Engineering sulfur storage in maize seed proteins without apparent yield loss

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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 (EcAPR) 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 lines PES and PES2 that express different levels of EcAPR exhibit a high-Met seed phenotype. In feeding trials with chicks, PES maize promotes significant weight gain compared with nontransgenic maize, with higher methionine and cysteine in serum and feces. Nutritional value of high-Met maize is comparable to synthetic Met. Inclusion of unnatural amino acids, such as proline and glutamine, provides suboptimal protein use and, in some cases, reduces growth rate. Maize with elevated Met content could obviate the need for supplementation of animal feed with synthetic Met.

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 amino acids such as cysteine and methionine. This sulfur amino acid (SAAs) deficiency is a significant nutritional problem, as the only essential sulfur amino acid missing in this mixture is methionine, which 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 sulfite 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.

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 sulfite 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.

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The transgenic maize kernels used in feed formulation enhanced the growth of chicks. These results represent a breakthrough in the nutritional quality of maize.

Results

Tissue-Specific Expression of EcPAPR. Transgenic plants that harbor one of four different chimeric constructs were obtained via Agrobacterium infection of immature maize embryos. The constructs included PaPAPR or EcPAPR, each under transcriptional control of the leaf- and cell-specific RbcS or PeP promoter (Fig. S1B). Transgenic events generated from each of the constructs showed no phenotypic abnormalities, and some exhibited high accumulation of S-rich storage proteins, SSPs (Fig. S1 C–F). Because the objective of the research was to examine the effect of increased S assimilation on SSP expression, further analysis focused on only two EcPAPR transgenic lines, PES and RE3, showing the highest accumulation of the Met-rich 10-kDa δ-zein.

Both events, having single copies of the transgene (Table S1), were backcrossed to four different inbred lines (A654, B101, B73, or Mo17) for two to five generations. Subsequent analysis of SSP accumulation after every generation of backcrossing showed that transgenic events PES and RE3 introgressed into the inbred B101 (PE5-B101 and RE3-B101) had consistently high accumulation of the 10-kDa δ-zein, and PES had high accumulation of the 10-kDa δ-zein in different genetic backgrounds compared with the other transgenic events (Fig. S1 C–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. S1G). Therefore, EcPAPR expression in these lines did not negatively affect 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 (Table 1). The seeds also showed elevated fat and reduced fiber contents (Table S2). Transgenic plants displayed no apparent yield loss, as kernels had increased weight and kernel number per ear was not significantly different from that of the nontransgenic control (Table 1 and Fig. S1H). Actual yield would have to be determined with genotype x environment (GXE) performance by introgressing the transgene into elite lines and growth in different geographic field locations.

EcPAPR 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. 1A). EcPAPR protein localization showed that the PeP promoter directed specific expression in mesophyll cells, but the RbcS promoter resulted in EcPAPR expression in bundle sheath cells as well as leafy expression in the mesophylls (Fig. S2). EcPAPR transcripts in PE5-B101 and RE3-B101 accumulated at similar levels in mature leaves (Fig. 1B and C), however, the protein was much more abundant for RE3-B101 (Fig. 1D).

Glutathione (GSH) acts as transport and storage form of reduced S, and its biosynthesis is limited by Cys concentration (23). GSH accumulated by more than twofold in the leaves of both transgenic events (Fig. 1E). Because plant APR transcription is known to be particularly sensitive to down-regulation by an endproduct of S assimilation, expression of endogenous maize APR would be expected to decrease if S assimilation had been derepressed by EcPAPR expression. The maize genome contains two putative APR-like proteins ZmAPlR2 and ZmAPlR3 (GenBank accession nos. AT737296 and AT737296) (24). Both PES-B101 and RE3-B101 show decreased abundance of ZmAPlR1 and ZmAPlR2 transcripts (Fig. 1F). These results indicate that EcPAPR expression has resulted in deregulation of sulfate reduction, but without a negative effect on plant growth and yield (Table 1 and Fig. S1 G and H), likely because of the leaf-specific promoters that were used compared with the constitutive promoter employed in prior studies (16).

Variation in Zein Expression Is a Function of Genetic Background. The EcPAPR transgene is stable and heritable. PCR analysis of segregating plants of PES and RE3 introgressed into different backgrounds (Table S1) indicates the segregation ratios of transgenic versus the null segregants were about 1:1, suggesting the presence of a single copy of the EcPAPR 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 EcPAPR also induced expression of the S-rich δ–, β-, and γ-zeins dependent on the genetic background (Fig. S1 C–F).

Varying expression levels of the Met-rich zeins were observed in events PES and RE3 (Fig. 2A–G). Both PES and RE3 in the B73 and Mo17 backgrounds show global increases in the levels of the S-rich zeins (Fig. 2B and C), whereas in the B101 background, only the 10-kDa δ- and 15-kDa β-zeins were increased (Fig. 24). PE5-B101 has 14.4% more kernel Met (Fig. 2D) than those from an F3 ear of the PE5 event, illustrating that specific maize inbreds can be exploited to enhance Met level. The nonfunctional 10-kDa δ-zein gene in A654 (Fig. 2D) 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 is shown in Fig. 2E–G. Elevated levels of the 15-kDa β- and 16-kDa γ-zeins were differentially regulated (Fig. 24 and Fig. S3) and observed only when the 10-kDa δ-zein was increased (Fig. S1 C–F). In the absence of the 10-kDa δ-zein, β-zein acted as the primary sink of Met among the SSPs (Fig. 2D).

Amino Acid Analysis of Transgenic Maize Kernels. Cys (Fig. 2H) and Met (Fig. 2I) 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 with the B101 control. Cys was increased 39.4% and 17.7% in PE5-B101 and RE3-B101; expression of Cys-containing nonzein proteins were also increased in the PES event (Fig. S4). Total S content of transgenic seeds from PE5-B101 and RE3-B101 was increased by 38.6% and 36.4%, respectively (Table 1). In contrast, transgenic seeds expressing the S-rich sunflower seed albumin either had unchanged or slightly increased levels of Met (Table S3).

Table 1. Kernel composition analysis

<table>
<thead>
<tr>
<th>Mean (SD)</th>
<th>B101</th>
<th>PES-B101</th>
<th>RE3-B101</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, %</td>
<td>10.58 (0.28)</td>
<td>12.54 (0.20)**</td>
<td>12.86 (0.21)**</td>
</tr>
<tr>
<td>Nitrogen, %</td>
<td>1.937 (0.029)</td>
<td>1.947 (0.025)</td>
<td>1.990 (0.030)</td>
</tr>
<tr>
<td>Sulfur, %</td>
<td>0.140 (0.002)</td>
<td>0.194 (0.002)***</td>
<td>0.191 (0.001)***</td>
</tr>
<tr>
<td>100-kernel wt., g</td>
<td>19.65 (1.28)</td>
<td>24.75 (1.26)***</td>
<td>22.02 (1.61)**</td>
</tr>
<tr>
<td>Kernel number per ear</td>
<td>459.83 (50.82)</td>
<td>494.50 (49.01)</td>
<td>483.67 (46.81)</td>
</tr>
</tbody>
</table>

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.
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). Serine was reduced the most, amounting to only 14.1% of the B101 control in PES-B101 seeds (Fig. 2). 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. S1A). 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 PES. Ultimately, the usefulness of increased seed methionine must be judged on whether it improves nutritional value. PES-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 PES-B101 without Met supplementation, and a reference diet composed of corn meal from null segregants derived from PES-B101 without Met supplementation. Chicks receiving the normal diet had the biggest weight gain, although this is not significantly different from those fed with PES-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 PES-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 Met-rich 10-kDa δ-zein gene, 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 \( \beta \)-, 16-kDa \( \gamma \)-, and 27-kDa \( \gamma \)-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 \( \delta \)-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 \( \delta \)-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...
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 plants such as maize can be co-opted, with the use of an ectopic expression construct, genotyping, and qRT-PCR analysis are listed in Table S5.


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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, insertion 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 δ- and γ-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 S 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.

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 ± SD of three replicates with five animals per replicate.

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


