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REVIEW PAPER

A Review on Characterization of Zein Multigene Family and its Regulatory Elements

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ABSTRACT

Maize (*Zea mays*) is one of the most productive crops in the world. Corn seeds, in particular, are attractive for a variety of reasons, including ease of storage and transportation, and the ability to store proteins stably for a long time and are easy to process (Howard and Hood, 2005). Maize is also a common food source for humans and animals, making it a convenient container for biopharmaceuticals that contain orally administered products. The growing prevalence of transgenes in crop improvement and biopharmaceuticals requires the exploration of ways to regulate transgene expression. Regulation can occur indifferent stages of gene expression and may be particularly important during transcription. Promoters that drive transgene expression provide this control. Promoters are regions of DNA upstream of the coding region of the gene which contains specific sequences recognized by proteins involved in transcription initiation. Transcription initiation for most protein-coding genes involves the binding and activation of RNA polymerase II. Variation in gene expression occur when other distinct, semi-conserved sequence elements are present in the regulatory regions of the gene, usually upstream or 5′ of the RNA polymerase binding site. These elements bind to protein factors involved in controlling the level and pattern of gene expression. The availability of a wide range of promoters that differ in their ability to regulate the temporal and spatial patterns of transgene expression could significant increase in the success of the application of transgenic technology. Studies on the regulatory elements of genes encoding key regulatory enzymes in the starch biosynthetic pathway and genes encoding storage proteins will be useful to find promoters and highly expressed compounds.

HIGHLIGHTS

- Endosperm is an ideal platform for the production of recombinant proteins.
- Shortage of strong endosperm-specific promoters for the expression of genes in cereal endosperm.
- Zeins, the major storage proteins of maize seed, are encoded by a multigene family.
- *Opaque2* (O2) gene encodes a basic leucine zipper (bZIP) transcription factor that binds to a promoter element in the 22 kDa class of zein genes to activate their expression.

Keywords: Maize, zein, endosperm, Opaque 2, promoter

Plant genetic transformation is an extremely powerful application for studying gene expression in plants. This has contributed to the understanding of genetic regulation and plant development. It has enabled the manipulation and analysis of biochemical processes and the integration and study of genes that cannot be manipulated in conventional breeding. This has led to an explosion of value-added crops with the potential to increase food

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security in developing countries and developing pharmaceutical and nutritional products to improve the health of people around the world. Despite the socio-economic prospects and ecological advantages of this technology, plant genetic engineering has also become one of the most controversial issues in agriculture. Researchers and the interested public have raised valid questions and challenges regarding the consumer and environmental safety of GM crops (Eaglesham *et al*. 2001). It has also become increasingly clear that in order to minimize the potential adverse effects of genetically engineered transgenic plants on beneficial or non-target organisms, it is necessary to establish appropriate levels of gene expression in the appropriate parts of the plant, both in space and time. Promoters that stimulate the expression of transgenes can be part of the answer to questions about the safety of transgenic organisms. The benefits of controlling gene expression in a temporal, spatial, and even intentionally controlled manner have been recognized by scientists studying the regulation and development of transgenic organisms with organizations for many years, as shown by the diversity of promoters in development (Buchanan *et al*. 2000).

Studies on the regulatory elements of genes encoding key regulatory enzymes in the starch biosynthetic pathway and on genes encoding storage proteins will be helpful in finding highly expressed and tissue-specific promoters. Promoters plays the most important role in determining the temporal and spatial pattern of expression and the level of gene transcription, although the final amount of gene product is determined at the transcriptional and post-transcriptional level. So far, some strong constitutive promoters, such as Cauliflower35S mosaic virus and maize ubiquitin-1 promoter (Christensen *et al*. 1992) are widely used in plant biotechnology research. In addition, the sustained high expression of the foreign gene in all tissues can have detrimental effects on the host plant (Cheon *et al*. 2004).

Special characteristics of the endosperm expression in maize

The endosperm is ideal production platform for recombinant proteins. The process of double fertilization leads to the formation of an embryonic and endospermic tissue (Fig. 1). Double pollination of maize after in-plant pollination has been extensively studied both cytologically and ultra structurally (Mól *et al*. 1994) and during in vitro ovarian culture (Schel and Kieft, 1986). The development of the maize endosperm can be divided into four stages, as described, for example, by Clore *et al*. (1996).

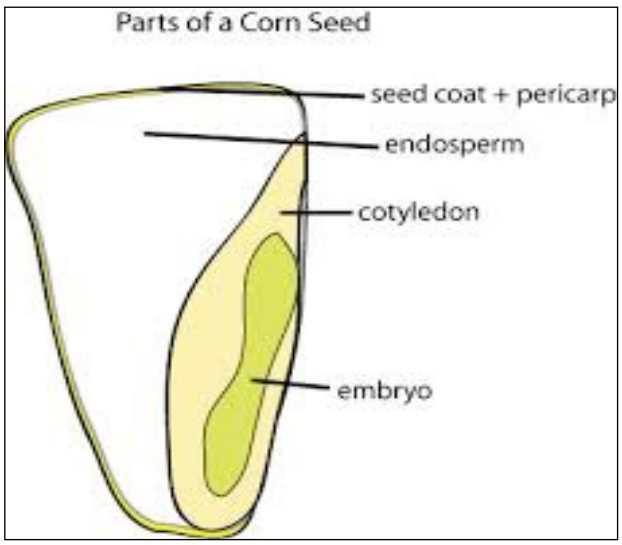

Fig. 1: Cross section of a maize kernel

In phase I (after central cell fertilization up to 3 days post pollination [DAP]) rapid nuclear division occurs without cell wall formation (syncytia formation). A cell wall forms around a single nucleus, resulting in tissue with stage II mononuclear cells (cellularization, 3 to 5 DAP), followed by stage III, characterized by mitotic divisions of up to ~12 DAP in central cells and up to 20-25 DAP in peripheral tissues. Starch granules and proteins accumulate in the center of the tissue. This process starts in phase III at 10 DAP and continues in phase IV when the corn kernels desiccate and endosperm cell death occurs. Compared to other expression systems, using the endosperm has many advantages, such as: Cost savings, production scale that is easy to control, large storage capacity, high storage safety of recombinant proteins and no animal pathogens. Furthermore, its use does not raise any ethical issues (Takaiwa *et al*. 2007). Significant advances have been made on a rice seed system used as a bioreactor for recombinant protein production (Yang *et al*. 2007). However, there is a lack of specifically strong endosperms expression promoters to drive the expression of recombinant protein genes in the endosperm remain an important constraint

in achieving the required level and pattern of expression.

Regulation of zein gene expression in the endosperm

A mature maize kernel contains a much larger embryo and endosperm surrounded by an envelope. In typical dent corn varieties, the endosperm consists of several distinct regions with different physical properties. The outermost layer, the aleurone, consists of specialized cells that secrete hydrolytic enzymes during germination. Below the aleurone are starchy endosperm cells that are filled with starch and storage proteins. These cells form two distinct regions called the "vitreous" or vitreous endosperm and the "starchy" endosperm. Glassy endosperm transmits light while the starchy endosperm does not. Typically, the endosperm is about 90% starch and 10% protein. Although corn kernels contain albumins, globulins, prolamins, and glutelins (Landry and Moureaux, 1970), two types of storage proteins are predominant in seeds: the embryo contains 7S-globulin, similar to that found in dicotyledonous embryos (Kriz, 1999), and the endosperm, the main site of accumulation of storage proteins, contains mainly prolamines, the so-called zein fraction. Almost 70% proteins are made up of different types of prolamin proteins called zeins. They form insoluble outgrowths called protein bodies in the lumen of the rough endoplasmic reticulum (ER), and as the nucleus matures, the protein bodies become tightly packed between the starch granules in the glassy regions of the endosperm (Gibbon and Larkins, 2005).

Most of the proteins in maize endosperm are found in outgrowths in the ER called protein bodies. These structures are composed of different protein cells, each with different structural and chemical properties. A characteristic of zeins is that they are hydrophobic and dissolve in highly concentrated alcohols such as ethanol or butanol. During endosperm development, the first zein proteins deposited in the ER are cysteine-rich β- and γ-zeins. Subsequently, the hydrophobic 19 kDa and 22 kDa α-zeins are secreted in the ER and accumulate in the body's protein center. The mechanism by which α-zein proteins pass through β- and γ-zein proteins is not known. The mature protein body is a sphere with β- and γ-zeins on its surface and a center filled with α-zeins. At some point during the development or desiccation of the testicles, the cysteine remains. The β- and γ-zeins bind to each other and to other proteins, leading to the hypothesis that these cysteine-rich zeins form a kind of "mortar" that binds together the starch granules in the glassy endosperm (Chandrashekar and Mazhar, 1999).

Prolamine genes

Maize storage proteins, called zeins, are encoded by five different classes of genes, which differ in the molecular weight of their protein products (Thompson and Larkins, 1989). Zeins are a group of four structurally distinct types of alcohol-soluble proteins (prolamins) that are synthesized to coordinate the development of the corn seed endosperm. The synthesis of α-zein (19 kDa and 22 kDa), β-zein (14 kDa), γ-zein (16 kDa and 27 kDa) and δ-zein (10 kDa) begins 10 to 12 days after pollination and sets progressed throughout the seed development period (Guo *et al*. 2013). The main reserves proteins of maize are contained in the prolamin fraction called zein. Prolamines are hydrophobic and rich in glutamine, leucine and proline.

The α-Prolamines have a primary structure consisting of an N-terminal signal peptide followed by highly conserved tandem repeats of a α-helical domain of 15-20 amino acids surrounded by a polyglutamine chain and with a short C-terminal. Both 22kDaand 19-kDa α-zein have a conserved primary structure, but while the 22-kDa protein has ten tandem α-helical repeats, the 19-kDa protein contains only nine such domains. Modeling of the three-dimensional structures of these two α-zeins confirmed that the essential difference between them is the lack of α-Helix domain in the 19 kDa protein (Garrat *et al*. 1993).

Sorghum and coix contain prolamines, called kaffirins and coixins, respectively, and share similar structural relationships and solubility in alcoholic solutions to corn. Although there are two classes of α-prolamines in corn, 22-kDa and 19-kDa zein, sorghum and coke, there are only the larger homologues of the α-prolamines, 22-kDa caphyrin and 22-kDa, respectively kDa-α-Coxin , have been described so far (Ottoboni *et al*. 1993). The α-prolamines are encoded by large polygenic

families, while the β-, γ-, and δ-prolamines are encoded by genes with only one or two copies in the genome (Woo *et al*. 2001). The β-prolamines from maize, sorghum and koix are the products of a single gene encoding a polypeptide with an SDS-PAGE mobility of approximately 15 kDa (Coleman and Larkins, 1999). The γ-prolamines are also characterized by a conserved tandem hexapeptide, PPPVHL, followed by a series of proline residues (Leite *et al*. 1999). The δ-Prolamines are the least abundant of these storage proteins in corn and are made up of two10 and 18 kDa polypeptides (Woo *et al*. 2001). Also, unlike other classes of prolamines, there is no evidence of δ-prolamines in Coix. The 22 and 19 kDa α-prolamines contain unusual amino acidsa primary acid structure consisting of an N-terminal signal peptide of approximately 50-60 amino acids followed by highly conserved tandem repeats of an α-helical domain of 15-20 amino acids flanked by polyglutamine chains and a short C-terminus (Leiteet *et al*. 1999).

The main difference between the 22kD and 19kD α-prolamines is that the 19kD protein lacks one of these α-helix domains. Alpha-zein genes have long been mapped the short arms of chromosome 4, the short arm of chromosome 7, the long arm of chromosome 10 and near the centromere of chromosome 1. Although the alpha cell gene families are large and complex, only a small one number of genes identified are transcribed at detectable levels in the endosperm. Several identified genes have in-frame stop codons (Song and Messing, 2003) and it has been hypothesized that many alpha-zein genes are in fact pseudogenes. Knowledge of these gene families is important for understanding corn seed storage proteins, as alpha-zeins are the most abundant storage proteins in the seed kernels. The differences between different inbred lines observed in these families will prove challenging when attempting to sequence the maize genome. Many breeds and hybrids require research to fully understand the effects of the development of these gene families in maize. Cysteine-rich gama-zeins can be assigned to a single locus on the long arm of chromosome 7. There is a single copy of the 16 kDa gamma zein gene and one or two copies of the 27 kDa gamma zein protein gene (Das and Messing, 1987).

Beta-zein contains high concentrations of methionine (11%) and cysteine. It contains less glutamine, leucine and proline than alpha-zeins, but like them no lysine and tryptophan. The 15-kDa beta-zein protein is encoded by a single copy of the gene on the short arm of chromosome 6 (Coleman and Larkins, 1999). Delta-zeins are structurally unrelated to other zeins, but have an amino acid composition similar to Beta-zein. With a molecular weight of 10 kDa, delta-zein has the highest methionine content of all zeins, with 22.5% of the protein being methionine residues. The gene that encodes it exists as a single copy of the gene found on the short arm chromosome 7. Zein delta, with a molecular weight of 18 kDa, is unique in that it is the only known zein protein that contains lysine and tryptophan and is found as a single gene copy on the long arm of chromosome 6 (Swarup *et al*. 1995).

Regulatory elements of zein genes

Coordinated transcription of zein genes suggests that regulatory features are common to all genes encoding different types of zein proteins. One of the largest contiguous maize genome sequences ever identified, it represents a remarkable array of tandemly arranged gene family members involved in the synthesis of endosperm storage proteins. Although these genes are scattered between unrelated sequence fragments, they are relatively densely organized with an average spacing of 6 kb, assuming approximately 2 kb for each gene. Although gene size may not be accurate, they have already been shown to be non-zein promoters using natural variants and promoter deletion assaysgreater than 1 kb (Ueda *et al*. 1994). Since these genes have no introns, their transcribed region is also very small, 0.9 kb.This is in contrast to the P1 gene on maize chromosome 1, which spans approximately 25 kb according to DNAseI hypersensitivity mapping (Lund *et al*. 1995). DNA sequences involved in the control of seed-specific transcription have been identified in the 5′ flanking regions of these seed protein zein genes. In many cases, these seed-specific regulatory sequences are located in the proximal region of the promoter immediately upstream of the transcription start site (Marzábal *et al*. 1998). Sequence comparisons between prolamin promoters reveal a limited number of conserved motifs, the most conserved

being the 25 bp element located about 300 bp upstream of translation start point (Kreis *et al*. 1985). This consensus sequence, called the "endosperm box" or "–300 element", consists of two motifs.

In addition to the CCAAT and TATA blocks typical of many eukaryotic RNA polymerase II promoters, there is a conserved 5′,5′-TGTAAAG-3′ motif known as a prolamin (P-) or endosperm motif (EM model) at the top of the α, β and γ-zein genes. This 7 bp sequence is also found in the 5′ flanking regions of prolamins gene of Oats (Shotwell *et al*. 1990), wheat (Colot *et al*. 1987), barley (Marris *et al*., 1988) and Millet (DeRose *et al*. 1989). These conserved sequences are found in larger regions of DNA that bind to nuclear proteins *in vitro*. Maier *et al*. (1990) found that a 22 bp region containing the TGTAAAG sequence, ranging from -339 to -318 of the α-zein genomic clone, was specific against DNase I digestion by proteins from maize endosperm kernel extracts was protected. In addition, core proteins isolated from maize endosperm have been shown to bind to the 29 bp region (-144 to -114) containing the putative CCAAT box.

Functional assays of the 5′ flanking sequences of α-zein genes show that DNA sequences containing upstream protein binding sites are important for high-level transcription. Deletion analysis of the 5′ flanking region of α-zein clone demonstrated that the region -394 to -189 is essential for high levels of CAT activity in carrot protoplasts (Roussell *et al*. 1988). The α-zein promoter deletion assay in tobacco plants generated endosperm-specific GUS activity when the 5′ flanking -174 were deleted (Matzke *et al*. 1990). To analyze the α-zein promoter sequences important for transcriptional activation, they constructed chimeric genes from the 5′ flanking region with internal deletions between the -483 to -8 nucleotides spindles to the GUS reporter gene. Analysis of these genes in transient expression assays using carrot and maize somatic protoplasts quantified the effect of the 5′ flanking sequences of the zein gene (Ueda *et al*. 1994). Transient expression assays of zein gene promoter activity in maize endosperm suspension culture cells (Quayle and Feix, 1992) suggest that the P box plays a positive role in the coordinated activation of zein gene expression during endosperm development. Interestingly, in the 22-kDa zein gene promoter, the P-box is located just 20 nucleotides upstream of the O2-binding site, suggesting that O2 may interact with P-binding factors to promote the 22-kDa to activate the zein gene (Schmidt *et al*. 1992).

Opaque2 (O2) -zein gene transcriptional activator

Preservation of cereal seed proteins are the major source of protein in the diet of people around the world and have long been the subject of intense research, and our understanding of the molecular mechanisms that regulate their expression is limited. Much of what is known about this process in maize is based on molecular, genetic and Biochemical analysis of the opaque locus2 (O2). Opaque 2 protein is specifically expressed in maize endosperm, the triploid tissue of the grain where storage products accumulate during seed development. There is always more the critical evidence of O2 in controlling various pathways involved in nitrogen and sugar metabolism in the developing endosperm (Kemper *et al*. 1999). O2 expression is subject to both spatial and developmental control, and O2 function is regulated at multiple levels. The O2 gene is only expressed in the endosperm 10 to 45 days after pollination (DAP) in parallel with accumulation α-zeins (Gallusci *et al*. 1994). Translational control of O2 expression is exercised by three short uORFs located in the mRNA leader sequence (Lohmer *et al*. 1993). Post-translational phosphorylation of O2 protein modulates its binding affinity and provides clear evidence that O2 activity is downregulated during the night through O2 transcript reduction and O2 protein hyperphosphorylation (Ciceri *et al*. 1997).

The Opaque2 (O2) gene encodes a basic leucine zipper (bZIP) transcription factor that binds to the promoter element of the 22 kDa class of zein genes to activate the expression of them. Only the 22 and 15 kDa zein gene promoters contain O2 binding sites (Cord-Neto *et al*. 1995). Therefore, additional regulatory factors and promoter elements must be involved in the coordinated activation of all classes of zein genes during endosperm development. Schmidt *et al*. (1992) reported that the prolamin box (P-box) is a good candidate for this cis-action of a regulatory element present in the promoters of all zeins and many genes related to grain storage proteins. The p-box was initially identified both by its highly conserved nucleotide sequence

(5′-TGTAAAG-3′) and by its position (region -300) with respect to translation initial codon of prolamin genes (Brown *et al*. 1986). Endosperm core factors have been shown to bind to the P box present in the 19, 22, and 27 kDa zein gene promoters (Ueda *et al*. 1994). Vicente-Carbajosa *et al*. (1997) reported that P-box binding factor (PBF) specifically interacts with the Opaque2 (O2) bZIP protein, a binding site *invitro* is linked to GCN4-like motifs and is present only 20 bp downstream of the p-box in the 22 kDa maize α-zein promoter dependent activation in maize. The O2 mutation differentially inhibits transcription of the 22 kD α-zein component, which is significantly reduced in the endosperm (Kodrzycki *et al*. 1989). This mutation causes an increase in the concentration of lysine in the cell nucleus through a decrease in one of the main fractions of storage proteins, the 22 kDa alpha-zeins (Jones *et al*. 1977). Opaque2 grains have a low zein to gluten ratio and nearly 70% more lysine than normal endosperm. The opaque2 mutation partially inhibits zein synthesis and thus alters the distribution of protein fractions (Landry and Moureaux, 1982). A decrease in zein mRNA was observed in brittle2-opaque2 double mutants, and other studies showed a significant decrease in zein mRNA in opaque2 mutants, and no 22 kDa mRNA was detected (Pedersen *et al*. 1980).

Although the 22 kDa mRNA could not be detected, the complexity of the zein mRNA remained similar to that of wild corn, suggesting that the mRNA levels were likely reduced but their RNAs were not entirely absent. The other mutations like opaque7 and floury2 have also been shown to alter protein levels by lowering the level of zein present. They differ from opaques2 in that they reduce the content of all classes of zein equally, rather than reducing a specific proportion (Soave *et al*. 1976). Although the opaque2 and opaque7 mutations showed an additive effect on zein expression, they appear to be epistatic with respect to floury2. Studies performed on these three mutations indicate that the opaque2 product is not regulated by either opaque7 or floury2. The opaque2 mutation generally causes a greater decrease in 22 kDa than 19 kDa zeins. The mechanism by which 19 kDa zeins are reduced in opaque nuclei2 is not fully understood as no promoter sequences for the 19 kDa zeins have been found binds to O2. This suggests that the opaque2 mutation affects the 19 kDa zein promoters through an indirect interaction. These studies demonstrate that O2 transcription can be both directly and indirectly regulated interaction with zein gene promoters (Aukerman *et al*. 1991). Sequence comparison identified a cis element, TTTACGT, in the 27 kD γ-zein promoter, which is O2 box-like (TCCACGT) in α-zein 22kD promoters; therefore it was called the O2 box. O2 does not have strong O2-like box-binding affinity, consistent with its constitutive expression in the o2 mutant. However when the O2-like box was modified to generate the exact O2-binding sequence, transcription of the 27 kD promoter-driven γ-zein reporter gene was significantly enhanced when co-expressed with O2 (Ueda *et al*. 1994). The 22 kD α-zein and 27 kD γ-zein promoters appear to be both contain a two-factor motif consisting of a closely connected P-box and an O2-like or O2-like box (Wu and Messing, 2012), resembling a similar regulatory apparatus in which an unknown bZIP TF targeting an O2-like box and co-transaction of the 27 kD γ-zein gene by PBF.

DISCUSSION

Very little is known about the biochemical composition of the endosperm tissue of many species. Constituents such as storable and non-storable proteins, phytin, oils, carotenoids, polysaccharides, free amino acids and phenolic compounds are known to exist in a variety of parts in the endosperm. A better understanding of their cellular distribution, functional roles, and implications for nutritional quality, storage, and seed germination will provide insights into how seed quality and utilization can be improved. Improving the nutritional quality of grain is hampered by the lack of knowledge of endosperm proteins containing nutritionally desirable amino acids. The development of highly nutritious cereals has traditionally been based on the use of mutations with undesirable pleiotropic effects. The development of biochemical tests that enable efficient characterization of endosperm components in large numbers of offspring promises to help breeders better examine existing genetic variations to develop improved strains. Availability of different genes specifically expressed in the endosperm, and the development of efficient reporter gene systems

and transformation techniques have been useful to study gene regulation and cellular function.

Zein proteins are the main storage proteins in the endosperm of maize. In certain classes, different classes appear in different amounts with many genes contained in large gene families. It is clear that large zein-specific mRNAs are part of the complex expression mechanism used to achieve intensive zein synthesis. Transcript analysis of studies explains that the zein gene and the sequence of the 5′ non-coding region of the gene are therefore important in terms of zein synthesis. A striking feature of the zein gene family is its level of expression, with γ- and α-zeins being the most highly expressed subfamilies (Hunter *et al*., 2002; Chen *et al*. 2014).Storage protein genes provide excellent models to study endosperm-specific gene regulation expression. However, the mechanisms that regulate gene expression of storage proteins are only just beginning to be discovered. Recent research is mainly focused on identifying cis-acting DNA sequences and trans-acting proteins factors controlling the evolutionary and spatial expression of storage protein genes in developing seeds. O2-recognized promoter elements have been shown to be important for endosperm-specific expression, conferred by promoters of various grain storage protein genes and also from dicotyledonous plants (Lara *et al*. 2003). In maize, O2 is the first regulatory gene that plays an important role in controlling the expression of storage protein genes. Numerous pleiotropic effects factor genes and their cis-acting recognition sequences can dramatically alter expression levels or activity patterns of target genes.

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