The 1Dx5 high molecular weight subunit gene from wheat in transgenic maize

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The 1Dx5 high molecular weight subunit gene from wheat in transgenic maize

by

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For the Major Program
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ABSTRACT

We have developed a method for detecting a transgene and its protein product in maize endosperm that allows the kernel to be germinated after analysis. Our method involves first sampling the endosperm with a hand-held rotary grinder so that the embryo is preserved and capable of germination. This tissue is then serially extracted, first with SDS-PAGE sample buffer to extract proteins, then with an aqueous buffer to extract DNA. The product of the transgene can be detected in the first extract by SDS-PAGE with visualization by total protein staining or immuno-blot detection. The second extract can be purified and used as template DNA in PCR reactions to detect the transgene. This method is particularly useful for screening transgenic kernels in breeding experiments and in testing for gene silencing in kernels. We have produced transgenic maize plants containing a wheat Glu-1Dx5 gene encoding the high molecular weight glutenin subunit 1Dx5. Analysis by SDS-PAGE showed that a protein similar in size to the wheat 1Dx5 HMW glutenin subunit accumulates in the endosperm of transgenic maize from four independent transformation events. This protein reacts with a monoclonal antibody specific to the wheat 1Dx5 HMW glutenin subunit and was not detected in nontransgenic controls or in pollen, anthers, leaves, or embryos of plants grown from kernels expressing this protein in endosperm. Genomic Southern blot analysis is consistent with results from SDS-PAGE and indicates that the transgene integration sites are complex and are different in the four events studied. Using the presence of this protein as a phenotypic marker, we studied the inheritance of this gene through three sexual generations. Reciprocal crosses with nontransgenic plants and self-pollinations were performed, and
the resulting kernels were analyzed for the presence of the 1Dx5 HMW glutenin subunit. These data, together with PCR analysis for the transgene, suggest the transgene is inefficiently transmitted through pollen in all four events. The \textit{Glu-1Dx5} transgenic maize has non-mendelian inheritance. Therefore, the expressing (hemizygous) and non-expressing (null) near isogenic kernels were produced on the same ear to study of the effect of the \textit{Glu-1Dx5} transgene expression on endogenous seed storage protein in endosperm. Environmental variation was minimized by comparing expressing and non-expressing transgenic kernels derived from the same ear. Seed weight of \textit{Glu-1Dx5} transgenic maize was not different between expressing and non-expressing transgenic plants or kernels. The 1Dx5 HMW glutenin subunit contents were 30.04 and 37.14 ng/mg in expressing endosperm of two events of transgenic maize when measured by ELISA. This accounts for 0.003 \% and 0.0037\% of the endosperm respectively. The 1Dx5 HMW glutenin subunit in endosperm of transgenic maize accounted for from 0.92\% to 3.82\% of total zeins by HPLC analysis. The level of 1Dx5 HMW glutenin subunit in transgenic maize was lower than native 1Dx5 HMW glutenin subunit in wheat endosperm. The \textit{Glu-1Dx5} transgene expression in transgenic maize endosperm affected the levels of zeins and nitrogen content in both expressing endosperm and non-expressing kernels.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

The grass genomes are evolutionarily related and show some co-linearity of gene sequences. Given this relationship, genes from one grass species could be useful for improvement of some traits in other grass species. In addition, this knowledge should create new opportunities for us to understand how genes are expressed and how gene products function by comparing them in related grass species (Bennetzen 1999). The \textit{Glu-1Dx5} gene from wheat encodes the 1Dx5 HMW glutenin subunit seed storage protein in wheat endosperm and was cloned by Anderson et al. (1989). This native wheat gene is expressed only in wheat endosperm and not in other tissues of wheat (Lamacchia et al. 2001). This gene was transformed into wheat (Blechl et al. 1996; Barro et al. 1997; Alvarez et al. 2000; Popineau et al. 2001) and into tritordeum (Rooke et al. 1999) to improve the bread-making quality of dough made from wheat flour.

The \textit{Glu-1Dx5} gene from wheat has not been transferred to maize previously, so transformation gives the opportunity to examine the function and effects of this gene in the maize. To do this, it was necessary to develop an effective method to screen or analyze endosperm-specific transgene expression in transgenic maize. This method was used to characterize the function and effects of this gene in transgenic maize. Transgenic plants of many species have been developed using several different transformation methods. The transgenes in these plants have been reported to be transmitted by mendelian inheritance in some cases and by non-mendelian inheritance in others. The
inheritance of the wheat *Glu-1Dx5* transgene in maize was studied to provide basic information for breeding programs. It is interesting to know whether or not the wheat *Glu-1Dx5* gene affects the expression of endogenous maize genes. Therefore, the effect of the *Glu-1Dx5* transgene expression on the endogenous endosperm proteins was studied.

**Dissertation Organization**

This dissertation consists of the general introduction, three papers, the general conclusion, the references cited and acknowledgments. The title of the first paper is "Serial extraction of endosperm drilling (SEED)- a method for detecting transgenes and proteins in single viable maize kernels". This paper was published in Plant Molecular Biology Reporter (2001) 19: 151-158. The second paper is entitled "Expression and inheritance of the wheat *Glu-1Dx5* gene in transgenic maize". This is currently under review for publication in Theoretical and Applied Genetics. The title of the third paper is "The effect of *Glu-1Dx5* transgene expression on maize endosperm composition in expressing and non-expressing maize kernels derived from the same F₂ transgenic maize plants". This paper will be submitted for publication in Theoretical and Applied Genetics. Each of these papers was written in a manuscript format appropriate for the target journal. The final paper is followed by a General Conclusion including recommendations of future research. A literature cited for the General Introduction and the General Conclusion follows the General Conclusion.
Literature Review

Wheat Gluten Proteins

Wheat flour can be used to make many kinds of foods; breads, cakes, biscuits, pasta and noodles. Wheat flour is unique among cereal flours because it contains gluten that has the elasticity and viscosity required for good bread-making quality (Barro et al. 1997). Gluten is a polymer that is responsible for these functional properties of wheat dough (Shewry et al. 1995). Gluten consists of more than 50 different kinds of proteins (Shewry et al. 1994). The major storage proteins in wheat grain are alcohol-soluble prolamins that account for 50 to 60% of the total protein in the endosperm (Shotwell and Larkins 1989). Gluten proteins are divided into 2 major groups, gliadins and glutenins. The gliadins are monomeric proteins that are soluble in alcohol-water solutions, and the glutenins are polymeric proteins insoluble in alcohol-water solutions. The glutenin polypeptides are joined together by disulfide bonds to form polymers. Glutenins are classified into high molecular weight (HMW) and the low molecular weight (LMW) subunits. The HMW glutenin subunits have molecular masses of about 80,000-120,000 (Kasarda 1989). Defatted wheat flour contains about 6-16% protein, including 4-7% glutenin, 2-6% gliadin and 1-2% albumins and globulins (Huebner and Wall 1976). Bread wheat cultivars that express four HMW glutenin subunit genes contain about 8% of the protein in the form of HMW glutenin subunits. Lines that have 5 HMW glutenin subunit genes expression contain about 10% of the protein in the form of HMW glutenin subunits. Each HMW glutenin subunit contributes about 2% of the total grain protein.
(Halford et al. 1992). The HMW glutenin proteins in wheat endosperm play an important role in bread-making quality (Payne et al. 1980).

*Genetics of the high molecular weight subunits of wheat*

Bread wheat, *Triticum aestivum* L., is an allohexaploid and it contains 42 chromosomes from three genomes: A, B and D. Each genome contains seven pairs of chromosomes. Chromosomes from the A, B and D genomes are said to be homeologous because they are related to each other but they do not pair in meiotic cell division (Shotwell and Larkins 1989). Payne et al. (1981) studied the electrophoretic mobility of the HMW glutenin subunits from about 185 wheat varieties. About twenty HMW glutenin subunits were identified in this study. All of the HMW glutenin subunits are encoded by genes located on the homeologous group 1 chromosomes of the wheat genomes. Each variety normally contains between 3 to 5 HMW glutenin subunits: two of the HMW glutenin subunits are controlled by chromosome 1D; one or two of HMW glutenin subunits are controlled by chromosome 1B, and zero or one of them is controlled by chromosome 1A. Payne et al. (1982) concluded that the HMW glutenin subunits of wheat endosperm are located on the long arms of chromosomes 1A, 1B and 1D. *Glu-IA*, *Glu-IB*, and *Glu-ID* are the symbols of the loci that encode the HMW glutenin subunit genes located on chromosome 1A, 1B and 1D respectively. The recombination percentage between *Glu-IA*, *Glu-IB*, *Glu-ID* and their centromers are similar (7.6, 9.2 and 10.1% respectively). The HMW glutenin subunits encoded at each locus are
subdivided into x-type and y-type according to their mobilities in electrophoresis and their isoelectric points (Payne et al. 1981). There are two genes at each Glu-1 locus. One gene encodes for a higher molecular weight x-type subunit, and a second gene encodes a lower molecular weight y-type subunit (Harberd et al. 1986). The genes at each locus are tightly linked (Lawrence and Shepherd 1981). Therefore, hexaploid bread wheat contains six different HMW subunit genes: 1Ax + 1Ay, 1Bx + 1By and 1Dx + 1Dy and between 3 and 5 of the HMW subunit genes of these six genes are expressed. In all cultivars the 1Dx, 1Dy and the 1Bx genes are expressed, and in some cultivars the 1By and/or the 1Ax genes are expressed (Payne 1987). The 1Ay glutenin subunit is absent in most cultivars. Silencing of this gene is caused by transcriptional inactivation due to a mutation 280 bps upstream of the transcription start site (Halford et al. 1989). Payne and Lawrence (1983) concluded that there are about 3 alleles at locus Glu-A1, 11 alleles at locus Glu-B1 and 5 alleles at locus Glu-ID in 300 varieties of hexaploid wheat.

Molecular Cloning of Glu-1Dx5

Anderson et al. (1989) isolated a 1Dx5 HMW glutenin subunit gene from cv. Cheyenne. The complete nucleotide sequence of 1Dx5 HMW glutenin subunit gene and derived amino acid sequence has been determined. The 1Dx5 HMW glutenin subunit gene from Cheyenne is contained on an 8.7 kb EcoR1 fragment.
Gene structure of Glu-1Dx5

The Glu-1Dx5 gene which encodes 1Dx5 HMW glutenin subunit from wheat cv. Cheyenne includes the translation start site (at position +62), the transcription start site (position +1), TATA box (at position −30), HMW enhancer at (position −186 to −149), partial HMW enhancer (position −359), N box (position −530), E box (position −685) and reverse N box (position −1045). The Glu-1Dx5 promoter controls endosperm-specific expression of the Glu-1Dx5 gene starting at 10-12 days after anthesis (Lamacchia et al. 2001). The HMW glutenin subunit genes express only during endosperm development because the enhancer elements in their promoters are responsible for endosperm-specific gene expression (Thomas et al. 1990).

The coding sequence of Glu-1Dx5 including the signal peptide and two stop codons in tandem (TGA.TAG) contains 2550 bp. The 3’ untranslated region contains a polyadenylation signals at position +2601 (AATAAA) and at position +2654 (AATAAT) (Shewry et al. 1989).

Signal peptide of 1Dx5 HMW glutenin subunit

The mature 1Dx5 HMW glutenin subunit protein is derived from a precursor with a signal peptide of 21 residues. The signal peptide of the 1Dx5 HMW glutenin subunit is MAKRLVLFVAVVVALVALTVA. The signal peptide is cleaved from the precursor to yield the mature protein (Shewry et al. 1989)
Amino acid sequence of 1Dx5 HMW glutenin subunit

Shewry et al. (1989) studied the primary structures of three x-types and three y-types of HMW glutenin subunit proteins. As a result of this comparison, all of these HMW glutenin subunits were shown to have similar structures, which can be divided into 3 domains: an N-terminal domain, a repetitive central domain and a C-terminal domain. The N-terminal domain is non-repetitive and consists of 81-104 amino acid residues. The repetitive central domain of the x-type subunits consists of three kinds of repeated motifs: tripeptide (GQQ), hexapeptide (PGQGQQ) and nonapeptide (GYYPSTSPQQ). The y-type HMW subunits contain only 2 types of repeated motifs: hexapeptide (PGQGQQ) and nanopeptide (GYYPSTSLQQ). The C-terminal domain consists of 42 amino acid residues in both x-types and y-types. The x-types usually have 3 cysteine residues in the N-terminal domain and 1 cysteine in the C-terminal domain. The y-types contain 5 cysteine residues in the N-terminal domain, 1 cysteine in the C-terminal domain and have one more cysteine in the repetitive domain.

The mature 1Dx5 HMW glutenin subunit contains 827 amino acid residues and has a predicted molecular mass of 88,128. The N-terminal domain of 1Dx5 HMW glutenin subunit has 89 amino acid residues and a predicted molecular mass of 10,087. The repetitive domain has 696 residues and a predicted molecular mass of 73,724. The C-terminal domain has 42 residues and a predicted molecular mass 4,353. The repetitive and non-repetitive domains of 1Dx5 HMW glutenin subunit are quite different in amino acid composition because the non-repetitive domains contain many charged residues; glutamate, lysine and arginine. In addition, most of the cysteine residues are in the non-
repetitive domain, with three in the N-terminal domain, and one in the C-terminal domain. The repetitive domain contains only one cysteine residue and this region is rich in glutamine and glycine and poor in charged residues (Shewry et al. 1989).

**Bread-making quality**

Wheat flour can be used to make bread products because it has visco-elastic properties derived from gluten proteins. The HMW glutenin subunits of gluten are mainly responsible for the elasticity and the gliadins provide the viscosity. The amount and type of HMW glutenin subunits in each cultivar are an important determinant of bread-making quality (Shewry et al. 1995). Gluten structure is important for wheat flour used to make bread. Upon synthesis, the HMW glutenin subunits are translocated to the endoplasmic reticulum. The gluten polymer is formed by HMW glutenin subunits that have been cross-linked by intermolecular disulfide bonds. The LMW glutenin subunits are also assembled into the polymer. Thus, glutenin has visco-elasticity derived from the complex interaction between the HMW glutenin subunits and the LMW glutenin subunits in the gluten polymer (Maizeec et al. 1994). The molecular mass of this polymer can reach several million Daltons (Shani et al. 1994). The molar ratio of LMW and HMW glutenin subunits in the gluten polymer of wheat flour is about six molecules of LMW glutenin subunits for every one HMW glutenin subunit. The HMW glutenin subunits have a greater effect on dough strength and elasticity of gluten than LMW glutenin subunits (Gupta et al. 1995). Popineau et al. (1994) concluded that qualitative and quantitative
variation of the HMW glutenin subunit composition affects the visco-elastic properties of dough by changing the glutenin polymer size distribution and aggregative character of glutenin in gluten. Gupta and MacRitchie (1994) reported that the 1Dx5-1Dy10 HMW glutenin subunits had a greater effect on dough strength than 1Dx2-1Dy12 HMW glutenin subunits. Halford et al. (1992) reported that the 1Ax1 HMW glutenin subunit is also associated with good quality of bread wheat flour.

**Genetic engineering of the HMW glutenin subunit**

The bread-making quality of wheat dough depends on the visco-elasticity of gluten. The HMW glutenin subunits are mainly important for dough elasticity. Therefore, the bread-making quality of wheat cultivars is determined in part by the amount and combination of the HMW glutenin subunits (Altpeter et al. 1996). One way to improve the bread-making quality of bread wheat dough is to increase the amount of the good HMW glutenin subunits by transforming additional HMW glutenin subunit genes into wheat (Shewry et al. 1995). HMW glutenin subunits have been used in a number of genetic engineering experiments.

Galili (1989) cloned the entire coding region of the Glu-1Dx2 gene from wheat in a bacterial expression vector and transformed *E. coli* with this vector. The mature 1Dx2 HMW glutenin subunit was produced in *E. coli*. This protein and the native 1Dx2 HMW glutenin subunit protein co-migrated in SDS-PAGE and had the same pI and solubility characteristics. Moreover, the *E coli* expressed 1Dx2 HMW glutenin protein that was purified from *E. coli* formed inter-molecular disulfide bonds like the native 1Dx2 HMW glutenin subunit in wheat endosperm.
Robert et al. (1989) transformed the HMW glutenin subunit 12 gene including its own promoter into tobacco. This transgene was transcribed to produce a 2.2 Kb mRNA and this mRNA was translated to produce the HMW glutenin subunit 12 protein in tobacco kernels. This transgenic protein and the native HMW glutenin subunit 12 protein exhibited the same characteristics in molecular weight and solubility. The HMW glutenin subunit 12 protein from tobacco did not show signs of protein degradation. This protein accounted for about 0.1% of the total protein in tobacco endosperm.

Altpeter et al. (1996) transformed the entire Glu-1Axl gene that is correlated with good bread-making quality into the Bob White cultivar, which lacks the native Glu-1Axl gene. From 21 transformed lines, 9 lines expressed the 1Axl HMW glutenin subunit from the transgene. The 1Axl HMW glutenin subunit levels in the transgenic lines varied from 0.6% to 2.3% of total protein, and it contributes to 71% of the total HMW subunits. Expression of this transgene was stable and the transgene was transmitted to the R₃ kernel generation. The segregation of transgene followed mendelian laws.

Blechl et al. (1996) transformed a fusion of the Glu-IDy10 and Glu-IDx5 genes into wheat. In this fusion, Glu-IDy10 provides the promoter, 5’ untranslated region and the first 145 codons of coding region. Glu-IDx5 provides codons 130–848 and 3’ untranslated region. The expression of the hybrid HMW glutenin subunit in transgenic wheat was stable through three generations.

Barro et al. (1997) reported that the Glu-1Axl and/or Glu-1Dx5 genes were transformed into bread wheat to increase the amount of the HMW glutenin subunits to improve bread-making quality. The transgenic HMW glutenin subunits in some lines
accumulated to higher levels than the endogenous HMW glutenin subunits. The expressing kernels were analyzed for bread-making quality. The results showed that introducing transgenic 1Ax1 and 1Ax5 HMW glutenin subunits results in an increase in dough strength.

Rooke et al. (1999) reported that the bread-making quality of tritordeum was improved by increasing the amount of HMW glutenin subunits using particle bombardment to transform tritordeum with Glu-1Ax1 and Glu-1Dx5 genes from wheat. The bread-making quality of T2 transgenic kernels was evaluated using a small-scale mixograph. The lines that expressed the Glu-1Dx5 gene showed significant improvements in dough strength and stability.

Alvarez et al. (2000) reported that the Glu-1Ax1 and Glu-1Dx5 genes from wheat were transformed into wheat. Over-expression of the Glu-1Dx5 transgene did not change the expression of endogenous proteins in transgenic wheat. SDS-PAGE analysis showed that a new protein that is of lower molecular weight than the native 1Dx5 HMW glutenin subunit accumulates.

Popineau et al. (2001) reported that over expression of the 1Dx5 HMW glutenin subunit from a transgene affected the glutenin aggregation more than expression of the 1Ax1 HMW glutenin subunit from a transgene. Wheat flour containing the 1Dx5 HMW glutenin subunit expressed from a transgene has high strength and causes abnormal dough mixing properties such as the lack of a peak of torque.
Maize products

Maize can be used to make many products, such as: starch, dextrins, maize syrup, dextrose, maize-sugar, steepwater products, steepwater for feed, maize oil meal, maize gluten feed, maize gluten meal, maize oil and maize oil free fatty acids (Corn Industries Research foundation 1937). Modified zein films derived from evaporated ethanol extracts can be used in the food and drug industries for such uses as to coat medical pills so that the medicine will be released slowly in the stomach (Payne and Rhodes 1982). Ethanol is a clean fuel that is safe for the environment. Ethanol is made from starch-based dextrose that is derived from maize (Erdmann 1995).

Maize kernel composition

Maize endosperm cells are triploid, and these cells accumulate starch and protein. The starch and proteins are broken down into sugars and amino acids that are used by the seedling in early developmental stages following germination (Soave and Salamini 1984). Maize starch contains 24% amylose and 76% amylopectin. Starch is stored in amyloplasts (Badenhuizen 1969). Kernel storage proteins are classified into four groups by solubility: albumin, globulin, glutelin and prolamin (Osborne 1924). The major maize kernel proteins are extracted by alcohol solutions and are, therefore, classified into the prolamin group. The prolamins of maize are called zeins. Zeins account for about 50 percent of the total protein in maize kernels (Shotwell and Larkins 1989). Relative to the nutritional requirements of animals, zeins are deficient in the essential amino acids lysine,
tryptophan and methionine. The high percentage of zeins in maize kernels causes this
deficiency to be reflected in the amino acid composition of the grain. (Kirihara et al.
1988a).

**Classification of zeins**

Zeins are classified into four groups based on their structure and solubility. The
first one is alpha (α) zein, in which composed of M_r 22-kD and the M_r 19-kD proteins.
The second class is beta (β) zein composed of a M_r 14-kD protein. The third one is
gamma (γ) zein composed of M_r 27-kD and M_r 16-kD proteins, and the fourth one is delta
(δ) zein, a M_r 10-kD protein (Thompson and Larkins 1994). Alpha, beta and gamma
zeins account for 80%, 15% and 5-10%, respectively, of total zeins (Esen 1986).

Zeins have been also classified into two subclasses based on their solubility in
alcohol. The first subclass is zein-1 that is soluble in alcohol without reducing agent. The
second subclass is zein-2 that is soluble in alcohol with reducing agent. Zein-1 is
separated into the M_r 19-kD and M_r 22-kD classes. The zein-2 fraction can be resolved by
SDS gel electrophoresis into M_r 10-kD, 15-kD, 16-kD, and 27-kD proteins (Gianazza et
al. 1977).

**Zein synthesis and storage**

Zeins are synthesized specifically in endosperm between 10 to 45 days after
pollination during endosperm development (Lee et al. 1976). Zeins are synthesized on
polysomes, translocated to the lumen of endoplasmic reticulum and deposited into protein bodies. (Burr and Burr 1976; Lending and Larkins 1989). Immunolocalization studies have shown that the alpha zeins are located in the center of protein bodies and the beta and gamma zeins are located at the periphery of protein bodies (Lending et al. 1988; Lending and Larkins 1989).

**Genetics and primary structure of zeins**

**Alpha zeins (19- and 22-kD)**

\(M, 19\text{-kD} \text{ and } M, 22\text{-kD} \text{ zeins (zein-1)}\) are encoded by a large multigene family. 70-80 zein sequences in this multigene family were detected by Southern blot analysis (Wilson and Larkins 1984). The genes of the zein-1 family are located on chromosomes 10, 7 and 4 in the maize genome (Soave et al. 1981; Soave et al. 1982; Valentini et al. 1979).

Thompson et al. (1992) have cloned two 22-kD zein genes that are closely linked. One gene is functional and the other gene is not. The open reading frame of functional clone gZ22.8 contains 801 bp and does not contain introns. The coding region of this gene encodes a 22-kD zein polypeptide of 266 amino acids including 21 amino acid residues encoding a signal peptide. The regulatory region of this gene contains TATA box located at 32 to 35 nucleotides from the 5' end of the transcription initiation site. There are two putative CCAAT boxes in the regulatory region located at 21 bp and 91 bp
from TATA box. The clone contains two consensus polyadenylation signals in the 3’ flanking region.

Pedersen et al. (1982) reported that the nucleotide sequence of one 19-kD zein gene encodes 235 amino acids, including a signal peptide of 21 amino acids. There are no introns in the coding region. This gene is a member of a multigene family.

Immature zein-1 proteins can be divided into 4 regions. Region 1 contains the signal peptide of about 21 amino acids. Region 2 contains the amino acid terminus of about 36-37 amino acid residues. Region 3 contains 9-10 tandem repeats of a 20 amino acid motif. Region 4 contains a carboxyl terminus of about 10 amino acids (Messing et al. 1983).

Marks et al. (1985) reported that when the polypeptides from among 22-kD zein clones and among 19-kD zein clones were compared, there was 92% homology among 22- kD zein clones and about 75-95% homology among 19- kD zein clones. When polypeptides from 22-kD zein clones were compared to polypeptides from 19-kD zein clones, there is 60-65% homology.

**Beta zeins (15-kD)**

Wilson and Larkins (1984) reported that about 2 or 3 genes per haploid genome encode 15-kD zeins. Pedersen et al. (1986) reported that the 15-kD zein gene encodes a sulfur-rich zein protein and it contains 180 amino acids including a signal sequence of 20 amino acids. This protein contains 18 methionine residues that are separated into three clusters. This means that the 15-kD zein contains about 11% methionine (Kirihara et al.
It also contains 7 cysteine residues. There is no intron in the coding region of this gene. The polypeptides encoded by the 15-kD zein clones do not have homology to the polypeptides encoded by 22-kD zein and 19-kD zein clones (Mark et al. 1985; Pedersen et al. 1986).

**Gamma zeins (27- and 16-kD)**

Wang and Esen (1986) concluded from isoelectric focusing experiments that the 27-kD zein is unlikely to be encoded by multigene family. Das et al. (1991) reported that the maize inbred lines A188 and W22 contain two 27-kD zein genes that are located within tandem duplications of 12 kb. The maize inbred line W 64A contains only one 27-kD zein gene. Reina et al. (1990a) reported that the Zc2 gene encodes a 28-kD zein protein. The coding region of this gene contains 671 bp and no introns are found in the coding region. Wang and Esen (1986) reported that the γ-zein contains 204 amino acid residues. The predicted molecular weight of this protein is about 27-kD. The amino terminal domain of this protein contains eight identical hexapeptide repeats of Pro-Pro-Pro-Val-His-Leu and two repeats of the octapeptide Gln-Pro-His-Pro-Cys-Pro-Cys-Gln. This zein is rich in cysteine.

Reina et al. (1990b) reported that Zc1 gene encodes a 16-kD zein protein. The coding region contains 551 bp and there are no introns in this gene. Part et al. (1987) reported that the 16-kD zein gene has sequence homology to the 28-kD zein gene. The 16-kD and 28-kD zein genes are expressed at the same developmental stage, between 15 and 40 days after pollination.
**Delta zein (10-kD)**

Benner et al. (1989) reported that the structural gene of the M, 10-kD zein is located on chromosome 9 and there are only one or two copies as estimated by southern blot analysis. A gene that regulates the expression of this gene is located on chromosome 4. Kirihara et al. (1988a) reported that the maize genome contains one or two of 10-kD zein genes. The coding region of 10-kD zein gene contains 449 bp. This gene contains no introns. The promoter of this gene contains a TATA box but no CAAT or AGGA boxes. There is a 15 bp repeated sequence located at -330, and this sequence is conserved in all of the zein genes. There are two polyadenylation signals in 3' region of this gene. The predicted polypeptide contains 129 amino acids, preceded by 21 amino acids of signal sequence. The 10-kD zein polypeptide is unique because it has an extremely high methionine content (22.5%).

**Gene mutations effect zeins**

There are at least three genes with mutant alleles that cause changes in the amount of the storage proteins in maize: opaque-2 (o2), opaque-7 (o7), and floury-2 (fl2). These mutant genes affect the amount of zeins in maize endosperm (Burr and Burr 1982).

The *Opaque-2* (*O2*) wildtype gene encodes a bZIP transcription factor that regulates transcription of most of the zein genes (Schmidt at al. 1992). The *opaque-2* mutant (*o2*) is a recessive gene. In maize with the *o2* genotype, synthesis of zeins is
decreased when compared to wild type (Gianazza et al. 1976). This decrease is due in part to the fact that the onset of synthesis of zeins is delayed for several days compared to the normal genotype. The amount of zeins is reduced about 50% relative to the wild-type genotype. The 22-kD zein is rarely detected in o2 maize (Pedersen et al. 1980) because O2 is a transcription factor that controls endosperm-specific expression of the 22-kD zein genes (Lohmer et al. 1991). The nutritional quality of the normal maize kernel is low in part because of low lysine content. In the opaque-2 mutant, the lysine content is increased significantly because zein synthesis is reduced, coupled with an increased synthesis of other proteins that are relatively high in lysine, such as elongation factor 1 alpha (eEF 1A) (Wang et al. 2001). Thus, opaque-2 mutant contains a higher amount of albumins and globulins but a lower amount of zeins than the wild type genotype (Dierks-Ventling 1981). The opaque-2 mutant has a higher lysine and tryptophan level than normal corn but is poor in yield, endosperm hardness and disease resistance. Therefore, it has not been accepted for commercial production. The International Maize and Wheat improvement Center (CIMMYT) has improved the opaque-2 phenotype by introducing modifier genes from many varieties of maize germplasms to improve kernel quality and agronomic performance while still maintaining high lysine and tryptophan levels. These new maize varieties are called Quality Protein Maize (QPM). The soft, floury endosperm texture of the opaque-2 phenotype is changed to a hard and vitreous endosperm texture by the opaque-2 modifier genes. The activity of the opaque2-modifier genes results in increasing gamma zein gene expression by two- to threefold relative to unmodified opaque-2 maize
varieties (Geetha et al. 1991). The vitreous hard endosperm in QPM is correlated with high expression of gamma zein (the 27-kD zein) (Wallace et al. 1990).

We would like to make maize with novel flour property. The *Glu-1Dx5* gene encoding for 1Dx5 HMW glutenin subunit that contributes elasticity to wheat dough was transformed into maize. We developed the methods to detected the transgene and its expression, studied inheritance of this transgene, and studied the effect of this transgene on zeins, major seed storage protein in maize. We will study the effect of 1Dx5 HMW glutenin subunit on maize flour property.
CHAPTER 2. SERIAL EXTRACTION OF ENDOSPERM DRILLINGS
(SEED)-A METHOD FOR DETECTING TRANSGENES AND PROTEINS IN
SINGLE VIALBE MAIZE KERNELS


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Abstract

We have developed a method for detecting a transgene and its protein product in
maize endosperm that allows the kernel to be germinated after analysis. This method
should have broad utility in several monocots and dicots. Our method involves first
sampling the endosperm with a hand-held rotary grinder so that the embryo is preserved
and capable of germination. This tissue is then serially extracted, first with SDS-PAGE
sample buffer to extract proteins, then with an aqueous buffer to extract DNA. The
product of the transgene can be detected in the first extract by SDS-PAGE with
visualization by total protein staining or immuno-blot detection. The second extract can
be purified and used as template DNA in PCR reactions to detect the transgene. This
method is particularly useful for screening transgenic kernels in breeding experiments and to test for gene silencing in kernels.

Keywords: ELISA, gene expression, PCR, transgene, *Zea mays* L.

**Introduction**

Because of recent advances in plant transformation research, transgenic crops are being incorporated into many breeding and research programs. A major difficulty with working with transgenic plants is gene silencing (Kumpatla et al., 1998). While transgenes are often inherited in a predictable manner, the expression of the transgene often varies in different lines due to position effects or epigenetic effects. Thus, it is necessary to monitor the inheritance and the expression of the transgene each generation to verify that the transgene functions properly in the conditions tested. Inexpensive, high-throughput methods are needed to meet the demand of breeding programs for screening large numbers of plants.

We have developed a method that allows detection of transgenes and their protein products in the endosperm of individual maize kernels. The embryo is not damaged so that the kernels can be analyzed, stored, and subsequently germinated. Nonlethal sampling methods that retain kernel viability are frequently used to screen kernel traits. Our method couples nonlethal sampling with serial extraction of the excised tissue to obtain extracts suitable for several types of analyses. Serial extraction was one of the first methods used to separate and classify kernel components (Osborne, 1924) and is
based on the differential solubility of the components being separated. Coupled with microsampling and analysis methods, serial extraction is a useful tool in molecular breeding.

Protocol

The protocol is outlined schematically in Figure 1. All liquid handling steps are carried out in 96-well format, which lends itself to automation for high-throughput. All chemicals are molecular biology grade or equivalent. The protocol presented here is optimized for analysis of the wheat Glu-IDx5 transgene in maize. The parameters of SDS-PAGE, ELISA, PCR, and immuno-blotting will require optimization for detection of other transgenes and proteins.

Grinding apparatus

Maize kernels are ground using a hand-held rotary grinder (Sears Craftsman variable speed rotary tool, model #61053, Sears Roebuck and Co., Chicago, Illinois, USA) with a flexible shaft attachment. If contamination with maternal tissue is a problem, a 3 mm² patch of pericarp can be removed with sand paper prior to grinding. The kernels are then placed on a small piece of weighing paper and kept in place by the operator's thumbnail. The kernels are held embryo side down with the pedicel underneath the thumb. The rotary grinder is used at approximately 50% full speed with a
#105 Dremel grinding bit. The endosperm is carefully ground to avoid damaging the embryo or removing too much of the endosperm. Typical yields range from 10 to 20 mg of finely ground endosperm. This powder is transferred to a 1.5 ml round-bottom 96-well plate using a small glass funnel. Drill bits, funnels, and fingers are thoroughly washed between samples to avoid contamination. A fresh sheet of weigh paper is used for each kernel. To increase the efficiency of the process, several funnels and drill bits are used so that they do not have to be washed between every kernel.

Protein extraction for SDS-PAGE and immuno-blot analysis

Protein extraction buffer (0.0625N Tris-HCl, pH 6.8, 3.3% (W/V) SDS, 5% (v/v) 2-mercaptoethanol, 10% glycerol, 0.002% Bromphenol blue) is added to preweighed samples in a ratio of 10 to 1 μl/mg. One hundred to two hundred microliters of protein extraction buffer are added to 10-20 mg of ground endosperm in the ratio of 10 μl of buffer per 1 mg of ground endosperm. The 96-well plate is mixed for 10 minutes using a vortex mixer followed by 2 hours of shaking at 1400 RPM at room temperature. The 96-well plate is then centrifuged for 5 minutes at 4000 × g, and the supernatants are transferred to a new 96-well plate. These extracts are heated to 95 °C for 5 minutes and stored at -20 °C prior to analysis. The extracted endosperm pellet is used for DNA extraction.
**SDS-PAGE**

Extracted proteins are separated by electrophoresis on a 12% SDS-PAGE (Acrylamide/bis 37.5:1) gel (Laemmli, 1970) using a Mini-Protean II Electrophoresis Cell from BIO-RAD with a 20-well comb. Five microliters of protein extract is loaded in each well. Electrophoresis is carried out at 100 volts for 1.5 hours. Gels are stained with 0.1% Coomassie Blue-R-250 in 1% acetic acid and 40% ethanol for 0.5 hours and destained with 40% ethanol and 10% acetic acid for 1-3 hours.

**Immuno-blot analysis**

Immuno-blot detection is carried out using a Bio-Rad Trans-Blot apparatus according to the manufacturer's directions. Proteins are detected using a monoclonal antibody that specifically binds to the transgene product.

**DNA extraction**

DNA is extracted and purified from the endosperm pellet remaining in the 96-well plate after extraction of the proteins for SDS-PAGE. The DNA is extracted in 300 μl of cell lysis buffer from the Puregene DNA purification kit (Gentra), and purification is carried out using the Puregene protocol according to the manufacturer's directions. Since the purification is carried out in microplate format, centrifugation is conducted at 4000 x g for 5 minutes in a swinging bucket microplate rotor. DNA resulting from this
procedure is resuspended in 100 μl of 1 mM Tris HCl, pH 8.0, 0.1 mM EDTA. The DNA is allowed to resuspend by first heating to 65 °C for 60 minutes, then shaking at 300 RPM and 50 °C overnight in a shaking incubator.

**Polymerase chain reaction**

The transgene is detected by PCR using 5 μl of the purified DNA as template and primers specific to the transgene. A touchdown PCR protocol is used (Senior and Huen, 1993). Products were separated on 2% Metaphor (FMC Bioproducts) agarose gels and visualized by UV fluorescence of the ethidium bromide-stained DNA.

**Alternative protocol for ELISA analysis**

An alternative protocol can be used to detect the protein by ELISA rather than immuno-blot analysis. The protein extraction procedure for ELISA analysis is the same as that used for SDS-PAGE and immuno-blot analysis, except that our transgene product requires a different protein extraction buffer (70% ethanol, 61 mM Sodium Acetate, and add 5% (v/v) 2-mercaptoethanol before use), and 20-30 mg of endosperm are extracted in a ratio of 10 μl to 1 mg. The resulting extract can be analyzed by SDS-PAGE and immuno-blot detection as well. The extracted endosperm pellet was dried overnight at 4°C and then used for DNA extraction.
ELISA analysis is conducted according to standard protocols (Harlow and Lane, 1999). Standards of purified transgene product were included in a range of 5 to 120 ng/50 ul.

**Kernel germination**

Because the sampling process damages the pericarp, we took precautions to protect the kernels from fungal infection. Prior to planting in sterile soil, the kernels were treated with Chlorothalonil (tetrachloroisophthalonitrile 0.087%, Fung-Onil, Earl May). Kernels were germinated in 3” peat pots in the greenhouse and transplanted to the field.

**Results**

We have applied this method to analyze over 2000 kernels from populations of maize segregating for a transgene from wheat, the HMW-Glutelin Glu-IDx5 gene (Anderson et al., 1989). In wheat, expression of the glutenins is normally tissue specific (Shewry, 1995) with expression confined to the endosperm. We anticipated a similar expression pattern in maize given the genetic similarity of these organisms (Bennetzen and Freeling, 1993).
Protein analysis

In order to determine if the transgene was functional in maize endosperm, we tested for the accumulation of the protein it encoded using SDS-PAGE (Laemmli, 1970). This method is sensitive enough that the drillings from a single mature kernel (about 20 mg) can be analyzed. The transgene product was sufficiently abundant to be visible by Coomassie Brilliant Blue staining (Figure 2A). In order to confirm that the bands in the Coomassie-stained gel were the product of the transgene, we ran duplicate gels and visualized the one gel using immuno-blots detection (Figure 2B) using a monoclonal antibody specific to wheat high molecular weight glutenin subunits. A band in the position of the prominent Coomassie-stained band was detected by this method, as well as a weaker band just below it. This lower band may be a product of proteolysis.

The variation of the protocol utilizing ELISA analysis to detect the transgene product is valuable because it yields a quantitative measure of the target protein (Table 1). Using ELISA, we were able to detect the transgene product in kernels that were scored negative by SDS-PAGE (for example, Table 1, sample 5).

Genotype analysis

A common goal is to develop transgenic lines that are homozygous for the transgene. As we advance plants in this breeding program, there are at least two reasons why a kernel may not express the transgene. First, it may not have inherited the gene, and second, the gene may have been silenced. To distinguish between these two possibilities,
it was necessary to determine if the transgene was present in the kernels lacking expression of the transgene as measured by SDS-PAGE. After extraction for protein analysis, the kernel drillings contained enough DNA to extract and use for PCR analysis. We found that all kernels expressing the protein were positive in the PCR analysis, but some kernels that did not contain the protein contained the gene, while others did not. We concluded that kernels lacking both the gene and the protein did not inherit the transgene, while in kernels containing the gene without detectable protein the transgene was silenced. Caution must be used when interpreting PCR results, because the endosperm tissue will normally be contaminated with small amounts of pericarp tissue, which could contain the transgene when the corresponding endosperm does not. Because pericarp tissue is present in very small quantities, it should be possible to adjust the PCR conditions to distinguish between bands resulting from endosperm DNA and those resulting from pericarp DNA.

**Germination of analyzed kernels**

The sampled kernels were planted in sterile soil in a greenhouse prior to transplanting to the field. Of 236 kernels planted, 191 (81%) germinated. Our normal germination rate is about 90%, so we conclude that our sampling procedure has a minor impact, if any, on the ability of sampled kernels to germinate in carefully controlled conditions.
Discussion

In most high-throughput kernel analysis strategies, grinding the kernel requires a substantial amount of labor. The serial extraction strategy employed by this method minimizes the grinding requirement by using the same ground material for several types of analysis. This method will be particularly useful for the analysis of transgenes targeting cereal kernel quality traits, which often direct expression specifically in the endosperm. The method is applicable to any transgene containing a unique DNA sequence and encoding a product that is detectable in kernels by ELISA, immunoblotting or total protein staining of SDS-PAGE. ELISA has the advantages of being quantitative and the throughput is higher than with immuno-blot analysis. Nontarget proteins that react with the antibody can interfere in this type of assay, so it is important to verify the specificity of the antibody. The throughput of the method is sufficiently high to be useful in a breeding program. One person can analyze 192 kernels in 4-5 days (two 96-well plates). Grinding the tissue takes the majority of the time.

Nondestructive kernel sampling methods have been in use for many years with several plant species because they are rapid, and planting decisions can be based on the results of the analysis. This method includes these advantages, and in addition, the serial extraction allows it to give information both about the inheritance and the expression of the transgene. This is particularly useful in situations where gene silencing is common.
We have tested the method with maize; however, it should be applicable to other cereals.

It would be interesting to determine if this method could be applied to large-kernelled dicots by sampling the cotyledons in place of the endosperm.

Acknowledgments

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References


Figure 1. Schematic diagram of the SEED method.
Figure 2A: Characterization of proteins from endosperm drillings by SDS-PAGE with Coomassie Brilliant Blue staining. Lane 1: nontransgenic maize kernel. Lanes 2-11: transgenic maize kernels. Lane 12: wheat variety L88-6, which contains the 1Dx5 subunit. The same endosperm drillings were used to make Figures 2B and 2C. 2B. Immuno-blot detection of SDS-PAGE-separated proteins. Lanes are labeled as in Figure 2A. 2C. Detection of the transgene by PCR, agarose gel electrophoresis, and ethidium bromide staining. Lanes are labeled as in Figures 2A and 2B, except Lane 12 contains plasmid DNA of the construct used in transformation. DNA was purified from the same endosperm drillings used to make Figures 2A and 2B.
Table 1. Analysis of segregating F$_4$ kernels using ELISA in the kernel protocol.*

<table>
<thead>
<tr>
<th>Samples</th>
<th>lDx5 concentration (ng/mg kernel ± SD)$^b$</th>
<th>SDS-PAGE</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-14.5 ± 3.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>50.3 ± 7.3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>-9.3 ± 3.6</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>55.5 ± 7.3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>6.2 ± 3.6</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>42.5 ± 3.6</td>
<td>+</td>
<td>+</td>
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<tr>
<td>7</td>
<td>55.5 ± 7.3</td>
<td>+</td>
<td>+</td>
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<td>8</td>
<td>-9.3 ± 3.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-11.95 ± 0.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-9.3 ± 3.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>-9.3 ± 3.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>-9.3 ± 3.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>55.5 ± 7.3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>-6.7 ± 0.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15 nontransgenic maize</td>
<td>-9.3 ± 3.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16 wheat</td>
<td>50.3 ± 7.3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17 plasmid</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*These samples are different from those used in Figure 2.

$^b$We interpret negative values to indicate no detectable expression.
CHAPTER 3. EXPRESSION AND INHERITANCE OF THE WHEAT Glu-1Dx5 GENE IN TRANSGENIC MAIZE

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Abstract

We have produced transgenic maize plants containing a wheat Glu-1Dx5 gene encoding the high molecular weight glutenin subunit 1Dx5. Analysis by SDS-PAGE showed that a protein similar in size to the wheat 1Dx5 subunit accumulates in the endosperm of transgenic maize from four independent transformation events. This protein reacts with a monoclonal antibody specific to the wheat 1Dx5 subunit and was not detected in nontransgenic controls or in pollen, anthers, leaves, or embryos of plants grown from kernels expressing this protein in endosperm. Genomic Southern blot analysis is consistent with results from SDS-PAGE and indicates that the transgene
integration sites are complex and are different in the four events studied. Using the presence of this protein as a phenotypic marker, we studied the inheritance of this gene through three sexual generations. Reciprocal crosses with nontransgenic plants and self-pollinations were performed, and the resulting kernels were analyzed for the presence of the 1Dx5 subunit. These data, together with PCR analysis for the transgene, suggest the transgene is inefficiently transmitted through pollen in all four events.

Key words: Zea mays L., transgenic plants, HMW glutenin, gene expression

Introduction

Wheat flour is different from other cereal flours, including maize, because it contains gluten that gives it the elasticity and extensity required for bread-making (Barro et al. 1997). Gluten consists mainly of two types of kernel storage proteins, the glutenins and the gliadins. Glutenins are classified into high molecular weight (HMW) subunits and low molecular weight (LMW) subunits. Although the HMW glutenins contribute only about 5% of the total protein in mature wheat kernels (Shewry et al. 1989), the elasticity of wheat dough depends mainly on the HMW glutenins, so they determine bread-making quality (Payne et al. 1979, 1981). Payne et al. (1987) reported that most good bread wheat cultivars contain the 1Dx5-1Dy10 HMW glutenin combination. This suggests that bread-making quality is associated with the presence of these combinations of HMW glutenins.
Genes encoding several HMW glutenins have been cloned. Anderson et al. (1989) isolated a Glu-1Dx5 gene from cv. Cheyenne contained on an 8.7 kb EcoRI fragment, and determined that it encodes a protein of 88,128 Da with 827 amino acid residues. The availability of cloned HMW glutenin genes allows plant transformation approaches to altering HMW glutenin content.

Cloned HMW glutenin genes have been shown to be functional when introduced into E. coli (Galili et al. 1989), tobacco (Roberts et al. 1989), wheat (Blechl and Anderson, 1996, Altpeter et al. 1996; Barro et al. 1997; Alvarez et al. 2000) and triordium (Rooke et al. 1999). Detailed inheritance studies of these transgenes have not been reported, although it has been reported that the transgene is stable through three generations in wheat (Altpeter et al. 1996).

Dough from maize flour lacks extensibility and elasticity. A probable cause for this is that maize endosperm lacks the proteins responsible for this trait. Because the HMW glutenins have a large impact on dough quality in wheat, we set out to determine if a wheat HMW glutenin gene could be used to develop maize with novel dough characteristics. Our goals were to determine if this gene is expressed in maize, to examine tissue specificity of expression, and to characterize the inheritance of this gene in transgenic maize.
Materials and method

Plasmids

Two plasmids were used for co-transformation of maize. The first plasmid, named pHMW1Dx5, is derived from pUC9 and contains an 8.7 kb EcoRI genomic DNA fragment from hexaploid bread wheat that includes the 1Dx5 coding sequence (Anderson et al. 1989), 3.2 kb of 5' flanking sequence and 1.2 kb of 3' flanking sequence (Halford et al. 1992). The construct pBAR184 consists of the maize ubiquitin promoter, first exon, and first intron, which drives the Streptomyces hygroscopicus bar gene with the Agrobacterium tumefaciens nos terminator (Frame et al., 2000).

Plant transformation

Plant transformation was carried out at the Iowa State University Plant Transformation Facility using their standard method (Frame et al., 2000). Briefly, embryogenic callus from the genotype Hi-II was co-bombarded with the plasmids pBAR184 and pHMW1Dx5. Bialaphos-resistant calli were screened by polymerase chain reaction (PCR) for the presence of the 1Dx5 coding sequence. Callus lines containing this sequence were regenerated to produce T0 plants.

Plant growth and maintenance

The F1 generation was produced from a cross between a T0 plant and the inbred line B73. Throughout this manuscript, the designation “F1” refers to material produced in
this way. The F₁ and F₃ generations were produced in a greenhouse with the temperature maintained at 26 °C during the day and 22 °C at night. Artificial light was used for 16 hours each day. Kernels were planted in 2-gallon pots prepared as follows. Pots were filled half-full with Universal Mix soil (Consumers Supply Corp. IA) mixed with about 5 g of Sierra 17-6-12 controlled-release fertilizer (Hummert International, MO, Cat#07-6375-1). Universal Mix soil was then added to fill the pot to 80 % of its capacity. Because the sampling process damages the pericarp, the kernels were treated with Chlorothalonil (tetrachloroisophthalonitrile 0.087%, Fung-Onil, Earl May) before planting. All other plants were grown in the field during the summer of 1999 at the Iowa State University Agronomy Farm in Boone, Iowa. Kernels were treated with Chlorothalonil and germinated in 3” peat pots containing Universal Mix soil (Consumers Supply Corp. IA), in a greenhouse. Two weeks after planting, the seedlings were transplanted to the field.

**SDS-PAGE**

Endosperm material from individual maize kernels was collected using a hand-held rotary grinder (Dremel) as described (Sangtong et al. 2001). Extracted proteins were separated by electrophoresis on a 12% SDS-PAGE (Acrylamide/bis 37.5:1) gel (Laemmli 1970) in the presence of β-mercaptoethanol using a Mini-Protean II Electrophoresis Cell from BIO-RAD with a 20-well comb. Five microliters of protein extract was loaded in each well, and electrophoresis was carried out at 150 volts for 1.5 hours. Gels were
stained with 0.1% coomassie blue-R-250 in 1% acetic acid and 40% ethanol for 0.5 hours and destained with 40% ethanol and 10% acetic acid.

**Immuno-blot analysis**

Immuno-blot detection was carried out using a Bio-Rad Trans-Blot apparatus according to the manufacturer’s directions. Proteins were detected using a monoclonal antibody that specifically binds to the N-terminal end of the repetitive regions of the 1Dx and 1Ax HMW glutenins (Barro et al. 1997).

**Polymerase chain reaction**

DNA was extracted and purified from the endosperm pellet remaining in the 96-well plate after extraction of the proteins for SDS-PAGE (Sangtong et al. 2001). Leaf DNA was prepared using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). PCR was carried out using 5 μl of the purified DNA as template and primers specific to the 1Dx5 high molecular-weight glutenin subunit (1Dx5F: 5’- gagatcagcatggctagggtagc-3’ and 1Dx5R: 5’- tgctgcggacaagttacact-3’) via a touchdown PCR protocol (Senior and Heum, 1993) (the amplified fragment is labeled “probe” in Figure 1). Products were separated on 2% Metaphor (FMC Bioproducts) agarose gels and visualized by UV fluorescence of the ethidium bromide-stained DNA.
**DNA isolation and Southern blot analysis**

Genomic DNA was extracted from leaves and analyzed by Southern hybridization using protocols of Veldboom et al. 1994. Blots were probed with the 362 bp PCR product corresponding to a portion of the 1Dx5 coding sequence that was used for PCR analysis (Figure 1).

**Results**

*The wheat Glu-1Dx5 gene is functional in transgenic maize*

The first step in determining the feasibility of using the wheat HMW glutenin gene in maize was to develop transgenic plants containing this gene and to determine if the gene is functional. We used a particle bombardment protocol (Frame et al. 2000) in which a plasmid containing a selectable marker, *bar*, is co-bombarded with a plasmid containing the wheat glutenin gene. The glutenin expression construct contains a wheat genomic DNA fragment including the 1Dx5 coding sequence and flanking sequences (Figure 1). Embryogenic callus derived from the genotype Hi-II was used as a substrate for bombardment, and herbicide resistance and presence of the *Glu-1Dx5* gene were used as the criteria to select callus for regeneration to *T*₀ plants (Frame et al. 2000). The *T*₀ plants from 12 independent events were crossed with B73 to produce 54 *F*₁ families. Most events yielded several *T*₀ plants that are likely to be clonally related, and each *F*₁ family is derived from a cross of a different *T*₀ plant with B73, so therefore there are several *F*₁ families from most events (Table 1). The endosperms of the *F*₁ kernels
resulting from these crosses were analyzed by SDS-PAGE with coomassie blue staining and immuno-blot analysis to detect the 1Dx5 HMW glutenin. Endosperm extracts of some F₁ transgenic kernels contained a protein that migrated near the 116 kD marker in SDS-PAGE in reducing conditions (Figure 2, Lanes 2-4). This is different that the 88 kD predicted from the cDNA sequence, but HMW glutenins are known to exhibit anomalous migration in SDS-PAGE (Galili, 1989). Western blot analysis with a monoclonal antibody specific to the N-terminus of the 1Dx5 HMW glutenin demonstrated that this protein was immunologically related to the 1Dx5 HMW glutenin (Figure 2). When protein extracts from nontransgenic endosperm were analyzed by the same methods, no protein is detected in the same position by SDS-PAGE (Figure 2A, lane 5), and there was no detectable immunological reaction with the 1Dx5 monoclonal antibody in western blot analysis (Figure 2B, lane 5). The immuno reactive bands in maize migrate closely with bands from wheat endosperm that react with the 1Dx5 monoclonal antibody as well (Figure 2A and 2B, lane 1). The larger of the two bands in wheat can be explained by cross reaction of the monoclonal antibody with the 1A HMW glutenin subunits (Mills et al, 2000). In maize, the two bands on the immuno-blot could result from partial degradation of the protein. Interestingly, the bands from maize do not line up precisely with the bands from wheat, suggesting that the protein may undergo slightly different processing in maize than in wheat. These data strongly suggest that the band in the transgenic maize is the product of the wheat Glu-1Dx5 transgene. In the subsequent analyses presented here, we used the presence or absence of this band to indicate the phenotype of the transgenic plants, positive or negative, respectively.
Inheritance of the **Glu-1Dx5** gene in the \( F_1 \) kernels from \( T_0 \) transgenic plants

About 3000 \( F_1 \) kernels of 54 \( F_1 \) families representing 12 independent transformation events were produced. SDS-PAGE was used to assess 1Dx5 HMW glutenin expression on five kernels from each \( F_1 \) family. By analyzing five kernels, there is a 95% probability that at least one positive kernel will be selected if 50% of the kernels in the family are positive. This would reflect the 1:1 genotypic ratio expected from this cross if **Glu-1Dx5** were inherited as a single dominant gene. Only four events (097, 144, 182, and 190) showed at least one positive kernel. Of the 15 \( F_1 \) families in these four events, 13 families had at least one positive kernel in the five kernels screened (Table 1).

In order to characterize the inheritance of the transgene in the 15 \( F_1 \) families from the four expressing events, the phenotypic segregation ratio of each family was determined by analyzing the remaining kernels using SDS-PAGE (Table 2). Chi-squared tests were performed to determine if the segregation ratios obtained for each \( F_1 \) family were consistent with a 1:1 segregation model. In each event, families with segregation ratios consistent with a 1:1 segregation model were identified. Nine \( F_1 \) families fit a 1:1 segregation model. In the six families that did not fit a 1:1 segregation model, an excess of negative progeny was observed. Only one family did not contain any positive kernels.

The absence of positive kernels in this family can be explained by differential transmission of the transgene from the male and female gametes. In event number 144, there are three \( F_1 \) families, and the progeny of two of these \( F_1 \) families fit a 1:1 segregation model. The remaining family had no kernels with detectable expression. Interestingly, this was the only \( F_1 \) family produced by using the \( T_0 \) plant as the male
parent when crossed with the inbred B73. The other 14 F₁ families in the four expressing events were produced using T₀ transgenic plants as the female parent and B73 as the male parent.

Because there was an excess of negative progeny in families that did not show a 1:1 segregation ratio, it was important to determine if the negative progeny inherited DNA sequences derived from the Glu-IDx5 gene. PCR-based assessment of the Glu-IDx5 gene from the three families indicated that their progeny segregated with a 1:1 (presence: absence) ratio for sequences derived from the Glu-IDx5 transgene. Endosperms of 16 F₁ kernels from 097-2 x B73 were analyzed for protein expression using SDS-PAGE, and then the genotypes of these same endosperms were determined by PCR. In the family 097-2, two kernels out of 16 had detectable protein expression, while the PCR analysis indicated that 10 kernels of these 16 kernels contained DNA derived from the transgene. By comparing these data with the SDS-PAGE data, it is clear that some progeny have inherited a nonfunctional version of the transgene, because some kernels with positive PCR results do not produce the 1DX5 protein. Two other F₁ families, 097-8 x B73 and 190-2 x B73, were subjected to a similar analysis. In these families, the genotypic ratios corresponded to the phenotypic ratio, which was not statistically different from 1:1 (see Table 2).
**Southern blot analysis of the Glu-1Dx5 transgene**

As an initial characterization of the transgene insertion site(s), genomic DNA was purified from plants grown from 1Dx5 expressing F1 kernels from four independent events and analyzed by Southern blot hybridization using a portion of the Glu-1Dx5 gene as a probe (Figure 3). The transgene insertions are different in each event. One event (182) contains relatively simple insertion sites, resulting in a single band on the blot. Comparison of the intensity of this band to the standards representing one and five genome equivalents suggests that this band is derived from multiple copies of the transgene. It is possible that this band results from a series of tandem integrations of the transgene at one insertion site. The other three events (097, 144, and 190) exhibit multiple bands, suggesting multiple copies of the transgene are present. The expected position for a band resulting from an intact copy of the construct is indicated for each restriction enzyme used in Figure 3. All events but 097 have a band in this area when genomic DNA is digested with EcoR1 or EcoRV.

**The Glu-1Dx5 gene exhibits maternal inheritance in transgenic maize**

Of the 15 families from events containing expressing kernels, only one (event 144, T0 plant 4, Table 2) failed to produce at least one kernel expressing Glu-1Dx5 when crossed with B73. This family was the only one in which the T0 plant was used as the male when crossed with B73. Families 144-3 and 144-8 are from the same event as 144-4 and produced positive progeny when their T0 plants were crossed as females with
B73. One possible explanation for this observation is that the *Glu-IDx5* gene exhibits maternal inheritance in maize. To test this explanation, we studied the inheritance and expression of *Glu-IDx5* in more detail in the next generation. Two different approaches were used. First, progeny of reciprocal crosses between B73 and F1 plants from events 097 and 190 were evaluated. Second, F1 plants from all the expressing events (097, 190, 182, and 144) were self-pollinated, and these same plants were also crossed as males to B73. All ears produced from these crosses had normal kernel set.

The results of SDS-PAGE analyses of kernels resulting from the reciprocal crosses between B73 and F1 plants from events 097 and 190 are summarized in Table 3. The 1DX5 protein was not observed in the progeny when the transgenic plant was the male parent. When a transgenic F1 plant was used as the female parent, however, about half of the resulting kernels contained 1DX5 HMW glutenin when analyzed by the same method. These data are consistent with a model in which the *Glu-IDx5* gene is transmitted in the nuclear genome of female gametes with no transmission by male gametes.

In the second inheritance study, five F2 families from four events were analyzed for expression of the 1DX5 HMW glutenin by SDS-PAGE. All families were significantly different from a 3:1 (positive: negative) ratio and were characterized by an excess of 1DX5 negative kernels. However, the results for three F2 families from events 097 and 190 fit a 1:1 segregation ratio. The segregation data for two F2 families from events 144 and 182 were not consistent with a 1:1 phenotypic segregation ratio due to an excess of nonexpressing kernels.
The segregation data of crosses to B73 (female) using the same 1Dx5-expressing plants that were self-pollinated are also summarized in Table 4. Three crosses with families from events 097 and 190 did not produce any 1Dx5-positive kernels. Two families from events 144 and 182 had some 1Dx5-positive kernels, but the ratio was significantly less than 1:1.

The genotypic ratios in families resulting from self-pollination and crossing with events 097 and 182 were determined by PCR amplification of a portion of the Glu-1Dx5 transgene. The results of this analysis (Table 4) corresponded well with the results of the SDS-PAGE analysis. One kernel out of 161 analyzed was positive by PCR but not by SDS-PAGE, and all SDS-PAGE-positive clones were PCR positive.

To confirm the stability of the inheritance pattern of this transgene, 16 F2 plants grown from 1Dx5 containing kernels from four events were self-pollinated to produce F3 kernels and analyzed by SDS-PAGE to detect 1Dx5 HMW glutenin in the endosperm. Thirteen of these F3 families fit a 1:1 (presence: absence of 1Dx5) phenotypic segregation model. Two F3 families from events 144 and 182 did not fit a 1:1 segregation model, due to an excess of negative kernels (Table 5). The phenotypic ratios were similar in the F2 and F3 generations, indicating that the inheritance of the transgene is stable through two generations.

Taken together, these data support a model in which the Glu-1Dx5 gene from families from events 097 and 190 is not transmitted through the pollen, and the Glu-1Dx5 gene from families from events 144 and 182 can be transmitted through pollen, but with reduced efficiency. Furthermore, in the lines from events 144 and 182 that produced an
excess of negative kernels when used as the female parent in crosses with B73, the transgene appears to be inefficiently passed through the female gamete as well. In these families, male transmission is much lower than female transmission.

Tissue specificity of the wheat *Glu-1Dx5* gene in transgenic maize

Given the unusual inheritance of the *Glu-1Dx5* gene in maize, it is important to understand the tissue specificity of this gene in this species, as an unusual expression pattern could explain the inheritance of this gene. We therefore used both SDS-PAGE and immuno-blot analysis to analyze several tissues in plants from families known to contain plants expressing 1Dx5 in endosperm. The results of these analyses are summarized in Table 6.

Endosperms and embryos of 85 mature kernels from eight F3 families were analyzed. Thirty-six of these kernels contained 1Dx5 HMW glutenin in the endosperm, but 1Dx5 HMW glutenin was not detected in any of the embryos. Leaf blades from 30 plants of six F3 families were harvested at 14 days after planting. Twelve of these plants were grown from kernels containing 1Dx5 in endosperm. In addition, leaf tissues from 14 F3 plants from eight F3 families were harvested at 58 days after planting. All plants were derived from kernels containing 1Dx5 in the endosperm. The 1Dx5 protein was not observed in any of the leaf samples. It was of particular interest to determine if the 1Dx5 protein is present in immature anthers, because this could affect the pollen transmission of the transgene. Anthers were harvested from 14 F3 plants at 58 days after planting.
These plants were grown from kernels containing 1Dx5 in the endosperm. The 1Dx5 protein was not detected in immature anthers. Pollen was harvested from the newly exerted anthers of four F2 plants and two F1 plants. These plants were grown from kernels containing 1Dx5 in the endosperm. The 1Dx5 protein was not detected in these pollen samples. Nine ears from nine F3 families were harvested before silk emergence. These plants were grown from expressing kernels. The 1Dx5 HMW glutenin was not detected by either SDS-PAGE or immuno-blot analysis in these ears.

Discussion

Dough made from wheat flour is elastic and extensible, making it suitable for many food products. Dough made from maize flour does not have these properties. The wheat high-molecular weight glutenins have a large impact on the physical properties of flour made from wheat, and this class of protein is lacking in maize. We have developed maize that produces a wheat high-molecular weight glutenin. This maize will be valuable for studying the impact of this protein on dough properties and could lead to the development of maize with improved utility for food products. Here we describe the inheritance of this transgene in maize.

From this study we conclude that (i) the wheat Glu-1Dx5 gene functions to produce the 1Dx5 protein in the endosperm of transgenic maize. The Glu-1Dx5 gene from wheat is genetically transmitted and specifically expressed in the endosperm of transgenic maize. (ii) The phenotypic and genotypic segregation ratios of the Glu-1Dx5
gene in the four events of transgenic maize that we studied are consistent with an inheritance model in which the transgene is incorporated into the nuclear genome and is transmitted with low efficiency through male gametes of maize.

The tissue specificity of Glu-1Dx5 expression is similar in maize to that observed in wheat. In both species, 1Dx5 has only been detected in endosperm. Also, in both species 1Dx5 is a major kernel protein, clearly visible on a coomassie blue stained SDS-PAGE gel. These observations suggest that the wheat promoter functions similarly in wheat and in maize, underscoring the similarities that exist among these cereal species in transcriptional machinery.

A number of plant transformation experiments have been reported that resulted in plants with a mixture of non-mendelian and mendelian inheritance of the transgene (Register et al. 1994; Walters et al. 1992; Spencer et al. 1992; Tomes et al. 1990; Christou et al. 1989; Hiei et al. 1994; Peng et al. 1995). In our experiments, none of the four events we studied exhibited efficient pollen transmission. In two events (097 and 190), pollen transmission was never observed, and in two others (182 and 144), pollen transmission occurred infrequently. There are several possible explanations for the poor transmission of the Glu-1Dx5 transgene through the pollen.

One possible explanation for the inefficient pollen transmission of the Glu-1Dx5 gene is that the transgene is ectopically expressed in maize in a tissue where the gene product interferes with pollen development or fertility. In wheat, expression of the Glu-1Dx5 gene has not been reported in any tissue other than endosperm and has not been associated with differential transmission to our knowledge. Further, expression of the
The Glu-1Dx5 gene has been confined to kernels in both transgenic wheat (Barro et al. 1997) and transgenic tobacco (Halford et al. 1989). Consistent with these observations, we failed to detect 1Dx5 HMW glutenin expression by SDS-PAGE and western blotting in embryo, leaves, pollen, and immature anthers of our transgenic maize.

A second possibility to explain the inefficient transmission of the Glu-1Dx5 transgene through pollen is that the gene Glu-1Dx5 may be inserted into the plastidic or mitochondrial genome. The segregation ratios of the F2 plants were not consistent with cytoplasmic/organellar inheritance. However, the F2 segregation ratios were typically 1:1 (Table 4), while a cytoplasmically inherited gene would show 100% transmission if all plastids contained the transgene.

A third possible explanation for the reduced transmission of the Glu-1Dx5 transgene through the pollen is that transformation produced chromosomal abnormalities. It has been shown in oat that particle bombardment transformation can generate insertion loci of several megabases, and may cause chromosomal rearrangements at the insertion locus (Svitashev et al. 2000). In maize, some chromosomal abnormalities may result in nonviable or noncompetitive gametes. (Phillips et al. 1971; Rhoades and Dempsey, 1973).

Characterization of the mechanism underlying the unusual inheritance of the Glu-1Dx5 transgene in maize will be particularly interesting. For example, in species where hybrid kernel production is important, male sterility systems can be useful for kernel production. Also, transmission of transgenes through pollen is an important issue concerning the inadvertent pollination of non-GMO crops or weeds. We have not
investigated the cause of the lack of expression in the nonexpressing events. Further characterization of these plants may lead to an understanding of the determinants of transgene expression.

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References


**Figure legends**

Figure 1. Schematic diagram of the *Glu-1Dx5* plant transformation construct. The upper bar represents the plasmid used in particle bombardment, presented in linear form using an arbitrary linearization site. Black areas represent wheat genomic DNA, and the cross-hatched arrow represents the 1Dx5 coding region. The stippled arrow is the ampicillin resistance gene from the plasmid pUC9. The bar below the construct represents the PCR product used for genotypic screens. The same PCR product was labeled and used as a probe in hybridization experiments, and the lines at the bottom indicate the expected sizes of hybridizing restriction fragments. *Xmal* cuts the plasmid at one site, so the expected fragment is the length of the plasmid.

Figure 2. A: Reducing SDS-PAGE analysis with coomassie blue R-250 stain of transgenic F₃ endosperm. B: Immuno-blot analysis of transgenic F₃ endosperm visualized with a monoclonal antibody specific to the 1Dx5 HMW glutenin. Two gels from the same batch were electrophoresed together in the same apparatus. One was blotted and the other was stained. Corresponding regions of the blot and the gel are shown. Lane 1:
Wheat containing *Glu-1Dx5*. Lane 2: Transgenic maize 144-8. Lane 3: Transgenic maize 190-4. Lane 4: Transgenic maize 097-6. Lane 5: Non-transgenic inbred B73. Lane 6: Molecular mass marker. The molecular mass of each band is indicated.

Figure 3. Genomic Southern blot analysis of transgenic F₁ plants. Genomic DNA of transgenic plants was digested with *Eco*RI, *Xma*I, or *Eco*RV and hybridized with a 362 bp PCR product amplified from the coding sequence of the *Glu-1Dx5* gene. Sample 1: F₁ plant of event 097-3. Sample 2: F₁ plant of event 144-3. Sample 3: F₁ plant of event 182-1. Sample 4: F₁ plant of event 190-2. Sample 5: Nontransgenic plant (B73). Sample 6: Marker. Sample 7: pHMW1Dx5 loaded at one genome equivalent concentration. Sample 8: pHMW1Dx5 loaded at five genome equivalents concentration. Arrowheads indicate the position of bands predicted to arise from integration of the intact hybridizing restriction fragment for each enzyme (see Figure 1).
Table 1. Screening of five F$_1$ kernels from crosses between a T$_0$ plant and B73 from each F$_1$ family in 12 events using SDS-PAGE to identify 1Dx5 HMW glutenin-expressing kernels in each F$_1$ family.

<table>
<thead>
<tr>
<th>Event number</th>
<th>Number of F$_1$ families in each event</th>
<th>Number of F$_1$ families with at least one positive kernel</th>
</tr>
</thead>
<tbody>
<tr>
<td>020</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>097</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>132</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>144</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>171</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>174</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>177</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>181</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>182</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>187</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>190</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>200</td>
<td>9</td>
<td>0</td>
</tr>
</tbody>
</table>

N = 12  Total = 54  Total = 13
Table 2. SDS-PAGE analysis of 1Dx5 HMW glutenin expression in every kernel from the 15 F₁ families containing positive kernels.

<table>
<thead>
<tr>
<th>Event</th>
<th>T₀ Plant</th>
<th>Analyzed kernels</th>
<th>Positive kernels</th>
<th>Phenotypic ratio&lt;sup&gt;b&lt;/sup&gt; 1 positive:1 negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>097</td>
<td>2</td>
<td>41</td>
<td>9</td>
<td>No**</td>
</tr>
<tr>
<td>&quot;</td>
<td>3</td>
<td>19</td>
<td>4</td>
<td>No*</td>
</tr>
<tr>
<td>&quot;</td>
<td>4</td>
<td>31</td>
<td>15</td>
<td>Yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>&quot;</td>
<td>5</td>
<td>16</td>
<td>1</td>
<td>No**</td>
</tr>
<tr>
<td>&quot;</td>
<td>7</td>
<td>28</td>
<td>2</td>
<td>No**</td>
</tr>
<tr>
<td>&quot;</td>
<td>8</td>
<td>35</td>
<td>19</td>
<td>Yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>144</td>
<td>3</td>
<td>12</td>
<td>4</td>
<td>Yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>&quot;</td>
<td>4</td>
<td>24</td>
<td>0</td>
<td>No**</td>
</tr>
<tr>
<td>&quot;</td>
<td>8</td>
<td>10</td>
<td>5</td>
<td>Yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>182</td>
<td>1</td>
<td>33</td>
<td>12</td>
<td>Yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>&quot;</td>
<td>2</td>
<td>59</td>
<td>29</td>
<td>Yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>&quot;</td>
<td>4</td>
<td>31</td>
<td>2</td>
<td>No**</td>
</tr>
<tr>
<td>&quot;</td>
<td>6</td>
<td>14</td>
<td>9</td>
<td>Yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>190</td>
<td>2</td>
<td>25</td>
<td>10</td>
<td>Yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>&quot;</td>
<td>4</td>
<td>15</td>
<td>7</td>
<td>Yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Italic print denotes a cross made with the transgenic plant as the male parent. All other crosses were made with the transgenic plant as the female parent.

<sup>b</sup>As determined by the chi-squared test. One and two asterisks indicate statistical significance at the 0.05 and 0.01 levels, respectively. A superscripted ns indicates a lack of statistical significance, that is, the ratio is not significantly different than 1:1.

n = 4  n = 15  Total = 409  Total = 136  No = 6 families  Yes = 9 families
Table 3. SDS-PAGE analysis of 1Dx5 HMW glutenin expression in kernels derived from reciprocal crosses between F\textsubscript{1} transgenic plants and B73.

<table>
<thead>
<tr>
<th>Female parent</th>
<th>Male parent</th>
<th>Analyzed kernels</th>
<th>Pos.</th>
<th>Neg.</th>
<th>Phenotypic ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(097-2 / B73) F\textsubscript{1}-11</td>
<td>B73</td>
<td>56</td>
<td>31</td>
<td>25</td>
<td>Yes**</td>
</tr>
<tr>
<td>B73</td>
<td>(097-2 / B73) F\textsubscript{1}-11</td>
<td>56</td>
<td>0</td>
<td>56</td>
<td>No**</td>
</tr>
<tr>
<td>(097-4 / B73) F\textsubscript{1}-6</td>
<td>B73</td>
<td>56</td>
<td>28</td>
<td>28</td>
<td>Yes**</td>
</tr>
<tr>
<td>B73</td>
<td>(097-4 / B73) F\textsubscript{1}-6</td>
<td>56</td>
<td>0</td>
<td>56</td>
<td>No**</td>
</tr>
<tr>
<td>(190-2 / B73) F\textsubscript{1}-18</td>
<td>B73</td>
<td>56</td>
<td>16</td>
<td>40</td>
<td>No*</td>
</tr>
<tr>
<td>B73</td>
<td>(190-2 / B73) F\textsubscript{1}-18</td>
<td>56</td>
<td>0</td>
<td>56</td>
<td>No**</td>
</tr>
</tbody>
</table>

* As determined by the chi-squared test. * and ** indicate statistical significance at the 0.05 and 0.01 levels, respectively. A superscripted ns indicates a lack of statistical significance, that is, the ratio is not significantly different than 1:1.
Table 4. Phenotypic and genotypic analyses of the *Glu-1Dx5* gene in kernels derived from self-pollination and out-crossing of F₁ transgenic plants to B73.

<table>
<thead>
<tr>
<th>Pedigree</th>
<th>Pollination</th>
<th>Phenotypic analysis</th>
<th>Genotypic analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n¹</td>
<td>Pos.</td>
</tr>
<tr>
<td>(097-2 / B73) F1-22-F2</td>
<td>Self</td>
<td>56</td>
<td>22</td>
</tr>
<tr>
<td>B73 // (097-2 / B73) F1-22</td>
<td>Cross</td>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td>(097-4 / B73) F1-7-F2</td>
<td>Self</td>
<td>56</td>
<td>30</td>
</tr>
<tr>
<td>B73 // (097-4 / B73) F1-7</td>
<td>Cross</td>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td>(190-2 / B73) F1-5-F2</td>
<td>Self</td>
<td>56</td>
<td>30</td>
</tr>
<tr>
<td>B73 // (190-2 / B73) F1-5</td>
<td>Cross</td>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td>(182-4 / B73) F1-22-F2</td>
<td>Self</td>
<td>55</td>
<td>15</td>
</tr>
<tr>
<td>B73 // (182-4 / B73) F1-22</td>
<td>Cross</td>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td>(144-3 / B73) F1-4-F2</td>
<td>Self</td>
<td>42</td>
<td>14</td>
</tr>
<tr>
<td>B73 // (144-3 / B73) F1-4</td>
<td>Cross</td>
<td>56</td>
<td>2</td>
</tr>
</tbody>
</table>

¹ Number of kernels analyzed.

² As determined by the chi-squared test. One and two asterisks indicate statistical significance at the 0.05 and 0.01 levels, respectively. A superscripted ns indicates a lack of statistical significance, that is, the ratio is not significantly different than 1:1.
Table 5. SDS-PAGE analysis of 1Dx5 HMW glutenin expression in kernels of 16 F₃ families representing four events.

<table>
<thead>
<tr>
<th>Pedigree</th>
<th>n</th>
<th>Positive</th>
<th>F3 Phenotypic ratio= 1:1ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>(097-2 / B73) F₁-3-F₂-27-F₃</td>
<td>35</td>
<td>18</td>
<td>Yes₃⁸</td>
</tr>
<tr>
<td>(097-6 / B73) F₁-1- F₂-20- F₃</td>
<td>20</td>
<td>9</td>
<td>Yes₃⁸</td>
</tr>
<tr>
<td>(097-6 / B73) F₁-1- F₂-17- F₃</td>
<td>15</td>
<td>10</td>
<td>Yes₃⁸</td>
</tr>
<tr>
<td>(097-6 / B73) F₁-1- F₂-17- F₃</td>
<td>15</td>
<td>10</td>
<td>Yes₃⁸</td>
</tr>
<tr>
<td>(144-3 / B73) F₁-3- F₂-37- F₃</td>
<td>20</td>
<td>2</td>
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<tr>
<td>(144-8 / B73) F₁-4- F₂-3- F₃</td>
<td>20</td>
<td>17</td>
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</tr>
<tr>
<td>(144-8 / B73) F₁-4- F₂-7- F₃</td>
<td>25</td>
<td>14</td>
<td>Yes₃⁸</td>
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<tr>
<td>(182-1 / B73) F₁-3- F₂-11- F₃</td>
<td>25</td>
<td>12</td>
<td>Yes₃⁸</td>
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<tr>
<td>(182-1 / B73) F₁-3- F₂-55- F₃</td>
<td>15</td>
<td>1</td>
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<tr>
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<td>35</td>
<td>10</td>
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<td>20</td>
<td>10</td>
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<tr>
<td>(190-4 / B73) F₁-2- F₂-131- F₃</td>
<td>15</td>
<td>8</td>
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</tr>
</tbody>
</table>

ᵃ Number of kernels analyzed.

ᵇ As determined by the chi-squared test. One and two asterisks indicate statistical significance at the 0.05 and 0.01 levels, respectively. A superscripted ns indicates a lack of statistical significance, that is, the ratio is not significantly different than 1:1.
Table 6. SDS-PAGE and immuno-blot analysis of 1Dx5 HMW glutenin expression in different tissues of transgenic maize.

<table>
<thead>
<tr>
<th>No. of events</th>
<th>No. of families</th>
<th>n²</th>
<th>Number of samples that have 1Dx5 HMW glutenin subunit expression</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Endosperm</td>
</tr>
<tr>
<td>4</td>
<td>8 F₁</td>
<td>85</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>6 F₁</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>8 F₁</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>14 F₁</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>4 F₂, 2 F₁</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>9 F₁</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

² Number of analyses, representing the indicated number of events and families.
Figure 1. Sangtong et al.

[Diagram showing EcoRI, EcoRV, probe, and EcoRV EcoRI digestion sites with distances and restriction enzymes mentioned: 1Dx5 coding region (2518 bp), OR, AP^R, 5201 bp EcoRV, 8248 bp EcoRV1, 10936 bp XmaI (single site produces linear plasmid).]
Figure 2. Sangtong et al.
Figure 3. Sangton et al.
CHAPTER 4. THE EFFECT OF *Glu-IDx5* TRANSGENE EXPRESSION ON MAIZE ENDOSPERM COMPOSITION IN EXPRESSING AND NON-EXPRESSING MAIZE KERNELS DERIVED FROM THE SAME F$_2$ TRANSGENIC MAIZE PLANTS

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Abstract

Wheat *Glu-IDx5* gene was transformed into maize. The non-mendelian inheritance of *Glu-IDx5* transgene in maize allowed us to produce expressing (hemizygous) and non-expressing (null) near isogenic kernels on the same ear to study the effect of the *Glu-IDx5* transgene on protein accumulation in kernels. Environmental variation was minimized by comparing expressing and non-expressing transgenic kernels derived from the same ear. Plant type and kernel weight of *Glu-IDx5* transgenic maize were not significantly different between expressing and non-expressing transgenic plants.
or kernels. The 1Dx5 HMW glutenin subunit contents were 30.04 and 37.14 ng/mg in expressing endosperm of two events when measured by ELISA. This accounts for 0.003 % and 0.0037% of the endosperm mass, respectively. In analysis of the total alcohol soluble proteins by HPLC, the 1Dx5 HMW glutenin subunit in endosperm of transgenic maize accounted for between 0.92% to 3.82% of the alcohol extractable proteins. The level of the 1Dx5 HMW glutenin subunit in transgenic maize was lower than the level of the native 1Dx5 HMW glutenin subunit in wheat endosperm. *Glu-1Dx5* transgene expression in transgenic maize endosperm affected nitrogen content and the levels of zeins in both expressing and non-expressing endosperms from ears containing expressing endosperms.

**Introduction**

The *Glu-1Dx5* gene from wheat encodes the 1Dx5 HMW glutenin subunit and was cloned by Anderson et al. (1989). The 1Dx5 HMW glutenin subunit is in the prolamin group of kernel storage proteins, as are the zeins, the major kernel storage proteins of maize (Shotwell and Larkins 1989). *Glu-1Dx5* and zein genes are expressed only in developing endosperm (Lee et al. 1976, Lamacchia et al. 2001). Dent maize kernels consist of endosperm, germ, pericarp, and tip cap and each accounts for 82.6%, 11.1%, 5.4% and 0.8% of the kernel weight, respectively (Watson 1977, Earle et al. 1946). A whole maize kernel consists of starch (73.5%) protein (9.0%) fat (4.3%), sugar (1.9%) and ash (1.5%). Proteins in germ account for 22% of the total protein in mature
maize kernel (Earle 1946). Maize endosperm contains 75-80% of the protein, 15% of the lipid and 98% of the starch in the whole maize kernel (Glover and Mertz 1987). Serial extraction of maize endosperm has resulted in division of maize endosperm proteins into 4 classes. First, albumins, globulins, and non-protein N were extracted by 0.5 M NaCl and they account for 4% total protein in endosperm. Second, zeins accounting for 41% of total protein in maize endosperm were extracted by 70% ethanol and 0.5% NaOAc. Third, alcohol-soluble reduced glutelins; water-soluble and water-insoluble accounting for 7% and 13% total protein in endosperm, respectively, were extracted by 70% ethanol, 0.5% NaOAC and 0.1 β-ME. The final residue fraction was alcohol-insoluble reduced glutenin accounting for 35% of total protein in endosperm. (Paulis and Wall 1977). Zeins are the major kernel storage proteins in maize endosperm. The composition of zeins is affected by mutants such as o2 (Mertz et al. 1964), o7 (Misra et al. 1972) and fll2 (Barbosa and Glover 1978). Paulis and Bietz (1986) used RP-HPLC to detect the differences in zein composition between o2 and sul. Paulis et al. (1991) used a rapid reversed-phase high-performance liquid chromatography (RP-HPLC) to detect differences in zein composition in o2, modified o2 and normal maize. Paulis et al. (1992) reported that RP-HPLC can detect the effects of the single mutants; amylose-extender, floury-1, soft starch, dull-1, shrunken-1, sugary-1, sugary-2 and waxy on zein composition. The opaque-2 and floury-2 mutations decreased the amount of A- and B-zeins. The double mutant had epistatic effects on zein composition. HPLC methods are rapid-and-sensitive, have exceptional reproducibility and high resolution (Bietz and Kruger 1994). HPLC has been used to characterize, qualify, isolate and compare the
cereal proteins such as zeins in maize and the HMW glutenin subunits in wheat. In addition, HPLC is used in genotype identification in maize; inbred and hybrid, single and double mutants in maize, and purification of plant varieties (Paulis and Bietz 1994). RP-HPLC has been also used to study the genetics of the gliadin and glutenin storage proteins in wheat. It is used for wheat breeding programs to predict bread-making quality of wheat (Huebner and Bietz et al. 1994, Lafiandra et al. 1994; Wieser et al. 1994).

In this study, we used RP-HPLC to study the effect of Glu-1Dx5 transgene expression on expressing and non-expressing endosperm derived from the same ear on the major kernel storage protein in maize, the zeins. In addition, we studied the difference in nitrogen content, carbon content and kernel weight between expressing and non-expressing kernels derived from the same ear of Glu-1Dx5 transgenic maize, and also we quantified the 1Dx5 HMW glutenin subunit content in these kernels by ELISA.

Materials and methods

Treatments

Because the Glu-1Dx5 gene exhibits non-mendelian inheritance in maize, kernels resulting from self-pollination of F2 plants segregate 1:1 for expressing and non-expressing. The two self-pollinations leading to the F3 kernels should result in an average genome homozygosity of about 87.5%. We examined endosperm of expressing and non-expressing kernels from three transformation events; F3-097-6, F3-144-8, and F3-190-4. These F3 transgenic kernels were produced in the greenhouse in the winter of 1999. Maize inbred lines B73 and B73o2 were used as non-transformed maize. Wheat L88-6
line contains five HMW glutenin subunits; lAxl, lDx5, lBx17, lBy18 and lDy10 and was also used in this study as a positive control for the presence of the lDx5 HMW glutenin subunit. The B73, B73o2 and wheat were produced in different years and environments. The profiles of zeins from maize endosperm determined by RP-HPLC are not greatly influenced by planting years and locations (Paulis and Bietz 1986; Smith and Smith 1986).

Sample preparation

One hundred kernels from each of 3 different F3 transformation events of transgenic maize, 25 kernels from B73 and 25 kernels from B73o2 were used for this analysis. Individual F3 transgenic kernels were weighed. Each kernel was ground with a hand-held rotary grinder to remove about 10 mg of endosperm. The endosperm from individual transgenic kernels was analyzed by SDS-PAGE to detect the expression of the Glu-lDx5 transgene. The null-expressing kernels were re-analyzed by ELISA to confirm the non-expression phenotype. ELISA is more sensitive to low levels of the lDx5 HMW glutenin subunit than SDS-PAGE. The method for serial extraction of endosperm for SDS-PAGE and ELISA that was used in this study was as described by Sangtong et al. (2001).

After sorting into expressing and non-expressing kernels, the remaining endosperm of the kernels from each ear was ground in bulk, to give six bulks representing expressing and non-expressing kernels from each of three events. Bulk
endosperm from B73 and B73o2 was prepared similarly. The bulk endosperm used to
detect the 1Dx5 HMW glutenin subunit content by ELISA was different than that used
for nitrogen and carbon content, and zein contents. However, these kernels were derived
from the same ears. Whole kernels of wheat, including the embryo and endosperm were
ground and used in these studies as a positive check.

*Nitrogen and carbon content analyzed by the dry combustion*

Nitrogen and carbon content of endosperm was analyzed by dry combustion. A
CHN-2000 analyzer (LECO, Corp., St. Joseph, MI) was used for C and N determination
in endosperm. The combustion temperature was 950 °C and 0.15g of endosperm was
used. Each sample was measured in duplicate. This analysis was done by Iowa State
University Soil Testing Laboratory, Ames, Iowa 50011.

*Alpha zeins, beta zein, gamma zein and 1Dx5 HMW glutenin subunit content by*

*HPLC*

HPLC was used to analyze the effect of 1Dx5 HMW on zein composition in
endosperm of expressing and non-expressing kernels. One hundred milligrams of
endosperm were added to 1 ml of extraction buffer that contained 75% EtOH, 61 mM
NaOAc and 5% β-ME and shaken at room temperature for 1 hour. The extract was then
centrifuged at top speed in a microcentrifuge for 10 min. Twenty-five microliters of the
supernatant was injected into the column. The HPLC consisted of the following Waters
components: two model 510 pumps, a model 770 autosampler, a model 996 photodiode array detector and Millennium Software. The column was a Vydac C18 with a pore size of 300 Å and it was run at 55°C. The absorbance of the eluent at 210 nm was monitored. Solution A and B were 0.1% TFA in Water and 0.1% TFA in ACN respectively. Peaks were assigned in comparison to positions reported in the literature (Beitz 1985). Paulis et al. (1991) reported the standard error of relative peak area of each sample is usually less than 1% with this method.

Results

Non-mendelian inheritance of the Glu-1Dx5 gene in transgenic maize

The non-mendelian inheritance of the wheat Glu-1Dx5 gene in F2 and F3 generation of transgenic maize (Sangtong et al., 2002) allowed us to produce ears on which the kernels segregated with two phenotypic and genotypic classes, hemizygous expressing kernels and non-expressing, or null kernels. The F3 expressing and non-expressing kernels in each family were derived from the same F2 plant. The phenotypic classification of F3 kernels in each of three families from different transformation events was analyzed by SDS-PAGE to detect the expression of the Glu-1Dx5 gene and then the non-expressing kernels were analyzed by ELISA to confirm the non-expressing phenotype. This was necessary because expression of this gene is occasionally not detected by SDS-PAGE because of protein degradation or low level of expression. The ELISA is more sensitive than SDS-PAGE (Sangtong et al. 2001). The observed phenotypic segregation ratios for all three F3 families were consistent with non-
mendelian inheritance of this transgene, as observed previously (Sangtong et al. 2002); i.e. 1 expresser: 1 non-expresser. To minimize the effects of residual heterozygosity, bulks of F3 endosperms derived from 25 expressing kernels and 25 non-expressing kernels were used in this study. Because the expressing and non-expressing kernels from each event were produced on the same ear, environmental differences were minimized, and differences between expressing and non-expressing kernels were most likely due to genetic effects.

*Kernel weight and agronomic characteristics of expressing and non-expressing maize*

Expressing and non-expressing kernels from the same ear cannot be visually distinguished. In addition, in the F1, F2 and F3 generations, plants derived from expressing and non-expressing kernels cannot be distinguished phenotypically. Some F1 families exhibited partial male sterility but, after selection, male sterile F2 and F3 plants were not detected. When plants derived from expressing kernels and plants derived from non-expressing kernel were used for cross- or self- pollination, the kernel set was similar. Finally, there is no significant difference in the average whole kernel weight of expressing and non-expressing kernels from the same ear in the three events examined (Table 2).
**ELISA analysis of the 1Dx5 HMW glutenin subunit concentration in expressing and non-expressing endosperm of transgenic maize**

The 1Dx5 HMW glutenin subunit concentration from endosperm of transgenic maize was analyzed by ELISA. A polyclonal antibody raised to 1Dx5 purified from wheat was used as primary antibody. Whole ground wheat kernels of line L88-6 containing five HMW glutenin subunits; lAx1, 1Dx5, 1Bx17, 1By18 and 1Dy10 was used for a positive check in ELISA, and all of these five HMW subunit reacted with the primary antibody. The concentration of five HMW subunits (not only 1Dx5 HMW glutenin subunit) from wheat L88-6 line was 60.49 ng/mg of whole kernel (Table 3). The concentration of 1Dx5 HMW glutenin subunit in the endosperm of expressing transgenic maize kernels derived from event 97-6 and 190-4 were 37.14 and 30.04 ng/mg of endosperm, respectively. These results are derived from a different bulk of expressing seeds than the bulk used for nitrogen, carbon, zein, and weight analysis. While the two bulks were prepared from the same ears, the ELISA result should not be compared directly to these results.

**Nitrogen and carbon content in expressing and non-expressing endosperm of transgenic maize by combustion analysis**

The nitrogen content the endosperms ranged from 1.14% to 1.99 % and the carbon content ranged from 40.97% to 41.85 % of the endosperm mass (Table 3). The difference in carbon content between expressing and non-expressing endosperms derived
from the same ear was within 1% in each of the three transgenic events analyzed (Table 6), although these differences were statistically significant (Table 4). The difference in nitrogen content between expressing and non-expressing endosperms from the same ear was also statistically significant for all three events (Table 4). The expressing endosperms of F3-097-6 contained 30.70% more nitrogen than non-expressing endosperms derived from the same ear, but the expressing endosperm of F3-144-8 and F3-190-4 contained 17.58% and 14.55% less nitrogen than non-expressing endosperm derived from the same ear, respectively (Table 6). The nitrogen content of the expressing endosperm of F3-097-6 and the expressing endosperm of F3-144-8 were not statistically different at the P=0.05 and 0.01 levels, respectively (Table 4). In addition, the carbon content of the expressing endosperm of F3-097-6 and the expressing endosperm of F3-144-8 was not statistically different at the P=0.05 level (Table 4).

Zeins and 1Dx5 HMW glutenin subunit concentration by HPLC in expressing and non-expressing endosperm of transgenic maize

In order to determine the effect of the Glu-1Dx5 transgene on zein expression, we used HPLC to quantify the zein content of the expressing and non-expressing endosperms. This method allowed us to quantify the 1Dx5 HMW glutenin subunit, in addition to the alpha, beta, and gamma zein classes. Total zein content was calculated by the addition of the peak areas of the alpha, beta and gamma zeins. The HPLC retention times of each zein and 1Dx5 HMW glutenin subunit were nearly constant in each sample
that was analyzed. In this analysis the retention time for a given peak varied between 0.10-0.21 min in a total run length of 110 minutes (Table 8). Between runs, the retention times of the same protein varied no more than 0.10 – 0.15 min (Bietz, 1985). The expressing endosperm of F3-097-6 contained higher total zeins, alpha, beta and gamma zein than non-expressing endosperm derived from the same ear, but the expressing endosperm of F3-144-8 and F3-190-4 had lower total zeins, alpha, beta and gamma zein than in non-expressing endosperm derived from the same ear (Table 5). The level of 1DX5 HMW glutenin subunit in wheat was higher than in transgenic maize (Table 6).

**Relationship between total zeins, 1DX5 HMW glutenin subunit, nitrogen and carbon content in expressing and non-expressing endosperm of transgenic maize**

There are two patterns of relationship between expressing and non-expressing endosperm of *Glu-1DX5* transgenic maize. Firstly, expressing endosperm of F3-097-6 contained higher total zeins, nitrogen and carbon content when compared with non-expressing endosperm of F3-097-6. Secondly, expressing endosperm of F3-144-8 and expressing endosperm of F3-190-4 contained lower total zeins and nitrogen content but higher carbon content when compared with non-expressing endosperm of F3-144-8 and non-expressing endosperm of F3-190-4 respectively (Table 6).

There were high correlations $(r$ greater than 0.8) between the total zeins and nitrogen content, total zeins and alpha zeins, and nitrogen content and alpha zeins. The
correlation coefficient between 1Dx5 HMW glutenin subunit content and nitrogen content, and total zeins are less than 0.4 (Table 7).

Discussion

The maternal inheritance of this transgene has allowed us to prepare near isogenic kernels for comparison of the effect of the transgene in fewer generations than would be required by a normal backcrossing program and environmental variation can be minimized when comparing expressing (hemizygous) and non-expressing (null) transgenic kernels derived from the same ear.

**Plant type and kernel weight of Glu-1Dx5 transgenic maize**

Kernel weight of Glu-1Dx5 transgenic maize were not different between expressing and non-expressing transgenic plants or kernels. This is similar to the report by Molvig et al. (1997) in which seed yields of a transgenic lupin line homozygous for a sunflower seed albumin transgene and the non-transgenic parent lupin cultivar were not significantly different in yield.

**The 1Dx5 HMW glutenin subunit content in endosperm of transgenic maize**

The concentrations of 1Dx5 HMW glutenin subunit were 30.04 and 37.14 ng/mg in expressing endosperm of two events of transgenic maize by using ELISA. This is
0.003% and 0.0037% of endosperm respectively. The peak area attributed to the 1Dx5 HMW glutenin subunit in the endosperm of transgenic maize accounted for from 0.92% to 3.82% of total zeins as measured by HPLC. The level of 1Dx5 HMW glutenin subunit in transgenic maize was lower than the native 1Dx5 HMW glutenin subunit in wheat endosperm. Blechl and Anderson (1996) transformed the hybrid HMW-GS gene; Dy10 and 1Dx5, into wheat. The level of hybrid HMW-GS from transgene was similar or higher than endogenous HMW glutenin subunits. The hybrid HMW-GS represented about 15% to 41% of the total HMW glutenin subunits. Barro et al. (1997) reported that in some lines of transgenic wheat the amount of 1Dx5 HMW glutenin subunit increased about 400% when compared with the amounts of other HMW glutenin subunits. He et al. (1999) transformed Glu-I Dx5 into 3 durum wheat cultivars. The level of transgene expression in transgenic wheat was similar or higher than native HMW glutenin subunit genes. Rooke et al. (1999) transformed Glu-I Dx5 into tritordeum. The expression levels of transgenes were similar or greater than the native HMW glutenin subunit genes. Alvarez et al. (2000) reported the expression of Glu-I Dx5 transgene caused the 1Dx5 HMW glutenin subunit in commercial transgenic wheat to increase from to 2% to 4.8% and 2.7% of total protein for 2 different events. Popineau et al. (2001) transformed Glu-I Dx5 into wheat L88-31 line and L88-6 where the expression of the transgene accounted for 18% and 29% of the total flour protein, respectively. In these two transgenic lines, the 1Dx5 HMW glutenin subunit made up about 70% of the HMW subunits.

The level of Glu-I Dx5 transgene expression in maize was lower than in wheat or closely related species. This may be because the regulatory elements in the promoter
region of Glu-1Dx5 gene cannot function fully with the transcription system of maize. Another possible explanation may be that the level 1Dx5 HMW glutenin subunit was reduced by degradation of 1Dx5 HMW glutenin subunit in the maize cell.

The expression of Glu-1Dx5 transgene effects the expression of zeins in endosperm of transgenic maize

Accumulation of zein, the main storage kernel protein of transgenic maize was affected by Glu-1Dx5 transgene. In all three events studied, there were substantial differences in zein content between the expressing and non-expressing kernels. In the F3-097-6 transgenic line, the expressing kernels contained higher nitrogen content (about 31%) and total zein content (about 78%) than the non-expressing kernels. One possible explanation for this is that the 1Dx5 HMW glutenin subunit in these kernels creates a nitrogen sink that pulls more nitrogen into these kernels, depriving the non-expressing kernels of nitrogen for zein biosynthesis.

In contrast to F3-097-6, F3-144-8 and F3-190-4, the expressing endosperm contained lower nitrogen (about 18% and 15% respectively) and total zeins content (about 50% and 47%, respectively) than non-expressing endosperm. A possible explanation for this is that the 1Dx5 HMW glutenin subunit perturbs protein body formation or interferes with zeins deposition in the expressing seeds in some other way. This reduces the overall zeins contents of these kernels. The effect of 1Dx5 transgene expression on total zein and nitrogen content in the expressing and non-expressing
kernel is event specific. Our results were different than those reported for the expression of *Glu-1Dx5* transgene in bread wheat, in which transgene expression did not affect any native HMW glutenin subunits gene in wheat (Barro et al. 1997). The over expression of *Glu-1Dx5 transgene* in commercial wheat did not cause major change in other native HMW subunits and gliadins (Alvarez et al. 2000). Popineau et al. (2001) reported that the expression of *Glu-1Dx5 transgene* in transgenic wheat increased the amount of 1Dx5 HMW glutenin subunit but did not affect the total protein content. Blechl and Anderson (1996) transformed the hybrid HMW-GS gene; Dy10 and 1Dx5, into wheat. The expression of the transgene did not affect the major kernel storage proteins; LMW-GS and gliadins, in wheat.

**Acknowledgments**

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Lafiandra D, Porceddu E, Colaprico G, Margiotta B (1994) Combined reversed phase high performance liquid chromatography (RP-HPLC) and electrophoretic techniques in genetics and breeding of wheat storage proteins. In: Kruger JE, Bietz JA (eds) High-


**Figure and table**

Table 1. The phenotypic ratio of 3 F3 families of *Glu-IDx5* transgenic maize analyzed by SDS-PAGE and ELISA

<table>
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<tr>
<th>Pedigree</th>
<th>Total kernels</th>
<th>Expressing</th>
<th>Non-expressing</th>
<th>Chi-square</th>
<th>Phenotypic ratio 1 expressing : 1 non-expressing</th>
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<tr>
<td>F3-097-6</td>
<td>100</td>
<td>44</td>
<td>56</td>
<td>0.16</td>
<td>yes</td>
</tr>
<tr>
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<td>100</td>
<td>49</td>
<td>51</td>
<td>1.44</td>
<td>yes</td>
</tr>
<tr>
<td>F3-190-4</td>
<td>100</td>
<td>48</td>
<td>52</td>
<td>0.04</td>
<td>yes</td>
</tr>
</tbody>
</table>
Table 2. Kernel weight of expressing and non-expressing of F3 families of *Glu-1Dx5* transgenic maize

<table>
<thead>
<tr>
<th>Pedigree</th>
<th>Classification</th>
<th>Average (n=20) Kernel weight (gram/kernel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F3-097-6</td>
<td>Expressing of 1Dx5</td>
<td>0.268 ± 0.001</td>
</tr>
<tr>
<td>F3-097-6</td>
<td>Non-expressing of 1Dx5</td>
<td>0.284 ± 0.007</td>
</tr>
<tr>
<td>F3-144-8</td>
<td>Expressing of 1Dx5</td>
<td>0.266 ± 0.016</td>
</tr>
<tr>
<td>F3-144-8</td>
<td>Non-expressing of 1Dx5</td>
<td>0.270 ± 0.006</td>
</tr>
<tr>
<td>F3-190-4</td>
<td>Expressing of 1Dx5</td>
<td>0.252 ± 0.012</td>
</tr>
<tr>
<td>F3-190-4</td>
<td>Non-expressing of 1Dx5</td>
<td>0.254 ± 0.06</td>
</tr>
<tr>
<td>Mean</td>
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</tr>
<tr>
<td>CV (%)</td>
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</tbody>
</table>
Table 3. The 1DX5 HMW glutenin subunit content by ELISA, the nitrogen and carbon content by dry combustion of expressing and non-expressing from F3 families of transgenic maize

<table>
<thead>
<tr>
<th>Pedigree</th>
<th>Classification</th>
<th>1DX5 HMW*(ng/mg)</th>
<th>% Total nitrogen</th>
<th>% Total carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>F3-097-6</td>
<td>Expressing</td>
<td>37.14 ± 2.87</td>
<td>1.49 ± 0.05</td>
<td>41.59 ± 0.11</td>
</tr>
<tr>
<td>F3-097-6</td>
<td>Non-expressing</td>
<td>-4.48 ± 1.44</td>
<td>1.14 ± 0.00</td>
<td>41.22 ± 0.05</td>
</tr>
<tr>
<td>F3-144-8</td>
<td>Expressing</td>
<td>-</td>
<td>1.50 ± 0.00</td>
<td>41.44 ± 0.06</td>
</tr>
<tr>
<td>F3-144-8</td>
<td>Non-expressing</td>
<td>-</td>
<td>1.82 ± 0.14</td>
<td>40.97 ± 0.40</td>
</tr>
<tr>
<td>F3-190-4</td>
<td>Expressing</td>
<td>30.04 ± 4.31</td>
<td>1.70 ± 0.01</td>
<td>41.85 ± 0.08</td>
</tr>
<tr>
<td>F3-190-4</td>
<td>Non-expressing</td>
<td>-</td>
<td>1.99 ± 0.01</td>
<td>41.62 ± 0.04</td>
</tr>
<tr>
<td>B73</td>
<td>Non transgenic</td>
<td>-5.50 ± 0.00</td>
<td>1.20 ± 0.02</td>
<td>40.40 ± 0.03</td>
</tr>
<tr>
<td>B7302</td>
<td>Non transgenic</td>
<td>-4.48 ± 1.44</td>
<td>1.40 ± 0.01</td>
<td>40.88 ± 0.01</td>
</tr>
<tr>
<td>Wheat L88-6</td>
<td>5 HMW subunits</td>
<td>60.49 ± 10.05</td>
<td>2.46 ± 0.01</td>
<td>40.66 ± 0.02</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>15.51</td>
<td>1.63</td>
<td>41.18</td>
</tr>
<tr>
<td>CV (%)</td>
<td></td>
<td>28</td>
<td>1.20</td>
<td>0.14</td>
</tr>
</tbody>
</table>

*The concentration of 1DX5 HMW glutenin subunit in endosperm of transgenic maize expressed as ng per mg of endosperm except the of L88-6 wheat line was total concentration of five HMW glutenin subunits; 1Ax1, 1DX5, 1Bx17, 1By18 and 1Dy10.
Table 4. Orthogonal contrast for 1Dx5 HMW glutenin subunit, total nitrogen and total carbon content

<table>
<thead>
<tr>
<th>Contrast</th>
<th>1Dx5a</th>
<th>% Total nitrogena</th>
<th>% Total carbona</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Expb- F3-097-6) vs (Nonc- F3-097-6)</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>(Exp- F3-144-8) vs (Non- F3-144-8)</td>
<td>-</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>(Exp F3-190-4-F3) vs (Non- F3-190-4)</td>
<td>-</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>(Non- F3-097-6) vs (Non- F3-144-8)</td>
<td>-</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>(Non- F3-097-6) vs (Non F3-190-4)</td>
<td>-</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>(Non- F3-144-8) vs (Non F3-190-4)</td>
<td>-</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>(Exp- F3-097-6) vs (Exp- F3-144-8)</td>
<td>-</td>
<td>ns</td>
<td>*</td>
</tr>
<tr>
<td>(Exp- F3-097-6) vs (Exp- F3-190-4-F3)</td>
<td>-</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>(Exp- F3-144-8) vs (Exp- F3-190-4-F3)</td>
<td>-</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>(Exp- F3-097-6) vs Wheat L88-6 line</td>
<td>**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(Exp- F3-144-8) vs Wheat L88-6 line</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(Exp- F3-190-4) vs Wheat L88-6 line</td>
<td>**</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

As determined by the orthogonal contrast. One and two asterisks indicate statistical significance at the 0.05 and 0.01 levels, respectively.

b Exp is abbreviation of expressing endosperm of

c Non is abbreviation of non-expressing endosperm of
Table 5. 1Dx5 HMW glutenin subunit and zeins content by HPLC of expressing and non-expressing endosperm of transgenic maize

<table>
<thead>
<tr>
<th>Pedigree</th>
<th>1Dx5</th>
<th>Total zein</th>
<th>Alpha zein</th>
<th>Beta zein</th>
<th>Gamma 27-kD zein</th>
<th>Gamma 16-kD zein</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1-097-6</td>
<td>369</td>
<td>40.306</td>
<td>32.553</td>
<td>1.668</td>
<td>3.548</td>
<td>1.916</td>
</tr>
<tr>
<td>Expressing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-expressing</td>
<td></td>
<td>22.610</td>
<td>17.106</td>
<td>860</td>
<td>2.791</td>
<td>1.580</td>
</tr>
<tr>
<td>F2-144-8</td>
<td>304</td>
<td>29.951</td>
<td>23.716</td>
<td>1.098</td>
<td>2.647</td>
<td>1.442</td>
</tr>
<tr>
<td>Expressing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-expressing</td>
<td></td>
<td>60.450</td>
<td>52.156</td>
<td>1.845</td>
<td>3.326</td>
<td>1.803</td>
</tr>
<tr>
<td>F2-190-4</td>
<td>1.241</td>
<td>32.527</td>
<td>26.516</td>
<td>908</td>
<td>2.538</td>
<td>1.357</td>
</tr>
<tr>
<td>Expressing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-expressing</td>
<td></td>
<td>61.236</td>
<td>54.211</td>
<td>1.346</td>
<td>2.803</td>
<td>1.535</td>
</tr>
<tr>
<td>B73 Non transgenic</td>
<td></td>
<td>54.249</td>
<td>43.887</td>
<td>2.280</td>
<td>3.895</td>
<td>2.540</td>
</tr>
<tr>
<td>Wheat L88-line</td>
<td>1.964</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Relative units derived from chromatographic peak areas
<table>
<thead>
<tr>
<th>Kernel composition</th>
<th>F₂-097-6 transgenic maize</th>
<th>F₂-144-8 transgenic maize</th>
<th>F₂-190-4 transgenic maize</th>
<th>Wheat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+¹</td>
<td>+/- *100</td>
<td>+</td>
<td>+/- *100</td>
</tr>
<tr>
<td>Total zeins</td>
<td>40,306</td>
<td>22,610</td>
<td>178</td>
<td>29,951</td>
</tr>
<tr>
<td>1Dx5 HMW</td>
<td>369</td>
<td>-</td>
<td>304</td>
<td>-</td>
</tr>
<tr>
<td>% Total nitrogen</td>
<td>1.49</td>
<td>1.14</td>
<td>130.7</td>
<td>1.50</td>
</tr>
<tr>
<td>% Total carbon</td>
<td>41.59</td>
<td>41.22</td>
<td>100.9</td>
<td>41.44</td>
</tr>
<tr>
<td>1Dx5 HMW / Zeins*100</td>
<td>0.92</td>
<td>-</td>
<td>1.01</td>
<td>-</td>
</tr>
<tr>
<td>1Dx5 in maize/1Dx5 in wheat*100</td>
<td>18.80</td>
<td>-</td>
<td>15.48</td>
<td>-</td>
</tr>
</tbody>
</table>

¹: expressing endosperm

²: non-expressing endosperm
Table 7. Correlation between total zeins, 1Dx5 HMW glutenin subunit, nitrogen content and carbon content of transgenic maize

<table>
<thead>
<tr>
<th>traits</th>
<th>Total* zein</th>
<th>1Dx5* HMW</th>
<th>% Total* nitrogen</th>
<th>% Total* carbon</th>
<th>Alpha* zeins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total zein</td>
<td>-</td>
<td>-0.37ns</td>
<td>0.87*</td>
<td>-0.20ns</td>
<td>0.999**</td>
</tr>
<tr>
<td>1Dx5HMW</td>
<td>-</td>
<td>-</td>
<td>0.06ns</td>
<td>0.73ns</td>
<td>-0.37ns</td>
</tr>
<tr>
<td>% Total nitrogen</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.20ns</td>
<td>0.87*</td>
</tr>
<tr>
<td>% Total carbon</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-0.19ns</td>
</tr>
</tbody>
</table>

*Values indicate of the correlation coefficient (r) for the indicated variables. One and two asterisks indicate statistical significance at the 0.05 and 0.01 levels, respectively, as determined by the t-test. A superscripted ns indicates a lack of statistical significance.
Table 8. HPLC Retention Times (min) of zeins and 1Dx5 in HPLC

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fy-097-6</th>
<th>Fy-144-6</th>
<th>Fy-190-4</th>
<th>B73</th>
<th>Wheat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expressing</td>
<td>Non expressing</td>
<td>Expressing</td>
<td>Non expressing</td>
<td>Expressing</td>
</tr>
<tr>
<td>Total zeins</td>
<td>18.40-76.67</td>
<td>18.44-76.74</td>
<td>18.41-76.68</td>
<td>18.40-76.67</td>
<td>18.41-76.68</td>
</tr>
<tr>
<td>Alpha</td>
<td>41.14-76.67</td>
<td>41.34-76.74</td>
<td>41.18-76.68</td>
<td>41.23-76.68</td>
<td>41.17-76.68</td>
</tr>
<tr>
<td>Beta</td>
<td>22.80</td>
<td>22.94</td>
<td>22.80</td>
<td>22.83</td>
<td>22.83</td>
</tr>
<tr>
<td>Gamma 27-kD</td>
<td>25.27</td>
<td>25.41</td>
<td>25.27</td>
<td>25.28</td>
<td>25.30</td>
</tr>
<tr>
<td>Gamma 16-kD</td>
<td>26.50</td>
<td>26.64</td>
<td>26.47</td>
<td>26.51</td>
<td>26.43</td>
</tr>
<tr>
<td>1Dx5 HMW</td>
<td>16.77</td>
<td>-</td>
<td>16.67</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
CHAPTER 5 GENERAL CONCLUSION

General conclusion

The Glu-1Dx5 gene from hexaploid wheat is contained on an 8.7 kb EcoRI genomic DNA fragment that includes the Glu-1Dx5 coding sequence (Anderson et al. 1989), 3.2 kb of 5’ flanking sequence and 1.2 kb of 3’ flanking sequence (Halford et al. 1992). The Glu-1Dx5 gene encoding the 1Dx5 high molecular weight glutenin subunit kernel storage protein that is specifically expressed only in wheat endosperm, and this protein is significantly responsible for the elasticity of wheat dough. Maize that was transformed by the Glu-1Dx5 gene gave us the opportunity to (i) develop efficient methods to screen or analyze phenotype and genotype of endosperm-specific transgenes without reducing the viability of the kernel, (ii) characterize the expression of this transgene in different organs of transgenic maize, (iii) study the inheritance of this transgene in transgenic maize to provide basic information for breeding programs, (iv) to study the effect of Glu-1Dx5 transgene expression on kernel composition of expressing and non-expressing kernel derive from the same ear.

In the first paper, efficient methods for determining the phenotype and the genotype of specific-endosperm transgene expression of individual maize kernel without destroying the viability of transgenic kernel are described. Kernels analyzed by these methods still have a high capacity for germination. The first step of the method is that part of the endosperm is removed from each kernel using a hand-held rotary grinder to prevent damage of the viable embryo. This way, we can control the amount of endosperm taken from the kernel-leaving enough stored nutrients in the kernel to get normal seedling
growth. The endosperm tissue is subjected to serial extraction; protein extraction for phenotypic analysis and DNA extraction for genotypic analysis. In phenotypic analysis, the endosperm is extracted with protein extraction buffer and the product of transgene expression is detected by SDS-PAGE or immuno-blotting. For an alternate protein analysis technique, the endosperm is extracted by the protein extraction buffer for ELISA. For genotypic analysis, the extracted endosperm is extracted to get DNA that is used as template DNA in PCR reactions to detect the transgene. This method is particularly useful for screening individual transgenic kernels in breeding programs and to test for gene silencing in each kernel.

In the second paper, we describe the production of transgenic maize plants containing a wheat *Glu-1Dx5* gene (including its regulatory region) encoding the 1Dx5 high molecular weight glutenin subunit. Analysis by SDS-PAGE demonstrated that a protein similar in size to the wheat 1Dx5 glutenin subunit accumulates in the endosperm of transgenic maize from four independent transformation events. This protein reacts with a monoclonal antibody specific to the wheat 1Dx5 HMW glutenin subunit but was not detected in non-transgenic controls or in pollen, anthers, leaves, young ears or embryos of plants grown from kernels expressing this protein in transgenic endosperm. Therefore, the characteristic of endosperm-specific *Glu-1Dx5* gene expression is the same as it is in wheat.

The phenotypic ratios of the wheat 1Dx5 HMW glutenin subunit in transgenic maize of four events were studied through three sexual generations. Reciprocal crosses with non-transgenic plants and self-pollinations were performed, and the resulting kernels
were analyzed for the presence of the 1Dx5 HMW glutenin subunit. These data, together with PCR analysis of two generations suggested that the transgene inheritance is non-mendelian. The transgene inheritance of 4 events is similar for three (F₁-F₃) generations. Two events had phenotypic ratio of 1:1 from the F₁ to F₃ generations. The transgene is inefficiently transmitted through pollen in all four events.

In the third paper, the maternal inheritance of Glu-1Dx5 transgene in maize allowed us to produce expressing (hemizygous) and non-expressing (null) near isogenic kernels on the same ear for study of the effect of the Glu-1Dx5 transgene protein on the kernel composition of transgenic kernels. Environmental variation was minimized by comparing between expressing and non-expressing transgenic kernels derived from the same ear.

Kernel weight of Glu-1Dx5 transgenic maize was not different between expressing and non-expressing transgenic plants or kernels. The 1Dx5 HMW glutenin subunit contents were 30.04 and 37.14 ng/mg in expressing endosperm of two events of transgenic maize when measure by ELISA. This accounts for 0.003 % and 0.0037% of the endosperm respectively. The 1Dx5 HMW glutenin subunit in endosperm of transgenic maize accounted for from 0.92% to 3.82% of total zeins by HPLC analysis. The level of 1Dx5 HMW glutenin subunit in transgenic maize was lower than native 1Dx5 HMW glutenin subunit in wheat endosperm. The Glu-1Dx5 transgene expression in transgenic maize endosperm affected the nitrogen content and the levels of zeins in both expressing endosperm and non-expressing kernels.
The results presented here illustrate the potential of using the Glu-1Dx5 gene from wheat for the improvement of maize. The transgenic plants exhibited some traits that could useful in maize, and some traits that were unexpected. It is clear that there is great potential in this technology, and equally clear that there is much to learn before genes from other species can be used routinely for crop improvement. It is my hope that these results will be valuable to others interested in using this technology.

**Recommendations for Future Research**

i) Highly inbred transgenic lines should be produced to compare transgenic kernels and non-transgenic kernels derived from the same ear.

ii) Immunolocalization of 1Dx5 HMW glutenin subunit protein should be performed to study where protein accumulates.

iii) Genome-wide expression studies should be conducted to determine the effect of the Glu-1Dx5 transgene on the expression of endogenous genes in maize.

iv) The effect of 1Dx5 HMW glutenin subunit on corn flour property should be studied.
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Corn Industries Research Foundation (1937) Corn in Industry. The Foundation, New York, 63 pp


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expressing 1Ax and 1Dx high molecular weight glutenin subunit transgenes. J Agric Food Chem 49:395-401


