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Full Length Research Paper

Molecular mechanism of methionine differentiation in high and low methionine maize lines

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Since maize is a primary food stuff for humans and livestock, its amino acid balance is important for proper nutrition. Methionine, an essential amino acid and a primary source of sulfur, is lacking in maize endosperm. Several maize populations were developed through breeding with enhanced methionine content in comparison with normal maize populations. BS31HM (high methionine) and BS31LM (low methionine) maize were among such populations created by the selection from the highest or lowest methionine content population from original BS31 maize. Candidate gene approach was adopted to determine the difference between the two populations at transcript level of the selected genes in the endosperm. The genes selected were mostly expressed in the endosperm and could be involved in enhanced methionine biosynthesis. The selected genes, that is, 15-kDa β-zein, 16-kDa γ-zein, 19-kDa α-zeinB1, 27-kDa γ-zein, 22-kDa α-zein and 18-kDa δ-zein were responsible for coding of endosperm storage proteins when analyzed through RT-PCR. Similarly, expression level relative to the high population ($2^{-\Delta\Delta ct}$) values were also calculated for BS31HM and BS31LM, respectively. These values were found as 1 and 0.25, 1 and 0.07, 1 and 0.10, 1 and 0.15, 1 and 0.33, 1 and 0.43 for 27-kDa γ-zein, 22-kDa α-zein, 18-kDa δ-zein, 15-kDa β-zein, 16-kDa γ-zein and 19-kDa α-zeinB1, respectively, in both populations. The p-values were determined by student's t-test at confidence level of 95%. The expression of 18-kDa δ-gene, 15-kDa β-gene and 16-kDa γ-gene were found to be significant ($p < 0.05$) in high methionine maize population when compared with low methionine maize population. Non significant ($p > 0.05$) differences in the expression level of 27-kDa γ-gene, 22-kDa α-gene and 19-kDa α-gene were observed in both HM and LM maize populations. From these results it can be concluded that all zein genes did not show expression equally in high and low methionine maize populations.

Key words: Maize, methionine, zein, storage protein, amino acid, real time PCR.

INTRODUCTION

Maize (*Zea mays* L.) is one of the important cereal crops of the world. Maize is a vital crop for the nutrition of people around the world. In Khyber Pukhtunkhwa Province (KPK) of Pakistan, it is the second most important cereal crop on the basis of daily intake and cultivation.

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Abbreviations: PCR, Polymerase chain reaction; **RT-PCR,** reverse transcriptase- polymerase chain reaction; **DAP,** days after pollination.

Maize is limited by essential amino acids like methionine, lysine and tryptophan. Essential amino acids like tryptophan and lysine are at lower level than minimum requirement established for the growth and development of human and monogastric animals (FAO/WHO/UNU, 1985). Maize is mostly used as animal and poultry feed around the world including Pakistan. Maize based legume diets are also deficient in sulfur containing amino acid methionine and cystine, therefore, nutritional value of maize needed to be improved. Maize being a diet without any proper protein supplementation is a source of nutrional deficiency (Glover and Mertz, 1987). Deficiency of macronutrients such as essential amino acids

methionine and lysine causes malnutrition and more than 40% of the world population are affected (Holger et al., 2004). Both methionine and lysine in poultry diets, has comparatively higher amount of supplemented methionine amino acid which has positive impact on growth (Featherstone and Rogler, 1978).

Modern breeding and plant genetic engineering techniques can improve the deficiencies of micrountrients, macrountrients and essential amino acids deficiencies in major crops to overcome malnutrition problems and other related health problems in the world (Welch and Graham, 2004; Wenefrida et al., 2009). Therefore, plant breeding programs should be started with main objectives to increase and balance the level of such limiting amino acids through their genetic improvement. Such nutritional improvement will reduce the cost of supplementation of synthetic essential amino acids in animal and poultry diets. Methionine is a sulfur rich essential amino acid that is limiting in maize based diets and recurrent selection strategies has been suggested as an efficient method for alteration of methionine content in maize grain (Scott et al., 2008). The maize endosperm consists of storage proteins where accumulation of amino acids take place (Larkins, 1986). These storage proteins are prolamins, composed of four different polypeptides which are alpha zein 19 and 22 kDa, beta zein 15 kDa, gamma zein 16, 27 and 50 kDa (Prat, 1987) and delta zeins 10 kDa (Larkins et al., 1993). Amino acid contents vary between each zein; the typical zein is particularly rich in proline, leucine, glutamine, alanine and serine, with these amino acids making up 70% of the total content. Zeins are synthesized in the rough endoplasmic reticulum and are deposited in the seed as relatively large, insoluble protein bodies that make up about 10 to 15% of the endosperm by volume (Larkins and Hurkman, 1978). These zeins can be separated using high performance liquid chromatography techniques which can precisely separate and characterize zeins in maize endosperm (Wilson, 1991). *In situ* hybridization analyses can determine the synthesis of gamma zein in endosperm before alpha and delta zeins (Woo et al., 2001), while beta zein have interaction with delta zein and beta zeins helps in deposition of delta and gamma zein protein bodies (Bagga et al., 1997). Conversely, the zeins are deficient in the essential amino acids lysine and tryptophan and alpha and gamma zeins are particularly low in methionine (Kirihara, 1988). The present study investigates maize population that have low and high methionine content by recurrent selection, their variation in mRNA expression of zeins genes in the endosperm quantitative RT-PCR analysis.

MATERIALS AND METHODS

Plant material

BS31HM (high methionine) and BS31LM (low methionine) maize populations were investigated and their endosperm tissue was collected from the kernels 14 days after pollination (DAP). The plant tissues were frozen in liquid nitrogen and stored at -80°C until used.

RNA extraction

The mRNA was isolated from approximately 100 g of each endosperm tissue using the methodology Promega PolyATtract 1000 kit (Promega Corp., Madison, WI), without modification. Isolated mRNA was quantified using a NanoDrop ND-1000 spectrophotometer and were stored at -80°C until amplification reactions was performed.

DNA extraction

DNA was isolated from 100 mg of leaf tissues. Ninety six wells PCR-Grade Genomic DNA Isolation Procedure for Cereal Leaf Tissues were used to isolate DNA from the leaf samples and were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Isolated DNA samples were stored at -80°C until amplification reactions were performed.

Candidate genes and primer designing

Six genes 27 kDa, 16 kDa gamma zein gene, 22 kDa α-zeinZ1 gene, 19 kDa gene, 18 kDa δ-zein and 15 kDa β-zein were selected as candidate genes that had a role in methionine deposition in endosperm at maturity. Primers were designed using online (Primer3) primer designing software on the basis of product size of each selected gene and were provided by Integrated DNA Technologies (IDT) USA.

Differential expression of candidate genes via quantitative real-time RT-PCR

Real-time QRT-PCR experiments were performed with 1 ng of endosperm mRNA with concentration of 0.5 ng μl^{-1} as an initial template. Primers were used to amplify the transcript levels of selected maize populations for high and low methionine content, whereas 18S primer was used as standard control. Stratagene Brilliant II QRT-PCR kit was used to assemble all reactions in 25 µl volumes according to manufacturer instructions with 500 µM forward and reverse primers. The fluorophore CyBr Green was included in the reactions to quantify the amplified product. Reference dye was also added in the reaction to confirm the amplification as described in the manual. Cycling protocols were performed in a Stratagene MX3000P (Stratagene, La Jolla, CA) thermocycler and consisted of a first strand synthesis of 50°C for 30 min, followed by 95°C for 10 min, 40 cycles of 95°C for 30 s, T_m °C, 59°C for 1 min, 72°C for 30 s, followed by a dissociation curve, that involved taking a fluorescence reading at every degree between 55 and 95°C to ensure that only one product of the correct annealing temperature was amplified. Using the average fluorescence of cycles 4 to 14, the Stratagene analysis software established a fluorescence threshold below which fluorescence levels were not statistically different than the background fluorescence levels. The cycle at which any reaction crosses this threshold level is referred to as its Ct (threshold cycle) value. All reactions were run in triplicate to assess consistency, and each sample was normalized against 18S (also in triplicate) to ensure that any resulting differential expression was not due to varying amounts of initial RNA template. The delta Ct values were calculated by subtracting the 18S Ct value from Ct value of each primer. Similarly, the delta-delta Ct values were calculated by subtracting the mean ct value of high population from high population and low population from high population. Statistical differences between populations was calcu-

lated by first determining the copy number of each individual plant using the population average and standard deviation and significance was calculated by using the Student's t-test assuming equal variances with a threshold of $p < 0.05$.

RESULTS

Figure (1A, B and C) represents the comparitve expression analysis of 27 kDa gamma zein and 16 kDa gamma zein, 22 kDa α-zeinZ1 known as floury-2 gene and 19kDa alpha gene, 18 kD δ-zein and 15 kD β-zein, respectively, in BS31 maize population contain high content of methionine (HM) and BS31 maize population has low methionine (LM) contents using mRNA extracted 18 days after pollination. The 27 kDa, 16kDa gamma zein gene, 22 kDa α-zeinZ1 gene, 19 kDa gene, 18 kDa δ-zein and 15-kDa β-zein transcripts were amplified through RT-PCR analysis for methionine contents in the endosperm and their mean values, standard deviation and expression level relative to the high population ($2^{-\Delta\Delta ct}$) values are presented in Table 1. The p-value (0.414) of 27 kDa gene was determined by student's t-test which was non significant ($p > 0.05$) at confidence level of 95%, while the p-value for 16 kDa gamma gene was 0.05 determined and it revealed significant differences among the two maize population at $p < 0.05$. Similarly, the p-value (0.206) of 22 kDa alpha gene was determined which was non significant ($p > 0.05$) at a confidence level of 95% and the p-value for 19 kDa alpha gene (0.33) was determined by student's t-test which also revealed non significant differences among the two maize population at $p > 0.05$. It is clear from our results that p-value of both maize populaton was estimated to be 0.002 which revealed significance between the two maize population (high and low methionine) for the expression of of Dz18 gene at $p < 0.05$ at confidence level of 95%. The p-value for bz15 gene (0.01) was determined by student's t-test which also revealed significant differences among the two maize population at $p < 0.05$.

DISCUSSION

The current study was conducted on high and low methionine containing maize populations and candidate gene approach was taken to determine the difference in both populations on the basis of transcript level of selected genes in the endosperm. All the selected genes, that is, 15 kDa, 16 kDa, 19 kDa, 27 kDa, floury2 and dzs18 were responsible for coding of endosperm storage proteins. Methionine is an essential amino acid in any protein, but their representation varies between proteins. Since zeins are so abundant in the endosperm, their amino acid content reflects the amino acid content of the endosperm. Zeins mRNAs are expressed in the maize endosperm (Boston and Larkins, 1987), therefore, candidate genes expression was measured in the endosperm only. Zeins tend to be low in methionine, but there were a few exceptions (dzs10 being methionine-rich). We were interested in knowing the function of other zeins, if all zeins were increased or just methionine-rich ones. It was clear from the results that expression of dzs18 was high in the high methionine maize population which suggested that it is also methionine-rich zein storage protein. The 18 and 10 kDa delta zein genes also contain coding information for lysine and tryptophan but they are the major determinant of methionine and are mostly present in landraces and wild type cultivars and needs breeding strategies to transform it into inbreed lines (Swarup et al., 1995). Similarly, the 15 kDa beta zein and 16 kDa gamma zein were also highly expressed in high methionine maize population, it was clear from our results that the expression of these genes were somehow linked with methionine biosynthesis and endosperm was the methionine deposition sink. The messenger RNA (mRNA) expression that encodes these storage proteins can determine their interaction which suggested their assembling protein bodies as strong interaction were observed between 16 kDa gamma zein and 15 kDa beta zein during their distribution analysis (Kim et al., 2002). The data further suggested that the 19 kDa alpha and 22 kDa alpha zeins were non significantly ($p > 0.05$) expressed in high methionine population. The 19 and 22 kDa alpha zeins seems to be linked with low protein quality of grain, while expression of these genes at RNA level were found to be low in transgenic maize designed for increased amount of lysine and tryptophan (Huang et al., 2006). Most of the alpha zeins genes expression is affected due to mutation found in these genes (Spena et al., 1983). Similarly, the expression of 27 kDa gamma zein was also significantly ($p < 0.05$) low in high methionine population, which was contrary to the earlier findings that gamma and delta zeins are encoded by smaller gene families where gamma zeins are highly expressed (Woo et al., 2001), while on the other hand, same findings supports our results in case of 16 kDa gamma and 18 kDa delta zein gene expression. Alpha genes and gamma genes were not highly expressed which shows a relationship between these two genes families. The results agree with those reported by Coleman et al. (1996) who revealed that stabilization of alpha zeins are related with continuous accumulation of gamma zeins as was observed in transgenic tobacco plants. Zein regulation is a complex process because it is controlled by several genes located on different chromosome and due to their complexity, they cannot be properly expressed in different transgenic plants because of differences at transcriptional level (Thompson and Larkins, 1989), or in different genetic backgrounds they are dependent on promoter response to several factors (Ciceri et al., 2000). Similarly, there was no significant ($p > 0.05$) effect on methionine levels in the leaves of transgenic alfalfa (Bagga et al., 2005). It was concluded that some zein genes were differentially expressed in high methionine maize population when

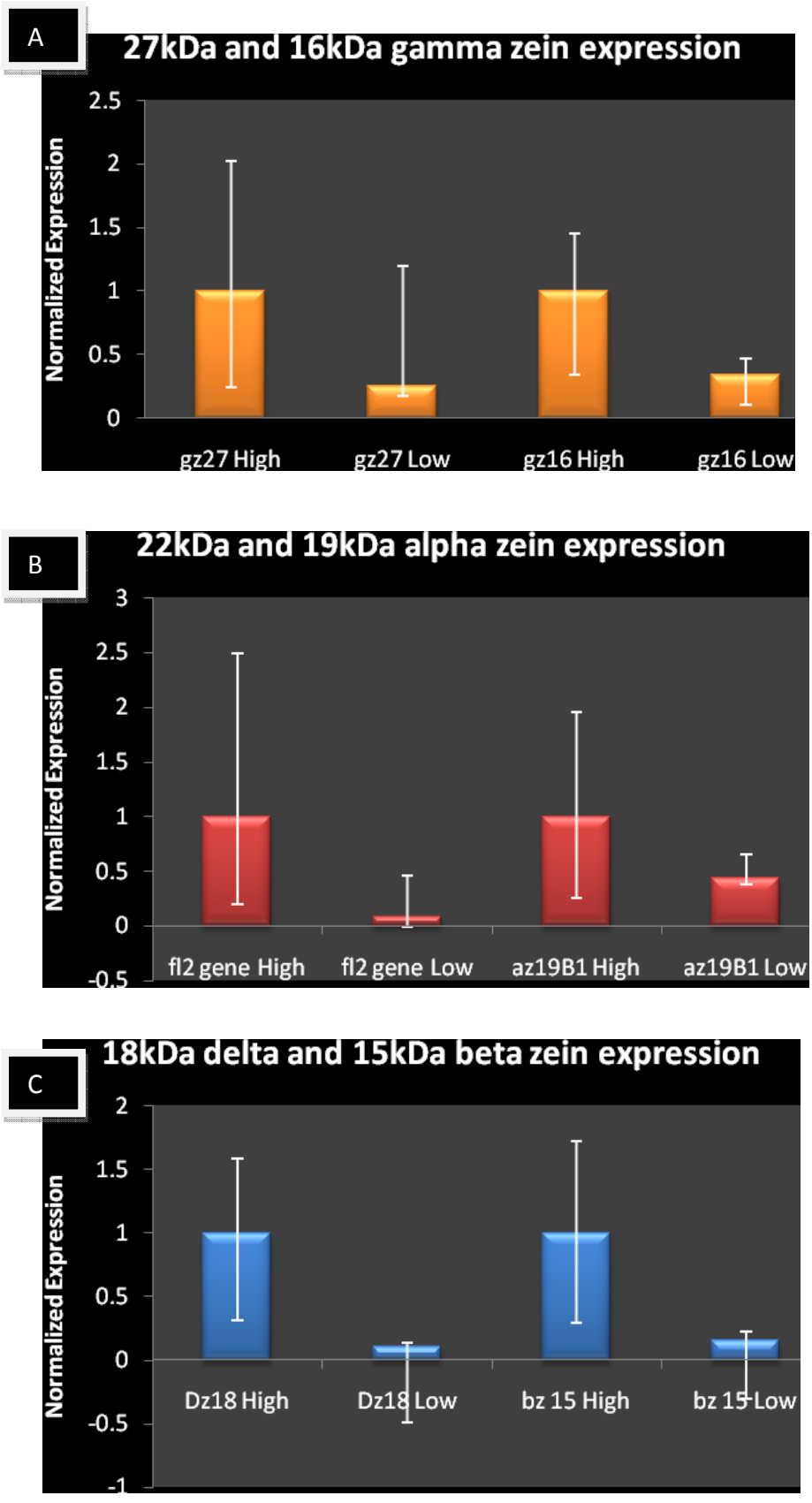

Figure 1. Expression profile of candidate genes in BS31 high methionine and BS31 low methionine endosperm.

Table 1. Comparitive value ($2^{-\Delta\Delta ct}$) of candidate genes in BS31 high and low methionine maize populations (the results are an average of three replicates).

Gene	Population	Mean	Standard deviation	($2^{-\Delta\Delta ct}$)	p-value
gz27	BS31 HM	7.78	2.68	1	0.414
	BS31 LM	9.76	5.48	0.25	
gz16	BS31 HM	7.25	1.41	1	0.05
	BS31 LM	8.82	1.18	0.33	
Floury-2 (fl2)	BS31 HM	6.21	3.48	1	0.206
	BS31 LM	9.86	6.16	0.07	
az19B1	BS31 HM	4.11	2.56	1	0.33
	BS31 LM	5.31	1.43	0.43	
Dz18	BS31 HM	3.47	1.76	1	0.002
	BS31 LM	6.75	1.01	0.10	
bz15	BS31 HM	14.34	2.07	1	0.01
	BS31 LM	17.02	1.31	0.15	

compared to low methionine maize populations which indicated linked coordination of methionine content with zein storage genes.

Conclusions

Enhanced methionine content is linked with higher expression of delta zeins and results in more accumulation of methionine. All zein genes do not express for high methionine content, some have neutral interaction with methionine. Expression of beta and delta zeins showed their interaction with methionine content and are important for methionine accumulation. Alpha zeins are linked with low quality of grain as their expression is low in high methionine lines which may be due to some mutation present in the genes. If gamma zeins are fully expressed, methionine content can be increased in the endosperm.

Recommendations

Delta zeins content in maize endosperm could be enhanced through plant genetic engineering or breeding techniques. Expression level of methionine synthesis genes could be also studied in vegetative parts. Also, different enzymes can be investigated for their detailed role in methionine biosynthesis. The 27 kDa gamma gene could likewise be investigated with further methionine enhanced maize lines.

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REFERENCES

Bagga S, Potenza C, Ross J, Martin MN, Leustek T, Sengupta-Gopalan C (2005). A transgene for high methionine protein is posttranscriptionally regulated by methionine. *In Vitro* Cell. Dev. Biol. 41: 731-741.

Bagga S, Adams H, Rodriguez F, Kemp JD, Sengupta-Gopalan C (1997). Coexpression of the maize delta-zein and beta-zein genes results in stable accumulation of delta-zein in endoplasmic reticulum-derived protein bodies formed by beta-zein. Plant Cell, 9: 1683-1696.

Boston RS, Larkins BA (1987). Control of maize zein gene expression. In JK Setlow,ed, Genetic Engineering, Principles and Methods. Plenum Press, New York. pp. 61-74.

Ciceri P, Castelli S, Lauria M, Lazzari B, Genga A, Bernard L, Sturaro M, Viotti A (2000). Specific combinations of zein genes and genetic backgrounds influence the transcription of the heavy-chain zein genes in maize opaque-2 endosperms. Plant Physiol. 124: 451-460.

Coleman CE, Herman EM, Takasaki K, Larkins BA (1996). The maize gamma-zein sequesters alpha-zein and stabilizes its accumulation in protein bodies of transgenic tobacco endosperm. Plant Cell, 8: 2335-2345.

FAO/WHO/UNU (1985). Energy and protein requirements. Report of a joint FAO/WHO/UNU Expert Consultation. WHO, Geneva.

Featherston WR, Rogler JC (1978). Methionine-cystine interrelations in chicks fed diets containing suboptimal levels of methionine. J. Nutr. 108: 1954-1958.

Glover DV, Mertz ET (1987). Corn. In: Olson RA, Frey KJ (Ed.). Nutritional quality of cereal grains: genetic and agronomy improvement. Madison: ASA. pp. 183-336.

Holger H, Kreft O, Maimann S, Zeh M, Hoefgen R (2004). Current understanding of the regulation of methionine biosynthesis in plants. J. Exp. Bot. 55: 1799-1808.

Huang S, Frizzi A, Florida CA, Kruger DE, Luethy MH (2006). High lysine and high tryptophan transgenic maize resulting from the reduction of both 19- and 22-kDa alpha-zeins. Plant Mol. Biol. 61: 525-535.

Kim CS, Woo YYM, Clore AM, Burnett RJ, Carneiro NP, Larkins BA (2002). Zein protein interactions, rather than the asymmetric distribution of zein mRNAs on endoplasmic reticulum membranes, influence protein body formation in maize endosperm. Plant Cell, 14: 655-672.

Kirihara JA, Petri JB, Messing JW (1988). Isolation and sequence of a gene encoding a methionine-rich 10-kDa zein protein from maize. Gene, 71: 359-370.

Larkins BA (1986). Modification of proteins encoded by seed storage protein genes. Tailoring genes for crop improvement: an agricultural perspective. pp. 163-167.

Larkins BA, Hurkman WJ (1978). Synthesis and deposition of zein in protein bodies of maize endosperm. Plant Physiol. 62: 256-263.
Larkins BA, Lending CR, Wallace JC (1993). Modification of maize seed protein quality. Am. J. Clin. Nutr. 58: 264S-269S.
Prat S, Perez-Grau L, Puigdomenech P (1987). Multiple variability in the sequence of a family of maize endosperm proteins. Gene, 52: 41-49.
Scott MP, Darrigues A, Stahly TS, Lamkey K (2008). Recurrent selection to alter grain methionine concentration and improve nutritional value of maize. Crop Sci. 48: 1705-1713.
Spena A, Viotti A, Pirotta V (1983). Two adjacent genomic zein sequences: Structure, organization, and tissue-specific restriction pattern. J. Mol. Biol. 169: 799-811.
Swarup S, Timmermans MC, Chaudhuri S, Messing J (1995). Determinants of the high-methionine trait in wild and exotic germplasm may have escaped selection during early cultivation of maize. Plant J. 8: 359-368.
Thompson GA, Larkins BA (1989). Structural elements regulating zein gene expression. Bioessays, 10: 108-113.
Welch RM, Graham RD (2004). Breeding for micronutrients in staple food crops from a human nutrition perspective. J. Exp. Bot. 55: 353-364.
Wenefrida I, Utomo HS, Blanche SB, Linscombe SD (2009). Enhancing essential amino acids and health benefit components in grain crops for improved nutritional values. Recent Pat. DNA Gene Seq. 3: 219-225.
Wilson CM (1991). Multiple zeins from maize endosperms characterized by reversed-phase high performance liquid chromatography. Plant Physiol. 95: 777-786.
Woo YM, Hu DW, Larkins BA, Jung R (2001). Genomics analysis of genes expressed in maize endosperm identifies novel seed proteins and clarifies patterns of zein gene expression. Plant Cell, 13: 2297-2317.