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Stability of Transgene Expression in Reduced Allergen Peanut (*Arachis hypogaea* L.) across Multiple Generations and at Different Soil Sulfur Levels

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ABSTRACT: Transgenic peanut (*Arachis hypogaea* L.) containing a gene designed for RNA interference (RNAi) showed stable complete silencing of Ara h 2 and partial silencing of Ara h 6, two potent peanut allergens/proteins, along with minimal collateral changes to other allergens, Ara h 1 and Ara h 3, across three generations (T_3, T_4, and T_5) under field conditions. Different soil sulfur levels (0.012, 0.3, and 3.0 mM) differentially impacted sulfur-rich (Ara h 2, Ara h 3, and Ara h 6) versus sulfur-poor (Ara h 1) proteins in non-transgenic versus transgenic peanut. The sulfur level had no effect on Ara h 1, whereas low sulfur led to a significant reduction of Ara h 3 in transgenic and non-transgenic seeds and Ara h 2 and Ara h 6 in non-transgenic but not in transgenic peanuts because these proteins already were reduced by gene silencing. These results demonstrate stability of transgene expression and the potential utility of RNAi in allergen manipulation.

KEYWORDS: *Arachis hypogaea, transgene stability, sulfur nutrition, allergen expression*

INTRODUCTION

The oil, protein, and carbohydrate contents in peanut (*Arachis hypogaea* L.) seeds are 40−60, 20−40, and 10−20%, respectively.[1] Although peanut is a rich and economical protein source, consumption of peanut proteins can cause allergic reactions in certain individuals and is a common cause of fatal anaphylaxis.[2] In the United States, approximately 0.8% of young children and 0.6% of adults are affected by peanut allergy,[3] which is becoming a major health concern in developed countries.[4] Ara h 1, Ara h 2, Ara h 3, and Ara h 6 are the major allergens among the 12 allergens identified in peanut.[5−7]

Developing hypoallergenic peanut varieties by conventional breeding is currently not possible because no null mutants for allergens could be identified from screening of peanut accessions,[8] with the exception of naturally occurring genotypes deficient in Ara h 1.[9] Although the 66 kDa Ara h 1 protein was absent in two Indonesian varieties (Bali-1 and Bali-2), no reduction in allergenicity was detected in a mediator release assay of rat basophilic leukemia cells.[9] A reverse genetic approach exploiting RNA interference (RNAi) was employed to silence *Ara h 2* and *Ara h 6* genes,[10] two major allergens that share 63% sequence similarity.[11] Ara h 2 is a 17−20 kDa protein belonging to the conglutin family and accounts for 5.9−9.3% of total seed protein.[12,13] Cultivated peanut has two homeologues of Ara h 2, Ara h 2.01 and Ara h 2.02.[11,14] Ara h 6 is a 14.5 kDa protein, also of the conglutin family, and together with Ara h 2 was the major elicitor of anaphylaxis in a mouse model system.[15] The RNAi line, B11.1.1/11, developed from "Georgia Green" by the biolistic transformation method showed nearly complete silencing of Ara h 2 and Ara h 6 in the T_2 generation.[10] Quantitative immunoblotting with seed proteins from the T_2 generation showed that Ara h 2 and Ara h 6 were reduced 2−4 orders of magnitude, respectively, in the transgenic line. IgE binding with peanut allergic patient sera showed differences only with respect to Ara h 2 and Ara h 6 between transgenic and non-transgenic lines.[10] Analysis of transgene stability in subsequent generations is needed before advancing this line for additional functional analysis and potential utilization.

Ara h 2 and Ara h 6 belong to the sulfur-rich conglutin family of proteins. Studies in crops have shown that external sulfur application may increase sulfur-containing proteins in seeds, changing seed protein profiles.[16−18] Although the protein profile of transgenic line B11.1.1 appeared to be the same as that of the wild type in western blotting,[10] collateral changes, such as upregulation of 13-lipoxygenase and 11S Ahy-3 proteins, were detected by more sensitive mass spectrometry analysis.[19] The present study was carried out to test the stability of silencing and expression of allergens in transgenic peanut line B11.1.1/11. Stability was assessed across three generations (T_3, T_4, and T_5) under field conditions and upon exposure to variable sulfur levels in the greenhouse. Any collateral effects, mainly on Ara h 1 and Ara h 3 allergen proteins, because of silencing of Ara h 2 and Ara h 6, were also tested in both the field and sulfur nutrition studies.

MATERIALS AND METHODS

The RNAi construct used to silence *Ara h 2* and *Ara h 6* genes was based on the plasmid pFGC1008 (AY310333) obtained from the *Arabidopsis* Biological Resource Center, The Ohio State University. It includes a 293 base pair (bp) sense fragment identical to nucleotides 122−414 of *Ara h 2.01* (GenBank ID L77197) and a 228 bp antisense

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fragment identical to nucleotides 192−414 of *Ara h 2.01*.[10] Upon transcription, a 222 bp hairpin is formed by the sense and antisense fragments. The same construct also targets *Ara h 6* because *Ara h 2* and *Ara h 6* share 63% sequence identity overall[11] and 81% identity across the region used for the inverted repeat.[10] The RNAi construct was delivered by particle bombardment to 9 month old embryogenic clusters developed from mature zygotic embryos of peanut cultivar Georgia Green.

Testing Stability of Seed Allergen Content Across Three Transgenic Generations. In the field study, to test the stability of seed allergen content by silencing of *Ara h 2* and *Ara h 6* genes, seeds for T_4 and T_5 transgenic generations were raised in the greenhouse starting with bulked seeds of the T_2 generation. Transgenic generations T_3, T_4, and T_5 were grown in the field at the University of Georgia, Tifton Campus, from June 2010 to Oct 2010. There were four replications of T_3, T_4, and T_5 generations and three replications of non-transgenic segregants (similar to "Georgia Green") as controls. Each plot was 1.95 × 0.9 m in dimension, leaving 0.6 m between plots. A total of 66 seeds were sown in each plot, 33 in each of two rows at 16.2 plants/m (5 seeds/ft). Seeds were coated with Vitavax fungicide before sowing. Non-transgenic plants bordered the entire experimental area. The field test was conducted under notification (USDA-APHIS-BRS number 10-116-101n).

Testing Effects of Sulfur on Seed Allergen Content in Transgenic Lines Grown in the Greenhouse. To test the effect of sulfur on seed allergen content in the T_3 generation, seeds were planted in polyethylene containers 90 × 60 × 20 cm in dimension filled with a mixture of sand, vermiculite, and perlite in the ratio of 2:1:1. Each container had three transgenic and three non-transgenic plants. The planting mixture was irrigated with deionized water before planting. The nutrient solution for irrigation was prepared in deionized water with constant concentrations of macro- and micronutrients, except for sulfur (Table 1). Three concentrations of sulfur (S) were provided as $MgSO_4$ at 3 mM (high S), 0.3 mM (medium S), and 0.012 mM (low S). The concentration of Mg was kept constant with $MgCl_2$ at 2.97 mM (in low S solution), 2.7 mM (in medium S solution), and 0 mM (in high S solution). The pH of the solution was adjusted to 5.8. There were five replications for each S treatment concentration (0.012, 0.3, and 3 mM). The plants were watered on alternate days with nutrient solution at 500 mL per plant. The potting medium was flushed with deionized water after every three applications of nutrient solution to prevent salt accumulation in the root zone.

Table 1. Composition of Nutrient Solution

chemical	molarity (mM)
KNO_3	1
$Ca(NO_3)$	4
NaH_2PO_4	1
$MnCl_2$	0.025
$ZnSO_4{\cdot}7H_2O$	0.006
$CuSO_4{\cdot}5H_2O$	0.006
H_3BO_3	0.1
Na_2MoO_4	0.001
$CoCl_2{\cdot}6H_2O$	0.1 μM
FeEDTA	0.02
$MgSO_4{\cdot}7H_2O$	
low S	0.012
medium S	0.3
high S	3.0
$MgCl_2{\cdot}6H_2O$	
low S	2.97
medium S	2.7
high S	0

Protein Extraction. In the study, to test stability across three transgenic generations, 15 seeds were pooled separately from three replications of T_3, T_4, and T_5 transgenic generations and non-transgenic control. To study the effect of sulfur on seed allergen expression, seeds were selected from each plant for protein extraction. Two seeds per plant were selected from low S treatment (0.012 mM) and pooled to obtain six seeds for each transgenic and non-transgenic genotype per replication. For medium (0.3 mM) and high (3 mM) S treatments, five seeds per plant were selected and pooled to obtain 15 seeds for each transgenic and non-transgenic genotype per replication. Thus, there were five pooled replicates from each sulfur treatment. Homogeneity of seed maturity within treatments was maintained by choosing the most mature seeds of nearly uniform size. A standard curve for quantitative westerns was derived using protein extracted from 15 seeds of field grown "Georgia Green". Seeds were weighed; testas were removed; and cotyledons were ground in liquid nitrogen. The seed powder was defatted with hexane at 1:35 (w/v) for 4 h, keeping the sample on ice. Defatted and air-dried seed powder was passed through a 30 mesh sieve. Seed protein was extracted using Tris-buffered saline (TBS, 150 mM NaCl and 50 mM Tris) at pH 7.4, containing ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor (Roche Diagnostics, Indianapolis, IN). The defatted and air-dried peanut flour was mixed with ice-cold TBS in the ratio of 1:10 (w/v) and mixed overnight at 4 °C.[20] The mixture was centrifuged at 16060*g* for 15 min at 4 °C, and crude protein extract (CPE) was collected, aliquoted, and stored at −80 °C. Quantification of protein in the crude protein extract was performed using the Pierce bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL) according to the protocol of the manufacturer.

Quantitative Western Blots. Seed protein was denatured at 95 °C for 5 min, resolved by running on 15% polyacrylamide gels for 1.5 h, and blotted onto Amersham Hybond-P polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Piscataway, NJ) by electro-transfer at 100 V for 1 h. The PVDF membrane was dried at 37 °C overnight. To test for equal loading of proteins, the membrane was stained with SYPRO Ruby (Invitrogen, Grand Island, NY) as per the protocol of the manufacturer. Briefly, proteins on the PVDF membrane were fixed with a solution containing 10% methanol and 5% acetic acid, washed with deionized water 4 times for 5 min each, stained with SYPRO Ruby, and washed. Fluorescence detection and imaging were performed with a STORM molecular imager (Amersham Biosciences, Piscataway, NJ) at 450 nm excitation/520 nm emission. For western blotting, the membranes were blocked with 5% non-fat dry milk in Tris-buffered saline and Tween 20 (TBST) for 1 h, followed by incubation with anti-Ara h 1 (1:5000), anti-Ara h 2 (1:8000), anti-Ara h 3 (1:5000), or anti-Ara h 6 (1:5000) primary antibodies custom manufactured by Sigma Immunosys (Woodlands, TX). Subsequent to three washes with TBST, the membranes were reacted with alkaline phosphatase (AP)-labeled rabbit anti-chicken/turkey secondary antibody (Invitrogen) at 1:10 000 for $^1/_2$ h. Signal detection was performed with enhanced chemifluorescence (ECF) substrate (GE Healthcare, Piscataway, NJ) treatment for 5 min. Fluorescence was detected with a STORM molecular imager (Amersham Biosciences) at 450 nm excitation/520 nm emission.

Quantification of Allergens in Seed Protein Extract. The standard curve for allergen quantification was derived from "Georgia Green" protein at concentrations of 20, 10, 5, and 2.5 μg obtained by loading 5 μL per well of protein at 4, 2, 1, and 0.5 μg/μL, respectively. For Ara h 1 and Ara h 3 quantification, 5 μg of total protein of test samples was loaded. For Ara h 2 and Ara h 6 quantification, 20 μg of total protein of test samples was loaded. The volume of each band was quantified with Image Quant software after background correction by the object average method. Allergen band volume calculated for test samples was normalized with that of "Georgia Green".

Because allergens are quantified relative to "Georgia Green" total protein concentration, the concentration of individual allergens will be proportional to the total protein concentration. The mean allergen concentration was compared by Tukey's *t* test at $p \leq 0.05$ using SAS software.[21]

Carbon−Nitrogen−Sulfur (CNS) Analysis. In the sulfur study, CNS analysis was conducted on the same seed samples used for quantitative westerns. Replicate seed samples, four each from low, medium, and high sulfur treatments, were used. Analysis was

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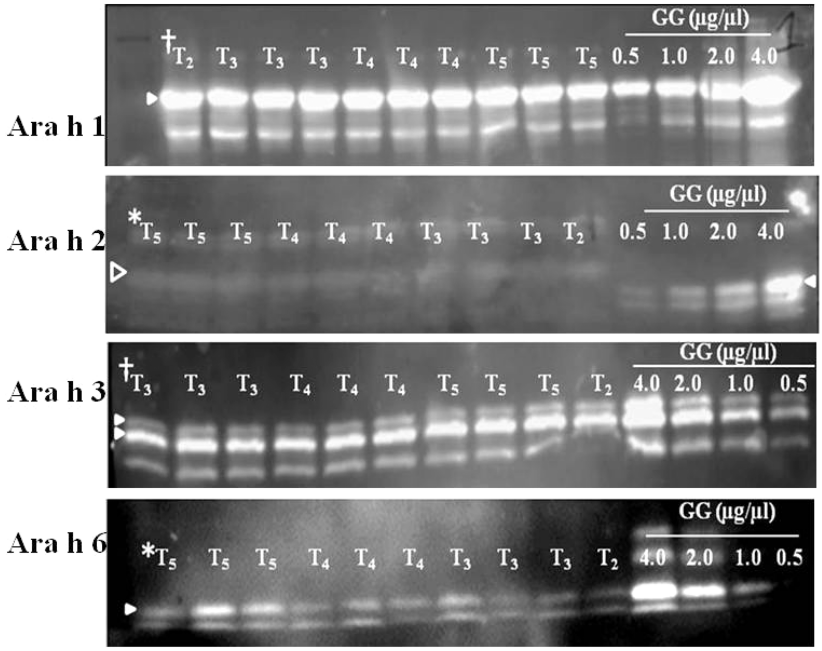

Figure 1. Western blot for allergen quantification in transgenic generations T_3, T_4, and T_5. A total of 5 μL of Tg and "Georgia Green" (GG) protein were loaded for SDS−PAGE. Western blotting was performed using a 1:5000 dilution of anti-Ara h 1, anti-Ara h 3, and anti-Ara h 6 and 1:8000 dilution of anti-Ara h 2 primary antibodies, followed by AP-labeled secondary antibody in 1:10 000 dilution. Signal detection of allergens was performed with ECF substrate with a STORM molecular imager at 450 nm excitation/520 nm emission. Allergen band selected for quantification was indicated by filled triangles. A standard curve was developed using the GG protein concentration and corresponding allergen band volume. The band volume for each allergen was calculated using Image Quant software. The control for protein loading in the Ara h 2 blot was performed with primary Ara h 3. A non-filled arrow in Ara h 2 blot corresponds to Ara h 3 detected with primary Ara h 3 antibody at 0.2:5000 dilution. (†) Transgenic (Tg) protein samples at 1 μg/μL. (∗) Transgenic protein samples at 4 μg/μL.

Table 2. Amount of "Georgia Green" Total Protein Equivalent Relative to Allergen Band Intensity in 1 μg/μL Total Protein from Transgenic Generations T_3, T_4, and T_5 and Non-transgenic (NTg) Null Segregants Grown in the Field

	"Georgia Green" protein equivalent corresponding to allergen band intensity (μg/μL)			
generation	Ara h 1[a,b]	Ara h 3[a,b]	Ara h 2[a,c]	Ara h 6[a,c]
T_3	1.451 ± 0.105 a[d]	1.697 ± 0.240 a	0 b	0.220 ± 0.057 b
T_4	1.204 ± 0.214 a	1.672 ± 0.102 a	0 b	0.218 ± 0.042 b
T_5	1.290 ± 0.126 a	1.664 ± 0.225 a	0 b	0.316 ± 0.075 b
NTg	1.158 ± 0.619 a	1.232 ± 0.404 a	0.954 ± 0.059 a	1.148 ± 0.095 a
observed p value	0.73	0.17	<0.0001	<0.0001

[a]Band volume calculated for each allergen in total protein, by quantitative western blotting, was normalized with "Georgia Green" protein concentration used to develop a standard curve. Each value represents the average ± standard deviation from two technical replications of each of three biological replicates. Means were analyzed by Tukey's t test at $p \leq 0.05$. [b]Loaded 5 μL of protein at 1000 ng/μL. [c]Loaded 5 μL of protein at 4000 ng/μL. [d]Values within a column represented by the same letter are not significantly different.

performed with the dry combustion method in a Leco CNS 2000 analyzer at the Laboratory for Environmental Analysis, University of Georgia, Athens, GA. Carbon and sulfur were detected as CO_2 and SO_2, respectively, by infrared absorption measurement. Nitrogen was determined by thermal conductivity.

Amino Acid Analysis. Free and total amino acid analysis was conducted with one pooled seed sample each, from low-, medium- and high-sulfur plants of transgenic and non-transgenic genotypes. Seed samples (0.05 g each) were pooled from four pooled replicates (same as the replicates used for quantitative western blots) from each sulfur treatment. Amino acid analysis was conducted at the Molecular Structure Facility, University of California, Davis, Davis, CA. Briefly, for total amino acid detection, approximately 10 mg of seed meal was dissolved in 1 N HCl and heated to 56 °C for 15 min. The mixture was defatted with CH_2Cl_2 (2-chloromethane), and the supernatant was collected, dried, and hydrolyzed with 6 N HCl at 110 °C for 24 h. NorLeu dilution buffer was added, and samples were analyzed on a L-8800 Hitachi instrument. For analysis of cysteine and methionine, performic acid oxidation was performed prior to acid hydrolysis. For free amino acid determination, approximately 10 mg of seed meal was dissolved in 1 N HCl and heated to 56 °C for 10 min, proteins were precipitated with 10% sulfosalicylic acid, and supernatant was collected, dried, dissolved in AE-cysteine buffer and analyzed on a L-8800 Hitachi instrument.

RESULTS AND DISCUSSION

Germination, Extractable Seed Protein, and Allergen Expression in T_3, T_4, and T_5 Generations. The germination percentage of T_3, T_4, and T_5 generations and the non-transgenic control varied from 82.95 to 93.2%, although no statistically significant variation was observed ($p \leq 0.05$). The percentage of the total seed extractable protein varied from 6.09 to 10.4%, and no significant difference was detected among three transgenic and non-transgenic generations. Protein extraction efficiency differs with buffer, meal/buffer ratio,[22] and pH of buffer.[23] Peanut seed protein ranged from 20.7 to

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28.1% in analysis of 64 peanut genotypes[24] and was reported as 21% in a comparative study of edible seed nuts.[25] Both of these studies measured protein by applying a conversion factor to total nitrogen content. In our study, only soluble protein was assayed, hence the variation from expected protein content per unit mass of defatted meal observed in this study.

No statistically significant difference was seen between "Georgia Green" and null segregants in quantitative western blots for Ara h 1, Ara h 2, Ara h 3, and Ara h 6 levels (results not shown). As shown by quantitative western blot (Figure 1 and Table 2), Ara h 1 and Ara h 3 levels in T_3, T_4, and T_5 generations were not significantly different from those of non-transgenics, although transgenics consistently showed numerically higher Ara h 1 and Ara h 3 than the null segregant. No signal for Ara h 2 proteins was detectable in RNAi lines, which was significantly different ($p \leq 0.0001$) from the non-transgenics. The concentration of Ara h 6 in all three transgenic generations was also significantly lower than in the null segregant ($p \leq 0.0001$).

The present study shows that inheritance and expression of the transgene are stable in T_3, T_4, and T_5 generations for both Ara h 2 and Ara h 6 under field conditions. This is in agreement with stable integration and inheritance of transgenes across multiple generations in other transgenic plants.[26,27] Because the transgene silencing of Ara h 2 was found to be stable across four generations (this study and the study by Chu et al.[10]), it is possible that the integration event may be near matrix attachment or hypomethylated chromosomal regions,[27] although this could only be determined with further molecular evidence.

Collateral changes in seed proteins were seen in soybean[28,29] and *Phaseolus vulgaris*,[30−32] where lines deficient in one seed protein showed an increase in other proteins and enzymes. The lack of a significant quantitative change in Ara h 1 and Ara h 3 probably would result in only minor changes in allergenicity because of these proteins. Mass spectrometry analysis of proteins from the same line did show a significant increase in 13-lipoxygenase and 11S Ahy-3 (Ara h 3-related) proteins.[19] This variation in detection efficiency is due to the sensitivity and ability of mass spectrometry to differentiate products of multiple gene families. In this study, a band was observed in transgenics alone when Ara h 3 primary antibody was used at dilution of 0.2:5000 with 4 μg/μL total protein (Ara h 2 in Figure 1). This band was not detectable with 1 μg/μL total protein used for Ara h 3 quantification in this study. A higher but statistically insignificant level of Ara h 3 in transgenics observed in this study could be attributed to differential detection of Ara h 3 isoforms by western blotting under the conditions employed in this study. Thus, the data from quantitative western blots of major allergens should be combined with more global proteomics platforms to develop a more comprehensive profile of differences between transgenic and non-transgenic lines.

An effect of the environment on silencing was not observed in this study, because both greenhouse-raised plants (T_3) and field-grown plants showed stable expression of the transgene. Environmental effects on transgene expression have been observed between field and greenhouse-grown *Gladiolus*,[33] in *Chrysanthemum* grown under different temperatures,[34] and in *Nicotiana* exposed to different light intensities.[35]

Of the four major allergens analyzed in this study, Ara h 1 and Ara h 2 constitute approximately 12−16 and 5.9−9.3% of the total seed protein, respectively.[12,13] Because there was no statistically significant difference in the extractable seed protein of field-grown transgenic compared to non-transgenic lines across generations, decreasing Ara h 2 and Ara h 6 proteins in seeds must be tempered by compensation with multiple proteins, including slight increases in Ara h 1 and Ara h 3.

Plant Morphology in the T_3 Generation under Varying Soil Sulfur Levels. Plants that received low sulfur nutrition (0.012 mM) showed clear symptoms of sulfur deficiency, including extensive yellowing, reduced vegetative growth, reduced seed number, and reduced seed weight, when compared to the plants grown under medium (0.3 mM) and high (3.0 mM) sulfur nutrition (Figures 2 and 3). Sulfur is an essential macronutrient for plants and impacts CO_2 assimilation, Rubisco enzyme activity, and photosynthesis.[36,37] Sulfur participates in several redox reactions and is essential for synthesis of amino acids methionine and cysteine, several proteins, co-enzymes, and iron−sulfur clusters. An adequate soil sulfur concentration is essential for nitrogen fixation.[38] Sulfur deficiency is associated with chlorosis, growth retardation, and reduced yield.[39,40] Reproductive growth was more affected than vegetative growth by sulfur deficiency in wheat.[40] Poor growth, reduced seed weight, and low yield under low sulfur were observed in this study and in other crops.[41−43]

Figure 2. Peanut plants 3 months after planting in two different soil sulfur levels.

Figure 3. Seed samples used for protein extraction and quantification of relative amounts of peanut allergens. For low S, two seeds were collected per plant from three plants per replication and pooled to obtain six seeds per replication of transgenic/non-transgenic seeds. For medium and high S, five seeds were collected per plant from three plants per replication and pooled to obtain 15 seeds per replication of transgenic/non-transgenic seeds.

Relative Concentrations of Allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6 in Peanut Transgenic Line B11.1.1 under Different Soil Sulfur Levels. Total extractable seed protein in greenhouse-grown seeds showed significant variation

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Table 3. Extractable Seed Protein in T_3 Generation Transgenic (Tg) and Non-transgenic (NTg) Plants Grown in the Greenhouse under Three Soil Sulfur Levels

		total protein[a] (%)	observed F	observed p	F critical
sulfur (S)	0.012 mM	4.35 ± 0.84 b[b]	5.35	0.01	$F(2, 24) = 3.40$
	0.3 mM	4.66 ± 0.80 ab			
	3.0 mM	5.47 ± 1.05 a			
genotype	Tg	5.27 ± 0.93 a	9.13	0.005	$F(1, 24) = 4.25$
	NTg	4.39 ± 0.88 b			

[a]Seed protein was extracted from six pooled seeds of low S and 15 pooled seeds of medium and high S from each of the five replications and quantified by a bicinchoninic acid assay. Total protein values represent the average ± standard deviation from two technical replications of each of the five biological replicates. [b]Means were compared by Tukey's t test, and values with the same letter within a row (S and genotype) are not statistically significant at $p \leq 0.05$.

between transgenic plants (5.27%) and non-transgenic plants (4.39%) and between low S (4.35%) and high S (5.47%) treatments (Table 3). Contrary to results from field-grown seeds, there was a significantly higher percentage of extractable protein in transgenic versus non-transgenic plants, suggesting a possible environmental effect.

As tested by two-way ANOVA, relative levels of Ara h 1 in non-transgenic and T_3 generation transgenic seeds were not influenced by genotype or sulfur nutrition. Sulfur nutrition did influence seed Ara h 3 levels [$F(2, 18) = 3.55$; $p < 0.0001$)], but no effect of genotype or genotype × sulfur interaction was observed. The relative Ara h 3 protein concentration in seeds of plants grown under low sulfur status was reduced 9−10-fold compared to plants grown with medium- or high-sulfur nutrition (Figure 4). Effects of genotype [$F(2, 18) = 3.55$; $p < 0.0001$)], sulfur [$F(1, 18) = 4.41$; $p < 0.0001$)], and genotype × sulfur interaction [$F(2, 18) = 3.55$; $p < 0.0001$)] were significant for seed Ara h 2 and Ara h 6 levels. No Ara h 2 signal was detected in Ara h 2-silenced peanut at any sulfur level by quantitative western blot experiments (Figure 4). Quantitative westerns detected Ara h 6 in transgenics grown under medium- and high-sulfur nutrition but at significantly lower amounts than in non-transgenics (Figure 4). Non-transgenic plants grown under low-sulfur nutrition showed significantly lower concentrations of Ara h 2 and Ara h 6 proteins, lowered by ∼6 and ∼22 times, respectively, than those grown at medium- and high-sulfur levels.

The levels of Ara h 1 in low-, medium-, and high-sulfur treatments among transgenic and non-transgenic plants showed no significant variation, as might be expected for a protein that is not sulfur-rich. On the other hand, low levels of sulfur caused an approximately 10-fold reduction in Ara h 3 compared to high-sulfur levels. Ara h 3 belongs to the glycinin family of proteins. Glycinins comprise major 11S storage proteins in soybeans.[44] Increased legumin (glycinin) mRNA levels and higher legumin mRNA stability have been reported under high S conditions in pea.[45,46] Transcription and transcript stability were not tested in the current study. Ara h 3, like soy glycinin, has acidic and basic subunits covalently linked by disulfide bonds. C88 of the acidic subunit (residues 1−325) and C338 of the basic subunit (residues 326−510) of Ara h 3 are linked by disulfide bonds, as shown by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and mass spectrometry analysis.[47,48] Disulfide bonding in glycinin is important for stabilizing protein conformation.[49] Low sulfur levels in soil were found to reduce the cysteine and methionine contents in barley[41] and *Arabidopsis*.[50] A similar reduction of the cysteine content (Table 4) in seeds of peanut under low-sulfur nutrition was observed, thus lowering the concentration of glycinin Ara h 3, which has also conserved cysteine residues in acidic and basic polypeptides.

Lines silenced for Ara h 2 showed no chemifluorescence signal for Ara h 2 proteins in seeds from the T_3 generation at any sulfur level. These results confirm the stability of RNAi silencing in the T_3 generation and that variation in S nutrition does not alter the stability of transgene expression, even though significant interaction was seen between the sulfur level and genotype. The level of Ara h 2 reduction is consistent with other studies for detection of Ara h 2 proteins in seeds from the T_2 generation.[10] Statistically significant low levels of Ara h 6 detected in transgenics at medium and high S levels compared to non-transgenics also showed stability of transgene expression to added S. At low S, the available S was insufficient for the synthesis of Ara h 6 in the transgenic lines because no chemifluorescent signal was detected. The low level of Ara h 6 in transgenic peanut at medium and high sulfur levels should be the consequence of efficient post-transcriptional mRNA degradation through RNAi, which probably is unaffected by sulfur nutrition.

Free and Total Amino Acids. Because developing seeds can modulate the amino acid and protein composition based on nutrient availability,[51] the amino acid profile of the plants grown at different sulfur levels was tested. The proportion of each amino acid in the free and total amino acid pool under various S treatments (Tables 4 and 5) and total and free amino acids per unit seed mass (Table 6) was determined to assess any differences in amino acid profile among treatments. These unreplicated data are helpful only in deriving the trend in amino acid profile changes under different S levels in genotypes tested. Accordingly, in the present study, the highest total amino acid content was shown by low-S treatment. Wide variation was seen between low S and the two higher S levels for all amino acids. Under low S, the majority of amino acids was found in low proportions, except for six, namely, histidine, arginine, asparagine, ornithine, hydroxyproline, and glutamine. Among these six, histidine, ornithine, hydroxyproline, and glutamine were measurable only under low-S conditions. At low S, both free and total arginine levels were higher than in medium- and high-S treatments. Arginine represented 68% of the free amino acid pool and ∼18% of the total amino acid pool. Under higher S levels, it represented ∼5−9 and ∼7−9% of free and total amino acid pools, respectively. Except for arginine and asparagine, total amino acids (Table 4) did not vary much, for most amino acids, between S levels compared to the variation seen for free amino acids (Table 5). Arginine levels also were higher in non-transgenics under medium and high S levels. Cysteine and methionine levels were higher under

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J. Agric. Food Chem. 2015, 63, 1788−1797

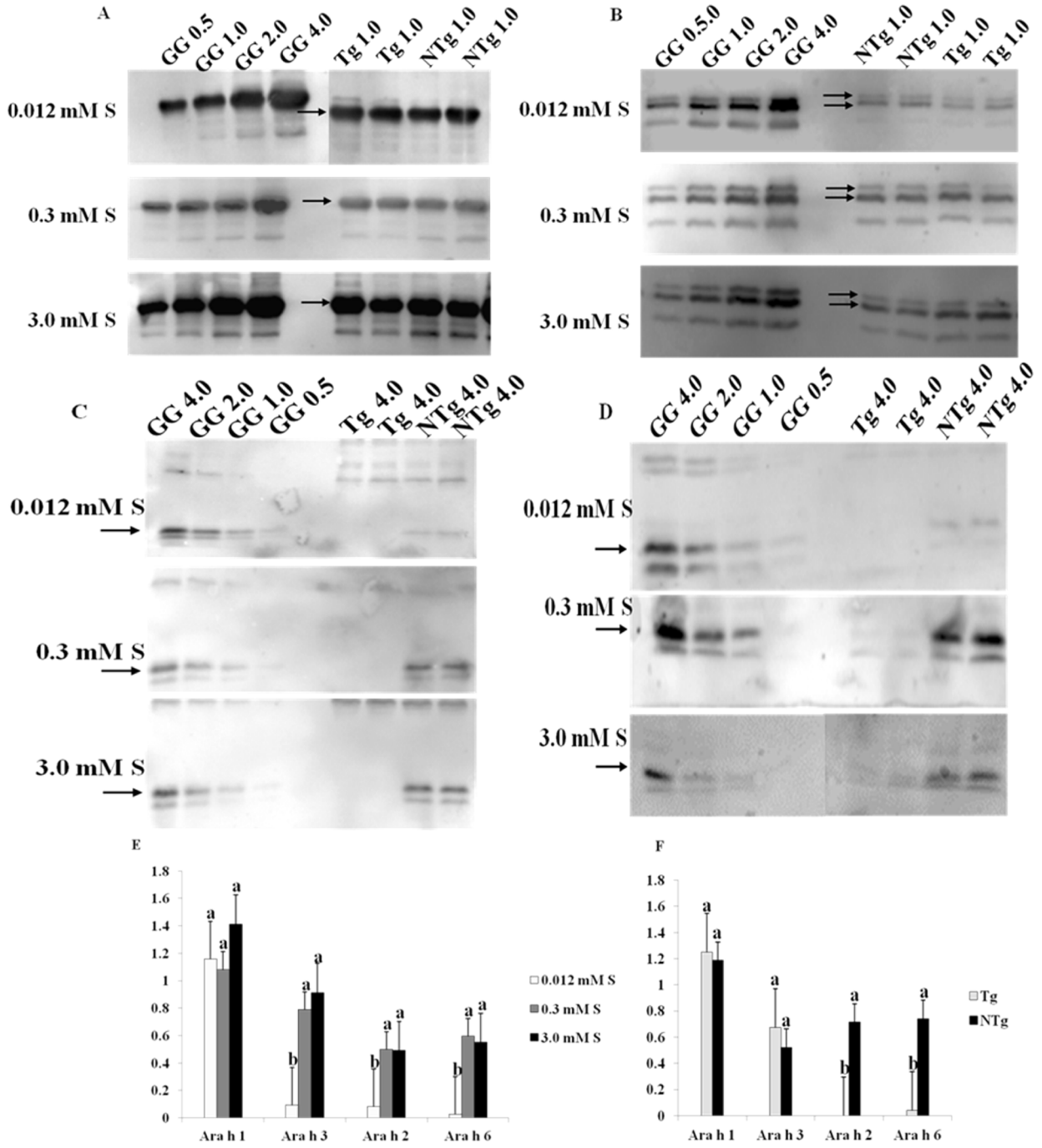

Figure 4. Western blot of allergens in T_3 generation transgenic (Tg) and non-transgenic (NTg) plants grown under three soil sulfur levels. A total of 5 μL of Tg, NTg, and "Georgia Green" (GG) protein was loaded for SDS−PAGE. Numbers corresponding to Tg, NTg, and GG indicate protein concentration in μg/μL. Western blotting was performed using (A) 1:5000 dilution of anti-Ara h 1 primary antibody, (B) 1:5000 dilution of anti-Ara h 3 primary antibody, (C) 1:8000 dilution of anti-Ara h 2 primary antibody, and (D) 1:5000 dilution of anti-Ara h 6 primary antibody, followed by AP-labeled secondary antibody in 1:10 000 dilution. Signal detection of allergen was performed using ECF substrate with STORM molecular imager at 450 nm excitation/520 nm emission. The allergen band selected for quantification is indicated by arrows in western blot images. The standard curve was developed using the GG protein concentration and corresponding allergen band volume. The band volume was calculated using Image Quant software. For Ara h1 and Ara h 3, band volume in 1 μg/μL of total protein was calculated. For Ara h 2 and Ara h 6, band volume in 4 μg/μL of total protein was calculated. (E) Effect of three soil sulfur levels on the allergen content. (F) Effect of genotype (Tg or NTg) on the allergen content. For panels E and F, error bars correspond to standard error, means were compared by Tukey's *t* test, and values with the same letter in bars for an allergen are not statistically significant at $p \leq 0.05$.

medium- and high-S treatments as well as higher in non-transgenics compared to transgenics under these two levels.

Abiotic stress[52−54] and unbalanced nitrogen metabolism[55−57] lead to accumulation of nitrogen-containing compounds, and members of the serine−arginine-rich protein family are found to be induced during abiotic stress.[58] This might explain the increased arginine and other amino acids in the free and total amino acid pools under low-S conditions, which can also lead

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J. Agric. Food Chem. 2015, 63, 1788−1797

Table 4. Percentage of Total Amino Acids in Seeds of T_3 Generation Transgenics (Tg) and Non-transgenic (NTg) Plants Grown in the Greenhouse under Three Soil Sulfur Levels

amino acid	L-Tg[a]	L-NTg[a]	M-Tg[a]	M-NTg[a]	H-Tg[a]	H-NTg[a]
asparagine/aspartic acid	13.5	13.1	10.3	11.8	10.7	12.0
threonine	2.5	2.5	3.1	2.6	3.0	2.7
serine	4.9	5.2	6.0	6.0	5.8	6.1
glutamine/glutamic acid	14.4	14.9	15.2	18.7	15.9	18.2
glycine	12.3	12.8	20.0	14.0	18.7	12.9
alanine	5.0	5.0	5.4	4.9	5.6	5.1
valine	3.6	3.6	4.1	3.7	4.2	4.1
isoleucine	2.8	2.8	2.8	2.8	2.9	3.0
leucine	5.0	5.2	5.2	6.1	5.4	6.3
tyrosine	2.8	3.0	2.9	2.7	2.8	2.9
phenylalanine	3.3	3.5	3.7	3.3	3.8	3.6
lysine	3.8	3.6	4.9	3.8	4.3	3.7
histidine	2.2	2.1	2.2	2.0	2.2	2.0
arginine	18.9	17.4	7.7	9.5	7.9	9.5
proline	4.4	4.5	4.2	4.5	4.5	4.6
cysteic acid	0.7	0.7	1.7	2.6	1.6	2.3
methionine sulfone	0.0	0.0	0.6	1.1	0.6	1.1

[a]Percentage of each amino acid calculated on the basis of amino acids (nanomoles) in 50 μL of final volume analyzed on a L-8800 Hitachi analyzer, in a single analysis of four pooled samples for each treatment. L, low S (0.012 mM); M, medium S (0.30 mM); and H, high S (3.0 mM).

Table 5. Percentage of Free Amino Acids in Seeds of T_3 Generation Transgenics (Tg) and Non-transgenic (NTg) Plants Grown in the Greenhouse under Three Soil Sulfur Levels

amino acid	L-Tg[a]	L-NTg[a]	M-Tg[a]	M-NTg[a]	H-Tg[a]	H-NTg[a]
aspartic acid	0.2	0.2	9.2	12.7	8.7	11.8
threonine	0.4	0.3	1.3	1.2	1.4	1.1
serine	0.5	0.5	1.5	1.4	1.4	1.4
glutamic acid	5.5	6.4	38.8	36.6	35.5	30.4
glycine	0.8	0.7	7.4	6.7	6.4	6.0
alanine	1.6	1.6	7.1	6.7	6.1	6.9
valine	0.3	0.3	2.1	2.1	2.1	2.4
isoleucine	0.1	0.1	0.7	0.7	0.7	0.7
leucine	0.1	0.1	0.9	0.9	0.9	1.1
tyrosine	0.1	0.1	0.7	0.6	0.7	0.9
phenylalanine	0.3	0.5	4.6	3.5	6.7	3.2
lysine	2.7	2.4	1.8	1.6	1.8	1.8
histidine	1.6	1.2				
arginine	68.4	68.2	6.0	4.7	9.1	8.7
proline	0.8	0.6	3.5	4.0	2.9	4.2
asparagine	14.5	13.7	6.4	10.0	6.3	14.6
ornithine	0.7	0.8				
hydroxyproline	0.2	0.2				
citrulline	0.4	0.6	8.0	6.6	9.2	4.7
glutamine	0.9	1.5				

[a]Percentage of each amino acid calculated on the basis of free amino acids (nanomoles) in 50 μL of final volume analyzed on a L-8800 Hitachi analyzer, in a single analysis of four pooled samples. L, low S (0.012 mM); M, medium S (0.30 mM); and H, high S (3.0 mM).

Table 6. Total (TAA) and Free (FAA) Amino Acids Per Unit Mass of Defatted Seed Meal in T_3 Generation Transgenics (Tg) and Non-transgenic (NTg) Plants Grown in the Greenhouse under Three Soil Sulfur Levels

sulfur (mM)	genotype	TAA[a] (nmol/mg)	FAA[a] (nmol/mg)
0.012	Tg	1644.5	269.2
	NTg	1833.7	237.16
0.3	Tg	836.3	23.43
	NTg	1275.9	25.7
3.0	Tg	1006.2	22.46
	NTg	1552.1	25.55

[a]TAA and FAA per unit mass of defatted seed mass represent single measurements of four pooled samples, analyzed with a L-8800 Hitachi analyzer.

to a high nitrogen content, as seen in this study. Free amino acids are critical for the roasted flavor of peanut. Free amino acids react with the sugars in seed to produce organic compounds, which impart the typical roasted peanut flavor through the Maillard reaction.[59,60] Typical roasted flavor is attributed by glutamic acid, glutamine, asparagine, phenylalanine, aspartic acid, and histidine. Free arginine, tyrosine, lysine, and threonine are responsible for the atypical flavors.[60,61] Arginine, although beneficial to human health in multiple ways,[62] can be detrimental at high concentrations with regard to peanut-roasting quality. Hence, sulfur nutrition is important for maintaining the quality of peanut because low S levels showed higher arginine, irrespective of the plant genotype. Low levels of cysteine and methionine, in the total amino acid pool, in transgenics is due to the silencing of sulfur-rich Ara h 2 and Ara h 6 in these lines.

Seed CNS Content. The percentage of carbon in dry defatted meal varied from 49% in low-S treatment to 56% in high-S treatment (Table 7). Seed carbon was influenced by sulfur (p = 0.000 12); however, no significant variation in seed carbon was seen between transgenic and non-transgenic plants, and no genotype × sulfur effect was observed. Both genotype (p < 0.0001) and soil sulfur (p = 0.0002) levels showed significant effects on seed nitrogen in two-way ANOVA, although no effect of interaction was observed. Seed nitrogen content was significantly higher in the low-S treatment (8.62%), and no significant difference was shown between medium (7.13%) and high (6.92%) sulfur. Significant variation was observed between transgenic and non-transgenic plants in seed nitrogen content, with non-transgenics having higher nitrogen (8.31%) than transgenics (6.80%). Seed sulfur was influenced by sulfur nutrition (p < 0.0001) and genotype (p = 0.000 15),

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Table 7. Seed CNS in T_3 Generation Transgenics (Tg) and Non-transgenics (NTg) Grown in the Greenhouse under Three Soil Sulfur Levels

		carbon (%)[a]	nitrogen (%)[a]	sulfur (%)[a]	N/S
sulfur (S)	0.012 mM	49.89 ± 1.35 b[b]	8.62 ± 0.08 a	0.18 ± 0.03 b	49.67 ± 6.36 a
	0.3 mM	53.52 ± 3.96 a	7.13 ± 1.35 b	0.26 ± 0.07 a	28.07 ± 4.40 b
	3.0 mM	56.54 ± 1.28 a	6.92 ± 0.90 b	0.27 ± 0.04 a	25.68 ± 2.39 b
observed F		15.36	13.92	17.57	66.32
observed p		0.00012	0.0002	5.86×10^{-5}	4.96×10^{-9}
F critical (2, 18) = 3.55					
genotype	Tg	52.79 ± 3.94 a	6.80 ± 1.12 b	0.20 ± 0.04 b	35.90 ± 12.39 a
	NTg	53.84 ± 3.49 a	8.31 ± 0.92 a	0.27 ± 0.07 a	33.04 ± 11.73 a
observed F		1.128	28.05	22.82	2.32
observed p		0.302	4.91×10^{-5}	0.00015	0.144
F critical (1, 18) = 4.41					

[a]CNS values represent averages of four biological replicates analyzed using 0.2 g of defatted and lyophilized seed meal in a Leco CNS analyzer. [b]Means were compared by Tukey's t test, and values with the same letter in columns within a row (S, genotype, and S × genotype) are not significantly different at $p \leq 0.05$.

and no genotype × sulfur interaction was observed. The sulfur content in seeds ranged from 0.18 to 0.27% in the three S nutrient regimes. Non-transgenic plants grown under three S treatments showed significantly higher seed S content (0.27%) than transgenics (0.2%). The seed N/S ratio was influenced by sulfur alone ($p < 0.0001$). Nitrogen/sulfur ratios varied from 25.68 in high-S conditions to 49.67 in low-S conditions. The N/S ratio was higher in low-S treatment, and no difference was seen between transgenic and non-transgenic plants (Table 7)

Seeds from lupin plants carrying the sunflower seed albumin transgene showed higher total S and S amino acids than non-transgenic plants.[63] In the current study, transgenic peanuts showed significantly low seed S, a result expected to be contrary to that of lupins, given the difference between overexpressing and silencing S-rich proteins. Under abiotic stress, nitrogen-containing compounds accumulate, with the most common being amino acids, such as arginine, proline, amides (glutamine and asparagine), citrulline, and ornithine, which are formed as products of detoxification of excess ammonium produced under stress.[52−54] In barley grown under low sulfur, a high proportion of aspartic acid and asparagine and low levels of methionine and cysteine were found.[41] The difference in the percentage of N between transgenic and non-transgenic plants can possibly be due to the differences in the amino acid content in the two genotypes, with the non-transgenic controls having a high total amino acid content compared to transgenics (Table 6). Low S and high nitrogen contents of seeds resulted in high and unfavorable N/S ratios under S deficiency and varied significantly from plants under medium- and high-S nutrition. This is in accordance with reports where higher seed and plant S was seen with higher S nutrition in both high and low N levels.[64,65] Carbon, nitrogen, and sulfur contents in seeds result from interactions between carbon, nitrogen, and sulfur metabolic pathways. Sulfur is a component of chloroplast Fe−S cluster proteins, and low S concentrations lead to reduced chlorophyll, Rubisco level, photosynthesis, and CO_2 fixation in rice and barley.[37,52] A low seed carbon content in seeds of low-S treatment in this study may be a consequence of the reduced growth and photosynthesis under S deficiency. Also, when S is limited, activity of the nitrate reductase enzyme is reduced, leading to reduced nitrogen assimilation,[66,67] because the enzymatic activity of nitrate reductase depends upon S−Mo biochemistry.[68] Nitrogenase activity and nitrogen fixation in roots of white clover were also reduced under S deficiency[55] because S is an essential component of the Fe−Mo cofactor of the nitrogenase protein.[56] The unbalanced nitrogen metabolism results in accumulation of nitrogen compounds, especially nitrogen-rich amino acids.[55,57] Reduced S also impairs synthesis of sulfolipids and *S*-adenosyl methionine, required for photosynthesis. Thus, S limitation leads to reduced metabolism by its influence on photosynthesis and the carbon assimilation pathway.[57]

In summary, the stability of allergen gene silencing by RNAi was tested in the present study. Concentrations of Ara h 1, Ara h 2, Ara h 3, and Ara h 6 were analyzed relative to "Georgia Green" by quantitative western blotting. Silencing of Ara h 2 and partial silencing of Ara h 6 were stably inherited across three generations. Also, relative levels of Ara h 1 and Ara h 3 were slightly but not significantly higher in transgenics compared to non-transgenics. Soil sulfur had no effect on levels of completely silenced Ara h 2 and partially silenced Ara h 6, although high-sulfur nutrition increases Ara h 2 and Ara h 6 as well as cysteine and methionine in non-transgenic peanuts. Western blotting should be coupled with more sensitive proteomics techniques to develop a detailed protein profile of transgenic peanut line B11.1.1 across generations and under different environmental conditions. This transgenic line can also serve as a good experimental material for deducing the sulfur assimilation and metabolism pathways in perturbed and non-perturbed systems.

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