

Engineering sulfur storage in maize seed proteins without apparent yield loss

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Contributed by Joachim Messing, September 1, 2017 (sent for review August 22, 2017; reviewed by L. Curtis Hannah and Ronald L. Phillips)

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 (EcPAPR) 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 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 with nontransgenic kernels. Therefore, increased source strength can improve the nutritional value of maize without apparent yield loss and may significantly reduce the cost of feed supplementation.

APS reductase | sulfur assimilation | sulfur-rich zeins | 10-kDa δ -zein

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 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, such as the racemic Met used in feed formulation to replace protein-bound amino acids, provides suboptimal protein use and, in some cases, reduces growth rate (1). 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 essential amino acids such as lysine, threonine, tryptophan, methionine, and tyrosine (2, 3). 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 (4) and the complex regulation of kernel Met accumulation in different maize inbred lines (5–8) complicate traditional breeding approaches for high-Met maize. As a consequence, 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 (9–13) or targeted reduction of S-poor SSPs by gene silencing (14). 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 (9–11). Knockdown of expression

of S-poor zeins by RNA interference increased lysine but did not increase Met (15).

Another transgenic approach might be to increase the supply of sulfur amino acids (SAAs) 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. S1A). 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 because of the accumulation of toxic intermediates (16). If this problem could be solved, then S assimilation could potentially increase the source of Met for accumulation in the seed (17).

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 (18, 19). To circumvent the many control points, bacterial APR homologs may be better targets for transgenic plant studies. Ec-topically expressed *Escherichia coli* and *Pseudomonas aeruginosa* enzymes EcPAPR and PaAPR, respectively, have both been shown to function in plants (16, 20). Although having differing substrate specificities (APS for PaAPR and the phosphorylated APS derivative 3'-phosphoadenosine-5'-phosphosulfate, PAPS, for EcPAPR; Fig. S1A), both enzymes are able to drive sulfate reduction (16). Because sulfate assimilation in maize is known to be compartmentalized in specific cell types (21), we achieved expression of the bacterial genes with two tissue-specific promoters: the mesophyll-specific *PepC* and the bundle sheath cell-specific *RbcS* promoters (22). 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.

Significance

Poultry feed is usually prepared as a corn-soybean mixture. Because the only essential sulfur amino acid missing in this mixture is methionine, it is chemically synthesized and added separately, increasing the cost of major food supply. It appears to be difficult to circumvent the regulatory aspects of sulfur metabolism, which is controlled at many levels, without damage to plant growth. By using tissue-specific promoters to express a bacterial enzyme that increases the efficiency of assimilative sulfate reduction, seed methionine accumulation can be increased without the concomitant accumulation of toxic metabolites. We show that even in maize inbred lines with repressed seed methionine levels, sink strength can be increased to the benefit of feed consumption efficiency in chicks.

Author contributions: J.P., T.L., and J.M. designed research; J.P. and X.X. performed research; J.P., T.L., and J.M. analyzed data; and J.P., T.L., and J.M. wrote the paper.

Reviewers: L.C.H., University of Florida; and R.L.P., University of Minnesota.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1714805114/-DCSupplemental.

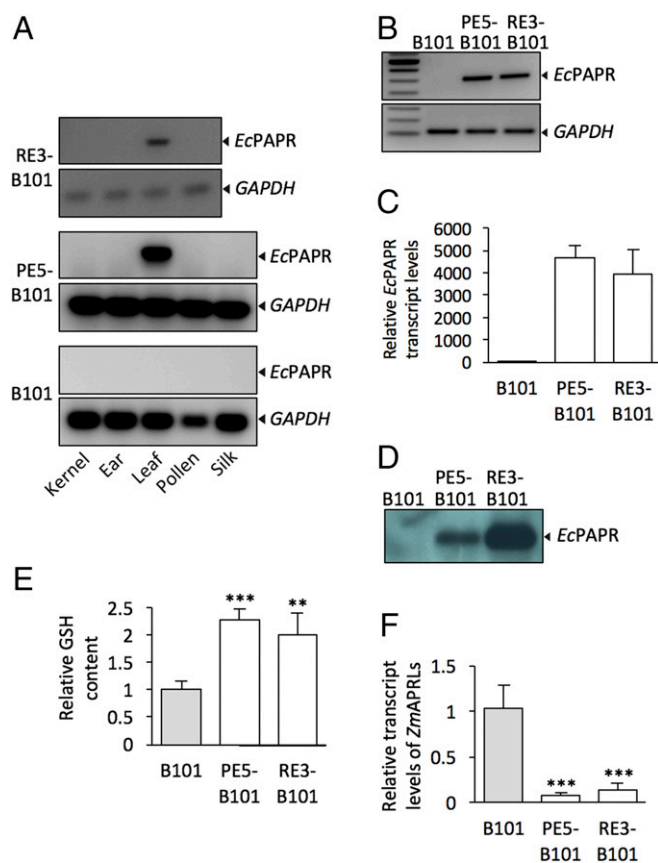


Fig. 1. Maize transformed with the bacterial assimilatory reductase *EcPAPR*. (A) Tissue-specific expression of *EcPAPR* under *PepC* and *RbcS* promoter control in transgenic maize. RT-PCR analysis was performed to detect *EcPAPR* expression in different maize tissues. First-strand cDNA from RE3-B101 was amplified for 30 cycles, whereas that from B101 and PE5-B101 were amplified for 40 cycles. (B) RT-PCR and (C) qRT-PCR analysis of *EcPAPR* transcript levels in mature leaves of PE5-B101 and RE3-B101. Data shown are means \pm SD of four determinations each from two biological replicates. (D) Western blot analysis of protein extracts from leaves, using an antibody against *EcPAPR*. (E) Relative glutathione (GSH) content and (F) transcript levels of ZmAPRLs in transgenic mature leaves. Primers targeting two putative APR-like genes in maize, *ZmAPRL1* and *ZmAPRL2* (GenBank accession nos. AY739296 and AY739297), were used for qRT-PCR analysis. Glyceraldehyde-3-phosphate dehydrogenase, GAPDH, was used as the reference gene. Data shown are means \pm 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.

lower Cys content than nontransgenic controls. In addition, total seed S content did not change, presumably because of reallocation of S reserves from endogenous proteins to the transgenic products (9–13) (Table S3).

Concomitant with increased Met and Cys, total aspartic acid, lysine, threonine, and serine decreased in the transgenic seeds (Fig. 2*J*). Serine was reduced the most, amounting to only 14.1% of the B101 control in PE5-B101 seeds (Fig. 2*J*). 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. S14). 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 (25, 26). The increase in proline content may be attributed to

increased expression of the 10-kDa δ -zein, of which 15.5% of its residues are proline (27).

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 4-wk feeding trial of chicks with a corn–soybean meal formulation that is deficient in Met (28) (Table S4). Three diet rations, consisting of different corn meals, were tested with 5-d-old chicks: a complete diet consisting of a yellow dent corn supplemented with synthetic Met, corn meal from PE5-B101 without Met supplementation, and 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. 3).

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 (16). In addition, different maize inbred lines exhibit variability in the amount of Met stored in the seed (4), and this variability appears to be mainly a result of the differential expression of the Met-rich 10-kDa δ -zein gene, *Dzs10*, with 22.5% Met codons (27), the same gene whose expression is up-regulated 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 (9–13), or by reducing expression of an S-poor SSP through antisense RNA expression (14). The overall increase we observed in the accumulation of the S-rich zeins indicates 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 already has the highest kernel Met content among common maize inbreds (4). 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 (29). This regulation was eliminated with a chimeric storage protein gene that contained only the coding region of the *Dzs10* gene (9). 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 Mo17 backgrounds, overexpression of *EcPAPR* can induce an increase in the accumulation of the S-rich zeins, indicating 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 with 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

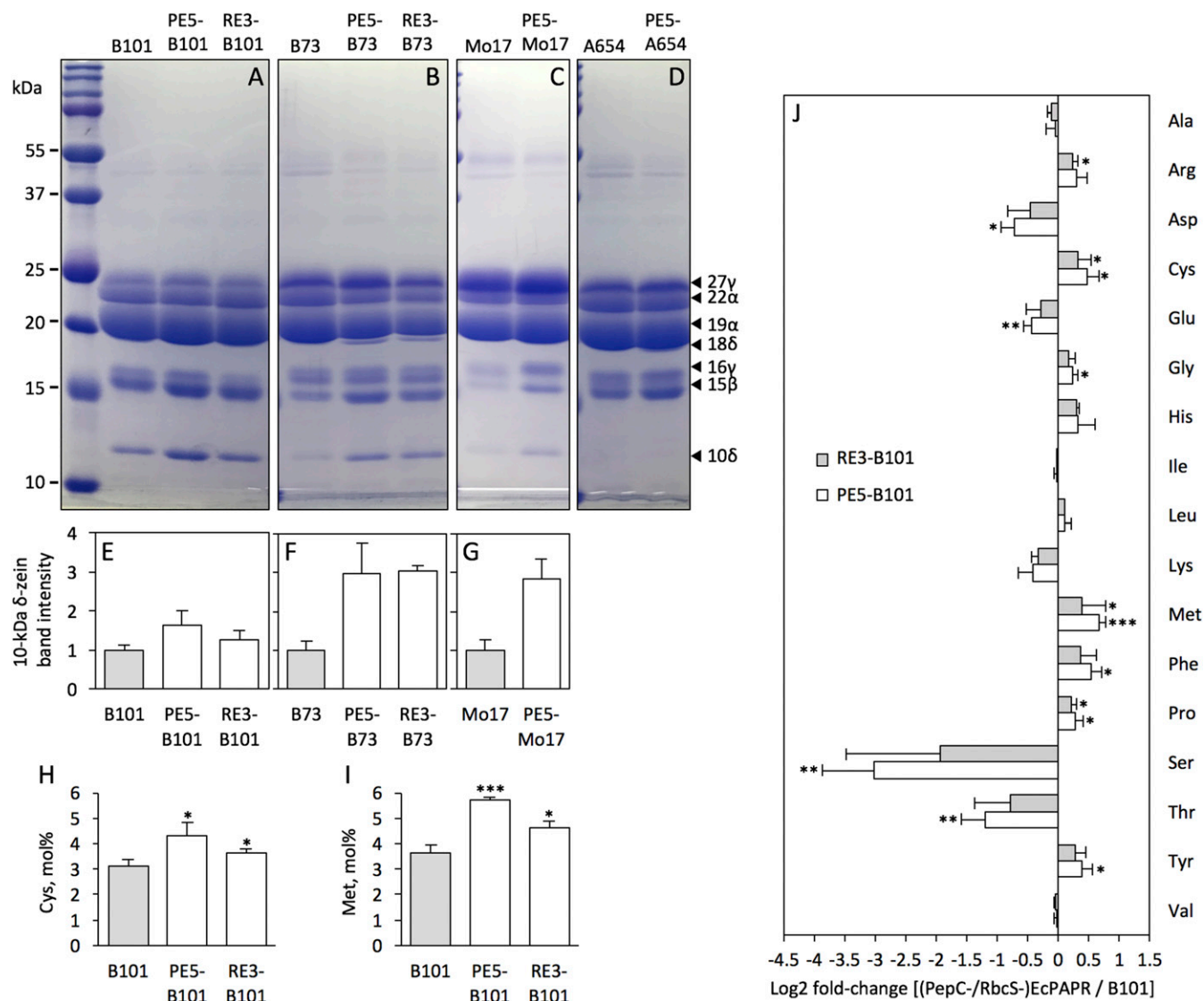
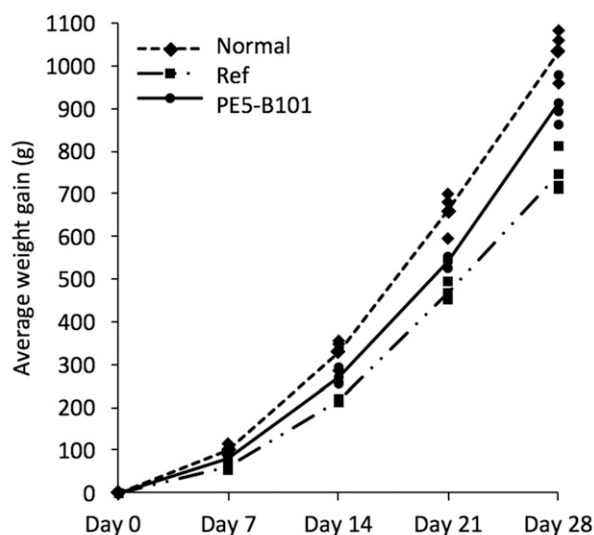


Fig. 2. Accumulation patterns of seed storage proteins in transgenic *EcPAPR* maize introgressed into different genetic backgrounds. (A–D) SDS/PAGE zelin profiles of the transgenic events PE5 and RE3 in different genetic backgrounds and (E–G) relative band intensity of the 10-kDa δ -zein in these events. 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 six to eight kernels (*SI Materials and Methods*), using 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 and 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 with 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 *EcPAPR* 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 nontransgenic B101 kernels. Data shown are means \pm SD of three replicates.

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 (8). 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 (21). For this work, we focused on obtaining transgenic plants with increased S assimilation that did not show negative effects on plant growth, to assess the effect of increased S assimilation on SSP. Therefore, we did not perform a rigorous comparison of *EcPAPR* and *PaAPR* under either the *PepC* or *RbcS* promoters. However, of all the transgenic events we have generated, *EcPAPR* plants appeared to accumulate more S-rich zeins than *PaAPR* plants. *EcPAPR* plants also showed the hallmarks of deregulated S assimilation, including the accumulation of glutathione and down-regulation of expression of endogenous APR. This result was unexpected, as maize has an APS reductase-type (*PaAPR*) sulfate assimilatory pathway, not the PAPS reductase type (*EcPAPR*) (30–32). Although maize, similar to other



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)

Fig. 3. Feeding trial with the transgenic high-Met PE5 maize. A 4-wk feeding trial with 5-d-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 chick at the conclusion of the feeding trial. Weight gain is calculated as the difference between the finishing and starting weights, and 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 \pm SD of three replicates with five animals per replicate.

flowering plants, can produce PAPS, used as a sulfate donor in sulfation reactions of some secondary metabolites (33, 34), it was until now unclear whether PAPS could be directed toward sulfate assimilation. Our results show that endogenous PAPS in higher plants such as 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 (16, 20), yet in the present study, *EcPAPR* expression was not associated with any apparent growth abnormalities. Thus, our results point to another aspect of metabolic engineering for enhanced crop value: using redundancies and alternative circuits for endogenous biosynthetic pathways to improve the nutritional value of crops. That Met accumulates in transgenic *EcPAPR* plants suggests Met synthesis in maize is not strictly controlled by the enzyme cystathionine γ -synthase responsible for synthesis of Met from Cys. Cystathionine γ -synthase is also not a limitation for Met synthesis in potato (35), whereas it is a bottleneck enzyme in *Arabidopsis* (36–38).

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 (39) 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 is the major form in which reduced S moves in the phloem (40). However, insertional mutants of *Arabidopsis* and maize in Met S-methyltransferase, the enzyme that catalyzes synthesis of S-methylmethionine, produced plants that grew and reproduced normally, and the mutant seeds from *Arabidopsis* had normal S contents. These results rule out an indispensable role for S-methylmethionine in S transport in *Arabidopsis* and maize, and the S transport form is probably fulfilled by other reduced S form in these species (41). 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 δ -zein decreases expression of the β - and γ -zeins, leading to rebalancing of protein S in the seeds (9). There seems to be two major limiting factors in the accumulation of Met and Cys in maize seeds: demand for S imposed by the S-rich zeins, and SAA availability or supply from the source tissues, which determines the uptake of SAA into the seeds (13). 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 (42).

Materials and Methods

Maize genetic stocks and methods to characterize the transgenic plants and kernels are described in *SI Materials and Methods*. Primers used for vector construction, genotyping, and qRT-PCR analysis are listed in *Table S5*.

ACKNOWLEDGMENTS. We thank Dr. Yongrui Wu of the Chinese Academy of Sciences for advice and comments, Marc Probasco of the Waksman Institute for field and greenhouse management and plant care, and Dr. Renate Scheibele for providing antibodies against *EcPAPR*. This research was supported by the Selman A. Waksman Chair in Molecular Genetics of Rutgers University (J.M.) and the U.S. Department of Agriculture National Institute of Food and Agriculture Hatch Project 202535 through the New Jersey Agricultural Experiment Station Hatch Project NJ12136 and the Professor Charles Gilvarg Memorial Fund for Plant Metabolic Engineering (T.L.).

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