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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 seeds 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 that the transgene is inefficiently transmitted through pollen in all four events.

Keywords *Zea mays* L. · Transgenic plants · HMW glutenin · Gene expression

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Introduction

Wheat flour is different from other cereal flours, including maize, because it contains gluten that gives it the elasticity and extensity required for breadmaking (Barro et al. 1997). Gluten consists mainly of two types of seed-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 are important determinants of 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 this combination 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 8.2-kb *Eco*RI 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 *Escherichia coli* (Galili 1989), tobacco (Roberts et al. 1989), wheat (Altpeter et al. 1996; Blechl and Anderson 1996; Barro et al. 1997; Alvarez et al. 2000) and tritordeum (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 methods

Plasmids

Two plasmids were used for co-transformation of maize. The first plasmid, pHMW1Dx5, is derived from pUC9 and contains an 8.7-kb *Eco*RI genomic DNA fragment from hexaploid bread wheat 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 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 the polymerase chain reaction (PCR) for the presence of a portion of the 1Dx5 coding sequence. Callus lines containing this sequence were regenerated to produce T_0 plants. In most cases, several T_0 plants resulted from each transformed callus. Pedigrees are based on the event and the T_0 plant; for example, 097-2 is the second T_0 plant derived from event 097.

Plant growth and maintenance

The F_1 generation was produced by matings between a T_0 plant and the inbred line B73. Throughout this manuscript, the designation "F_1" refers to progeny produced by such matings, and an F_1 family is the F_1 kernels from one ear, or plants derived from these kernels. The F_1 and F_3 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 h each day. Kernels were planted in 2-gallon pots prepared as follows. Pots were filled half-full with Universal Mix soil (Consumers Supply Corporation Iowa) 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 Corporation Iowa), in a greenhouse. Two weeks after planting, the seedlings were transplanted to the field.

SDS-PAGE

Endosperm material from individual 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 were loaded in each well, and electrophoresis was carried out at 150 V for 1.5 h. Gels were stained with 0.1% coomassie blue-R-250 in 1% acetic acid and 40% ethanol for 0.5 h and de-stained 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 with SDS-PAGE (Sangtong et al. 2001). Leaf DNA was prepared using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn.). PCR was carried out using 5 µl of the purified DNA as a template and primers specific to the 1Dx5 high-molecular-weight glutenin subunit (1Dx5F: 5′-gagatcgataccatggctaagcggttagtcc-3′ and 1Dx5R: 5′-tgctgcggacaagttacactt-3′) via a touchdown PCR protocol (Senior and Heun 1993) (the amplified fragment is labeled "probe" in Fig. 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 (See Fig. 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 plasmid contains a wheat genomic DNA fragment including the 1Dx5 coding sequence and flanking sequences (Fig. 1). Embryogenic callus derived from the genotype Hi-II was used as a substrate for bombardment. 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 ears containing F_1 kernels. The kernels from a single ear comprise an F_1 family. Most events yielded several T_0 plants that are likely to be clonally related and each F_1 family is derived from a cross between an individual T_0 plant and B73, 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 im-

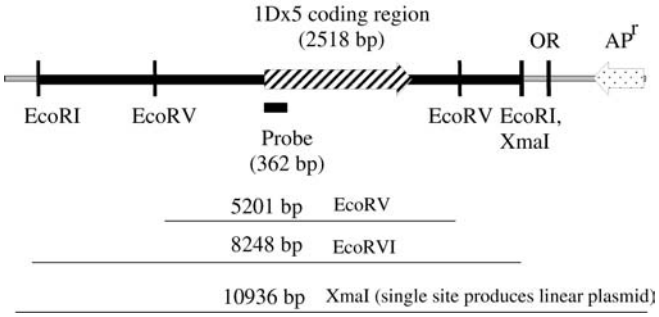

Fig. 1 Schematic diagram of the Glu-1Dx5 plant transformation construct. The upper bar represents the plasmid used in particle bombardment, presented in linear form using an arbitrary linearization site. *Black areas* represent wheat genomic DNA, and the *cross-hatched arrow* represents the 1Dx5 coding 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. *Xma*I cuts the plasmid at one site, so the expected fragment is the length of the plasmid

Table 1 Results of screening five F_1 kernels ears resulting from crosses between a T_0 plant and B73 using SDS-PAGE to identify 1Dx5 HMW glutenin-expressing kernels

Event number	Number of ears screened	Number of ears with at least one positive kernel
020	2	0
097	6	6
132	1	0
144	3	2
171	1	0
174	2	0
177	4	0
181	7	0
182	4	3
187	12	0
190	2	2
200	9	0
N = 12	Total = 54	Total = 13

muno-blot analysis to detect the 1Dx5 HMW glutenin. Endosperm extracts of some F_1 transgenic kernels contained a novel protein that migrated near the 116-kDa marker in SDS-PAGE in reducing conditions. This is different from the 88 kDa predicted from the cDNA sequence, but HMW glutenins are known to exhibit anomalous migration in SDS-PAGE (Shewry et al. 1992). 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. When protein extracts from nontransgenic endosperm were analyzed by the same methods, no protein is detected in the same position by SDS-PAGE, and there was no detectable immunological reaction with the 1Dx5 monoclonal antibody in Western-blot analysis. Similar results were obtained using kernels from the F_2 and F_3 generations (Fig. 2). The immunoreactive bands in maize migrate closely with bands from

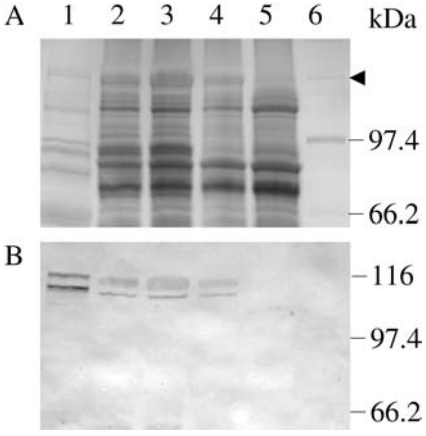

Fig. 2 **A** Reducing SDS-PAGE analysis with coomassie blue R-250 stain of transgenic F_3 endosperm. **B** Immuno-blot analysis of transgenic F_3 endosperm visualized with a monoclonal antibody specific to the 1Dx5 HMW glutenin. Two gels from the same batch were electrophoresed together in the same apparatus. One was blotted and the other was stained. Corresponding regions of the blot and the gel are shown. *Lane 1* Wheat containing Glu1-Dx5. *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. The *arrowhead* indicates the band that was scored for the 1Dx5 expression phenotype

wheat endosperm that react with the 1Dx5 monoclonal antibody as well (Fig. 2A and B, 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 corn 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 suggest that the band in transgenic maize is a product of the wheat Glu-1Dx5 transgene. In the subsequent analyses presented here, we used the presence or absence of the SDS-PAGE band indicated in Fig. 1 to identify 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 3,000 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 in five kernels from each F_1 family. By analyzing five F_1 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 a cross between a hemizygous T_0 plant (Glu-1Dx5/–) and B73 (–/–) if Glu1-Dx5 were inherited as a single dominant locus. 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 F_1 kernels using SDS-PAGE (Table 2). Chi-square tests were performed to determine if the segregation ratios obtained for each F_1 family were consistent with a 1:1 segregation ratio. 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.

Because there was an excess of negative progeny in F_1 families that did not show a 1:1 segregation ratio (Table 2), it was important to determine if the negative progeny inherited DNA sequences derived from the Glu1-Dx5 gene. Endosperms of 16 F_1 kernels from the cross 097-2 × 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 ten of these 16 kernels contained DNA derived from the transgene. Two other F_1 families, 097-8 × B73 and 190-2 × B73, were subjected to similar analyses. In these families, the genotypic ratios corresponded to the phenotypic ratio, which was not statistically different from 1:1. By comparing PCR data with the SDS-PAGE data, it is apparent that some F_1 progeny in the 097-2 family have inherited a nonfunctional version of the transgene, because some kernels with positive PCR results do not produce the 1Dx5 protein. Thus, PCR-based assessment of the Glu-1Dx5 gene from the three families (097-2, 097-8 and 190-2) indicated that their progeny segregated with a 1:1 (presence:absence) ratio for sequences derived from the Glu-1Dx5 transgene.

Table 2 SDS-PAGE analysis of 1Dx5 HMW glutenin expression in every kernel from the 15 F_1 families containing positive kernels[a]

Event	T_0 Plant	Analyzed kernels	Positive kernels	Phenotypic ratio[b] 1 positive: 1 negative
097	2	41	9	No**
	3	19	4	No*
	4	31	15	Yes^{ns}
	5	16	1	No**
	7	28	2	No**
	8	35	19	Yes^{ns}
144	3	12	4	Yes^{ns}
	4	*24*	*0*	*No***
	8	10	5	Yes^{ns}
182	1	33	12	Yes^{ns}
	2	59	29	Yes^{ns}
	4	31	2	No**
	6	14	9	Yes^{ns}
190	2	25	10	Yes^{ns}
	4	15	7	Yes^{ns}
n = 4	n = 15	Total = 409	Total = 136	No = 6 families Yes = 9 families

[a] 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

[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

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 F_1 kernels from four independent events and analyzed by Southern-blot hybridization using a portion of the Glu-1Dx5 gene as a probe (Fig. 3). The transgene insertions are different in each event. 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 that 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 Fig. 3. All events but 097 have a band in this area when genomic DNA is digested with *Eco*RI or *Eco*RV.

The Glu-1Dx5 gene exhibits reduced male transmission in transgenic maize

Of the 15 T_0 plants from events containing expressing kernels, only one plant (event 144, T_0 plant 4, Table 2) failed to produce at least one F_1 kernel expressing Glu-1Dx5 when crossed with B73. This was the only family in which the T_0 plant was used as the male when crossed with B73. The families derived from 144-3 and 144-8 are from the same event as 144-4 and produced F_1 progeny with 1:1 segregation ratios when their T_0 plants were crossed as females with B73. One possible explanation for this observation is that the Glu-1Dx5 gene exhibits maternal inheritance in maize. To test this explanation, we studied the inheritance and expression of Glu-1Dx5 in more detail in the next generation. Two different approaches were used. First, progeny of reciprocal crosses between B73 and 1Dx5 expressing F_1 plants from events 097 and 190 were evaluated. Second, 1Dx5 expressing F_1 plants from all the expressing events (097, 190, 182 and 144) were self-pollinated to produce F_2 kernels, and

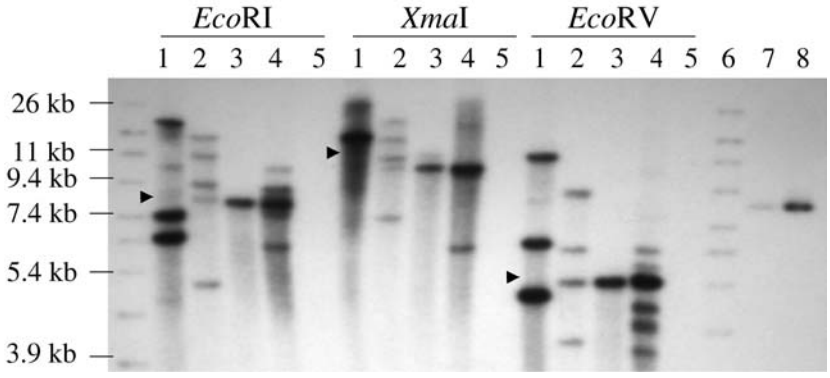

Fig. 3 Genomic Southern-blot analysis of transgenic F_1 plants. Genomic DNA of transgenic plants was digested with *Eco*RI, *Xma*I or *Eco*RV and hybridized with a 362-bp PCR product amplified from the coding sequence of the Glu-1Dx5 gene. *Sample 1* F_1 plant of event 097-3. *Sample 2* F_1 plant of event 144-3. *Sample 3* F_1 plant of event 182-1. *Sample 4* F_1 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 Fig. 1)

Table 3 SDS-PAGE analysis of 1Dx5 HMW glutenin expression in kernels derived from reciprocal crosses between F_1 transgenic plants and B73

Female parent	Male parent	Analyzed kernels	Pos.	Neg.	Phenotypic ratio[a] 1 positive: 1 negative
(097-2/B73) F_1-11[b]	B73	56	31	25	Yes^{ns}
B73	(097-2/B73) F_1-11	56	0	56	No**
(097-4/B73) F_1-6	B73	56	28	28	Yes^{ns}
B73	(097-4/B73) F_1-6	56	0	56	No**
(190-2/B73) F_1-18	B73	56	16	40	No*
B73	(190-2/B73) F_1-18	56	0	56	No*

[a] 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

[b] Pedigress indicate the F_1 family, for example (097-2/B73), and F_1 plant number, for example F_1-11

the same 1Dx5 expressing-F_1 plants were also crossed as males to B73 to produce back-cross F_1 kernels. All ears produced from these crosses had normal seed set.

The results of SDS-PAGE analyses of kernels resulting from reciprocal crosses between B73 and 1Dx5-expressing F_1 plants from events 097 and 190 are summarized in Table 3. The 1Dx5 protein was not detected in the progeny when the 1Dx5-expressing transgenic plant was the male parent. When a 1Dx5-expressing transgenic F_1 plant was used as the female parent, however, about half of the resulting kernels contained 1Dx5 HMW glutenin. These data are consistent with a model in which the Glu-1Dx5 gene is transmitted from a 1Dx5-expressing, heterozygous F_1 plant through female gametes but not by male gametes.

When 1Dx5-expressing F_1 plants were self-pollinated, F_2 progeny from five F_1 plants representing four events were analyzed for expression of the 1Dx5 HMW glutenin by SDS-PAGE (Table 4). All F_2 segregation ratios were significantly different from a 3:1 (positive:negative) ratio and were characterized by an excess of 1Dx5 negative kernels. However, the results for F_2 progeny of three F_1 families from events 097 and 190 fit a 1:1 segregation ratio. The segregation ratios for F_2 progeny of two F_1 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 using pollen from the same 1Dx5-expressing plants that were self-pollinated are also summarized in Table 4. Three crosses with 1Dx5-expressing F_1 plants from events 097 and 190 did not produce any 1Dx5-positive kernels. Two crosses with 1Dx5-expressing F_1 plants from events 144 and 182 had some 1Dx5-positive kernels, but the ratio was significantly less than 1:1.

The genotypic ratios in some of the kernels resulting from self-pollination of 1Dx5-expressing F_1 plants from events 097 and 182, and out-crossing 1Dx5-expressing F_1 plants from events 097 and 182 as males 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 F_2 plants grown from 1Dx5-containing seeds from four events were self-pollinated to produce F_3 kernels and analyzed by SDS-PAGE to detect 1Dx5 HMW glutenin in the endosperm. Thirteen of these F_3

Table 4 SDS-PAGE and PCR analyses of the Glu-1Dx5 gene in kernels derived from self-pollination and out-crossing of F_1 transgenic plants to B73

Pedigree	Pollination	SDS-PAGE analysis				PCR analysis			
		n[a]	Pos.	Neg.	Ratio[b] = 1:1	n	Pos.	Neg.	Ratio[b] = 1:1
(097-2/B73) F1-22-F2[c]	Self	56	22	34	Yes^{ns}	–	–	–	–
B73//(097-2/B73) F1-22	Cross	56	0	56	No**	–	–	–	–
(097-4/B73) F1-7-F2	Self	56	30	26	Yes^{ns}	31	15	16	Yes^{ns}
B73//(097-4/B73) F1-7	Cross	56	0	56	No**	32	0	32	No**
(190-2/B73) F1-5-F2	Self	56	30	26	Yes^{ns}	–	–	–	–
B73//(190-2/B73) F1-5	Cross	56	0	56	No**	–	–	–	–
(182-4/B73) F1-22-F2	Self	55	15	40	No*	48	12	36	No*
B73//(182-4/B73) F1-22	Cross	56	4	52	No**	50	3	47	No**
(144-3/B73) F1-4-F2	Self	42	14	28	No*	–	–	–	–
B73//(144-3/B73) F1-4	Cross	56	2	54	No**	–	–	–	–

[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
[c] Pedigress are as described in Table 4 with the addition of the F_2 plant number for self-pollinations, and the cross for cross-pollinations. For example, B73//(097-2/B73) F_1-22 indicates a cross of B73 with the F_1 plant (097-2/B73) F_1-22

families fit a 1:1 (presence:absence of 1Dx5) phenotypic segregation model. Three F_3 families did not fit a 1:1 segregation model, due to an excess of negative kernels (Table 5). The phenotypic ratios were similar in the F_2 and F_3 generations, indicating that the inheritance of the transgene is stable through two generations.

Thus, three observations are consistent with an inheritance model in which the Glu-1Dx5 transgene exhibits reduced male transmission. (1) Glu-1Dx5 expression was observed in resulting progeny when 1DX5-expressing F_1 plants were used as the male parent and not when the same plants were used as the male parent and not when the same plants were used as the female parent in reciprocal crosses between nontransgenic B73 and 1Dx5-expressing F_1 plants, (2) F_2 progeny resulting from self-pollination of 1DX5-expressing F_1 plants do not segregate for transgene expression with a 3:1 ratio, but do segregate with a 1:1 ratio in some families, and (3) crosses using pollen from the same 1DX5-expressing F_1 plants that were self-pollinated to produce the F_2 plants only occasionally result in expressing kernels. Pollen transmission of the Glu-1Dx5 gene from events 097 and 190 has never been observed, while pollen transmission of the Glu-1Dx5 gene from events 144 and 182 was observed to occur with reduced efficiency. Furthermore, because lines from events 144 and 182 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 in these events. Male transmission is reduced to a greater extent than female transmission in these events, however.

Table 5 SDS-PAGE analysis of 1Dx5 HMW glutenin expression in kernels of 16 F_3 families representing four events

Pedigree	n[a]	Positive	F3 Phenotypic ratio = 1:1[b]
(097-2/B73) F_1-3-F_2-27-F_3[c]	35	18	Yes^{ns}
(097-6/B73) F_1-1-F_2-20-F_3	20	9	Yes^{ns}
(097-6/B73) F_1-1-F_2-17-F_3	15	10	Yes^{ns}
(097-6/B73) F_1-1-F_2-17-F_3	15	10	Yes^{ns}
(144-3/B73) F_1-3-F_2-37-F_3	20	2	No**
(144-8/B73) F_1-4-F_2-3-F_3	20	17	Yes^{ns}
(144-8/B73) F_1-4-F_2-7-F_3	25	14	Yes^{ns}
(182-1/B73) F_1-3-F_2-11-F_3	25	12	Yes^{ns}
(182-1/B73) F_1-3-F_2-55-F_3	15	1	No**
(182-1/B73) F_1-3-F_2-8-F_3	35	10	No*
(182-1/B73) F_1-3-F_2-36-F_3	10	4	Yes^{ns}
(182-2/B73) F_1-4-F_2-6-F_3	20	10	Yes^{ns}
(190-4/B73) F_1-2-F_2-52-F_3	20	8	Yes^{ns}
(190-4/B73) F_1-2-F_2-43-F_3	25	11	Yes^{ns}
(190-4/B73) F_1-2-F_2-16-F_3	10	6	Yes^{ns}
(190-4/B73) F_1-2-F_2-131-F_3	15	8	Yes^{ns}

[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
[c] Pedigress are as defined in Table 4 with the addition of the F_2 plant number

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

We used both SDS-PAGE and immuno-blot analysis to analyze several tissues in plants from families known to contain plants expressing 1Dx5 in the endosperm. The results of these analyses are summarized in Table 6.

Endosperms and embryos of 85 mature kernels from eight F_3 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 F_3 families were harvested at 14 days after planting. Twelve of these plants were grown from kernels containing 1Dx5 in the endosperm. In addition, leaf tissues from 14 F_3 plants from eight F_3 families were harvested at 58 days after

Table 6 SDS-PAGE and immuno-blot analysis of 1Dx5 HMW glutenin expression in different tissues of transgenic maize

No. of events	No. of families	n[a]	Number of samples that have 1Dx5 HMW subunit expression						
			Endosperm	Embryo	14-day leaf	58-day leaf	58-day anther	Mature pollen	Young ear
4	8 F_3	85	36	0	–	–	–	–	–
3	6 F_3	30	12	–	0	–	–	–	–
4	8 F_3	14	14	–	–	0	–	–	–
4	14 F_3	14	14	–	–	–	0	–	–
3	4 F_2, 2 F_1	6	6	–	–	–	–	0	–
4	9 F_3	9	9	–	–	–	–	–	0

[a] Number of analyses, representing the indicated number of events and families

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 F_3 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 F_2 plants and two F_1 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 F_3 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 immunoblot 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 the wheat 1Dx5 high-molecular-weight glutenin in its kernels. 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.

From this study we conclude that: (1) 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, (2) 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 the 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 the endosperm. Also, in both species 1Dx5 is a major seed protein, clearly visible on a Coomassie blue-stained SDS-PAGE gel. These observations suggest that the wheat promoter functions similarly in wheat and in corn, underscoring the similarities that exist among these cereal species in transcriptional machinery.

A number of plant transformation experiments have reported plants with a mixture of non-Mendelian and Mendelian inheritance of the transgene (Christou et al. 1989; Tomes et al. 1990; Spencer et al. 1992; Walters et al. 1992; Hiei et al. 1994; Register 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 Glu-1Dx5 gene has been confined to seeds 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 embryos, 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 F_2 plants were not consistent with cytoplasmic/organellar inheritance, however. The F_2 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 *Avena* 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 less competitive 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 seed production is important, male sterility systems can be useful for seed 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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