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Keywords

kernel, maize, synthetic gene, targeting, α-lactalbumin

Disciplines

Agronomy and Crop Sciences | Plant Breeding and Genetics

Comments

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Expression of a synthetic porcine α-lactalbumin gene in the kernels of transgenic maize

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Abstract

The main nutritional limitation of maize used for feed is the content of protein that is digestible, bioavailable and contains an amino acid balance that matches the requirements of animals. In contrast, milk protein has good digestibility, bioavailability and amino acid balance. As an initial effort to create maize optimized as a source of swine nutrition, a codon-adjusted version of a gene encoding the milk protein porcine α-lactalbumin was synthesized. Maize expression vectors containing this gene under the control of the *Ubi-1* promoter and *nos 3′* terminator were constructed. These vectors were used to transform maize callus lines that were regenerated into fertile plants. The α-lactalbumin transgenes were transmitted through meiosis to the sexual progeny of the regenerated plants. Porcine α-lactalbumin was detected in callus and kernels from transgenic maize lines that were transformed by two constructs containing the 27-kDa maize gamma-zein signal sequence at the 5′ end of the synthetic porcine α-lactalbumin coding sequence. One of these constructs contained an ER retention signal and the other did not. Expression was not observed in kernels or callus from transgenic maize lines that were transformed by a construct that does not contain an exogenous protein-targeting signal. This suggests that the signal peptide might play an important role in porcine α-lactalbumin accumulation in transgenic maize kernels.

Introduction

Kernels of higher plants are a rich source of nutrition for humans and livestock. However, the major crops are deficient in one or more of the essential amino acids. For example, wheat, barley, maize and sorghum accumulate major storage proteins that are low in lysine, while storage proteins of legumes are deficient in sulfur-containing amino acids. Barley and sorghum are also low in threonine, and maize is low in tryptophan (Brar et al., 1995). These deficiencies limit the nutritional value of the grain. Therefore, alteration of the amino acid composition of kernels is a key step toward providing nutritionally balanced grain for human and livestock consumption. Tremendous efforts have been devoted to improving the quality and quantity of kernel storage proteins by traditional breeding in cereals (Payne, 1983) and soybean (Kitamura, 1993). With recent advances in genetic engineering in plants, efforts have been concentrated on genetic transformation (reviewed by Brar et al., 1995).

Milk proteins have been proposed as an attractive target for introduction into plants for nutritional improvements because they are a natural component of animal diets and have excellent nutritional parameters. Human α-lactalbumin has been produced in transgenic

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tobacco leaves (Takase & Hagiwara, 1998). Other human milk proteins have been expressed in dicot plants as well, including lactoferrin (Mitra & Zhang, 1994) and human casein (Chong et al., 1997).

Among milk proteins, α-lactalbumin is an attractive target for plant production because its small size is conducive to the construction of a synthetic coding region using plant-preferred codons. Porcine α-lactalbumin is expressed in the mammary gland and is a major protein in sow's milk. α-Lactalbumin is a component of the lactose synthetase complex. It modifies galactosyltransferase specificity by lowering the K_M of the complex for glucose, thereby promoting synthesis of lactose (Klee & Klee, 1970). A cDNA clone encoding porcine α-lactalbumin was isolated and sequenced (Das Gupta et al., 1992). The cDNA encoded a preprotein of 141 amino acids, including a leader peptide of 19 amino acids. Nutritionally, porcine α-lactalbumin has an excellent amino acid profile, a digestibility of 99%, biological value of 94% and protein efficiency ratio of 3.2 (Robinson, 1986).

Our goal is to improve the nutritional value of maize. Toward this goal, a codon-adjusted porcine α-lactalbumin gene was synthesized and used to construct plant expression vectors that were used to transform maize callus. Transgenic maize plants expressing porcine α-lactalbumin in the kernel have been generated.

Materials and methods

Gene construction

Synthesis of porcine α-lactalbumin coding sequences

A porcine α-lactalbumin cDNA was isolated and sequenced by Das Gupta et al. (1992). The amino acid sequence derived from this clone was reverse-translated into a nucleotide sequence using the maize-preferred codons (GCG, assembled by Mike Cherry, Stanford University) (Table 1). An α-lactalbumin coding sequence consisting of the mature protein with a methionine appended to the N-terminus was synthesized using a two-phase recursive PCR (Sardana et al., 1996). Eight partially overlapping oligonucleotides representing gapped sense and antisense strands of the synthetic porcine α-lactalbumin coding sequence were designed and synthesized. These oligonucleotides were 36–86 bp in length. When oligonucleotides were designed, the secondary structure of overlapping regions was minimized to ensure good primer specificity, and all overlaps had similar melting temperatures. Codons were selected to create unique restriction sites in every overlapping region. The first phase PCR (30 cycles of 1 min at 94°C, 2 min at 55°C and 2 min at 72°C, with 5 min of final extension at 72°C) was performed in a Rapidcycler thermal cycler (Idaho Technology, Inc.). The first phase PCR reaction contained 30 pmol of each oligonucleotide, 10 µlitre 10 × *Pfu* DNA polymerase buffer (Stratagene), 250 µM of each dNTP, 6 mM $MgSO_4$ and 2.5 unit *Pfu* DNA polymerase (Stratagene) in a 100 µl volume. Ten microlitre of the first phase PCR product was used as a template for the second phase PCR, and 30 pmol of the outer oligonucleotides, which contained a *Sma*I restriction endonuclease site at the 5′ end and a *Sac*I restriction endonuclease site at the 3′ end, were used as primers. The other PCR conditions were the same as the first phase PCR except for a 5 min, 94°C denaturation step at the beginning of the reaction. A slow-cooling method was applied prior to the first phase PCR to improve the yield of the expected fragment (Sardana et al., 1996).

A second version of the synthetic coding sequence was made that encoded mature porcine α-lactalbumin translationally fused to the maize 27-kDa gamma zein signal sequence. This was done by PCR amplification of the recursive PCR product using a 5′ oligonucleotide with an extension encoding the zein signal sequence (Prat et al., 1985). This PCR product also had a *Sma*I restriction endonuclease site at the 5′ end and a *Sac*I restriction endonuclease site at the 3′ end. A plasmid containing this construct was used as template in a PCR reaction to produce a third coding sequence. In this reaction, the 3′ primer was modified to append a sequence encoding KDEL to the 3′ end of the coding sequence.

Vector construction

PCR products were separated on a 1% (w/v) Seaplaque GTG low-melting temperature agarose gel (FMC Bioproducts) and extracted from the gel using a phenol extraction method (FMC Bioproducts). The gel-purified PCR products were treated with *Sma*I and *Sac*I and ligated into pGEM-4Z (Promega) digested with *Sma*I and *Sac*I, creating the plasmids pGEM-4Z/Lactal, pGEM-4Z/z-sig/Lactal and pGEM-4Z/z-sig/Lactal/KDEL. The nucleotide sequences of the cloned synthetic porcine α-lactalbumin coding regions were verified by DNA sequencing (Iowa State University DNA synthesis and sequencing facility),

Table 1. Comparison of codon usage patterns between the porcine α-lactalbumin cDNA and the synthetic coding sequence.

Amino acid	Codon	# In porcine gene	# In synthetic gene	Amino acid	Codon	# In porcine gene	# In synthetic gene
Ala	GCC	2	3	Leu	CTG	5	1
	GCA	1			CTG	4	11
					TTG	2	
					CTT	1	
Arg	AGA	1		Lys	AAG	5	12
	CGC		1		AAA	7	
Asn	AAC	2	6	Met	ATG	4	4
	AAT	4					
Asp	GAC	9	15	Phe	TTT	2	
	GAT	6			TTC	2	4
Cys	TGT	7		Pro	CCT	1	
	TGC	1	8		CCG		1
Gln	CAA	1	1	Ser	TCC	2	5
	CAG	5	5		AGT	1	
					AGC	1	
					TCA	2	
					TCT		1
Glu	GAG	2	6	Thr	ACC	2	7
	GAA	4			ACA	3	
					ACT	2	
Gly	GGC	4	6	Thr	TGG	4	4
	GGA	2					
	GGG	1	1				
His	CAT	3		Tyr	TAC	1	4
	CAC		3		TAT	3	
Ile	ATC	8	9	Val	GTG	2	2
	ATA	1					
	ATT	2	2				

and clones with the desired sequence were used for the construction of the plant transformation vectors.

Plant transformation vectors for expression of porcine α-lactalbumin in maize were constructed using standard cloning procedures (Sambrook et al., 1989). Three vectors were constructed, all based on pAHC25 (Christensen et al., 1992), a plant expression vector that contains the *gus*A and *bar* genes fused to the promoter, the 5′-untranslated region and first intron of a maize ubiquitin gene, and a nopaline synthase transcription terminator. In each vector, the *gus*A gene in pAHC25 was replaced by a *Sma*I-*Sac*I fragment containing the synthetic porcine α-lactalbumin coding sequence from pGEM-4Z/Lactal, pGEM-4Z/z-sig/Lactal, or from pGEM-4Z/z-sig/Lactal/KDEL. The resulting plasmids were called pUbi/Lactal, pUbi/z-sig/Lactal, and pUbi/z-sig/Lactal/KDEL, respectively.

Plant transformation and tissue culture

All plant transformation and tissue culture was done at the plant transformation facility at Iowa State University as described (Frame et al., 2000). Highly embryo-

genic, friable, Type II callus for bombardment was initiated from F_1 embryos of the Hi II genotype (Armstrong et al., 1991). Established Type II embryogenic callus lines were bombarded with either pUbi/Lactal, pUbi/z-sig/Lactal or pUbi/z-sig/Lactal/KDEL using particle bombardment-mediated transformation with a PDS-1000/HE Biolistic Particle Delivery System (Bio-Rad) following the manufacturer's recommendations. Transgenic calli expressing the *bar* gene were selected in media containing the herbicide Bialaphos (3 mg/l). Eight weeks after bombardment, bialaphos-resistant calli were screened for the presence of the transgene using PCR, and those containing the transgene were regenerated to plants. Plants and calli derived from transformation with pUbi/Lactal were designated P40, those derived from transformation with pUbi/z-sig/Lactal were designated P42, and those derived from transformation with pUbi/z-sig/Lactal/KDEL were designated P45. All T_0 plants were crossed with the inbred line B73 to produce F_1 kernels. Some of these kernels were planted, and the resulting plants were self-pollinated to produce F_2 kernels.

Production and testing of polyclonal antibodies specific to α-lactalbumin

Polyclonal antisera against human α-lactalbumin were produced in rabbits using standard procedures (Harlow & Lane, 1988) at the Iowa State University Hybridoma Facility. Commercially available human α-lactalbumin (Sigma) was used as an antigen. This serum reacted with commercial human α-lactalbumin, protein extracts from sow's milk and *Escherichia coli-* expressed porcine α-lactalbumin in immunoblot experiments.

Immunoblot analysis of transgenic callus

Protein was extracted by homogenizing 100 mg (fresh weight) of transgenic maize callus in 300 μl of 1x SDS sample buffer. The insoluble materials were removed by centrifugation at 13,000 rpm for 10 min in a microcentrifuge. The supernatant was boiled for 5 min before loading on a 15% SDS-PAGE gel (Laemmli, 1970). Gels were blotted onto a nylon-backed nitrocellulose membrane using a mini-transblot apparatus (Bio-Rad). The membrane was blocked with 1% ovalbumin and visualized according to the manufacturer's protocol for colorimetric visualization of an alkaline phosphatase-conjugated anti-rabbit IgG (Bio-Rad).

Immunoblot analysis of kernels

A small portion of endosperm (∼20 mg) was removed from kernels using a hand drill. Protein was extracted from this tissue for 30 min using 200 μl of SDS-PAGE sample buffer per 20 mg endosperm powder, and immunoblot analysis was performed as described above. To quantify α-lactalbumin, the band intensity of kernel α-lactalbumin was compared to the intensities of standards consisting of *E. coli*-expressed and purified porcine α-lactalbumin that was quantified by a dye binding assay (Bradford, 1976).

PCR analysis of F_1 plants

Kernels that were sampled for immunoblot analysis were planted in the greenhouse. About 3 weeks after germination, fresh leaf tissue (∼50 mg) was collected from plants. DNA was extracted from leaves using a Puregene DNA Isolation Kit (Gentra Systems) following the manufacturer's recommendations. The DNA pellet was resuspended in 200 μl 50 mM Tris, 10 mM EDTA, pH 8.0. All PCR reactions contained 1 μl of isolated DNA (∼50 ng), 250 μM each dNTP, 2.5 U Platinum TAQ DNA polymerase (Gibco BRL), 3 mM $MgSO_4$, 5 μl BSA solution (2.5 mg/ml), 5 μl 10x PCR buffer (Gibco BRL) and 25 pmol of forward and reverse primers in a 50 μl volume. The forward and reverse primers corresponded to sequences in the α-lactalbumin coding sequence and amplified a DNA fragment of 280 bp. PCR conditions were 35 cycles of 30 s at 94°C, 30 s at 58°C and 2 min at 72°C, with 5 min at 94°C prior to the reaction and 5 min of final extension at 72°C in a Rapidcycler (Idaho Technology, Inc.). PCR products were resolved using a 1.4% agarose gel, stained with ethidium bromide and photographed.

RNA-blot analysis of F_1 plants

Fresh leaf tissue collected from 6-week-old plants was flash-frozen in liquid nitrogen, ground to a fine powder and stored at −80°C. Total RNA was extracted from 300 mg of frozen tissue using the Ambion RNAasy kit (Ambion, Inc.). Ten micrograms of total RNA was size-fractionated on a 1.4% agarose gel containing ethidium bromide, 1.2% formaldehyde, 20 mM 3[N-morpholino]propanesulfonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA, pH 7.0. After electrophoresis, the gel was photographed and blotted onto positively charged nylon membrane (Micron Separations, Inc.) and baked for 1 h at 80°C.

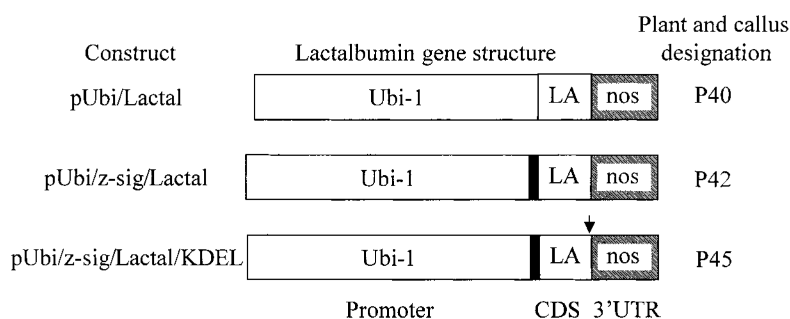

Figure 1. Gene structure of α-lactalbumin genes used in transformation. Black bars indicate the position of the 27-kDa gamma zein signal sequence, and the arrow indicates the position of the ER retention signal (KDEL). CDS is the synthetic α-lactalbumin coding sequence.

α-Lactalbumin RNA was visualized by hybridization to a labeled PCR product from the α-lactalbumin coding region. Twenty-five nanogram of the PCR product was labeled with [^{32}P]-dCTP using the random primer method (Rediprime 1633, Amersham Pharmacia Biotech). Hybridization of the membrane and visualization were done using standard methods (Sambrook et al., 1989).

Results

Construction of synthetic porcine α-lactalbumin genes by recursive PCR

In order to make an impact on the nutritional quality of the kernel, it is important for the porcine α-lactalbumin transgene to function efficiently in kernel tissues. The codon usage of the porcine α-lactalbumin cDNA was markedly different from the codon usage typically found in maize genes, and we were concerned that this could limit the accumulation of porcine α-lactalbumin in the plants. Increased levels of protein expression after codon usage adjustment have been reported (Perlak et al., 1991; Adang et al., 1993; Koziel et al., 1993; Sardana et al., 1996; Stewart et al., 1996; Hood et al., 1997; Rouwendal et al., 1997; Horvath et al., 2000), so we designed a codon-adjusted version of the porcine α-lactalbumin coding sequence. A comparison of the codon usage of the porcine α-lactalbumin coding sequence and our codon-adjusted version of the coding sequence is presented in Table 1.

An improved recursive PCR method (Sardana et al., 1996) was used for the synthesis of codon-adjusted synthetic porcine α-lactalbumin coding region of 373 bp. This protocol consists of a two-phase PCR reaction. The first phase was accomplished by the mutual extension of eight partially overlapping oligonucleotides representing both strands of a synthetic porcine α-lactalbumin coding sequence in the first phase of the PCR, followed by amplification of the first phase product by the outer oligonucleotides in the second phase PCR.

We investigated three different targeting strategies in order to optimize the production of this protein in plants. Three plant transformation vectors were constructed using the synthetic α-lactalbumin coding sequence. All vectors contained a modified porcine α-lactalbumin gene. The selectable marker gene, *bar*, fused to the maize *Ubi*-1 promoter was incorporated into each vector as well. Each α-lactalbumin gene consisted of the maize ubiquitin promoter and the synthetic porcine α-lactalbumin coding sequence followed by the nos 3′ untranslated region. The three vectors differed in their coding regions, with pUbi/Lactal encoding mature porcine α-lactalbumin translationally fused to an ATG translation initiation codon, pUbi/z-sig/Lactal encoding the mature porcine α-lactalbumin coding sequence translationally fused to the maize 27-kDa gamma zein signal sequence. pUbi/z-sig/Lactal/KDEL is identical to the second construct, but encodes an ER retention sequence (KDEL) at the C-terminal end of the protein. The structures of these three expression cassettes are shown in Figure 1.

Integration of the synthetic porcine α-lactalbumin gene into the genome of maize

The three transformation vectors were introduced into maize callus cells by the Iowa State University Plant Transformation Facility using their standard particle bombardment protocol (Frame et al., 2000). Seven to nine days after bombardment, calli were moved to selective media containing the Bialaphos. The transformation efficiencies were 9, 5.7 and 8.3

Bialaphos-resistant clones per 100 pieces of callus bombarded with pUbi/Lactal, pUbi/z-sig/Lactal and pUbi/z-sig/Lactal/KDEL, respectively. About 8 weeks after bombardment, DNA was extracted from callus lines resistant to Bialaphos and used as a template for PCR screening. Using primers corresponding to the synthetic porcine α-lactalbumin coding sequence and the nopaline synthase terminator, a PCR product of 448 bp was detected only in Bialaphos-resistant lines. Among calli transformed by the pUbi/Lactal vector, 56 of 61 callus lines that showed Bialaphos resistance contained the α-lactalbumin transgene as determined by this PCR analysis. For calli transformed by pUbi/z-sig/Lactal, 36 out of 40 callus lines that showed Bialaphos resistance contained the α-lactalbumin transgene by the same analysis. All PCR-positive callus lines were transferred to regeneration media for plant regeneration. Calli transformed with pUbi/z-sig/Lactal/KDEL were not screened by PCR, but were assessed for porcine α-lactalbumin accumulation by immuno-blot detection. All positive clones were transferred to regeneration media.

Female fertile T_0 plants were regenerated from eight different PCR positive Bialaphos-resistant calli transformed with pUbi/Lactal, from eight different PCR positive Bialaphos-resistant calli transformed with pUbi/z-sig/Lactal and from nine different immumoblot positive Bialaphos-resistant calli transformed with pUbi/z-sig/Lactal/KDEL. These plants were pollinated by the inbred line B73 to produce F_1 kernels.

Expression of porcine α-lactalbumin in maize callus and kernels

Polyclonal antiserum was used to detect porcine α-lactalbumin expressed in transgenic maize callus (Table 2). Porcine α-lactalbumin was detected in 20 out of 40 PCR positive P42 callus lines (transformed with pUbi/z-sig/Lactal), and in 26 of 30 bialaphos resistant P45 callus lines (transformed with pUbi/z-sig/Lactal/KDEL). Porcine α-lactalbumin was not detected in the 36 PCR positive P40 callus lines (transformed with pUbi/Lactal) tested.

Table 2. Expression of α-lactalbumin in callus and kernel by event.

Tissue screened	Callus		F1 Kernels	
	Screened	Positive	Screened[a]	Positive[b]
P40	36	0	8	0
P42	40	20	8	2
P45	30	26	9	5

[a]The results presented are the number of events screened. At least 10 seeds were screened for each event.
[b]An event was scored positive if at least one positive seed was identified among those screened.

Several F_1 plants were analyzed at the DNA, RNA and protein level to further characterize the inheritance and expression of the porcine α-lactalbumin transgene (Figure 2). This analysis demonstrated that the porcine α-lactalbumin transgene was transmitted to the F_1 generation in P40 and P42 plants (Figure 2A). Inheritance of the transgene in the P42s and P45s in the F_2 generation was verified by immuno-blot analysis (Figure 3). Note that the P45 protein is slightly larger than the P42 protein. We attribute this to the presence of the ER retention signal (KDEL) in the P45 protein.

Accumulation of the α-lactalbumin protein was confirmed by immuno-blot analysis of F_1 kernels resulting from crosses between T_0 plants and the inbred line B73. An example of this analysis is given in Figure 2C, and the results are summarized in Table 2. Among 160 P40 F_1 kernels (20 kernels from each of 8 events), none contained detectable α-lactalbumin. Among 80 P42 F_1 kernels (10 kernels from each of 8 events) α-lactalbumin was detected in the F_1 lines derived from two events. Among 215 p45 F1 kernels from 9 events, α-lactalbumin was detected in the F_1 lines derived from 5 events.

A possible explanation for the lack of accumulation of porcine α-lactalbumin in P40 calli and seeds is that the gene cannot be transcribed. We therefore sought to determine if the α-lactalbumin transgene is transcriptionally competent. Ideally this would be done in mature kernels, because this is the tissue that was analyzed for protein accumulation. It is difficult to recover RNA from mature kernel tissue, however, and because we wanted to establish transcriptional competence rather than draw conclusions about tissue specificity, we took advantage of the broad range of tissues in which the Ubi-1 promoter is active and characterized transcription in leaves. Several PCR positive P40 kernels were germinated and grown in the greenhouse. Northern blot analysis of F_1 leaf tissue from these plants was conducted to determine if the α-lactalbumin transcript was present. Several expressing and non-expressing P42 plants were analyzed similarly to serve as controls. A transcript similar to that in the positive controls was detected in P40 plants (Figure 2B).

The phenotypic segregation ratio for α-lactalbumin expression was determined for a cross between the T_0

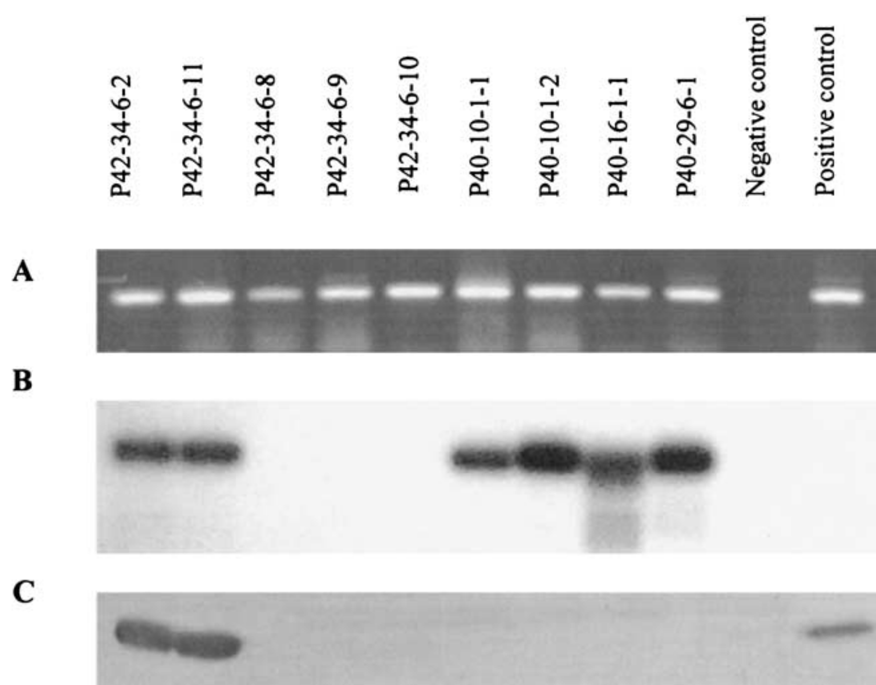

Figure 2. Analysis of gene expression in transgenic plants selected to illustrate the variety of expression patterns observed. In each panel, corresponding lanes represent the same plant, except for the positive and negative control lanes. The designation above each lane specifies an individual plant, giving the construct used (P40 or P42), the transgenic event, the clone and the F_1 family number. (A) PCR analysis to determine the presence of the transgene. Nontransgenic B73 DNA was used as template for the negative control, and 50 pg of a pGEM vector housing the synthetic α-lactalbumin gene was used as a positive control. (B) Northern blot analysis of leaves of F_1 transgenic plants. (C) Immunoblot analysis of seeds of transgenic plants. The negative control lane is from the inbred line B73, and the positive control lane is human α-lactalbumin.

Figure 3. Immunoblot analysis of kernel α-lactalbumin compared to *E. coli*-produced α-lactalbumin standards. Extracts from equal amounts of endosperm of non-transgenic B73, and transgenic P40, P42 and P45 kernels were loaded in the indicated lanes. The numbered lanes were loaded with the indicated number of nanograms of *E. coli*-expressed and purified α-lactalbumin.

expressing clone P42-34-6 and the inbred line B73. Immunoblot analysis revealed that the protein accumulated in 11 of 29 F_1 kernels. A single dominant locus in independent assortment would be expected to produce a 1 : 1 ratio of expressers to nonexpressers. The chi-square test does not confirm that this is a 1 : 1 ratio with a 90% probability. Thus, the phenotypic expression ratio is not consistent with the ratio expected from normal nuclear inheritance. This could be a consequence of sampling error or it could be that complex genetic factors such as gene silencing or gamete mortality are influencing the segregation ratio.

The level to which α-lactalbumin accumulates in the seed is an important factor in determining the nutritional impact of the protein in maize kernels. We determined the level of α-lactalbumin in transgenic kernels by comparing the intensities of bands on immuno blots. An example with the highest expressers from each construct is shown in Figure 3. Known amounts of *E. coli* expressed porcine α-lactalbumin were loaded as standards that were compared to endosperm extracts of single kernels from each expressing event on the same blot. The results are summarized in Table 3.

Discussion

Construction of the synthetic porcine α-lactalbumin genes

In spite of extensive precautions to avoid errors during PCR, it was necessary to screen many clones

Table 3. Approximate level of porcine α-lactalbumin in the kernels of F2 transgenic plants.

Event	Level of α-lactalbumin (μg/ g of kernel)[a]
P42–17	100
P42–34	500
P45–16	400
P45–11	400
P45–22	400
P45–03	200
P45–19	<60

[a]Level was determined by comparison of immunoblot band intensities to those of standards of known concentrations.

before one with the correct sequence was identified. The errors found in clones with incorrect sequences were typically rearrangements of primer sequences. These rearrangements may be due to the redundancy of certain codons within the synthetic gene, because only optimal codons were used at most of the positions in the synthetic coding sequence. This problem may be avoided by increasing the complexity of the sequence by using both the first- and second-most preferred codons for a given amino acid when designing synthetic genes.

When selecting a promoter, our goals were to obtain a high level of α-lactalbumin expression and to be able to analyze several tissues (including callus) for expression. We chose the maize *Ubi-1* promoter (Christensen et al., 1992) as a compromise between high expression level and broad tissue specificity. This promoter has been used successfully as an expression cassette in several monocot plants when coupled with the nopaline synthase 3′ terminator (Toki et al., 1992; Cornejo et al., 1993; Taylor et al., 1993; Weeks et al., 1993; Chaïr et al., 1996; Sardana et al., 1996; Hood et al., 1997; Nayak et al., 1997).

Comparison of callus and plant expression

Because transgenic callus is much easier to produce than transgenic kernels, it would be useful to use expression in callus as a predictor of the success for kernel expression of a construct. The Ubi-1 promoter is active in many plant tissues, allowing us to examine expression of porcine α-lactalbumin in callus, leaves and seeds. Each construct that produced α-lactalbumin expressing calli produced plants expressing α-lactalbumin in some events. Thus it appears that expression in callus may be useful as a general predictor of construct performance, but certainly not all successful callus lines give rise to successful transformation events. Callus and kernels are from different generations, so it is possible that meiosis affected expression of the gene. Another possible explanation is that these genes may be more active in callus than in kernel cells, so the weakest events produce detectable α-lactalbumin in callus but the level of α-lactalbumin in these events is below the level of detection in kernel cells.

Post-translational modification and protein targeting

Porcine α-lactalbumin was detected in several P42 and P45 calli and plants but not in P40 tissues, even though the transcript of each gene was detected at approximately equal levels and in a similar proportion in leaves of P40 and P42 plants. The only difference between the two constructs is that the latter encodes the signal peptide from 27-kDa maize gamma-zein at the 5′ end of the porcine α-lactalbumin gene. This suggests that the signal sequence is important for porcine α-lactalbumin accumulation in maize cells. Porcine α-lactalbumin is normally a secreted protein, but we have not included the porcine signal sequence in any of our constructs. With the absence of α-lactalbumin targeting information in P40 cells, α-lactalbumin would be expected to remain in the cytosol after translation. No protein was detected in calli or kernels from the P40 transformation events. It may be that accumulation of high levels of α-lactalbumin requires compartmentalization for protection from proteases, proper folding by endoplasmic reticulum-specific chaperones, post-translational modification or to prevent interference with cytosolic metabolism. These results are in agreement with several studies demonstrating that addition of sequences for protein targeting leads to increased levels of protein expression (Sijmons et al., 1990; Wandelt et al., 1992; Pen et al., 1993; Fiedler & Conrad, 1995; Schouten et al., 1996). It will be important to determine if the signal sequence of porcine α-lactalbumin is correctly processed in plant cells. There are examples that demonstrate that properly processed recombinant proteins can be produced in plants, as well as examples of incomplete processing (reviewed by Kusnadi et al., 1997).

The lactalbumin constructs in the P42 and P45 plants have the same signal sequences, and differ in the presence of an ER retention signal in the P45 construct. Both types of plants produced porcine

α-lactalbumin. Our data do not allow us to distinguish between these constructs regarding the level of α-lactalbumin produced or the frequency of expression. A more accurate method of quantifying α-lactalbumin and analysis of a larger number of events will be required to determine if there are differences in expression due to the ER retention signal.

Having demonstrated that this transgene functions to produce porcine α-lactalbumin in kernel tissues, it will be important to determine if this modification results in a nutritional improvement. We plan to analyze predictors of nutritional quality such as the amino acid balance in kernels producing high levels of α-lactalbumin. Comparison to literature in which foreign proteins produced in seeds have resulted in a changed amino acid balance (Altenbach et al., 1992; Molvig et al., 1997) suggests that the plants described here do produce enough α-lactalbumin to make a substantial change to the amino acid balance of the kernel. In order to increase level of α-lactalbumin in the kernel, we plan to investigate other protein expression strategies. It may be possible to improve the design of our synthetic coding sequence, and the use of kernel-specific promoters may be helpful as well.

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