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Sulphur and nitrogen nutrition influence the response of chickpea seeds to an added, transgenic sink for organic sulphur

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Abstract

In order to increase the concentration of the nutritionally essential sulphur amino acids in seed protein, a transgene encoding a methionine- and cysteine-rich protein, sunflower seed albumin (SSA), was transferred to chickpeas (*Cicer arietinum* L). Transgenic seeds that accumulated SSA contained more methionine and less oxidized sulphur than the controls, suggesting that additional demand for sulphur amino acids from the expression of the transgene stimulated sulphur assimilation. In addition, the activity of trypsin inhibitors, a known family of endogenous, sulphur-rich chickpea seed proteins, was diminished in transgenic, SSA-containing seeds compared with the non-transgenic controls. Together, these results indicate that the reduced sulphur sequestered into SSA was supplied partly by additional sulphur assimilation in the developing transgenic seeds, and partly by some diversion of sulphur amino acids from endogenous seed proteins. Growth of chickpeas on nutrient with a high sulphur-to-nitrogen ratio increased the total seed sulphur content and the accumulation of sulphur amino acids in the seeds, and partly mitigated the effect of SSA accumulation on the trypsin inhibitor amount. The results suggest that free methionine and *O*-acetylserine (OAS) acted as signals that modulated chickpea seed protein composition in response to the variation in sulphur demand, as well as in response to variation in the nitrogen and sulphur status of the plant.

Key words: Amino acid, metabolism, modification, nutritive value, plant, seed, sulphur.

Introduction

Chickpeas, like other grain legumes, contain seed protein that is relatively deficient in the sulphur amino acids, cysteine and methionine. Because methionine is one of the ten amino acids essential for animal nutrition, there has been sustained interest in increasing the concentration of methionine in plant material used for feed and food. Most reported attempts to increase seed sulphur amino acid concentration by adding a sulphur-rich sink protein have met with success, but some of these modified seeds have been reported to display alterations in endogenous protein composition similar to those triggered by sulphur nutritional deficiency (Jung *et al*., 1997; Tabe and Higgins, 1998; Tabe and Droux, 2002). An extreme example is rice (*Oryza sativa* L.) in which the addition of a foreign, sulphur-rich protein resulted in a dramatic modification of endogenous storage protein composition with no net

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Abbreviations: cv., cultivar; daa, days after anthesis; DW, dry weight; HN-HS, high nitrogen, high sulphur nutrition; HN-LS, high nitrogen, low sulphur nutrition; LN-LS, low nitrogen, low sulphur nutrition; LN-HS, low nitrogen, high sulphur nutrition; OAS, *O*-acetylserine; SSA, sunflower seed albumin; TIA, trypsin inhibitor activity.

increase in seed sulphur amino acids (Hagan *et al*., 2003). On the other hand, transformation with a seed-expressed gene encoding the sulphur-rich SSA was used successfully to increase the methionine concentration of seeds of narrow leaf lupin (*Lupinus angustifolius* L.) (Molvig *et al*., 1997). In the transgenic lupin seeds that accumulated high levels of SSA, total methionine concentration in the seed protein was increased, but total seed cysteine was not increased compared with non-transgenic controls. The evidence suggested that this was due to a compensatory decrease in the expression of genes encoding endogenous cysteine-rich seed-storage proteins, particularly conglutin delta, in the transgenic seeds (Tabe and Droux, 2002).

Grain legumes and cereals can modulate their seed storage protein composition in response to the relative availabilities of sulphur and nitrogen (reviewed in Tabe *et al*., 2002). In seeds grown with adequate nitrogen, but limiting sulphur, the relative abundance of sulphur-rich seed proteins decreases, while sulphur-poor proteins become over-represented. Conversely, when plants are grown with abundant sulphur, sulphur-rich proteins accumulate to high levels, with concomitant improvement in the protein amino acid balance in legume seeds (Blagrove *et al*., 1976; Randall *et al*., 1979; Gayler and Sykes, 1985; Sexton *et al*., 1998), and in the bread-making quality in wheat (*Triticum aestivum* L.) (Moss *et al*., 1981, 1983; Zhao *et al*., 1999). As well as modifying seed protein composition, sulphur and nitrogen nutrition also regulate the sulphur-assimilation pathway in plants. A relative deficiency of sulphur compared with nitrogen de-represses expression of root transporters responsible for sulphate uptake from the soil (Smith *et al*., 1995, 1997; Takahashi *et al*., 1997, 2000). In addition, transcript levels of genes encoding several enzymes of the pathway of sulphur reduction and assimilation are also increased (for reviews, see Leustek and Saito, 1999; Hawkesford, 2000; Saito, 2000). *O*-acetylserine, which supplies the amino acid skeleton for cysteine biosynthesis, has been proposed to be a central signal that mediates the effects of sulphur stress on plant sulphur metabolism (Smith *et al*., 1997; Koprivova *et al*., 2000), and seed protein composition (Kim *et al*., 1999).

In the current work, the methionine content of chickpea seeds has been increased by adding a seed-expressed transgene encoding SSA, containing 16% methionine residues and 8% cysteine residues (Kortt *et al*., 1991). The accumulation of SSA in chickpeas appeared to stimulate sulphur assimilation in the seeds, but also appeared to down-regulate synthesis of endogenous sulphur-containing seed proteins. The effects of SSA on seed protein and amino acid composition were modified by the sulphur nutrition of the plants, with the effect of sulphur depending on the level of nitrogen in the nutrient. The data indicate that the effects of SSA accumulation and the effects of plant nutrition on chickpea seed amino acid composition are mediated by similar mechanisms that involve free methionine and OAS as signals.

Materials and methods

Plant material and growth conditions

Two desi-type chickpea cultivars, Semsen and Amethyst, were obtained from Dr Ted Knights, New South Wales Agriculture, Tamworth, NSW, Australia. Chickpea plants were grown in the glasshouse in two different ways.

Soil: Seeds were sown in 25 cm pots (9.0 l) containing soil to which was added 0.6 g l^{-1} of a slow release fertilizer, 'Aboska', containing 15.2% (w/w) N, 6.9% (w/w) phosphorous, and 5.2% (w/w) potassium sulphate. Pots were placed in a uniformly shaded glasshouse and watered once a day. The temperature range was from 18 °C (night) to a maximum of 26 °C (day). For experiments involving developing seeds, chickpea flowers were tagged on the day they opened, and developing fruits were harvested at specified intervals after flowering.

Defined mineral nutrition: Seeds of non-transgenic chickpea, cultivar (cv.) Semsen, and transgenic chickpea line 45S were germinated on filter paper in darkness at 24 °C. The seedlings were transplanted into pots containing a mixture of 50% washed river sand, 25% vermiculite, and 25% perlite. Two plants of the same genotype were grown in each pot. The plants were maintained in a uniformly shaded glasshouse with a temperature range from 18°C (night) to a maximum of 26 °C (day).

A different mineral nutrient solution was applied to each of four groups of chickpea plants from the time of transplanting to the sand mixture (7 d after imbibition). The four treatments consisted of combinations of two levels of S nutrition (low, containing 0.2 mM SO_4 and high, containing 2 mM SO_4), and two levels of N nutrition (low, containing 2.5 mM NO_3 and high, containing 9 mM NO_3). All nutrient solutions contained the same concentrations of the following nutrients: 1 mM KH_2PO_4, 50 μM Fe-citrate, 50 μM Na_2-EDTA, 50 μM H_3BO_3, 10 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$, 0.5 μM Na_2MoO_4, 0.2 μM $CoSO_4$. The nutrient solutions with high N contained 1 mM KNO_3 and 4 mM $Ca(NO_3)_2$; the low N-nutrient solutions contained 0.5 mM KNO_3 and 1 mM $Ca(NO_3)_2$. The nutrient solutions with high S contained 2 mM $MgSO_4$, the low S-nutrient solutions contained 0.2 mM $MgSO_4$. The Mg concentration was balanced with $MgCl_2$. The four treatments are referred to as: high N, high S (HN-HS), high N, low S (HN-LS), low N, high S (LN-HS), and low N, low S (LN-LS). There were 10 non-transgenic plants in each of the LN-LS and LN-HS groups, and 11 non-transgenic plants in the HN-LS and HN-HS groups. There were nine plants of transgenic line 45S in the LN-LS group, 10 in the LN-HS group, and 11 in each of the HN-LS and HN-HS groups. Two groups of seven plants of transgenic line 161S were also grown at HN-LS and HN-HS nutrition, respectively. The nutrient solutions were made fresh from stock solutions every two days and applied to the plants once a day in the morning. Pots were flushed with excess de-ionized water every afternoon to prevent any build-up of salts in the potting medium.

Compositional analysis

Freeze-dried organs were pulverized using a puck mill, mature seeds were milled in a cyclone mill with a 0.5 mm screen. Total nitrogen was determined using an autoanalyser after Kjeldahl digestion of the finely ground flour (Heffernan, 1985). For determination of sulphur, powdered samples were compressed into aluminium planchettes or compressed on a backing of solid boric acid. Reduced sulphur and oxidized sulphur were determined using X-ray fluorescence

spectrometry (XRFS), as described by Pinkerton *et al*. (1989). The analysis of sulphur composition of seeds from soil-grown plants was performed as follows. A pool of approximately 25 mature seeds (around 6 g) was milled to fine flour for 11 individual plants of cv. Semsen, five T_4 plants of transgenic line 45S that were homozygous for the SSA transgene, nine T_2 plants of transgenic line 161S, all of which contained at least one SSA transgene locus; one plant of cv. Amethyst, and three T_1 plants of transgenic line 213A, all of which contained at least one SSA locus. XRFS analysis was performed in duplicate for each flour sample. The value for each sample was the mean of these technical replicates.

Analysis of amino acid composition of soil-grown seed flour was performed as follows. A pool of approximately 25 mature seeds (approximately 6 g) was milled to fine flour for three individual plants of the cv. Semsen control, one T_4 plant of transgenic line 45S that was homozygous for the SSA transgene, and two individual T_2 plants of transgenic line 161S that were confirmed to contain at least one transgene locus. Three values were determined for cv. Semsen plants in order to provide a measure of plant-to-plant variation in a single genotype. The values for the 161S plants are presented separately because there was independent segregation of two transgene loci in this line, producing some genetic heterogeneity. Sulphur amino acids were determined by an accredited analytical laboratory, using an amino acid analyser, after oxidation of cysteine to cysteic acid and methionine to methionine sulphone, and complete hydrolysis of the seed meal. The sulphur amino acid composition of mature chickpea seeds grown in controlled mineral nutrition was determined in the same way on pools of flour representing three to four individual plants from each experimental group.

Glutathione was determined in acid extracts after labelling with monobromobimane (mBBr) and separation by reverse phase HPLC as previously described (Droux *et al*., 1995; Tabe and Droux, 2001). Extraction was performed on samples of the pooled, powdered, freeze-dried organs from mid-maturation pods at 25 d after anthesis (daa). Samples of 20 mg of embryos, 40 mg of testa, or 40 mg of pods, were extracted into 1 ml of 25 mM HCl by vortexing several times at room temperature for 15 s over a 30 min period. OAS and free methionine were determined by HPLC after derivatization with *O*-phthaldialdehyde, as previously described (Kim *et al*., 1999).

Protein extraction and SDS-PAGE

Total protein was extracted from approximately 20 mg samples of flour from pools of approximately 6 g of mature seeds from each plant by vortexing for 30 s several times over a period of 10 min in 1.0 ml of seed protein extraction buffer (SPEB) consisting of 0.5 M NaCl, 1 mm EDTA, and 0.1 M TES (*N-tris*[hydroxymethyl]methyl-2-amino-ethanesulphonic acid)-NaOH, pH 7.8. Insoluble material was removed by centrifugation in a bench microfuge at 13 000 rpm for 10 min and protein concentration in the supernatant was determined by the method of Bradford (1976) with bovine serum albumin as a standard. Albumins were purified using the method of Schroeder (1982).

Samples were prepared for electrophoresis by dissolving 50 μg of total protein in SDS sample buffer consisting of 0.125 M TRIS-HCl (pH 6.7), 2% (w/v) SDS, 1% (v/v) β-mercaptoethanol, 5% (w/v) glycerol, 0.1% (w/v) bromophenol blue, and heating at 100 °C for 5 min. Proteins were fractioned on a slab gradient polyacrylamide (15% to 30%, w/v) gel as described previously (Spencer *et al*., 1980). The staining of proteins in SDS-polyacrylamide gels with mBBr followed a protocol described by Buchanan *et al*. (1997). Western blotting was performed as previously described (Molvig *et al*., 1997).

Protease inhibitor assay

Proteins were extracted from 20 mg samples of flour from pools of approximately 6 g of mature seeds from each plant into 30 vols of SPEB, and quantified as described above. Uninhibited trypsin activity was determined by measuring the increase in absorbance at 247 nm in a volume of 1 ml of the artificial substrate Na-*p*-tosyl-L-arginine methyl ester (TAME) dissolved at 1 mM in 10 mM $CaCl_2$, 10 mM TRIS-HCl pH 8.0 (Walsh, 1970). The assay was performed on a recording spectrophotometer using 1.0 μg of bovine pancreas trypsin type III (10 600 units mg^{-1} protein from Sigma) per assay. Inhibition of trypsin by seed extracts was determined by mixing 10 μg of seed protein with 1.0 μg of trypsin in 30 μl of 0.5× SPEB and preincubating at room temperature for 3 min. The mixture was then added to 1 ml of TAME assay buffer pre-equilibrated at 24 °C, and the change in A_{247} was recorded over a 5 min period. The velocity of each reaction was calculated as change in A_{247} min^{-1}, and trypsin inhibitor activity (TIA) μg^{-1} of total seed protein was calculated for each seed extract using the following formula:

$$\text{TIA} = \frac{[\text{uninhibited trypsin velocity}(V^{\text{T}}) - \text{residual trypsin velocity}(V^{\text{R}})] \times 100}{V^{\text{T}} \times 10(\mu\text{g seed protein})}$$

Gene constructs and plant transformation

Three plasmids, all containing identical versions of a chimeric gene encoding sunflower seed albumin (SSA) were used for plant transformation. The transferred DNA in plasmid pBSF16 (Molvig *et al*., 1997) contained three genes; a herbicide tolerance selectable marker gene, *bar*, controlled by a cauliflower mosaic virus (CaMV) 35S promoter; a screenable marker gene, *uidA*, encoding β-glucuronidase, also controlled by a CaMV 35S promoter, and a gene encoding SSA controlled by the seed specifically expressed promoter from a pea vicilin gene. The transferred DNA in plasmid pBSF19 contained the same *bar* and *ssa* genes as pBSF16, but did not contain the *uidA* gene. Both plasmids were based on the binary vector pTAB10 (Tabe *et al*., 1995). Plasmid pBSF101 contained a CaMV 35S-driven *bar* gene and the same seed-specific *ssa* gene as pBSF16 and 19. In pBSF101 both genes were inserted between the T-DNA borders of the pPZP201 binary vector (Hajdukiewicz *et al*., 1994). Full details of plasmid construction are available on request. *Agrobacterium*-mediated chickpea transformation was as described by Sarmah *et al*. (2004).

Statistical analysis

Seed concentrations of reduced sulphur, oxidized sulphur, and TIA were compared for cv. Semsen and lines 45S and 161S plants grown in soil using one-way ANOVA with genotype as the blocking variable. *Post hoc* comparisons of lines 45S and 161S with cv. Semsen were performed using Dunnett's *t*-tests. In data from nutrient-fed plants, the effects of genotype, nitrogen nutrition (N), and sulphur nutrition (S) on seed concentrations of reduced sulphur and oxidized sulphur were investigated using general linear model analysis with genotype, N and S used in a three-way factorial treatment design for the data relating to cv. Semsen and transgenic genotype 45S. Each model contained main effects, all two-way interaction terms as well as the Genotype×N×S three-way interaction term. *Post hoc* tests comparing group means were made using the Tukey–Kramer adjustment for multiple comparisons. Data relating to transgenic genotype 161S were compared with those for cv. Semsen using Student's *t*-test to compare means for 161S with the means for cv. Semsen in each of the two experimental groups.

The effect of genotype (Semsen versus line 45S), S and N on TIA was determined using three-way ANOVA; *post hoc* tests were performed using Tukey's adjustment for multiple comparisons. Since data were available for line 161S in high nitrogen treatments, a second analysis was performed with genotype (Semsen, 45S and 161S) and S as blocking variables in a two-way ANOVA with TIA as the dependent variable. Finally, to test whether the difference between

TIA levels of Semsen and line 161S was dependent on the level of S, a two-way ANOVA, with genotype (Semsen and 161S) and S as blocking variables, was conducted. The corresponding genotype×S interaction term was evaluated to determine whether the TIA difference was related to level of S. All statistical analyses were performed using SAS version 8.02 (SAS Institute, Cary, NC, USA).

Results

Increasing sulphur amino acid accumulation in chickpea seeds by transfer of a gene encoding SSA

A chimeric gene encoding SSA and controlled by a strong, seed-specific promoter from a pea vicilin gene (Higgins *et al*., 1988) was transferred to chickpea. Several transgenic lines were generated, and three lines that expressed SSA at high levels in their seeds were chosen for further study. Lines 45S and 161S were derived from transformation of chickpea cv. Semsen, with binary plasmids pBSF16 and pBSF19, respectively, while line 213A was derived from the transformation of chickpea cv. Amethyst with binary plasmid pBSF101. Southern blotting analysis and segregation data showed that line 45S contained two copies of the T-DNA at a single locus, while line 161S contained at least two transgene loci. Segregation data indicated that line 213A contained a single transgene locus. T_1 generation mature seeds of all these lines accumulated SSA, as demonstrated by western blotting analysis. The levels of SSA in T_1 seeds of lines 161S and 213A were estimated to constitute approximately 6–12% (w/w) of total seed protein, depending on the zygosity of the transgene loci. SSA was estimated to constitute approximately 3% of total seed protein in T_3 seeds of a homozygous, transgenic 45S progeny line that was used in all the experiments in this study (data not shown). Where plants of transgenic lines 161S or 213A were analysed, the presence of at least one transgene locus was confirmed by the activity of the selectable marker, and subsequently by the presence of SSA in the mature seed (data not shown).

Sulphur in mature, soil-grown seeds of transgenic lines 45S and 161S was compared with seed sulphur in the parental genotype, cv. Semsen. ANOVA using genotype as a treatment variable demonstrated a highly significant effect of the SSA transgene on both reduced ($P<0.001$), and oxidized sulphur ($P<0.001$). The seed concentrations of reduced sulphur were significantly higher (using Dunnett's *t*-test) than the parental control for both transgenic genotypes 45S ($P<0.05$) and 161S ($P<0.05$, Table 1), consistent with an additional accumulation of sulphur amino acids in the transgenic SSA protein. The increases in the amounts of reduced sulphur in the seeds were accompanied by decreases of approximately the same magnitude in the oxidized sulphur in the seeds ($P<0.05$ for both transgenic genotypes). Seed sulphur was also determined in transgenic line 213A and the parental genotype, cv. Amethyst. The concentrations of reduced sulphur in mature seed of three T_1 progeny plants of line 213A were 87, 88, and 95.6 μmol g^{-1} dry weight (DW). Oxidized sulphur was undetectable in all three seed samples. By comparison, the reduced sulphur concentration in mature seed from a non-transgenic, azygous control plant of the parental cv. Amethyst, contained 76.2 μmol g^{-1} DW, and the oxidized sulphur was 15 μmol g^{-1} DW. Therefore, in three independent transgenic chickpea lines expressing SSA, there were complementary changes in oxidized and reduced sulphur concentrations relative to the appropriate non-transgenic controls. Total seed sulphur concentrations were not consistently different between transgenic and control genotypes (Table 1).

Sulphur amino acid composition was determined for mature seeds of cv. Semsen and of the two SSA transgenic lines derived from it. Because of the cost of these analyses, within-genotype variation was assessed by duplicate determinations only for the non-transgenic cv. Semsen, for which analysis was performed for three separate plants. The values for the 161S plants are presented separately because there was independent segregation of two transgene loci in this line, producing some genetic heterogeneity. The presence of SSA protein was confirmed in the seeds of both plants of 161S analysed (data not shown). The 45S plant was homozygous for the SSA transgene. The methionine concentrations were higher in all three seed samples of the two transgenic lines than in the control seed (Table 2). Because the SSA protein contained a high proportion of

Table 1. *Sulphur composition of soil-grown chickpeas expressing SSA*

Sulphur in reduced (Red S) and oxidized (Ox S) forms was determined in flour from a sample of 6 g of mature seed for each plant. Total S is the sum of these figures. The values for each genotype are the means ±SE of the values for each plant (n=5–11 plants per genotype). Tg; transgenic line.

Chickpea genotype	Seed sulphur content		
	Red S (μmol g^{-1} DW)	Ox S (μmol g^{-1} DW)	Total S (μmol g^{-1} DW)
cv. Semsen	63.6±1.08	16.4±1.56	80
Tg 45S	72.0±1.58	6.4±1.26	78.4
Tg 161S	82.5±2.11	2.2±1.00	84.7

Table 2. *Sulphur amino acid composition of soil-grown chickpeas expressing SSA*

The values for the non-transgenic control, cv. Semsen, are means of determinations on mature seed flour from each of three individual plants. The SE for both methionine and cysteine measurements was less than 1%. Tg; transgenic line. 161S/1 and 161S/2 were different T_2 plants from transgenic line 161S.

Chickpea genotype	Methionine (% of total amino acid)	Cysteine (% of total amino acid)	Met+Cys (% of total amino acid)
cv. Semsen	1.7	2.0	3.7
Tg 45S	2.1	1.8	3.9
Tg 161S/1	3.3	1.7	5.0
Tg 161S/2	3.0	1.8	4.8

cysteine (8% of total amino acid residues) as well as methionine (16% of total), it was expected that cysteine would also be increased in the transgenic seeds. Instead, the seed cysteine concentrations were consistently slightly lower for the transgenic seed flour than for the non-transgenic controls. The total seed sulphur amino acids were slightly higher than the control levels in the sample from line 45S and were more clearly higher in the samples from line 161S (Table 2).

Reallocation of sulphur amino acids in transgenic seeds accumulating SSA

Analysis of total seed protein of transgenic lines 45S and 161S by SDS–PAGE revealed the presence of SSA (confirmed by western blotting with a specific antibody, results not shown) and, in addition, showed some differences in the relative abundance of a number of prominent proteins in the transgenic seeds, particularly 161S, relative to the control (Fig. 1A). This was clearest in the albumin fraction, in which a protein that co-migrated with the 6.5 kDa marker was obviously under-represented in the transgenic seeds (Fig. 1B). Similar results were seen when the total protein from seeds of transgenic line 213A was compared with that from the control seeds of cv. Amethyst. In this case, it was demonstrated by staining with the thiol-specific reagent, mBBr, that a protein that was under-represented in the transgenic line was rich in cysteine residues (Fig. 1C).

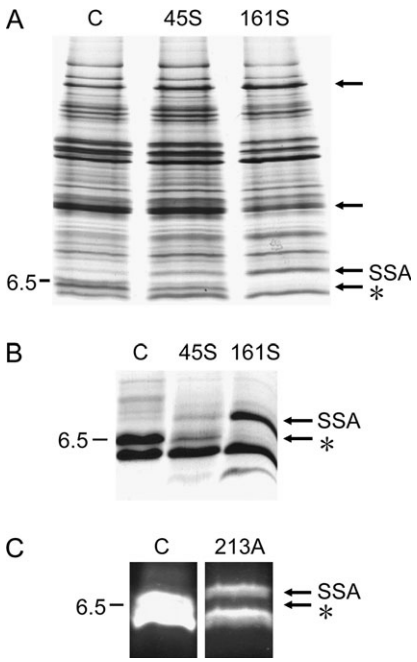

Fig. 1. Protein composition of soil-grown chickpeas expressing SSA. (A) Samples of 50 μg of protein extracts from mature seeds of control chickpea cv. Semsen 'C', and two transgenic lines that expressed SSA at different levels (45S and 161S) were resolved on SDS-PAGE, and stained with Coomassie Blue. The labelled arrow indicates SSA, unlabelled arrows indicate proteins whose relative abundance is different in the transgenic versus the control extracts. The arrows labelled with an asterisk mark the position of a protein with the approximate predicted mobility of the chickpea trypsin inhibitor. The position of the 6.5 kDa marker is indicated on the left. (B) Albumins were extracted from samples of the same seed flour, and 50 μg samples were resolved on SDS-PAGE and stained with Coomassie Blue. (C) Total protein extracted from mature seeds of an SSA-negative control line of cv. Amethyst 'C', and from an SSA positive transgenic line (213A) was reacted with mBBr and 50 μg samples of the labelled protein were resolved on SDS-PAGE. The fluorescence of each band is proportional to the cysteine content of the protein and its relative abundance.

Chickpea seeds contain a family of small, cysteine-rich protease inhibitors (Belew *et al*., 1975). A single protein of approximately 8 kDa that contains 21% cysteine residues accounts for approximately 50% of the protease inhibitory activity extracted from chickpea seeds (Belew *et al*., 1975; Belew and Eaker, 1976). The abundance of this known class of cysteine-rich proteins was assessed by measuring TIA in extracts from seeds of control and transgenic chickpeas expressing SSA. Non-transgenic chickpeas of both parental cvs contained readily detectable inhibitory activity against trypsin in an *in vitro* assay. ANOVA demonstrated a highly significant effect of the SSA transgene on TIA in the case of the lines derived from cv. Semsen ($P<0.001$). TIA was significantly lower in both line 45S ($P<0.05$) and line 161S ($P<0.05$) than in cv. Semsen (Table 3). Similarly, TIA in seeds of one plant of transgenic line 213A was strongly diminished compared with that in one plant of cv. Amethyst. The TIA in seed from line 213A was 3.0 units μg^{-1} protein compared with 9.4 units μg^{-1} protein in cv. Amethyst.

The effects of plant sulphur and nitrogen nutrition on the composition of chickpea seeds with different sink strengths for organic sulphur

The decreased levels of some endogenous sulphur-rich proteins in transgenic SSA chickpea seeds grown in soil resembled the adaptations of pulse seeds to sulphur nutritional stress (Randall *et al*., 1979; Gayler and Sykes, 1985; Higgins *et al*., 1986). Therefore, the transgenic chickpeas were grown in conditions of controlled mineral nutrition to compare the effects of nutritional stress with the effects of SSA accumulation, and to determine whether manipulating

Table 3. *Trypsin inhibitor activity (TIA) in soil-grown chickpeas expressing SSA*

Total protein was extracted from mature seed flour of each chickpea genotype. The activity of trypsin against a synthetic substrate was assayed either alone, or in the presence of seed protein extracts or BSA, the latter of which produced no detectable inhibition of trypsin in this assay. The results are the mean ±SE of assays on protein extracts from seed of three plants for each genotype. Tg; transgenic line.

Chickpea genotype	TIA (units μg^{-1} protein)
cv. Semsen	9.5±0.10
Tg 45S	5.7±0.26
Tg 161S	2.4±0.53

plant nutrition could influence the composition of the transgenic seeds with increased sink strength for sulphur. The experiment tested the effects of two levels of nitrogen nutrition combined with two levels of sulphur nutrition on seed composition of the non-transgenic cv. Semsen and the homozygous transgenic line 45S (see Materials and methods for details of nutritional regimes). The concentrations of nitrogen, reduced sulphur, oxidized sulphur, total sulphur amino acids, and trypsin inhibitors in mature whole seeds were measured. In addition, putative signalling metabolites were quantified in extracts from developing cotyledons of the Semsen and 45S genotypes at mid-maturation (approximately 25 daa). Transgenic line 161S, which expressed a higher level of SSA than line 45S was also grown in two of the nutritional treatments (HN-LS and HN-HS). Inclusion of this genotype in the other treatments was not possible because of the low number of viable seed available. Because of this imbalance in the experimental design, data for line 161S were not included in the ANOVA analysis for the data in Fig. 2. In this section, the results relating to the Semsen and 45S genotypes will be considered first, then any additional points relating to genotype 161S.

The results of the mature seed composition analysis are presented as concentrations in order to reflect processes at the biochemical level in the seeds. However, the response of the plants to nutrient availability was also expressed at the level of plant size, seed yield, and seed size. Seed yields were least for the LN-LS treatment, and increased to a maximum for the HN-HS treatment (Table 4). There were no consistent differences between the transgenic lines and the cv. Semsen controls in yield or seed weight, although both tended to be lower for the 45S genotype than for the other two genotypes. Plant nutrition also influenced the total seed nitrogen content, with seed nitrogen being higher in the HN treatments. For cv. Semsen, the mean seed nitrogen concentrations were 2.14 (0.09) mmol g^{-1} DW at LN-LS, 2.46 (0.05) mmol g^{-1} DW at LN-HS, 2.78 (0.04) mmol g^{-1} DW at HN-LS, and 2.91 (0.06) mmol g^{-1} DW at HN-HS. Figures in brackets are standard errors, n=3–9 plants per group. The nitrogen concentrations in seeds of the two transgenic lines did not differ consistently from those of the non-transgenic controls. SSA accumulation was estimated at approximately 3% of total seed protein in mature seeds of transgenic line 45S in all nutritional treatments. Similarly, SSA was expressed at a uniform level in seeds of transgenic line 161S grown in the two HN treatments demonstrating that expression of the SSA gene did not respond to plant nutrition (data not shown).

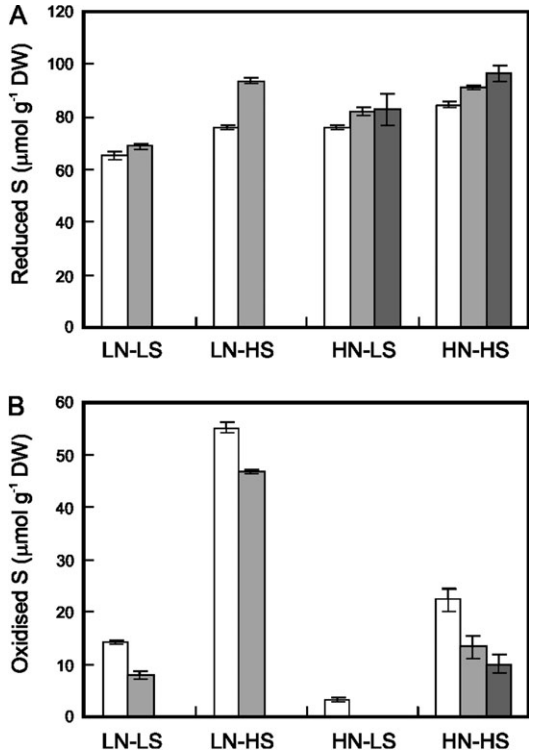

Fig. 2. Effects of sulphur and nitrogen nutrition and SSA genotype on concentrations of reduced sulphur (A), and oxidized sulphur (B), in mature chickpea seeds from cv. Semsen (open bars); transgenic line 45S (light grey bars); and transgenic line 161S (dark grey bars). Values are the mean (±SE) of measurements on seed flour from 9–11 plants in each experimental group.

Reduced sulphur concentration in the seeds was strongly affected by SSA genotype, and this effect depended on the nutritional treatment (Genotype×S×N interaction $F_{1,75}$=20.83, P<0.001). Seed reduced sulphur was significantly higher (P<0.01) in line 45S than in cv. Semsen in all but the LN-LS treatment (P=0.45, Fig. 2A). Plant sulphur nutrition also had a direct influence ($F_{1,75}$=280.1, P<0.001), with seed reduced sulphur being significantly higher (P<0.001) for both 45S and Semsen in HS treatments than in the LS treatments. In addition, the magnitude of the effect of higher sulphur nutrition depended on the level of nitrogen in the nutrient (N×S interaction $F_{1,75}$=31.37, P<0.001), with the effect of higher sulphur nutrition being greater at low nitrogen levels in the nutrient (Fig. 2A). Thus, the increase in reduced sulphur concentration in seeds of line 45S compared with cv. Semsen was greatest in the LN-HS treatment in which the ratio of sulphur to nitrogen concentrations in the plant nutrient was highest (molar ratio 0.8). Independent pair-wise comparisons showed that the concentrations of reduced sulphur in seeds of line 161S were significantly higher than for cv. Semsen in both HN-LS (P<0.05, Student's t-test) and HN-HS groups (P<0.001).

Oxidized sulphur concentration in the seeds was most dramatically affected by plant nutrition, but also by SSA genotype. The effect of genotype was consistent across nutritional treatments and statistically significant in all but the HN-LS treatment, in which oxidized sulphur concentrations were extremely low in seeds of cv. Semsen and mostly undetectable in seeds of line 45S (Genotype×S interaction $F_{1,75}$=4.28, P<0.05; Genotype×N and

Table 4. *Seed yields and seed weights for chickpeas grown with controlled mineral nutrition*

All values are means ±SE of values for 7–11 plants per treatment.

Treatment	Seed yield (g plant^{-1})			Mean seed weight (mg seed^{-1})		
	Semsen	45S	161S	Semsen	45S	161S
LN-LS	6.9±0.86	5.0±5.3	–	141±5.7	119±6.9	–
LN-HS	11.1±1.09	8.4±1.33	–	200±6.9	166±8.1	–
HN-LS	17.9±2.12	17.5±1.96	24.5±5.82	189±9.2	172±6.9	185±6.4
HN-HS	29.9±3.84	25.5±2.65	42±5.44	212±8.0	212±10.7	229±6.2

three-way interactions not significant). Seed oxidized sulphur was significantly lower ($P \leqslant 0.05$) in line 45S than in cv. Semsen in all but the HN-LS treatment (P=0.75, Fig. 2B). Plant sulphur nutrition had a strong influence ($F_{1,75}$=846.8, P<0.001) on oxidized sulphur concentration in seeds of both genotypes, and this effect depended on the level of nitrogen in the nutrient (N×S interaction $F_{1,75}$=152.6, P<0.001). Oxidized sulphur concentration was very much higher in seeds of cv. Semsen and line 45S in the LN-HS treatment than in seeds of either genotype in any of the other treatments (P<0.001). Thus, in the LN-HS treatment, the levels of seed oxidized sulphur were the highest of any of the experimental treatments, but the difference between the seeds of the two genotypes was essentially the same as in the other treatments. Independent pair-wise comparisons showed that the concentrations of oxidized sulphur in seeds of line 161S were significantly lower than for cv. Semsen in both HN-LS (P<0.001, Student's t-test) and HN-HS groups (P<0.001).

Seed sulphur composition was further investigated by determining the total sulphur amino acid concentrations in seed flour pooled from three to four plants for each experimental group, including the plants of transgenic line 161S. Plants whose seed size, and nitrogen and sulphur concentrations, most closely approximated the group means for these parameters, were chosen for analysis. The effects of variation in nitrogen concentration and seed size were avoided by considering amino acid concentrations as percentages of total amino acids. Because the cost of the analyses prevented replication of the measurements, statistical analysis of the results was not possible, therefore, the data represent the trends in amino acid composition only. The trends were the same when the data were expressed as sulphur amino acid content per seed (data not shown).

The proportion of methionine in the total seed protein was higher in the flour samples from the 45S and 161S genotypes than in the flour from the non-transgenic genotype in all treatments, indicating that seed total methionine content was increased in the transgenic lines across all the nutritional regimes (Fig. 3A). By contrast, seed cysteine was not consistently different in the transgenic compared with the control samples, as was observed for soil-grown plants (cf. Fig. 3B with Table 2). Cysteine concentration was actually lower in the transgenic flour samples than in the cv. Semsen flour sample in the HN-LS treatment, in which the sulphur-to-nitrogen ratio in the plant nutrient medium was lowest (molar ratio 0.02). Total sulphur amino acids (Met+Cys) were increased in all the transgenic flour samples compared with the cv. Semsen samples, except in the HN-LS treatment (Fig. 3C) indicating that the effects of SSA genotype on seed sulphur amino acid content were modified by both nitrogen and sulphur nutrition. This was particularly clear for the flour sample from line 161S that expressed SSA at a high level.

In order to examine the effects of SSA genotype and plant nutrition on a specific, endogenous cysteine-rich protein, TIA was compared in the experimental groups (Fig. 4). The most important effect in the full ANOVA model was that of genotype ($F_{1,16}$=183.24, P<0.001), demonstrating a dominant effect of the expression of the SSA gene on seed TIA. However, although TIA was lower in line 45S than in cv. Semsen in all treatments (P<0.05, Fig. 4), the magnitude of this difference was related to the levels of both nitrogen and sulphur in the nutrient (Genotype×N×S interaction $F_{1,23}$=12.77, P<0.01). The difference between TIA of cv. Semsen, and line 45S was least in the LN-HS treatment (1.88 units μg^{-1} protein, Fig. 4), in which the sulphur-to-nitrogen ratio in the nutrient was highest. Genotype also had the dominant effect in the reduced ANOVA model in which TIA of cv. Semsen and lines 45S and 161S grown at HN-LS and HN-HS were compared ($F_{2,12}$=153.6, P<0.001). At HN, differences among genotypes were weakly dependent on level of S (Genotype×S interaction $F_{2,12}$=6.66, P<0.05). Both transgenic lines had lower levels of TIA than cv. Semsen in both treatments (P<0.01), but TIA in line 161S (that expressed the higher level of SSA) was significantly less than in line 45S only in the low S treatment. Finally, there was some evidence that the difference between cv. Semsen and line 161S was higher in the low S treatment (5.89 units μg^{-1} protein) than in the high S treatment (4.93 units μg^{-1} protein, Fig. 4), although this effect was of only marginal significance (P=0.06).

Sulphur and nitrogen metabolites in non-transgenic and transgenic chickpeas

Potential signalling metabolites were quantified in embryos of developing chickpea seeds at mid-maturation (25 daa).

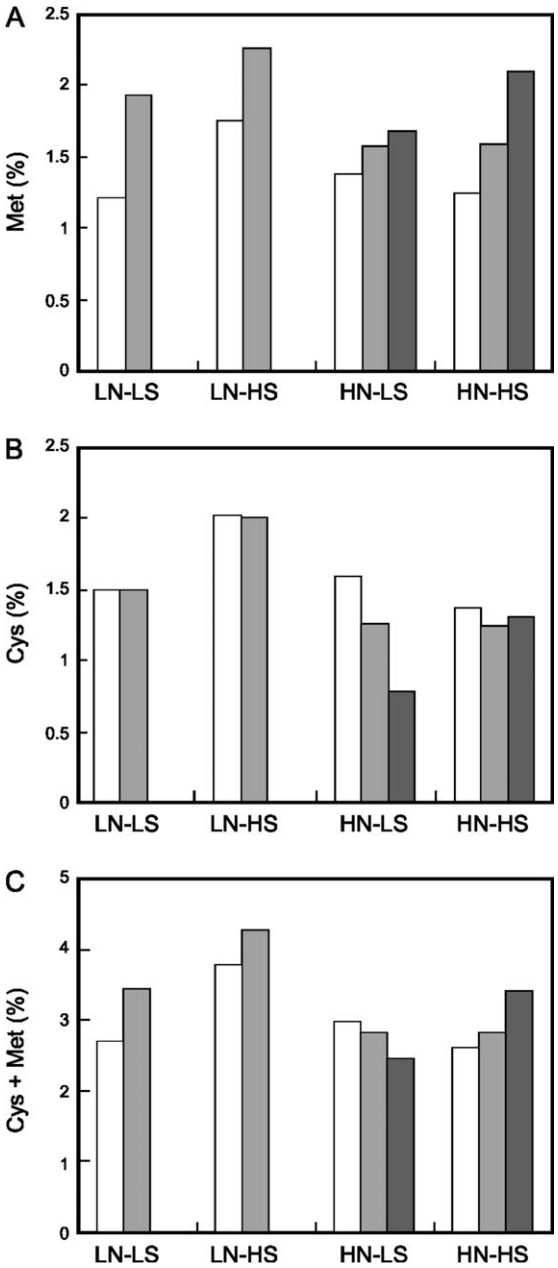

Fig. 3. Effects of sulphur and nitrogen nutrition and SSA genotype on the total methionine (A), cysteine (B), and sulphur amino acid (C) composition of mature chickpea seeds from cv. Semsen (open bars), transgenic line 45S (light grey bars), and transgenic line 161S (dark grey bars). Each value represents a single determination on a pooled flour sample from mature seed from three to four individual plants from each experimental group.

Free methionine and OAS were determined independently in extracts from three separate cotyledons from a pooled sample of mid-maturation embryos for each experimental group for cv. Semsen and transgenic line 45S (Fig. 5). Because it could not be guaranteed that individual cotyledons from the pooled sample were from different plants, statistical analysis of the data was not possible. The following trends were observed. OAS was consistently

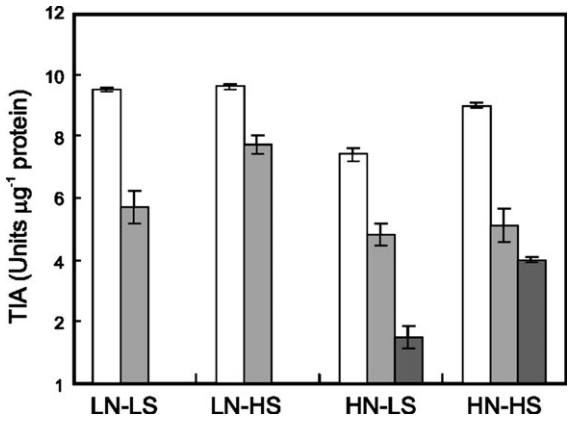

Fig. 4. Effects of sulphur and nitrogen nutrition, and SSA genotype on the TIA in mature chickpea seeds from cv. Semsen (open bars), transgenic line 45S (light grey bars), and transgenic line 161S (dark grey bars). Each value is the mean (±SE) of determinations on seed flour from three individual plants.

higher in the 45S samples compared with the non-transgenic controls in all experimental groups (Fig. 5A). This difference seemed to be greatest in the LN-LS treatment. The effects of plant nutrition on OAS levels in the developing embryos seemed to be relatively minor. In the non-transgenic genotype, OAS tended to be highest in cotyledons from the LN-HS treatment, but varied relatively little between the other nutritional treatments. Free methionine was not consistently different in the transgenic samples compared with the cv. Semsen samples, but its levels in embryos of both genotypes were highest in the LN-HS samples (Fig. 5B). Glutathione was measured in pools of powdered, freeze-dried 25 daa embryos, and in extracts from samples of the same pooled, mature seed flour that was used for the determination of total amino acid composition. The concentration of glutathione did not vary in a consistent pattern between the control and transgenic genotypes, or with plant nutrition, although it appeared to be lowest in mature seeds from the HN-LS treatment, reflecting the low sulphur status of those plants (Table 5).

Discussion

The introduction of a seed-expressed transgene for a methionine- and cysteine-rich protein into chickpea led to increased accumulation of sulphur amino acids in mature seeds of the transgenic lines. This result indicates that, at a given level of sulphur supply to the plants, sulphur sink strength limited the accumulation of sulphur amino acids in the storage protein of the unmodified control seeds. Furthermore, it suggests that developing chickpea seeds have some capacity to increase their rate of sulphur assimilation and sulphur amino acid biosynthesis in response to an added demand. Despite this, there were changes in the abundance of endogenous, cysteine-rich proteins in the transgenic chickpea seeds that were reminiscent of responses to sulphur stress.

The results presented here demonstrate the generality of similar effects previously reported for another grain legume, narrow leaf lupin, transformed with SSA (Tabe and Droux, 2002). In the case of lupins, evidence was presented that the developing embryos were capable of sulphur assimilation. Chickpea developing embryos also contain high activities of the two enzymes of the cysteine synthase complex, serine acetyltransferase and OAS (thiol) lyase (P Chiaiese, unpublished results). Thus, in both chickpeas and lupins, the expression of a heterologous sulphur-rich protein was associated with an increase in the concentration of sulphur amino acids in seed protein, most probably due to the up-regulation of sulphur amino acid biosynthesis in the developing seed. Sulphur was apparently supplied from existing oxidized sulphur pools in the legume seeds, rather than by increased sulphur transport into the seed, because total seed sulphur concentration was not increased in the transgenic seeds. As well as additional reduced sulphur accumulation, there were also apparent sulphur-stress responses in the composition of endogenous storage proteins in both types of SSA-containing legume seeds. It has previously been reported that accumulation of SSA in a transgenic cereal, rice, was also associated with major alterations in the relative abundance of endogenous seed storage proteins; however, in the case of the transgenic SSA rice, there was no increase in total sulphur amino acid concentration in the seed protein. It therefore seems that SSA expression triggered a sulphur-stress response in seed protein composition in rice, but not an increase in the capacity for the import or biosynthesis of sulphur amino acids. Unlike the lupins and chickpeas, mature, non-transgenic rice grains did not contain detectable pools of oxidized sulphur that could potentially provide additional reduced sulphur for incorporation into SSA (Hagan *et al*., 2003). Further investigation of other cereals will be needed to determine whether these observations represent fundamental differences between different types of seeds.

The interactions reported here between the effects of plant sulphur and nitrogen nutrition and the effects of SSA genotype on chickpea seed composition provide support for the hypothesis that seed responses to both sulphur sink manipulation and to sulphur nutritional stress are mediated by the same signal transduction pathway. Firstly, it was found that there was significant interdependence of the effects of sulphur and nitrogen nutrition on chickpea seed composition. This was most dramatically illustrated by the high concentrations of oxidized sulphur in chickpea seeds

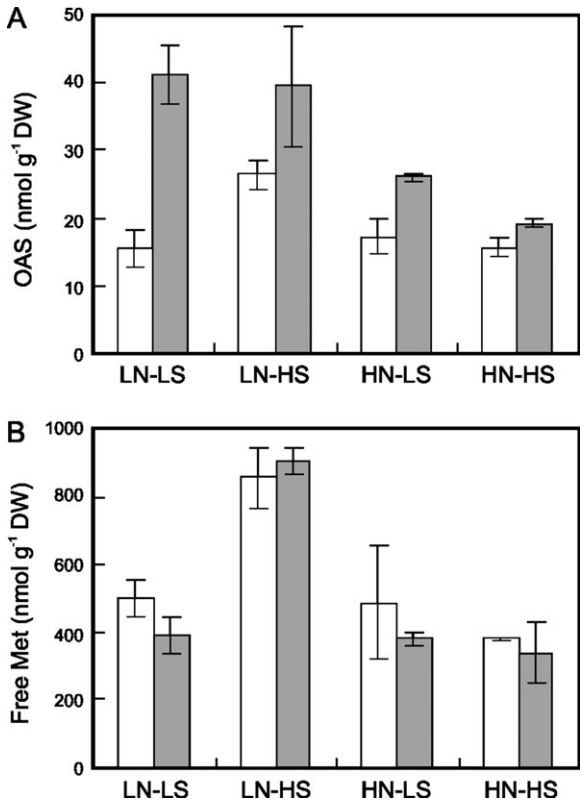

Fig. 5. Effects of sulphur and nitrogen nutrition and SSA genotype on concentrations of OAS (A), and free methionine (B), in mid-maturation chickpea embryos from cv. Semsen (open bars) and transgenic line 45S (light grey bars). Values are the mean (±SE) of determinations on three individual cotyledons for each experimental group.

Table 5. *Glutathione in mid-maturation embryos and mature seed of transgenic chickpeas expressing SSA*

Glutathione was quantified in a pool of freeze-dried developing embryos using reverse phase HPLC after derivatization with mBBr. Each value represents the mean ±SE of determinations on three different extractions from a single pool of freeze-dried material consisting of 25–35 developing embryos at 25 daa (nm, not measured). Glutathione was also determined in flour from the same mature seed pools (representing three to four plants for each genotype and treatment) that were analysed for total amino acid composition (Fig. 3). Each value is the mean ±SE of three measurements on one extract.

Treatment	Glutathione (μmol g^{-1} DW)					
	Developing embryos			Mature seeds		
	Semsen	45S	161S	Semsen	45S	161S
LN-LS	nm	nm		2.0±0.16	2.1±0.29	
LN-HS	nm	nm		2.3±0.12	2.7±0.23	
HN-LS	2.2±0.10	3.9±0.16	2.4±0.38	1.50±0.21	1.5±0.36	1.4±0.20
HN-HS	2.2±0.09	3.1±0.10	4.6±0.12	2.39±0.40	2.0±0.08	2.0±0.17

grown in the nutrient with the highest ratio of sulphur to nitrogen (LN-HS treatment). Total seed sulphur (the sum of reduced and oxidized sulphur) was clearly highest in the seeds from the LN-HS treatment. Total sulphur concentrations for seeds of cv. Semsen were 79.5, 130.7, 79.0, and 106.8 μmol g^{-1} DW, respectively, for LN-LS, LN-HS, HN-LS, and HN-HS treatments, with similar respective levels for line 45S. These results imply that transport of sulphur into the developing seeds of both genotypes was greater in the LN-HS treatment than in the other nutritional environments. Oxidized sulphur levels were similarly high in other organs of the LN-HS plants (data not shown) indicating that this effect was systemic, rather than being restricted to the developing seed. The finding that seed total sulphur concentration was higher at LN-HS than at HN-HS indicates that this was an effect of the sulphur-to-nitrogen ratio rather than the sulphur level in the nutrient per se. The proportions of total sulphur amino acids in seed protein also tended to be higher in seeds of control and transgenic genotypes in the LN-HS treatment than in any of the other treatments (Fig. 3). This indicates that plant sulphur and nitrogen nutrition had a dominant effect on total seed sulphur content, whereas SSA affected the distribution of this sulphur between different pools in the seed.

There were strong effects of the SSA genotype on seed sulphur composition, and these were influenced by plant nutrition. The concentrations of reduced sulphur in the chickpea seeds of transgenic line 45S were higher than those in the cv. Semsen seeds in all four nutritional regimes. However, these differences were greatest in the LN-HS treatment in which the sulphur-to-nitrogen ratio in the nutrient was highest (Fig. 2). Interactions of SSA genotype and plant nutrition were also evident in the proportions of methionine and cysteine in the seed protein of the high SSA expressing line, 161S, for which the increase in seed protein methionine relative to the non-transgenic controls appeared to be greater at HN-HS than at HN-LS. Conversely, seed protein cysteine appeared to be decreased in line 161S compared with the non-transgenic controls to a greater extent in the HN-LS treatment than in HN-HS (Fig. 3). TIA, representing a single type of endogenous cysteine-rich protein, proved to be a sensitive indicator of interactions between the effects of SSA genotype, sulphur nutrition, and nitrogen nutrition. The decreases in TIA in the transgenic seeds, compared with the non-transgenic controls, depended on both the levels of sulphur and nitrogen in the nutrient in the case of line 45S, and on the level of sulphur in the nutrient in the case of line 161S (for which data were not available in the LN treatments; Fig. 4).

The modification of seed responses to the presence of SSA by plant sulphur and nitrogen nutrition suggested that similar metabolic signals may mediate both kinds of responses. The concentrations of metabolites that have been reported to act as signals in the transduction pathway that mediates plant response to sulphur nutrition, were investigated. Free methionine has been reported to act as a signal of sulphur sufficiency that enhanced expression of sulphur-rich seed storage proteins and decreased expression of the sulphur-poor β-subunit of β-conglycinin in cultured soybean cotyledons (Holowach *et al*., 1984*a*, *b*, 1986). On the other hand, OAS was reported to act as a signal of sulphur deficiency that enhanced expression of the soybean conglycinin β-subunit, and decreased the expression of the sulphur-rich glycinin (Kim *et al*., 1999). Glutathione has been reported to act as a signal of plant sulphur sufficiency that down-regulates sulphur assimilation and sulphur uptake by roots (Lappartient and Touraine, 1997; Lappartient *et al*., 1999).

A striking result of this study was the consistently increased levels of OAS in mid-maturation transgenic embryos expressing SSA (Fig. 5), which correlated generally with the consistent decreases in TIA in the mature transgenic seeds (Fig. 4). These results suggest that OAS has a role in mediating the changes in the abundance of endogenous cysteine-rich proteins that accompany SSA accumulation in transgenic chickpea seeds. OAS has also been reported to vary with plant sulphur nutrition. On the basis of reports that OAS was elevated in siliques of *Arabidopsis thaliana* plants grown in a hydroponic medium with high nitrogen and low sulphur (Kim *et al*., 1999), it had been expected that OAS would be highest in chickpea embryos in the HN-LS treatment. Instead, OAS in the non-transgenic embryos varied relatively little between nutritional treatments. This apparent discrepancy may be explained by the different relative levels of sulphur and nitrogen used in the two studies. In the current study, the low sulphur concentration was 0.2 mM while the high nitrogen concentration was 9 mM (molar ratio of sulphur to nitrogen=0.02). In the work of Kim *et al*. (1999), the lowest sulphur concentration was 0.03 mM, while the highest nitrogen concentration was 5 mM (molar ratio of sulphur to nitrogen=0.006). An additional difference between the two studies was that the current work measured OAS in developing chickpea embryos, which may be more buffered against nutritional changes in the rest of the plant than whole siliques. By contrast with OAS, free methionine varied relatively little between transgenic chickpea embryos expressing SSA and the corresponding non-transgenic controls. On the other hand, free methionine concentrations were quite clearly higher in the embryos of both chickpea genotypes grown in the LN-HS nutrient that had a high sulphur-to-nitrogen ratio. Glutathione levels did not vary consistently between control and transgenic developing embryos, or mature seeds (Table 5), but tended to be lower in mature seeds of both genotypes in the HN-LS nutritional regime.

The following model is proposed to explain the effects of increased sulphur demand in the transgenic lines, and of sulphur and nitrogen nutrition on chickpea seed composition.

It is suggested that synthesis of SSA in seeds of the transgenic lines sequestered free cysteine and methionine, generating a signal of sulphur deficiency in the transgenic seeds. This immediate signal was most probably a drop in the concentration of one of the free sulphur amino acids, or possibly in glutathione. No consistent drop in the concentration of glutathione was detected in the transgenic embryos compared with the controls, but slight changes, transient changes, or changes in specific subcellular compartments, cannot be excluded. Slight decreases in free methionine were observed in the transgenic seed extracts in most nutritional treatments, but the statistical significance of these could not be established. Free cysteine was quantified in extracts from the mid-maturation embryos, but the concentrations were below the reliable detection limit of this analysis. Sulphur nutritional deficiency has previously been reported to have a stronger negative effect on free cysteine than on free methionine concentration in developing pea seeds (Macnicol, 1983).

Recently, it has been proposed that, in the case of sulphur nutritional deficiency, an unknown primary signal stimulates a change in cytosolic calcium concentration, which results in the desensitization of cytoplasmic serine acetyltransferase to feedback inhibition by cysteine, leading to over-accumulation of OAS (Saito, 2000). This study's data for demand-generated sulphur deficiency are consistent with this model, with the primary signal being the depletion of a reduced sulphur metabolite. Alternatively, depletion of cytoplasmic cysteine by SSA expression could lead directly to an increase in OAS via de-represssion of serine acetyltransferase. Elevated OAS would then mediate downstream changes in the expression of genes of the sulphur-assimilation pathway and of genes encoding endogenous storage proteins. The decrease in levels of the representative, endogenous, cysteine-rich protein, TIA in transgenic chickpea seeds was mitigated to some extent by growing the plants in nutrient with a high sulphur-to-nitrogen ratio. Free methionine was clearly higher in chickpea embryos of both transgenic and non-transgenic genotypes in this nutritional environment, suggesting this metabolite as a mediator of the effects of plant sulphur and nitrogen nutrition on seed responses to SSA. It is, therefore, suggested that the level of TIA and, by inference, of other sulphur-rich proteins in transgenic chickpea seeds with altered sulphur demand, would be regulated by both OAS and free methionine in a converse manner to that suggested for the sulphur-poor β-conglycinin in recent studies using cultured, developing soybean cotyledons, and transgenic *A. thaliana* with altered sulphur supply (Hirai *et al*., 2002). In this study, the dominant influence on the proportion of methionine in total seed protein was SSA itself, thereby masking any effect of these proposed regulatory mechanisms on endogenous methionine-rich proteins.

In the future, the role of OAS in mediating responses to sulphur sink manipulation in transgenic seeds will need to be validated in other experimental systems. Towards that end, preliminary data from developing rice endosperm indicate that OAS is greatly elevated in transgenic rice grains expressing SSA, compared with non-transgenic controls (L Tabe, N Ohkama-Ohtsu, unpublished results). A role for free methionine in modulating responses to sulphur sink manipulation in transgenic seeds is supported by a brief report that addition of free methionine to cultured cotyledons reversed the apparent sulphur-stress responses in endogenous seed protein composition that accompanied expression of a sulphur-rich Brazil nut protein in transgenic soybean (Jung *et al*., 1997). It has recently been reported that *S*-adenosylmethionine, not methionine itself, regulates methionine biosynthesis by modulating the activities of cystathionine γ-synthase, and threonine synthase, via distinct, post-transcriptional mechanisms (Kim *et al*., 2002; Chiba *et al*., 2003; Curien *et al*., 1998). Although there is some contrary evidence from studies with methionine analogues in cultured soybean cotyledons (Creason *et al*., 1985), the possibility that *S*-adenosylmethionine is involved in the regulation of storage protein gene expression should also be reconsidered.

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