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ABSTRACT

Seed tissues (endosperm, embryo, and pericarp) are often separated into tissue-enriched fractions by wet- or dry-milling methods for use in food, feed, and industrial products. Seed tissue markers that are sensitive and readily quantifiable would be useful to optimize fractionation processes. To meet this need for tissue markers, we set out to produce and characterize different transgenic maize lines, each containing green fluorescent protein (GFP) in either endosperm or embryo. We examined mRNA transcripts using expressed sequence tag (EST) profiles of several major seed proteins and selected several with strong seed tissue preferences. Stably transformed maize lines were produced, and visual observation of fluorescence confirmed the presence of GFP in the desired tissues. To establish the utility of this grain for evaluating the effectiveness or separation efficiencies of fractionation processes, transgenic kernels were hand-dissected into pericarp, endosperm, and embryo fractions and the GFP concentration in each fraction was determined. The GFP distribution between fractions of each transgenic event was calculated from GFP concentration and mass balance, which enabled the determination of GFP yield based on the hand-dissection fractionation data and the amount of tissue contamination in each fraction. Our transgenic lines exhibited strong tissue preference for either embryo or endosperm. These lines should be useful for assessing separation efficiencies in maize fractionation processes.

Maize grain is an important commodity used for food, feed, energy, and industrial products. These different uses utilize different fractions and properties of the grain. For example, ethanol production for motor fuels benefits from high fermentable starch content; food production from corn flaking grits and brewer's grits benefits from high content of hard endosperm; and feed production benefits from high protein and high oil contents. Maize kernels are composed of several tissues including embryo, endosperm, aleurone, and pericarp (Kiesselbach 1949). These tissues serve different biological functions and therefore have different chemical compositions. One way to increase the value of maize grain is to separate the kernel components into tissue-enriched fractions by milling. These fractions can then be used for different purposes for which they are well-suited.

To optimize the efficiency of separating different maize seed tissues, researchers have used seed tissue markers such as oil and density (Yildirim et al 2002), biochemical markers such as phenolic acid (Antoine et al 2004), or ash content (Pomeranz 1987). One problem with using native compounds as markers is that they often are not strictly tissue-specific, limiting their use for evaluating fractionation processes. To improve our ability to separate seed tissues, a seed tissue marker with improved tissue specificity that is sensitive and easily quantifiable is necessary.

Green fluorescent protein (GFP) is a versatile marker protein requiring no substrate to fluoresce that has been expressed in many organisms and tissue types. GFP fluorescence is directly proportional to the amount of GFP present and can be easily quantified (Southward and Surette 2002). GFP is commonly used in molecular biology studies involving protein trafficking, subcellular structure identification, protein interactions, and intracellular protein targeting (Ehrhardt 2003). In addition, GFP has been used as a marker protein in plant transformation and as a tool to measure transgene spread in the environment (Stewart 2005). It is well-suited to serve as a seed tissue marker in grain fractionation studies.

The development of a tissue marker gene requires a strong promoter with tissue preference to drive expression. Maize endosperm contains families of distinct proteins called zeins that account for 70% of maize endosperm protein content (Zarkadas et al 2000). Transcription of the genes encoding these proteins is specific to the endosperm (Bianchi and Viotti 1988). The most abundant and well-characterized zeins, separated based on molecular weight, are α-zeins, consisting of the 19,000 Da zeins (19zn), the 22,000 Da zeins (22zn), and the γ-zeins which include the 27,000 Da zeins (27zn) (Coleman and Larkins 1999). Promoters of these genes were used previously to express marker genes (Russell and Fromm 1997) and recombinant proteins (Chikwamba et al 2003).

The embryo contains globulin storage proteins that accumulate in protein bodies during early seed development (Liu and Kriz 1996). The globulins accumulate 10–20% of the protein content in mature embryo, with Globulin-1 (Glb1) accumulating the highest levels and comprising one-half of the total globulin content (Kriz 1989). Unlike the large gene families of globulins in other cereal crops such as wheat and oats, the maize Glb1 is present in one copy in the genome (Belanger and Kriz 1989). Expression of Glb1 occurs in the embryo, with a small amount of Glb1 expressed in the endosperm and pericarp/aleurone layer (Kriz 1999). The Glb1 promoter is attractive for use in the development of a tissue marker because of its strong preference for embryo expression, its developmental accumulation, and its high expression level despite having only one copy in the genome.

In the present study, our objectives were to create and characterize new maize transgenic lines that produce GFP preferentially in either embryo or endosperm and to determine whether GFP in these lines will be useful as a tissue marker in grain fractionation studies. By making transcriptional fusions to well-characterized maize seed storage protein promoters, we created transgenic plants that expressed GFP in either the embryo or endosperm tissue. To illustrate the utility of these lines as tissue markers in grain fractionation studies, we hand-dissected kernels from these lines, separated bran, embryo, and endosperm, and examined the GFP concentration of the fractions. With this information, we were able to determine the efficiency of fractionation of the hand-dissection procedure. In addition to being useful as tissue markers in grain fractionation studies, these lines will be useful for studies of kernel development and protein deposition.

MATERIALS AND METHODS

Expression Analysis by Comparing EST Frequency

Expression analysis was performed on expressed sequence tags (EST) from tissue-specific cDNA libraries located at MaizeSeq (http://www.maizeseq.com). These libraries contain large collections of sequences from various tissues, and EST frequencies can be calculated and compared with each other. A table of frequencies of EST correlating to major maize seed storage genes was

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made by determining the proportions of EST found by using Blastn with a 95% threshold for a specific gene in a specific tissue.

DNA Constructs

Plasmid pAct1IsGFP-1 (Cho et al 2000) was used to prepare all constructs used for transformations. pAct1IsGFP-1 contains the synthetic green fluorescent protein (sGFPs65T) gene (Chiu et al 1996) and nos terminator sequences. The promoters containing important elements necessary for transcription and ending with the start codon were amplified by polymerase chain reaction (PCR) using genomic DNA from inbred line Va26 (19zn primers: forward TCTGTGTCACAACTCAACTGTC; reverse CACCATGG TTTGGCTGCC, 22zn primers: forward GGATCCGATCCGGCG C AG; reverse GGAGCATTGTGGAATAATG; 27zn primers: forward CGATCGTCCCGTCCGCGTCAATA; reverse GGTGTCGATCGG GTTCTTCTG). The Glb1 promoter was amplified from a Glb1 containing construct obtained from J Widholm (University of Illinois) (Glb1 primers: forward GCTTGCCGAGTGCCATCCT TG; reverse GGGTTGGCTGTATGCAGAAG). The 19zn (GenBank accession # EF061091), 22zn (GenBank accession # EF061092), 27zn (GenBank accession # EF061093), and the Glb1 (GenBank accession # EF061094) PCR products were then inserted in pActIsGFP-1 using restriction sites XhoI and NcoI so that each maize gene was fused to GFP at the translational start codon (Fig. 1).

Plant Transformation and Plant Material

Stable transformation of maize plants was accomplished at the Plant Transformation Facility at Iowa State University using a previously described microprojectile bombardment method (Frame et al 2000). Herbicide resistant T0 callus cells were screened for the presence of the transgene by PCR (Glb1 primer: forward CCA CCATTAGCTCTCCTGTTTAG; GFP reverse CGTCCAGCTCGA CCAGGATG; 27zn primer: forward CTTAACAACTCACAGAA CATCAAC; GFP reverse CGTCCAGCTCGACCAG GATG; 19zn primer: forward GTGGAAAATAGCCAAACCAA GC; GFP reverse CGCCGTAGGTGAAGGTGGTC; 22zn primer: forward GCATT CTAGGATTTCAATTAGTC; GFP reverse CGTCCAGCTCGAC-CAGGATG) using the GoTaq Master Mix (Promega, Madison, WI) and transgene positive calli were regenerated to plants. T0 plants were crossed to the inbred B73 to create F1 kernels. F1 kernels were self-pollinated or back-crossed to B73 to obtain F2 kernels or BC1F1 kernels, respectively. F3 kernels were created by selfing F2 kernels. After three generations of selfing or backcrossing and visual evaluation of GFP expression, nine events were selected for plant breeding and analysis. F3 kernels were used for visual and quantitative fluorescence analysis.

Evaluation of Transgene Copy Number

Quantitative real-time PCR analyses were performed using the MX3000P real-time PCR system (Stratagene, La Jolla, CA). A PCR reaction containing 12 µL of Brilliant SYBR Green master mix (Stratagene, La Jolla, CA), 12 µL of dd H_2O, 1 µL of each primer (0.5 µ*M* final concentration), and 1 µL of DNA (6 ng total DNA) was conducted at conditions of 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 60 sec, and 72°C for 30 sec. Each reaction was run in triplicate.

Quantification of transgene copy number was performed using the relative quantitative method in which a target gene is compared with a known endogenous gene (Ginzinger 2002). The target genes in the present study were the GFP transgene (primers: forward CCTCGTGACCACCTTCACCTA; reverse ACCATGTGA TCGCGCTTCT) and the endogenous genes used for comparison were Glb1 present at one copy in the genome (Kriz 1989) (primers: forward CACTGTGGAACACGACAAAGTCTG: reverse CTCACCATGCTGTAGTGTCACTGTGAT) and the 27zn which is present at 1–2 copies in the genome (Das and Messing 1987) (primers: forward ATTGCACGTCAAGGGTATTGG; reverse TCTT GTGTTCTATGCCACCGA). PCR efficiencies were >90% using standard curves of a dilution series for the GFP transgene and endogenous Glb1 and 27zn genes by using the method of Bubner et al (2004).

Quantification of Fluorescence in Stably Transformed Kernels

Twenty F3 kernels from each transformation event were selected at random and ground together to a fine consistency for determination of fluorescence levels. Three 28-mg samples from each event were each placed into a well of a black, 96-well flat-bottom assay plate (Corning, Corning, NY). Fluorescence of dry ground samples in 96-well plates were measured using a spectrafluorometer (Tecan, Mannedorf/Zurich, Switzerland) at 485 nm excitation wavelength and 535 nm emission wavelength. Mean fluorescence levels were calculated for each event and the nontransgenic control inbred line B73.

Conservative Sampling of Transgenic Maize Seed Tissues

To determine the fluorescence levels of endosperm and embryo tissues in the transgenic maize lines, we took conservative samples of each tissue and measured the fluorescence. These conservative samples consisted of a small amount of tissue that was taken from the center of the tissue to be as free as possible of contamination from other tissues. Conservatively sampled embryo and endosperm tissues harvested from the 19zn (event 228-3), 27zn (event 230-3), and Glb1 (event 231-23) transgenic lines and the B73 nontransgenic inbred line control were ground, and three

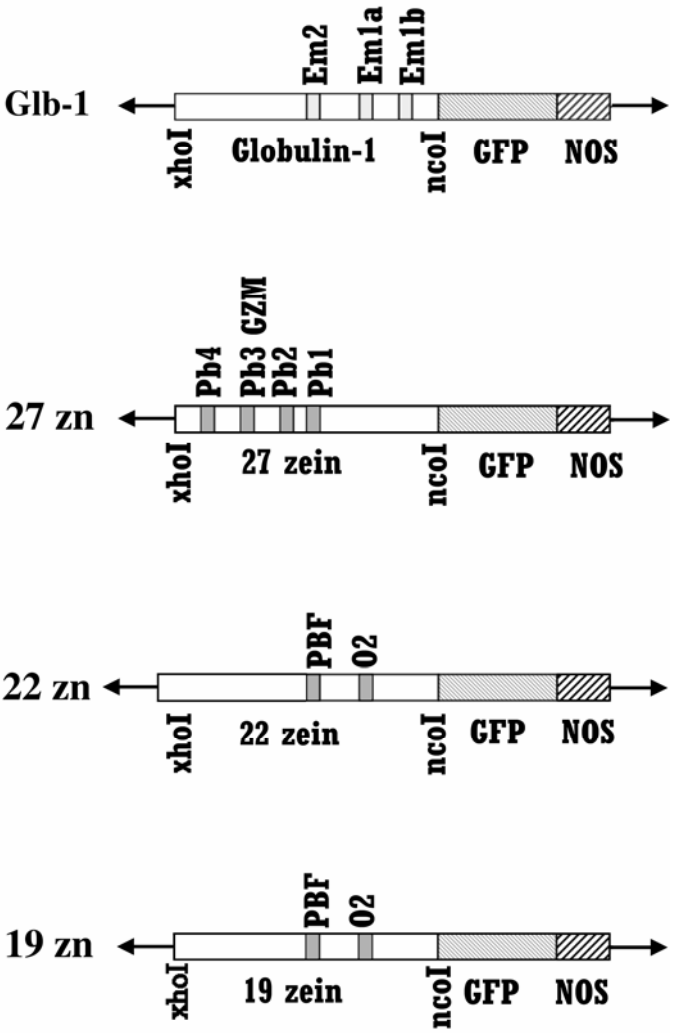

Fig. 1. Constructs used in stable transformation experiments. Detailed are known transcription factor binding sites that direct expression to embryo (Glb1) and endosperm (27zn, 22zn, 19zn) of maize seed tissues.

10-mg portions were each placed into a well of a black, 96-well flat-bottom assay plate (Corning). To each well, 100 μL of GFP extraction buffer was added and the fluorescence was measured as described above. Fluorescence values were converted to mass of GFP by using a standard curve consisting of B73 inbred line tissue spiked with known amounts of recombinant GFP (Clontech, Mountain View, CA).

Hand-Dissection and Analytical Procedures

Whole kernels, soaked overnight in water, were fractionated by hand-dissection to produce as pure as possible pericarp, embryo, and endosperm fractions. Unlike the conservative sampling procedure described above, in hand-dissection, the entire kernel was divided into fractions, and therefore some degree of contamination between tissues was unavoidable. This procedure more closely reflects commercial grain processing procedures than does the conservative sampling procedure. Fractions were dried at 34°C for two days, ground to a fine consistency, and moisture and GFP contents of the dried samples were measured. GFP fluorescence was quantified as described above for the stably transformed kernels. The moisture contents of the fractions were determined according to Approved Method 44-19 (AACC International 2000). Crude free-fat contents were determined according to Approved Method 30-25 (AACC International 2000). All measurements were performed in duplicate, except for moisture, which was measured once. Mass-balance data were determined on a moisture-free basis.

TABLE I
Expression Level of Major Seed Storage Proteins in Maize Using Expressed Sequence Tag (EST) Frequency

	EST Frequency (%)				
Name	Endosperm	Embryo	Immature Leaf/Seedling	Tassel	Leaf
Glb1	0.03	0.45	0.05	0.05	0.01
Glb2	0.02	0.59	0.24	0.00	0.00
Oleosin	0.08	0.65	0.00	0.17	0.00
α-Globulin	0.24	0.00	0.01	0.00	0.07
19zn	0.15	0.0	0.01	0.00	0.00
22zn	0.13	0.01	0.00	0.00	0.02
27zn	0.12	0.01	0.00	0.00	0.01
Actin-1	0.05	0.03	0.02	0.07	0.01

TABLE II
Constructs and Transformation Events of Green Fluorescent Protein (GFP) Transgenic Lines

Construct	Event	No. of Events	Selected Positives
19zn	P228	18	3
22zn	P229	13	2
27zn	P230	15	1
Glb1	P231	16	3

TABLE III
Transgene Copy Number Estimated by Real-time Quantitative Polymerase Chain Reaction (PCR)

Construct	Event	Copy Number	SD[a]
19zn	P228-3	3	0.21
19zn	P228-29	2.5	0.44
19zn	P228-49	2	0.40
27zn	P230-3	2	0.52
27zn	P230-71	1	0.31
Glb1	P231-23	3	0.32
Glb1	P231-24	13	0.15
Glb1	P231-27	1	0.09
22zn	P229-18	2	0.12

[a] Standard deviation.

RESULTS

Expression Analysis of Major Seed Storage Proteins Based on EST Frequencies

We investigated Maize EST (MaizeSeq, http://www.maizeseq.com) and counted the number of EST of each seed storage protein gene in libraries from embryo, endosperm, leaf, immature leaf, and tassel (Table I). The frequency of EST was the percentage of EST from the combined libraries that correlate with each seed storage gene based on the Blastn results. In this analysis, the frequency of the EST represented the expression level of each gene.

One of the key questions regarding maize seed storage protein genes was whether the genes are tissue-specific. The presence of EST can indicate whether the corresponding gene was expressed. The native Glb1 message is present in embryo and endosperm tissue (Kriz 1989) and this was confirmed by the EST analysis of tissue-specific EST libraries (Table II). Interestingly, EST of the native Glb1 gene were also found in the immature leaf and in the tassel tissues of the maize plant, tissues not reported to express Glb1, indicating Glb1 was not expressed only in the seed. From the EST analysis, however, Glb1 expression was clearly embryo-preferred. The native 19zn, 22zn, and 27zn genes were expressed mostly in the endosperm tissue as shown in Table II, yet very low expression levels were present in the embryo, immature leaf, and leaf. Other seed-expressed genes such as oleosin, α-globulin, and globulin-2 were analyzed for EST frequency in several tissue-specific libraries. These genes have expression preferences for either embryo or endosperm tissue; however, they are also expressed in other tissues such as immature leaf, tassel, or leaf tissues. These data show that seed storage protein genes do not have strictly tissue-specific expression but have tissue-preferred expression, with expression occurring in multiple tissues in the plant.

Selection of GFP Expressing Clones from Stable Maize Transformation

To create transgenic maize plants expressing GFP in the seed tissues, the constructs illustrated in Fig. 1 were transformed into maize plants using biolistic transformation (Frame et al 2000). Kernels from each independent transformation event were evaluated visually for the presence of GFP fluorescence using a Dark Reader hand lamp (Clare Chemical, Dolores, CO) and positive kernels were selected based on the level of visual fluorescence. Kernels identified as positives were selected and planted in the field and were self-pollinated or back-crossed to the maize inbred line B73. The resulting F2 or BC1F1 generation seeds were evaluated and a second round of visual selection for high levels of GFP fluorescence was performed. However, other positives existed with lower levels of fluorescence that were not chosen to continue in our breeding program. Third-generation kernels were harvested and GFP positive kernels were sorted based on visual evaluation of fluorescence. Kernel cross-sections were analyzed under normal and fluorescent lights to determine GFP tissue localization (Fig. 2). Distinctive patterns of GFP localization occurred in the seed tissues as predicted by previous promoter characterizations. Thus, Glb1 GFP could be seen in the embryo and 27zn GFP could be seen in the endosperm. Glb1 expression has been reported to occur at low levels in the endosperm and aleurone layer of the kernel (Kriz 1999). However, we were unable to visually detect expression in this tissue.

Transgene Copy Number of Transformation Events

Transgene copy number can affect accumulation of transgene product so we determined the transgene copy number of each transformation event. This was done in heterozygous plants produced by crossing transgenic plants with a nontransgenic inbred line. We used the relative quantification method for determining gene copy number by using quantitative real-time PCR. In this procedure, a control endogenous gene was compared with the gene

of interest within a single DNA sample (Ginzinger 2002). Transgene copy number estimations were calculated using the relative standard curve quantitation method with 27zn or Glb1 coding sequences as endogenous controls (Yang et al 2005).

The Glb1 gene was present as one copy in the genome (Kriz 1989) and the 27zn gene was present in most cases as one or two copies in the genome (Das and Messing 1987). Therefore, the native 27zn and Glb1 genes were desirable for endogenous gene

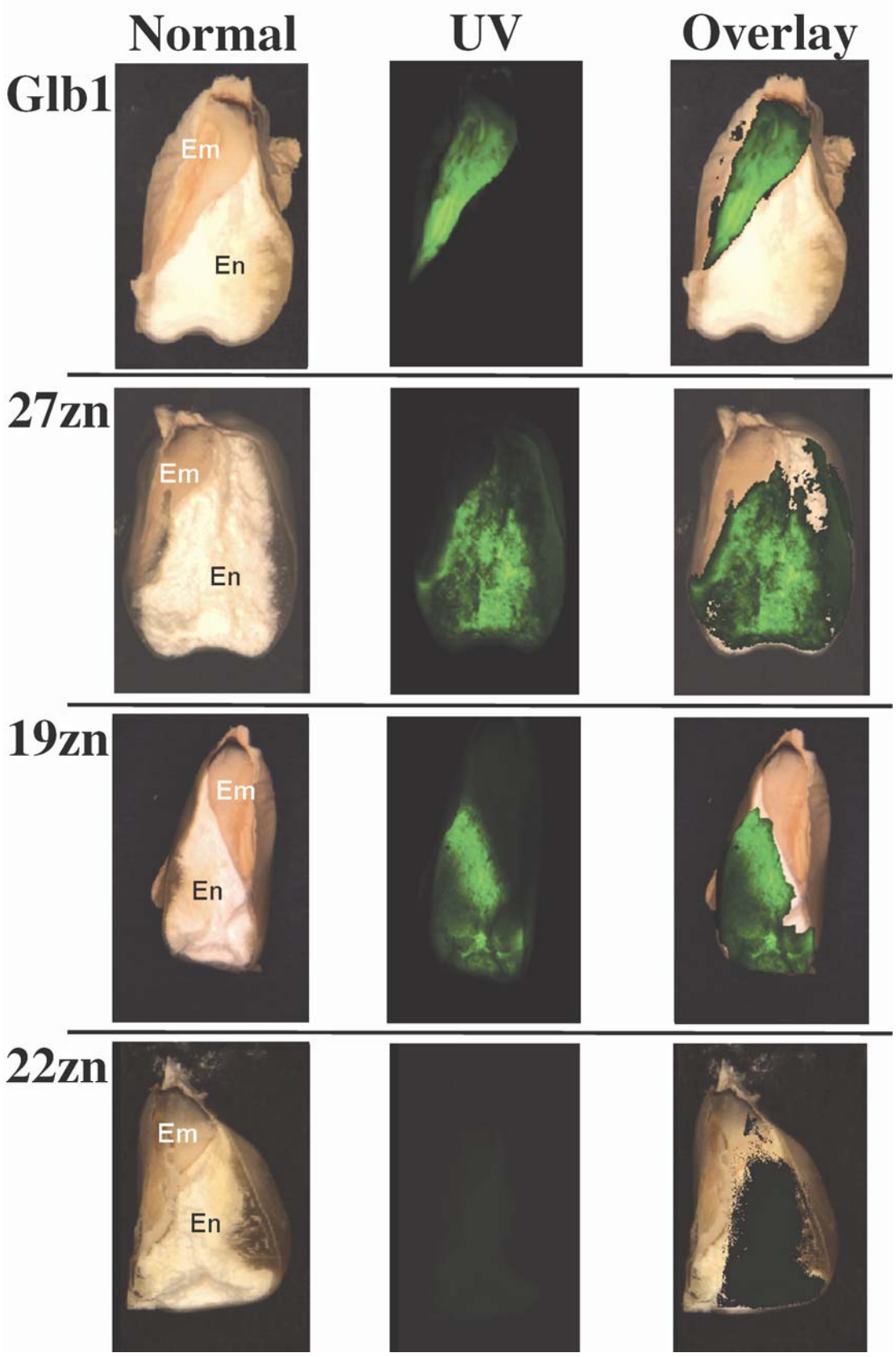

Fig. 2. Stable GFP expression in cross-sections of maize kernels seen under normal light and UV light. Images are overlaid to localize fluorescence in the kernel. Em, embryo tissue. En, endosperm tissue.

controls. PCR efficiencies, important for correlation of results of all PCR reactions (Song et al 2002), were measured by performing a fivefold serial dilution of genomic DNA isolated from each transgenic event. PCR efficiencies for the endogenous gene and transgene reactions were measured by comparing amplification threshold-cycle numbers to those of a standard curve and, in all cases, the efficiencies for the reactions were >0.90.

Endogenous gene copy number was assumed to be constant in all of the transgenic events, while transgene copy number changed with biolistic transformation event (Table III). Therefore, the threshold-cycle values of the native Glb1 or the 27zn gene can be compared directly with that of the transgene to get copy number estimation. The copy number of each transformation event was measured in triplicate and the average Ct values were compared with standard curves of the endogenous gene to calculate transgene copy number. All events had three or fewer copies with the exception of Glb1 event P231-24, which had 13 copies.

GFP Fluorescence Levels in Different Transformation Events

To determine the GFP fluorescence levels in the stable transformation events, plants from nine events were back-crossed to B73 in two successive generations and kernels were harvested, ground, and the fluorescence of the ground kernels was measured (Fig. 3). Nontransgenic B73 used as a negative control had fluorescence not significantly different from the event that contained the 22zn promoter. The events containing the 27zn promoter sequences had the highest expression levels of all the events in endosperm tissue. When evaluating fluorescence levels of transformation events containing the Glb1 promoter, it was important to consider that the results were expressed on a kernel mass basis and that the embryo comprised only about one-fifth of the kernel mass. This was one reason why the fluorescence levels in Glb1 constructs were lower than the fluorescence levels in the 19zn and 27zn constructs. The 19zn events had fluorescence levels in stable transformants that were generally intermediate to those containing the Glb1 promoter and those containing the 27zn promoter (Fig. 3). Expression of the transgene in this experiment showed that the transgene was stably inherited through three generations.

Conservative Sampling for GFP in Target Tissues

Visual examination of transgenic kernels suggested that GFP accumulated in one seed tissue in each line. The EST results indicated that the promoters we used to control GFP expression may have been transcriptionally active in other seed tissues. Therefore, it was possible that GFP accumulated in these tissues at levels too low to be visually detected. To determine whether this was the case, we evaluated transgene fluorescence levels in embryo and endosperm from one event each containing either the Glb1, 27zn, or 19zn constructs by using a conservative sampling technique in which tissue was removed by hand from the central part of the tissue of interest. This method minimized contamination from tissues other than the tissue being sampled. In the 19zn and 27zn transgenic lines, 100% of the GFP fluorescence was in the endosperm tissue (Table IV). In the Glb1 transgenic line, the ratio of GFP concentration in embryo-to-endosperm was 94.5:5.5 based on equal mass of tissue (Table IV). The Glb1 promoter has activity in the endosperm and, now that we know the tissue expression ratio, we can account for this in future experiments. In grain fractionation studies of Glb1 grain, the embryo-to-endosperm expression ratio can be considered to be a theoretical limit to fractionation efficiency because this is the expression ratio that would be expected for a perfect fractionation.

TABLE IV
Conservative Sampling of Transgenic Lines

Line or Transgene and Tissue	GFP Concentration (μg GFP/g tissue)	GFP Distribution(%)[a]
B73		
Endosperm	1.3	0
Embryo	–1.6	0
Total		0.3
Glb1		
Endosperm	12.3	5.5
Embryo	207.7	94.5
Total		220.0
27zn		
Endosperm	261.8	100.0
Embryo	0	0
Total		261.8
19zn		
Endosperm	234.4	100.0
Embryo	0.0	0.0
Total		234.4

[a] Green fluorescent protein (GFP) concentration of tissue divided by combined GFP concentration of the embryo and endosperm of the transgenic line.

Characterization of Transgenic Seeds by Hand-Dissection

To establish the utility of using GFP as a tissue marker for grain fractionation studies, kernels were hand-dissected to separate pericarp, embryo, and endosperm tissues. This procedure differs from the conservative sampling process described above in that the entire kernel was fractionated, whereas in conservative sampling, only a small part of the kernel is sampled. While conservative sampling can yield pure tissue, hand-dissection results in a small but unavoidable contamination of fractions with different tissues. Pericarp, embryo, and endosperm fractions were analyzed for GFP and moisture contents (Table V). The endosperm fraction contained 100% of GFP when endosperm-preferred GFP expressing lines were fractionated. In contrast, in the Glb1 event ≈67.8% of the GFP was found in the embryo fraction and 32.3% was found in the endosperm fraction.

GFP concentration (μg of GFP/g of tissue) was determined for each hand-dissected fraction and the observed GFP distribution from hand-dissection on a tissue mass basis was derived from GFP concentration by expressing this distribution as a percentage (Table VI). The theoretical GFP distribution was the GFP distribution between embryo and endosperm that represented a perfect fractionation and was derived from conservative sampling results. The deviation of the observed GFP distribution from that of the theoretical GFP distribution (which represents a perfect hand-

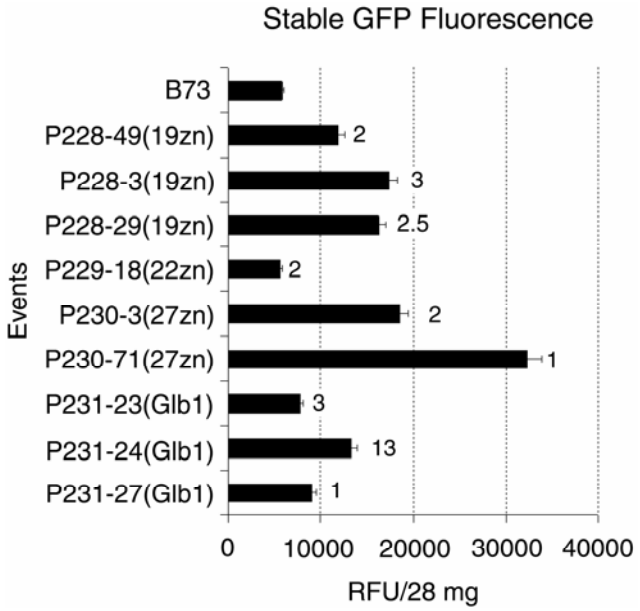

Fig. 3. Fluorescence measurements of GFP in stable transformants per kernel mass. Error bars show standard deviation of the mean. Numbers following error bars are transgene copy numbers for that event.

dissection) was the error in hand-dissection fractionation. In the Glb1 event, the observed GFP distribution from hand-dissection was 93.1% in the embryo and 6.9% in the endosperm on a per tissue mass basis. This ratio deviated from the theoretical distribution by –1.4% in the embryo and 1.4% in the endosperm tissue. Converting this error into GFP yield (based on the mass % of each fraction) resulted in an observed GFP yield from hand-dissection of 73 and 27% for the embryo and endosperm, respectively. Thus, the deviation from the theoretical GFP yield was –5.2% for embryo and 5.2% for endosperm. From this, we concluded that after hand-fractionation 5.2% of total grain embryo mass was present in the endosperm fraction. In the 19zn and 27zn events, observed GFP distribution from hand-dissection equaled the theoretical GFP distribution, meaning that hand-dissection of endosperm tissue was essentially perfect. In addition, because there was no GFP detectable in the pericarp fraction in any of the GFP lines, we concluded that the pericarp fraction was not contaminated with embryo or endosperm.

TABLE V
Composition and Yield of Hand-Dissected Transgenic Kernels

Promoter/ Fraction	Mass Yield (%)	GFP Concentration (µg GFP/g tissue, db)	GFP Yield (%)[a]
27zn			
Embryo	11.9	4.7	0.2
Endosperm	81.4	406.7	99.8
Pericarp	6.7	0.0	0.0
Total	100.0	–	100.0
19zn			
Embryo	10.5	0.0	0.0
Endosperm	83.5	321.7	100.0
Pericarp	6.0	0.0	0.0
Total	100.0	–	100.0
Glb1			
Embryo	12.6	117.3	67.8
Endosperm	80.8	8.7	32.3
Pericarp	6.7	0.0	0.0
Total	100.0	–	100.0
B73			
Embryo	11.4	0.0	0.0
Endosperm	86.8	0.0	0.0
Pericarp	1.7	0.0	0.0
Total	100.0	–	–

[a] Green fluorescent protein (GFP) yield corrected for GFP yield due to expression and represents only the GFP yield resulting from hand-dissection.

DISCUSSION

Tissue markers can be used to evaluate and improve grain fractionation processes. While markers such as native seed proteins, biochemical markers, and even fiber, oil, and ash contents have been used, a transgene expressing the fluorescent protein GFP in the embryo or endosperm is attractive because it can be accurately and easily measured in mature fractionated maize seed tissues. One objective of this study was to develop transgenic maize lines containing GFP as an embryo or an endosperm marker. These transgenic lines were developed and characterized with respect to their GFP accumulation to determine whether they were suitable as tissue markers.

We created constructs based on promoters with strong tissue preference for embryo or endosperm, and these constructs were used to develop stably transformed GFP expressing plants. Several independent transformation events were generated with each construct. Expression levels varied by transformation event and gene copy number. The reasons for these differences are unknown but may have involved differences in transgene integration sites into the genome that affect transcription rates. Also, while event 231-24 had 13 copies of the transgene and the highest fluorescence level among the Glb1 constructs, the fluorescence was not proportional to the increase in copy number compared with the other Glb1 constructs. Reasons for this finding are unknown as well, but may have been due to a physiological limit on transcription from the Glb1 promoter.

The 22zn promoter used in the present study had no detectable activity in the transgenic plants. The GFP measurements in whole kernels showed the 22zn transformants were not significantly different from the B73 inbred control kernels. The 22zn gene family contains as at least 15 copies in the maize genome, however, some of these copies are inactive due to mutations (Song et al 2001). The 22zn promoter that we used in these experiments was sequenced and found to be similar to the 22zn promoter that produced a low percent of the EST observed by Woo et al (2001).

To understand the tissue specificity of the native seed storage proteins including the Glb1, 19zn, 22zn, and 27zn genes, we compared EST frequencies derived from tissue-specific EST libraries. EST frequency is a measure of the abundance of mRNA levels. Messenger RNA levels are not the sole determinant of the tissue in which a protein accumulates in maize endosperm (Chourey and Taliercio 1994; Chourey et al 2006) but it is difficult or impossible to predict genetic interactions or aspects of protein deposition that may affect tissue specificity. This is particularly true when producing a foreign protein in a tissue. Native proteins have mech-

TABLE VI
Green Fluorescent Protein (GFP) Distributions and the GFP Yields in Embryo and Endosperm of Each Transgenic Line

Promoter/ Fraction	Theoretical GFP Distribution (%)[a]	Observed GFP Distribution from Hand-Dissection (%)[b]	Deviation from Theoretical GFP Distribution (%)[c]	Theoretical GFP Yield (%)[d]	Observed GFP Yield from Hand-Dissection (%)[e]	Deviation from Theoretical GFP Yield (%)[f]
Glb1						
Embryo	94.5	93.1	(1.4)	73.0	67.8	(5.2)
Endosperm	5.5	6.9	1.4	27.0	32.2	5.2
19zn						
Embryo	0.0	0.0	0.0	0.0	0.0	0.0
Endosperm	100.0	100.0	0.0	100.0	100.0	0.0
27zn						
Embryo	0.0	0.0	0.0	0.0	0.0	0.0
Endosperm	100.0	100.0	0.0	100.0	100.0	0.0

[a] Theoretical green fluorescent protein (GFP) distribution from Table IV represents GFP distribution in perfect fractionation derived from conservative sampling experiment.
[b] Derived from the GFP content (µg of GFP/g of tissue) data in Table VI.
[c] Derived from the difference between theoretical GFP distribution and observed GFP distribution from hand-dissection.
[d] Theoretical GFP derived yield was from Table IV and represents GFP yield in a perfect fractionation.
[e] Calculated by multiplying the observed GFP distribution from hand-dissection by the mass yield in Table V.
[f] Derived from the difference between theoretical GFP yield and observed GFP yield from hand-dissection.

anisms to promote stability such as assembly into complexes and subcellular targeting, and these mechanisms are not likely to function with a foreign protein. In spite of this, we felt that a prediction of tissue specificity based on EST frequency was better than no prediction at all. EST from seed storage protein genes were located in tissues other than the seed (i.e., leaf, immature leaf, and tassel) in almost all cases, indicating that seed storage protein genes are not tissue-specific or seed-specific. Expression level was higher in either embryo or endosperm seed tissue in all seed storage protein genes. This result implied that expression of seed storage proteins is seed-tissue-preferred, not seed-tissue-specific. For the Glb1 and Glb2 genes, EST frequency analysis indicated expression in the immature leaf as well as endosperm and embryo, although expression in the immature leaf did not occur in all leaf libraries. This result may be dependent on genotype, but the expression level dependence on genotype is not understood and needs to be tested further.

The value of a tissue marker is proportional to the tissue specificity of the marker. To characterize the seed tissue specificity of GFP in the transgenic events, we performed a conservative sampling experiment in which the fluorescence level of each major seed tissue was determined. The extent of fluorescence in non target tissues was important to establish a base level of fluorescence for that tissue. We show conclusively that no GFP fluorescence was present in the nontarget embryo tissue of the 19zn and 27zn transgenic lines. GFP fluorescence in the nontarget endosperm tissue of the Glb1 transgenic line was higher than that of the negative control. This was probably due to Glb1 promoter activity in this tissue, which is consistent with the observation that Glb1 EST were detected in endosperm tissue. With these results, however, we can establish a baseline of fluorescence for further experiments.

A major objective of the present study was to establish the feasibility of using the transgenic grain containing tissue-preferred markers in grain fractionation studies. Transgenic kernels (≈100 g) for each event were hand-dissected to separate the pericarp, embryo, and endosperm tissues. For 19zn and 27zn endosperm GFP specific events, the GFP was almost exclusively present in the endosperm fraction, similar to the conservative sampling results above, suggesting that there was little or no endosperm contamination in the embryo or pericarp fractions. For the Glb1 event, 68% of GFP was found in the embryo and 32% was present in the endosperm. Because we knew the expected ratio of GFP in endosperm and embryo from our conservative sampling experiment, we used these values to calculate that 5.2% of the total embryo mass was present in the hand-dissected endosperm fraction. The pericarp was free of embryo and endosperm contamination. However, we did not have a good marker for pericarp, so we could not determine the extent of pericarp contamination in either the embryo or endosperm fractions. From this information, we concluded that comparisons can be made between tissues using GFP as a marker protein.

One objective of this work was to determine whether GFP could be developed as a tissue marker for grain fractionation studies. GFP is superior to biochemical markers that have been used previously for this purpose from the standpoint that it is easier to quantify. When driven by a zein promoter, it is also superior as an endosperm marker because it exhibits a higher degree of tissue specificity than other markers. As an embryo marker, the lack of complete specificity is a drawback that is shared with other markers that have been used.

CONCLUSIONS

We determined from the outset of our research that these transgenic lines must have three criteria to meet our objective of producing maize containing tissue markers for grain milling studies. First, the marker protein must be expressed at a high level. Second, the marker protein must be stable through generations. Third, the marker protein must be quantifiable in each tissue. From our results, it is clear that these criteria were met. GFP-containing maize lines developed in this study will be valuable as tissue-specific markers for the evaluation and optimization of milling processes as well as for a variety of research, including developmental studies and recombinant protein production studies. Research using these genetic resources will require compliance with federal regulations regarding use of transgenic plant material.

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