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Nucleic acid (cDNA) and amino acid sequences of the maize endosperm protein glutelin-2

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ABSTRACT

The cDNA coding for a glutelin-2 protein from maize endosperm has been cloned and the complete amino acid sequence of the protein derived for the first time. An immature maize endosperm cDNA bank was screened for the expression of a β-lactamase:glutelin-2 (G2) fusion polypeptide by using antibodies against the purified 28 kd G2 protein. A clone corresponding to the 28 kd G2 protein was sequenced and the primary structure of this protein was derived. Five regions can be defined in the protein sequence: an 11 residue N-terminal part, a repeated region formed by eight units of the sequence Pro-Pro-Pro-Val-His-Leu, an alternating Pro-X stretch 21 residues long, a Cys rich domain and a C-terminal part rich in Gln. The protein sequence is preceded by 19 residues which have the characteristics of the signal peptide found in secreted proteins. Unlike zeins, the main maize storage proteins, 28 kd glutelin-2 has several homologous sequences in common with other cereal storage proteins.

INTRODUCTION

The sequences of cereal storage proteins such as gliadins from wheat (1,2) and hordeins from barley (3,4) show homology at both protein and mRNA levels. The main exception to this rule are zeins, the most abundant proteins in maize endosperm (5). These proteins accumulate in protein bodies accounting for 50% of total endosperm proteins. DNA sequences of cDNA and genomic clones corresponding to zein polypeptides have been reported (5) and the complete amino acid sequences of different components elucidated. A model for packaging of zeins into the protein body has been proposed (6).

The second largest protein fraction in maize endosperm is glutelin-2, accounting for about 15% of total endosperm protein. When analyzed by SDS-polyacrylamide gel electrophoresis, glutelin-2 separates into a major component around 28 kd. This protein has also been referred to as a component of the alcohol-soluble reduced glutelin (ASG) and a partial sequence of its N-terminus has been reported (7).

Recent work done in our laboratory has shown that glutelin-2 is present

in the endosperm protein bodies, bordering the inner part of the membrane of the organelle (8). It was also shown that their accumulation in the endosperm occurs in parallel to zeins, being synthesized between 15 and 37 days after pollination. In order to increase our knowledge about this protein fraction, cDNA clones corresponding to glutelin-2 have been obtained and sequenced. It appears that this protein has a well-defined domain structure and that homology can be found between its sequence and that of other cereal storage proteins.

MATERIALS AND METHODS

Enzymes

DNA restriction endonucleases were purchased from Amersham or New England Biolabs. AMV reverse transcriptase was from Life Sciences Inc.. Calf intestinal alkaline phosphatase and DNA polymerase I Klenow fragment were obtained from Boehringer Mannheim. Terminal transferase and polynucleotide kinase were purchased from Bethesda Research Laboratories.

Isolation of mRNA and cDNA cloning

Total polysomal RNA was extracted from kernels 20 days after pollination of the double hybrid inbred line E-10 as described by Wienand and Feix (9). Poly A+ mRNA was purified by two successive passages through an oligo-dT cellulose chromatographic column (10). Complementary DNA and double-stranded cDNA were prepared by a one step procedure modified from Wickens et al. (11). Cloning of the cDNA was done at the PstI site of pBR322 by dG/dC tailing (12).

Subcloning of representative cDNA clones was done by purification of the cDNA inserts by electroelution onto DEAE-cellulose paper (Whatman DE81) after agarose gel electrophoresis using a simplified version of the Dretzen et al. method (13), and cloning at the PstI site of pUC8.

Immunodetection of clones

Tetracycline-resistant, ampicillin-sensitive clones were screened for β-lactamase:G2 fusion protein expression with anti-G2 antibodies elicited in rabbits by injection of the purified 28 kd G2 protein (8). Bacterial colonies grown onto nitrocellulose filter sheets (BA 85 Schleicher and Schuell) were precipitated with 25% TCA, incubated with 1/50 diluted anti-G2 antiserum, washed with 1M NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.5), 0.05% Nonidet P-40, 0.02% NaN_3 and detected by ^{125}I labelled Protein A (Amersham) and autoradiographed (14).

Secondary screening was done by colony hybridization (15) using as

probes ^{32}P nick-translated cDNA inserts of immunodetected clones.

Hybrid-selection and "in vitro" translation

Purified plasmid DNA (10 μg) from selected clones was linearized by EcoRI digestion, denatured and fixed onto nitrocellulose filters (4x4 mm.) to be used for selection of specific mRNA according to Ricciardi et al. (16). Before hybridization, filters were prehybridized for 1 hour at 52°C with 40 μg/ml of poly(rA) in the hybridization buffer as described by Viotti et al. (17), in order to avoid non-specific retention of the mRNA.

"In vitro" translation experiments were done in micrococcal nuclease treated rabbit reticulocyte lysate (kindly provided by Dr. A. Boronat, Centro de Biologia Molecular, Madrid) using ^{35}S-Methionine as radioactive label. Optimal incorporation was obtained at 125 mM KCl and 2.0 mM $MgCl_2$.

Immunoprecipitation of the newly synthesized proteins was done essentially according to Borgese and Gaetani (18). Electrophoresis was performed according to Laemmli (19) in 15% polyacrylamide gels. Acrylamide/bis-acrylamide ratio was 150/1. Gels were dried in an LKB slab gel drier and autoradiographed using En^{3}Hancer (NEN) as scintillation medium. Preflashed RP-X1 (MAFE) films were used.

DNA sequencing

Chemical degradations were performed according to Maxam and Gilbert (20), using the modified A+G reaction described by Bernard and Gough (21). 5'-recessed ends were digested with Klenow fragment of DNA polymerase I prior to labelling with polynucleotide kinase. Polyacrylamide (6% and 20%) gels (55 cm x 19 cm x 0.3 mm) thermostatized at 55°C were used in a LKB sequencing system. Computer analysis of the sequence was done by Dr. Martin Caballero (Centro de Biologia Molecular, Madrid).

RESULTS

cDNA cloning and detection of G2 clones

The cDNA inserts at the PstI site of pBR322 are under control of the β-lactamase promotor (12). Therefore we would expect the expression in some of the host cells of a β-lactamase:G2 fusion protein when total polysomal polyA+ RNA is used as a template for the cDNA synthesis. By this method, five clones (termed pME116-pME120) out of the 400 Tc^R Amp^S clones screened with anti-G2 antibodies were detected (Fig.1a). This is in the range of the expected proportion of positive clones, taking into account the abundance of the protein (15%) and that only the cDNA inserts in the appropiate orientation and reading phase (one sixth) may in principle synthesize

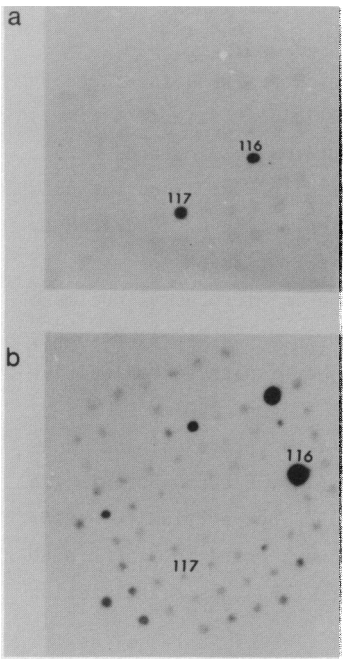

Figure 1: Screening of cDNA clones. (a)immunodetection using anti-G2 antibodies; the picture shows two of the immunodetected clones; (b)colony hybridization using one of the previously detected clones (pME116 insert) as a probe.

polypeptides reacting with the antibody.

Additional clones were detected by colony hybridization with cDNA inserts from the immunodetected clones. The insert from clone pME116 was ^{32}P-labelled and hybridized to the same cDNA bank and 18 positive clones were detected. However, neither the pME117 nor the pME118 clones gave a positive signal with this probe (Fig. 1b). The insert from clone pME117 was then prepared and a secondary screening done. This probe hybridized to 17 additional clones that had not been detected with the pME116 probe. Weak hybridization was also observed with some of the clones previously detected with pME116 probe. The cDNA bank was also screened with probes from zein clones A-20 and B-49 (kindly provided by Drs. B. and F. Burr, Brookhaven) which contain inserts coding for the 19 kd and 22 kd zein components respectively. No cross-reaction was observed with any of the clones detected with anti-G2 antibodies or by the subsequent screening.

Plasmid DNA from all positive clones was prepared and the sizes of the inserts were estimated by running agarose gel electrophoresis of DNA linearized by restriction with EcoRI. Clones that had been detected with the antibodies showed in general longer insert size (670 b.p. for clone pME117, 695 b.p. for clone pME120, and 870 b.p. for clone pME119) as

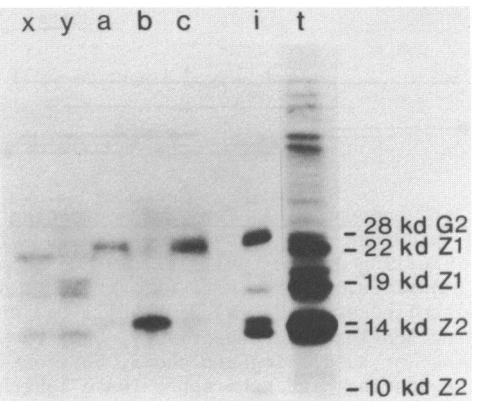

Figure 2: SDS-PAGE of hybrid-selected translation products. (t)translation products of maize endosperm poly A+ RNA; (i)translation products immunoprecipitated by the anti-G2 antibody (x,y,a-c)translation products of RNAs selected by hybridization to the zein cDNA clones B-49(x), A-20(y), and to the cDNA clones pME116(a), pME117(b), and pME119(c).

compared to those detected by hybridization.

Identification of the cDNA clones

Hybridization results suggest the existence of two families of cDNA clones, both detected with the anti-G2 antibody. Immunoblotting of maize endosperm protein fractions with anti-G2 antibodies showed strong reaction not only with 28 kd G2 but also with 14 kd zein-2 (8). Translation of hybrid-selected mRNA was carried out in order to investigate whether each family of clones corresponded to one of these proteins. Figure 2 shows the electrophoretic separation of the "in vitro" translation assay of the mRNAs selected by three of our cDNA clones, pME116, pME117, and pME119, and two zein clones, A-20 and B-49. These are compared with translation of total endosperm mRNA. Translation products immunoprecipitated by the anti-G2 antibodies are also included.

Total endosperm mRNA translates into a characteristic pattern of proteins. The most prominent bands correspond to zeins, with an altered electrophoretic mobility due to the presence of a signal peptide (22). Low molecular weight proteins (14 kd) show a very strong signal probably as a result of their high content of methionine, the amino acid used for labelling. The 28 kd G2 protein is not well resolved from the 22 kd zein polypeptides. Immunoprecipitation of whole translation products was carried out in order to identify this protein. As occurs with immunoblotting of

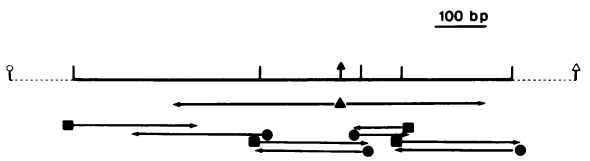

Figure 3: Restriction endonuclease map and sequencing strategy of clone pME119. Nucleotide sequence was determined either directly from clone pME119 by 5' end-labelling at the KpnI site (▲) and secondary cleavage at BglI (○) or PvuI (△) sites, or from PstI (⊥) fragments subcloned in pUC8. Subclones were end-labelled at EcoRI (■) or HindIII (●) sites, and secondly cleaved at HindIII or EcoRI sites respectively. Only the restriction sites used in end-labelling or subcloning are shown. Horizontal arrows indicate the direction of sequencing and the approximate length of the sequences obtained.

purified proteins (8), two bands react with the antibodies having electrophoretical mobilities that allow to identify them as 28 kd G2 and one of the 14 kd Z2 components. It was difficult to avoid the presence of zein fraction in the immunoprecipitates due to their low water solubility. This is probably the reason for the traces of highly labelled zein protein fractions (19 kd and lower Mw 14 kd component) which coprecipitate with the immunocomplexes.

Clones pME116 and pME119 both selected mRNA that translates into 28 kd G2 while clone pME117 only selects the 14 kd Z2 mRNA. Therefore, it can be concluded that the two families of cDNA clones detected correspond to two different types of messages that code for the different proteins reacting with anti-G2 antibodies. As expected from the lack of hybridization of the corresponding plasmids, no zein messages were selected by glutelin clones. In the same way, A-20 and B-49 zein clones did not select G2 messages, but selected their corresponding zein polypeptides. They also select one of the two components of the 14 kd fraction, different from the one corresponding to clone pME117 which showed a slightly lower mobility. Although this component has been previously considered as a zein-like polypeptide preliminary data indicate a high homology at the protein level to the 28 kd G2 protein. This homology may explain the immunocrossreaction of this 14 kd component with the 28 kd G2 antibody.

DNA sequencing of plasmid pME119

"In vitro" translation results indicate that the clones corresponding to glutelin-2 proteins are those of the pME116, pME119 family. Therefore plasmid pME119 containing the longest cDNA insert was selected for

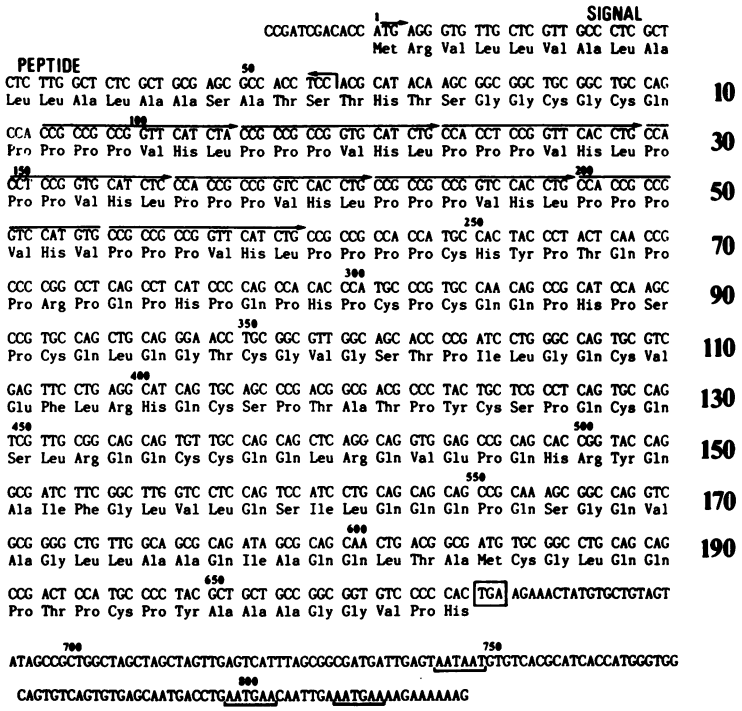

Figure 4: Nucleotide sequence and derived protein sequence of cDNA clone pME119. Nucleotides are numbered starting with the first base of the ATG initiation codon. Signal peptide is also shown. Arrows in residues 30-78 denote position of tandem repeat units. Presumptive polyadenylation signals are bracketed.

sequencing. Restriction map and sequencing strategy for clone pME119 are shown in figure 3. The Maxam and Gilbert method (20) was used. Nearly all sequences were determined on both strands, and most of them were sequenced several times.

The complete nucleotide sequence of the pME119 cDNA insert consisting of 836 nucleotides between the dG/dC tails is shown in figure 4. This insert has a 669-nucleotide long open reading frame, coding for a 223 amino acid long protein. The complete amino acid sequence of a 28 kd G2 protein can be deduced from the clone pME119 nucleotide sequence (Figure 4). The known partial amino acid sequence (7) agrees very well with the amino acid sequence deduced from the nucleotide sequence. Consequently, unambiguous identification of pME119 as corresponding to a G2 mRNA can be established.

The first ATG initiation codon in the pME119 sequence precedes the

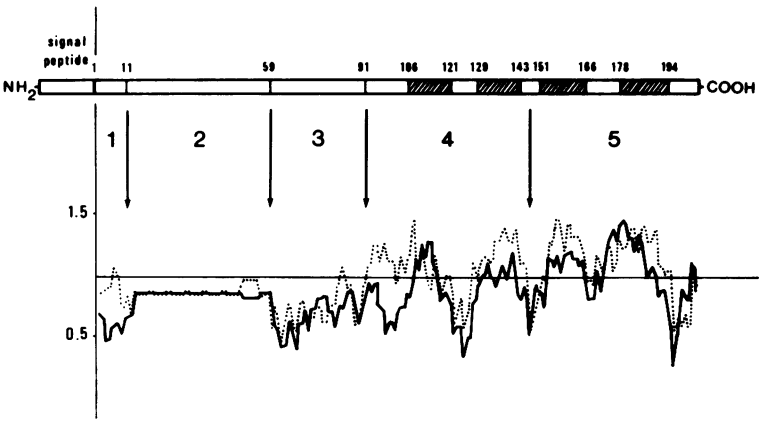

Figure 5: Secondary structure prediction plot of the 28 kd G2 protein. Dashed and solid line curves correspond to the predicted β-structure and α-helix values respectively. The different regions are indicated: 1) N-terminal domain; 2) tandem repeats; 3) Pro-X domain; 4) Cys-rich domain; 5) C-terminal domain. Hatched boxes represent the homology modules as compared to gliadin and hordein (see figure 6)

N-terminal protein sequence by 19 amino acids. This additional sequence probably corresponds to a signal peptide as it has the characteristic features of eukaryotic signal peptides: 18-24 amino acids long, a basic residue near the N-terminal end, and highly hydrophobic amino acid composition (23).

The sequence of the mature protein comprises five distinct regions: the N-terminal portion, 11 amino acids long; a repeated region, composed by the hexapeptide Pro-Pro-Pro-Val-His-Leu repeated eight times with only one replacement in its seventh unit; an alternating Pro-X sequence between residues 70 and 91; and a C-terminal part, rich in Gln which can be divided into two domains. A fourth domain rich in Cys and containing some degree of internal homology can be defined from residue 92 to 148. Indeed the heptapeptide Gln-Cys-X-Glx-X-Leu-Arg repeated twice and the related Gln-Cys-Gln-Ser-Leu-Arg are found in this region. The fifth domain from residue 148 to the C-terminal end does not show any internal homology. The definition of these domains is clearly confirmed when the Chou and Fasman (24) parameters for secondary structure prediction are applied (Figure 5).

The molecular weight of the 204 residues protein deduced from the pME119 sequence (discounting the 19 residues signal peptide) is 21,780 dalton. This value is different from that estimated from mobility in

TABLE I: Codon uage in clone pME119.

Phe	TTT	0	Ser	TCT	0	Tyr	TAT	0	Cys	TGT	0
	TTC	2		TCC	2		TAC	4		TGC	14
Leu	TTA	0		TCA	0	stop	TAA	-	stop	TGA	1
	TTG	5		TCG	2		TAG	-	Trp	TGG	0
	CTT	0	Pro	CCT	6	His	CAT	9	Arg	CGT	0
	CTC	8		CCC	5		CAC	7		CGC	0
	CTA	1		CCA	11	Glu	CAA	4		CGA	0
	CTG	11		CCG	29		CAG	26		CGG	3
Ile	ATT	0	Thr	ACT	2	Asn	AAT	0	Ser	AGT	0
	ATC	3		ACC	3		AAC	0		AGC	6
	ATA	1		ACA	1	Lys	AAA	0	Arg	AGA	0
Met	ATG	2		ACG	4		AAG	0		AGG	3
Val	GTT	5	Ala	GCT	5	Asp	GAT	0	Gly	GGT	1
	GTC	7		GCC	3		GAC	0		GGC	10
	GTA	0		GCA	1	Glu	GAA	0		GGA	1
	GTG	5		GCG	7		GAG	2		GGG	1

SDS-gel electrophoresis. This is not surprising considering the unusual repeat in its N-terminal region, and the highly variable mobility of the protein that migrates as a diffuse band ranging from 21,000 to 28,000 dalton.

The dG+dC content in the coding region of the clone is 69%, whereas in the non-coding part it is only 41%. The codon usage (Table I) is in agreement with the nucleotide composition, and shows a preference for the third nucleotide being G or C. This is especially clear for the most abundant amino acids, such as Pro, Gln, Leu, Gly, and Cys. The main exception to this rule is His that has nine CAT codons compared to seven CAC codons. The codon usage is in general very different from the one found for zeins (25). The cDNA insert of pME119 extends 153 nucleotides from the stop codon at its 3'-end and three potential polydenylation signals are found, two of them being AATGAA and the other AATAAT.

DISCUSSION

In this paper the cloning of a cDNA corresponding to a 28 kd glutelin-2 protein from maize endosperm is presented for the first time. An immunological procedure was chosen to identify pBR322 recombinant clones from a cDNA bank of immature maize endosperm, taking into account that the protein represents a high proportion (around 15%) of total endosperm protein content. This method has proven to be useful even if cross-reaction of the antibodies with other proteins from the same tissue exists. In our case further screening with plasmid DNA revealed the presence of two

families of clones which by hybrid-selection experiments were shown to correspond to the two proteins reacting with the antiserum. To our knowledge this is the first time that this technique has been used in a plant system.

Cloning of the 28 kd G2 cDNA allowed us to deduce the total sequence of the protein. From this sequence several features can be discussed. A 19 residues peptide can be observed at the N-terminus of the deduced protein sequence, which is not present in the partial sequence obtained by protein sequencing (7). This peptide has the properties of the signal peptides present in proteins secreted through a membrane (23). This appears to be a general feature of seed storage proteins (26). The presence of a signal peptide is, on the other hand, in agreement with the localization of glutelin-2 inside the protein bodies from maize endosperm (27,8).

Five well-defined regions can be observed in the protein sequence. The mature protein starts with an 11 residue segment, followed by eight perfect repeats of a Pro-Pro-Pro-Val-His-Leu sequence containing only one amino acid substitution. Other tandem repeats have been observed in cereal storage proteins. In α/β and γ-gliadin (1,2,28) and B-hordein (3,4) they are also located in the N-terminal part of the protein, while in zeins (5) they are spread along the sequence. However, in the 28 kd G2 the conservation of the repeated sequence is very high as compared with the other proteins. The repetitive sequence is followed by a stretch of 21 residues between amino acids 70 and 92 with a Pro-X repetition with only one interruption. The last two domains of the protein have a more balanced composition with Pro residues spaced along the sequence. Most of the glutamine residues (25 out of 29) are located in this zone and 19 of them in the form of doublets, triplets, or Gln-X-Gln sequences. Prediction methods show that secondary structure may be located in the fourth and fifth domains with a general predominance of β-structure probability except in the C-terminal half of the last domain.

In many respects 28 kd G2 protein resembles more closely gliadins (1,2) and B-hordein (3) polypeptides than zeins. It has already been mentioned that these proteins have a repeat in their N-terminal part as occurs with gliadins although in the case of glutelins the repeat is shorter and perfect. Some other features are in common between these proteins. These include several motives at the C-terminal half of the protein that are present in glutelin, gliadin and hordein as shown in figure 6. Interestingly the four homologous stretches observed correspond

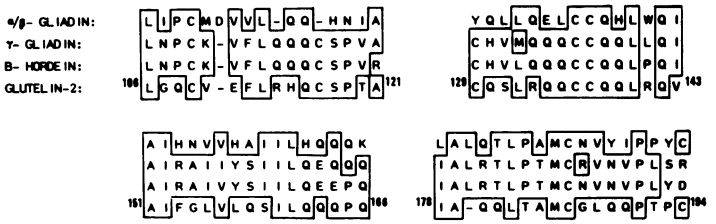

Figure 6: Homology regions of glutelin, gliadin and hordein. The homology regions of the 28 kd G2 protein as compared to α/β-gliadin (1), γ-gliadin (2), and B-hordein (3) are shown.

to the regions of highest predicted secondary structure in 28 kd G2 protein (Figure 5). These sequence elements are not present in zein polypeptides.

The partial nucleotide sequence obtained from our clone indicates some characteristic features that distinguish this protein from zeins. Those are the high dG+dC content and the structure of the 3' end of the clone. The percentage of dG+dC is 69% in the coding region of pME119 compared with the 41% of zein clones. The 3' end closely resembles other cereal storage plant genes. It extends at least 153 nucleotides (as compared with 80 nucleotides in zeins) from the stop codon. It includes three hexanucleotides placed at positions similar to the polyadenylation signals in gliadin and hordein genes and having only one base replacement from the canonical AATAAA sequence. At the 5' end the sequence around the initiation codon agrees with the CCACCATGA/G consensus sequence proposed by Kozak (29).

From the results discussed above we can draw the conclusion that glutelin-2 from maize endosperm has several characteristic features in common with storage proteins from other cereals, both at protein and DNA level. In fact it appears to be the first member found in maize of this superfamily of plant proteins. Zeins, the most abundant storage proteins from maize, do not show homology with maize glutelins or with other cereal proteins. This may indicate that, while glutelins probably belong to the same evolutionary family as gliadins or hordeins, zeins have independently evolved and adopted in maize the function of main storage protein.

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