

# Transgenic maize endosperm containing a milk protein has improved amino acid balance

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Received: 7 August 2006 / Accepted: 30 January 2007  
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**Abstract** In order to meet the protein nutrition needs of the world population, greater reliance on plant protein sources will become necessary. The amino acid balance of most plant protein sources does not match the nutritional requirements of monogastric animals, limiting their nutritional value. In cereals, the essential amino acid lysine is deficient. Maize is a major component of human and animal diets worldwide and especially where sources of plant protein are in critical need such as sub-Saharan Africa. To improve the amino acid balance of maize, we developed

transgenic maize lines that produce the milk protein  $\alpha$ -lactalbumin in the endosperm. Lines in which the transgene was inherited as a single dominant genetic locus were identified. Sibling kernels with or without the transgene were compared to determine the effect of the transgene on kernel traits in lines selected for their high content of  $\alpha$ -lactalbumin. Total protein content in endosperm from transgene positive kernels was not significantly different from total protein content in endosperm from transgene negative kernels in three out of four comparisons, whereas the lysine content of the lines examined was 29–47% greater in endosperm from transgene positive kernels. The content of some other amino acids was changed to a lesser extent. Taken together, these changes resulted in the transgenic endosperms having an improved amino acid balance relative to non-transgenic endosperms produced on the same ear. Kernel appearance, weight, density and zein content did not exhibit substantial differences in kernels expressing the transgene when compared to non-expressing siblings. Assessment of the antigenicity and impacts on animal health will be required in order to determine the overall value of this technology.

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**Keywords** Transgene · Maize ·  $\alpha$ -Lactalbumin ·  
Lysine · Nutrition · Grain

## Introduction

Maize (*Zea mays* L.) is one of the most important sources of food and feed in the world. In 2002, the world maize supply was 630 million metric tons and 98% of this was used for either food or feed (FAO 2003). Maize is widely grown, including in parts of world with poor food security such as Southeast Asia and sub-Saharan Africa. Thus, improving the nutritional value of maize grain would improve the quality of the world food supply.

An important nutritional limitation of maize endosperm is its amino acid balance. Deficiency in certain amino acids reduces the availability of others present in abundance. Comparison of maize protein to the FAO provisional pattern of amino acids required for human growth and development (FAO/WHO/UNU 1985) reveals that the limiting amino acid in maize is lysine.

Another limitation of plant proteins is that they can be poorly digested by animals or can cause allergic reactions (Nordlee et al. 1996; Scott and Bicar 2003). Milk proteins have been proposed as alternative proteins for expression in plants for nutritional improvement (Chong et al. 1997; Yang et al. 2002) because they are a natural component of animal diets and they have excellent nutritional parameters (Renner 1988; Matthews and Hughes 1993). Alpha-lactalbumin is a calcium binding milk protein with a  $M_R$  of 14,200 Da and a pI of 4–5 (Permyakov and Berliner 2000). It is rich in lysine, complementing the deficiency in maize. In addition, some forms of  $\alpha$ -lactalbumin have other properties that could be beneficial, such as bactericidal activity (Hakansson et al. 2000) and induction of apoptosis in tumor cells (Hakansson et al. 1995; Svensson et al. 1999). A cDNA clone encoding a porcine  $\alpha$ -lactalbumin preprotein of 141 amino acids, including a leader peptide of 19 amino acids, has been isolated and sequenced (Das Gupta et al. 1992). Functional  $\alpha$ -lactalbumin has been successfully produced in tobacco (Takase and Hagiwara 1998).

Recent advances in genetic engineering and the improvements in transformation technology have provided new opportunities to improve the amino acid balance of crops. Transgenes that express plant proteins have been successfully used

to alter the amino acid balance of several crop species. Grain methionine levels have been increased by introduction of plant methionine-rich albumin genes (Altenbach et al. 1989; De Clercq et al. 1990; Altenbach et al. 1992; Molvig et al. 1997), or by manipulation of the content of native seed storage proteins (Lai and Messing 2002). In addition, the total protein content in potato tubers (Chakraborty et al. 2000) and maize kernels (Yu et al. 2004) has been increased with a concomitant increase in essential amino acid levels by the addition of proteins from other plant species using genetic transformation.

Alpha-lactalbumin has been produced in maize grain using a synthetic coding sequence coupled to the maize *ubi-1* promoter (Yang et al. 2002). Versions containing either a zein signal sequence or a zein signal sequence and an endoplasmic reticulum (ER) retention motif both caused  $\alpha$ -lactalbumin to accumulate in grain, while a version with no subcellular targeting information did not (Yang et al. 2002). The  $\alpha$ -lactalbumin transgene was functional with expression levels between 0.01 to 0.05 g  $\alpha$ -lactalbumin per 100 g endosperm, but changes in the amino acid composition as a result of  $\alpha$ -lactalbumin expression were not examined (Yang et al. 2002).

The objective of this study was to determine if production of porcine  $\alpha$ -lactalbumin in the kernels of transgenic maize is a viable approach to altering the amino acid composition of the endosperm. This was accomplished by transforming maize with a new construct to create transgenic maize plants that express high levels of  $\alpha$ -lactalbumin specifically in endosperm tissue. Kernels from these plants were characterized genetically and biochemically.

## Materials and methods

### Expression vector construction

A synthetic, modified porcine  $\alpha$ -lactalbumin coding sequence (Yang et al. 2002) was used in the transformation construct. In this construct, the synthetic  $\alpha$ -lactalbumin coding sequence was transcriptionally fused to the maize 27 kDa gamma zein promoter and the *nos* 3' untranslated

region. The coding region encodes mature porcine  $\alpha$ -lactalbumin that is translationally fused to the maize 27 kDa gamma zein signal sequence at the N-terminus and an ER retention sequence (KDEL) at the C-terminus of the protein. The expression vector, designated P64, contains 4505 bp.

#### Plant transformation and tissue culture

Plant transformation and tissue culture was done at the Plant Transformation Facility at Iowa State University as described elsewhere (Frame et al. 2000). Embryogenic Type II callus was initiated from embryos of the Hi II genotype (Armstrong et al. 1991). This callus was transformed by particle bombardment with the  $\alpha$ -lactalbumin expression construct together with pBAR184 (Frame et al. 2000), a plasmid containing the selectable marker gene *BAR* driven by the maize *ubi-1* promoter. Plants resulting from a single callus are considered to contain a unique transformation event. These plants were designated as T0 plants and were transplanted from regeneration media into soil.

#### Development of segregating populations

The F1 generation was produced by crossing a T0 plant and the inbred line B73 using the T0 plants as females. F1 kernels were evaluated for the presence of the transgene by western blotting. Plants grown from five  $\alpha$ -lactalbumin positive F1 kernels from each event were backcrossed as males to B73 to obtain BC1F1 kernels. In addition, the same F1 plants were self-pollinated to obtain F2 generation kernels. From each self-pollinated ear on an F1 plant, positive F2 kernels were identified and planted and the resulting plants were self-pollinated to obtain F3 kernels. The T0 plants and F1 generation kernels were produced in the greenhouse while successive generations were produced in the field.

#### PCR analysis

The F1 kernels that were positive for  $\alpha$ -lactalbumin by western analysis were planted in the field. Three weeks after germination, leaf tissue was

collected from these plants. DNA was extracted from the fresh leaf tissue using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) following the manufacturer's recommendations. The DNA pellet was resuspended in 50  $\mu$ l of 50 mM Tris, 10 mM EDTA, pH 8.0. PCR was carried out using 1  $\mu$ l of isolated genomic DNA (~50 ng) in a 20  $\mu$ l total reaction volume containing 100  $\mu$ M of each dNTP, 2  $\mu$ l of 10 $\times$  PCR buffer, 1 U Platinum Taq DNA Polymerase (Gibco BRL, Carlsbad, CA), 2.85 mM of MgCl<sub>2</sub> and 0.2  $\mu$ M of each primer. The forward primer (LA-F = AAGCAGTTCACCAAGTGCGAGC) and the reverse primer (LA-R = TCTTCTTGGCGCACATCATGTC) corresponded to the  $\alpha$ -lactalbumin coding sequence and amplified a 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 after the reaction at 72°C in a Rapidcycler (Idaho Technologies, Inc., Salt Lake City, UT). PCR products were analyzed on 1.5% (w/v) agarose gels stained with ethidium bromide and photographed.

#### Evaluation of $\alpha$ -lactalbumin accumulation by western blot analysis of kernels

The presence or absence of  $\alpha$ -lactalbumin in all kernels used in this study was determined by western blot analysis. A portion of the endosperm (~10 mg) was removed from each kernel using a hand-held drill. Protein was extracted from this tissue with 100  $\mu$ l of SDS-PAGE sample buffer (0.5 M Tris-HCl, pH 6.8; 10% SDS; 10% glycerol; 5%  $\beta$ -mercaptoethanol) per 10 mg of finely ground endosperm. The samples were then mixed in a vortex shaker for 30 min and insoluble material was removed by centrifugation at 13,000 rpm for 5 min. The supernatant was heated to 95°C for 5 min before loading onto a 15% SDS-PAGE gel (Laemmli 1970). Gels were blotted onto a nylon-backed nitrocellulose membrane (0.45  $\mu$ m) using a mini-transblot apparatus according to the manufacturer's directions (Bio-Rad, Hercules, CA). The membrane was blocked with 1% ovalbumin in PBS-Tween buffer (0.14 M NaCl; 2.7 mM KCl; 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>; 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4; 0.1% Tween 20) for 1 h,

allowed to react for 8–10 h with a polyclonal antibody raised against human  $\alpha$ -lactalbumin in rabbits and visualized according to the manufacturer's protocol for colorimetric visualization of an alkaline phosphatase-conjugated anti-rabbit IgG (Bio-Rad, Hercules, CA).

#### Southern blot analysis

DNA was prepared from leaf tissue by the CTAB method (Saghai-Marooof et al. 1984). Genomic DNA was digested with *Sca1*, which cut the plasmid once outside the  $\alpha$ -lactalbumin transgene and then fractionated by electrophoresis on a 0.8 % agarose gel. Transfer to nylon membranes (Hybond, Amersham, Piscataway, NJ) was performed (Southern 1975). Approximately 5  $\mu$ g of genomic DNA were used per digest. Hybridization was carried out using a  $^{32}$ P-labeled probe at 65°C for 12–16 h. The probe was obtained by amplifying the  $\alpha$ -lactalbumin coding region of the P64 construct using PCR. The amplified band was resolved in a low melting point agarose gel, excised and labeled with ( $\alpha$ - $^{32}$ P) dCTP (Feinberg and Vogelstein 1983).

#### Quantitation of $\alpha$ -lactalbumin

Ten milligrams of finely ground endosperm were extracted with 100  $\mu$ l of SDS–PAGE sample buffer. Fifty microliters of endosperm extract were loaded into each well of a 96-well microtiter plate. Protein from the endosperm extract was captured for 16–18 h at 37°C. The plates were then blocked with 1% ovalbumin for 1 h at room temperature to prevent non-specific antibody binding. The  $\alpha$ -lactalbumin from endosperm extract was allowed to react for 4 h at room temperature with a rabbit polyclonal antibody against commercially prepared human  $\alpha$ -lactalbumin (Sigma, St. Louis, MO). Alkaline phosphatase-conjugated anti-rabbit IgG (Bio-Rad, Hercules, CA) was then added and colorimetric visualization was carried out according to the manufacturer's protocol. Between each step, wells were washed 3 or 4 times with 1 $\times$  PBS-Tween buffer. Absorbance at 405 nm was measured spectrophotometrically over a 2 h period in a Dynatech MRX Plate Reader (Dynex

Technologies, Chantilly, VA). The maximum change in absorbance was converted to concentrations of  $\alpha$ -lactalbumin by comparison to the values produced by standards of known  $\alpha$ -lactalbumin concentrations (50, 40, 30, 20, 10 ng/ $\mu$ l) using linear regression.

#### Amino acid analysis and nitrogen analysis

Amino acid concentrations were determined at the University of Missouri Experiment Station Laboratories using the standard AOAC Method (AOAC 2002), which consists of acid hydrolysis of the ground tissue followed by chromatographic separation and quantitation of the amino acids. Total nitrogen content was determined at the Iowa State University Plant and Soil Analysis Laboratory using combustion analysis. All comparisons reported were between pairs of kernels produced the same ear, with each pair consisting of one positive and one negative for  $\alpha$ -lactalbumin expression.

#### Evaluation of kernel phenotypes

The impact of  $\alpha$ -lactalbumin accumulation on kernel morphology, density, and zein profiles was assessed using kernels from ears segregating for  $\alpha$ -lactalbumin accumulation in each of the four events. Twenty randomly selected sibling kernels from one ear representing each event were photographed and volume and weight measurements were taken for estimates of density. Following measurements, kernels were screened by dot blot immuno assay as described previously (Scott et al. 2007) to identify which kernels contained  $\alpha$ -lactalbumin.

#### Evaluation of zein content of $\alpha$ -lactalbumin-containing kernels

Alcohol soluble proteins were extracted from approximately 25–30 mg endosperm tissue in 250–300 ml buffer containing 70% ethanol, 61 mM sodium acetate, and 5%  $\beta$ -mercaptoethanol by shaking for one hour at 37°C. Proteins were resolved by reverse phase HPLC according to the method of Wilson (1991). Peak assignments were taken from this reference.

## Results

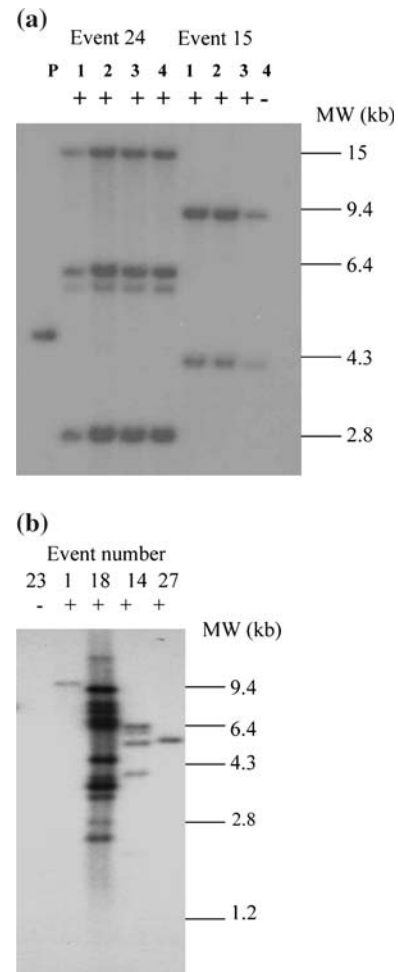
### Integration of the synthetic porcine $\alpha$ -lactalbumin gene into the maize genome

For a protein to impact amino acid balance, it must be abundant in the tissue of interest. Because  $\alpha$ -lactalbumin accumulated to relatively low levels when controlled by the maize *ubi-1* promoter (Yang et al. 2002), we designed a new construct, P64, incorporating the maize 27 kDa gamma zein promoter in place of the *ubi-1* promoter (Yang et al. 2002). The maize 27 kDa gamma zein promoter is the strongest of the zein promoters (Woo et al. 2001), and is therefore one of the strongest seed-specific promoters. This construct was used to transform maize by the Iowa State University Plant Transformation Facility using their standard protocol for particle bombardment (Frame et al. 2000). The resulting T0 plants were then crossed as females to the non-transformed inbred line B73 to obtain F1 kernels. F1 kernels were obtained from eight different T0 plants, each representing a different, independent transformation event.

PCR analysis confirmed the presence of the transgene into the genome of the F1 plants. Using primers corresponding to the synthetic porcine  $\alpha$ -lactalbumin coding sequence, a PCR product of the expected size (280 bp) was detected in F1 plants derived from six of the eight T0 plants (data not shown). Because the F1 plants were obtained from a cross between T0 and non-transformed B73 plants, the presence of  $\alpha$ -lactalbumin DNA sequences in the F1 plants from six of the eight events indicated that in six events the porcine  $\alpha$ -lactalbumin transgene was sexually transmitted to the F1 generation.

Southern blot analyses were performed on several F1 plants from each event and their F2 and F3 progenies in order to characterize the transgene integrations and to assess transgene stability through meiosis. Plants from the same transformation event had identical banding patterns characteristic of that transformation event, and this banding pattern was maintained through the F3 generation (Fig. 1a). No evidence of transgene rearrangement was detected. Additionally, since the restriction enzyme used to digest

the genomic DNA cut only once in the P64 construct, the number of transgene copies can be estimated from the number of the bands on the blot. Between 1 and 12 copies of the transgene were detected in the events examined (Fig. 1b).

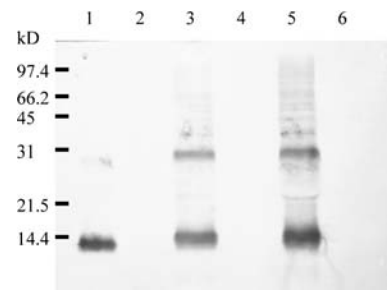


**Fig. 1** (a) Southern blot analysis of F1 plants and their corresponding F2 and F3 progenies from 2 events. Numbers above the lanes indicate the events, and + or - indicates whether  $\alpha$ -lactalbumin was identified in the kernel from which the analyzed plant was produced. Genomic DNA was digested with *Sca1* and probed with  $\alpha$ -lactalbumin coding sequence. Lane P contains 5 pg of plasmid DNA representing 1 copy per diploid genome. Lane 1 contains DNA from an F1 plant. Lane 2 contains DNA from an F2 plant. Lanes 3 and 4 contain DNA from 2 F3 sibling progenies. M = molecular weight standards in kilobases (kb). (b) Genomic Southern blot hybridization profiles of F1 plants from different events

## Transgene expression and inheritance

Having established that the transgene was successfully integrated into the maize genome, accumulation of the  $\alpha$ -lactalbumin protein was assessed by western blot to determine whether the synthetic  $\alpha$ -lactalbumin transgene functions in the plant system. Accumulation of  $\alpha$ -lactalbumin in F1 kernels was detected by western blot in six of the eight events (Table 1). In two events,  $\alpha$ -lactalbumin was not detected in any of the kernels analyzed, possibly because the transgene was silenced or was somehow disrupted in the process of transformation. As shown in Fig. 2, immunologically reactive protein bands can be detected in some transgenic kernels while no bands were present in the B73 control. A prominent band comigrated with the human  $\alpha$ -lactalbumin positive control, suggesting that  $\alpha$ -lactalbumin was produced and accumulated in the endosperm tissue. The western blot results were consistent with the PCR results described earlier. These results demonstrated that not only was the  $\alpha$ -lactalbumin transgene transmitted to the F1 generation but that the transgene functioned to produce immunologically detectable  $\alpha$ -lactalbumin in F1 kernel endosperm.

The expression and inheritance of transgenes in the first generation of transgenic plants are not



**Fig. 2** Western blot analysis  $\alpha$ -lactalbumin in the endosperm of transformed maize kernels. Lane 1. Human  $\alpha$ -lactalbumin; 2. P64-18 (negative); 3. P64-18 (positive); 4. P64-14 (negative); 5. P64-14 (positive); 6. Untransformed maize inbred B73

always good predictors of transgene stability in subsequent generations because problems with transmission and expression of a transgene may arise after several sexual generations (Register et al. 1994). To characterize the expression and inheritance of the synthetic  $\alpha$ -lactalbumin transgene, transgene expression was monitored by western blot analysis for three generations. Four of six events examined demonstrated stable inheritance of the synthetic porcine  $\alpha$ -lactalbumin gene in the F2 generation, consistent with a single dominant locus inheritance model (Table 1). In the F3 generation, the homozygous class is somewhat underrepresented (Table 1). This

**Table 1** Segregation of a modified  $\alpha$ -lactalbumin transgene among the  $\alpha$ -lactalbumin-positive independent events in maize based on the presence and absence of the  $\alpha$ -lactalbumin protein as detected by western blot analysis

Event Number	Generations			
	F1 (+/-)	BC1F1 (+/-)	F2 (+/-)	F3 (+/-)
18	4/1	5/5 ns	46/14 ns	14/5 ns; 15/5 ns; 14/6 ns; 15/5 ns; 12/5 ns; 12/4 ns; 11/6 ns; 10/3 ns; 12/5 ns; 9/5 ns
14	3/2	5/5 ns	49/18 ns	9/6 ns; 13/4 ns; 14/3 ns; 10/7 ns; 12/5 ns; 10/7 ns; 11/5 ns; 12/5 ns; 10/7 ns; 14/3 ns; 14/3 ns; 13/4 ns; 14/3 ns; 12/4 ns; 17/0 H; 11/7 ns; 11/6 ns;
15	4/1	5/5 ns	34/12 ns	11/6 ns; 12/5 ns; 11/6 ns; 13/4 ns; 17/0 H; 13/4 ns; 14/2 ns
1	5/0		50/16 ns	14/3 ns; 10/7 ns
24	1/0	4/6 ns	39/27**	17/0 H; 15/2 ns; 16/1 ns; 20/0 H; 17/3 ns
27	2/3		23/43**	0/17**; 9/8 ns; 0/17**; 0/17**; 0/17**
23	0/5			
13	0/5			

ns = not significantly different from 3:1 phenotypic ratio for a single dominant locus model in the F2 and F3; 1:1 ratio for BC1F1; \*\* significantly different;  $\chi^2$  (0.01, 1df) = 6.64; H = homozygous for  $\alpha$ -lactalbumin protein expression. Numbers in each generation column are the proportion of kernels expressing (+) / not expressing (-) the  $\alpha$ -lactalbumin protein in the endosperm. F1 kernels were produced by crossing a T0 plant and B73. Positive plants resulting from these kernels were crossed to B73 or selfed to get BC1F1 or F2 kernels, respectively. Positive F2 plants were self-pollinated to get F3 kernels

could be the result of aberrant transmission of the transgene or poor germination of the transgenic seeds. In subsequent experiments with plants that have been backcrossed into agronomically adapted genetic backgrounds, we have produced homozygous lines and have not observed poor germination, so the underrepresentation of the homozygous class in the F3 generation may be a consequence of genetic background. In any case, germination and transmission will need to be monitored carefully in the course of development of commercial products containing this transgene.

#### Levels of porcine $\alpha$ -lactalbumin in the endosperm of transformed kernels

To quantify the level of porcine  $\alpha$ -lactalbumin protein in the endosperm, ELISA was performed using a polyclonal antibody against human  $\alpha$ -lactalbumin. Variation in the amounts of  $\alpha$ -lactalbumin in an event was observed among sibling kernels that were produced on the same ear. The  $\alpha$ -lactalbumin levels in 5–15 positive kernels in an event were averaged to obtain the mean  $\alpha$ -lactalbumin value for that event. The average amount of  $\alpha$ -lactalbumin in the endosperm on a per unit dry weight basis ranged from 0.001 to 0.039 g per 100 g endosperm tissue in F2 kernels and from 0.003 to 0.095 g per 100 g endosperm tissue in F3 kernels (Table 2). For comparison, the level of some individual zein subunits was about 1 g per 100 g endosperm tissue.

**Table 2** Levels of porcine  $\alpha$ -lactalbumin protein in the endosperm of transformed maize kernels among different events of P64 constructs as measured by ELISA

Events	$\alpha$ -lactalbumin $\pm$ SE (g/100g)	
	F2	F3
P64-18-5	0.025 $\pm$ 0.001	0.089 $\pm$ 0.020
P64-14-4	0.039 $\pm$ 0.007	0.095 $\pm$ 0.020
P64-24-4	0.007 $\pm$ 0.001	0.012 $\pm$ 0.002
P64-1-4	0.001 $\pm$ 0.000	0.003 $\pm$ 0.000
P64-15-6	ND	0.074 $\pm$ 0.025

<sup>a</sup> Values are means of 5 to 15 positive sibling kernels. F2 and F3 kernels are derived from a cross between a T0 plant and the maize inbred, B73, and were produced in different environments. ND = Not Determined

#### Amino acid composition of endosperm of kernels expressing $\alpha$ -lactalbumin

To determine if the expression of the porcine  $\alpha$ -lactalbumin protein in the endosperm led to changes in amino acid composition, complete amino acid analysis was performed on dissected endosperms from two events expressing high levels of  $\alpha$ -lactalbumin, P64-14 and P64-18. In order to minimize environmental effects and differences due to different female parents, we compared kernels that were produced on the same ears. Ears segregating for the transgene were produced from the F2 and F3 generations of selected events and positive and negative sibling kernels were compared using the standard AOAC method (AOAC 2002) for amino acid analysis. These samples were divided and subjected to combustion analysis of nitrogen as well.

Several statistically significant changes in amino acid composition were observed (summarized in Table 3, details given in Tables 1 and 2 in the Appendix). The level of lysine was significantly higher in positive kernels than in their negative siblings. This increase ranged from 29 to 47% and was accompanied by changes in levels of other amino acids. Aspartic acid and isoleucine both were increased significantly in transgenic kernels relative to their negative siblings in three out of the four observations made. In the modified porcine  $\alpha$ -lactalbumin coding sequence, aspartic acid is the most frequent amino acid (15 of 141) followed by lysine (12 of 141). Isoleucine is also abundant in this protein (11 of 141) (Yang et al. 2002). Thus, the changes observed in the amino acid content of the transgenic grain generally reflect the amino acid composition of  $\alpha$ -lactalbumin. The positive and negative sibling kernels did not exhibit obvious differences for other traits and are otherwise indistinguishable from each other.

#### Nitrogen content of endosperm of kernels expressing $\alpha$ -lactalbumin

To compare the allocation of carbon and nitrogen between transgenic and nontransgenic kernels, carbon and nitrogen levels were determined by combustion analysis. Significant differences in

**Table 3** Percent difference in amino acid and protein content between  $\alpha$ -lactalbumin positive and negative sibling kernels from events P64-14 and P64-18

Amino acid	% difference <sup>a</sup>			
	Event 14 F2	Event 14 F3	Event 18 F2	Event 18 F3
Asp	7.14 ns	12.31 *	24.59 *	13.89 *
Thr	6.06 *	2.86 ns	15.63 *	5.41 ns
Ser	7.69 ns	-6.38 *	22.50 *	6.00 ns
Glu	-0.85 ns	0.00 ns	11.01 *	-2.86 ns
Pro	-3.70 ns	-5.79 *	-1.87 ns	-8.00 ns
Gly	0.00 ns	0.00 ns	17.39 *	4.00 ns
Ala	-1.14 ns	-1.05 ns	12.94 *	-0.97 ns
Cys	8.70 *	4.00 ns	4.35 ns	-4.35 ns
Val	3.85 ns	1.92 ns	6.12 ns	-7.27 ns
Met	6.90 ns	-3.23 ns	21.43 *	8.00 ns
Ile	9.76 *	17.95 *	15.79 *	-4.26 ns
Leu	-0.60 ns	-0.55 ns	12.35 *	-0.49 ns
Tyr	-2.56 ns	-2.56 ns	22.22 *	10.00 *
Phe	1.61 ns	1.52 ns	15.25 *	-1.33 ns
His	0.00 ns	0 ns	-3.85 ns	-10.71 ns
Lys	35.00 *	29.41 *	47.06 *	33.33 *
Arg	2.68 ns	2.78 ns	14.29 ns	0.00 ns
Total Nitrogen <sup>b</sup>	-0.89 ns	1.71 ns	10.58 *	4.13 ns

<sup>a</sup> Each entry is calculated as ((positive kernel value/negative kernel value)-1)  $\times$  100

<sup>b</sup> Determined by combustion analysis

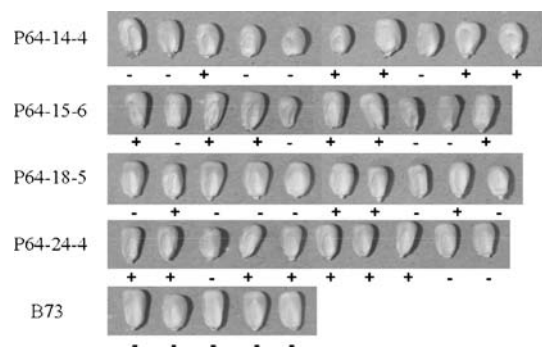
\* = Difference between positive and negative values is significant in a t-test at  $\alpha = 0.05$ ; ns = not significant

carbon content between  $\alpha$ -lactalbumin positive and negative sibling kernels were not detected in the events examined. In nitrogen content, however,  $\alpha$ -lactalbumin positive kernels in P64-18 F2 showed a significant 11% increase compared to their negative siblings (Table 3). No significant change in total nitrogen was detected between  $\alpha$ -lactalbumin positive and negative sibling kernels in event 14 or in the F3 generation of event 18. Since this change in total nitrogen content was only seen in one of the four analyses, it is unlikely to be a fundamental consequence of the presence of the transgene. Since the change in total nitrogen was considerably smaller than the change in the levels of certain amino acids, these results indicate that the amino acid balance was altered favorably in endosperm of kernels containing the transgene relative to sibling kernels lacking the transgene.

#### Effect of the transgene on kernel phenotypes

We next set out to determine if the  $\alpha$ -lactalbumin transgene had an effect on kernel mass or density. For this experiment we started with heterozygous kernels from plants that were back-crossed to B73 at 2 or 3 three times. These kernels were planted and the resulting plants were crossed to B73. The resulting ears would be expected to segregate 1:1

for the transgene. As before, we compared positive kernels to negative kernels from the same ears to minimize maternal and environmental effects. Kernels were photographed, kernel weight and density were determined, and then kernels were scored for  $\alpha$ -lactalbumin content using the tissue print blotting method. There were no obvious visual differences between  $\alpha$ -lactalbumin positive and  $\alpha$ -lactalbumin negative kernels (Fig. 3). Mean kernel masses and densities of  $\alpha$ -lactalbumin positive kernels were compared to



**Fig. 3** Randomly selected kernels for measurements of weight and density that were then scored for tissue blot immuno blot assay and zein extraction. All kernels representing one event were produced on the same ear. Positive and negative signs indicate the result of subsequent screening for the presence of  $\alpha$ -lactalbumin

**Table 4** Comparison of kernel weight and density within and among four P64 events and B73 by tissue blot immuno assay score (positive or negative for  $\alpha$ -lactalbumin)

Event	Score	Number of kernels	Weight (g)	Std Dev	Probability of > F	Mean density (g/cm <sup>3</sup> )	Std Dev	Probability of > F
P64-14-	pos	8	0.170	0.031	0.508 <sup>a</sup>	1.20	0.131	0.435
4	neg	12	0.162	0.024		1.27	0.242	
P64-15-	pos	12	0.159	0.023	0.489	1.24	0.151	0.132
6	neg	8	0.168	0.037		1.36	0.192	
P64-18-	pos	9	0.201	0.014	0.787	1.24	0.113	0.067
5	neg	11	0.202	0.009		1.13	0.134	
P64-24-	pos	12	0.135	0.008	0.946	1.26	0.167	0.161
4	neg	8	0.136	0.014		1.11	0.279	
Overall	pos	41	0.163	0.031	0.411	1.23	0.145	0.624
	neg	39	0.169	0.032		1.21	0.230	
B73	neg	5	0.223	0.022		1.08	0.044	

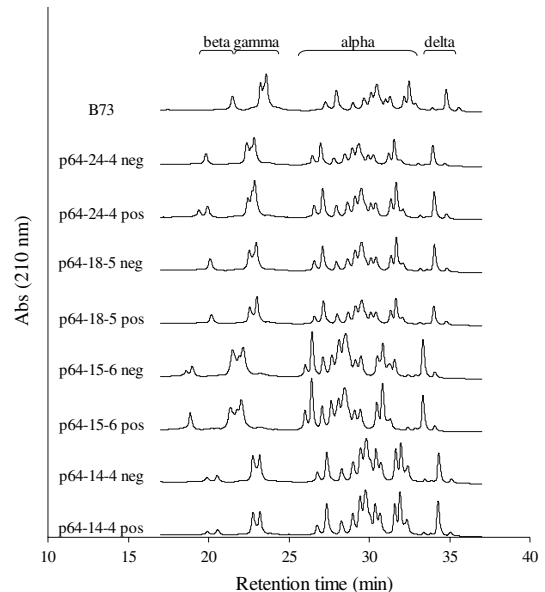
<sup>a</sup> Probability of obtaining the observed difference between positive and negative kernels by chance evaluated by one-way ANOVA. Values >0.05 indicate the observed difference between positive and negative kernels is probably (95% likelihood) due to random variation in the measurements rather than an actual difference in the means of the two groups

those of  $\alpha$ -lactalbumin negative kernels (Table 4). While significant differences in kernel mass were found between the ears of different events (analysis not shown), there were no significant differences between transgene positive and negative kernels within any of the events. There were no significant differences in kernel density as well.

#### Effect of the transgene on zein accumulation

Zeins are the main nitrogen storage depot in the seed, so changes caused by accumulation of a foreign protein such as  $\alpha$ -lactalbumin could be reflected in changes in the accumulation of zeins. Additionally, because the transgene contains a zein promoter, it is possible that the transgene would compete for transcription factors with native zein genes or cause gene silencing of zein genes and thereby cause a change in zein profiles. To investigate these possibilities, we analyzed the alcohol-soluble protein content by reverse-phase HPLC (Wilson 1991). This procedure resolves many of the zein polypeptides into discrete peaks. As before, we compared kernels within ears segregating for the transgene to minimize maternal and environmental effects. There were clear differences in zein content between kernels from different ears and in some cases there were differences between  $\alpha$ -lactalbumin positive and  $\alpha$ -lactalbumin negative kernels (Fig. 4). None of these differences was consistent in all events

studied. Thus, we conclude that  $\alpha$ -lactalbumin may have minor event-specific impact on accumulation of some zeins; however, the effect on zein accumulation as a whole was small.



**Fig. 4** HPLC traces of kernels from four P64 events and non-transgenic B73. Neg and pos in the trace label indicate the presence or absence, respectively, of  $\alpha$ -lactalbumin in the kernels analyzed. The approximate positions of zein families are indicated at the top of the figure

## Discussion

The objective of this work was to increase the level of lysine in grain by expressing the milk protein  $\alpha$ -lactalbumin in the kernels of transgenic maize. Two events, P64-14 and P64-18, contained the highest levels of  $\alpha$ -lactalbumin in endosperm as measured by ELISA and these events displayed consistently higher lysine levels in a complete amino acid composition analysis using F2 and F3 kernels. These plants were produced in the field with the different generations produced in different years. The endosperm lysine content was increased by 35 and 29% in F2 and F3 kernels of P64-14, respectively, and by 47 and 33% in F2 and F3 kernels of P64-18, respectively. These results suggest that expression of milk proteins in grain is a potentially useful strategy to improve maize grain quality.

While the expression of the modified porcine  $\alpha$ -lactalbumin gene resulted in significant differences in lysine content, the accumulation of  $\alpha$ -lactalbumin in transgenic grain samples was not sufficient to explain the observed changes in amino acid balance. For example, in P64-18 F2 kernels, the amount of  $\alpha$ -lactalbumin protein in this grain as measured by ELISA was 0.025 g per 100 g of endosperm tissue (Table 2). The difference in lysine content between kernels containing  $\alpha$ -lactalbumin and those not containing  $\alpha$ -lactalbumin was 0.08 g lysine per 100 g endosperm tissue, more than three times greater than the measured amount of  $\alpha$ -lactalbumin in the kernels. It is possible that our  $\alpha$ -lactalbumin assay underestimates the level of  $\alpha$ -lactalbumin in the grain, perhaps because  $\alpha$ -lactalbumin was not extracted efficiently. This possibility is supported by the observation that changes in amino acid balance caused by  $\alpha$ -lactalbumin expression reflected the amino acid balance of  $\alpha$ -lactalbumin. A second possibility is that  $\alpha$ -lactalbumin production caused changes in gene expression and/or metabolism that resulted in increased expression of high lysine proteins or production of elevated levels of free lysine.

It is encouraging that significant differences in kernel appearance, weight, density and zein content between positive and negative kernels were not found. The use of mutations such as

*opaque2* to increase the lysine content results in changes in these traits that offset the value of the increased lysine content. Our results suggest that the  $\alpha$ -lactalbumin transgene does not perturb native gene expression sufficiently to cause detectable changes in these traits.

The levels of  $\alpha$ -lactalbumin protein detected in the endosperm varied among events. The mean levels of  $\alpha$ -lactalbumin among events ranged from 0.0014 to 0.062 g per 100 g of endosperm tissue in F2 kernels and from 0.003 to 0.095 g per 100 g of endosperm tissue in F3 kernels (Table 2). Because of this variation, it may be possible to identify transformation events that produce higher levels of  $\alpha$ -lactalbumin by examining more transformation events. Variation in the levels of transgene protein products among events has been observed in several transgenic crops and has been postulated to be partly due to variation in the chromosomal location where transgenes are integrated into the genome and to differences in copy number (Ye and Signer 1996). Independent transformation events clearly had different transgene integration sites, as indicated by their unique Southern blot hybridization patterns (Fig. 1). It is possible that in some events the  $\alpha$ -lactalbumin transgene might have integrated near hypermethylated chromosomal regions which can result in reduced transgene expression (Zhong et al. 1999). Also, Southern blot analysis indicated that each event differed in  $\alpha$ -lactalbumin copy number (Fig. 1). The levels of  $\alpha$ -lactalbumin in F3 kernels were 2- to 4-fold higher compared to the  $\alpha$ -lactalbumin levels in F2 kernels (Table 2). In other studies, advanced generations have been shown to accumulate higher levels of the novel protein when compared to the F1 generation (Zhong et al. 1999). In this study, however, the levels of  $\alpha$ -lactalbumin accumulated in F2 and F3 kernels are confounded with the environmental effects. The F2 and F3 kernels were produced in different environmental conditions, therefore direct comparison of  $\alpha$ -lactalbumin levels between the F2 and F3 kernels is not appropriate. The fact that lysine levels were higher in the positive kernels across environments suggests however, that the presence of the  $\alpha$ -lactalbumin transgene was the

critical determinant of improved lysine levels in the endosperm.

In our analyses of  $\alpha$ -lactalbumin levels and amino acid content, we compared positive and negative kernels, but it is likely that the positive kernels were a mixture of homozygous and heterozygous kernels. It will be important to determine if there is a gene dosage effect because if there is, then homozygous kernels should contain more  $\alpha$ -lactalbumin than heterozygous kernels. If this were the case, then it may be possible to achieve greater improvements than reported here using homozygous plants. The analysis presented here therefore represents a conservative estimate of the gain possible with this transgene.

This work demonstrates the feasibility of manipulating grain lysine content by introduction of a foreign protein in the grain, providing a valuable model system for studies aimed at producing foreign proteins in the grain. However, this germplasm is not being considered for

commercial release because important information is lacking. Perhaps most important is the antigenicity of this grain. Bovine  $\alpha$ -lactalbumin is a known human allergen, and while porcine  $\alpha$ -lactalbumin has not been tested, Taylor indicates “Milk from other animals is likely to be allergenic to individuals with cow’s milk allergy” (1992). In addition, it will be important to evaluate the effects of this modification on other aspects of animal health as well as on agronomic traits such as seed quality and grain yield.

**Acknowledgements** The authors wish to thank Erik Mottl and Merinda Struthers for technical assistance. Names are necessary to report factually on the available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may be suitable. This work was funded in part by an Iowa Corn Promotion Board grant to MPS and ML and by the Raymond F. Baker Center for Plant Breeding. EHB was supported by a fellowship from Pioneer Hibred International awarded to EHB and ML.

**Appendix**

**Table 1** Endosperm amino acid composition and total protein content of  $\alpha$ -lactalbumin protein positive and negative sibling kernels in event 14 as measured in a complete amino acid analysis assay

Amino acid	F2		% Difference	F3		% Difference
	Mean (g/100g tissue) $\pm$ SE <sup>a</sup>			Mean (g/100g tissue) $\pm$ SE <sup>a</sup>		
	Positive	Negative		Positive	Negative	
Asp	0.75 $\pm$ 0.023	0.70 $\pm$ 0.046	7.14 ns	0.73 $\pm$ 0.015	0.65 $\pm$ 0.003	12.31*
Thr	0.35 $\pm$ 0.005	0.33 $\pm$ 0.005	6.06*	0.36 $\pm$ 0.005	0.35 $\pm$ 0.003	2.86 ns
Ser	0.42 $\pm$ 0.012	0.39 $\pm$ 0.014	7.69 ns	0.44 $\pm$ 0.003	0.47 $\pm$ 0.008	-6.38*
Glu	2.34 $\pm$ 0.000	2.36 $\pm$ 0.059	-0.85 ns	2.58 $\pm$ 0.020	2.58 $\pm$ 0.020	0.00 ns
Pro	1.04 $\pm$ 0.006	1.08 $\pm$ 0.029	-3.70 ns	1.14 $\pm$ 0.018	1.21 $\pm$ 0.008	-5.79*
Gly	0.26 $\pm$ 0.006	0.26 $\pm$ 0.012	0.00 ns	0.25 $\pm$ 0.006	0.25 $\pm$ 0.000	0.00 ns
Ala	0.87 $\pm$ 0.003	0.88 $\pm$ 0.023	-1.14 ns	0.94 $\pm$ 0.008	0.95 $\pm$ 0.006	-1.05 ns
Cys	0.25 $\pm$ 0.000	0.23 $\pm$ 0.006	8.70*	0.26 $\pm$ 0.000	0.25 $\pm$ 0.003	4.00 ns
Val	0.54 $\pm$ 0.012	0.52 $\pm$ 0.010	3.85 ns	0.53 $\pm$ 0.013	0.52 $\pm$ 0.006	1.92 ns
Met	0.31 $\pm$ 0.008	0.29 $\pm$ 0.013	6.90 ns	0.30 $\pm$ 0.003	0.31 $\pm$ 0.006	-3.23 ns
Ile	0.45 $\pm$ 0.006	0.41 $\pm$ 0.012	9.76*	0.46 $\pm$ 0.005	0.39 $\pm$ 0.012	17.95*
Leu	1.65 $\pm$ 0.008	1.66 $\pm$ 0.052	-0.60 ns	1.82 $\pm$ 0.025	1.83 $\pm$ 0.020	-0.55 ns
Tyr	0.38 $\pm$ 0.008	0.39 $\pm$ 0.005	-2.56 ns	0.38 $\pm$ 0.012	0.39 $\pm$ 0.008	-2.56 ns
Phe	0.63 $\pm$ 0.003	0.62 $\pm$ 0.023	1.61 ns	0.67 $\pm$ 0.010	0.66 $\pm$ 0.008	1.52 ns
His	0.28 $\pm$ 0.006	0.28 $\pm$ 0.008	0.00 ns	0.29 $\pm$ 0.005	0.29 $\pm$ 0.003	0 ns
<b>Lys</b>	<b>0.27 <math>\pm</math> 0.014</b>	<b>0.20 <math>\pm</math> 0.005</b>	<b>35.00*</b>	<b>0.22 <math>\pm</math> 0.008</b>	<b>0.17 <math>\pm</math> 0.003</b>	<b>29.41*</b>
Arg	0.38 $\pm$ 0.008	0.37 $\pm$ 0.012	2.68 ns	0.37 $\pm$ 0.008	0.36 $\pm$ 0.000	2.78 ns
Crude Protein		11.15 $\pm$ 0.156		11.27 $\pm$ 0.181-0.89 ns		
		11.90 $\pm$ 0.054	11.79 $\pm$ 0.139	1.71 ns		

<sup>a</sup> Mean values are from three sibling kernels from each positive and negative class  $\pm$  standard error

\* = difference between positive and negative values is significant in a *t*-test at  $\alpha = 0.05$ ; ns = not significant

F2 and F3 are consecutive sexual generations

**Table 2** Endosperm amino acid composition and total protein content of  $\alpha$ -lactalbumin protein positive and negative sibling kernels in event 18 as measured in a complete amino acid analysis assay

Amino acid	F2			% Difference	F3		
	Mean (g/100g tissue) $\pm$ SE <sup>a</sup>		Positive		Mean (g/100g tissue) $\pm$ SE <sup>a</sup>		Positive
	Positive	Negative			Negative	% Difference	
Asp	0.76 $\pm$ 0.054	0.61 $\pm$ 0.020	24.59*	0.82 $\pm$ 0.023	0.72 $\pm$ 0.020	13.89*	
Thr	0.37 $\pm$ 0.020	0.32 $\pm$ 0.008	15.63*	0.39 $\pm$ 0.011	0.37 $\pm$ 0.000	5.41 ns	
Ser	0.49 $\pm$ 0.020	0.40 $\pm$ 0.014	22.50*	0.53 $\pm$ 0.017	0.50 $\pm$ 0.017	6.00 ns	
Glu	2.52 $\pm$ 0.040	2.27 $\pm$ 0.029	11.01*	2.72 $\pm$ 0.047	2.80 $\pm$ 0.075	-2.86 ns	
Pro	1.05 $\pm$ 0.031	1.07 $\pm$ 0.014	-1.87 ns	1.15 $\pm$ 0.027	1.25 $\pm$ 0.039	-8.00 ns	
Gly	0.27 $\pm$ 0.020	0.23 $\pm$ 0.003	17.39*	0.26 $\pm$ 0.014	0.25 $\pm$ 0.005	4.00 ns	
Ala	0.96 $\pm$ 0.026	0.85 $\pm$ 0.013	12.94*	1.02 $\pm$ 0.016	1.03 $\pm$ 0.029	-0.97 ns	
Cys	0.24 $\pm$ 0.010	0.23 $\pm$ 0.003	4.35 ns	0.22 $\pm$ 0.008	0.23 $\pm$ 0.003	-4.35 ns	
Val	0.52 $\pm$ 0.018	0.49 $\pm$ 0.011	6.12 ns	0.51 $\pm$ 0.011	0.55 $\pm$ 0.023	-7.27 ns	
Met	0.34 $\pm$ 0.015	0.28 $\pm$ 0.005	21.43*	0.27 $\pm$ 0.012	0.25 $\pm$ 0.005	8.00 ns	
Ile	0.44 $\pm$ 0.014	0.38 $\pm$ 0.008	15.79*	0.45 $\pm$ 0.003	0.47 $\pm$ 0.020	-4.26 ns	
Leu	1.82 $\pm$ 0.030	1.62 $\pm$ 0.020	12.35*	2.05 $\pm$ 0.043	2.06 $\pm$ 0.079	-0.49 ns	
Tyr	0.44 $\pm$ 0.020	0.36 $\pm$ 0.010	22.22*	0.44 $\pm$ 0.013	0.40 $\pm$ 0.005	10.00*	
Phe	0.68 $\pm$ 0.023	0.59 $\pm$ 0.005	15.25*	0.74 $\pm$ 0.010	0.75 $\pm$ 0.023	-1.33 ns	
His	0.25 $\pm$ 0.008	0.26 $\pm$ 0.006	-3.85 ns	0.25 $\pm$ 0.005	0.28 $\pm$ 0.012	-10.71 ns	
<b>Lys</b>	<b>0.25 <math>\pm</math> 0.040</b>	<b>0.17 <math>\pm</math> 0.005</b>	<b>47.06*</b>	<b>0.24 <math>\pm</math> 0.016</b>	<b>0.18 <math>\pm</math> 0.008</b>	<b>33.33*</b>	
Arg	0.40 $\pm$ 0.028	0.35 $\pm$ 0.008	14.29 ns	0.36 $\pm$ 0.015	0.36 $\pm$ 0.008	0.00 ns	
Crude Protein		11.55 $\pm$ 0.470		10.47 $\pm$ 0.046	10.58*		
	12.63 $\pm$ 0.375	12.15 $\pm$ 0.276	4.13 ns				

<sup>a</sup> Mean values are from three sibling kernels from each positive and negative class  $\pm$  standard error.

\* = difference between positive and negative values is significant in a *t*-test at  $\alpha = 0.05$ ; ns = not significant.

F2 and F3 are consecutive sexual generations.

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