Overexpression of serine acetyltransferase in maize leaves increases seed-specific methionine-rich zeins

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Summary
Maize kernels do not contain enough of the essential sulphur-amino acid methionine (Met) to serve as a complete diet for animals, even though maize has the genetic capacity to store Met in kernels. Prior studies indicated that the availability of the sulphur (S)-amino acids may limit their incorporation into seed storage proteins. Serine acetyltransferase (SAT) is a key control point for S-assimilation leading to Cys and Met biosynthesis, and SAT overexpression is known to enhance S-assimilation without negative impact on plant growth. Therefore, we overexpressed Arabidopsis thaliana AtSAT1 in maize under control of the leaf bundle sheath cell-specific rbcS1 promoter to determine the impact on seed storage protein expression. The transgenic events exhibited up to 12-fold higher SAT activity without negative impact on growth. S-assimilation was increased in the leaves of SAT overexpressing plants, followed by higher levels of storage protein mRNA and storage proteins, particularly the 10-kDa δ-zein, during endosperm development. This zein is known to impact the level of Met stored in kernels. The elite event with the highest expression of AtSAT1 showed 1.40-fold increase in kernel Met. When fed to chickens, transgenic AtSAT1 kernels significantly increased growth rate compared with the parent maize line. The result demonstrates the efficacy of increasing maize nutritional value by SAT overexpression without apparent yield loss. Maternal overexpression of SAT in vegetative tissues was necessary for high-Met zein accumulation. Moreover, SAT overcomes the shortage of S-amino acids that limits the expression and accumulation of high-Met zeins during kernel development.

Introduction
Maize (Zea mays L.) is a primary grain commodity with 1053.8 million metric tons projected worldwide production in 2016/2017 (USAD/FAS report January 2017, Grain: World Markets and Trade). By 2050, maize production is expected to increase by 10%, whereas per capita consumption is expected to increase by 63% (http://maize.org/why-maize/). A key shortcoming of maize as a food source is that it is deficient in the essential amino acids such as lysine (Lys), tryptophan (Trp) and methionine (Met). For this reason, formulation of maize-based livestock feed includes the addition of soya bean, which complements Trp and Lys deficiency. However, soya bean does not supply sufficient Met, which is added to feed as a racemic mixture. The current total worldwide Met market is 685–700 KMT and is expected to increase by 27% in 2018 (http://www.feedinfo.com/files/novus-white-paper.pdf). Therefore, a means to increase the Met content of maize would be a significant benefit for the animal feed market. Nutritionally enhanced maize would also benefit subsistence farmers in developing countries who rely on maize as a staple food.

The amino acid content of maize kernels is mainly determined by the expression of a set of seed storage proteins termed zeins that are produced during grain development and are deposited in endosperm into structures, termed protein bodies. Zeins belong to the superfamily of prolamins and have evolved into four classes based on their chemophysical properties, termed alpha (α), beta (β), gamma (γ) and delta (δ) zeins (Xu and Messing, 2009). The α-zein genes have many copies in the genome that can vary in numbers between different inbred lines, whereas the others range between one and two copies (Dong et al., 2016). The amino acid composition of the specific zeins differs markedly. Most zeins have very few Met and cysteine (Cys) residues. Only five zeins have higher numbers of Cys and Met residues. The 50-kDa, 27-kDa and 16-kDa γ-zeins contain 6.48%, 7.84% and 9.20% Met + Cys residues, respectively, the 15-kDa β-zein contains 15.63% Met+Cys, and the 18-kDa and 10-kDa δ-zeins contain 26.84% and 26.3% Met + Cys (Wu et al., 2012). Even though these zeins determine the Met + Cys content of maize kernels, they normally make up only a small proportion of the total complement of seed storage proteins. The level of Met in kernels varies significantly among different inbred lines ranging from sufficient to insufficient levels in animal feed. This genetic variation is controlled by quantitative trait loci (QTLs). However, none of the QTLs have yet been cloned (Deng et al., 2017).

Maize zeins are also essentially devoid of two other essential amino acids, Lys and Trp. The opaque2 and flouny2 mutant seeds have a higher content of these amino acids resulting from elevated expression of nonzein proteins, combined with reduced expression of the most abundantly expressed α-zeins (Mertz et al., 1964). Reduced α-zeins render kernels soft, making them unfit for commercial production. Hard endosperm of opaque2 mutant lines has been restored by breeders, which is referred to
as quality protein maize (QPM) that has higher Lys and Trp (Vasal et al., 1980). But QPM maize is still Met-deficient (Cromwell et al., 1967).

One potential method of manipulating the S-amino acid composition of maize kernels is to engineer overexpression of Met-rich seed storage proteins. Overexpression of the 10-kDa a-zein significantly increased the Met content, but at the expense of b- and g-zein expression and reduced Cys content (Lai and Messing, 2002; Wu et al., 2012), indicating that the availability of S-amino acids limits the total accumulation of the S-amino acids in zeins. A similar phenomenon of S-amino acid reallocation resulting from a limitation of S-amino acids was described by others who have attempted to overexpress S-rich storage proteins in crop plants (Chiaise et al., 2004; Hagan et al., 2003).

Another potential method of increasing S-amino acid content of maize kernels is to reduce the expression of specific seed storage proteins. When b- and g-zein expression is silenced through RNAi, the 10-kDa a-zein accumulates to a higher level, resulting in a significant increase in the total seed Met content (Wu and Messing, 2010; Wu et al., 2012). These results illustrate that it is possible to shift the amino acid content of kernels by altering expression of individual zeins that change the allocation of S-amino acids. Nevertheless, S-amino acid supply remains a limitation to production of zeins with high Met + Cys content.

Cys and Met are the end products of the sulphur (S) assimilation pathway 0 (Takahashi et al., 2011). In brief, S-assimilation occurs when sulphide reacts with O-acetyl-L-serine (OAS) to form Cys, catalysed by OAS-thiol-lyase (OASTL). Met is formed from Cys in three additional steps. The primary biosynthetic control point for Cys includes sulphate reduction by APS reductase (APR) and OAS formation by serine acetyltransferase (SAT). Met synthesis is controlled by cystathionine-g-synthase in some, but not all species (Haas et al., 2008; Kim et al., 2002; Leustek and Saito, 1999; Leustek et al., 2000; Tabe et al., 2010).

Transgenic A. thaliana overexpressing APR accumulated intermediates of S-assimilation including sulphite (SO42-), sulphide (S2-), thiosulphate (S-SO32-) as well as the pathway end products Cys and glutathione (Tsakraklides et al., 2002). The same transgenic strategy in maize produced a marked increase in S-assimilation, but at the expense of plant vigour, which was attributed to the accumulation of toxic S intermediates (Martin et al., 2005). Moreover, the accumulation of the S intermediates suggested that sulphate reduction outpaced OAS production in the APR overexpressing plants. However, these negative growth effects can be overcome by expressing E. coli APR in leaf cells (Planta et al., 2017).

Transgenic overexpression of SAT, necessary for OAS synthesis, has also been shown to increase S-assimilation in a variety of crop plant species (Blassczyk et al., 1999; Harms et al., 2000; Nguyen et al., 2012; Tabe et al., 2010), but without the negative effects on plant growth observed with APR overexpression (Martin et al., 2005; Tsakraklides et al., 2002). The stimulation of S-assimilation was attributed to a twofold effect: (i) increased OAS providing the direct substrate for increased Cys synthesis, and (ii) OAS also functions as a positive regulator of expression of S-reduction genes (Ohkama-Ohtsu et al., 2004), resulting in increased S2- necessary for reaction with OAS to form Cys.

Here, we describe the analysis of transgenic maize lines overexpressing SAT. In C4 plants such as maize, S-assimilation is known to occur in bundle sheath cells (Kopriva and Kopriva, 2005). Therefore, SAT overproduction was engineered using the bundle sheath cell-specific Rubisco small subunit (rbcS7) promoter. (Sattarzadeh et al., 2010). The effect of overexpressing SAT in bundle sheath cells was to increase S-assimilation without apparent negative impacts on plant growth. In addition, we found that this transgenic strategy resulted in the elevated accumulation of the 10-kDa a-zein, as well as the 27-kDa g-zein and 15-kDa b-zein proteins in both developing and mature seeds. In particular, the expression of the 10-kDa a-zein is known to be a prime contributor to Met content of kernels (Wu and Messing, 2010; Wu et al., 2012). The net result was a significant increase in protein-bound Met in mature kernels and an increase in the nutritional value measured in a chicken feeding trial. In total, overexpression of SAT in maize has the effect of, not only enhancing S-assimilation, but also, indirectly impacting expression of high-Met seed storage proteins.

**Results**

**Transformation of maize with AtSAT1**

To investigate overexpression of SAT in maize, the A. thaliana SAT1 (AtSAT1) coding sequence was placed under the control of the rbcS1 promoter, which drives expression specifically in bundle sheath cells (Sattarzadeh et al., 2010). Because the AtSAT1 protein lacks a transit peptide, it should result in cytosolic accumulation of the protein. AtSAT1 was chosen as the target of overexpression because of prior studies by the senior authors' laboratory with the A. thaliana enzyme (Murillo et al., 1995; Sors et al., 2005). The rbcS1p-AtSAT1 expression cassette was cloned into binary vector pTF102 (Frame et al., 2002) (Figure 1a), and this plasmid was used to transform immature embryos of F1 progenies of the cross between the maize Hill lines of B and A (Hill Parent B x Parent A F1).

**Identification of AtSAT1 expression lines**

The primary phosphinothricin-resistant transgenic plants from independent transformation events (derived from different immature embryos) were tested for the presence of the transgene by PCR amplification using genomic DNA as template. Six of the nine transgenic events that were tested contained the AtSAT1 transgene (Figure 1b). Those plants were grown and a fully expanded leaf used for the measurement of SAT activity and SAT protein by immunoblotting. All lines showed significantly higher SAT protein level as measured by immunoblotting than the parent maize line (Figure S1a), and in addition, all of the transgenic plants showed significantly higher SAT activity (Figure S1b).

Two lines, OE1 and OE3, derived from transgenic event #1 and #3 (Figure 1b), were selected for further analysis. These T1 plants were grown to maturity, backcrossed for two generations with maize inbred line B73, then self-pollinated for selection of transgenic nonsegregating lines (T3 generation). Unless noted otherwise, the T3 nonsegregating plants were used for all subsequent analyses. Both OE1 and OE3 were used to measure AtSAT1 mRNA level by qRT-PCR, and the same plants were used to measure SAT enzyme activity. Figure 2 shows that the expression of AtSAT1 mRNA (Figure 2a) and SAT enzyme activity (Figure 2b) was much higher in OE1 and OE3 lines than parental B73. In addition, the plants derived from OE1 showed higher AtSAT1 mRNA and higher SAT enzyme activity than OE3. It should be noted that the measured enzyme activity is a combination of endogenous SAT and that derived from expression of AtSAT1 (Figure 2b), whereas only AtSAT1 mRNA was measured in Figure 2a. These results show that AtSAT1 was stably expressed over multiple generations.
Sulphur assimilation end products accumulate in leaves of AtSAT1 transgenic maize

The leaves of 2-month-old OE1 and OE3 plants were examined for the contents of S metabolites. When compared with parental B73, free Cys and Met were found to be approximately twofold higher in OE1 and OE3 (Figure 3a and b). Total (free and protein-bound) Cys was slightly, but significantly increased, and Met was up to fourfold higher (Figure 3c and d). Also, glutathione was found to be twofold to threefold higher (Figure 3e). These results show that overexpression of AtSAT1 has resulted in an increase in S-assimilation end products in the transgenic lines. That Met accumulates in OE1, and OE3 suggests that Met synthesis in maize is not strictly controlled by the enzyme cystathionine-γ-synthase (CGS). CGS is also not a limitation for Met synthesis in potato (Kreft et al., 2003), whereas it is a bottleneck in Arabidopsis (Chiba et al., 1999; Kim et al., 2002; Lee et al., 2005).

Sulphate reduction is increased in leaves of AtSAT1 maize

When SAT was overexpressed in potato and A. thaliana, a pleiotropic increase in APR was observed (Hopkins et al., 2005; Hubberten et al., 2012; Sirko et al., 2004). The increase in APR expression was attributed to elevated OAS, the product of the SAT enzyme, which, in addition to its role in Cys synthesis, also functions as a positive signal for expression of sulphate reduction genes. To assess whether SAT overexpression has the same effect in maize as in A. thaliana and potato, OAS and sulphite were measured as was the activity of APR. OAS was significantly higher in leaf of OE1 and OE3 compared with the parental B73 (Figure 3f). APR activity was also significantly higher (Figure 4a) as was the content of sulphite (Figure 4b). Sulphite is the product of APR and might be expected to accumulate given that APR activity was higher in the AtSAT1 plants. Therefore, we conclude that SAT overexpression in maize has a similar effect as it does in other plant species (Hubberten et al., 2012).

Mature kernels of AtSAT1 overexpressing maize show changes in zein expression profile

S-amino acid supply was thought to limit accumulation of high-Met zeins and the storage of S-amino acids in maize seed (Lai and Messing, 2002). Therefore, the seed storage protein profile of the seeds produced by the collection of T1 AtSAT1-expressing primary transgenic maize was analysed by SDS-PAGE. The seeds from all of the transgenic plants showed increased expression of the 10-kDa δ-zein (Figure 5c, d), whereas the other zeins were unaffected (although a slight increase in the 16-kDa γ-zein, 15-kDa β-zein was noted). The 10-kDa δ-zein has the highest content of Met + Cys residues (26.84%, Wu et al., 2012). In order to determine whether the increase in the 10-kDa δ-zein in AtSAT1 expressing maize is heritable, the zein profiles of seeds produced by nonsegregating T3 plants OE1 and OE3 were analysed. The profile of zeins is shown in Figure 5a, and the densitometric quantification of the protein bands is shown in

Figure 1 AtSAT1 transformation of maize. (a) Schematic diagram of the SAT overexpression construct. The construct components include the T-DNA right border, RB; and left border, LB; the bundle sheath cell-specific Rubisco small subunit 1 promoter, rbcS1 promoter; the A. thaliana serine acetyltransferase1 coding sequence, AtSAT1; the CaMV 35S terminator, T35S, and the phosphinothricin acetyltransferase cassette consisting of the 35S promoter; tobacco etch virus translational enhancer, TEV; the bar gene; and the soya bean vegetative storage protein terminator, Tvsp. (b) PCR confirmation of T1 transformants. The DNA templates used for PCR amplification include the vector plasmid, or genomic DNA from each of nine transformants or the nontransformed line BXA HII, which is the maize line used for transformation.

Figure 2 Maize plant expression of AtSAT1. (a) Quantitative RT-PCR of two independent transgenic lines OE1 and OE3. Both OE1 and OE3 for this and all subsequent experiments, unless noted otherwise, were the result of two backcrosses to the maize inbred line B73 and then selection of nonsegregating plants. For this reason, B73 was used as the nontransgenic control. RNA was extracted from young leaves of two-month-old plants, and AtSAT1 was amplified with specific primers. The Actin primers were used as reference gene control. (b) SAT activity in the leaves of OE1 and OE3. The data in graphs (a) and (b) represent the mean of three measurements from different plant samples±SD. The specific activity of crude extracts is given in nmol CoA produced per min and mg total protein. Asterisks indicate significant differences between B73 and transgenic plant lines using the one-way ANOVA function of GraphPad Prim (P < 0.001).
Figure 5b. The results reveal that the 10-kDa \(\delta\)-zein is markedly increased in both transgenic lines. The 15-kDa \(\beta\)-zein and 16-kDa \(\gamma\)-zein, two other zeins are also increased in both transgenic lines. Other changes in the zein profile included small, but reproducible increase in the 27-kDa \(\alpha\)-zein, and slight but reproducible decreases in the 19-kDa \(\alpha\)-zein. One of the proteins with the lowest Met + Cys content, was unchanged. The effect of \(AtSAT1\) expression on the zein profile change appears to be specific to the high Met + Cys class of proteins because low Met + Cys zeins expression was unaffected as was the nonzein protein profile of endosperm and embryos (Figure S2).

\(AtSAT1\)-associated changes in zein profile improve nutritional value

Given the increased expression of 10-kDa, 15-kDa and 16-kDa zeins in \(AtSAT1\) kernels, it was of interest to determine whether kernel nutritional value is improved. The amino acid content of mature kernels represents the culmination of gene expression throughout seed development. To assess the effects of \(AtSAT1\) overexpression on maize kernel amino acid composition, the total amino acids content in hydrolysed kernel flour from OE1 and OE3 were compared with B73. Table 1 shows that the Met and Cys contents from both OE1 and OE3 were significantly increased. In OE1, Met was 1.40-fold higher compared with kernels from B73 and Cys was 1.32-fold higher. In OE3, Met was 1.20-fold higher, and Cys was 1.32-fold higher. The content of all other amino acids was found to be the same or very slightly reduced.

To examine whether the increase in total Met correlates with an increase in bioavailable Met, a Met biosensor assay was used. The biosensor assay measures the bioavailable Met used for growth of a Met-auxotrophic \(Escherichia coli\) strain, so it is a measure of the nutritional value of the kernel samples. Unfortunately, an equivalent \(E. coli\) Cys biosensor does not exist. The results in Figure 6 show that Met content is significantly increased specifically in the zein fraction derived from OE1 kernels compared to null sergeant or the B73 parent. By comparison, the bioavailable Met content of the nonzein fraction was nearly identical in B73 and OE1, indicating that the increase in Met is specific to the zein protein fraction and that the Met content of the zein fraction is in a bioavailable form.

To further test the nutritional value of OE1 kernels, a chicken feeding trial was performed. The growth of chickens is a
model for testing Met nutritional value of animal feed (Lai and Messing, 2002; Messing and Fisher, 1991). Two different maize flour-based diets were formulated to feed Erlang Mountain chicks, one with flour from B73 kernels and the second with flour from OE1 kernels. Aside from the different plant sources of the maize flour, the formulated diets were prepared identically. The chicks were fed for up to 21 days and their weights periodically measured. Compared with the feed that was formulated with normal B73 kernels, chicks fed a diet formulated with OE1 kernels grew significantly faster as evidenced by weight measurements performed at 14 days and 21 days (Figure 7a and Table S1). Adequate dietary Met is required for robust feather growth. Indeed, diminished feather growth is an early symptom of dietary Met deficiency in chickens. Figure 7b, c shows that wing feathers are much longer from chicks fed the OE1-kernel diet compared with the B73 kernel diet.

To achieve common use of this new high-Met trait, it is necessary that the transgene does not negatively impact yield. Examination of the OE1 transgenic line revealed that there was no significant change compared with null segregants in respect to several parameters including average grain yield per plant, seed weight, ear length, plant height, number of rows per ear and kernels per row (Figure S3).

Reciprocal crosses reveal that zein profile correlates with maternal AtSAT1 expression

Given that the increase in Met content and nutritional value of kernels from plants expressing AtSAT1 was found to result from changes in zein profile, it was of interest to define the constraints.

### Table 1 Amino acid composition analysis of maize kernels

|        | AAab (%) | B73 | OE1 | OE3 |
|--------|----------|-----|-----|-----|
| Methionine | 0.25 (0.02) | 0.35 (0.05) | 0.30 (0.02) |
| Cysteine     | 0.19 (0.02) | 0.25 (0.02) | 0.25 (0.01) |
| Lysine       | 0.35 (0.02) | 0.34 (0.02) | 0.37 (0.02) |
| Phenylalanine | 0.60 (0.02) | 0.61 (0.05) | 0.62 (0.07) |
| Leucine      | 1.41 (0.05) | 1.40 (0.16) | 1.42 (0.14) |
| Isoleucine   | 0.38 (0.02) | 0.40 (0.06) | 0.40 (0.05) |
| Threonine    | 0.47 (0.04) | 0.45 (0.03) | 0.40 (0.03) |
| Valine       | 0.62 (0.04) | 0.60 (0.06) | 0.67 (0.05) |
| Histidine    | 0.31 (0.03) | 0.31 (0.04) | 0.36 (0.04) |
| Arginine     | 0.52 (0.06) | 0.43 (0.05) | 0.45 (0.06) |
| Glycine      | 0.42 (0.04) | 0.41 (0.05) | 0.47 (0.03) |
| Aspartic Acid | 0.76 (0.05) | 0.75 (0.07) | 0.88 (0.11) |
| Serine       | 0.62 (0.03) | 0.60 (0.07) | 0.67 (0.07) |
| Glutamic Acid | 2.65 (0.19) | 2.41 (0.58) | 2.63 (0.60) |
| Proline      | 0.98 (0.05) | 1.03 (0.13) | 1.03 (0.02) |
| Alanine      | 0.94 (0.03) | 0.88 (0.10) | 0.96 (0.10) |
| Tyrosine     | 0.37 (0.02) | 0.36 (0.05) | 0.38 (0.03) |
| Total        | 11.84 (0.57) | 11.58 (0.46) | 12.26 (0.36) |

Amino acid values are expressed as the percentage of total amino acids in the sample (AAab) with standard deviations in parentheses. The values are the averages from three independent measurements ±SD in parentheses. Each assayed sample was from kernels taken from a single ear of nonsegregating OE1 and OE3 in B73 background.

Figure 5 Zein accumulation in transgenic kernels. (a) Kernels from OE1 and OE3 were harvest from field plants. The kernels were fully mature, and protein profiles from three different kernels harvested from different plants are shown. Protein from 300 μg dry weight of endosperm sample was loaded in each lane. M, protein markers from top to bottom being 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa. The mass of each zein is indicated to the right of the figure. (b) The relative abundance of zein proteins was analysed from SDS-PAGE analysis from three different kernels harvested from different plants using the densitometry function of ImageJ software. The band intensities were normalized using the 22-kDa zein and the results plotted as relative values ±SD. Asterisks indicate significant differences from B73 (Student’s t-test, P < 0.05).

Figure 6 Met content measured with a bacterial biosensor. The Met content of maize zein and nonzein proteins was measured from B73 and plants derived from a segregating population of OE1. Each value is the mean of three individual plants ±SD. Asterisks indicate significant differences from B73 (Student’s t-test, P < 0.05).
of the relationship between SAT and zeins. Self-pollinated and reciprocal crosses of OE1 and parental B73 were studied to determine the inheritance of the change in zein profile associated with AtSAT1 expression. Self-pollinated OE1 was compared to crosses of parental line B73 and OE1 in which the transgenic plant was either the maternal (OE1 × B73) or paternal (B73 × OE1) parent. The dynamics of zein accumulation was analysed at three time points in the kernels produced by the F1 seed. The results show that the 15-kDa β-zein and 10-kDa δ-zein are strongly overproduced in kernels of either self-pollinated OE1, or when OE1 was the maternal parent (Figure 8a), but not when OE1 was the paternal parent. To determine whether accumulation of these proteins correlates with an increased abundance of the respective mRNA, qRT-PCR analysis was carried out on endosperm samples from developing kernels at 18 DAP. A range of zein mRNAs were measured. Figure 8b shows that the mRNAs corresponding to the 10-kDa, 15-kDa and 18-kDa zeins were significantly more measured. Figure 8b shows that the mRNAs corresponding to the 10-kDa, 15-kDa and 18-kDa zeins were significantly more abundant in OE1. Interestingly, the 18-kDa zein is another high Met Cys zein. Although the mRNA for this protein was observed to increase when AtSAT1 is maternally expressed (Figure 8b), this protein is not possible to detect in the gel shown in Figure 8a because it is obscured by the more abundant 19-kDa α-zein.

qRT-PCR analysis of AtSAT1 mRNA expression in embryo and endosperm from 18-DAP kernels showed that AtSAT1 accumulates mainly in leaf, but not in endosperm (Figure S4), as would be expected under transcriptional control of the rbcS promoter. We observed low-level expression of AtSAT1 in the seeds; therefore, the accumulation of these zeins is likely the function of AtSAT1 expression in the vegetative tissues of the maternal plant.

Maternal expression of AtSAT1 overcomes the limitation in S-amino acid supply needed for zein accumulation

One possibility for the pleiotropic effect of SAT overexpression on zein profile is that increased S-amino acid supply influences accumulation of specific zein proteins in developing seed. Based on results from transgenic maize overexpressing the 10-kDa δ-zein (line Dzs10OE), it was earlier hypothesized that S-amino acid supply limits the accumulation of high-Met zeins (Lai and Messing, 2002; Wu et al., 2012). If so, then S-amino acid supply limitation in Dzs10OE should be overcome by AtSAT1 expression. The results of this experiment are shown in Figure 9. A representative protein gel is shown in Figure 9a, and the densitometric quantification of the 10-kDa δ-zein is shown in Figure 9b. As Wu et al. (2012) previously reported, when the 10-kDa δ-zein is transgenically overexpressed in maize (Dzs10OE), the 10-kDa δ-zein accumulates, but the amounts of the 15-kDa β- and 16-kDa γ-zeins are reduced (Figure 9a, compare null with Dzs10OE). This observation was interpreted by Wu et al. (2012) to be a hallmark of S-amino acid supply limitation. As we have reported here, AtSAT1 expression results in increased 10-kDa δ-zein (compare null with OE1). The F1 progeny of the cross OE1 × Dzs10OE produced seed in which expression of the 15-kDa β- and 16-kDa γ-zeins are restored compared with the null. In addition, the 10-kDa δ-zein is increased further compared with the Dzs10OE and OE1 transgenic parents (Figure 9b). This result strongly implies that S-amino acid supply limits the accumulation of high-Met zeins and that AtSAT1 overcomes this limitation by increasing S-amino acid supply.

Discussion

In this study, we found that deregulation of S-assimilation by ectopic overexpression of SAT in maize not only increases S-assimilation (Figures 2–4 and S1, Table 1), but also increases the nutritional value of transgenic kernels (Figures 6 and 7). The increase in nutritional Met content resulted from a pleiotropic induction of expression of specific zeins (Figure 5) that are among those with the highest number of Met residues, including the 10-kDa δ- and 15-kDa β-zeins. Maize also contains additional high Cys zein including the 50-kDa, 27-kDa and 16-kDa γ-zeins, but these were not increased to the same extent in SAT overexpressing lines (Figure 5). The high Met + Cys zeins usually make up only a small proportion of the total complement of seed storage proteins. It is for this reason that maize kernels are an insufficient
source of Met necessary for a complete animal diet (Wu et al., 2012). As animals are able to metabolize Cys from Met, but are unable to perform the reverse reaction, Met is essential in diet but Cys is nonessential (Messing and Fisher, 1991).

The level of Met in kernels of \( \text{AtSAT1} \) line OE1 is as high as the inbred line BSSS53 that has been shown to contain sufficient Met to serve as a complete source for animal feed (Lai and Messing, 2002). The reason that other maize lines have lower levels of the 10-kDa \( \delta \)-zein than BSSS53 is due to repression of the 10-kDa \( \delta \)-zein mRNA by alleles of the transacting factor \( \text{dzr1} \) (Schickler et al., 1993). The alleles can exhibit either a dosage effect due to the triploid nature of the endosperm or are silenced due to imprinting (Chaudhuri and Messing, 1994). The imprinted allele can be activated, when maternally transmitted. Still in the hybrid seed business, repression by \( \text{dzr1} \) alleles in elite lines prevents the accumulation of sufficient levels of Met storage, making supplementation of feed with pure Met necessary, as has previously been shown (Rudra and Chowdhury, 1950).

When the 15-kDa \( \beta \)-zein was inserted into the potato genome, it increased both free Met content and the zein containing Met content of the transgenic tubers (Noctor et al., 1996). The current assumption is that \( \beta \)-zein is post-transcriptionally regulated by free Met (Bagga et al., 2005), although the mechanism is not understood, indicating that in other cases sulphur-rich seed proteins need to be expressed in the appropriate background (Galili and Hofgen, 2002; Tabe and Droux, 2002). The present results show that S-amino acid production in maternal parts of the plant can drive changes to the zein profile in the kernels of maize, indicating that S must be transported as a reduced organic form, and not in an oxidized inorganic form. This observation is supported by reciprocal cross-experiments (Figure 8), and the observation that \( \text{AtSAT1} \), engineered under transcriptional control of the \( \text{rbcS} \) promoter, is expressed in leaves, but only at very low level in developing kernels (Figure S4). Importantly, our results do not rule out that Cys synthesized in seeds may contribute to seed storage protein synthesis. Therefore, whereas sulphate is an abundant translocated S-compound in plants

Figure 8: Maternal \text{AtSAT1} is necessary for accumulation of 10-kDa \( \delta \), 15-kDa \( \beta \) and 18 kDa \( \delta \)-zeins. (a) SDS-PAGE analysis of zein proteins in endosperm of B73xOE1, OE1xB73 and self-pollinated OE1. The samples were harvested at 18 DAP, 25 DAP and 35 DAP. Endosperm was isolated, and zein was extracted. Total zein loaded in each lane was equal to 300 \( \mu \)g of maize fresh weight. (b) Zein mRNA was measured in endosperm sampled 18-DAP by quantitative RT-PCR. Relative expression of zein genes in OE1 compared to B73. The data are the mean from three independent plants per transgenic line \( \pm \)SD. Asterisks indicate significant differences from B73 (Student’s \( t \)-test, \( P < 0.01 \)).
expected to reach US$5.1 billion world market by 2024, so the production for the formulation of livestock and poultry feed is general applicability in commercial maize lines. Synthetic Met

Future optimization must be performed to determine the content in corn kernels in elite, commercial line of maize. Described here may provide a simple means to increase Met and poultry feed market. The genetic engineering approach nutritional requirements for the enormous worldwide livestock capacity in maize could be a critical approach for improving seed development.

Seeds where Cys and Met become incorporated into zeins during some other derived metabolite(s) are transported to developing reduced and assimilated into Cys in leaf cells. Then, Cys, Met or depicted in Figure S5, wherein sulphate, taken up by roots, is (Tabe et al., 2010). In total, our results support the model pathway known as the SMM cycle via the enzyme methionine S-methyltransferase (MMT) (Ranocha et al., 2001). However, more recently, it was shown that SMM cannot be a major transported S-metabolite in maize because insertional mmt mutants grew normally, and seeds of the comparable A. thaliana mutant had normal S content (Kocsis et al., 2003). Therefore, the transport form of S must be some other reduced S-compound. Another possibility is either Cys, cystine or both as has previously been shown in maize (Burgener et al., 1998), or glutathione as has been shown in rice (Kuzuhara et al., 2000). In this regard, grain crops may be different from legumes, where S-amino acids for storage protein synthesis are likely synthesized in situ in seeds (Tabe et al., 2010). In total, our results support the model depicted in Figure S5, wherein sulphate, taken up by roots, is reduced and assimilated into Cys in leaf cells. Then, Cys, Met or some other derived metabolite(s) are transported to developing seeds where Cys and Met become incorporated into zeins during seed development.

In summary, this study shows that increasing S-assimilation capacity in maize could be a critical approach for improving nutritional requirements for the enormous worldwide livestock and poultry feed market. The genetic engineering approach described here may provide a simple means to increase Met content in corn kernels in elite, commercial line of maize. Future optimization must be performed to determine the general applicability in commercial maize lines. Synthetic Met production for the formulation of livestock and poultry feed is expected to reach US$5.1 billion world market by 2024, so the technology provides a viable alternative for animal feed production. In addition, from a basic research standpoint, our results point to a regulation mechanism whereby the supply of S-amino acids controls the production and accumulation of specific S-sink proteins, the high Cys + Met zeins. This finding provides the impetus for mechanistic studies into the regulation mechanism.

**Experimental procedures**

**Plasmid construction and plant transformation and initial analysis**

The DNA primers used in this study are listed in Table S2. The A. thaliana SAT1 cDNA (Gene Bank accession number BT008309.1) was used as template for PCR amplification with primers ATS1AT1PF1 and ATS1AT1PR1. This primer pair introduced two restriction enzyme sites, BamH I and Sac I. A DNA fragment including the rbcS1 promoter plus the rbcS1 5’UTR was amplified from pPTN533 (Sattarzadeh et al., 2010) with primers RbcsPF and RbcsFR. The primers used for the rbcS1 promoter PCR included the restriction sequences for EcoR I and Bcl I. Isocaudameric Bcl I and BamH I sites on the rbcS1 promoter and SAT1 fragments were ligated, and the expression cassette was cloned into the EcoR I and Sac I restriction sites of binary vector pTF102. The construct was vetted by sequencing, and a confirmed isolate was used to transform Agrobacterium tumefaciens strain EHA101.

Maize transformations using Hill Parent B x Parent A F1 immature embryos were carried out by Agrobacterium-mediated transformation as described in Frame et al. (2002). The primary transgenic plantlets were verified by PCR amplification with the primer combination ATSAT1750PF and ATSAT1750PR using genomic DNA as template.

The plants used for transformation were grown in a greenhouse, and the immature embryos were harvested at 12 DAP when they were between 1.5 mm and 2.0 mm long. Embryo cultures were infected with EHA101 harbouring the plasmid, and
Soluble and total amino acid content of leaf and kernel samples was analysed by the Beijing Mass Spectrometry Medical Research Co. Ltd. Samples were pretreated with performic acid to yield the acid stable derivatives of Cys and Met, cysteic acid and methionine sulfoxide. Samples were acid hydrolysed to yield total amino acid content.

Biosensor measurement of kernel Met was carried out with a fluorimeter (Synergy 2, Bio-Tek Instruments, http://www.biotek.com/) as described (Bertels et al., 2012), with the following modifications. Zein and nonzein protein fractions were adjusted to 300 μg/mL with water. In a 0.8 mL reaction, 0.1 mL of protein sample was combined with a protease mixture consisting of 22 μm Units Type XIV protease (part number P5147, Sigma-Aldrich, St. Louis, MO) and Aminopeptidase M (part number 164598, Sigma-Aldrich) in 10 mΜ sodium phosphate buffer pH 7.0. The reaction was incubated at 37 °C for 2 h. M9 minimal medium was used in place of MMA8 medium. Met content is presented as per cent amino acid (%AA) calculated by dividing moles of Met by moles of amino acids in the protein sample, assuming an average amino acid molecular weight of 110.

Chicken feeding experiment

Mature dry maize seeds were harvested from field-grown plants (13.6 Kg of B73 and 12 Kg of OE1) and ground into powder. The samples were used as the cornmeal source in a feeding trials carried out at Sichuan Agricultural University (Chengdu, China).

The cornmeal was incorporated into a feed that was formulated as follows: each 100 g of feed contained 55.4 g corn meal, 36.2 g of soybean meal, 0.08 g of L-Lys, 0.25 g of NaCl, 3.9 g of corn oil, 1.0 g of vitamin mixture (Guangdong Dahanuang Animal Health Products Co., Ltd., Xincheng, China), 80 mg of choline, 1.2 g of calcium carbonate, 1.92 g of calcium hydrophosphate. Using commercial cornmeal, this diet has been shown to produce Met deficiency (Messing and Fischer, 1991). Newly hatched Erlang mountain chicks were randomly divided into five groups of four chicks per feed formulation and were fed with B73 corn formulation, or the formulation made from OE1. Feeding continued for 21 days, and the chicks were weighed at the start of the experiment and then at 7, 14 and 21 days. At 21 days, the chicks were photographed to document feather quality.

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References

Bagga, S., Potenza, C., Ross, J., Martin, M.N., Leustek, T. and Sengupta-Gopal, C. (2005) A transgene for high methionine protein is
posttranscriptionally regulated by methionine. In Vitro Cell. Dev. Biol. Plant, 41, 731–741.
Bertels, F., Merker, H. and Kost, C. (2012) Design and characterization of auxotrophy-based amino acid biosensors. PLoS ONE, 7, e41349.
Blaszczyk, A., Brodzik, R. and Sirko, A. (1999) Increased resistance to oxidative stress in transgenic tobacco plants overexpressing bacterial serine acetyltransferase. Plant J. 20, 237–243.
Bourjis, F., Roje, S., Nuccio, M.L., Fisher, D.B., Tarczynski, M.C., Li, C., Herschbach, C. et al. (1999) S-Methylmethionine plays a major role in phloem sulfur transport and is synthesized by a novel type of methyltransferase. Plant Cell, 11, 1485–1497.
Buchner, P., Parmar, S., Kriegel, A., Carpenter, M. and Hawkesford, M.J. (2010) The sulfate transporter family in wheat: tissue-specific gene expression in relation to nutrition. Mol. Plant, 3, 374–389.
Burgener, M., Suter, M., Jones, S. and Brunold, C. (1998) Cysteine is the transport metabolite of assimilated sulfur from bundle-sheath to mesophyll cells in maize leaves. Plant Physiol. 116, 1315–1322.
Chaudhari, S. and Messing, J. (1994) Allele-specific parental imprinting of dzr1, a posttranscriptional regulator of zein accumulation. Proc. Natl Acad. Sci. USA, 91, 4867–4871.
Chiaiese, P., Ohshima-Ohtsu, N., Molvig, L., Godfere, R., Dove, H., Hocart, C., Fujiwara, T., et al. (2004) Sulphur and nitrogen nutrition influence the response of chickpea seeds to an added, transgenic sink for organic sulphur. J. Exp. Bot. 55, 1889–1901.
Chiba, Y., Ikikawa, M., Kijima, F., Tyson, R.H., Kim, J., Yamamoto, A., Nambara, E. et al. (1999) Evidence for autoregulation of cysteine γ-synthase mRNA stability in Arabidopsis. Science, 286, 1371–1374.
Cromwell, G.L., Pickett, R.A. and Beeson, W.M. (1967) Nutritional value of opaque-2 corn for swine. J. Anim. Sci. 26, 1325–1331.
Deng, M., Li, D., Luo, J., Xiao, Y., Liu, H., Pan, Q., Zhang, X. et al. (2017) The genetic architecture of amino acids dissection by association and linkage analysis in maize. Plant Biotechnol. J. 15, 1250–1263.
Dong, J., Feng, Y., Kumar, D., Zhang, W., Zhu, T., Luo, M.C. and Messing, J. (2016) Analysis of tandem gene copies in maize chromosomal regions reconstructed from long sequence reads. Proc. Natl Acad. Sci. USA, 113, 7949–7956.
Frame, B.R., Shou, H., Tyson, R.H., Kim, J., Yamamoto, A., Nambara, E. et al. (2002) Agrobacterium tumefaciens-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol. 129, 13–22.
Galili, G. and Hofgen, R. (2002) Metabolic engineering of amino acids and storage proteins in plants. Metab. Eng. 4, 3–11.
Haas, F.H., Heeg, C., Queiroz, R., Bauer, A., Wirtz, M. and Hell, R. (2008) Mitochondrial serine acetyltransferase functions as a pacemaker of cysteine synthesis in plant cells. Plant Physiol. 148, 1055–1067.
Hagen, N.D., Upadhyaya, N., Tabe, L.M. and Higgins, T.J.V. (2003) The redistribution of protein sulfur in transgenic potato plants overexpressing bacterial serine acetyltransferase. Plant Physiol. 131, 1808–1815.
Kopriwa, S. and Kopriwova, A. (2005) Sulfate assimilation and glutathione synthesis in C4 plants. Photosynth. Res. 86, 363–372.
Kreft, O., Hoefgen, R. and Hesse, H. (2003) Functional analysis of cystathionine γ-synthase in genetically engineered potato plants. Plant Physiol. 131, 1843–1854.
Kuzuhara, Y., Isobe, A., Awazuhara, M., Fujiwara, T. and Hayaishi, H. (2000) Glutathione levels in phloem sap of rice plants under sulfur deficient conditions. Soil Sci. Plant Nutr. 46, 265–270.
Lai, J. and Messing, J. (2002) Increasing maize seed methionine by mRNA stability. Plant J. 30, 395–402.
Lee, M., Martin, M.N., Hudson, A.O., Lee, J., Muhihtch, M.J. and Leustek, T. (2005) Methionine and threonine synthesis are limited by homoserine availability and not the activity of homoserine kinase in Arabidopsis thaliana. Plant J. 41, 685–696.
Leinweber, F.-J. and Monty, K.J. (1987) Sulfite Determination: fuchsin Method. Methods Enzymol. 143, 15–17.
Leustek, T. and Saito, K. (1999) Sulfate transport and assimilation in plants. Plant Physiol. 120, 637–644.
Leustek, T., Martin, M.N., Bick, J.A. and Davies, J.P. (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51, 141–165.
Livak, K. and Schmittgen, T.D. (2001) Analysis of relative gene expression using real-time quantitative PCR and the 2−(ΔΔCt) Method. Methods, 25, 402–408.
Martin, M.N., Tarczynski, M.C., Shen, B. and Leustek, T. (2005) The role of 5′-adenylylsulfate reduc-tase in controlling sulfate reduction in plants. Phototroph. Res. 86, 309–323.
Mertz, E.T., Bates, L.S. and Nelson, O.E. (1964) Mutant that changes protein composition and increases lysine content of maize endosperm. Science, 145, 279–280.
Messing, J. and Fisher, H. (1991) Maternal effect on high methionine levels in hybrid corn. J. Biotechnol. 21, 229–238.
Murillo, M., Foglia, R., Diller, A., Lee, S. and Leustek, T. (1995) Serine acetyltransferase from Arabidopsis thaliana can functionally complement the cysteine requirement of a cysteine mutant strain of Escherichia coli. Cell. Mol. Biol. Res. 41, 425–433.
Nguyen, H.C., Hoefgen, R. and Hesse, H. (2012) Improving the nutritive value of rice seeds: elevation of cysteine and methionine contents in rice plants by ectopic expression of a bacterial serine acetyltransferase. J. Exp. Bot. 63, 5991–6001.
Noctor, G., Strohm, M., Joaunin, L., Kerner, K.J., Foyer, C.H. and Rennenberg, H. (1996) Synthesis of glutathione in leaves of transgenic poplar overexpressing γ-glutamylcysteinyl synthetase. Plant Physiol. 112, 471–482.
Ohkama-Ohtsu, N., Kasajima, I., Fujiwara, T. and Naito, S. (2004) Isolation and characterization of an Arabidopsis mutant that overaccumulates O-Acetyl-L-serine. Plant J. 36, 3209–3222.
Planta, J., Xiang, X., Leustek, T. and Messing, J. (2017) Engineering sulfur storage in maize seed proteins without apparent yield loss. Proc. Natl Acad. Sci. USA 114, 11386–11391.
Ranocha, P., McNeil, S.D., Ziemak, M.J., Li, C., Tarczynski, M.C. and Hanson, A.D. (2001) The S-methylmethionine cycle in angiosperms: ubiquity, antiquity and activity. Plant J. 25, 575–584.
Rudra, M.N. and Chowdhury, L.M. (1950) Methionine concentration of cereals and legumes. Nature, 166, 568–569.
Sattarzadeh, A., Fuller, J., Moguel, S., Wostrikoff, K., Sato, S., Covshoff, S., Clemente, T. et al. (2010) Transgenic maize lines with cell-type specific expression of fluorescent proteins in plastids. Plant Biotechnol. J. 8, 112–125.
Schickler, H., Benner, M.S. and Messing, J. (1993) Repression of the high-methionine zein gene in the maize inbred line Mo17. J. Exp. Bot. 3, 221–229.
Sirko, A., Blaszczyk, A. and Lisiewska, F. (2004) Overproduction of SAT and/or OASTL in transgenic plants: a survey of effects. J. Exp. Bot. 55, 1881–1888.
Sors, G.T., Ellis, D.R., Magi, N., Lahner, B., Lee, S., Leustek, T., Pickering, I.J. et al. (2005) Role of sulfur assimilating enzymes in selenate reduction, tolerance and accumulation in Astragalus. Plant J. 42, 785–797.
Tabe, L.M. and Droux, M. (2002) Limits to sulfur accumulation in transgenic lupin lines expressing a foreign sulfur- rich protein. Plant Physiol. 128, 1137–1148.
Tabe, L., Wirtz, M., Molvig, L., Droux, M. and Hell, R. (2010) Overexpression of serine acetyltransferase produced large increases in O-acetyls erine and free cysteine in developing seeds of a grain legume. J. Exp. Bot. 61, 721–733.
Takahashi, H., Kopriva, S., Giordano, M., Saito, K. and Hell, R. (2011) Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. *Ann. Rev. Plant Biol.* 62, 157–184.

Tsakraklides, G., Martin, M., Chalam, R., Tarczynski, M.C., Schmidt, A. and Leustek, T. (2002) Sulfate reduction is increased in transgenic *Arabidopsis thaliana* expressing 5'-adenylylsulfate reductase from *Pseudomonas aeruginosa*. *Plant J.* 32, 879–889.

Vasal, S.K., Villegas, E., Bjarnason, M., Gelaw, B. and Goertz, F. (1980) Genetic modifiers and breeding strategies in developing hard endosperm opaque2 materials. In *Improvement of quality traits of maize for grain and silage use* (Pollmer, W.G. and Phipps, R.H., eds), pp. 37–73. London: Martinus Nijhoff.

Wu, Y. and Messing, J. (2010) RNA interference-mediated change in protein body morphology and seed opacity through loss of different zein proteins. *Plant Physiol.* 153, 337–347.

Wu, Y. and Messing, J. (2012) RNA interference can rebalance the nitrogen sink of maize seeds without losing hard endosperm. *PLoS ONE*, 7, e32850.

Wu, Y., Wang, W. and Messing, J. (2012) Balancing of sulfur storage in maize seed. *BMC Plant Biol.* 12, 77.

Xu, J.H. and Messing, J. (2009) Amplification of prolamin storage protein genes in different subfamilies of the Poaceae. *Theor. Appl. Genet.* 119, 1397–1412.

Zhang, B., Pasini, R., Dan, H., Joshi, N., Zhao, Y., Leustek, T. and Zheng, Z.-L. (2014) Aberrant gene expression in the Arabidopsis SULTR1;2 mutants suggests a possible regulatory role for this sulfate transporter in response to sulfur nutrient status. *Plant J.* 77, 185–197.

Zhang, Z., Yang, J. and Wu, Y. (2015) Transcriptional regulation of zein gene expression in maize through the additive and synergistic action of opaque2, prolamine-box binding factor, and O2 heterodimerizing proteins. *Plant Cell*, 27, 1162–1172.

**Supporting information**

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1** SAT enzyme activity, *AtSAT1* immunoblot, and zein profile of *AtSAT1* transgenic maize.

**Figure S2** Analysis of nonzein proteins in endosperm and embryo.

**Figure S3** Performance of transgenic line OE1 under field conditions.

**Figure S4** *AtSAT1* expression pattern.

**Figure S5** Diagram of a flowering maize plant illustrating the relationship between SAT and zein accumulation.

**Table S1** Growth performance of chickens fed with corn meal from OE1.

**Table S2** Primers used in this study.