UDC 575:633.15
DOI:10.2298/GENSR1001023M
Original scientific paper

GENE DISCOVERY FOR IMPROVEMENT OF KERNEL QUALITY-RELATED TRAITS IN MAIZE

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Motto M., C. Balconi, H. Hartings and V. Rossi (2010): *Gene discovery for improvement of kernel quality-related traits in maize*.- Genetika, Vol 42, No. 1, 23 - 56.

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

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potential that the new technologies of cell and molecular biology will provide for the creation of new variation in the future are also indicated and discussed.

Key words: endosperm development, endosperm mutants, starch and protein synthesis, genetic variability, gene discovery

INTRODUCTION

Maize kernel is a major source of food and feed for animal and human nutrition and it provides raw materials for manufacturing many industrial products, including biofuels (WHITE and JOHNSON 2003). Although, plant breeding has been extremely successful at improving the yield of maize, quality has received less attention; however, important advances were made by breeders in this area as well, resulting in maize with a wide range of compositions. In fact, by exploiting genetic variation, the composition of the kernel was altered for both the quantity and quality (structure and chemical diversity) of starch, protein, and oil throughout its development. Furthermore, the ability of plant scientists to use existing genetic variation and to identify and manipulate commercially important genes will open new avenues to designing novel variation in kernel composition. This will provide the basis for the development of the next generation of speciality maize and of new products to meet future needs.

This paper provides an overview of current knowledge on gene discovery, exploitation, and genetic variation known to affect the development, composition, and structure of maize kernels. In addition, we will provide further possibilities, using new technologies of cell and molecular biology, for the creation of genetic variation for the development of novel speciality in maize kernels better suited to its various end uses.

ENDOSPERM DEVELOPMENT

The economic and nutritional value of the kernel is mostly derived from the endosperm, a starch-rich tissue that supports the embryo at germination. The developing endosperm and embryo are enclosed by a maternally derived pericarp to form the kernel. In maize, endosperm makes up the majority of kernel dry matter (70-90%) and is the predominant sink of photosynthate and other assimilates during reproductive growth (see SABELLI and LARKINS 2009, for a recent review); therefore, factors that mediate endosperm development to a large extent also determine grain yield.

Endosperm development and function has been the subject of several recent reviews (*i.e.* OLSEN 2004, SABELLI and LARKINS 2009). The main findings emerging from studies indicate that endosperm is an organizationally simple structure containing four major tissues. Despite the apparent simplicity of the mature tissue, endosperm development is complex and has various distinct phases that can overlap largely: early development, comprising double fertilization, syncitium formation,

and cellularization, differentiation, which includes the formation of the main cell types (transfer cells, aleurone, starchy endosperm, and embryo surrounding cells), the periods of mitosis and endoreduplication, and the accumulation of storage compounds. It also shows several unique innovations 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). As the kernel reaches maturity starch-filled cells senescence, apparently undergoing a form of programmed cell death, whereas aleurone cells acquire desiccation tolerance and remain viable in the dry seed (YOUNG and GALLIE 2000, KLADNIK *et al*. 2004).

Because the endosperm is a remarkable emerging sink, an important aspect of its development concerns the role of carbon (C) metabolism, sugar partitioning and signaling, nutrient fluxes, and the regulation of energy status (BORISJNK *et al*. 2004). However, the inter-relationships with developmental and energetic processes remain largely unclear. High-throughput genomic approaches (LAI *et al*. 2004, VERZA *et al*. 2005, LIU *et al*. 2008, PRIOUL *et al*. 2008) are providing new tools for a better understanding of the genomic and biochemical networks operating during kernel development. Furthermore, PRIUL *et al*. (2008), by a joint transcriptomic, proteomic, and metabolism analyses of starch filling, have provided an overview of the regulation of carbohydrate metabolism during maize endosperm development in relation to generals kernel mebabolism. Collectively, the transcriptome and proteome maps constitute a powerful tool for physiological studies and are the first step for investigating maize endosperm development. This information is useful for identifying distinctive, previously uncharacterised, endosperm-specific genes; in addition, it provides both further research material for academic laboratories, and material for plant breeders and food processors to include in their respective research or product pipelines. The endosperm can also serve as a valuable system to addressing fundamental questions related to the improvement of seed size in crops. For example, WANG *et al*. (2009) have recently provided evidence that genetic studies of endosperm development can be impaired via the use of functional genetic mapping. This approach that may detect the temporal expression pattern of dynamic QTLs during endosperm development is a valuable information for understanding the interplay of endosperm development.

Differentiation of starch-filled cells

Studies of genotypes, differing in endosperm size and in environmental treatments, that affect endosperm growth 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 relevant roles in determining the subsequent grain-filling rate and duration of storage product deposition.

Key pathways relevant to differentiation of starch-filled cells involve the initiation of starch granules in amyloplasts, the start of zein storage protein synthesis in protein bodies, the enlargement of nuclei and cell cycle regulation (OLSEN 2001).

Interestingly, the transitions through syncitial, cellular, and endopolyploid phases make the starch-filled endosperm an attractive model for cell-cycle studies (ROSSI and VAROTTO 2002).

It was shown that enlargement of the maize endosperm relies upon two cellular processes: cell division and cell expansion, which is, in turn, related to endoreduplication of DNA (LARKINS *et al*. 2001). It is currently believed that endoreduplications (around 8-10 Days After Pollination, DAP) 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. It has been proposed that endoreduplication in maize endosperm function primarily to provide a store of nucleotides during embryogenesis and/or germination (SABELLI and LARKINS 2008).

It is well established that endoreduplication, like cell number, is under maternal genetic control and is sensitive to environmental stresses or exogenous application of abscisic acid (ABA) (e.g. SABELLI *et al*. 2005). Cell cycle-related genes are also suggested to play a central role in the regulation of the chromosomal endoreduplication in maize endosperm. In this plant, initiation of endoreduplication is associated with a decrease of the activities of CDKs that bind to $p13^{suc1}$ (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). Mutants with suppressed endoreduplication have not yet been isolated. The *dek* mutants have been frequently considered candidates for genes involved in the mutation and endoreduplication cell cycle. Their molecular analysis should allow a clarification of the defects in the mechanism controlling endoreduplication. However, it has been documented that endoreduplication of the endosperm is correlated positively with phosphorylation of the maize retinoblastoma homologues (ZmRBR1, GRAFI *et al*. 1996, SABELLI and LARKINS 2006), termed RBR1 and RBR3. In this context, it was shown that RBR3 expression is repressed by RBR1, suggesting a compensating interplay between *RBR1* and *RBR3* genes due to functional differences between them (SABELLI *et al*. 2005, SABELLI and LARKINS 2006). Further genetics research that modulates the expression of RBR1 and RBR3 might elucidate their roles.

In other plant species evidence was provided that DNA replication licensing components, such as CDC6 and CDT1, are up-regulated when extra endocycles are triggered (CASTELLANO *et al*. 2004). Additionally, destruction of M-phase regulatory proteins by the anaphase promoting complex is required to initiate endoreduplication (VINARDELL *et al*. 2003) and is negatively affected by the activity of an atypical E2F (E2Fe/DEL) protein, a negative regulator that antagonizes the E2F pathway (VLIEGHE *et al*. 2005). It was also shown that during endoreduplication there is a reduction in chromatin condensation and accumulation of a HMGA-type protein (non-histone chromatin proteins); this protein binds AT-rich regions to causes an open chromatin structure (ZHAO and GRAFI 2000), thus assisting the binding of transcriptional regulators (GRASSER 2003). In addition to the activity of key cell cycle regulators, both the cell cycle and the development of the endosperms depends on hormonal and environmental factors (SABELLI *et al*. 2007).

A link between the control of G1/S transition in cell cycle and factor modulating chromatin structure through histone modification has been studied in this laboratory (ROSSI *et al.* 2003, VAROTTO *et al.* 2003). In these papers, it was found that maize RBR1 (ZmRBR1) can recruit Rpd3-type histone deacetylase (ZmRpd3I/hda101) and cooperate in repressing gene transcription. In addition, they observed that 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* and augments their association. The results of these studies, together with previous published findings regarding 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, RBR/E2F pathway is active in regulating the commitment for cell division, it can be hypothesized that the RBR1/ZmRbAp1/hda101 complex also plays a role in the control of endoreduplication. Most 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 determined also various morphological and development defects and affected expression of genes involved in vegetative to reproductive transition and in meristem function, suggesting a function of *hda101* in mediating developmental programs. Moreover, CASTRO *et al.* (2008), by applying a metabolomic approach to analyze *hda101* mutants, reported differences in the accumulation of several metabolites during kernel development and highlighted the changes occurring in the modified seed confirming the idea that this gene affects various metabolic pathways.

Genetics of endosperm formation

The maize endosperm has been extensively investigated by analysis of mutants affecting kernel development and appearance (CONSONNI *et al.* 2005). Based on a statistical analysis of ethyl methane sulphonate mutagenesis, NEUFFER and SHERIDAN (1980) estimated that at least 300 maize genes condition visible endosperm phenotypes. The *Robertson's Mutator* transposon system of maize generates a similar spectrum of seed phenotypes (SCANLON *et al.* 1994). However, only a small fraction of the known endosperm mutants - broadly referred as *defective endosperm* and *kernel* (*de* and *dek*) - have been up to now molecularly analyzed.

Transposition-based approaches are valuable tools to identify novel mutations affecting endosperm development and for cloning the mutated genes. 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 were cloned: *discolored1* (*dsc1*; SCANLON and MYERS 1998), and *empty pericarp* (*emp2*; FU *et al.* 2002). For *Dsc1* no function has been yet 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 kernel 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, the characterization of the maize endosperm transcriptome (LAI *et al*. 2004; VERZA *et al*. 2005), and 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 carbohydrate synthesis appear to affect endosperm growth and development. Example of such mutations include *miniature1* (*mn1*), in which a loss of the cell wall invertase INCW2 activity is associated with reduced mitotic activity (VILHAR *et al*. 2002), and 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). 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.

Aleurone differentiation and gene expression

The aleurone consists of a uniform single layer of cells that have large vacuoles and accumulates protein, oil, and anthocyanins to high concentrations. These cells are involved in the breakdown and mobilization of storage product upon germination. Aleurone 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 (LID *et al*. 2002, and references therein).

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*, *Mosaic1 (Msc1)*, *collapsed2* (*cp2), opaque-12 (o12),* and *white2* (*w2)* 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 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 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). Most recently, SUZUKI *et al.* (2008) have reported that *Viviparous8 (Vp8)* locus encodes a putative ALTERED MERISTEM PROGRAM1-like peptidase, that regulates ABA accumulation and coordinate embryo and endosperm development.

Anthocyanins are attributed with many biological activities, including heath-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 both regulation of anthocyanin biosynthesis, and in the acquisition of seed dormancy, in which its action depends on the presence of the phitohormone ABA (MCCARTY *et al.* 1991). Molecular analysis of VP1 protein has shown it to represent a plant-specific class of transcription factor, which interacts 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 found that VP1 binds 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). *C1* and *R1* loci encode myb and helix-loop-helix transcription factors, respectively, that interact and specifically activate structural genes in the anthocyanin biosynthetic pathway (GOFF *et al.* 1992, SAINZ *et al.* 1997).

Solute transfer in the seed

Several cell layers of the endosperm near the placent stop dividing and differentiate early into basal endosperm transfer layer (BETL) cell layer. For this layer, formed by of 2 - 3 strata of highly specialized transfer cells (DAVIS *et al.* 1990), one of the often postulated functions is related to acquisition of nutrients from maternal post-phloem regions in the pedicel and high metabolic rates are required during differentiation of the transfer cells. (reviewed in OFFLER *et al.* 2002, and references therein). In this context, MAITZ *et al.* (2000) reported a *reduced grain filling* (*rgf1*) locus that is associated with reduced expression of BETL markers and a loss of 70% seed weight at maturity. Similarly, the *empty pericarp4* (*emp4*; GUTIERREZ-MARCOS *et al.* 2007) and *baseless1* (GUTIERREZ-MARCOS *et al.* 2006a) mutants exhibit abnormal BETL at an early stage of seed development and, ultimately, the aborted seed lethal phenotypes. The nuclear *Emp4* gene codes for a novel type of a mitochondrion-targeted pentatricopeptide repeat protein that is necessary in the proper regulation of expression of a small subset of mitochondrial

transcripts in various parts of the plant, including a 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 mitochondrial deficiency can lead to reduced wall-in-growth (WIG) formation in these cells.

Further studies in this field showed 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). These gene products resemble antimicrobial proteins, suggesting a role in protecting the kernel from potential pathogenic invaders. BETL1 and BAP2 expression appears to be transactivated by a MYB-related gene, ZmMRP-1, which is expressed before the BETL genes in the basal area of the coenocytic endosperm (GOMEZ *et al*. 2002). 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).

In vitro experiments with cultured maize endosperm have reinforced previous views that development of the basal transfer cell layers requires a contribution from maternal sporophytic tissue (GRUIS *et al*. 2006). Moreover, the contribution of the transfer layer to seed development can be deduced from the phenotype of mutants in which these cells are defective, such as *mn1* (MILLER and CHOUREY 1992). This mutant shows a drastic reduction in endosperm cell size and cell number compared with that of the wild-type, *Mn1*, with the weight of the mature *miniature* endosperm being only 20% that of the wild-type. The causal basis of the *mn1* seed phenotype is, as previously reported, the loss of INCW2 localised entirely in the basal endosperm transfer cells (VILHAR *et al*. 2002). Furthermore, KANG *et al.* (2009), reported that INCW2 is crucial for normal assembly and function of WIGs. The same authors 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.

Maternal control of endosperm development

The extent to which maternal tissue is essential for kernel formation is still unclear: somatic embryogenesis and endosperm development can occur *in vitro* in the absence of maternal tissue (KRANZ *et al.* 1998). In addition, molecular evidence has shown that female sporophytic and gametophytic genes govern early endosperm development (GARCIA *et al.* 2003, and references therein); for instance, a number of female-gametophytic mutations in *Arabidopsis thaliana* severely affect development of the seed, particularly the endosperm (KOHLER *et al.* 2003, and references therein). The molecular characterization of these mutations revealed the existence of a set of proteins, closely related to the *Drosophila melanogaster* Polycomb-group (PcG) proteins, that are important in early seed development: Medea, Fertilization-independent Seed2, and Fertilization-independent endosperm.

In plants, as in flies, PcG proteins aggregate into complexes (KOHLER *et al.* 2003) that are required for the establishment of the anterior-posterior axis in the

endosperm (SØRENSEN *et al.* 2001) and repression of precocious embryo and endosperm development until fertilization (LUO *et al.* 2000; SPILLANE *et al.* 2000). Interestingly, the expression of these genes in the endosperm is restricted to the maternal alleles by genomic imprinting (e.g. LUO *et al.* 2000). In maize, like in *A. thaliana*, a genome-wide imprinting mechanism that ensures maternal control of early seed development has been postulated. In fact, a large number of genes are transiently silenced during early embryo and endosperm development upon pollen transmission (GRIMANELLI *et al.* 2005).

To determine the extent to which post-fertilization gene expression in the maize endosperm is regulated by imprinting, GUTIERREZ-MARCOS *et al.* (2004) experimented to identify endosperm genes expressed from either maternal or paternal alleles. In these studies, they have identified a novel gene, *Maternally expressed gene1 (Meg1),* that is specifically expressed in the endosperm transfer cell region and is subject to transient parent-of-origin effects. This may be taken as evidence indicating the existence of a group of transfer cell-specific genes whose expression is under maternal control. Moreover, the expression of *Meg1* depends on *ZmMRP1*, a *Myb* transcription factor, which induces transcription specifically in the basal endosperm transfer layer (GOMEZ *et al.* 2002).

It has been proposed that parent-of-origin expression is accomplished through epigenetic modification such as DNA methylation. The analysis of specific alleles of seed storage protein genes (α-zeins) has provided evidence of differentially methylated regions (DMRs) in plants. It has been suggested that both coding and non-coding regions of 19- and 22-kD α-zein genes are hypomethylated upon maternal transmission, whereas the paternal counterparts are heavily methylated (LAURIA *et al.* 2004, and references therein). Further studies have indicated that DMRs are endosperm-specific, while embryos or seedlings are unaffected. Similarly, DMRs have been found in other maize genes, including α-tubulin, *R, DZR1, FIE1 and FIE2,* and *Meg1* loci (GUO *et al.* 2003, GUTIÉRREZ-MARCOS *et al.* 2004, MAGNARD *et al.* 2004, and references therein). It was also shown that the expression of *FIE1 and FIE2* is differentially regulated in maize, suggesting diversification of function during endosperm development (GUTIÉRREZ-MARCOS *et al.* 2006b, HERMON *et al.* 2007).

Embryo surrounding region (ESR)

In maize, the embryo surrounding cells 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 ESR is still unknown; however, 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 ESR 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 ESR 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 OF STORAGE PRODUCTS

The storage products of the maize kernel, mainly starch and proteins, are synthesised, beginning around 15 DAP, in the subaleurone and starchy cell layers of the endosperm (reviewed in MOTTO *et al*. 2005). Their synthesis continues until metabolic activity is prevented by desiccation at seed maturity (after 40 DAP).

The structure and biochemical properties of kernel storage compounds have been widely investigated over the past 30 years due to their abundance, complexity, and impact on the overall nutritional value of the maize seed. A great deal is now known about the compounds that are made and stored in the kernel, as well as how they are hydrolyzed and absorbed by the embryo. For more detailed reviews describing the nature and biochemistry of maize endosperm storage products, we refer the reader to a number of recent reviews (e.g. HANNAH 2007, HOLDING and LARKINS 2008).

Storage protein

The primary storage proteins in the maize grain are prolamines called "zeins". Specifically, the zeins are the most abundant protein storage component (>60%) in developing endosperm tissues and are constituted by alcohol-soluble compounds with a characteristic amino acid composition, being rich in glutamine, proline, alanine, and leucine, and almost completely devoid of lysine and tryptophan (GIBBON and LARKINS 2005). Zeins have also unique functional and biochemical properties that make them suitable for a variety of food, pharmaceutical, and manufactured goods (LAWTON 2002).

Based on their evolutionary relationships, zeins are divided into four protein subfamily of α- (19 and 22-kDa), β- (15 kDa), γ- (16-, 27-, and 50-kDa), and δ-zeins (10- and 18-kDa), that are encoded by distinct classes of structural genes (HOLDING and LARKINS 2008). The large α-zein component - 70% of all zein proteins - is composed of multiple active genes clustered in several chromosomal locations. (SONG and MESSING 2002). From a nutritional point of view, the exceedingly large proportion of codons for hydrophobic amino acids in α-zeins is mostly responsible for the imbalance of maize protein reserves. Therefore, the reduction in α-zein protein accumulation with biased amino acid content could provide a correction to this imbalance.

Several endosperm mutants altering the timing and the rate of zein synthesis have been described (reviewed by BALCONI *et al.* 2007). The mutants altering the timing of zein synthesis exhibit a more or less defective endosperm and have a lower than normal zein content at maturity. Many of these genes have been mapped to chromosomes and their effect on zein synthesis has been described (Table 1). All mutants confer an opaque phenotype to the endosperm, and, as zein synthesis is reduced, the overall lysine content is elevated, giving potential for use in the development of "high-lysine" maize.

Table 1. Some features of maize mutants affecting zein accumulation

Genotype	Inheritance	Effect on zein accumulation	Molecular bases
Opaque-2 (o2)	Recessive	22-kD elimination 20-kD reduction	Transcript. activator
Opaque-6 (o6)	Recessive	general reduction	
Opaque-7 (o7)	Recessive	20- kD reduction	
Opaque-15 (o15)	Recessive	27-kD reduction reduction in γ-zein	
Opaque-2 modifiers	Semidominant	27-kD overproduction	
Floury 1 (fl1)	Semidominant	general reduction	Trasmemb. protein
Floury-2 (fl2)	Semidominant	general reduction	Defect 22- kDa
Floury-3 (fl3)	Semidominant	general reduction	
*Defective Endosperm B30 (De*B30)*	Dominant	22-kD reduction	Defect 22-kDa
Mucronate (Mc1)	Dominant	general reduction	Defect 22-kDa
Zpr10(22)	Recessive	10-kD reduction	

In this context genetics has played an important role in discovering a series of opaque endosperm mutants and demonstrating their effects on genes mediating zein deposition (PIRONA *et al.* 2005). For example, the recessive mutation *opaque-2* (*o2*) and *opaque-7* (*o7*) induce a specific decrease in the accumulation of 22- and 19-kDa α-zeins, respectively, while the *opaque-15* (*o15*) mutation exerts its effect primarily on the 27-kDa γ-zein. 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 more general reduction in accumulation of all zein classes. It was shown that several 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 protein body and lead to the increased expression of cellular stress response genes (HUNTER *et al.* 2002) consistent with the expression of a mal-folded, endosperm reticular (ER), localised protein. More recently, HOLDING *et al.* (2007) have found that *Fl1* encodes a transmembrane protein that is located in the protein body ER membrane, and

appears to be involved with targeting the 22-kDa α-zein to a location at the interface between the γ-zein rich periphery and the core of the protein body.

The *o2* mutation has been widely studied at the genetic, biochemical, and molecular levels (e.g. MOTTO *et al*. 2005). *O2* encodes a basic leucine zipper (bZIP) transcriptional regulator that is specifically expressed in the endosperm and 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. It was also shown that O2 also regulates directly or indirectly a number of other non-storage protein genes, including *b-32*, encoding a type I ribosome-inactivating protein, *cyPPDK1*, one of the two cytosolic isoforms of the pyruvate orthophosphate dikinase gene, and b-70, encoding a heat shock protein 70 analogue, possibly acting as a chaperonin during protein body formation (MOTTO *et al*. 1997). Furthermore, O2 regulates the levels of lysine-ketoglutarate reductase (BROCHETTO-BRAGA *et al*. 1992) and aspartate kinase1 (AZEVEDO *et al*. 1997). These broad effects suggest that *O2* plays an important role in the developing grain as a coordinator of the expression of genes controlling storage protein, and nitrogen (N) and C metabolism.

Despite efforts to develop opaque mutations that are commercially useful, its inherent phenotypic deficiencies - *i.e.* soft endosperm texture, lower yield, increased seed susceptibility to pathogens and mechanical damages - have limited their use. To overcome these drawbacks Quality Protein Maize (QPM) strains were created by selecting genetic modifiers that convert the starchy endosperm of an *o2* mutant to a hard, vitreous phenotype. Genetic studies have shown that there are multiple, unlinked *o2* modifiers (*Opm*), but their identity and mode of action are elusive (review in GIBBON and LARKINS 2005). However, using two independently developed QPM lines, HOLDING *et al*. (2008) have mapped various major *Opm* QTLs to chromosomes 1, 7, and 9. Additionally, the previous workers, using a microarray hybridization, performed with RNA obtained from true breeding *o2* progeny with vitreous and opaque kernel phenotypes, identified a small group of differentially expressed genes, some of which map at or near the *Opm* QTLs. Moreover, they observed that several of those genes are associated with ethylene and ABA signaling and suggested a potential linkage of *o2* endosperm modification with programmed cell death.

An alternative approach to understand the relationship between zein synthesis and the origin of the opaque endosperm phenotype was to perturb zein accumulation transgenically. Recently, a number of laboratories have reported a reduction in 22-kD (SEGAL *et al*. 2003) and 19-kD α-zeins (HUANG *et al*. 2004) by RNA interference (RNAi), and by seed-specific expression of lysine rich protein (RASCON-CRUZ *et al*. 2004, YU *et al*. 2004). More recently, HOUMARD *et al*. (2007) have reported the increase in maize grains by specific suppression of lysine catabolism via RNAi. An important observation from these studies was that the lysine content was increased in the transgenic lines by 15-20% to 54.8%. These results showed that transgenic approaches, in addition to investigating relationships

between zein synthesis and opaque endosperm, may be useful to increase kernel lysine content.

The expression of zein genes is regulated co-ordinately and zein mRNAs accumulate to high concentrations during early stages of endosperm development (reviewed in MOTTO *et al.* 2005). From these studies, it was also noted that the coordinate expression of zein genes in maize is controlled primarily at the level of transcription according to specific spatial/temporal patterns. Therefore, attention has turned to understanding the regulatory mechanisms responsible for zein gene expression. Highly conserved *cis*-regulatory sequences have been identified in the promoter of prolamine genes and corresponding *trans*-activity factors (reviewed in MOTTO *et al.* 2005). Zein gene expression can also be affected by other regulatory mechanisms, such as methylation and amino acid supply (reviewed in MOTTO *et al.* 1997). In this context, LOCATELLI *et al.* (2009) have recently provided evidence that *O2*-mediated transcriptional activation occurs in two-phases, first a potentiated and second a transcriptional activated phase, both characterized by a specific profile of chromatin modifications. The dependency on *O2* activity in the establishment of these chromatin states was different for distinct sub-sets of *O2* targets, indicating a gene-specific interaction of *O2* with chromatin modifying mechanisms in driving transcription.

Although maize endosperm storage protein genes have been studied for many years, many questions regarding their sequence relationships and expression levels have not been solved, such as structure, synthesis and assembly into protein bodies, and their genetic regulation (HOLDING and LARKINS 2009), The development of tools for genome-wide studies of gene families has allowed a comprehensive analysis of storage protein gene expression in maize endosperm and the identification of novel seed proteins that were not described previously (WOO *et al.* 2001).

To advance our understanding of the nature of the mutations associated with an opaque phenotype, HUNTER *et al.* (2002) have assayed, by profiling endosperm mRNA transcripts, the patterns of gene expression in a series of opaque endosperm mutants. 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, by gene expression profiling, was carried out by HARTINGS *et al.* (2009) by profiling endosperm mRNA transcripts at 14 DAP. Their result, based on a unigene set composed of 7,250 ESTs, allowed to identify a series of mutant related up-regulated (17.1%) and down-regulated (3.2%) transcripts. In addition, the same authors identified several differentially expressed ESTs, homologous to gene encoding enzymes involved in amino acid synthesis, carbon metabolism (TCA cycle and glycolysis), in storage protein and starch metabolism, in gene transcription and translation processes, in signal transduction, and in protein, fatty acid, and lipid synthesis. These results demonstrate that the mutants investigated are pleiotropic and play a critical role in several endosperm metabolic processes. Pleiotropic effects were less evident in the *o7* mutant, but severe in the *o2* and *o2o7* backgrounds, with large changes in gene expression patterns, affecting a

broad range of endosperm-expressed genes involved in several metabolic pathways. Although, these data are descriptive and more work is necessary to define gene function and dissect the complex regulation of gene expression, the gene isolated and characterized might give an intriguing insight to understand the biochemical and metabolic pathways involved in the developing endosperm.

Carbohydrate synthesis

Maize starch is one of the most plant products and has various direct and indirect applications in food, feed, and industries. For this reason attempts to increase its accumulation have received a great deal of attention by plant breeders.

In the maize endosperm, as in other cereals, sucrose is converted to glucose and then into starches that normally account for 75% of total kernel weight. Roughly three-quarters of the total starch is amylopectin, which consists of branched glucose chains that form insoluble, semi-crystalline granules. The remainder of the starch is amylose, which is composed of linear chains of glucose that adopt a helical configuration within the granule (MYERS *et al*. 2000).

The maize kernel is a suitable system for studying the genetic control of starch biosynthesis. A number of mutations that cause defects in various steps in the pathway of starch biosynthesis in the kernel have been described. These studies have greatly contributed to the understanding of starch synthesis (reviewed in BOYER and HANNAH 2001). In addition, they 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 the cloned maize genes and the 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.

It has been shown that starch biosynthesis in seeds is also dependent upon several environmental, physiological, and genetic factors (HANNAH 1997, BOYER and HANNAH 2001). The regulation of the pathway is likely to be complex and takes place at different levels. Although mechanisms of gene regulation based on trans-acting regulatory proteins have been identified, as mentioned in the previous sections in pathways leading to storage protein synthesis, seed pigmentation, and seed dormancy, similar mechanisms have not been reported for starch biosynthesis. This is surprising, considering the number and variety of starch mutations identified so far, which may indicate that nutrient flow is the key regulatory stimulus in carbohydrate interconversion. In this connection, it has been argued that glucose also serves as a signal molecule in regulating gene expression, in some cases, different sugars or sugar metabolites might act as the actual signal molecules (reviewed in KOCH 2004). There is evidence that regulation of major grain-filling pathway is highly integrated in endosperm and gene responses to sugars and C/N balance have been implicated. For instance, MORAIS DE SOUZA (2008) have recently identified a gene for *Sorbitol dehydrogenase1* (*Sdh1)* in maize They showed that this gene is highly expressed early in kernel development throughout the endosperm, with greatest levels in the basal region, compatible with SDH involvement in the initial metabolic steps of carbohydrate metabolism. The same authors also presented

genetic, kinetic, and transient expression evidence for regulation at the transcriptional level by sugars and hypoxia. These findings suggest that SDH activity may represent an adaptation to the high-sugar, low-oxygen environment of the endosperm. Moreover, many pleiotropic defective kernel (*dek*) mutations that fail to initiative or complete grain-filling have been identified, but not yet studied in detail. These are likely to include mutations in "housekeeping genes" as well as important developmental mutants. A key challenge is to devise molecular and genetic strategies that can be used to effectively analyse this large, complex phenotypic class.

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

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

[a] Changes relative to normal.
↑, ↓ = increase or decrease, respectively.
Sugars = the alcohol-soluble sugars
Adapted from BOYER and HANNAH (2001) and BALCONI *et al*. (2007).

Future research in this area is also needed to identify direct interaction among starch biosynthetic enzymes, as well as modifying factors that regulate enzyme activity. In this respect, WANG *et al*. (2007) described a study in which a bacterial *glgC16* gene, which encodes a catalytically active allosteric-insensitive enzyme, was introduced into maize. The results of this study showed that developing transgenic maize seeds exhibited higher AGPase activity (a rate limiting step in glycogenesis and starch synthesis), in the presence of an inhibitory level of Pi in vitro, compared with the untransformed control. More interestingly the kernel weight of transgenic plants was increased significantly.

Furthermore, tools for genome-based analyses of starch biosynthesis pathway are now available for maize and other cereals. This may eventually help to explain species differences in starch granule shape and size, and thus provide the

potential for agricultural advances. Recently, PRIOUL *et al*. (2008) have provided information on carbohydrate metabolism in maize kernels by comparing gene expression at three levels - transcripts, proteins, and enzyme activities - in relation to substrate or product during kernel development from 10 to 40 DAP. They identified two distinct patterns during kernel development: invertases and hexoses were predominant at the beginning, whereas enzyme patterns in the starch pathway, at the three levels, anticipated and paralleled starch accumulation, suggesting that, in most cases, transcriptional control is responsible for the regulation of starch biosynthesis.

Lipids

Plant oil is an important renewable resource for biodiesel production and for dietary consumption by humans and livestock. The mature maize embryo is approximately 33% lipid in standard hybrids and contains about 80% of the kernel lipids (VAL *et al*. 2009). The accumulation of lipids during development parallel the growth of the embryo, beginning at about 15 DAP and continuing until maturity (WATSON 1987). Triacylglycerol (TAG) is the storage oil used by the seedling for energy during early seedling growth, and comprises 70-85% of embryo lipids (TAN and MORRISON 1979). Other lipids in the embryo include dlglycerides and steryl esters. Approximately half of the endosperm lipids are associated with starch, and these lipids are composed primarily of free fatty acids (60%) and lysophospholipid (25%) (WEBER 1987). Lipids in the aleurone and adjacent starchy endosperm layers are similar to those of the embryo, and include approximately 60% of the endosperm TAG (WEBER 1987).

The primary determinant of amount of lipids in maize kernels is the genetic makeup (LAMBERT 2001). Studies in maize through genetic mapping of the oil traits reported that multiple (> 50) quantitative trait loci (QTLs) are involved in lipid accumulation (LAURIE *et al*. 2004), making yield improvement through conventional breeding difficulty. High-oil varieties of maize were developed at the University of Illinois through successive cycles of recurrent selection (DUDLEY and LAMBERT 1992). These lines have an improved energy content for animal feeding applications, but poor agronomic characteristics, including disease susceptibility and poor standability, precluding their commercial introduction on broad hectarage.

In spite of a good understanding of the oil biosynthetic pathway in plants and of the many genes involved in oil pathway have been isolated, the molecular basis for oil QTL is largely unknown. However, ZHENG *et al*. (2008) have recently found that a oil QTL (*qHO6*) affecting maize seed oil and oleic-acid content, encodes an acyl-CoA:diaglycerol acytransferase (DGAT1-2), which catalyze the final step of oil synthesis. A phenylalanine insertion in *DGAT1-2* at position 469 (F469) was responsible fro the increased oil and oleic- acid content. Ectopic expression of the high-oil *DGAT1-2* allele was effective to increase oil and oleic-acid content up to 41% and 107%, respectively. This work 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.

As far as the composition in concerned, maize oil is mainly composed of palmitic, stearic, oleic, linoleic, and linolenic fatty acids. Evidence has shown that genetic variation existed also for the fatty acid composition of the kernel (LAMBERT 2001). A single-gene *linoleic acid1* with a recessive allele, *ln1*, which conditions high linoleic acid levels, was identified in genetic studies involving Illinois High Oil strains. Single-gene inheritance has also been identified in other reports (PONELEIT and ALEXANDER 1965). Additionally, *oleic acid 1* (*olc1*), which reduces further desaturation of oleic acid to linoleic acid was identified and mapped to chromosome 1 (WRIGHT 1995). Other studies, using monosomic lines, have identified genes controlling oleic and linoleic acid composition on chromosomes 1, 2, 4, and 5 (WINDSTROM and JELLUM 1984). High stearic acid and high oleic acid contents were reported to be under the control of one major gene (WRIGHT 1995). In essentially all studies, research has suggested that major gene effects were being modulated by modifier genes for oil composition. Although, it seems that sources of major genes for composition of maize oil can be utilized, other studies indicate that the inheritance of oleic, linoleic, palmitic, and stearic acid content when considered together is complex and under multigenic control (SUN *et al*. 1978). In maize, molecular characterization of fatty acid desaturase-2 (*fad2*) and fatty acid desaturase-6 (*fad6*) indicates that *fad2* and *fad6* clones were 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 gene that may influence flux via enzymes encoded by *fad2* or *fad6*. Additional studies are needed to more precisely identify the genes and enzymes involved in determining the composition of maize oil. Application of powerful new technologies, such as transcription profiling, metabolic profiling, and flux analyses, should prove valuable to achieving this scope, In addition, identification of transcription factors or other regulatory proteins that exert higher level control of oil biosynthesis or embryo development will be particularly attractive candidate for biotechnology approaches in the future for improving oil composition and concentration.

Carotenoid pigments

Carotenoids is a complex class of isoprenoid pigments providing nutritional value as provitamin A and nonprovitamin A. Furthermore, their varied colours provide additional commercial value as colorants in foods and nutrient supplementation (reviewed in MATTHEWS and WURTZEL 2007).

Yellow maize kernels contains carotenoids (0.25 ug g^{-1} dry weight) with provitamin A activity (β-cryptoxanthin, α- and β-carotene, which can be converted to vitamin A) is typically small (15%-18% of the total carotenoids fraction) compared to lutein or zeaxanthin (~ 45% and 35%, respectively; KURLICH and JUVIK 1999, BRENNA and BERARDO 2004). Thus, there is considerable attention for breeding maize with an enhance provitamin A concentrations by shifting carotenoid biosynthesis to favour provitamin A versus other types of carotenoids and thereby increasing nutritional value of the grain. Substantial variation in the levels of specific forms and in total levels of carotenoids has been shown (WEBER 1987a). Moderate to

high heritability estimates indicate that breeding for increased levels of both carotenes and xanthophylls should be feasible (e.g. BLESSING *et al.* 1963).

While the carotenoid biosynthetic pathway is well characterized in several organisms (SANDMANN 1991), in maize it is not yet fully described and some of the genes encoding certain enzymes still need to be identified. Characterization of the carotenoid biosynthetic pathway in maize has been facilitated by the analysis of mutants associated with reduced levels of carotenoids. In fact, by using this approach three genes controlling early steps in the carotenoid pathway in maize have been cloned.

The *y1* mutant is a white endosperm mutant with greatly reduced levels of kernel carotenoids. BUCKNER *et al.* (1996) demonstrated a relationship between the *y1* gene and phytoene synthase. This enzyme is involved in the first dedicated step of carotenoid biosynthesis, the conversion of two molecules of geranylgeranyl pyrophosphate to phytoene. Phytoene desaturase is the second enzyme in the carotenoid biosynthetic pathway and is responsible for a two-step desaturation, taking phytoene to zeta (ζ)-carotene. Phytoene desaturase (PDS) has been associated with the mutant *viviparous 5* (*vp5*), a white endosperm mutant deficient in both carotenoids and ABA, cloned and mapped to ben 1.02 in maize (LI *et al.* 1996). ζ-carotene desaturase (ZDS) is the third enzyme in the carotenoid biosynthetic pathway and is responsible for a two-step saturation from ζ-carotene to lycopene. Another white endosperm mutant of maize has been associated with *viviparous 9* (MATTHEWS *et al.* 2003). Other important genes in the carotenoid biosynthetic pathway of maize still need to be cloned and made available, most notably lycopene β-cyclase, and ε-cyclase, which convert the straight-chain lycopene into β- and α-carotene (CUNNINGHAM *et al.* 1996) by adding two β-rings to β-carotene, and one each of an ε- and β-ring to α-carotene.

The use of these cloned genes as probes on mapping populations will enable the candidate gene approach to be used for studying the genetic control of quantitative variation in carotenoids. Accordingly, WONG *et al.* (2004) have detected major QTL affecting accumulation of β-carotene and β-cryptoxanthin indicating that these QTLs could be selected to increase levels of pro-vitamin A structures. CHANDER *et al.* (2008), using a RIL population found 31 QTL including 23 for individual and 8 for total carotenoid accumulations. Moreover, HARJES *et al.* (2008), via association mapping, linkage mapping, expression analysis, and mutagenesis, showed that variation in *lycopene epsilon cyclase* (*lcyE*) locus alters flux down α-carotene versus β-carotene branches of the carotenoid pathway. Another gene in the pathway, *carotene hydroxilase enzyme* (*crt-B1*), has also been cloned and analysed (J. YAN, unpublished).

The cloning of carotenogenic genes in maize and in other organisms are opening up the possibility of modifying and engineering the carotenoid biosynthetic pathways in plants. Although question remains about the rate-controlling steps that limit the predictability of metabolic engineering in plants, transgenic strategies can also be used as tools to complement breeding techniques in meeting the estimated levels of provitamin A. In this respect, ALURU *et al.* (2008) have reported that the

overexpression of the bacterial genes *crtB* (for phytoene synthase) and *crtI* (for the four desaturation steps of the carotenoid pathway catalyzed by phytoene desaturase and ζ-carotene desaturase in plants), resulted in an increase of total carotenoids of up to 34-fold with a preferential accumulation of *β*-carotene in the maize endosperm. The levels attained are approaching 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) have produced transgenic maize plants in which the level of 3 vitamins, including vitamin A, were increased specifically in the endosperm via that simultaneous modification of 3 separate metabolic pathways. The transgenic kernels contained 169-fold the normal amount of *β*-carotene, 6-fold, and twice the normal amount of ascorbate and folate, respectively. This finding, which largely exceeds any realized thus far by conventional breeding alone, opens the way for the development of nutritional complete cereals to benefits the consumers in developing countries.

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. Metabolic engineering of maize has been relatively slow due to the difficulty of maize transformation. Maize transformation with *Agrobacterium* (reviewed in JONES 2009) is now more efficient than currently used particle gun transformation. In addition, larger DNA fragments can be inserted with *Agrobacterium* than those previously reported by other methods. Furthermore, a strategy for targeted genome modification through the use of designed zinc finger nucleases that induce a double stranded break at their target locus has been recently developed (SHUKLA *et al.* 2009). The ability to routinely insert metabolic pathway quantities of DNA into the maize genome will further speed up maize metabolic engineering.

Maize-based diets (animals or human) require lysine and tryptophan supplementation for adequate protein synthesis. Tryptophan is also the precursor for the synthesis of some neurotransmitters and for niacin (HEINE *et al.* 1995, MORRIS and SANDS 2006). The development of high-lysine maize for use in improved animal feeds illustrates the challenges that continually interlace metabolic engineering projects. From a biochemical standpoint, the metabolic pathway for lysine biosynthesis in plants is very similar to that in many bacteria. The key enzymes in the biosynthetic pathway are aspartakinase (AK) and dihydrodipicolinic acid synthase (DHDPS), both of which are feedback inhibited by lysine (GALILI 2004). FALCO *et al.* (1995) isolated bacterial genes encoding lysine-insensitive forms of AK and DHDPS from *Escherichia coli* and *Corynebacterium*, respectively. A deregulated form of the plant DHDPS was created by site-specific mutagenesis

(SHAVER *et al*. 1996). The expression of the bacterial DHPS in maize kernels overproduced lysine, but they also contained higher level of lysine catabolic products then their wild-type parents (MAZUR *et al*. 1999) despite the fact that lysine catabolism was suggested to be minimal in this tissue (ARRUDA *et al*. 2000). Likewise, a gene corresponding to a feedback-resistant form of the enzyme anthranilate synthase (AS) has been cloned from maize and re-introduced via transformation under the control of seed-specific promoters. This altered AS has reduced sensitivity to feedback inhibition by tryptophan; thus, tryptophan is overproduced and accumulates to higher than normal levels in the grain. This strategy has been successful in reaching commercially valuable levels of tryptophan in the grain (ANDERSON *et al.* 1997). Most recently, REYES *et al*. (2009), using RNAi, have produced transgenic maize lines that had LKR/SDH suppressed in the embryo, endosperm or both. These authors noted a synergist increase in free lysine content in the mature kernel when LKR/SDH was suppressed in both embryo and endosperm; these results have also suggested new insights into how free lysine level is regulated and distributed in developing grains.

A different approach to enhance the level of a given amino acid in kernels is to improve the protein sink for this amino acid (KRIZ 2009). This can be achieved by transforming plants with genes encoding stable proteins that are rich in the desired amino acid(s) and that can accumulate to high levels. Among a variety of natural, modified or synthetic genes that were tested, the most significant increases in kernel 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 kernel proteins and when introduced together with a bacterial DHPS, resulted in a very high elevation of a total lysine to over 0.7% of kernel 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 of *Amaranth* plants, which is known to be balanced in its amino acid content, increases from 8 to 44% essential amino acid content. BICAR *et al*. (2007) have developed transgenic maize lines that produce milk α-lactalbumin in the endosperm; they noted that the lysine content of the lines examined was 29-47% greater in endosperm from transgene positive kernels. Furthermore, WU *et al*. (2007) have provided a novel approach to enrich the lysine content (up to 26%) in the maize grain by endosperm-specific expression of an *Arabidopsis* lysyl tRNA synthate. Combining these traits with seed-specific reduction of lysine catabolism offers an optimistic future for commercial application of high-lysine maize.

Advances in understanding the starch biosynthetic pathway provide new ways to redesign starch for specific purposes, such for ethanol production. Alteration in starch structure can be achieved by modifying genes encoding enzymes responsible for starch synthesis, many of which have more than one isoform (BOYER and HANNAH 2001). Transgenic lines with modified expression of specific starch synthases, starch branching enzymes or starch debranching enzymes, are being

generated in attempts to produce starch granules with increased or decreased crystallinity, and thus altered susceptibility to enzymatic digestion (M. JAMES, personal communication). Another strategy is to reduce the energy requirements for the starch to ethanol conversion process. For example, gelatinization is the first step in bioethanol production from starch. It is conceivable that a modified starch with decreased gelatinization temperature might require less energy for the conversion process. Recent research has shown that expression of a recombinant amylopullulanase in rice resulted in starch that when heated to 85°C was completely converted into soluble sugars (CHIH-MING 2005). The expression of microbial genes in transgenic plants represents also an opportunity to produce renewable resources of fructans. Transgenic maize expressing the *Bacillus amyloliquefaciens SacB* gene accumulates high-molecular weight fructose in mature seed (CAIMI *et al.* 1996). This could potentially be exploited within the high-fructose maize syrup market.

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

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

Several mutants with low levels of phytate have been isolated, including *lpa 1-1*, *lpa 2-1,* and *lpa 241*, and their loci mapped in maize (RABOY 2009). The *lpa1* mutant does not accumulate *myo*-inositol monophosphate or polyphosphate intermediates. It has been proposed that *lpa1* is a mutation in *myo*-inositol supply, the first part of the phytic acid biosynthesis pathway (RABOY *et al*. 2000). The *lpa2* mutant has reduced phytic acid content in kernels and accumulates *myo*-inositol phosphate intermediates. Maize *lpa2* gene encodes a *myo*-inositol phosphate kinase that belongs to the Ins(1,3,4)P3 5/6-kinase gene family (SHI *et al*. 2003). The *lpa3*

mutant seeds have reduced phytic acid content and accumulate *myo*-inositol, but not *myo*-inositol phosphate intermediates was found to encode *myo*-inositol kinase (SHI *et al*. 2005).

Despite efforts to elucidate and manipulate phytic acid biosynthesis, low phytic acid mutants have limited value to breeders because of adverse effects on agronomic traits: 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 Glb promoter produced low-phytic acid, high phosphate transgenic maize kernels that germinate normally and do not show any significant reduction in kernel dry weight. To increase in maize the amount of bioavailable iron, DRAKAKAKI *et al*. (2005) have generated transgenic maize plants expressing aspergillus phytase and iron-binding protein ferritin. This strategy has proven effective for increasing iron availability and enhancing its absorption. However, much work is still to be done to transfer this technology to tropical and subtropical maize genotypes normally grown in the areas of greatest need for enhanced iron content maize.

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

CONCLUSION AND FUTURE PERSPECTIVES

Currently more than 70% of maize production is used for food and feed; therefore, knowledge of genes involved in protein, starch, and lipids production is relevant for improving the nutritional and food-making properties of maize kernels. However, developing plants with improved grain quality traits involves overcoming a variety of technical challenges inherent in metabolic engineering programs.

Advances in plant genetics and genomic technologies are contributing to the acceleration of gene discovery for product development. In the past few years there has been much progress in the development of strategies to discover new plant genes. In large part, these developments derive from four experimental approaches: firstly, genetic and physical mapping in plants and the associated ability to use map-

based gene isolation strategies; secondly, transposon tagging which allows the direct isolation of a gene via forward and reverse genetic strategies as well as the development of the Targeting Induced Local Lesions IN Genomes (TILLING) technique; thirdly, protein-protein interaction cloning, that permits the isolation of multiple genes contributing to a single pathway or metabolic process. Finally, through bioinformatics/genomics, the development and use of large expressed sequence tags (ESTs) databases (*http://www.maizegdb.org*) and, DNA microarray technology to investigate mRNA-level controls of complex pathways. Moreover, new technologies and information continue to increase our understanding of maize; for instance, the complete DNA sequence of the maize genome (SCHNABLE *et al.* 2009), along with comprehensive transcriptome, proteome, and metabolome information, is also a key resource for advancing fundamental knowledge of the biology of seed development and quality-related traits to be applied in molecular breeding and biotechnology. These additional layers of information should help to further unravel the complexities of how genes and gene networks function to give plants including quality-traits. This knowledge will drive to improved predictions and capacities to assemble gene variation through molecular breeding as well as more optimal gene selection and regulation in the development of future biotechnology products. Although, conventional breeding, molecular marker assisted breeding, and genetic engineering have already had, and will continue to have, important roles in maize improvement, the rapidly expanding information from genomics and genetics combined with improved genetic engineering technology offer a wide range of possibilities for the improvement of the maize grain.

ACKNOWLEDGEMENTS

We apologize to all those whose contributions we did not include in this review because of space constraints, personal preferences, or simple oversight. In addition, we would like to thank the members of the laboratory for their research contributions which are described here. The work was supported by Ministero per le Politiche Agricole, Alimentari e Forestali, Rome, as a special grant "Zeagen".

Received February 13th, 2010
Accepted April 12th, 2010

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OSOBINE KUKURUZA VEZANE ZA KVALITET

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I z v o d

Stvaranje biljaka kukuruza poboljšanog kvaliteta semena uključuje mogućnost korišćenja postojeće varijabilnosti u identifikaciji i manipulaciji ekonomski značajnih gena. Ova istraživanja će otvoriti put za dizajniranje novog variranja u sastavu zrna i stvoriti osnovu za razvoj sledeće generacije kukuruza specifičnog kvaliteta. U radu je dat pregled sadašnjih znanja u identifikaciji i korišćenju gena koji utiču na sastav i strukturu zrna kukuruza sa posebnim naglaskom na mehanizme rasta i razvoja endosperma, diferencijaciju ćelija u kojima se deponije skrob, biosintezu skroba, rezervnih protein, lipida i karotenoida.Potencijal novih tehnologija u biologiji ćelije i molekularnoj biologiji, koje će obezbediti stvaranje nove varijabilnosti u budućnosti su naglašene i diskutovane

Primljeno 13. II. 2010.
Odobreno 12. IV. 2010.