it was found that major seed size controls are provided by genes that define the development of the maternal integument and the new-generation embryo and endosperm, many of the mechanisms controlling this process remain elusive. In maize several genes disrupting embryo pattern formation have been described. They embrace the defective seed (*de*) and embryoless (*emb*) mutants both of them are included into the large class of defective kernels (*dek*) mutants that impaired either the endosperm or the embryo or both of them. For example, the viable embryos of the pleitropic *semaphore1* (*sem1*) mutants are smaller than wild type embryos and have fewer if any leaf primordia. Studies suggest that *Sem1* is an intermediate in a signalling cascade leading from auxin to *Knox* genes, which encode a class of related homodomain proteins, that in turn are required for the correct initiation of leaf primordial (Scanlon et al, 2002). More recently, Suzuki et al (2008) have found that the *Viviparous8* (*Vp8*) gene encodes a putative ALTERED MERISTEM PROGRAM1-like peptidase, that regulates abscisic acid (ABA) accumulation and coordinate embryo and endosperm development.

Though not yet well defined in maize, a number of female-gametophytic mutations are known in *Arabidopsis thaliana* that severely affect development of the seed, particularly the endosperm (reviewed in Raissig et al, 2011). The molecular characterization of these mutations revealed the existence of a set of proteins, encoded by the *Medea* (*MEA*), *Fertilization-independent Seed2* (*FIS2*), and *Fertilization-independent endosperm1* (*FIE1*), as well as *MSI1* genes, components of a chromatin-associated polycomb complex playing a crucial role in early seed development. Moreover, in this model species a number of factors affecting seed size by acting in the maternal and/or zygotic tissues have been identified (Berger et al 2006). Among the zygotically acting factors, a small group of genes, comprising the *HAIKU1*, *HAIKU2*, and *MINISEED3*, promote endosperm growth. On the maternal side, several factors are required to increase or limit final seed size, such as the WRKY transcription factor TRANSPARENT TESTA GLABRA2, the transcription factors APETALA2 and AUXIN RESPONSE FACTOR2, and the ubiquitin interaction motif-containing DA1 protein limiting seed size. This trait is also affected by the cytochrome P450 *KLUH* (*KLU*) gene (Adamsky et al, 2009). Therefore, the level of KLU-dependent growth factor signaling determines size in ovules and seeds, suggesting this pathway as a target for crop improvement.

Endosperm growth and development

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 may serve as a valuable system to address fundamental questions related to the improvement of seed size in crops.

In grasses, maize included, endosperm development and function has been the subject of several recent reviews (e.g. Sabelli and Larkins, 2009). Collectively these studies have shown the existence of three major key phases including a total of five successive events. The “lag phase” takes place from a few hours following pollination to approximately 12 days after pollination (DAP) and consists of four stages: coenocytic, cellularization, cell fate specification, and differentiation resulting in rapid endosperm expansion. The subsequent period ranging from 12 to 40 DAP is devoted to grain filling. Biochemically, it consists in the conversion of imported nutrients, mainly sucrose and amino acids, into starch and storage proteins. Afterwards, the maturation-desiccation phase occurs, leading to the mature kernel at 70 DAP. As the kernel reaches maturity starch-filled cells senescence, apparently undergoing a form of programmed cell death (PCD), whereas aleurone cells acquire desiccation tolerance and remain viable in the dry seed (Young and Gallie, 2000a, b; Kladnik et al, 2004). PCD progression is accompanied by an increase in nuclease activity and internucleosomal degradation of nuclear DNA, hallmarks of this process. The onset and progression of PCD during maize endosperm is controlled by two hormones, ie. ethylene and ABA, which have a contrasting action (Gallie and Young, 2004). Other findings emerging from those studies indicate that the endosperm is an organizationally simple structure containing four major tissues: aleurone, starchy endosperm, basal endosperm transfer layer (BETL), and embryo surrounding region (ESR). It shows several unique mechanisms in the regulation of cell cycle, cytokinesis, and cytoskeletal functions and is surprisingly plastic, with aleurone cell fate decisions occurring dynamically throughout the course of kernel development (Becraft and Asuncion-Crabb, 2000). Understanding the regulation of these programs and their transitions should allow to define important links between cell cycle, cell differentiation, and development, as well as provide tools for the manipulation of seed yield.

High-throughput genomics and post-genomics approaches are providing new tools for understanding the genetic and biochemical networks operating during kernel development. Recently, large databases of expressed maize genes 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, allowing to analyze more thoroughly gene expression in developing maize en-

dosperm 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). In maize, excluding storage protein genes, at least 5,000 different genes are expressed during development (Liu et al, 2008). About 35% of them are orphan genes, whose functions remain elusive, possibly corresponding to endosperm specific genes. Furthermore, Méchin et al (2004) have established a proteome reference map for the maize endosperm that indicates that metabolic processes, protein destination and synthesis, cell rescue, defence, cell death and ageing are the most abundant functional categories.

The transcriptome and proteome maps taken together constitute a powerful tool for physiological studies and are the first step for investigating maize endosperm development. In fact, this information is useful for identifying distinctive, previously uncharacterised, endosperm-specific genes. In addition, it provides further research material for academic laboratories and material for plant breeders and food processors to include in their respective research or product pipelines.

Differentiation of starch-filled cells

Studies of genotypes differing in endosperm size and in response to environmental treatments have indicated that cell number, cell size, and starch granule number are correlated with endosperm mass at maturity (Jones et al, 1996). Thus, the regulation of these pre-grain fill processes may play important roles in determining the subsequent grain-filling rate and duration of storage product deposition.

The enlargement of the maize endosperm relies upon two cellular processes: cell division and cell expansion, which is, in turn, related to DNA endoreduplication (Larkins et al, 2001). Endoreduplications (ca 8-10 DAP) may provide high level of gene expression in a tissue where intense gene activity is required and where there are strong limitations in term of space or time (Sabelli and Larkins, 2009). It is also well established that endoreduplication, like cell number, is under maternal genetic control and is sensitive to environmental stresses or exogenous application of ABA (Sabelli and Larkins, 2009). A direct link has been established between water stress and decreased expression of genes involved in cell cycle regulation, i.e., β-*tubulin*, *CDKA*, *MCM5*, histone *H2B*, *RPA1*, and *RNR* (Yu and Setter, 2003). However, a subset of these genes becomes up-regulated upon recovery from the stress, indicating specific responses of different cell cycle regulators with respect to regimes of water stress and recovery.

Cell cycle-related genes seem to play a central role in the regulation of the chromosomal endoreduplication in maize endosperm In this compartement, initiation of endoreduplication is associated with a decrease in the activities of cyclin-dependent kinases (CDKs) that bind to p13suc1 (Grafi and Larkins, 1995; Coelho et al, 2005), and with high levels of expression of the ZmWee1/CDK-inhibitor protein kinase (Sun et al, 1999). Thus, modulation of CDK activity appears crucial in regulating the transition from a mitotic to an endoreduplication cell cycle, with multiple mechanisms converging to reduce CDK activity during endoreduplication. Mutants with suppressed endoreduplication have not yet been isolated. However, the *dek* mutants have been frequently considered candidates for genes involved in this type of mutations and endoreduplication cell cycle. Their molecular analysis should allow a clarification of the defects in the mechanism controlling endoreduplication.

Besides the activity of key cell cycle regulators, both the cell cycle and the development of the endosperm depend significantly on hormonal and environmental factors (John 2007; Brugiere et al, 2008; Rijavec et al, 2009). In these studies it was reported that plant hormones, such as cytokinins and auxins, might play a critical role in the expression of key cell cycle-controlling genes related to cell division, kernel filling, and sink strength. In this context, Liu et al (2008), by genome-wide analysis of gene expression profiles during seed development of maize, provide evidence of differential expression of genes involved in plant hormone signaling pathways; this finding has confirmed that phytohormones might play a critical role in the kernel developmental process. Moreover, more recent evidence indicates that a reduced accumulation of auxins in the maize *defective endosperm*B18* mutant, due to down regulation of *Pinformed1*, a member of the PINFORM family of auxin efflux carriers, leads to a reduction in dry matter accumulation in the seed (Forestan et al, 2010).

Evidence suggests that the RETINOBLASTOMA-RELATED (RBR)–E2F pathway shows a pivotal function in early maize endosperm development (reviewed in Sabelli and Larkins, 2009). Collectively these investigations suggest that the evolutionary conserved *RBR* gene family, represented by *RBR1*, *RBR2*, and *RBR3* loci, plays a key role in inhibiting cell cycle activity, primarily by repressing expression of many genes, regulated by the E2F family of transcription factors, whose activity is required for the expression of many S-phase genes. Forward genetics experiments that modulate the expression of *RBR1* and *RBR3* should help to elucidate their precise roles. However, results obtained in tissue culture indicated a surprisingly positive role of RBR3 in controlling DNA replication and the expression of the minochromosome maintenance (MCM) family of DNA replication licensing factors (Sabelli et al, 2009). It will be important to establish whether the RBR1/ RBR3/E2F/MCM paradigm is applicable to the developing endosperm.

A link between the control of G1/S transition in cell cycle and factor modulating chromatin structure through histone modification has been reported by a number of workers (Rossi et al, 2003; Varotto et al, 2003). It was found that maize *RBR1* (*ZmRBR1*) can

recruit Rpd3-type histone deacetylase (ZmRpd3l/hda101) and cooperate in repressing gene transcription. In addition, *ZmRbAp1* (Retinoblastoma Associated Protein-1), a maize member of the MSI/RbAp family of WD-repeat proteins (Rossi et al, 2001), interacts with both *RBR1* and *hda101* enhancing their association. The results of these studies, together with previous published findings on the components of the plant RBR/E2F pathway, suggest a model that highlights the role of histone acetylation in the control of G1/S progression. Because, as mentioned above, the RBR/E2F pathway is active in regulating the commitment for cell division, it can be hypothesized that also the RBR1/ZmRbAp1/hda101complex plays a role in the control of endoreduplication. More recently the analysis of transgenic plants with up and down-regulation of *hda101* expression provided evidence that HDA101 alters gene expression and participates in modulating the histone code (Rossi et al, 2007). Perturbation of *hda101* expression leads also various to morphological and development defects and affected expression of genes involved in vegetative to reproductive transition and in meristem processes, suggesting a function of *hda101* in mediating developmental programs. Moreover, Castro et al (2008), by applying a metabolomic approach to analyze *hda101* mutants, reported also differences in the accumulation of several metabolites during development and highlighted the changes occurring in the modified seed conferming the idea that this gene affects various metabolic pathways.

Although in maize no transcription factor involved in endosperm growth control has been yet identified, Cavel et al (2011) have recently found in Arabidopsis that a plant-specific TFIIB-related protein 2, pBRP2, is implied involved in this process. Specifically these authors have shown that *pBRP2* activity in both male and female gametophytes is important for nuclear proliferation during the syncitial stage of endosperm development, Thus as a consequence of pBRP2-dependent transcription, progeny originating bi-parentally probably receive maximum resource allocation to the seed. This is reminiscent of the role of genomic imprinting in the mammalian embryo, which acts to enforce relatively strict bi-parental reproduction, and represents the first description on of a converging strategy in plants.

The study of cell cycle regulation in the context of endosperm development represents an opportunity for understanding how key transitions between different cell types are temporally and spatially coordinated and how they have effect on gene expression, cell size, synthesis and accumulation of storage metabolites, homeostasis, and cell death. Information on the cross-talk between cell cycle regulation and epigenetic mechanisms is only beginning to emerge and much remains to be done; however, given the rate of recent progress and tools currently available, there is no doubt that the future will bring results to unravel the relationship between cell cycle regulation and endosperm development.

Genetics of endosperm formation

The maize endosperm has been extensively investigated by analysis of mutants affecting kernel development and appearance (Neuffer et al, 1997). However, only a small fraction of the known endosperm mutants - broadly referred as defective endosperm and kernel (*de* and *dek*) - have been molecularly analyzed. Furthermore, Wang et al (2009) have provided evidence that genetic studies of endosperm development can be impaired via the use of functional genetic mapping. This approach allowing the detection of the temporal expression pattern of dynamic QTLs during endosperm development, represents a valuable information for understanding the interplay of endosperm development.

In most of the *dek* mutants all tissues form regularly, but the degree of filling in the starchy endosperm is drastically reduced (Lid et al, 2002). Two of those genes, i.e. *discolored1* (*dsc1*; Scanlon and Myers, 1998) and *empty pericarp2* (*emp2*; Fu et al, 2002) were cloned, via transposition-based approaches. For *Dsc1* no function has yet been assigned to the cloned genomic sequence, whereas *emp2* is an embryo-lethal dek mutant encoding a heat-shock like binding protein1, although its function in seed development has yet to be clarified. The *globby1* (*glo1*) mutant is an example of a mutation that interferes with syncytial nuclear division and cellularization patterns in early endosperm development (Costa et al, 2003). The *disorgal1* (*dil1*) and *disorgal2* (*dil2*) mutants appear to create aberrant regulation of the mitotic division plane, resulting in a disorganized aleurone layer (Lid et al, 2004). Although, the identity of genes affected by the *glo1* and *dil1/2* mutations is presently unknown, the mutants represent a valuable tool for dissecting the genetic pathway controlling cell division of the endosperm tissue. Additionally, mutations of crucial cell cycle regulators can now be specifically identified by screening T-DNA insertion mutant collections which may reveal useful to associate phenotypes (Ebel et al, 2004). Moreover, studies of *in vitro* fertilised isolated maize central cells (Kranz et al, 1998), may further improve our understanding of the molecular mechanisms regulating endosperm development.

Because cell proliferation requires a large supply of energy, mutations in house-keeping genes or genes involved in polysaccharide synthesis appear to affect endosperm growth and development. Example of such mutations include the *defective kernel1* (*dek1*) mutant which has a defect in a membrane-anchored, calpain-like cysteine proteinase and is devoid of the aleurone cell layer (Wang et al, 2003) and *miniature1* (*mn1*), in which a loss of the cell wall invertase *INCW2* activity is associated with reduced mitotic activity (Vilhar et al, 2002). Kang et al (2009), reported that *INCW2* is crucial for normal assembly and function of

the wall ingrowths (WIGs) and concluded that a defective WIG formation, in the *mn1* endosperms, may result from rate limiting levels of monosaccharides that are essential for cell wall polysaccharide synthesis and glycosylation reactions. Moreover, LeClere et al (2010), have found that reduced IAA levels in *mn1* endosperms correspond to reduced transcript levels of *ZmYUCCA* (*ZmYUC*), a newly identified homolog of the Arabidopsis gene *YUCCA*. They further documented that ZmYUC catalyzes the N-hydroxylation of tryptamine and that sugar levels regulate transcript levels of *ZmYUC*, These results indicate that developing seeds may modulate growth by altering auxin biosynthesis in response to sugar concentrations. Similarly, developing *empty pericarp4* (*emp4*) seeds are retarded in their growth compared to wild-type seeds and display severe morphological abnormalities as well as altered patterns of gene expression within the endosperm transfer tissue (Gutierrez-Marcos et al, 2007). The *Emp4* gene codes for a novel type of a mitochondrion-targeted pentatricopeptide repeat protein required for the expression of a small subset of mitochondrial transcripts in various plant compartments, including the developing endosperm. Loss of the EMP4 protein in the *emp4* mutant is associated with fewer mitochondria and irregular differentiation of transfer cells in the BETL, consistent with the observation that the normal BETL cells are metabolically active and that mitochondrial deficiencies can lead to reduced wall-in-growth (WIG) formation in these cells. Other mutants that delay the initiation of dry matter accumulation at various stages of kernel development may contribute to the genetic control of endosperm development.

Maternal control of endosperm development

In maize, maternal effect mutants have been described that affect the endosperm and the embryo (i.e. maternal effect *lethal1*, *baselles1*, and *Dappled 1* (*Dap1*) (see Raissig et al, 2011, for a review). The extent to which maternal tissue is essential for seed formation is still unclear: somatic embryogenesis and endosperm development can occur *in vitro* in the absence of maternal tissue (Kranz et al, 1998). Molecular evidence has shown that female sporophytic and gametophytic genes govern early endosperm development (Garcia et al, 2003, and references therein). Phillips and Evans (2011), by studying the maize recessive maternal effect mutant *stunter1* (*stt1*), that displays viable, miniature kernels, reported that both parental alleles are active after fertilization in both the endosperm and embryo. This analysis also indicates that embryo development until the globular stage in maize can proceed without endosperm development and is likely supported directly by the diploid mother plant. It has been proposed that parent of origin expression is complex and several epigenetic mechanisms involving DNA methylation (e.g. DNA-methyltransferase *METHYLTRANSFERASE1*, *MET1* and the DNA-glycosylase *DEMETER*, *DME*), histone modifications, and noncoding RNAs are recruited to define the silent versus active state of parental alleles (reviewed in Koerner and Barlow, 2010; Bauer and Fisher, 2011).

To date, 11 genes in maize have been reported as imprinted or potentially imprinted (reviewed in Raissig et al, 2011). From these studies it was noted that imprinted genes (e.g. α-*zeins*, β-*tubulin*, *R*, *DZR1*, *FIE1*, *FIE2*, *Meg*, and *Mee1*) encode a wide range of molecular functions, ranging from the regulation of pigmentation, protein storage, transcriptional regulation, chromatin modification, and cytoskeletal function to mRNA regulation. It was also shown that in maize the expression of *FIE1* and *FIE2* is differentially regulated, suggesting diversification of function during endosperm development (Gutiérrez-Marcos et al, 2006; Hermon et al, 2007; Jahnke and Scholten, 2009). Furthermore, these experiments clearly illustrate that DNA methylation is highly dynamic and not always correlated with expression. Due to its dynamics, it is not possible to infer the DNA methylation state in the gametes based on analyses performed at later stages of seed development.It was also found that despite lacking a paternal effect on seed development these mutations often have a gametophytic effect on pollen development. For instance, five recently described potentially imprinted genes, for which only transcripts from one parental allele were detected in the endosperm (Gehring et al, 2009), encode transcription factors of the homeodomain and MYB classes. The function of these genes is currently unknown, and future studies will clarify whether they have parent-of-origin–specific roles during endosperm development. In contrast with the endosperm, very little is known about the role of imprinted genes during embryo development, although embryonic phenotypes were originally described for mutants of the fis class and in MPCRNA interference lines (Raissig et al, 2011).

Aleurone differentiation and function

The aleurone layer that represents the outer cell layer(s) of the endosperm plays a cardinal role in the mobilization of storage compounds stored in the endosperm during germination (reviewed in Becraft and Yi, 2011). Its formation is ensured by at least three mechanisms: i) positional signals, that specify the outer most layer of endosperm cells as aleurone, ii) controls of plane of aleurone cell divisions, being restricted either to the periclinal or anticlinal planes, and iii) control of the rate of periclinal divisions in later development stages (reviewed in Becraft and Yi, 2011).

Several genes that affect aleurone development have been cloned. These include the *dek1*, *crinkly4* (*cr4*), *Dappled 1* (*Dap1*), *dek1-d*, and *paleface* (*pfc*) mutants that cause mosaicism on the abgerminal face of the kernel, leaving aleurone layers to develop on the germinal face. Moreover, it has been found that *Dap1*, *Mosaic*, *collapsed2*, *opaque-12*, and *white2* mutants produce balanced mosaicism throughout kernels (Becraft, 2001). Peripheral cells

of *dek1* mutants retain the storage endosperm identity instead of specializing into aleurone (Becraft and Asuncion-Crabb, 2000; Lid et al, 2002). The analysis of *Dap1* mutants indicates that aleurone cell fate and cell differentiation are two genetically separate processes (Gavazzi et al, 1997).

Knowing which cellular processes and genes are regulated by the *dek1* gene product would contribute valuable information. *Dek1* appears to control different cellular-developmental processes depending on the cellular context (Becraft et al, 2002; Lid et al, 2002). Similarly, the *Cr4* locus, encoding a receptor-like kinase (Becraft et al, 1996), is important for the aleurone cell fate decision: mutations in this gene disrupt aleurone development (Becraft and Asuncion-Crabb, 2000; Jin et al, 2000). Furthermore, Shen et al (2003) have cloned a novel gene, *Superal1* (*Sal1*), which when mutated causes multiple layers of aleurone cells in maize endosperm. The *Sal1* gene encodes a member of the class E of vacuolar sorting proteins, raising the possibility that endosome trafficking is involved in aleurone cell fate signalling (Tian et al, 2007).

Anthocyanins are associated with many biological activities, including health-related beneficial effects (Hagiwara et al, 2001; Pedreschi and Cisneros-Zevallos, 2007). The biosynthesis of anthocyanins is the best-understood pathway specific to aleurone cell fate. Key regulators of this pathway include *Viviparous 1* (*Vp1*), *C1*, and *R1* genes. *Vp1* is involved in the regulation of anthocyanin biosynthesis and in the acquisition of seed dormancy, its action depending on the presence of the phitohormone ABA (McCarty et al, 1991). The analysis of VP1 protein has shown that it represents a plant-specific class of transcription factor interacting with DNA as part of a multicomponent complex. *Vp1* is expressed in both the aleurone and in the maturing embryo (Hattori et al, 1992). It has been shown that VP1 activates anthocyanin synthesis by binding to the promoter of the anthocyanin-regulatory gene *C1* at the Sph-box, an RY-motif containing sequence (Suzuki et al, 1997). In addition, Vp1 represses germination specific α-amylase genes (Hoecker et al, 1995). Other pleiotropic aspects of the vp1 phenotype suggest a still broader role in aleurone gene expression (Dooner et al, 1991).

Endosperm transfer cell

In maize, the endosperm epithelial cells facing the placenta chalazal area differentiate into endosperm transfer cells (ETCs), highly modified plant cells specialized in the transport of solutes, i.e. amino acids, sucrose, and monosaccharides (see Zheng and Wang, 2010, for a recent review). Wall ingrowths are a distinguishing feature of transfer cells and serve to amplify (up to 20-fold) the plasma membrane surface area available for solute transport. This makes ETCs very efficient in the uptake of nutrients from adjacent maternal vascular tissue to the endosperm.

Mutants specifically affecting this process have been identified. For example, Maitz et al (2000) reported a *reduced grain filling1* locus associated with reduced expression of BETL markers and a loss of 70% seed weight at maturity. Similarly, the *baseless1* (Gutierrez-Marcos et al, 2006) and *emp4* (see above; Gutierrez-Marcos et al, 2007) mutants exhibit abnormal BETL at an early stage of seed development leading to seed lethal phenotypes. Moreover, studies in this field have shown that three groups of maize genes are preferentially expressed in transfer cell layers, i.e. *BETL*, *BAP*, and *EBE* (Serna et al, 2001; Magnard et al, 2003). Their products resemble antimicrobial proteins, suggesting a role in the protection of the kernel from potential pathogenic invaders. There is also evidence that BETL secretes peptides that may have signaling function (Gutierrez-Marcos et al, 2004) or provide regulatory signals between the dead placento-chalazal cells in the maternal pedicel and filial cells in the endosperm (Kladnik et al,2004).

It is currently unknown how apical-basal polarity is established or why the peripheral cells at the base of the endosperm differentiate into transfer instead of aleurone cells. However, in maize endosperm, the process of ETCs differentiation is gradual and regulated by the cell position on the main two axes (reviewed in Royo et al, 2007). Studies of the maize ETCs differentiation have been facilitated by the identification of genes specifically expressed in these cells. The first ETC-specific transcriptional activator identified is the maize-transcription factor Myb-related protein1 (*ZmMRP1*; Gomez et al, 2002). This protein contains a MYB-related DNA binding domain presents in several DNA binding proteins belonging to the SHAQK(Y/F)F subfamily. Additionally, *ZmMRP1* regulates the expression of several ETC-specific genes, namely, *BETL-1* and *BETL-2* (Gomez et al, 2002, 2009), *Meg-1*, and *TCRR-1* (*Transfer Cell Response Regulator1*; Muniz et al, 2006), through its interaction with a specific sequence in the corresponding promoters (Barrero et al, 2006). It also activates the *ZmTCRR-1* and *ZmTCRR-2* two response regulators whose expression is cell specific and developmentally regulated (Muniz et al, 2010). These findings suggest that *ZmMRP1* might be a key player in the ETC differentiation process. Gomez et al (2009) have shown that the ectopic expression of *ZmMRP*, in epidermal cells committed to develop into aleurone cells, is sufficient to temporarily transform them into ETCs. These transformed cells acquire distinct transfer cell features, such as cell wall ingrowths and an elongated shape. In addition, these workers have shown that expression of *MRP1* is required to maintain the transfer cell phenotype. Later in development a reduction in the ectopic expression of *ZmMRP1* is followed by the reversion of the transformed cells, which then acquire aleurone cell features.

In vitro experiments with cultured maize endosperm have reinforced previous views that development of the basal transfer cell layers requires a contri-

bution from maternal sporophytic tissue (Gruis et al, 2006). The contribution of the transfer layer to seed development is supported by the phenotype of mutants in which these cells are defective, such as mn1 (see above). In this mutant the drastic reduction in endosperm cell size and cell number, compared with that of the wild-type, is attributable to the poor differentiation of BETL.

Embryo surrounding region

In maize, the embryo surrounding cells (ESRs) are identifiable by their dense cytoplasmic contents (Kowles and Phillips, 1988) and by the cell-specific expression of three different embryo surrounding region-1 to -3 (Esr-1 to -3) transcripts (Opsahl-Ferstad et al, 1997), *Zea mays androgenic1* (*ZmAE1*), and *ZmAE3* (Magnard et al, 2000) genes. ESR protein localizes to ESR cell walls (Bonello et al, 2002).

The function of the ESRs is still unknown. It is believed that it may have a role in embryo nutrition or in establishing a physical barrier between the embryo and the endosperm during seed development. Additional potential roles for the ESRs include defense from pathogens and signaling at the embryo-endosperm interface. Evidence of the former comes from at least two genes expressed in the ESR, *ZmAE3* and *ZmEsr6*, which have broad-range antimicrobial activities (Balandin et al, 2005). Support for a role of ESRs in mediating signaling between embryo and endosperm comes from the *ZmEsr1-3* gene family, which potentially encodes receptor ligands similar to Arabidopsis CLV3 (Bonello et al, 2002). In maize the ESR may also play an important role in establishing the so-called embryiogenic cavern (Cossegal et al, 2007). The observation that the endosperm of embryoless mutants forms a normal size embryo cavity suggests that the endosperm has an intrinsic program to form this structure.

Accumulation and synthesis of storage products

The structure and biochemical properties of seed storage compounds have been widely investigated over the past three decades 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 the seed, 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 and Larkins, 2009; Val et al, 2009).

Storage proteins

The primary storage proteins in the maize seed are prolamines called “zeins”. Specifically, the zeins are the most abundant protein storage component (>60%) in developing endosperm tissues and consist of alcohol-soluble compounds with a characteristic amino acid composition, rich in glutamine, proline, alanine, and leucine, and almost completely devoid of lysine and tryptophan (Gibbon and 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-zeins in 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 subfamilies of α- (19 and 22-kDa; 60% to 70% of total zeins), β- (15 kDa; 5% to 10% of total zeins), γ- (16-, 27-, and 50-kDa; 20% to 25% of total zeins), and δ-zeins (10- and 18-kDa; <5% of total zeins), that are encoded by distinct classes of structural genes (Holding and Larkins, 2009). In this context, 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.

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 originate 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 and Larkins, 1989). Alterations in size, shape or number of PBs generally determine the opaque phenotype (Holding and Larkins, 2009), with the exception of *floury1* (*fl1*), an opaque mutant with no alterations in PB size or shape (Holding et al, 2008). Recently, maize storage protein mutants obtained through RNAi showed that γ-zein RNA interference (RNAi) mutant lines exhibit slightly altered protein body formation and that a more drastic effect is observed in the β-γ- combined mutant, where protein bodies show an irregular shape, particularly in their periphery (Wu and Messing, 2010). In further studies Llop-Touset et al (2010) found that the N-terminal proline-rich domain of γ-zein plays an important role in PB formation. Washida et al (2009) identified the cis-localization elements of the 10-kDa δ-zein 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 play a role in mediating the deposition of storage protein in the endomembrane reticulum in plants.

The targeting of zeins to specific types of PBs is regulated by complex mechanisms (Herman and Larkins, 1999). ER-resident chaperones, such as

terminal binding protein (BiP) and protein disulphide isomerase, are also involved in seed storage protein targeting. In the ER lumen, newly synthesized secretary polypeptides are folded and assembled by aid of many chaperones and folding enzymes before sorting. These pathways are regulated by quality control (QC) and are closely linked with unfolded protein response (Vitale and Boston, 2008). BiP acts as a sensor of ER stress in QC. Interestingly, Reyes et al (2011) have recently discvered that genes encoding zeins, β-*globulin*, and l*egumin-1* are transcribed not only in the starchy endosperm but also in aleurone cells. Unlike the starchy endosperm, aleurone cells accumulate these storage proteins inside protein storage vacuoles instead of the ER.

Endosperm mutants altering storage protein synthesis

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 zein synthesis being reduced, the overall lysine content is elevated, giving opportunities for their use in the development of "high-lysine" maize.

The recessive mutation *opaque-6* (*o6*) and the dominant or semi-dominant mutations *Defective endosperm*B30* (*De*B30*), *floury-1* (*fl1*), *floury-2* (*fl2*), and *Mucronate* (*Mc*), cause a general reduction in accumulation of all zein classes. The phenotypic characterization of the *opaque-7* (*o7*) mutant endosperm revealed, in addition to a dramatic decrease in PB number and size, a significant reduction in seed proteins, particularly for major α-zeins. Differently the recessive mutation *opaque-2* (*o2*) induces 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-zein. Some of the opaque-class mutants, such as *De*B30* and *fl2*, are caused by mutant signal peptide in α-zeins (Coleman et al, 1997; Kim et al, 2004), while the *Mc* mutant encodes an abnormal 16-kDa γ-zein (Kim et al, 2006). These mutations disrupt the organization of α- and γ-zeins in the PBs and lead to the increased expression of cellular stress response genes (Hunter et al, 2002) consistent with the expression of a mal-folded, ER, localised protein. The *floury1* (*fl1*) mutation is somehow different, because it does not affect the amount or composition of zeins 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. In *opaque5* (*o5*) the mutant phenotype is caused by a reduction in the galactolipid content of the maize endosperm, with no changes in zein proteins (Myers et al, 2011). Accordingly, the *O5* gene encodes the monogalactosyldiacylglycerol synthase1 (MGD1) and specifically affects galactolipids necessary for amyloplast and chloroplast function. Similarly the *mto140* mutant does not affect specific zeins (Holding et al, 2010). The general reduction in storage protein accumulation and the elevated lysine phenotype typical of other opaque endosperm mutants is rather due to a disruption in amino acid biosynthesis: *mto140* was identified as a member of the small *arogenate dehydrogenase* gene (*zmAroDH-1–4*) family.

Among mutations affecting regulatory genes, 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). *O2* specifically 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* 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 seed 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 strategies and confirmed by transgenic functional complementation (Miclaus et al, 2011; Wang et al, 2011). Its sequence analysis indicated that the *O7* gene shows similarities with members of acyl-CoA synthetase-like genes (ACS) family, although its 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 accounting for the vitreous phenotype of the kernel. Alternatively, Wang et al (2011) have suggested, by analysis of amino acids and key metabolites, that the *O7* gene function might impair amino acid biosynthesis by affecting α-ketoglutaric and oxaloacetic acids. In this respect, Hartings et al (2011), through transcriptomic analyses, indicated that the *o2* and *o7* mutations alter gene expression in a number of enzymatic steps, such as the tricarboxylic acid cycle (TCA) and glycolysis pathways that are of central importance for the amino acid metabolism in devel-

Table 1 - Some features of maize mutants affecting zein accumulation (adapted from Motto et al, 2009).

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	recessive	10-kDa reduction	

oping 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.

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). The specific spatial/temporal pattern is the result of the coordinate expression of zein genes primarily controlled at the level of transcription. Highly conserved cis-regulatory sequences in the promoter of prolamine genes and corresponding trans-activity factors have been described. Zein gene expression can also be affected by other regulatory mechanisms, such as methylation, aminoacid supply and phosphorylation.(cf. Motto et al, 2005). In this context, Locatelli et al (2009) provided evidence that the O2-mediated transcriptional activation occurs in two-phases, i.e. a first potentiated and a second 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.

Genetic applications and perspectives

Despite the efforts to develop commercially useful opaque mutants, their 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 *o2* genetic modifiers (Opm) that convert the starchy endosperm of an *o2* mutant to a hard, vitreous phenotype. Genetic studies have shown that there are multiple unlinked Opm, identified as disperse quantitative trait loci (QTLs; review in Gibbon and Larkins, 2005). Although their molecular identities remain unknown, QTLs are correlated with 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 Opm genes, affect 27-kDa γ-zein gene expression. The first of these is associated with increased expression of *o2* 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 could be critical in keeping 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 reinforces the idea that different zeins play distinct roles in endosperm development.

Although maize endosperm storage protein genes have been intensively studied, many questions still remain regarding their sequence relationships and expression levels, such as structure, synthesis and assembly into protein bodies, and their genetic regu-

lation (Holding and Larkins, 2009), The development of tools for genome-wide studies of gene families make feasible the analysis of storage protein gene expression in maize endosperm with the identification of novel seed proteins (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 the identification of 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). 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, presence of the recessive *o2* mutant allele, and the modifiers *o2*, (Mo2s). Accordingly, to overcame these difficulties 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 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.

Starch synthesis

Maize, like other cereals, accumulate starch in the 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 attention by plant breeders and plant scientists. Starch biosynthesis accomplished by a multiplicity of conserved enzymatic activities is a central function in plant metabolism (see Hannah and James, 2008, for a review). Roughly three-quarters of the total starch is amylopectin, consisting of branched glucose chains that form insoluble, semi-crystalline granules, whereas the remaining one-quarter is amylose, composed of linear chains of glucose that adopt a helical configuration within the granule (Myers et al, 2000). Briefly starch synthesis requires two fundamental activities represented by starch synthase, which catalyzes the polymerization of glucosyl units into α(1/4)-linked "linear" chains, and a 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. 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 inferred 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.

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 (cf. 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

Table 2 - Summary of mutant effects in maize where an associated enzyme lesion has been reported (adapted from Motto et al, 2009). a Changes relative to normal. ↑, ↓ = increase or decrease, respectively. sugars = Alcohol-soluble sugars.

Genotype	Mayor biochemical changesa	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-oligo saccharide 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	↑ Phytoglycogen branching enzyme ↓ Starch-bound starch syntase
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

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.

Many biochemical and molecular studies on starch synthesis have 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* (*Bt2*) 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), showed 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 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 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 the developing transgenic kernels exhibited higher *Bt2* and *Sh2* gene expression, higher AGPase activity, higher seed weight, and accumulated more starch compared with non-transgenic plants. The plants over-expressing either *Bt2* or *Sh2* enhanced AGPase activity, seed weight (+15%) and starch content compared with the wild type, while still lower content content was noted in plants transformed with both genes. Collectively, these results indicate that over-expression of those genes in transgenic maize plants might improve kernel traits and provide a feasible approach for enhancing starch content and seed weight in maize.

Regulation of starch biosynthesis

In spite of all the studies on the 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, suggesting that nutrient flow is the key regulatory stimulus in carbohydrate interconversion. In this connection, it has been proposed that glucose plays a roles as a signal molecule in regulating gene expression, although in some cases, different sugars or sugar metabolites might act as the actual signal molecules (reviewed in Koch, 2004). Gene responses to sugars and C/N balance have been implicated in the regulation of the major grain-filling pathway. In this context, Sousa et al (2008) have recently identified in maize a gene for *Sorbitol dehydrogenase1* (*Sdh1*) highly expressed in early seed development throughout the endosperm, with greatest levels in the basal region, suggesting an involvement of *SDH* in the initial 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.

As far as transcription factors are concerned, Fu

and 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. It is expected that similar orthologous loci will be soon identified in maize; this will provide informative clues on regulatory mechanisms affecting starch biosynthesis useful to facilitate the improvement of seed quality and nutrition.

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 compared with the untransformed seed control. More interestingly, 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 specific differences in starch granule shape and size thus providing 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 substrates or products 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

The intensive use of the maize kernel is due not only to its high starch content, but also to the oil stored in the embryo. Oil, in fact, is the most valuable co-product from industrial processing of maize grain through wet milling or dry milling and iit represents a source of high-quality oil for humans.

Research in this field (see Val et al, 2009, for a review) indicate that i) the mature embryo contains approximately 33% lipids in commercial hybrids, representing 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.

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. 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).

Lipid biosynthetic pathway and genetic inheritance

Studies on the biosynthesis of oil indicate that plant oil is synthesized from glycerol-3-phosphate and fatty acyl-CoA in the endoplasmic reticulum as triacylglycerols (TAGs, esters of fatty acids and glycerol) (reviewed in Baud and Lepiniec, 2010; Barthole et al, 2011). Fatty acids are synthesized from acetyl-CoA in plastid and then transported to the cytoplasm in the form of fatty acyl-CoA. In the endoplasmic reticulum, they are used for the acylation of the glycerol-3-phosphate backbone either by the relatively straightforward Kennedy pathway or by acyl exchange between lipids. The resulting TAGs are stored in specialized structures called oil bodies.

The primary determinant of lipids content in maize kernels is the genetic makeup (Lambert and Hallauer, 2001). In maize genetic mapping of oil traits indicates that multiple (>50) QTLs are involved in lipid accumulation (Laurie et al, 2004), making difficult yield improvement through conventional breeding. High-oil varieties of maize were developed at the University of Illinois through successive cycles of recurrent selection (Moose et al, 2004). Although these strains have an improved energy content for animal feeding applications, they have poor agronomic characteristics, including disease susceptibility and poor standability. These deficiencies precluded their commercial adoption on a broad hectarage.

As far as the composition in concerned, maize oil is mainly composed of palmitic, stearic, oleic, linoleic, and linolenic fatty acids. Evidence has been obtained that genetic variation exists also for the fatty acid composition of the kernel (reviewed in Lambert, 2001) and many studies indicate that the inheritance of oleic, linoleic, palmitic, and stearic acid content is complex and under multigenic control. 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 involve variants of fad2 and fad6, but rather involve other genes that may influence the flux via enzymes encoded by *fad2* or *fad6*. In spite of a good understanding of the oil biosynthetic pathway in plants and of the many genes involved in oil pathway so far isolated, the molecular basis for oil QTL is largely unknown. 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.

Application of new technologies, such as transcription profiling, metabolic profiling, and flux analyses, should prove valuable to more precisely identify the genes and enzymes determining the composition of maize oil. 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 for biotechnology approaches in the future. Actually the orthologs in maize of Arabidopsis of *LEAFY COTYLEDON1-2* (i.e. *ZmLEC1*) that activate genes involved in TAG metabolism and storage in embryos and of 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, have been identified (Pouvreau et al, 2011). 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). Furthermore, transcriptomic analyses of ZmWRI1a over-expressing lines led to the identification of 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 similar in action. 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 seeds did not only induce a significant increase in saturated and unsaturated fatty acids with 16 to 18 C atoms but also a significant increase in 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 activates genes coding for enzymes in late glycolysis, fatty acid, CoA, and TAG biosynthesis, and 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.

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 genome. For example, maize-based diets (animals or human) 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 generated by site-specific mutagenesis (Shaver et al, 1996). The expression of the bacterial *DHPS* in maize seeds overproduced lysine, even though the seeds had higher level of lysine catabolic products then their wild-type parents (Mazur et al, 1999) and 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). Morover, Houmard et al (2007) reported an increase in lysine content (from 15 to 55%)in maize seeds by specific suppression of lysine catabolism via RNAi. Similarly, Reyes et al (2008), using RNAi, have produced transgenic maize lines that had LKR/SDH suppressed either in the embryo or in the endosperm or both with a synergist increase in free lysine content in the mature kernel. These experiments showed that transgenic approaches, in addition to enlight the relationships between zein synthesis and opaque endosperm, may be succesfully applied to increase kernel lysine content.

An alternative approach to increase the content of limiting amino acids in maize endosperm, in addition 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 by RNA a reduction in 22-kDa (Segal et al, 2003) and 19-kDa α-zeins (Huang et al, 2004) accompanied by an increase in lysine and tryptophan. Furthermore, another strategy to enhance the level of a specific 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 rich in the desired amino acid(s) 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 over 0.7% of 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 essential amino acid content from 8 to 44%. Bicar et al (2008) have developed transgenic maize lines that produce milk α-lactalbumin in the endosperm. They noted that the lysine content in the endosperm of the lines examined was 29-47% greater. 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 synthase. 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 varieties such as “waxy” (waxy endosperm mutants) that can result in 99% amylopectin and "amylomaize" (amylose extender endosperm mutants) that have kernels with up to 20% amylopectin and 80% amylose. These varieties are already of interest for commercial purposes in starch industry, such as food ingredients, sweeteners, adhesives, and for the development of thermoplastics and polyurhetanes. Moreover, advances in understanding the starch biosynthetic pathway will 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 have 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. Gelatinization is the first step in bioethanol production from starch. Therefore, to reduce the energy requirements for the starch to ethanol conversion process a modified starch with decreased gelatinization temperature might be obtained. Recent 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), a trait that could be exploited for 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 for commercial production with economic and environmental benefits.

Efforts to increase oil content and composition in maize kernels through breeding has been considerable successfully, but unfortunately high oil lines have significant reduced yield (cf. Moose et al, 2004). Alternatively to enhance the oil content in maize kernels the relative proportion of the oil-rich embryonic tissue within the grain might be increased It has been recently reported that embryo size and oil content can be increased. In transgenic lines expressing the wheat Purindoline a and b (*PINA* and *PINB*) genes, the total oil content of the kernel was increased by 25% (Zhang et al, 2010). Despite the significance of the result, the molecular mechanism responsible for the increase remains to be clarified, and, 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 genotypes. 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 carbon 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 and Lambert,1991), which makes NADP-ME an attractive target for engineering high oil concentrations in maize embryos. Furthermore, in oilseed species, numerous biotechnological approaches have been devised to maximize 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 embryo specific 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 identification of transcriptional regulators of the oil biosynthetic network in maize has also opened the way to design and test new biotechnological strategies. A recent study showed that the seed-specific expression of ZmWRI1 enhanced oil accumulation in transgenic maize without detectable abnormalities (Pouvreau et al, 2011), whereas expression of ZmLEC1 under similar conditions severely affected growth and development of the resulting transgenic maize plants (Shen et al, 2010). 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 and 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 and coworkers (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. However, no successful attempt has been so far reported, even though the use of ZmWRI1 as a biotechnological tool for improving oil content in embryos of maize seems promising (see above), over-expression of ZmWRI in the starchy endosperm was not sufficient to trigger oil accumulation in this compartment (Shen et al, 2010). Because there is no evidence that WRI1 regulates TAG assembly, it is not surprising that over-expression of ZmWRI1 only proves to be efficient in tissues already accumulating oil, and thus already expressing the TAG biosynthetic machinery. In this regards, it is worth noting that the structure and size of maize kernels may impair large accumulation of oil in the endosperm.

Along with their essential role in photosynthesis, carotenoids are of significant commercial 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). 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) small (15% to 18% of the total carotenoids fraction) compared to lutein or zeaxanthin (45% and 35%, respectively; Kurlich and Juvik, 1999; Brenna and Berardo, 2004). Moreover, moderate to high heritability estimates indicate that breeding for increased levels of both carotenes and xanthophylls should be feasible. In this context, the use of these cloned genes as probes on mapping populations will also enable to use genetic variation in strategies aimed at improving carotenoids. Accordingly, Harjes et al (2008) showed, by studies of association mapping, expression analysis, and mutagenesis, that variation in the *lycopene epsil cyclase* (*lcyE*) gene alters flux from α-carotene versus β-carotene branches of the carotenoid pathway. Additional evidence obtained by Yan et al (2010) has proved that the gene encoding β-carotene hydroxylase1 (*crtRB1*) underlies a principal QTL associated with β-carotene concentration and conversion in maize kernels. The most favorable *crtRB1* alleles, rare in frequency and unique to temperate germplasm, were introgressed, via marker-assisted selection, into tropical maize germplasm adapted to human diet. Similarly, studies on natural maize genetic diversity carried out by Vallabhaneni and coworkers (2009), identified hydroxylation genes associated with reduced endosperm provitamin A content. Therefore, a simple PCR assay to track and identify *Hydroxylase3* alleles appears valuable for predicting nutritional content in genetically diverse cultivars found worldwide. In addition, Toledo-Ortiz et al (2010) have recently identified in Arabidopsis seedlings transcription factors of the phytochrome-interacting factor (PIF) family that 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 required 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, maize included, for the production of varieties with enhanced agronomical, industrial, or

nutritional value.

The cloning of carotenogenic genes in maize and in other organisms has opened the way to the manipulation of carotenogenesis in plants. Engineering high levels of specific carotenoids require controlled enhancement of total carotenoid levels (enhancing pathway flux, minimizing degradation, and optimizing sequestration) in addition to 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, the rate-controlling steps that limit the predictability of metabolic engineering in plants remain an open question. In this connection, transgenic strategies have been used as a tool to complement breeding techniques to estimate levels of provitamin A. 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), increase total carotenoids up to 34-fold with a preferential accumulation of β-carotene in the maize endosperm. Those levels are close to 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 the simultaneous modification of three 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 exceeding those thus far obtained by conventional breeding, opens the way for the development of nutritional complete cereals to benefits the consumers in developing countries and represents a very important proof of the feasibility of genetic manipulations of distinct metabolic pathways.

There is evidence indicating that tocopherols, in particular γ-tocopherol the predominant form of vitamin E in seeds, are indispensable for protection of the polyunsaturated fatty acid in addition to industrial applications (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-tocopherol to γ-tocopherol. This result has suggested that breeders can use natural varieties, molecular marker assisted selection strategies and transgenic technologies to alter overall level of tocopherols and ratio of α- to γ-tocopherol. However, current nutritional research on the relative and unique benefits of α- to γ-tocopherol should be considered in developing breeding strategies.

Another area in which transgenic approaches may help solve an important problem with maize as a feed grain is 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 significantly contributes 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 (Raboy, 2009). For example, 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 biosynthetic 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 the efforts to elucidate and manipulate phytic acid biosynthesis, *lpa* 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 was 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 and Howard, 2009 for a review). The long-term commercial expectations for this use of "plants as factories", often also called "molecular farming", are great. Transgenic maize seeds have 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 from 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.

Conclusions 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.

The rapidly expanding information from genomics and genetics, combined with improved genetic engineering technology, are contributing to the acceleration of gene discovery of seed quality-related traits in maize and offer a wide range of possibilities for their improvement. In large part, these developments derive from four experimental approaches: i) genetic and physical mapping in plants and the associated ability to use map-based gene isolation strategies; ii) 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; iii) protein-protein interaction cloning, that permits the isolation of multiple genes contributing to a single pathway or metabolic process. In addition, new technologies and information continue to increase our understanding of maize genome. For instance, the complete DNA sequence of the its genome (Schnable et al, 2009), 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 for the development of future commercial products.

Although metabolism is one of the most important networks within biological systems, advances in the understanding of metabolic regulation still suffer from insufficient research concerning the modular operation of such networks (Sweetlove et al, 2008). Thus, it is expected that genomics will bolster plant biochemistry as researchers seek to understand the metabolic pathways for the synthesis of commercial compounds. Moreover, metabolic engineering of maize has been relatively slow due to the difficulty of transformation of this plant. Maize transformation with Agrobacterium is now more efficient than currently used particle gun transformation (Jones, 2009; Reyes et al, 2010) and 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, are promising tools for genetic applications (Shukla et al, 2009; Saika et al, 2011). Their use may lead to both targeted mutagenesis and targeted gene replacement at remarkably high frequencies, thus allowing the application of useful information, acquired from structural- and computational-based protein engineering, to molecular breeding of crops, including metabolic engineering. Additionally, targeted expression will be used to channel metabolic flow into new pathways, while gene-silencing tools will reduce or eliminate undesirable compounds or traits.

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