ALTERATIONS IN THE ACCUMULATION PATTERNS OF SEED STORAGE PROTEINS ENHANCE THE NUTRITIVE VALUE OF MAIZE KERNELS

by

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ABSTRACT OF THE DISSERTATION

Alterations in the accumulation patterns of seed storage proteins enhance the nutritive value of maize kernels

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The phenotypic plasticity of the zeins, the major seed storage proteins in maize, allows for manipulation of the quality of amino acids to enhance the nutritive value of maize kernels. As the primary sink of nitrogen (N) and sulfur (S) for the germinating seedling and not as a repository of specific amino acids, it can tolerate significant alterations in its amino acid composition. Using transgenic approaches to improve the essential amino acid (EAA) content of maize, my dissertation is formatted into two chapters and focuses on disparate mechanisms that we have utilized to increase methionine and/or lysine in the kernels.

The first chapter relates to the generation and characterization of a high-methionine maize obtained by metabolic engineering of the cysteine biosynthetic pathway. Under limiting S availability, transgenic maize kernels overexpressing its own high-methionine 10-kDa δ-zein displays redistribution of protein S in the seeds, a sink

tissue, whereby the increased accumulation of the δ-zein is accompanied by a concomitant decrease in the cysteine-rich β- and γ-zeins. This reflects the limitation on S availability in the maize kernels. Increasing S availability or supply from the leaf, a source tissue, by overexpression of the assimilatory reductase *Ec*PAPR, 3'-phosphoadenosine-5'-phosphosulfate reductase enzyme from *Escherichia coli*, overcame the rebalancing of S storage in the zeins. Transgenic high-methionine kernels have high levels of the δ-zein without the accompanying loss of protein S from other zeins. In addition, cysteine-rich non-zein proteins in the transgenic kernels were also upregulated. This overall increase in the accumulation of the S-rich zeins and cysteine-rich non-zein proteins enhanced the kernel methionine and cysteine contents. One transgenic event, PE5, promoted weight gains in chicks when formulated as an animal feed without synthetic methionine supplementation. Increased expression of the S-rich zeins under enhanced sulfur assimilation in source tissues constitutes a newly-described aspect of regulation of S amino acid accumulation in maize kernels.

The second chapter involves the combination of two different mechanisms – deregulation of the cysteine biosynthetic pathway, as mentioned, and reduction of zeins by RNA interference – to produce maize kernels with enhanced lysine and methionine contents. Reduction in zeins are generally associated with increased nutritional quality of maize kernels, and this is exemplified by the classical *opaque-2* (*o2*) mutant of maize that conditions a severe reduction in the abundant α-zeins. *o2* has high nutritional value as determined by feeding trials with rats and infants and a biological value that approaches that of milk. However, O2 as a transcription factor recognizes several genes, including the 22-kDa a-zeins, and its loss results to pleiotropic changes in gene expression in the *o2* seeds, leading to the opaque, or soft endosperm, phenotype. The opaque phenotype

renders the kernel susceptible to insect and fungal damage and the soft texture makes the kernels prone to breakage during handling and storage. In combination with genetic modifiers that can restore the vitreous endosperm in *o2* kernels, quality protein maize (QPM) was developed that has the high-lysine trait of *o2* but with a hard endosperm. However, the complexity of introducing multiple, unlinked loci of these modifiers into an *o2* background makes the process of generating QPMs laborious and complicated.

To explore QPMs with elevated levels of methionine, PE5 maize was crossed with different transgenic zein reduction lines (RNAi lines that target zeins of classes: α, β, γ, α + γ, or γ + β). Although it was already known that loss of the abundant α-zeins redistributed N to the non-zein protein fraction, thereby increasing the synthesis of some lysine-rich non-zein proteins. Addition of the PE5 event to α-RNAi further increased the lysine content. Although an additional reduction in γ-zeins in PE5;α-/γ- resulted in a reduction of lysine, the methionine level were nearly as good as with PE5 alone. Despite this favorable outcome, PE5;α-/γ- shares the same problems as α-RNAi alone in that it had an opaque kernel phenotype. Surprisingly, leaving off α-RNAi still gave elevated levels of lysine and methionine over classical mutants without the opaque phenotype.

Aside from downregulation of zein proteins, upregulation of the S-rich zeins as displayed in the PE5 kernels show that altering zein synthesis in the maize endosperm can indeed significantly improve the nutritional quality of maize kernels. Combining PE5 with the loss of γ-zeins produced kernels with superior Met and Lys contents and hard endosperm compared to *o2* seeds. From our observations, a common mechanism in the regulation of EAA accumulation in maize kernels appears to be the redistribution of macronutrients, particularly N and S, under limiting nutrient availability. We show here that this redistribution occurs due to the altered synthesis of zeins and can improve the

amino acid quality of the seeds. This redistribution of nutrients leads to rebalancing of nutrient storage by altering the synthesis of other proteins. Increasing nutrient availability from the source tissues by deregulation of the corresponding metabolic pathway can overcome this restriction on EAA accumulation in the sink.

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Chapter 1 of this dissertation was submitted as an article to the journal Proceedings of the National Academy of Science and is currently in press. Chapter 2 was submitted as an article to the journal Genetics and is currently under review.

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LIST OF ABBREVIATIONS

APS, adenosine 5'-phosphosulfate

APR, APS reductase

Cys, cysteine

***Pa*APR**, *Pseudomonas aeruginosa* APS reductase

EAA, essential amino acid

PAPS, 3'-phosphoadenosine 5'-phosphosulfate

PAPR, PAPS reductase

***Ec*PAPR**, *Escherichia coli* PAPS reductase

GSH, glutathione

Lys, lysine

mBBr, monobromobimane

Met, methionine

N, nitrogen

o2, opaque-2

PB, protein body

PE#, event from transformation of maize with the PepC-EcPAPR expression cassette

PP#, event from transformation of maize with the PepC-PaAPR expression cassette

RE#, event from transformation of maize with the RbcS-EcPAPR expression cassette

RP#, event from transformation of maize with the RbcS-PaAPR expression cassette

S, sulfur

SAA, sulfur amino acid

SSA, sunflower seed albumin

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CHAPTER 1: ENGINEERING SULFUR STORAGE IN MAIZE SEED PROTEINS WITHOUT APPARENT YIELD LOSS

1.1. ABSTRACT

Sulfur assimilation may limit the pool of methionine and cysteine available for incorporation into zeins, the major seed storage proteins in maize. This hypothesis was tested by producing transgenic maize with deregulated sulfate reduction capacity achieved through leaf-specific expression of the *Escherichia coli* enzyme 3'-phosphoadenosine-5'-phosphosulfate reductase (*Ec*PAPR) that resulted in higher methionine accumulation in seeds. The transgenic kernels have higher expression of the methionine-rich 10-kDa δ-zein and total protein sulfur without reduction of other zeins. This overall increase in the expression of the S-rich zeins describes a new facet of regulation of these proteins under enhanced sulfur assimilation. Transgenic line PE5 accumulates 57.6% more kernel methionine than the high-methionine inbred line B101. In feeding trials with chicks, PE5 maize promotes significant weight gain compared to non-transgenic kernels. Therefore, increased source strength can improve the nutritional value of maize without yield loss and may significantly reduce the cost of feed supplementation.

1.2. INTRODUCTION

Maize is one of the most important agricultural commodities with its production amounting to 1,065.1 million metric tons in the trade year 2016/2017, far exceeding that of wheat and rice. About 60% of this global production was used for animal feed (https://apps.fas.usda.gov/psdonline/circulars/grain-corn-coarsegrains.pdf). To provide for amino acid balance in a corn-based diet, the addition of soybean corrects the deficiency of corn in certain essential amino acids (EAAs) such as lysine and tryptophan. This corn-soybean formulation, however, is still deficient in the sulfur (S)-containing methionine (Met). Therefore, feeds are supplemented with synthetic Met. Inclusion of unnatural amino acids, like the racemic Met used in feed formulation to replace protein-bound amino acids provides suboptimal protein utilization and, in some cases, reduces growth rate (Sveier et al., 2001). Maize with elevated Met content could obviate the need for supplementation of animal feed with synthetic Met.

The major seed storage proteins (SSPs) in maize, called zeins, are synthesized in the endosperm and serve as a reservoir of amino acids for the germinating seedling. The proline- and glutamine-rich zeins make up about 60% of the total seed proteins and are mostly devoid of EAAs such as lysine, threonine, tryptophan, methionine, and tyrosine (Messing, 1983; Shewry and Halford, 2002). Zeins such as the 10-kDa δ-zein have a higher proportion of Met, but they normally make up only a small proportion of the total zeins. Therefore, most maize inbred lines have low Met content. The variability of Met levels (Phillips and McClure, 1985) and the complex regulation of kernel Met accumulation in different maize inbred lines (Chaudhuri and Messing, 1994; Cruz-Alvarez et al., 1991; Schickler et al., 1993; Swarup et al., 1995) complicate traditional breeding approaches for

high-Met maize. Consequently, Met accumulation in maize seeds has been tested using transgenic means.

Two direct transgenic approaches that have met with less-than-optimal results involved seed-specific expression of a Met-rich protein (Chiaiese et al., 2004; Hagan et al., 2003; Lai and Messing, 2002; Molvig et al., 1997; Tabe and Droux, 2002) or targeted reduction of S-poor SSPs by gene silencing (Kohno-Murase et al., 1995). Introduction of S-rich proteins in developing seeds resulted in simultaneous reduction in levels of endogenous S-rich proteins, suggesting a reallocation of protein S in seeds brought about by limitations of S availability (Chiaiese et al., 2004; Hagan et al., 2003; Lai and Messing, 2002). Furthermore, heterologous expression of naturally S-rich proteins such as the 2S albumin from Brazil nut in canola (Altenbach et al., 1992) and the sunflower seed albumin (SSA) in lupin seeds (Molvig et al., 1997) produced seeds that were not suitable for consumption due to their allergenic properties. In another case, the Met content of rape seeds, along with lysine and cysteine (Cys), were improved by reducing the abundance of endogenous S-poor cruciferin using an antisense transgene (Kohno-Murase et al., 1995). However, knockdown of expression of S-poor zeins in maize by RNA interference increased lysine but it did not increase Met (Segal et al., 2003).

Another transgenic approach might be to increase the supply of SAA by deregulating assimilative sulfate reduction. In this pathway plants take up inorganic sulfate, reduce it to sulfide, and then assimilate it into cysteine (Cys) (Fig. 1.1A). A major metabolic control point in Cys synthesis is the enzyme adenosine 5'-phosphosulfate reductase (APS reductase or APR) which has been shown to increase flux through the pathway when constitutively overexpressed in maize. However, the plants were stunted due to the accumulation of toxic intermediates (Martin et al., 2005). If this problem could

be solved, then S assimilation could potentially increase the source of Met for accumulation in the seed (Wu et al., 2012).

Given the complexity of S metabolism it becomes critical for us to further consider the flux of S under conditions of limiting availability (Mugford et al., 2011). Cys serves as the reservoir of bioavailable S in the cell and as such is the precursor of S-containing cellular compounds, including Met, glutathione (or GSH), coenzymes, and other secondary metabolites. Synthesis of Cys from sulfate, termed assimilatory sulfate reduction, requires five enzymes (Fig. 1.1A: ATP sulfurylase, APS reductase, sulfite reductase, serine acetyltransferase, and O-acetylserine thiol-lyase) and can be subdivided into three major steps: (1) sulfate uptake via transporters, (2) sulfate reduction and synthesis of the carbon and nitrogen backbone for Cys, and (3) S assimilation into the backbone to produce Cys (Bick et al., 2001; Martin et al., 2005; Mugford et al., 2011; Tsakraklides et al., 2002; Vauclare et al., 2002; Wirtz and Droux, 2005).

Previous work has focused on the role of APS reductase (APR) as a major metabolic control point in Cys synthesis, and subsequently of Met biosynthesis in plants. Plant APR exerts a strict control over the metabolic flux through S assimilation, but it is also more susceptible to regulatory control than other enzymes in the assimilative reduction pathway (Kopriva et al., 2001; Vauclare et al., 2002). To circumvent the many control points, bacterial APR homologs may be better targets for transgenic plant studies. Expression of Arabidopsis APR isoforms in PAPS reductase mutant strains of *E. coli,* lacking the *CysH* gene product or *Ec*PAPR, was able to restore the ability to synthesize Cys (Setya et al., 1996). Ectopically expressed *Escherichia coli* and *Pseudomonas aeruginosa* enzymes *Ec*PAPR and *Pa*APR, respectively, have both been shown to function in plants (Martin et al., 2005; Tsakraklides et al., 2002). Although having differing substrate

specificities (APS for *Pa*APR and the phosphorylated APS derivative, 3'-phosphoadenosine-5'-phosphosulfate, PAPS, for *Ec*PAPR; Fig. 1.1A), both enzymes are able to drive sulfate reduction (Martin et al., 2005).

Collectively called (P)APRs, *Ec*PAPR and *Pa*APR are able to efficiently use plant thioredoxins for catalysis as opposed to plant APRs that require GSH for electron transfer (Bick et al., 2001). Both bacterial forms are independent of post-translational mechanisms that regulate one of the Arabidopsis APR isoforms (Bick et al., 2001; Tsakraklides et al., 2002). Although *Ec*PAPR requires PAPS as substrate, it could function when expressed in maize because of the presence of APS kinase in plants that is able to convert APS to PAPS (Mugford et al., 2009) (Fig. 1.1A). Constitutive overexpression of (P)APRs in maize showed that either enzyme is equally capable of deregulating the S metabolic pathway (Martin et al., 2005).

Because sulfate assimilation in maize is known to be compartmentalized in specific cell types (Burgener et al., 1998), we achieved expression of the bacterial genes with two tissue-specific promoters, the mesophyll-specific *PepC* and the bundle sheath cell-specific *RbcS* promoters (Sattarzadeh et al., 2010). The results showed a marked increase in seed Met sequestered in S-rich zeins regardless of APR or promoter used. Recurrent backcrosses of the transgenic plants to the high-Met maize inbred B101 exhibited a stable, high-Met seed phenotype. The transgenic maize kernels used in feed formulation enhanced the growth of chicks. These results represent a breakthrough in the nutritional quality of maize.

1.3. RESULTS

1.3.1. Tissue-specific expression of *Ec*PAPR

Transgenic plants that harbor one of four different chimeric constructs were obtained via Agrobacterium infection of immature maize embryos. The constructs included *Pa*APR or *Ec*PAPR, each under transcriptional control of the leaf- and cell-specific *RbcS* or *PepC* promoters (Fig. 1.1B). Transgenic events generated from each of the constructs (Table 1.1) showed no phenotypic abnormalities, and some exhibited high accumulation of S-rich SSPs (Fig. 1.1C-F). Because the objective of the research was to examine the effect of increased S assimilation on SSP expression, further analysis focused only on two *Ec*PAPR transgenic lines PE5 and RE3 showing the highest accumulation of the Met-rich 10-kDa δ-zein. Both events, having single copies of the transgene (Table 1.2), were backcrossed to four different inbred lines – A654, B101, B73, or M017 – for two to five generations (Table 1.1). Subsequent analysis of SSP accumulation after every generation of backcrossing showed that transgenic events PE5 and RE3, introgressed into the inbred B101 (PE5-B101 and RE3-B101), had consistently high accumulation of the 10-kDa δ-zein and PE5 had high accumulation of the 10-kDa δ-zein in different genetic backgrounds compared to the other transgenic events (Fig. 1.1C-F). PE5-B101 and RE3-B101 were backcrossed to the inbred B101 for four and five generations, respectively, and resembled B101 with respect to plant height, tassel morphology, and anthesis-silking interval (Fig. 1.1G). Therefore, *Ec*PAPR expression in these lines did not negatively impact plant growth or development. In addition to elevated 10-kDa δ-zein expression, the seeds had higher total protein content and no increase in total nitrogen. The seeds also showed elevated fat and reduced fiber contents (Table 1.3). Transgenic plants displayed no apparent yield loss

as kernels had increased weight and the kernel number per ear are not significantly different from the non-transgenic control (Table 1.3; Fig. 1.1H).

*Ec*PAPR mRNA was detected in leaves but not in the silks, pollen, ears, and immature kernels of PE5-B101 and RE3-B101, demonstrating leaf-specific expression (Fig. 1.2A). *Ec*PAPR protein localization showed that the *PepC* promoter directed specific expression in mesophyll cells, but the *RbcS* promoter resulted in EcPAPR expression in bundle sheath cells as well as leaky expression in the mesophylls (Fig. 1.3). *Ec*PAPR transcripts in PE5-B101 and RE3-B101 accumulated at similar levels in mature leaves (Fig. 1.2B, C), however, the protein was much more abundant for RE3-B101 (Fig. 1.2D).

Glutathione (GSH) acts as transport and storage form of reduced S and its biosynthesis is limited by Cys concentration (Noctor et al., 1998). GSH accumulated by more than two-fold in the leaves of both transgenic events (Fig. 1.2E). Because plant APR transcription is known to be particularly sensitive to downregulation by an end-product of S assimilation, expression of endogenous maize APR would be expected to decrease if S assimilation had been deregulated by *Ec*PAPR expression. The maize genome contains two putative APR-like proteins *Zm*APRL1 and *Zm*APRL2 (NCBI Accessions AY739296 and AY739296) (Houston et al., 2005). Both PE5-B101 and RE3-B101 show decreased abundance of *Zm*APRL1 and *Zm*APRL2 transcripts (Fig. 1.2F). These results indicate that *Ec*PAPR expression has resulted in deregulation of sulfate reduction, but without negative impact on plant growth and yield (Table 1.3; Fig. 1.1G, H), likely due to the leaf-specific promoters that were used, compared with the constitutive promoter employed in prior studies (Martin et al., 2005).

1.3.2. Variation in zein expression is a function of genetic background

The *Ec*PAPR transgene is stable and heritable. PCR analysis of segregating plants of PE5 and RE3 introgressed into different backgrounds (Table 1.2) indicates that the segregation ratios of transgenic versus the null segregants were about 1:1, suggesting the presence of a single copy of the *Ec*PAPR transgene in these transgenic events. Backcrosses of transgenic events to maize inbreds that differ in their accumulation of the Met-rich 10-kDa δ-zein revealed that *Ec*PAPR also induced expression of the S-rich δ-, β-, and γ-zeins dependent on the genetic background (Fig. 1.1C-F).

Varying expression levels of the Met-rich zeins were observed in events PE5 and RE3 (Fig. 1.4A-G). Both PE5 and RE3 in the B73 and M017 backgrounds show global increases in the levels of the S-rich zeins (Fig. 1.4B, C), whereas in the B101 background only the 10-kDa δ- and 15-kDa β-zeins were increased (Fig. 1.4A). PE5-B101 has 14.4% more kernel Met (Fig. 1.4I) than those from an F3 ear of the PE5 event, illustrating that specific maize inbreds can be exploited to enhance Met level. The non-functional 10-kDa δ-zein gene in A654 (Fig. 1.4D) resulted in an increase of only the 15-kDa β-zein in PE5. Of the S-containing zeins, the 10-kDa δ-zein appears to be the most responsive to enhanced sulfate assimilation. The relative accumulation of the 10-kDa δ-zein in both transgenic events backcrossed to different inbreds are shown in Fig. 1.4E-G. Elevated levels of the 15-kDa β- and 16-kDa γ-zeins were differentially regulated (Fig. 1.4A; Fig. 1.5) and observed only when the 10-kDa δ-zein was increased (Fig. 1.1C-F). In the absence of the 10-kDa δ-zein, β-zein acted as the primary sink of Met among the SSPs (Fig. 1.4D).

1.3.3. Amino acid analysis of transgenic maize kernels

Cys (Fig. 1.4H) and Met (Fig. 1.4I) were both increased in mature dry seeds of PE5-B101 and RE3-B101. Met was increased 57.6% in PE5-B101 and 27.8% in RE3-B101 compared to the B101 control. Cys was increased 39.4% and 17.7% in PE5-B101 and RE3-B101; expression of Cys-containing non-zein proteins were also increased in the PE5 event (Fig. 1.6). Total S content of transgenic seeds from PE5-B101 and RE3-B101 was increased by 38.6% and 36.4%, respectively (Table 1.3). In contrast, transgenic seeds expressing the S-rich sunflower seed albumin either had unchanged or slightly lower Cys content than non-transgenic controls. In addition, total seed S content did not change, presumably because of reallocation of S reserves from endogenous proteins to the transgenic products (Chiaiese et al., 2004; Hagan et al., 2003; Lai and Messing, 2002; Molvig et al., 1997; Tabe and Droux, 2002) (Table 1.4).

Concomitantly with increased Met and Cys, total aspartic acid, lysine, threonine, and serine decreased in the transgenic seeds (Fig. 1.4J). Serine was reduced the most, amounting to only 14.1% of the B101 control in PE5-B101 seeds (Fig. 1.4J). It is not clear why these amino acids are reduced, but it is interesting to note that lysine, threonine, and isoleucine use aspartic acid as a precursor for their synthesis and serine is used for Cys synthesis (Fig. 1.1A). Therefore, one might expect the free level of these amino acids to decline in plants engineered to increase Cys and Met biosynthesis. Other noteworthy changes included an increase in phenylalanine, tyrosine and proline. Elevated phenylalanine and tyrosine were previously reported to correlate with higher Met levels in transgenic seeds (Cohen et al., 2014; Song et al., 2013). The increase in proline content may be attributed to increased expression of the 10-kDa δ-zein, of which 15.5% of its residues is proline (Kirihara et al., 1988).

1.3.4. Chick feeding trials with the high-Met PE5

Ultimately, the usefulness of increased seed methionine must be judged on whether it improves nutritional value. PE5-B101 kernels were used in a four-week feeding trial of chicks with a corn-soybean meal formulation that is deficient in Met (Messing and Fisher, 1991) (Table 1.5). Three diet rations, consisting of different corn meals, were tested with five-day-old chicks: (1) a complete diet consisting of a yellow dent corn supplemented with synthetic Met; (2) corn meal from PE5-B101 without Met supplementation, and; (3) a reference diet composed of corn meal from null segregants derived from PE5-B101 without Met supplementation. Chicks receiving the normal diet had the biggest weight gain, although this is not significantly different from those fed with PE5-B101, whereas those fed the reference diet had the lowest weight gain (Fig. 1.7).

1.4. DISCUSSION

Deregulation of the sulfate assimilation pathway in the source tissues led to increased accumulation of protein-bound S in seeds resulting from the accumulation of specific S-rich zeins. Although previous work showed that deregulation of the reductive sulfate assimilation pathway by overexpression of the assimilatory reductases could be used to increase S flow from uptake to storage in seeds, it had no practical application because of detrimental plant phenotypes resulting from the accumulation of toxic intermediates, which could not be efficiently metabolized during plant development (Martin et al., 2005). In addition, different maize inbred lines exhibit variability in the amount of Met stored in the seed (Phillips and McClure, 1985) and this variability appears to be mainly due to the differential expression of the Met-rich 10-kDa δ-zein gene, *Dzs10*, with 22.5% Met codons (Kirihara et al., 1988), the same gene whose expression is upregulated by increased S assimilation.

Remarkably, transgenic kernels from S-deregulated plants showed no apparent rebalancing of protein S that was previously observed for overexpression of the 10-kDa δ-zein gene and the overexpression of S-rich proteins in other species (Chiaiese et al., 2004; Hagan et al., 2003; Lai and Messing, 2002; Molvig et al., 1997; Tabe and Droux, 2002) or by reducing expression of an S-poor SSP through antisense RNA expression (Kohno-Murase et al., 1995). The overall increase that we observed in the accumulation of the S-rich zeins indicate another facet of the regulation of zeins achieved by altering the supply of SAA. The present work illustrates that by genetically engineering increased biosynthesis of SAA, seed development can be altered to increase the ability to accumulate and fix the SAA into SSP to produce seeds with improved nutritional quality.

The introgression events PE5-B101 and RE3-B101 accumulated more Met and Cys than B101, which has already the highest kernel Met content among common maize inbreds (Phillips and McClure, 1985). In prior work, it was shown that when B101 was crossed with other inbreds or used as a high-Met male donor parent, the high expression level of the *Dzs10-B101* allele was lost, suggesting the presence of more than one genetic factor that affects expression of the gene in *trans* (Olsen et al., 2003). This regulation was eliminated with a chimeric storage protein gene that contained only the coding region of the *Dzs10* gene (Lai and Messing, 2002). Thus, it appears that B101 exemplifies the maximum natural threshold of maize grain Met accumulation under limiting SAA availability. This threshold apparently can be overcome with increased S reduction and assimilation during photosynthesis. Even in the B73 and M017 backgrounds, overexpression of *Ec*PAPR can induce an increase in the accumulation of the S-rich zeins, indicating that SAA supply or availability is a critical limiting factor in maize seed Met accumulation.

Under increased S supply, the 10-kDa δ-zein has higher accumulation compared to the other S-containing zeins. This preferential accumulation of the 10-kDa δ-zein is probably a function of its content of SAAs (22.5% Met and 3.9% Cys). Based on the zein profiles of the different transgenic events, the 10-kDa δ-zein seems to be the most responsive to enhanced assimilative sulfate reduction followed by the 15-kDa β-, 16-kDa γ-, and 27-kDa γ-zein, respectively. This order also follows the number of SAA residues in these zeins. Therefore, it would seem that the higher the SAA residues of the zeins, the more responsive it would be to increased S supply. We do not consider here the 18-kDa δ-zein, although it is exceptionally rich in the SAAs, as its expression is highly variable across inbred lines and most inbred lines have very low levels of expression of this protein

(Swarup et al., 1995). As suggested by our data on transgenic events introgressed into different genetic backgrounds, the 10-kDa δ-zein seems to be the primary, and foremost, sink for Met in the seeds.

In maize, a C4 plant, Cys synthesis is localized to the bundle sheath cells and exhibits spatial separation from synthesis of glutathione, a downstream metabolite produced from Cys in mesophyll cells (Burgener et al., 1998). For this work, we focused on obtaining transgenic plants with increased S assimilation that did not show negative impacts on plant growth, to assess the effect of increased S assimilation on SSP. Therefore, we did not perform a rigorous comparison of *Ec*PAPR and *Pa*APR under either the *PepC* or *RbcS* promoters. However, of all the transgenic events that we have generated, *Ec*PAPR plants appeared to accumulate more S-rich zeins than *Pa*APR plants. *Ec*PAPR plants also showed the hallmarks of deregulated S assimilation including the accumulation of glutathione and downregulation of expression of endogenous APR. This result was unexpected, as maize has an APS reductase-type (*Pa*APR) sulfate assimilatory pathway, not the PAPS reductase type (*Ec*PAPR) (Bick and Leustek, 1998; Hopkins et al., 2004; Setya et al., 1996). Although maize, like other flowering plants, can produce PAPS, used as a sulfate donor in sulfation reactions of some secondary metabolites (Koprivova and Kopriva, 2016; Mugford et al., 2009), it was until now unclear whether PAPS could be directed toward sulfate assimilation. Our results show that endogenous PAPS in higher plants like maize can be co-opted, with the use of an ectopic PAPS reductase, for reductive sulfate assimilation. Moreover, in prior studies it was shown that APS reductase overexpression in maize and Arabidopsis produces growth defects (Martin et al., 2005; Tsakraklides et al., 2002), yet in the present study *Ec*PAPR expression was not associated with any apparent growth abnormalities. Thus, our results point to another aspect of

metabolic engineering for enhanced crop value – utilizing redundancies and alternative circuits for endogenous biosynthetic pathways to improve the nutritional value of crops. That Met accumulates in transgenic *Ec*PAPR plants suggests that Met synthesis in maize is not strictly controlled by the enzyme cystathionine γ-synthase (CGS) responsible for synthesis of Met from Cys. CGS is also not a limitation for Met synthesis in potato (Kreft et al., 2003), whereas it is a bottleneck enzyme in Arabidopsis (Chiba et al., 1999; Kim et al., 2002; Lee et al., 2005).

It is known that APR is expressed in leaf mesophyll or bundle sheath cells, not in developing kernels, indicating that changes in S metabolism in the leaf parenchyma are sufficient to drive increased Met accumulation in the kernel. Therefore, the evidence is consistent with the hypothesis that S assimilated in the leaf is transported to the kernel. Our evidence does not rule out the possibility that S can be assimilated in the kernel (Tabe and Droux, 2001) or vascular cells resulting from low level expression of APR. Still, the prevailing hypothesis is that S is transported in a S transport form from the leaf to the ear via the phloem sap. In wheat, S-methylmethionine (SMM) is the major form in which reduced S moves in the phloem (Bourgis et al., 1999). However, insertional mutants of Arabidopsis and maize in Met S-methyltransferase (MMT), the enzyme that catalyzes synthesis of SMM, produced plants that grew and reproduced normally, and the mutant seeds from Arabidopsis had normal S contents. These rule out an indispensable role for SMM in S transport in Arabidopsis and maize, and the S transport form is probably fulfilled by other reduced S form in these species (Kocsis et al., 2003). Whether there is an in situ biosynthesis of SAAs in the maize phloem sap is currently unknown.

Depending on source availability of S, two distinct features of the regulation of SAA levels in the seed emerge from our study. Enhanced S assimilation in maize, in which

SAA is not limiting, leads to an overall increase in the expression of the β-, γ-, and δ-zeins. In the default state, in which SAAs are limiting, increased expression of the 10-kDa β-zein decreases expression of the β- and γ-zeins, leading to rebalancing of protein S in the seeds (Lai and Messing, 2002). There seems to be two major limiting factors in the accumulation of Met and Cys in maize seeds: (1) demand for S imposed by the S-rich zeins, and (2) SAA availability or supply from the source tissues, which determines the uptake of SAA into the seeds (Tabe and Droux, 2002). The demand, or S sink strength, is itself responsive to the SAA supply. These limitations constitute a conservative mechanism in the seeds that senses SAA availability from the source tissues and accordingly adjust the sink strength for SAA.

We have shown that by enhancing sulfate assimilation in the leaf by transgenic means coupled with traditional backcross breeding into desirable genetic backgrounds, maize kernels with high Met content were produced that was of significant increased nutritional value to livestock. Increased Met sequestered in the S-rich zeins was bioavailable in the diet fed to chicks and can supplant synthetic Met supplementation needed for optimal growth. From a nutritional point of view, increasing Met rather than Cys is beneficial because, although animals are not able to synthesize Met from Cys, they are able to convert Met to Cys (Finkelstein and Mudd, 1967).

1.5. MATERIALS AND METHODS

1.5.1. Cloning of the *PepC* and *RbcS* promoters to drive tissue-specific expression of the bacterial assimilatory reductases *Ec*PAPR and *Pa*APR in maize

The bacterial assimilatory reductases *Pa*APR from *Pseudomonas aeruginosa* and *Ec*PAPR from *Escherichia coli*, collectively called (P)APR, were cloned from plasmids obtained from our own collection (Martin et al., 2005; Tsakraklides et al., 2002). Plasmids in a pBlueScript vector backbone designated as strain file #788 and #793 were used for cloning the *Ec*PAPR and *Pa*APR fragments, respectively. The 1.6-kb maize mesophyll-specific phosphoenolpyruvate carboxylase (*PepC*) and 1.0-kb bundle sheath-specific Rubisco small subunit 1 (*RbcS*) promoters were cloned from the binary vectors pPTN512 and pPTN533, respectively, and were kindly provided by Dr. David Stern of the Boyce Thompson Institute at Cornell (Sattarzadeh et al., 2010). The expression cassettes were assembled in the standard binary vector pTF102 that contains the bar selectable marker gene (Frame et al., 2002). Primers used for the restriction enzyme-based cloning of the binary vectors are listed in Table 1.6.

Cloning of the binary vectors pTF102-PepC-EcPAPR, pTF102-PepC-PaAPR, pTF102-RbcS-EcPAPR, and pTF102-RbcS-PaAPR entailed two major steps: (i) cloning of *Ec*PAPR and *Pa*APR into the binary vector pTF102, generating the destination vectors pTF102-EcPAPR and pTF102-PaAPR, respectively, and (ii) subsequent incorporation of the *PepC* and *RbcS* promoter fragments into the destination vectors. The promoter-(P)APR cassettes replaced the P35S-GUS cassette in pTF102 (Fig. 1.8).

Fragments amplified by high-fidelity PCR using the Platinum *Taq* DNA Polymerase (Invitrogen) were gel-purified using the NucleoSpin Gel and PCR Clean-up Kit (Takara Bio USA). Plasmid vectors were transformed by electroporation into electrocompetent

ElectroMAX DH10B cells (Life Technologies). For vectors containing the *RbcS* promoter fragment, chemically competent and methylation-deficient *E. coli* K12 ER2925 (New England BioLabs) strain was used for heat-shock transformation of the *RbcS*-containing vectors. The *RbcS* promoter fragment contains a recognition site for *Bcl*I, a restriction enzyme that is blocked by *dam* methylation. Sanger sequencing was performed after every cloning step to verify the insert sequence.

Cloning of the destination vectors pTF102-(P)APRs. To generate the vector pTF102-EcPAPR, the *Ec*PAPR fragment from plasmid #788 was amplified by PCR using the forward primer EcpFOR bearing an *Eco*RI/*Sma*I/*Bam*HI sites and the reverse primer EcpREV with a *Sac*I site (Table 1.6). The resulting 900-bp amplicon was subcloned into the pGEM-T Easy vector (Promega), digested with *Eco*RI/*Sac*I, and ligated into the 9,043-bp fragment of pTF102 resulting from digestion with the same enzyme set. For pTF102-PaAPR, ligation was performed between the 9,043-bp and 1.030-bp fragments obtained from *Eco*RI and *Sac*I digestion of pTF102 and plasmid #793, respectively.

Cloning of the binary vectors pTF102-promoter-(P)APRs. The *PepC* promoter fragment was amplified from pPTN512 using the forward primer PepcFOR with an *Eco*RI site and the reverse primer PepcREV with a *Bgl*II site (Table 1.6). A 1,612-bp amplicon was subcloned into the pGEM-T Easy vector, digested with *Eco*RI/*Bgl*II, and ligated into an *Eco*RI-/*Bam*HI-linearized pTF102-(P)APR vectors. These cloning steps assembled the pTF102-PepC-EcPAPR and pTF102-PepC-PaAPR binary vectors. For the remaining two binary vectors, pTF102-RbcS-EcPAPR and pTF102-RbcS-PaAPR, the *RbcS* promoter was also introduced upstream of the (P)APR fragments. *RbcS* was amplified from pPTN533

using the forward primer RbcSFOR containing an *Mfe*I site and reverse primer RbcSREV with a *Bcl*I site. A 1,030-bp amplicon was subcloned into the pGEM-T Easy vector, digested with *Mfe*I/*Bcl*I, and ligated into a linearized pTF102-(P)APR vectors digested with *Eco*RI and *Bam*HI. The cloning scheme for generation of the four binary expression vectors is shown in (Fig. 1.8).

Verification of construct integrity. Generated T-vectors with the *Ec*PAPR, *PepC*, or *RbcS* fragments were verified by sequencing with M13 primers while binary vectors were both sequenced and digested with several restriction enzymes. Single or double digestion of the vectors pTF102-PepC-EcPAPR and pTF102-PepC-PaAPR were done with *Eco*RV/*Spe*I and *Sal*I/*Xho*I while both pTF102-RbcS-PaAPR and pTF102-RbcS-EcPAPR were digested with *Sac*I and *Sac*I/*Pvu*II. In addition, pTF102-RbcS-PaAPR was also digested with *Hind*III while pTF102-RbcS-EcPAPR was digested with *Nde*I/*Pst*I.

The four binary vectors transformed into *E. coli* and *Agrobacterium* were genotyped by PCR and sequenced using the primer pair ExpVec_F/ExpVec_R. In addition, EcPAPR binary vectors were genotyped and sequenced with the ExpVec_F/ExpVec_Ec_R primers while PaAPR binary vectors were genotyped and sequenced with the ExpVec_F/ExpVec_Pa_R primers (Table 1.6).

1.5.2. *Agrobacterium*-mediated transformation of immature maize embryos

Electrocompetent *Agrobacterium tumefaciens* EHA101 was prepared as described elsewhere (Wise et al., 2006) and transformed with the binary vectors pTF102-PepC-EcPAPR, pTF102-PepC-PaAPR, pTF102-RbcS-EcPAPR, or pTF102-RbcS-PaAPR. Maize transformations using immature embryos were carried out using an Agrobacterium-

mediated transformation protocol as described elsewhere (Frame et al., 2002). Immature ears of either genotype Hi-II A x B hybrid or the F2 of a Hi-II A x B hybrid were harvested 10 to 12 days post-pollination, as dictated by the size of the embryos. Transformation efficiency was 1-2%.

1.5.3. Introgression of the transformation events into different maize inbred lines

Maize genetic stocks for backcross breeding. B73 and the Hi-II A and B inbred lines were from our laboratory stocks. A654, M017, and B101 were obtained from the North Central Regional Plant Introduction Station of the United States National Plant Germplasm System (Ames, Iowa). T1 plants were obtained from crosses of the To plants with the inbred A654, B101, B73, and M017 and the Hi-II A x B hybrid, depending on the availability of usable pollen. Backcrosses were subsequently done using the inbred A654, B101, B73, and M017 as the recurrent paternal parents. Eight independent transgenic events encompassing the four constructed binary vectors were backcrossed to the four selected inbred lines for two to five generations, with some events having additional one to two generations of self-pollination (Table 1.1).

After the first backcross generation with A654 as the recurrent parent, kernels were screened by genotyping and phenotyping for *the dzs10-A654* allele, a nonfunctional allele of the 10-kDa δ-zein that contains a Misfit transposon insertion (Wu et al., 2009). Kernels homozygous for the *dzs10-A654* allele were propagated and further backcrossed with A654 as the recurrent paternal parent.

Designations for the transgenic events are made up of two letters followed by a number. The first letter, either “P” or “R”, refers to the promoters *PepC* or *RbcS*, respectively, while the second letter, “E” or “P”, refers to the bacterial reductase enzyme

that the promoter controls, *Ec*PAPR or *Pa*APR, respectively. The number indicates the order that a callus was picked, showed resistance to the phytotoxin bialaphos, and grown to maturity. For example, PE5 is a transgenic event that expresses the *Ec*PAPR enzyme under the control of the *PepC* promoter and was the fifth callus from which a transgenic plant was regenerated.

Genotyping. Genomic DNA were extracted from maize leaf tissues at the 4- or 5-leaf stage using a modified CTAB extraction method (Sawa et al., 1997). Transgenic events generated from transformation with the binary vectors pTF102-PepC-EcPAPR, pTF102-PepC-PaAPR, pTF102-RbcS-EcPAPR, and pTF102-RbcS-PaAPR were genotyped by PCR using the primer pairs rPepC_For/rEcPAPR_Rev, rPepC_For/rPaAPR_Rev, rRbcS_For/ rEcPAPR_Rev, and rRbcS_For/ rPaAPR_Rev, respectively (Table 1.6). PCR conditions involved an initial denaturation step at 95°C for 3 min; 40 cycles of 95°C for 30 sec; particular annealing temperatures for 30 sec, and 72°C for 1 min; and a final elongation step at 72°C for 7 minutes. Annealing temperatures for transgenic events bearing the transgenes for pTF102-PepC-EcPAPR, pTF102-PepC-PaAPR, pTF102-RbcS-EcPAPR, and pTF102-RbcS-PaAPR are 56°C, 57°C, 55°C, and 55°C, respectively.

1.5.4. Biochemical and molecular biological analysis of transgenic events

RNA extraction, reverse transcription polymerase chain reaction (RT-PCR), and quantitative reverse transcription PCR (RT-qPCR). Several maize tissues were used for RNA extraction: immature unpollinated ears two to three days after silk emergence; silks that were cut off and separated from the immature ears; kernels at 12 days post-pollination (dpp) or 16 dpp; leaf at the 4- or 5-leaf stage and at ~45 days post-germination,

and; pollen at two to three days after pollen shedding. Samples that were not immediately processed for extraction were submerged in RNAlater (Invitrogen) and stored at -20°C.

Samples were homogenized in liquid nitrogen and processed for RNA extraction using the column-based NucleoSpin RNA Plant kit (Takara Bio USA). DNase treatment of the eluates used Turbo DNA-free kit (Ambion) for efficient removal of contaminating genomic DNA. RNA quantitation was done using Nanodrop (Thermo Scientific) and cDNA was synthesized from one microgram of RNA with PrimeScript RT Reagent kit (Takara Bio USA) using the poly-dT primer in a 20-µl reaction.

Semi-quantitative RT-PCR was performed to determine the localization of expression of *Ec*PAPR in five different tissues: immature developing ears, silks, immature kernels at 12 dpp, pollen, and mature leaf. First-strand cDNA was synthesized as described and 2 µl of this was used for PCR with primers EcPAPR_for and EcPAPR_Rev. Thermocycling conditions were as follows: 95°C for 2 min; 30 or 40 cycles of 95°C for 20 sec, 60°C for 20 sec, and 72°C for 25 sec; and, a final extension at 72°C for 7 min. 40 cycles were used for B101 and PE5 while RE3 cDNA was amplified for 30 cycles. A control reaction for the ubiquitin or the glyceraldehyde-3-phosphate dehydrogenase (Fletcher, 2014) gene was performed under the same conditions using the primers 5'-ubq/3'-ubq or ZmGAPDH_f/ZmGAPDH_r (Table 1.6), respectively. Both reference control genes returned similar values when used for RT-qPCR. To check for genomic DNA contamination of the RNA, PCR was performed with the primers JP-ZmAPRL_for and JP-ZmAPRL_rev (Table 1.6), which spans an intron in the *Zm*APRL1 (NCBI Accession AY739296) and *Zm*APRL2 (NCBI Accession AY739297) genes.

For quantitative real-time PCR (RT-qPCR) using StepOnePlus Real-Time PCR system (Applied Biosystems), cDNA samples were diluted twice and 4 µl of this was used

in 10-ul reactions with PowerUp SYBR Green Master mix (Applied Biosystems) and each primer at 400 nM. Reactions were run in the fast cycling mode (50°C for 2 min; 95°C for 2 min; 40 cycles of 95°C for 3 sec, 60°C for 30 sec) with a default dissociation step after the real-time PCR run. Four technical replicates from pooled tissues were run per sample, and each sample was analyzed using two or three biological replicates. No template control reactions were included and returned no C_T values. Reference gene used the glyceraldehyde-3-phosphate dehydrogenase, GAPDH (Fletcher, 2014), and was amplified using primers ZmGAPDH_f and ZmGAPDH_r (Table 1.6). Nonspecific amplification was checked using the dissociation curves. For relative quantitation, the $\Delta\Delta C_T$ or the comparative C_T method was used, which gave values in fold-change of expression.

Sequence annotations of APR-like transcripts from maize (Houston et al., 2005) turned up two sequences, *Zm*APRL1 (NCBI Accession AY739296) and *Zm*APRL2 (NCBI Accession AY739297), highly likely to be 5'-adenylylysulfate reductases (APS reductases or APRs). Primers specific for *Zm*APRL1 and *Zm*APRL2, JP-ZmAPRL_for and JP-ZmAPRL_rev (Table 1.6), were used to quantitate the transcript accumulation of APRs in mature maize leaves. RNA was extracted from the endosperm of 16-dpp immature kernels for quantitation of accumulation of the 15-kDa β-zein and 16-kDa γ-zein transcripts using the primers q15-kDa_for/ q15-kDa_rev and q16-kDa_for/ q16-kDa_rev, respectively.

Copy number determination of transgenic events by RT-qPCR. Transgene copy number in events PE5-B101 and RE3-B101 was determined by qRT-PCR (Shepherd et al., 2009). Genomic DNA were extracted from the leaves of PE5-B101 and RE3-B101 at BC_4F_1 and BC_5 generations, respectively. 2.5-fold serial dilutions of genomic DNA at 100, 40, 16, 6.4, and 2.56 ng were prepared and 4 ul of these were run in 10-µl reactions with PowerUp

SYBR Green Master mix (Applied Biosystems) and 0.4 µl of each primer at 10 µM. DNA from the transgenic events were amplified with the bar primers bar_F_2 and bar_R_2 (Grohmann et al., 2009) and the copy number control gene GAPDH (Fletcher, 2014) with primers ZmGAPDH_f and ZmGAPDH_r (Table 1.6). Four technical replicates were run per sample, with each sample analyzed using two biological replicates. Reactions were run in the standard cycling mode (50°C for 2 min; 95°C for 2 min; 40 cycles of 95°C for 15 sec, 60°C for 1 min) with a default dissociation step after the real-time PCR run. Calculations for the PCR efficiency and the ratio of the copy number of the bar gene to the reference GAPDH gene were calculated as in Shepherd et al (2009).

Western blotting. Total protein from two to three mature maize leaf discs was extracted following the procedure of Conlon and Salter (2007) and immunoblotting was performed using an antibody against *Ec*PAPR kindly provided by Dr. Jens Schwenn (Krone et al., 1991). Ten microgram of total protein was separated in a 12% Tris-glycine SDS-PAGE gel subjected to 200 V for 50 min. The secondary antibody is a goat anti-rabbit peroxidase conjugate (Sigma-Aldrich) and was used at a 1 : 60,000 dilution while the primary anti-*Ec*PAPR antibody was used at a 1 : 4,000 dilution. Membranes were developed with ECL Western Blot Detection Reagent (Amersham) and exposed on a Blue Basic Autorad Film (BioExpress). Western blotting was performed after each backcross generation to test for stability and heritability of the transgene. Three biological replicates were analyzed for Western blotting and datum was presented from a representative blot.

Total glutathione assay. Total glutathione was assayed using the Glutathione assay kit (Cayman Chemical Company). Ten leaf discs were frozen in liquid nitrogen, homogenized,

and resuspended in 275 µl of ice-cold MES buffer. For deproteination, an equal volume of 10% w/v metaphosphoric acid (Sigma-Aldrich) was added to the homogenate. The suspension was vortexed, incubated at room temperature for 5 min, and centrifuged at 2,000 x g for 5 min. For samples that were not immediately assayed, the supernatant was stored at -20°C without any degradation of GSH or GSSG for a few months. Prior to assaying the sample, 50 µl of 4 M triethanolamine (Sigma-Aldrich) per ml of supernatant was added and then vortexed immediately. This assay reflects both GSH and its oxidized form, the disulfide dimer GSSG.

1.5.5. Characterization of transgenic kernels

Protein extraction from mature maize kernels and SDS-PAGE analysis. Total protein from pooled endosperm samples of mature maize kernels were extracted with an alkaline borate extraction buffer, with the alcohol-insoluble fraction from this extract constituting the non-zein proteins (Wallace et al., 1990). The alcohol-soluble zein protein fraction from the endosperm of mature maize kernels was extracted in a 70% ethanol / 2% β-mercaptoethanol solution (Wu et al., 2012). 2.25 µl of zein extracts, equivalent to 562.5 µg of endosperm flour, was separated in a 15% Tris-glycine SDS-PAGE gel at 200 V for 60 minutes. Three pooled samples constituting four to five kernels from each ear were run and a representative gel is shown in the figure.

Densitometric analysis of accumulation of the 10-kDa δ-zein protein. Densitometric analysis of the 10-kDa δ-zein was performed using the Image Studio Lite software (LI-COR Biotechnology). Zein proteins were extracted from six to eight different kernels from a single ear of a genotype and separated in an SDS-PAGE gel. Both the signal intensities of

the 10-kDa δ-zein and 19-kDa α-zein were determined from the gel. To normalize for protein loading, the value for the 10-kDa δ-zein was divided by the value for the 19-kDa α-zein. The relative intensity of the 10-kDa δ-zein of the transgenic kernel compared to the non-transgenic control was calculated by dividing the normalized value of the transgenic kernel with that of the non-transgenic control.

Thiol labeling with monobromobimane. Monobromobimane (mBBr) is a nonfluorescent dye when unconjugated and selectively reacts with low molecular weight thiols, including cysteine, yielding highly stable and fluorescent thioether conjugates (Fahey and Newton, 1987).

Protein labeling with mBBr followed the protocol described by Buchanan et al. (1997) and modified as follows. Alcohol-insoluble non-zein protein fraction from pooled samples of mature maize endosperm were extracted as previously described. Protein from this extraction was precipitated with acetone. Four volumes of ice-cold acetone were added to the protein sample, the mixture vortexed and incubated for 60 minutes at -20°C. Centrifugation followed at 15,000 x g for 10 minutes. The protein pellet was dried by allowing the acetone to evaporate at room temperature for 30 minutes and then dissolved in a pH 7.5 buffer of 50 mM Tris-Cl. Labeling of the protein extract with mBBr was performed in a 110 µl-reaction consisting of 4.4 µl of 50 mM bromobimane (Sigma-Aldrich), 40 µg total protein, and 10 µl of 0.5 M Tris-Cl, pH 7.5. The reaction was incubated in the dark at room temperature for 60 minutes and the protein was precipitated with acetone. Protein separation was performed in a 12% SDS-PAGE gel for 50 minutes at 200 V and the fluorescent conjugates viewed under UV light.

Hydrolyzed amino acid composition analysis of maize kernels. Pooled mature dry seeds were ground to fine powder and about 10 mg were used for amino acid composition analysis conducted by the Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center, St. Louis, MO, USA. Samples were pretreated with performic acid prior to acid hydrolysis, yielding the acid stable forms cysteic acid and methionine sulfoxide of cysteine and methionine, respectively, that can be measured by separation on a UPLC column. Tryptophan was not detected following acid hydrolysis.

Protein, fat, fiber, and sulfur content determination. Pooled mature dried kernels were sent to a commercial laboratory (New Jersey Feed Lab Inc, Trenton, NJ) for analysis of protein, fat, fiber, and sulfur content. Test methods followed the official AOAC methods 990.03, 920.39, 978.10, and 923.01 for protein, fat, fiber, and sulfur content determination. Three independent measurements were done per sample.

Determination of kernel weight and number per ear. The average kernel weight and number per ear were determined from 10 replicates of a 100-kernel sample and six ears, respectively. Ears that were well-filled and of similar size were chosen for determining the kernel number.

Statistical analysis. GraphPad Prism version 7.00 for Mac (GraphPad Software, La Jolla, California, USA) was used to determine the statistical significance of the differences observed between the control non-transgenic and transgenic plants.

1.5.6. Chick feeding trials

The research protocol (Protocol No. 16-049) for chick feeding trial was approved by Rutgers University's IACUC and followed the guidelines contained in the Guide for the Care and Use of Laboratory Animals (National Research Council, USA) and applicable provisions of the Animal Welfare Act.

Day-old male broiler chicks (Cornish Cross) were housed in the brooder barn in pens provided with overhanging heaters maintained at 90ºF for the first two weeks and reduced to 85ºF on the third week. A 14-hour fluorescent illumination was provided per day and feed and water were supplied ad libitum. The birds were acclimatized to the barn conditions until day 4. On day 5, 45 chicks within a weight range of 77–87 g were randomly assigned to 9 pens of 5 chicks each, covering three dietary treatments of a corn-soybean meal preparation (Table 1.5) (Messing and Fisher, 1991). Each experimental diet was fed to three replicate pens over the course of four weeks. Group body weights and feed intake per pen were recorded at weekly and daily intervals, respectively. A notable difference was observed in the efficiency of feed utilization, which is the feed conversion ratio (Fig. 1.7), between the three experimental diets. Chicks fed the normal and reference diets consumed the same amount of feed that were converted into body weight, whereas those subsisting in PE5-B101 consumed less amount of feed for the same amount of weight gain.

Corn meals from PE5-B101 and its null segregant were obtained from ears collected from about 1,000 plants for each genotype. Ears were obtained from plants that were either selfed or backcrossed to the inbred B101 and were grown in the greenhouse during the winter of 2015-2016 and the summer of 2016.

1.6. FIGURES

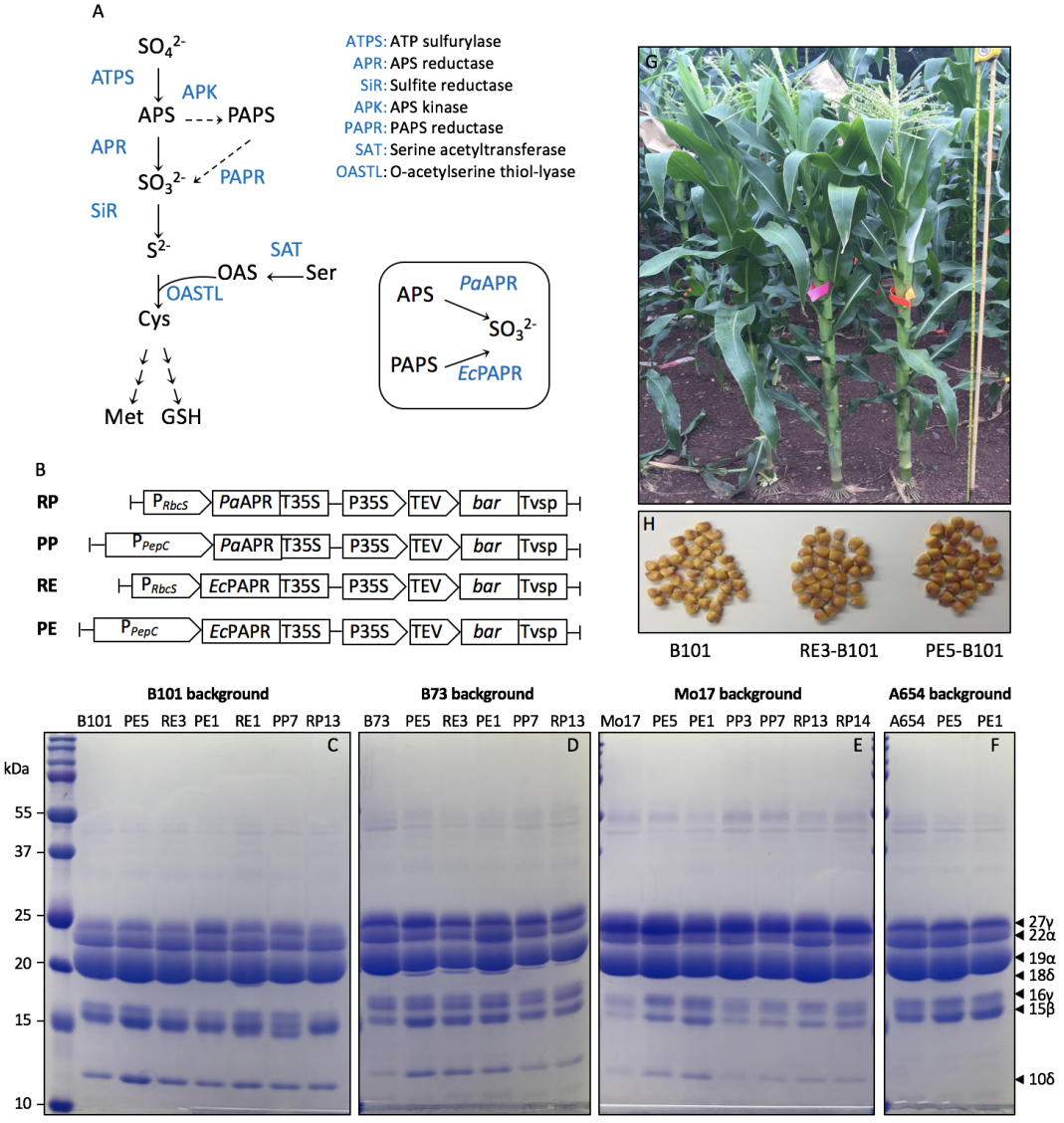


Figure 1.1. Accumulation patterns of the S-rich zeins in transgenic maize expressing the assimilatory reductases *Ec*PAPR or *Pa*APR.

(**A**) Assimilative sulfate reduction in plants. Sulfate is reduced to sulfite either by APS reductase (APR) via the primary (solid arrows) or an alternative secondary sulfate assimilation pathway (dashed arrows) with PAPS reductase (PAPR). Boxed are the *P. aeruginosa* APR, *Pa*APR, and *E. coli* PAPR, *Ec*PAPR, that had been demonstrated to deregulate sulfate assimilation in Arabidopsis and maize (Martin et al., 2005; Tsakraklides et al., 2002). Multiple arrows between steps indicate intervening steps in the pathway. (**B**) Transgenic expression cassettes of *Ec*PAPR and *Pa*APR under *PepC* or *RbcS* promoters used for *Agrobacterium* transformation of the Hi-II A x B maize immature embryos. SDS-PAGE zein profiles of the transgenic events backcrossed to the (**C**) B101, (**D**) B73, (**E**) Mo17, and (**F**) A654 genetic backgrounds for 2-5 generations. The two-letter designations for the transgenic events indicate: P – *PepC*, R- *RbcS*, E – *Ec*PAPR, and P – *Pa*APR. (**G, H**) Events PE5 and RE3 introgressed for four and five generations, respectively, into the B101 background (PE5-B101 and RE3-B101) exhibit normal plant development and kernel phenotypes.

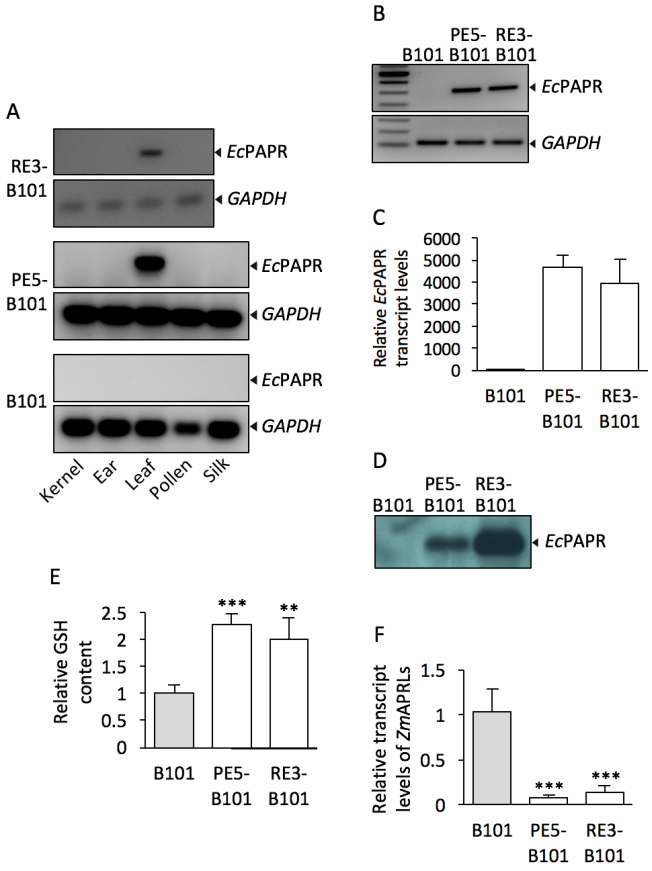


Figure 1.2. Maize transformed with the bacterial assimilatory reductase *Ec*PAPR.

(**A**) Tissue-specific expression of *Ec*PAPR under *PepC* and *RbcS* promoter control in transgenic maize. RT-PCR analysis was performed to detect *Ec*PAPR expression in different maize tissues. First-strand cDNA from RE3-B101 was amplified for 30 cycles while that from B101 and PE5-B101 were amplified for 40 cycles. (**B**) RT-PCR and (**C**) qRT-PCR analysis of *Ec*PAPR transcript levels in mature leaves of PE5-B101 and RE3-B101. Data shown are means ± SD of four determinations each from two biological replicates. (**D**) Western blot analysis of protein extracts from leaves using an antibody against *Ec*PAPR. (**E**) Relative glutathione (GSH) content and (**F**) transcript levels of ZmAPRLs in transgenic mature leaves. Primers targeting two putative APR-like genes in maize, *Zm*APRL1 and *Zm*APRL2 (NCBI Accessions AY739296 and AY739297), were used for qRT-PCR analysis. Glyceraldehyde-3-phosphate dehydrogenase, GAPDH, was used as the reference gene. Data shown are means ± SD of three measurements per three biological replicates. Statistical analysis was performed with the Student's *t* test: significantly different from the B101 control at $P < 0.01$ (**) and $P < 0.001$ (***).

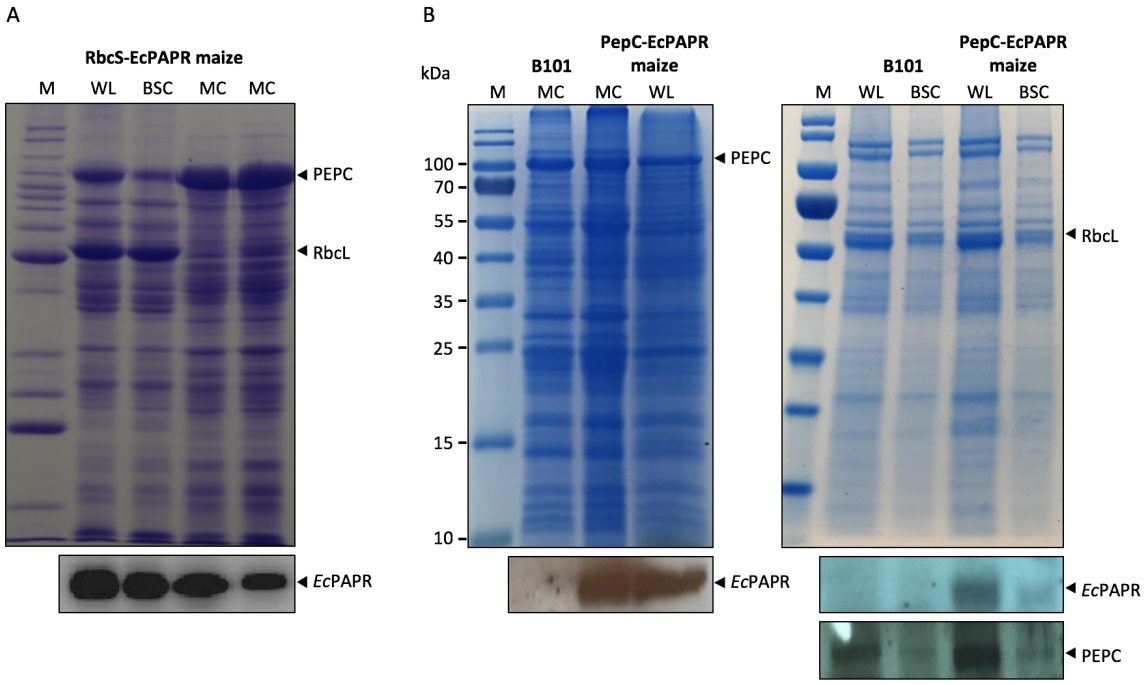


Figure 1.3. Non-specificity and specificity of the *RbcS* and *PepC* promoters, respectively, used for tissue-specific protein localization of *Ec*PAPR in transgenic maize leaves.

Total proteins isolated from whole leaf (WL), bundle sheath cells (BSC) and mesophyll cells (MC) from RbcS-EcPAPR (**A**) and PepC-EcPAPR (**B**) maize. SDS-PAGE analysis of protein extracts from the indicated cellular preparation are shown in the upper panel while the lower panels show immunoblotting of these protein extracts with antibodies indicated on the right. Data from Fig. 1.3A was obtained from the unpublished data of Xiang, et al. (2015).

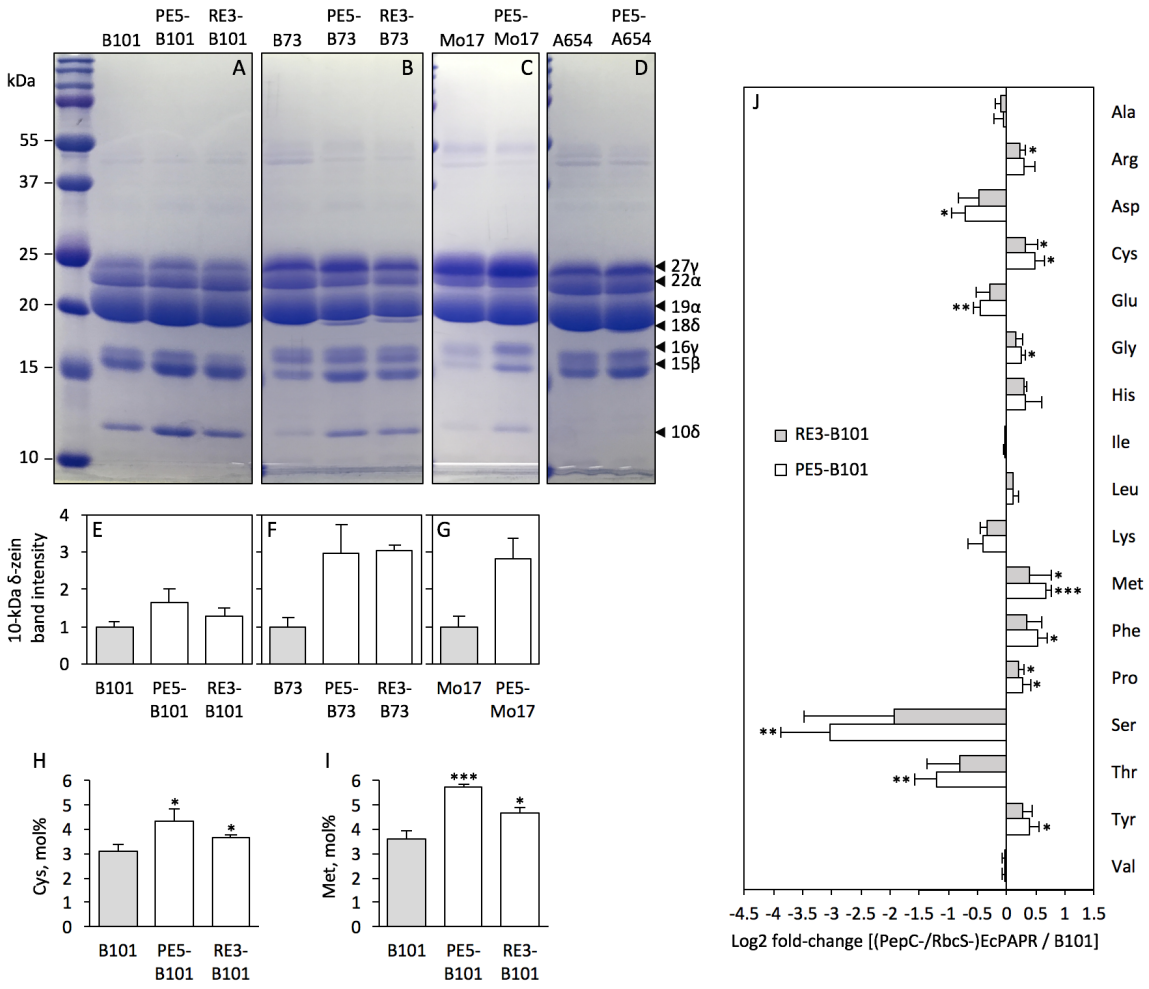


Figure 1.4. Accumulation patterns of seed storage proteins in transgenic *Ec*PAPR maize introgressed into different genetic backgrounds.

(**A**-**D**) SDS-PAGE zein profiles of the transgenic events PE5 and RE3 in different genetic backgrounds and (**E**-**G**) relative band intensity of the 10-kDa *δ*-zein in these events. Events were backcrossed to the (**A**) B101, (**B**) B73, (**C**) Mo17, and (**D**) A654 genetic backgrounds for at least four generations of backcrossing. Quantification of the relative band intensity of the 10-kDa δ-zein in 6-8 kernels (see Materials and Methods) used the Image Studio Lite software. Relative accumulation levels of the 10-kDa *δ*-zein in transgenic events in the B101, B73, and Mo17 backgrounds are shown in (**E**), (**F**), and (**G**), respectively. Sulfur amino acid contents (**H, I**) and changes in the composition of protein-bound amino acids (**J**) in mature dry seeds of PE5-B101 and RE3-B101. (**H**) Cys and (**I**) Met contents in mol% determined after protein hydrolysis and separation in a UPLC column. (**J**) Fold-changes in amino acid levels in transgenic seeds compared to B101 were log2-transformed and plotted in the bar graph. Bars to the left and right indicate a reduction and increase, respectively, in the amino acid content of the *Ec*PAPR plants relative to B101. Student *t* test at $P < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were used to determine the statistical difference between the transgenic PE5-B101 and RE3-B101 and non-transgenic B101 kernels. Data shown are means ± SD of three replicates.

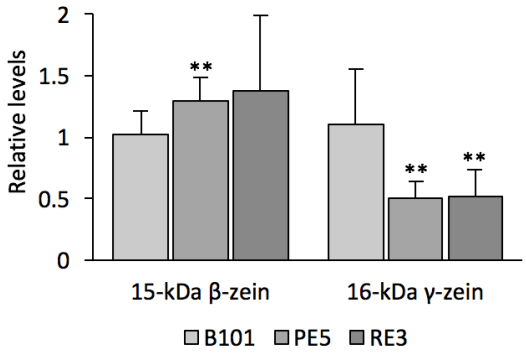


Figure 1.5. Relative expression levels of the 15-kDa β- and 16-kDa γ-zein transcripts in the endosperm of 16-day post-pollination B101-introgressed PE5 and RE3 kernels.

Statistical analysis was performed with the student's t test: significantly different from the B101 control at $P < 0.01$ (**). Data shown are means ± SD of three determinations each from two biological replicates.

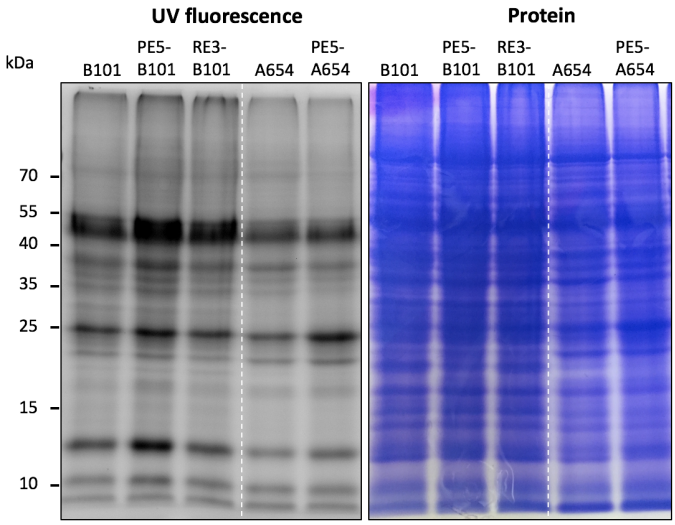
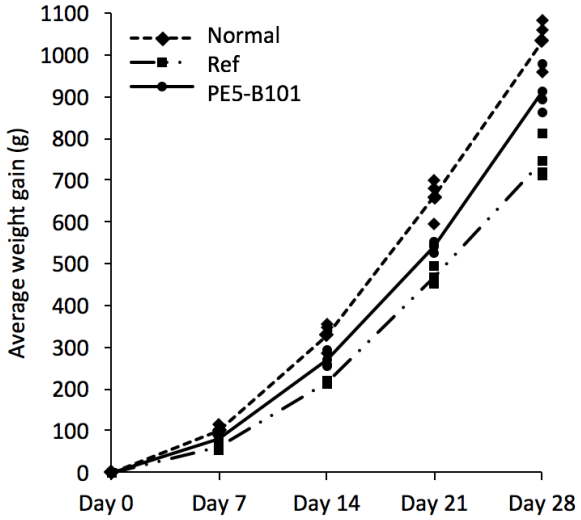


Figure 1.6. Protein S distribution in the kernels of transgenic high-Met maize.

Accumulation patterns of cysteine-containing non-zein proteins in transgenic kernels. Non-zein proteins were derivatized with monobromobimane and separated in a 12% SDS-PAGE gel. Band intensity under UV fluorescence is proportional to the cysteine content and relative abundance of the protein.



Diet	Ave. weight gain (g)	Ave. feed consumed (g)	Feed conversion ratio
Normal	1,032.6 ± 66.4 (a)	1,928.2 ± 49.9 (a)	1.87 ± 0.08 (a)
Ref	747.1 ± 56.2 (b)	1,444.7 ± 83.7 (b)	1.95 ± 0.12 (a)
PE5	911.7 ± 60.0 (a)	1,387.4 ± 58.4 (b)	1.53 ± 0.13 (b)

Figure 1.7. Feeding trial with the transgenic high-Met PE5 maize.

A four-week feeding trial with five-day-old chicks was carried out with three types of diets consisting of yellow dent corn supplemented with synthetic methionine (normal group), PE5-B101 without synthetic methionine, and the null transgenic segregant from PE5-B101 without methionine supplementation (reference group). Shown in the graph is the average weight gain, denoted by the lines, during the course of the experiment and the table shows the weight gain and feed intake per chicks at the conclusion of the feeding trial. Weight gain is calculated as the difference between the finishing and starting weights while the feed conversion ratio is the amount of food consumed per gained weight. Statistical analysis was performed with two-way ANOVA at $p < 0.05$ and significant differences between samples are indicated by different letters. Data shown are means ± SD of three replicates with five animals per replicate.

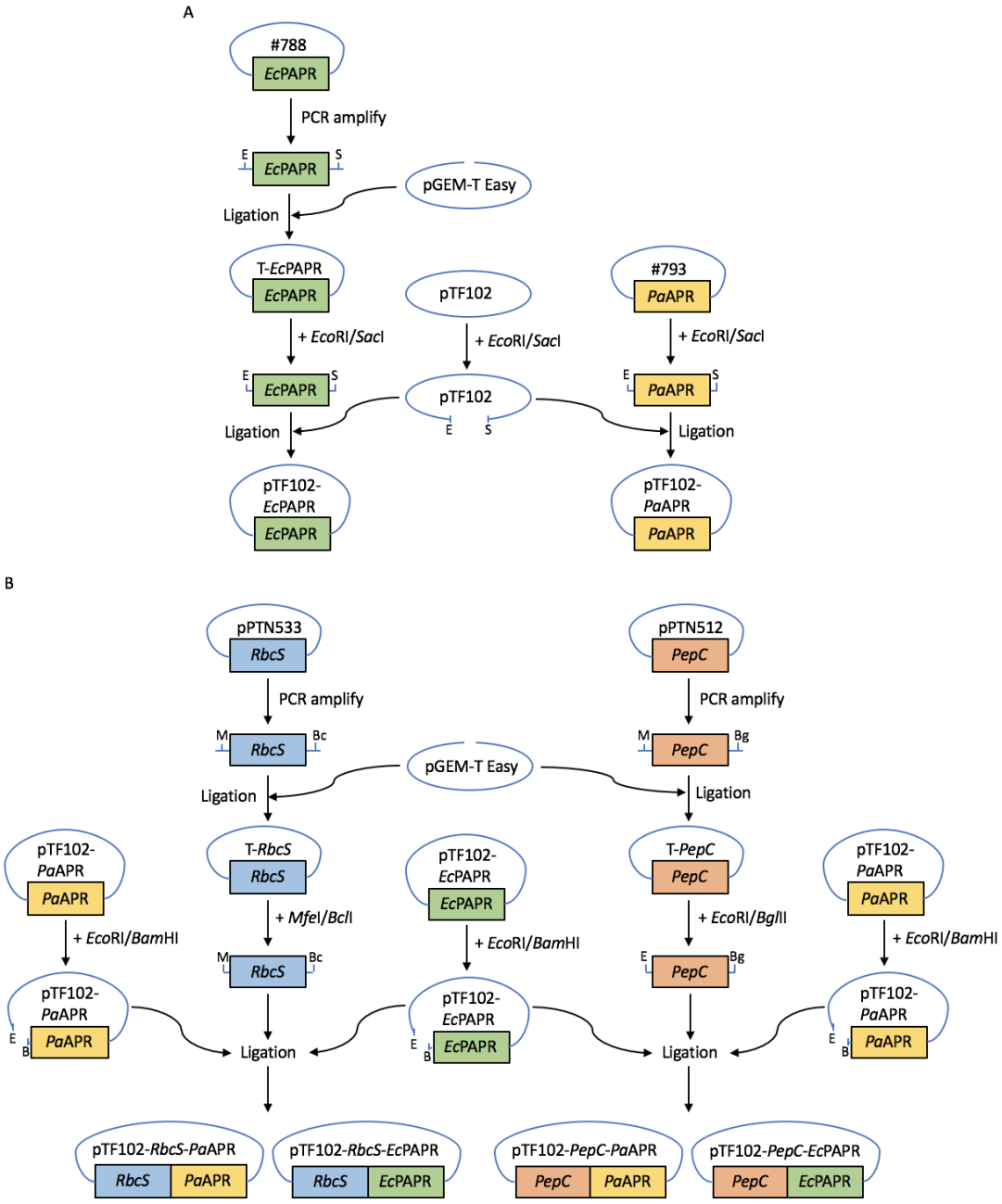


Figure 1.8. Cloning scheme for generation of the (A) pTF102-EcPAPR and pTF102-PaAPR and (B) pTF102-RbcS-PaAPR, pTF102-RbcS-EcPAPR, pTF102-PepC-PaAPR, and pTF102-PepC-EcPAPR expression vectors.

Plasmids #788 and #793 were from the collection of Dr. Thomas Leustek while the expression vectors pPTN533 and pPTN512 (Sattarzadeh et al., 2010) were kindly provided by Dr. David Stern. E – *Eco*RI, S – *Sac*I, M – *Mfe*I, Bc – *Bcl*I, B – *Bam*HI, Bg – *Bgl*II.

1.7. TABLES

Table 1.1. List of transgenic (P)APR events introgressed into the inbred lines A654, B101, B73, and Mo17.

Transgenic event	Introgression lines from transgenic events backcrossed to different maize inbreds			
	A654	B101	B73	Mo17
PE1	BC_4(A654 x (AB x PE1))	BC_5S_1(B101 x PE1)	BC_2((AB x PE1) x B73)	BC_3((AB x PE1) x Mo17)
PE5	BC_4(A654 x (AB x PE5))	BC_4S_2(B101 x PE5)	BC_4S_1(B73 x PE5)	BC_4S_1(Mo17 x PE5)
RE1	-	BC_5(B101 x RE1)	-	-
RE3	-	BC_5S_1(B101 x RE3)	BC_4S_1(B73 x RE3)	-
PP3	-	-	-	BC_2S_1(PP3 x Mo17)
PP7	-	BC_3S_1(B101 x PP7)	BC_3(PP7 x B73)	BC_3S_1(PP7 x Mo17)
RP13	-	BC_3S_1(B101 x RP13)	BC_2S_1(RP13 x B73)	BC_3(RP13 x Mo17)
RP14	-	-	-	BC_3S_1(RP14 x Mo17)

Table 1.2. Genetic characterization of selected transgenic maize lines overexpressing *Ec*PAPR.

Event	Characteristics of the standard curves[1]					Ave. $2^{\Delta Ct}$ ± SD[2]	Estimated copy number[3]	Transgene segregation (+/-)[4]
	Genes	Efficiency	R^2	Slope	Intercept			
PE5-B101	*GAPDH*	1.0024 ± 0.0309	0.9953 ± 0.0064	-3.3175 ± 0.0738	40.7405 ± 0.5947			51/48
	Bar	1.0710 ± 0.0318	0.9901 ± 0.0091	-3.1638 ± 0.0668	41.0330 ± 1.6136	1.1020 ± 0.0410	1	
RE3-B101	*GAPDH*	1.0026 ± 0.0039	0.9968 ± 0.0035	-3.3158 ± 0.0093	40.5855 ± 0.1068			45/51
	Bar	0.9805 ± 0.0390	0.9950 ± 0.0061	-3.3715 ± 0.0972	42.9820 ± 0.863	0.6090 ± 0.0086	1	
PE5-B73				ND[5]				29/25
RE3-B73				ND				10/8
PE5-Mo17				ND				9/6
PE5-A654				ND				11/11

[1]The standard curve was generated with the SYBR Green qRT-PCR and used for determination of the transgene copy number.
[2]Ratio of the copy number of the transgene (*Bar,* selectable marker gene for bialaphos resistance) to the reference gene (*GAPDH*, glyceraldehyde-3-phosphate dehydrogenase) was calculated using the equation: Ratio = 2^($Ct_{reference}$ – $Ct_{transgene}$).
[3]DNA from leaf tissues of PE5-B101 and RE3-B101 were from germinated S_1BC_4 and BC_5 seeds, respectively.
[4]"+" and "-" denote the number of plants that tested positive and negative for the *Ec*PAPR transgene. These transgenic events were characterized at the BC_3 generation except for RE3-B101 which was analyzed at the BC_4 generation.
[5]ND, not determined.

Table 1.3. Kernel composition analysis.

Mean (SD)	B101	PE5-B101	RE3-B101
Protein, %	10.58 (0.28)	12.54 (0.20)**	12.86 (0.21)***
Nitrogen, %	1.937 (0.029)	1.947 (0.025)	1.990 (0.030)
Sulfur, %	0.140 (0.002)	0.194 (0.002)****	0.191 (0.001)****
Fat	3.80 (0.10)	4.72 (0.04)***	4.25 (0.12)**
Fiber	2.67 (0.14)	2.24 (0.14)*	2.25 (0.05)**
100-kernel wt., g	19.65 (1.28)	24.75 (1.26)****	22.02 (1.61)**
Kernel no. per ear	459.83 (50.82)	494.50 (49.01)	483.67 (46.81)

Mature kernels were pooled and measured and values (SD) represent the average of three measurements for protein, nitrogen, and sulfur contents; average kernel weight and number per ear were determined from 10 replicates of a 100-kernel sample and six ears, respectively. Statistical analysis was performed with the student's t test: significantly different at $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****).

Table 1.4. Reports on transgenic seeds with elevated Met content expressing the Met-rich sunflower seed albumin or the 10-kDa δ-zein.

Plant	Tissue-specific expression of a Met-rich protein	Sulfur content of transgenic seeds	Ref.
Lupins	Seed-specific expression of a SSA gene	• No difference in total S content between control and transgenic seeds • Marked differences in the distribution of S between the oxidized (sulfate) and the reduced C-bonded S fraction (cysteine and Met)	(Molvig et al., 1997; Tabe and Droux, 2002)
Chickpea	Seed-specific expression of a SSA gene	• Total seed S concentrations were not consistently different between transgenic and control genotypes • Transgenic seeds have significantly higher reduced S than the parental control	(Chiaiese et al., 2004)
Rice	Grain-specific expression of SSA	• S content does not change in the transgenic grain • Level of reduced S was not significantly different between control and transgenic lines	(Hagan et al., 2003)
Maize[1]	Seed-specific overexpression of its own 10-kDa δ-zein	• The null segregant kernels have 0.132% (0.002)[2] while the transgenic kernels have 0.138% (0.002)[2] sulfur contents	(Lai and Messing, 2002)

[1]This transgenic maize is from our own laboratory stock.
[2]The sulfur contents of the maize kernels are shown as means (SD) of three replicates. Statistical analysis performed with the student's *t* test showed that the value of the transgenic kernels is significantly different from the null segregant kernels at $P < 0.01$.

Table 1.5. Composition of the experimental diets used in the chick feeding trials[1].

Ingredient	Amount		
	Normal	Reference	PE5-B101
Corn meal (g)[2]	717.5	720.0	720.0
Soy protein (g)[3]	150.0	150.0	150.0
DL-Met (g)[4]	2.5		
Cellulose, BW200 (g)	30.0	30.0	30.0
Corn oil (g)	30.0	30.0	30.0
Vitamin mixture (g)[5]	10.0	10.0	10.0
Salt mixture (g)[6]	60.0	60.0	60.0
Total (g)	1,000	1,000	1,000

[1]Cornish male chicks were used in a four-week feeding trial starting with five-day-old chicks in triplicate groups of five per diet formulation.

[2]Three diet rations with different corn meals were used in the feeding experiment: (i) yellow dent corn supplemented with Met (normal group), (ii) null transgenic segregant from event PE5 without synthetic Met supplementation (reference group), and (iii) high-Met transgenic event PE5 without synthetic Met supplementation (PE5).

[3]Supro isolated soy protein (DuPont) contains 1.1% Met and 1.1% cysteine.

[4]DL-Met was added to the diet at a concentration of 2.5 g/kg (NRC, 1994).

[5]Supplied per kg of diet: Vitamin A palmitate, 500,000 IU/g, 8 mg; Vitamin D3, 100,000 IU/g, 10 mg; Vitamin E acetate, 500 IU/g, 50 mg; menadione sodium bisulfite, 62.5% menadione, 3 mg; biotin, 1%, 20 mg; cyanocobalamin, 0.1%, 10 mg; folic acid, 2 mg; nicotinic acid, 70 mg; calcium panthothenate, 15 mg; pyridoxine-HCl, 10 mg; riboflavin, 5 mg; thiamine-HCl, 5 mg; sucrose, 9,792 mg.

[6]Supplied per kg of diet: $CaCO_3$, 900 mg; $Ca_3(PO_4)_2$, 30 g; MgO, 1,002 mg; $C_6H_5K_3O_7$, 17.28 g; K_2SO_4, 1.65 g; NaCl, 4.5 g; $Cu_2(OH)_2CO_3$, 13.80 mg; KIO_3, 660 µg; $C_6H_5FeO_7$, 376.80 mg; $MnCO_3$, 126 mg; Na_2SeO_3, 420 µg; $ZnCO_3$, 144 mg; sucrose, 4,006.32 mg.

Table 1.6. List of primers used.

Primer name	Sequence (5' to 3')	Usage	Available restriction sites	Ref.
For expression vector construction[1]				
EcpFOR[2]	GGGgaattcccgggatccAAG CTTCATGGCGCCCACC	Amplification of *Ec*PAPR fragment from #788	*Eco*RI, *Sma*I, *Bam*HI	
EcpREV[2]	GGGgagctcTTACCCTTCG TGTAACCCACAT		*Sac*I	
RbcsFOR[3]	CCCCcaattgGAGCTCGGT ACCCGGGGATCC	Amplification of *RbcS* fragment from pPTN533	*Mfe*I	
RbcsREV[3]	CCCtgatcaGCCTGGCTGC CTAGTATGTATGTACTC		*Bcl*I	
PepcFOR[3]	ATGATTACgaattcGAGCT CGGTACCC	Amplification of *PepC* fragment from pPTN512	*Eco*RI	
PepcREV[3]	GCCATagatctATCATAGA AGCCATAGATCC		*Bgl*II	
For genotyping				
ExpVec_Ec_R	CTTCCGCCGTTGCTGACG TTGCCGAG	Verification of construct integrity after ligation		
ExpVec_Pa_R	GAAGAGAGCATAGAGGA AGCCATTGT			
ExpVec_F	AGGCTTTACACTTTATGC TTCCGGCTCGTATG			
ExpVec_R	CTGGGAACTACTCACACA TTATTCTGGA			
rPepC_For	CTCCCCATCCCTATTTGAA CCC	Genotyping of transgenic (P)APR events		
rRbcS_For	CCGCTTCCTCCTATCTACA AGT			
rEcPAPR_Rev	GGTAGGTTTCCGGGAAC AAGTA			
rPaAPR_Rev	GAAGGAGATCCACAGCT CGTC			
For RT-PCR and RT-qPCR				
bar_F_2	TGCACCATCGTCAACCAC TACATCGAG	Determination of transgene copy number		(Grohmann et al., 2009)
bar_R_2	CAGGCTGAAGTCCAGCT GCCAGAAAC			
ZmGAPDH_f	GACAGCAGGTCGAGCAT CTTCGA	Reference gene for RT-PCR or qRT-PCR		(Fletcher, 2014)
ZmGAPDH_r	GTCGACGACGCGGTTGC TGTA			
EcPAPR_for	TTACTTGTTCCCGGAAAC CTACC	qRT-PCR analysis of *Ec*PAPR transcript levels		
EcPAPR_rev	AATCGGCAGCACTTTAAA TACGC			
q15-kDa_for	CATGGGTGGACTCTACCA GTACC	qRT-PCR analysis of zein transcript levels		

q15-kDa_rev	CATGATAATGTGTGTCGT CTTACTGC	
q16-kDa_for	GCGGTGTCTACTACTGAG GAAACT	
q16-kDa_rev	CATTCAGGTCATTGCTCA CACT	
JP-ZmAPRL_for	CAGGGCTACGTGTCCATC GGGTG	qRT-PCR analysis of maize APR-like transcript levels
JP-ZmAPRL_rev	TTGTGGAGGCCGCACTCC TTGG	

[1]The cloning scheme for generation of the expression vectors involved two consecutive steps: first, generation of the pTF102-(P)APR vectors by ligation of *Eco*RI- and *Sac*I-digested fragments of *Ec*PAPR or *Pa*APR into pTF102 and second, ligation of the *PepC* or *RbcS* promoter fragments into *Eco*RI- and *Bam*HI-digested pTF102-(P)APRs.
[2]*Ec*PAPR fragment was PCR-amplified from plasmid #788 while the *Pa*APR fragment was digested out of plasmid #793 with *Eco*RI and *Sac*I.
[3]Promoter fragments were digested with available restriction sites on their flanking primers and ligated into *Eco*RI- and *Bam*HI-digested pTF102-(P)APRs.

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CHAPTER 2: QUALITY PROTEIN MAIZE BASED ON INCREASED SULFUR REDUCTION IN LEAF CELLS

2.1. ABSTRACT

Low levels of the essential amino acids lysine (Lys) and methionine (Met) in a maize-based diet are a major cost in world food supply. Lys deficiency is due to the abundance of Lys-poor α-zeins in maize endosperm. However, a maize variant was discovered with a mutation rendering the transcription factor inactive that controlled the expression of α-zeins, known as O2 due to the opaque appearance of the maize kernel. Although *o2* maize has sufficient levels of Lys, its soft kernel rendered it unfit for storage and transportation. Breeders overcame this problem by selecting quantitative trait loci (QTLs) restoring kernel hardness in the presence of *o2*, a variety called Quality Protein Maize (QPM). Although some QTLs act by enhancing the expression of γ-zeins, we could surprisingly achieve rebalancing of the Lys content and a vitreous kernel phenotype with low levels of γ-zeins and without the o2 mutation by crossing two previously generated transgenic events. Levels of γ-zeins were reduced with RNA interference (RNAi). The other transgenic event, named PE5, expresses leaf-specifically the *E. coli* enzyme 3'-phosphoadenosine-5'-phosphosulfate reductase, an enzyme involved in reductive sulfate assimilation. The stacked transgenic events produced a vitreous phenotype and a higher Lys level than the classical opaque W64A*o2* variant. Moreover, due to the increased sulfur reduction in leaf cells, even the level of methionine is elevated in the seed. Such a combination of genes produces hybrid seeds superior to classical QPMs that would neither require a costly feed mix nor synthetic Met supplementation, thereby revolutionizing food production.

2.2. INTRODUCTION

In most developing countries, maize serves as the sole source of nutrition in human and animal diet, although its low protein content and quality necessitates its use in conjunction with other protein sources. Cereals like maize are limiting in the essential amino acids (EAAs) lysine (Lys), methionine (Met), and tryptophan, whereas legume crops like soybean are deficient in Met. Therefore, corn is usually supplemented with soybean and synthetic free methionine to provide a balanced amino acid diet for animal feed. A lot of research efforts has been expended on genetic improvements to the amino acid balance of maize kernels, either through conventional selection breeding or the use of recombinant DNA technology.

Like other cereal grains, the maize kernel is made up of 6% pericarp, 12% germ, and 82% endosperm (Watson, 1987). The bulk of the protein in a mature maize kernel resides in the endosperm, although the germ has a superior protein quality and higher protein content. Zeins in the endosperm make up the bulk of the protein in the mature maize kernel and possess an amino acid imbalance towards proline, glutamine, alanine, and leucine residues. The non-zein protein fraction in the endosperm (glutelins, globulins, and albumins) are relatively balanced in their amino acid composition (Prasanna et al., 2001). The abundance of zeins effectively dilutes the contribution of other endosperm proteins to the kernel Lys and tryptophan contents. It is not unexpected that alterations in the accumulation of zeins have led to the identification of mutants with altered nutritional quality. By increasing the accumulation of the β-, γ-, and δ-zeins relative to the more abundant α-zeins (Table S1), we could increase the sulfur amino acid contents of kernels, effectively increasing its nutritional value (Planta et al., in press). On the other hand, reduction of α-zeins either through a transcriptional mutation (e.g., with the

Opaque-2 or O2 protein) or a transgene that reduces its transcript accumulation through RNA interference (RNAi) resulted in more nutritionally-balanced endosperm proteins.

One of the thoroughly studied zein-reduction mutants, the recessive *o2* mutant has about a 50% reduction in zein proteins (Tsai et al., 1978) and approximately double the amount of Lys compared with normal genotypes (Mertz et al., 1964). It primarily affects the synthesis of α-zeins, the 22-kDa α-zeins is barely detectable, whereas the 19-kDa α-zeins are greatly reduced (Jones et al., 1977). The *o2* mutant, aside from a reduced Lys-poor zein content, has a compensating increase in the levels of Lys- and tryptophan-rich non-zein proteins. However, the soft and starchy endosperm producing the opaque phenotype observed in *o2* kernels makes it susceptible to fungi and insect infestation, both in storage and in the field (NRC, 1988). Identification of *o2* modifiers (m*o2*) (suppressors) that can restore the normal kernel phenotype while maintaining the increased EAA content of *o2* permitted the development of a new type of maize germplasm, known as quality protein maize (QPM) (Prasanna et al., 2001).

On the other hand, transforming maize with a transgene targeting the α-zeins through RNAi resulted in maize kernels with enhanced levels of Lys and tryptophan (Huang et al., 2004; Huang et al., 2006; Segal et al., 2003). The observed increase in Lys and tryptophan in the α-zein-reduced kernels was also due to the replacement of the Lys-poor zeins with the Lys-containing non-zein proteins (Huang et al., 2006). Doubling the Lys levels without changing the protein content in corn could add up to $480 million in annual gross value to US corn in the global feed market (Johnson et al., 2000).

Among the opaque mutants that affect the accumulation of the zeins, only two have been reported that alter the synthesis of the γ-zeins. The maize *Mucronate* mutation is a deletion in the 16-kDa γ-zein that produces an abnormal 16-kDa γ-zein, whereas

opaque-15 reduces the 27-kDa γ-zein synthesis and appears to be a mutation of an *o2* modifier gene (Dannenhoffer et al., 1995; Kim et al., 2006). Near-isogenic lines of several high-Lys opaque mutants in the W64A genetic background showed that *o2* has the highest kernel Lys content among the opaque mutants (Hunter et al., 2002). Efforts to improve the protein quality of maize seeds have focused on *o2* seeds as other opaque mutants offer no additional advantage over *o2* in terms of Lys content and nutritional quality.

Given these results, we wanted to generate a sole crop diet of corn that is enriched in both Lys and Met. Using two disparate transgenic mechanisms of deregulating an amino acid biosynthetic pathway and zein reduction, we crossed the high-Met maize line PE5 (Planta et al., in press) with several zein RNAi lines. The RNAi lines used target the α-, β-, γ-, α-/γ-, or γ-/β-zeins. Kernels of the genotypes PE5;α-/γ- and PE5;γ- have Lys and Met contents higher than the mutant *o2* in the W64A maize genetic background (W64A*o2*). Although the opaque PE5;α-/γ- kernels have higher Met and Lys contents than the vitreous PE5;γ- kernels, a vitreous phenotype would be worth the compromise for commercial applications. Moreover, PE5 with a single dominant RNAi-inducing transgene of γ-zein would be a superior QPM variety because of higher Lys and elevated Met levels.

2.3. RESULTS

The high-Met transgenic maize PE5 event (Planta et al., in press) was crossed with an α-zein RNAi line (Wu and Messing, 2011). Crosses of PE5 with suppression lines of β- and γ-zeins were also performed as redistribution of protein sulfur in seeds occurs when accumulation of sulfur-containing zeins are reduced (Wu et al., 2012) or when a sulfur-rich protein is synthesized in transgenic maize kernels (Lai and Messing, 2002).

Reduction lines of α-, β-, and γ-zeins had been achieved through RNAi (Fig. 2.1). Transgenes for the RNAi lines have inverted repeats of the target sequence or sequences separated by a partial GFP linker. The αRNAi construct has a tandem cassette of a 19- and 22-kDa α-zein genes and targets both α-zeins, γRNAi has an inverted repeat of the 27-kDa γ-zein gene but cross reacts with the 16-kDa γ-zein to target both 16- and 27-kDa γ-zeins, whereas βRNAi targets the 15-kDa β-zein (Wu and Messing, 2010; Wu and Messing, 2011).

PE5 is a high-Met transgenic maize that ectopically and leaf-specifically expresses the *PepC* promoter-driven *E. coli* enzyme 3'-phosphoadenosine-5'-phosphosulfate reductase, or *Ec*PAPR (Planta et al., in press). *Ec*PAPR is an enzyme involved in reductive sulfate assimilation (Martin et al., 2005) This transgenic PE5 line exhibits an increased kernel Met content when used as the maternal parent (Fig. 2.2a). PE5 plants were therefore crossed with the zein reduction lines as the pollen donor to manifest the increased seed Met phenotype in resulting ears.

2.3.1. Rebalancing of the protein S in different stacked events

Five types of distinct ears were obtained from the crosses of PE5 with the different zein reduction lines: (1) PE5;α-, (2) PE5;γ-, (3) PE5;β-, (4) PE5;γ-/α- and (5) PE5;γ-/β-. At least 14 kernels from each transgenic ear were analyzed individually by phenotyping with

SDS-PAGE or genotyping for the RNAi transgenes. Kernels were then pooled depending on whether they were segregating or non-segregating for the RNAi transgenes (Fig. 2.2b).

Loss of β- and γ-zeins promote a much higher increase in the accumulation of the Met-rich 10-kDa δ-zein compared to the reduction in the S-poor α-zeins (Fig. 2.2b). This increase in the accumulation of the 10-kDa δ-zein was previously demonstrated in transgenic maize expressing the β- and γ-zein RNAi transgenes (Wu et al., 2012), although it was not as high as when these RNAi transgenes were stacked with the PE5 event (Fig. 2.2b, upper panel). Reduction in both β- and γ-zeins channels even more protein sulfur to the 10-kDa δ-zein than the loss of either β- or γ-zein. We can confirm here that enhanced assimilation of sulfur in the source leaf tissues increases accumulation of the S-containing zeins in the seed sink tissues, but also show that loss of the S-containing zeins reallocate the protein S to the remaining or available S-containing zeins.

2.3.2. PE5 influences kernel opacity depending on zein gene expression

As the loss of α- and γ-zeins by RNAi induces a full or partial opaque seed phenotype, respectively, (Segal et al., 2003; Wu and Messing, 2010), hybrid kernels were inspected whether the PE5 transgenic event affects endosperm modification. Opacity of the hybrid PE5;zein RNAi kernels were phenotyped with a light box (Fig. 2.3). Loss of β-zeins did not change the phenotype of the vitreous kernel, as indicated by the thick outer layer of the vitreous endosperm in sliced kernels (Fig. 2.3g). Reduction in the γ-zeins produced a partial opaque phenotype, where the regular pattern of endosperm modification extends from the crown midway to the base of the kernel (Fig. 2.3d), whereas reduction in both β- and γ-zeins produced complete opaque kernels (Fig. 2.3f). However, combining PE5 with reduction in both β- and γ-zeins completely restored the vitreous

kernel phenotype (Fig. 2.3i). This reversion to a more vitreous phenotype was also observed in PE5;γ- kernels (Fig. 2.3e). As βRNAi kernels are vitreous, combining it with PE5 did not change its kernel phenotype (Fig. 2.3h). Stacking of PE5 with αRNAi (Fig. 2.3b) or α/γRNAi (Fig. 2.3c) did not alter the opacity of the hybrid kernels. The PE5 transgenic event in PE5;γ- or PE5;γ-/β- can influence endosperm modification depending on which RNAi event is used.

Kernels from a cross of PE5 with αRNAi showed increased level of the 27-kDa γ-zein (Fig. 2.2b). This increase in the γ-zein was also observed in crosses of αRNAi with other transgenic events overexpressing *Ec*PAPR (Fig. 2.4) and seems to be a response to the decreased levels of α-zeins. Segal et al. (2003) and (Huang et al., 2004), however, reported in their zein reduction lines varying and reduced levels, respectively, of the 27-kDa γ-zein. They attributed this to segregation of the 27-kDa γ-zein gene from the A and B lines used for transformation (Segal et al., 2003) and gene silencing of the 27-kDa γ-zein gene due to the use of the γ-zein promoter in the transgenic expression cassettes (Huang et al., 2004).

QPMs have reduced levels of the 22-kDa α-zein but have two- to three-fold increase in the 27-kDa γ-zein (Ortega and Bates, 1983; Wallace et al., 1990). The increase in the 27-kDa protein correlates, and is necessary, if not sufficient, for endosperm modification (Lopes and Larkins, 1991; Wu and Messing, 2010). Depending on the genetic background, maize inbreds can have one or two copies of the 27-kDa γ-zein gene (Das and Messing, 1987; Das et al., 1991). Therefore, the increased synthesis of the 27-kDa protein is that suppressors of the opaque phenotype in the seeds correspond to additional copies of the γ-zein genes. To explore this possibility, DNA was isolated from PE5 and αRNAi, their hybrid, and their parental lines to determine the nature of the γ-zein alleles (Fig. 2.5).

Two highly-similar tandemly-duplicated genes of the 27-kDa γ-zein, "A" and "B", in inbreds like W22 and A188, have different *Pst*I recognition sites which was utilized for the cleaved amplified polymorphic sequence assay (CAPS; Fig. 2.5a). The inbred line, A188, can have a single rearranged B gene (Rb) originating from a homologous recombination at the highly conserved 5' regions of the two repeats (Das and Messing, 1987; Das et al., 1991).

The DNA gel profile in Fig. 2.5b shows the utility of the assay in determining allelic variations in the 27-kDa γ-zein genes from different maize inbred lines. Hi-II A x B, B101, PE5, αRNAi, and kernels from a PE5 x αRNAi cross (Fig. 2.5b-c) have a single copy of the γ-zein gene. There seems to be no copy number variation in the hybrids and inbreds related to PE5 and αRNAi, and thus, unlikely that modifiers of the opaque phenotype are associated with copy number variations of the γ-zein genes in our crosses. Indeed, γ-/β- is opaque (Fig. 2.3f), but PE5;γ-/β- is vitreous (Fig. 2.3i), exemplifying restoration of the normal phenotype in the absence of the γ-zeins, but in the presence of PE5. On the other hand in the absence of PE5, a duplication of the 27-kDa γ-zein gene in QPMs enhances its expression and promotes endosperm modification (Liu et al., 2016).

2.3.3. Protein accumulation patterns in PE5;zein RNAi kernels

To determine whether the protein accumulation patterns are different between the transgenic zein reduction kernels and non-transgenic controls, kernels from an ear of a PE5 x RNAi cross were pooled depending on the segregation of the RNAi transgenes. Total protein and the non-zein protein fractions were extracted from these kernels and separated in an SDS-PAGE gel (Fig. 2.6). Aside from the reduction in zeins due to the RNAi transgenes, PE5;γ- and PE5;β- kernels had similar protein accumulation profiles compared to their PE5;non-RNAi controls (Fig. 2.6a). On the other hand, reduction in α-

zeins in kernels of PE5;α- and PE5;α-/γ- had different seed protein accumulation patterns than their corresponding PE5;non-RNAi kernels (Fig. 2.6a, b). The loss of α-zeins condition an increase in the accumulation of non-zein proteins in the kernel. Proteins in the range of 60–70 and 45–55 kDa were differentially upregulated in the α-zein-mutant kernels.

Two protein bands with sizes of about 60- and 65-kDa (red arrows in Fig. 2.6a) were particularly increased in PE5;α- and PE5;α-/γ- kernels, with the latter having more accumulation of these upregulated proteins than the former. These protein bands were identified by mass spectrophotometric analysis. Spectral quantitation showed that the upper band had 52.1% (316 out of 606) and the lower band had 29.8% (909 out of 3,048) of its identified peptides to be fragments of the GLB1 (globulin-1) protein. GLB1 has no known enzymatic function, and just like zeins, is thought to function as a storage protein (Kriz, 1989). Pulse-chase labeling and *in vitro* translation studies showed that the primary translation product of *Glb1* undergoes at least three post- and/or cotranslational processing steps to produce the mature GLB1 protein (Kriz and Schwartz, 1986). The lower band of ~60 kDa would therefore represent the mature GLB1, whereas the upper band of ~65 kDa would be the processing intermediate GLB1'. GLB1 is mostly an embryo-specific protein that is estimated to account for 10-20% of the total embryo protein along with GLB2 (Kriz, 1989). As the deduced amino acid composition of GLB1 has 4.11% Lys (Belanger and Kriz, 1989), an increased accumulation of this Lys-rich protein could contribute to an increase in the content of protein-bound Lys in the kernel. Although the 27-kDa γ-zein was increased in PE5;α- kernels, the increase in kernel Lys could not be attributed to over-accumulation of this protein as γ-zein is devoid of Lys (Table 2.3.1).

Loss of α-zeins redistributes the nitrogen, primarily stored in asparagine and glutamine in α-zeins (Table 2.3.1), to the non-zein protein fraction by compensatory increases in proteins in this fraction. Puckett and Kriz (1991) and Hunter et al. (2002) showed that GLB1 is upregulated in *o2* mutants, along with other Lys-rich proteins that contribute to an increase in the kernel Lys content. Proteins that were upregulated in *o2* kernels, such as GLB1 (Hunter et al., 2002; Puckett and Kriz, 1991) and the Lys-rich (>8% Lys residues) glyceraldehyde-3-phosphate dehydrogenase (Damerval and le Guilloux, 1998), were also confirmed in the transgenic α-zein reduction lines (Frizzi et al., 2010).

2.3.4. Amino acid composition analysis of PE5;zein RNAi kernels

The *o2* mutant has about twice the Lys content compared to normal phenotype (Mertz et al., 1964) and mutant *o2* alleles in different backgrounds display variations in Lys contents and penetrance of the opaque phenotype (Balconi et al., 1998; Hunter et al., 2002). Of the crosses of PE5 with different RNAi lines, only PE5;α-, PE5;γ-, and PE5;α-/γ- kernels have statistically significant higher Lys content over the normal A x B kernels (Fig. 2.7a). Both PE5;α- and PE5;α-/γ- kernels have higher Lys contents, with 7.25 and 5.29 mol%, respectively, than the high-Lys mutant W64A*o2*. The Lys contents in these two genotypes were 60% and 128.2% higher, respectively, compared to their corresponding PE5;non-RNAi controls (Table 2.3.2). This discrepancy in the variation of the Lys contents from their respective controls and the actual values suggests that the hybrid genetic backgrounds of these two groups have an impact on Lys accumulation. Compared to the A x B kernels, PE5;α- kernels had 151.9% and PE5;α/γ- kernels had 83.8% more Lys. As previously reported, our stock of αRNAi line had only a 26.7% increase in Lys content compared to the parent non-transgenic control (Wu and Messing, 2011), suggesting that

the high-Met PE5 maternal background also influences Lys accumulation in the seeds. Due to the well-established correlation between the levels of tryptophan and Lys in maize protein (approximately 1 to 4), either amino acid can be used as a single parameter for evaluation of protein quality (Hernandez and Bates, 1969; Villegas et al., 1984).

2.3.5. PE5;γ- kernels have significant Met and Lys contents and are vitreous

W64A02 had reduced levels of kernel Met due to reduced amounts of the 10-kDa δ- and 15-kDa β-zeins (Hunter et al., 2002). PE5;α-/γ- kernels have an increase of 26.4% more Met than its PE5;non-RNAi control (Table 2.2; Table 2.3), though it was not as high as in the parental PE5 introgressed into the B101 background (Fig. 2.7b). Therefore, positive regulators of expression of the 10-kDa δ-zein in the B101 background might have been lost in the hybrid kernels. Still, PE5;α-/γ- kernels have higher levels of both Met and Lys compared to the A x B and PE5;non-RNAi controls (Table 2.2).

Both Met and Lys can be increased by the synergistic effect of a maternal high-Met PE5 background and a combined α-/γ-zein reduction, with the hybrid genetic background of the kernel progenies affecting accumulation of these amino acids. Compared to other reports regarding increased Lys by transgenic zein reduction in maize (Table 2.4), our combination of PE5 with reduction in the α-zeins seems to promote the highest increase in Lys content. Although the higher Met and Lys contents of PE5;α-/γ- would make it a better animal feed alternative than the W64A02 kernels (Fig. 2.7), its opaque phenotype (Fig. 2.3) would preclude its general use. Therefore, the higher Met and Lys contents of the vitreous PE5;γ- would be a better alternative than the W64A02 kernels in terms of its nutritional quality and grain characteristics.

Knockdown of zeins by RNAi not only changes the accumulation profile of zeins (Fig. 2.2) but also the accumulation of amino acids sequestered in the reduced zeins (Fig. 2.8; Table 2.3). PE5;α- has more changes in the kernel amino acid composition compared to PE5;γ- and PE5;β- (Fig. 2.8a-c). The changes in the amino acid composition of PE5;α- kernels were exacerbated by stacking it with γRNAi (Fig. 2.8d; Table 2.5). Surprisingly, the loss of the γ- and β-zeins in PE5;γ-/β- kernels (Fig. 2.8e; Table 2.5) had less variation in its amino acid composition compared to other PE5;zein RNAi kernels. The levels of seed amino acid composition in PE5;γ-/β- were like its PE5;non-RNAi control (Fig. 2.8; Table 2.5) and this might have contributed to the restoration of the vitreous phenotype in PE5;γ-/β- kernels.

PE5;γ-/β- kernels had the least similarity in terms of amino acid changes with other kernel genotypes (Fig. 2.8; Table 2.5). Of the 17 amino acids that were analyzed in PE5;α-/γ- kernels, only three amino acids were significantly not different from the PE5;non-RNAi control, whereas PE5;β-/γ- kernels only had leucine as statistically different from normal kernels. In comparison to wheat grain, maize has a high leucine content which contributes to its relatively poorer nutritional performance in human feeding trials (Kies and Fox, 1972). An excess of dietary L-leucine acts as an antimetabolite of isoleucine as rats fed an excess of L-leucine in a low-protein diet or diets deficient in isoleucine exhibited growth retardations (Harper et al., 1955). Only PE5;α- and PE5;α-/γ- kernels had significantly reduced leucine contents (Fig. 2.5; Table 2.5). The αRNAi lines, as well as *o2*, also had reduced leucine contents (Huang et al., 2004; Huang et al., 2006; Segal et al., 2003). Zeins in general, particularly α-zeins, are exceptionally rich in leucine (Table 2.1), and therefore, reduction in α-zeins would also decrease leucine accumulation in the kernels.

2.4. DISCUSSION

The phenotypic plasticity of the seed storage proteins in maize were exploited to generate maize kernels that have enhanced accumulation of the EAAs Lys and Met. To explore a sole crop animal diet of corn, transgenic zein reduction lines were crossed with the high-Met line PE5. As seed storage proteins mainly serve as the reservoir of nitrogen in the germinating maize seedling and not so much of specific amino acids, it has been proposed that seeds are functionally able to accommodate a wide range of variations in amino acid composition (Shotwell and Larkins, 1991). These properties of the seed storage proteins would make it a good target for improving the nutritional quality of maize either through a direct manipulation of zein synthesis (e.g., by RNAi) or by increasing the amino acid supply to the kernels due to increased source strength or through a combination of both methods.

Out of the five genotypes that were tested for enhanced accumulation of Met and Lys, only PE5;α-, PE5;γ-, and PE5;α-/γ- kernels have higher Lys and Met contents than the high-Lys W64A*o2*. These kernels, however, have Met levels that are lower than that of PE5 (Fig. 2.7). PE5;α- and PE5;α-/γ- have opaque kernels whereas PE5;γ- has vitreous kernels (Fig. 2.3).

Amino acid composition of maize kernels varies widely across different genetic backgrounds. Balconi et al. (1998) and (Hunter et al., 2002) reported that the opaque mutant *o1* exhibits different Lys levels in different maize genetic backgrounds. The *o1* mutant has a Lys content that approximates that of the normal level, when the allele is in a W64A background (Hunter et al., 2002). However, it increased the Lys content of the inbred line A69Y as much as W64A*o2* (Balconi et al., 1998), indicating a differential response of these backgrounds to the same mutant allele. Met accumulation in PE5 also

varies when this transgenic event is in different backgrounds. Introgression of PE5 to the high-Met inbred line B101 (in at least four generations of backcrosses) has higher Met content than when it is in an A x B hybrid background at the F3 generation (Planta et al., in press). Results reported here refer to hybrid genetic backgrounds resulting from a combination of crosses of PE5 with the transgenic zein reduction lines (see Materials and Methods).

In QPMs, the RNAi-induced loss of the 27-kDa γ-zein abrogates the ability of the *mo2*s to restore kernel hardness, suggesting that it is necessary for endosperm modification (Wu et al., 2012). Because the 27-kDa gene does not require the O2 transcription factor for expression (Schmidt et al., 1992), it is not surprising that in absence of O2 QTLs of *mo2*s in QPMs can be linked to the 27-kDa γ-zein locus (Holding et al., 2008). In one example, gene duplication is implicated in the enhanced expression of the 27-kDa γ-zein for endosperm modification (Liu et al., 2016). Alternatively, enhanced mRNA transcription or stability, rather than gene amplification, was hypothesized to be the reason for enhanced expression of the γ-zein as one or two copies of the gene occurs in modified and non-modified *o2* genetic backgrounds that were studied (Geetha et al., 1991). However, cleaved amplified polymorphisms exhibited no variation in the 27-kDa γ-zein gene copy number of the genetic background that were used in this study (Fig. 2.5). Therefore, increased levels of γ-zeins in PE5;α- (Fig. 2.2b) and αRNAi (Fig. 2.9) is probably due to post-transcriptional regulation of gene expression as previously described (Geetha et al., 1991)

Whereas the increase in 27-kDa γ-zein expression in the presence of αRNAi occurred independently of the *o2* mutation, it can result in kernel modification in certain genetic backgrounds (Fig. 2.9). It appears that kernel modification in reduced levels of α-

zeins requires at least two factors: increased accumulation of the 27-kDa γ-zein and genetic modifiers of the opaque phenotype. The modifiers in M017 are probably dominant as it was used as the paternal parent in a cross with the maternal αRNAi line. Different genotypes studied for inheritance of modified endosperm in *o2* backgrounds exhibited a complex system of genetic control involving gene dosage effects in the triploid endosperm, cytoplasm effects, and unstable and incomplete penetrance of the modifier genes. These modifier genes have either dominant, semidominant, synergistic, or recessive modes of action (Belousov, 1987; Lopes and Larkins, 1991).

One explanation for the increased expression of the native 27-kDa γ-zein protein could be the selection pressure on the 27-kDa γ-zein promoter used for expression of the αRNAi cassette, in a phenomenon proposed as proxy selection (Bodnar et al., 2016). In proxy selection, the increased activity of a transgene under the control of a native promoter can enhance the protein levels of the native gene with the same promoter. One complication in the use of αRNAi lines is the use of sequences or promoter of the 27-kDa gene that drive the RNAi expression cassette, resulting in variations of γ-zein levels in kernels of the progenies of these RNAi lines. In transgenic α-zein reduction lines, gene silencing could occur or accumulation of variable levels of expression of the γ-zein due to segregation of its alleles (Huang et al., 2004; Segal et al., 2003).

The increased accumulation of non-zein proteins in both the endosperm and embryo of PE5;α- and PE5;α-/γ- kernels (Fig. 2.6) is a response to α-zein reduction, as also evident in *o2* (Hunter 2002). Because some of these non-zein proteins contain some amount of Lys residues, the effective kernel Lys is increased (Kriz, 2009). Because α-zeins make up more than 30% of the total seed proteins, its loss would also entail a major reduction in the levels of N-transport amino acids like glutamine. However, *o2*-converted

lines showed only a minor decrease in the protein content compared with the analogous normal inbred lines (Gupta et al., 1974), implying a protein N redistribution from zein to non-zein proteins. We have found that GLB1 has higher accumulation in our transgenic α-zein reduction lines, similar to what was observed in *o2* (Puckett and Kriz, 1991).

Reduction in γ-zeins can also induce the opaque seed phenotype, albeit at a less severe degree than the loss of α-zeins. Opacity of the β/γRNAi kernels is caused by incomplete embedding of the starch granules in the outer, vitreous endosperm rather than a reduction in the vitreous area observed in αRNAi kernels (Wu and Messing, 2010). Kernels with reduced levels of γ-zein in PE5;γ- and PE5;γ-/β-, but not in PE5;α-/γ- kernels, have the vitreous phenotype (Fig. 2.3). This endosperm modification is probably an indirect effect of the PE5 transgenic event, where it can overcome the opaque phenotype mediated by γRNAi but not by αRNAi or α/γRNAi. Zeins confer the distinct shape to PBs and can form intra- and intercellular disulfide bonds with other proteins. α-zeins are postulated to serve as the "brick" and the γ-zeins the "mortar" in the seed during maturation and desiccation. During desiccation, the rough ER associated with the protein bodies breaks down, mixing the zeins with other components of the cytosol and associating directly with the starch granules. The peripheral 27-kDa γ-zein then serves as the "mortar" that bonds the starch granules in a proteinaceous matrix in the outer vitreous zone of the kernel, imparting the hard endosperm phenotype to the kernel (Chandrashekar and Mazhar, 1999).

As a transgenic event in tobacco leaves, the 10-kDa δ-zein can form novel protein bodies, which is unlikely in PE5;γ-/β- as the δ-zein has a strong interaction with the α-zeins (Bagga et al., 1997; Kim et al., 2002). It is more likely that the 15-kDa β-zein has a redundant function with the 27-kDa γ-zein in terms of embedding the starch granules in a

proteinaceous matrix because of its cysteine content. We have previously shown that PE5 increases expression of cysteine-rich non-zein proteins (Planta et al., in press). It is possible that one of these proteins is an accessory protein that associates with protein bodies and promotes its structural integrity.

Although QPMS have been proven effective, the complexity of introducing multiple, unlinked loci of m02s into a defined *o2* background has slowed the creation and widespread use of QPMs (Gibbon and Larkins, 2005). Here we report a superior strategy to generate QPM with an additional high-Met content without a reduction of α-zeins, we call QPM+. PE5;γ- kernels could be generated in one generation of crossing, whereas QPMs entail generations of backcrossing the *o2* mutant allele into a desirable germplasm and subsequent backcrosses of the *o2*-converted germplasm with the m02s. It would take about 17 generations to convert a desirable germplasm into a QPM (Wu and Messing, 2011). Even if both the γRNAi and PE5 lines are introgressed into a desirable germplasm, the eight generations it would take to make an introgression line with both the PE5 and γRNAi transgenes are still about half the time it takes to generate a classical QPM.

2.5. MATERIALS AND METHOD

2.5.1. Genetic stocks

The α-, γ-, and βRNAi transgenic plants were from our laboratory stocks and have been described elsewhere (Wu and Messing, 2010; Wu and Messing, 2011; Wu et al., 2012). Both the γRNAi and βRNAi are homozygous for *A654-Dzs10*, a nonfunctional allele of the 10-kDa δ-zein gene from the inbred A654. The βRNAi plant used for crosses is homozygous for the RNAi transgene as all kernel progenies tested positive for βRNAi genotyping, whereas the γRNAi is hemizygous, as about half of the tested kernel progenies from crosses with γRNAi had the RNAi transgene. αRNAi, on the other hand, is in an A x B hybrid background and is hemizygous for the RNAi transgene.

The transgenic event PE5 was backcrossed twice to the high-Met inbred line B101 prior to being crossed with the RNAi lines. It ectopically expresses the *Escherichia coli* enzyme 3'-phosphoadenosine-5'-phosphosulfate reductase, designated as *Ec*PAPR (Martin et al., 2005), driven by the *PepC* promoter (Sattarzadeh et al., 2010). *Ec*PAPR is involved in assimilatory sulfate reduction and maize plants expressing this enzyme shows increased kernel Met content when used as the maternal parent (Martin et al., 2005; Tarczynski et al., 2003).

Crosses between the maternal PE5 and the paternal RNAi lines were performed during the summer of 2014 and 2015. PE5; γ- plants generated during the summer of 2014 were crossed with αRNAi during the summer of 2015. The rest of the crosses of γ/βRNAi, βRNAi, and αRNAi with PE5 were made during the summer of 2014. All in all, these crosses gave five distinct ears that were used for analysis: (1) PE5;αRNAi, (2) PE5;β-, (3) PE5;γ-, (4) PE5;γ-/β-, and (5) PE5;γ-/α-.

2.5.2. Genotyping

Genomic DNA was isolated from maize leaves at the 3- to 4-leaf stage using a modified CTAB extraction method (Sawa et al., 1997). For extraction of genomic DNA from mature maize kernels, a portion of the kernel that is mostly endosperm with no embryo tissues were ground to a fine powder and subjected to DNA extraction with the Nucleospin Plant II kit (Takara Bio USA). Transgenic plants were screened for the presence of both RNAi and *Ec*PAPR transgenes using the primer pairs 5'-ACAACCACTACCTGAGCAC-3' / 5'-ATTAAGCTTTGCAGGTCACTGGATTTTGG-3' (Wu and Messing, 2010) and 5'- CTCCCCATCCCTATTTGAACCC-3' / 5'-GGTAGGTTTCCGGGAACAAGTA-3', respectively. PCR amplification for the RNAi transgenes produced amplicons of the sizes 365, 913, and 1,096 bp corresponding to α-, β-, and γRNAi lines, respectively, whereas screening for PE5 yields a 696-bp product. Kernels from an ear segregating and non-segregating for the RNAi transgenes were pooled separately and used for DNA extraction and genotyping.

The 27-kDa γ-zein gene displays allelic variation and differential expression and exists as a tandemly-duplicated or single-copy gene depending on the genetic background (Das and Messing, 1987). To determine possible allelic variation between different maize genotypes, amplified DNA fragments of the 27-kDa γ-zein gene were digested with *Pst*I to display restriction fragment length polymorphisms (Konieczny and Ausubel, 1993). Primers 5'- CCACCTCCACGCATACAAG-3' and 5'- ATGGACTGGAGGACCAAGC-3' were used to amplify a 487-bp fragment of the 27-kDa γ-zein gene spanning positions 50 to 546 of the coding region (Das et al., 1991). Digestion with *Pst*I would produce three DNA fragments (487, 292, and 195 bp), when the gene exists as a tandem copy or only two fragments for a single-copy gene (292 and 195 bp).

2.5.3. Analysis of transgenic plants

Protein extraction, SDS-PAGE analysis, and Western blotting. Total protein from pooled endosperm samples of mature maize kernels were extracted with an alkaline sodium borate extraction buffer (Wallace et al., 1990), whereas the alcohol-soluble zeins from the endosperm of mature maize kernels were fractionated and separated in SDS-PAGE as previously described (Wu and Messing, 2010).

Mature maize kernels were imbibed in water for two days to facilitate easier separation of the embryo from the endosperm. Proteins were isolated from embryos macerated in an SDS sample buffer [10% (v/v) glycerol, 2.3% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 62.5 mM Tris-Cl, pH 6.8] at a ratio of 50 mg tissue/ml of buffer and the extracts processed as described previously (Belanger and Kriz, 1989; Puckett and Kriz, 1991).

Total protein from three mature maize leaf discs was extracted following the procedure of Conlon and Salter (2007). Ten microgram of total protein was separated in a 12% Tris-glycine SDS-PAGE gel and immunoblotted with an antibody against *Ec*PAPR kindly provided by Dr. Jens Schwen (Krone et al., 1991). For immuno-detection, the secondary antibody is a goat anti-rabbit peroxidase conjugate (Sigma-Aldrich) and was used at a 1:60,000 dilution while the primary anti-*Ec*PAPR antibody in a TBST buffer with 0.5% BSA was used at a 1:4,000 dilution.

Protein identification. Protein bands that have differential accumulation in kernels segregating for the α-zein RNAi transgene were excised out of the SDS-polyacrylamide gel and analyzed by trypsin-nano-LC-MS using Q Exactive HF hybrid quadrupole-Orbitrap

mass spectrometer (Thermo Scientific). Proteins from the samples were identified at the Biological Mass Spectrometry Facility at Rutgers University.

Amino acid composition analysis. Transgenic mature kernels were ground to fine powder and about 10 mg were used for amino acid composition analysis conducted by the Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center, St. Louis, Missouri. Samples were pretreated with performic acid prior to acid hydrolysis, yielding the acid stable forms cysteic acid and Met sulfone that can be measured by separation on a UPLC column. Tryptophan was not detected following acid hydrolysis.

2.6. FIGURES

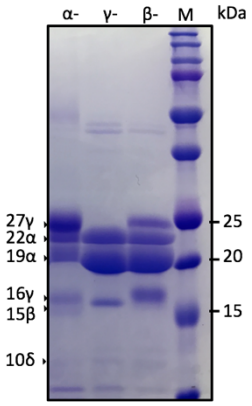

Figure 2.1. Differential accumulation of the zeins in 15% SDS-PAGE gel in different zein reduction lines.

α- has an RNAi that targets the 19- and 22-kDa α-zeins, γRNAi (γ-) targets both the 16- and 27-kDa γ-zeins while βRNAi (β-) promotes knockdown of expression of the 15-kDa β-zein. γRNAi and βRNAi are in a hybrid Hi II A x B and A654 backgrounds while αRNAi is in a Hi II A x B background.

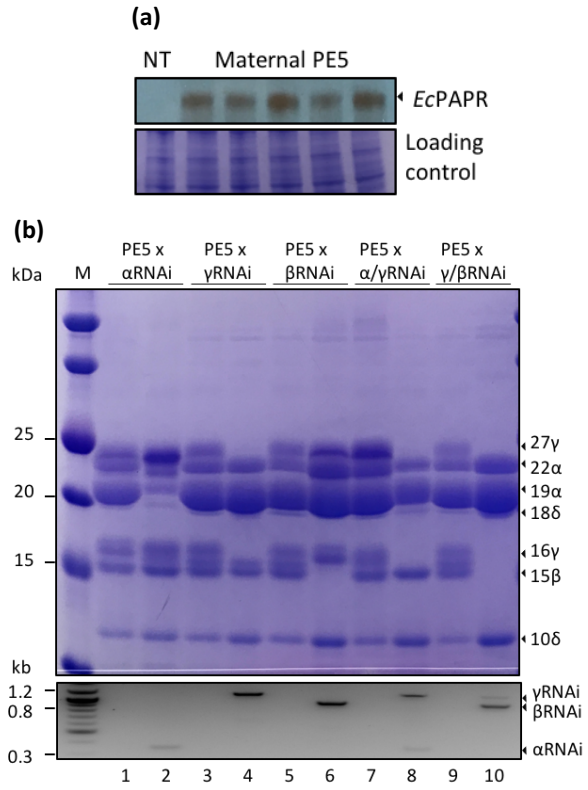

Figure 2.2. Zein profiles of kernels from crosses of PE5 with RNAi lines targeting the α-, γ-, β-, α-/γ-, or γ-/β-zeins.

(a) Western blotting for detection of exogenous expression of the bacterial *Ec*PAPR in leaf tissues of PE5 plants, which were used as the maternal parents for crossing with the zein reduction lines. (b) SDS-PAGE zein profiles (upper panel) of segregating populations of kernels from PE5 x zein RNAi crosses. Kernels non-segregating (odd-numbered lanes) and segregating (even-numbered lanes) for the RNAi transgenes were pooled and used for analysis. The RNAi transgenes in these segregating populations were detected by AGE (lower panel).

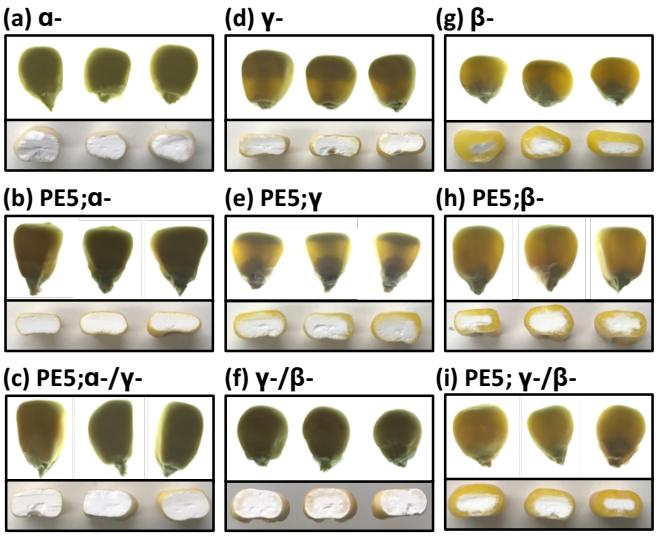

Figure 2.3. Endosperm phenotypes of transgenic zein reduction kernels (upper panels) sliced in half to reveal the degree of vitreous endosperm (lower panels).

(a, d, f, g) Zein RNAi and (b, c, e, h, i) PE5;zein RNAi lines. γRNAi and βRNAi are in a hybrid Hi II A x B and A654 backgrounds, αRNAi is in a Hi II A x B background, and PE5 was backcrossed twice to B101 prior to being crossed with the RNAi lines. PE5;α- is in a Hi II A x B and B101 backgrounds while remaining PE5;zein RNAi lines are in a hybrid background of Hi II A x B, B101, and A654 (see Materials and Methods). Restoration of the semi-vitreous or vitreous phenotypes by the high-Met maternal background of PE5 is observed in kernels with reduction of γ-zeins and γ-/β-zeins, respectively.

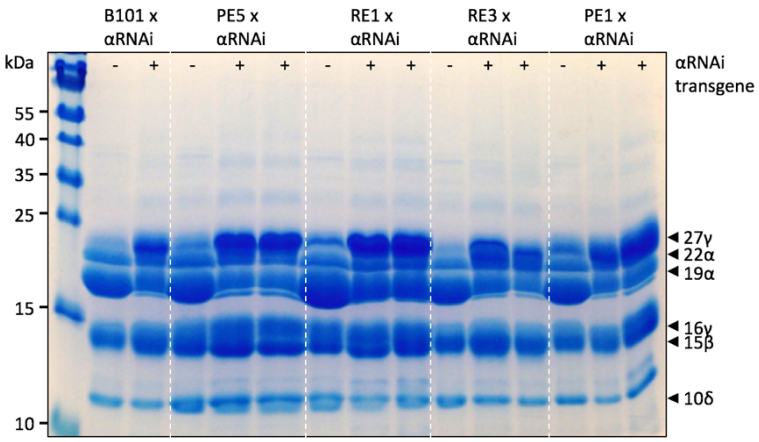

Figure 2.4. Zein profiles of segregating populations of kernels from crosses of α-zein RNAi with different *Ec*PAPR transgenic events.

Kernels segregating (+) and non-segregating (-) for the αRNAi transgene were pooled and separated in a 15% SDS-PAGE gel. The presence of the αRNAi transgene in the kernel increased accumulation of the 27-kDa γ-zein.

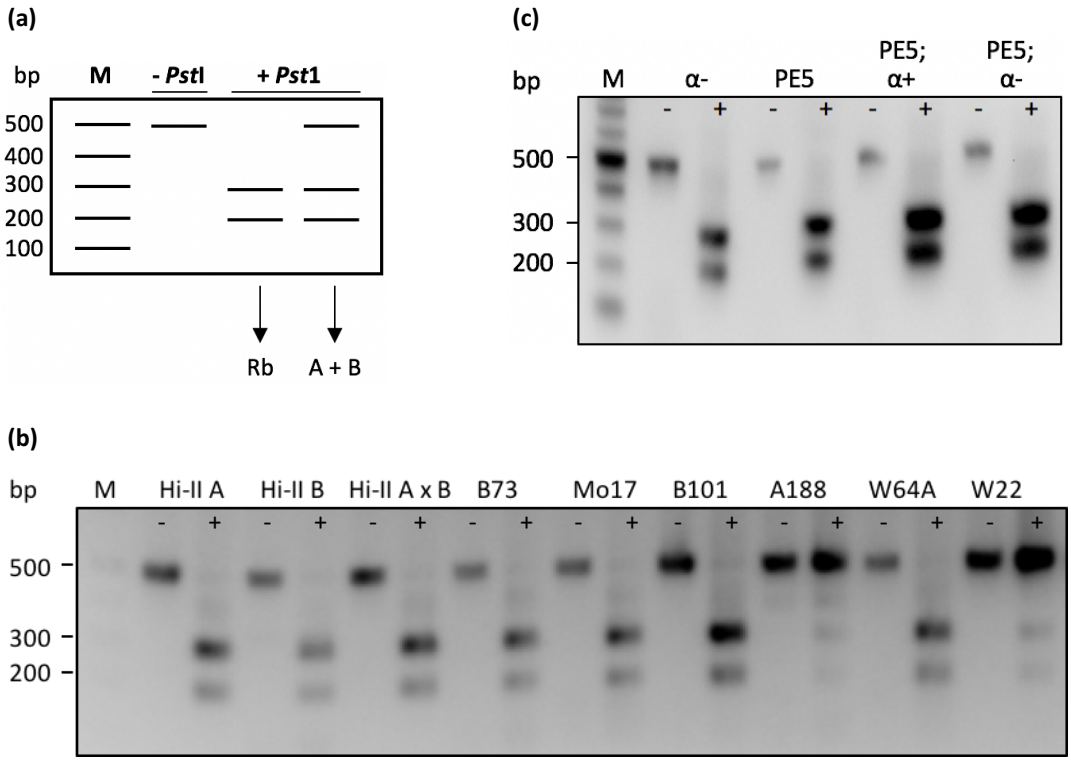

Figure 2.5. Cleaved amplified polymorphic sequence (CAPS) assay to determine allelic variation of the 27-kDa γ-zein gene in different maize genotypes.

(a) Diagram showing amplified DNA fragments of the 27-kDa γ-zein gene displaying restriction fragment length polymorphisms when digested with PstI, depending on the allelic variation of the gene (see Materials and Methods). The single-copy Rb allele resulted from recombination of the tandemly-duplicated A and B alleles of the 27-kDa γ-zein gene (Das et al., 1991). (b) Different maize inbred lines and the hybrid Hi-II A x B were tested with the CAPS assay to determine the variation in their 27-kDa γ-zein gene. The inbred lines A188 and W22 have tandemly-duplicated copy while the remaining maize genotypes tested have the single-copy allele of the gene. (c) Maize genotypes corresponding to the parental PE5 and α-zein RNAi lines and the resulting progeny kernels from the cross have the same allele of the 27-kDa γ-zein gene. PE5;α+ and PE5;α- refer to kernels resulting from the cross of the maternal PE5 with the α-zein RNAi and that are non-segregating and segregating for the α-zein RNAi transgene, respectively.

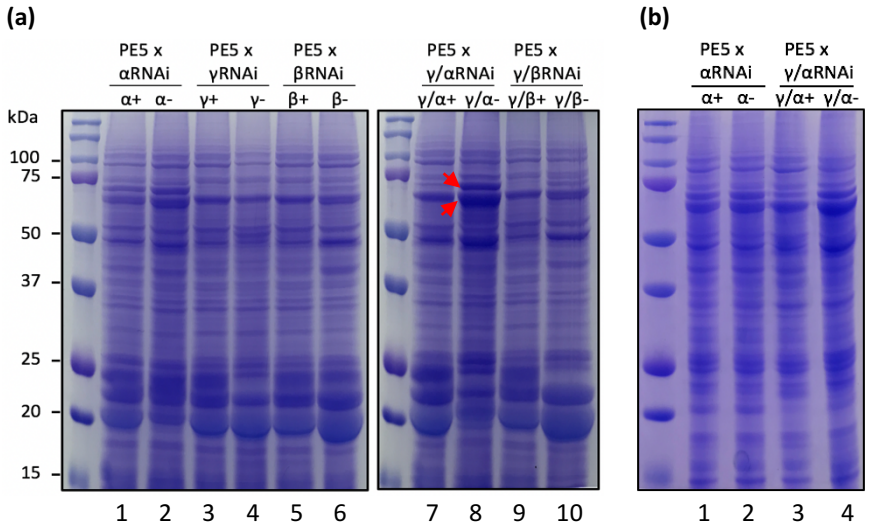

Figure 2.6. Total seed proteins (a) and non-zein proteins (b) from transgenic progeny kernels from crosses of PE5 with RNAi lines targeting the α-, β-, γ-, β-/γ-, or α-/γ-zeins separated in a 12% SDS-polyacrylamide gel.

Kernels non-segregating (odd-numbered lanes) and segregating (even-numbered lanes) for the RNAi transgenes were pooled and used for analysis. Red arrows indicate proteins in the ~ 65-70 kDa range that have increased accumulation in kernels segregating for the α- and α-/γ-zein RNAi transgenes. The identities of these protein bands were determined by MS analysis.

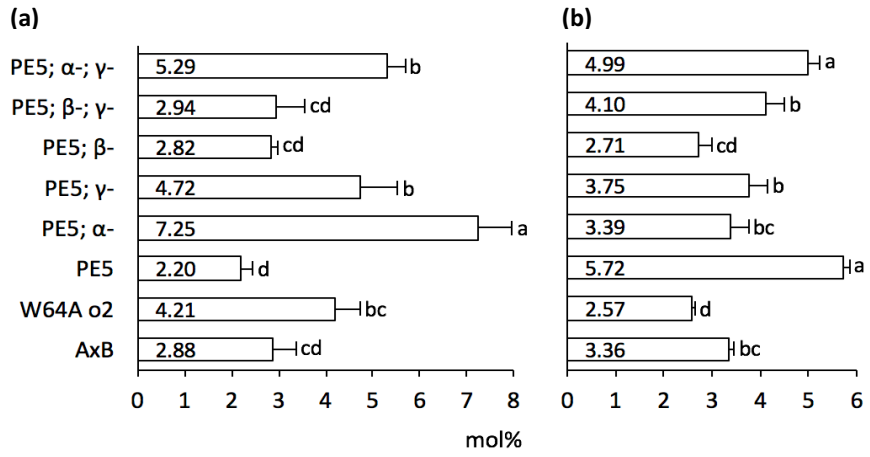

Figure 2.7. Total seed Lys (a) and Met (b) content.

Values are means from three independent measurements of pooled samples and error bars indicate standard deviation. Statistical analysis was performed by using the one-way analysis of variance with post-hoc Tukey HSD test; significant differences between samples are indicated by different letters.

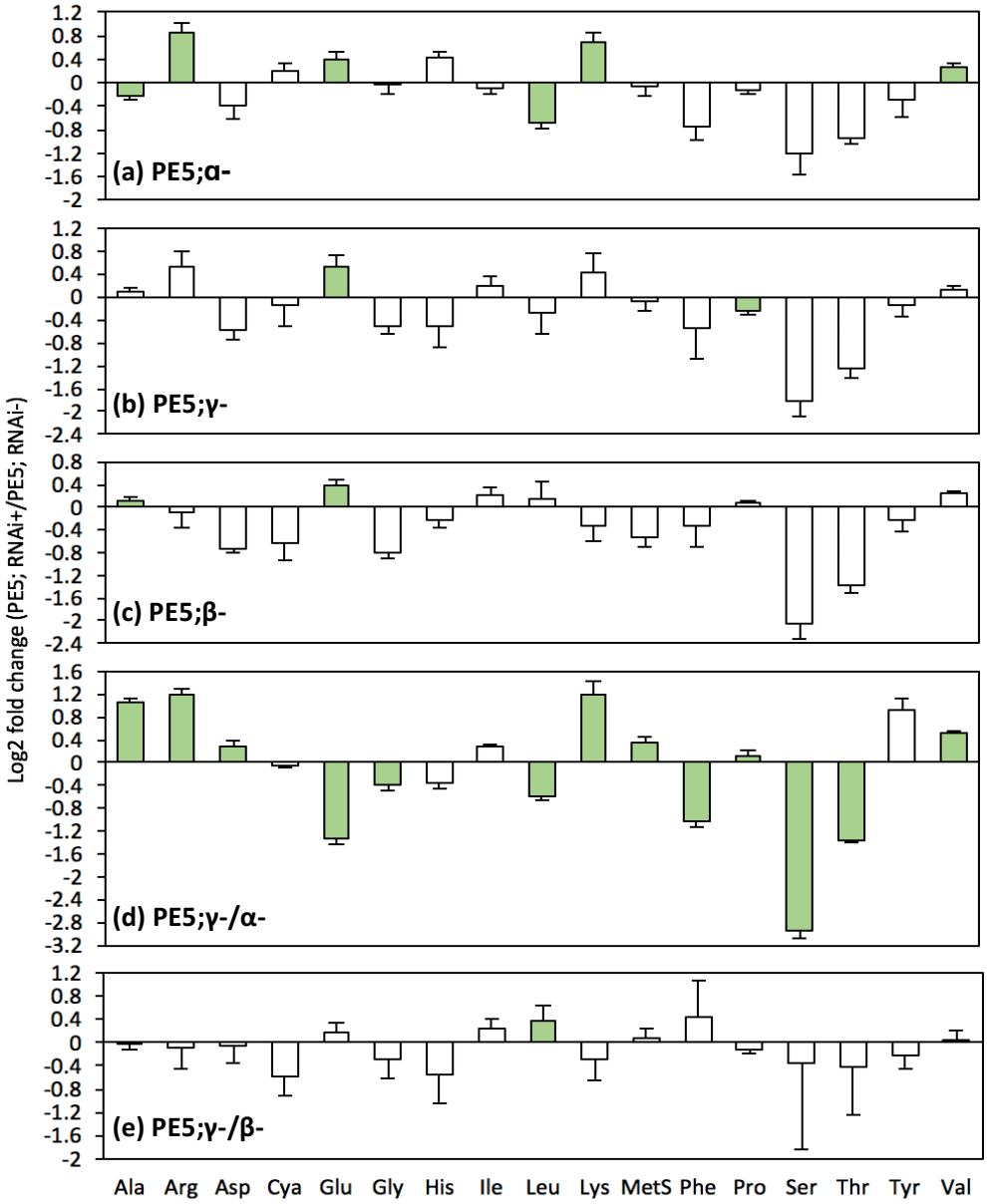

Figure 2.8. Amino acid composition analysis of segregating kernels from a cross of the maternal PE5 with the zein RNAi lines. Kernels from an ear resulting from the cross and segregating or non-segregating for the RNAi transgene(s) were pooled and used for analysis.

Shown are changes in the amino acid composition of (a) PE5;α-, (b) PE5;γ-, (c) PE5;β-, (d) PE5;γ-/α-, and (e) PE5;γ-/β- relative to the corresponding controls of PE5; RNAi- kernels. Cya and MetS refer to cysteic acid and Met sulfone, respectively, the acid stable forms of cysteine and Met produced after performic acid treatment of the sample. Values are means from three independent measurements of pooled samples and error bars indicate standard deviation. Boxes in green denote that values for the PE5; RNAi+ kernels are statistically different from the corresponding control at $p < 0.01$.

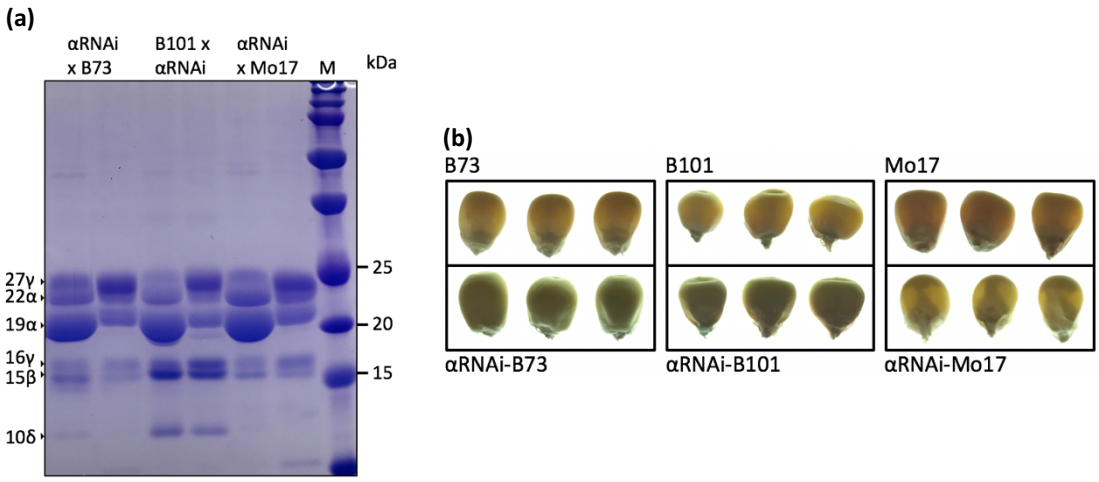

Figure 2.9. Protein accumulation profiles and phenotypes of hybrid kernels.

(a) Zein profiles of segregating populations of kernels from crosses of the α-zein RNAi line with the inbred lines B73, B101, and Mo17. The presence of the RNAi transgene in the kernel increased accumulation of the 27-kDa γ-zein. (b) Endosperm phenotypes of kernels segregating for the α-zein RNAi transgene (lower panels), as viewed over a light box, resulting from a cross of the α-zein RNAi with the inbreds B73, B101, and Mo17. Kernels that do not segregate for the RNAi transgene from these crosses are on the upper panels. Restoration of the vitreous phenotype is observed in the hybrid αRNAi-Mo17 kernels.

2.7. TABLES

Table 2.1. Number of amino acid residues in mature zeins, the seed storage proteins of maize.

Included are zeins that are soluble in a 70% ethanol/2% beta-mercaptoethanol solution and easily discernible in a 15% SDS-PAGE gel.

Amino acid	Number of residues					
	22-kDa α-zein	19-kDa α-zein	16-kDa γ-zein	27-kDa γ-zein	15-kDa β-zein	10-kDa δ-zein
Alanine	30 – 36	27 – 35	13	10	24	7
Arginine	2 – 3	1 – 5	3	5	6	0
Asparagine	12 – 13	10 – 13	1	0	0	3
Aspartic acid	0 – 1	0 – 1	0	0	5	1
Cysteine	1	1 – 2	12	15	7	5
Glutamic acid	1 – 2	1 – 2	3	2	3	0
Glutamine	43 – 53	39 – 47	31	30	21	15
Glycine	3 – 4	2 – 6	15	13	16	4
Histidine	2 – 7	1 – 4	4	16	8	3
Isoleucine	8 – 12	9 – 12	1	4	6	3
Leucine	40 – 44	38 – 52	14	19	17	15
Lys	0 – 1	0 – 1	0	0	0	0
Met	3 – 6	0 – 2	3	1	12	29
Phenylalanine	8 – 9	12 – 14	7	2	1	5
Proline	19 – 22	18 – 24	25	51	15	20
Serine	14 – 17	9 – 17	9	8	12	8
Threonine	7 – 9	5 – 10	6	9	3	5
Tryptophan	0 – 1	0	1	0	3	0
Tyrosine	7 – 8	8 – 10	8	4	7	1
Valine	13 – 17	5 – 13	8	15	5	5
Total mature residues[1]	233 - 246	212 - 246	164	204	171	129
Gene model / NCBI Accession[2]	GRMZM2G397687 GRMZM2G160739 GRMZM2G044625 GRMZM2G346897 GRMZM2G044152 GRMZM2G088365 GRMZM2G088441	GRMZM2G059620 GRMZM2G008913 GRMZM2G008341 GRMZM2G353272 GRMZM2G053120 GRMZM2G353268 GRMZM2G404459 AF546188.1_FG005 AF546188.1_FG007 AF546187.1_FG007 AF546187.1_FG001	GRMZM2G060429	GRMZM2G138727	GRMZM2G086294	AF371266
Gene name / Subfamily	z1C1_1; z1C1_5; z1C1_8; z1C1_10; z1C1_12; z1C1_20; z1C2	z1A1_2; z1A1_4; z1A1_5; z1A1_6; z1A1_7; z1A2_1; z1A2_2; z1B4; z1B6; z1D2; z1D4	16-kDa γ-zein	27-kDa γ-zein	15-kDa β-zein	10-kDa δ-zein
% transcript abundance in the endosperm[3]	6.0	24.0	2.9	5.4	4.7	0.5
% total zeins[4]	20	40	<5	20	10	<5

[1]Mature peptides were predicted from the full-length primary translation products by the signal peptide cleavage site prediction software
SignalP (Petersen et al., 2011)
[2]Full-length transcripts (containing both start and stop codons and with no premature stop codon) that were detected in the developing endosperm of B73 were culled from the transcriptome data of Woo et al. (2001) and Chen et al. (2014).
[3](Woo et al., 2001)
[4]Percent abundance relative to total zeins (Thompson and Larkins, 1994).

Table 2.2. Percent changes in the Lys and Met contents of the hybrid PE5;RNAi+ kernels relative to the PE5;RNAi- and non-transgenic A x B kernels.

Data shown are means (SD) of three pooled replicates.

Amino acid	% variation from									
	PE5;RNAi- kernels (SD)					Non-transgenic A x B kernels (SD)				
	PE5;α-	PE5; γ-	PE5;β-	PE5; γ/α-	PE5; γ/β-	PE5;α-	PE5;γ-	PE5;β-	PE5; γ/α-	PE5; γ/β-
Lys	60 (0.043)	32.83 (0.129)	-20.54 (0.030)	128.16 (0.163)	-17.26 (0.061)	151.85 (0.236)	63.89 (0.151)	-1.97 (0.030)	83.80 (0.112)	2.08 (0.076)
Met	-3.97 (0.018)	-4.12 (0.017)	-30.64 (0.010)	26.38 (0.009)	4.64 (0.020)	0.79 (0.013)	11.61 (0.014)	-19.35 (0.008)	48.61 (0.006)	22.12 (0.015)

Table 2.3. Amino acid composition of transgenic maize kernels from a cross of the maternal PE5 plant with the zein RNAi lines (α-, γ-, β-, γ-/α-, or γ-/β-zein RNAi).

Ground corn meal of bulked samples were from kernels segregating for the zein RNAi. Data shown are means (SD) of three pooled replicates. Values in bold for the RNAi kernels are statistically different from their corresponding controls ($p < 0.01$) of kernels that do not segregate for the RNAi transgene(s). The high-Lys opaque-2 mutant in W64A background, W64A *o2*, is added for comparison.

Amino Acid[a]	mol % (SD)					
	W64A*o2*	PE5;α-	PE5;γ-	PE5;β-	PE5;γ-/α-	PE5;γ/β-
Ala	10.06 (0.110)	**24.38 (1.000)**	29.24 (0.855)	**29.54 (1.915)**	**23.99 (0.398)**	26.83 (1.692)
Arg	3.70 (0.133)	**6.91 (0.745**)	5.01 (0.395)	3.22 (0.090)	**5.05 (0.351)**	3.31 (0.751)
Asp	9.83 (0.613)	3.28 (0.439)	2.88 (0.332)	2.58 (0.098)	**7.63 (0.110)**	4.22 (0.949)
Cya	3.21 (0.218)	5.36 (0.438)	3.57 (0.796)	2.49 (0.219)	3.58 (0.131)	2.51 (0.330)
Glu	15.09 (0.266)	**9.79 (0.870)**	**10.20 (1.339**)	**9.16 (0.160)**	**7.40 (0.625)**	7.89 (0.792)
Gly	9.28 (0.140)	3.30 (0.384)	2.38 (0.104)	1.98 (0.076)	**5.22 (0.408)**	2.87 (0.704)
His	2.93 (0.172)	2.59 (0.163)	1.35 (0.329)	1.64 (0.042)	2.24 (0.178)	1.35 (0.468)
Ile	3.07 (0.032)	3.32 (0.240)	4.36 (0.523)	4.48 (0.454)	3.68 (0.067)	4.57 (0.578)
Leu	7.94 (0.410)	**5.03 (0.331)**	8.30 (1.855)	11.00 (1.695)	**7.09 (0.269)**	**12.86 (1.591)**
Lys	4.21 (0.519)	**7.25 (0.711)**	4.72 (0.799)	2.82 (0.165)	**5.29 (0.389)**	2.94 (0.624)
MetS	2.57 (0.087)	3.39 (0.371)	3.72 (0.371)	2.69 (0.301)	**4.95 (0.275)**	4.06 (0.389)
Phe	2.54 (0.111)	0.71 (0.118)	1.05 (0.361)	1.18 (0.272)	**1.42 (0.060)**	2.11 (0.846)
Pro	9.48 (0.514)	14.36 (0.944)	**13.95 (0.767)**	17.31 (0.551)	**12.31 (0.374)**	14.97 (0.442)
Ser	5.38 (0.085)	0.21 (0.049)	0.13 (0.007)	0.11 (0.006)	**0.69 (0.070)**	0.69 (0.070)
Thr	3.96 (0.050)	0.50 (0.017)	0.44 (0.038)	0.40 (0.015)	**1.32 (0.030)**	0.88 (0.439)
Tyr	0.23 (0.025)	0.42 (0.085)	0.35 (0.015)	0.34 (0.035)	0.30 (0.036)	0.34 (0.032)
Val	6.46 (0.000)	**9.27 (0.555)**	8.38 (0.150)	9.03 (0.139)	**7.79 (0.119)**	7.71 (1.343)

[a]Acid hydrolysis of the sample yields the acid stable forms Cya (cysteic acid) and MetS (Met sulfone) from cysteine and Met, respectively.

Table 2.4. Reports on approaches to increasing kernel Lys content in maize by transgenic α-zein reduction.

Transgenic approach	% increase in Lys	References
22-kDa α-zein RNAi	18.5	(Segal et al., 2003)
19-kDa α-zein antisense silencing	43.9	(Huang et al., 2004)
Combination of 19-kDa α-zein RNAi and 22-kDa α-zein antisense silencing	105.2	(Huang et al., 2006)
19-kDa and 22-kDa α-zein RNAi	26.7	(Wu and Messing, 2011)

[a]Expression of a deregulated Lys biosynthetic enzyme.

Table 2.5. Percent changes in the amino acid contents of the hybrid PE5;RNAi+ kernels relative to the PE5;RNAi-segregating kernels.

Data shown are means (SD) of three pooled replicates.

Amino Acid[a]	% variation (SD) from PE5; RNAi- segregating kernels				
	PE5;α-	PE5;γ-	PE5;β-	PE5;γ-/α-	PE5;γ-/β-
Ala	-14.81 (0.001)	6.78 (0.002)	7.86 (0.006)	110.10 (0.005)	-2.02 (0.004)
Arg	82.72 (0.051)	44.06 (0.083)	-7.47 (0.030)	126.61 (0.038)	-4.79 (0.077)
Asp	-24.25 (0.016)	-32.87 (0.007)	-39.86 (0.001)	22.92 (0.008)	-1.71 (0.051)
Cya	16.09 (0.011)	-8.39 (0.077)	-36.13 (0.020)	-3.33 (0.002)	-35.62 (0.024)
Glu	32.06 (0.019)	45.92 (0.051)	31.09 (0.012)	-59.72 (0.001)	12.92 (0.021)
Gly	-1.49 (0.019)	-30.41 (0.005)	-42.01 (0.003)	-23.81 (0.004)	-16.08 (0.048)
His	35.30 (0.013)	-29.37 (0.037)	-13.81 (0.011)	-21.61 (0.006)	-29.2 (0.067)
Ile	-6.12 (0.005)	14.24 (0.020)	17.29 (0.015)	22.15 (0.001)	19.65 (0.024)
Leu	-37.57 (0.002)	-16.28 (0.055)	11.03 (0.065)	-33.72 (0.001)	29.8 (0.074)
Lys	60 (0.043)	32.83 (0.129)	-20.54 (0.030)	128.16 (0.163)	-17.26 (0.061)
Phe	-40.45 (0.010)	-28.47 (0.080)	-19.13 (0.058)	-51.15 (0.001)	44.19 (0.411)
MetS	-3.97 (0.018)	-4.12 (0.017)	-30.64 (0.010)	26.38 (0.009)	4.64 (0.020)
Pro	-7.33 (0.004)	-15.20 (0.003)	5.25 (0.002)	9.39 (0.005)	-9.00 (0.002)
Ser	-56.69 (0.016)	-72.22 (0.003)	-76.3 (0.002)	-87.03 (0.000)	53.33 (0.109)
Thr	-47.74 (0.002)	-58.28 (0.003)	-61.46 (0.002)	-61.06 (0.000)	-16.24 (0.184)
Tyr	-17.65 (0.037)	-10.92 (0.018)	-14.29 (0.023)	91.49 (0.088)	-15.13 (0.021)
Val	20.7 (0.006)	9.79 (0.002)	18.35 (0.002)	43.88 (0.001)	1.05 (0.032)

2.8. REFERENCES

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