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RESEARCH PAPER

Free amino acid profiles suggest a possible role for asparagine in the control of storage-product accumulation in developing seeds of low- and high-protein soybean lines

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Abstract

Several approaches were taken to examine the role of N-assimilate supply in the control of soybean (*Glycine max*) seed composition. In the first study, developing seeds were grown *in vitro* with D-[U-^{14}C]sucrose (Suc) and different concentrations of Gln. Light stimulated carbon flux into oil and protein, and was required to sustain Suc uptake and anabolic processes under conditions of elevated nitrogen supply. High Gln supply resulted in higher transcript levels of β-conglycinin and oleosin. In the second study, analyses of soluble amino acid pools in two genetically related lines, NC103 and NC106 (low- and high-seed protein, respectively) showed that, in the light, NC106 accumulated higher levels of Asn and several other amino acids in developing cotyledons compared with NC103, whereas at the seed coat and apoplast levels both lines were similar. In the dark, NC103 accumulated Gln, Arg, and its precursors, suggesting a reduced availability of organic acids required for amino acid interconversions, while NC106 maintained higher levels of the pyruvate-derived amino acids Val, Leu, and Ile. Comparing NC103 and NC106, differences in seed composition were reflected in steady-state transcript levels of storage proteins and the lipogenic enzyme multi-subunit acetyl CoA carboxylase. In the third study, a positive correlation ($P \leqslant 0.05$) between free Asn in developing cotyledons and seed protein content at maturity was confirmed in a comparison of five unrelated field-grown cultivars. The findings support the hypothesis that high seed-protein content in soybean is determined by the capacity of the embryo to take up nitrogen sources and to synthesize storage proteins. Asn levels are probably tightly regulated in the embryo of high-protein lines, and may act as a metabolic signal of seed nitrogen status.

Key words: Amino acid profile, carbon partitioning, seed nitrogen supply, seed protein content, soybean.

Introduction

The agronomic productivity of soybean plants is dependent upon their capacity to partition a significant proportion of assimilates to the seeds, and the economic value of the crop is directly related to the seed composition. In particular,

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Abbreviations: BCCP, biotin carboxyl carrier protein; FAD2-1, omega 6-desaturase; FW, fresh weight; GABA, γ-amino butyric acid; MS-ACCase, multi-subunit acetyl CoA carboxylase; SPC, seed protein content; Suc, sucrose; TAG, triacylglycerol.

seed protein content (SPC) is often one of the main determinants of crop value. Most varieties of soybean grown in south-eastern United States have an SPC in the 40–42% range, but recurrent selection programmes have generated germplasm lines with 48–50% of SPC at maturity (Carter *et al.*, 1986). However, enhancement of SPC is associated with a decrease in oil concentration [mainly triacylgycerols (TAG)] and seed yield (Brim and Burton, 1979). Increasing SPC, while maintaining oil content and yield, is an important objective for soybean breeding programmes. A fundamental understanding of the biological mechanisms that control the accumulation of protein and oil during seed development may contribute to this goal. A major question is whether SPC is controlled by the maternal plant (e.g. supply of nitrogenous assimilates) or by the developing seed itself (e.g. intrinsic capacity to synthesize storage proteins versus oil). Even though molecular genetic approaches have been used to modify seed components in different species (Voelker *et al.*, 1996; Mazur *et al.*, 1999; Ye *et al.*, 2000), and the metabolic pathways of the major storage compounds (protein, oil, and starch) are well described, the mechanisms determining the differential partitioning of seed reserves into the major storage components remain largely unknown.

In the early 1980s, physiological and biochemical studies of assimilate transport into soybean fruits confirmed that the developing seed is physically disconnected from the maternal plant (Thorne, 1980, 1981). The seed coat (maternal tissue), with specialized transport and metabolic processes, releases assimilates to the apoplast for uptake into the embryonic cotyledons. Sucrose (Suc) and amino acids, mainly Asn and Gln, are the primary sources of carbon and nitrogen available to the developing soybean embryo *in planta* (Hsu *et al.*, 1984; Rainbird *et al.*, 1984). Gln is the preferred source of nitrogen for developing seeds grown *in vitro* [*c.* 50 mg fresh weight (FW) of seeds at the start of cultures], and the developing cotyledons have limited capacity for assimilation of other forms of nitrogen, and essentially no capacity for assimilation of inorganic nitrogen (Thompson *et al.*, 1977; Haga and Sodek, 1987).

Conversion of the amide amino acids, Asn and Gln, to the other amino acids required for storage-protein synthesis demands carbon skeletons derived from metabolism of Suc imported from the phloem. The breakdown of Asn is catalysed by asparaginase, which releases ammonia for reassimilation via the Gln synthetase reaction. In the case of Gln, its amide-N group must first be donated to a suitable C-skeleton acceptor molecule, such as 2-oxoglutarate, to form Glu, which can then be utilized further in the formation of other amino acids. Oil synthesis also utilizes metabolites derived from Suc breakdown. Thus, storage-protein synthesis and oil/starch synthesis both require C-skeletons derived from imported Suc during seed development, and the often observed negative relationship between seed storage protein and oil concentration may be related to the regulation of carbon flux between these competing synthetic pathways. A better understanding of how carbon flux between protein and oil synthesis is regulated may lead to the development of molecular strategies to improve soybean seed quality.

Previous physiological studies using soybean cultivars of different SPC have shown that the negative relationship between SPC and yield is related to a faster rate of nitrogen partitioning and dry matter allocation into seeds in high-protein cultivars, as well as a shorter duration of seed filling (Salado-Navarro *et al.*, 1985). Further studies using *in vitro* culture of soybean seeds have suggested that SPC is regulated by the supply of nitrogenous substrate available to the developing seed, and that soybean seeds have the intrinsic capacity to synthesize high protein concentrations if sufficient substrate is available (Saravitz and Raper, 1995). In a different study, Hayati *et al.* (1996) showed that a minimal supply of nitrogen is required to sustain maximum growth rate of soybean cotyledons grown *in vitro* for up to 21 d. They also observed that a high-protein genotype accumulated higher nitrogen content in cotyledons than a normal genotype at any level of nitrogen supplied in the media, suggesting that differences in SPC are regulated by the embryo.

The overall goal of the present study was to elucidate the biological mechanisms that regulate protein and oil biosynthesis in developing soybean seeds with a specific focus on the role of amino acids. In particular, the aim was to determine whether N-assimilate supply could influence the use of carbon derived from Suc for protein versus oil synthesis, and, if so, whether SPC was related to the supply of N-assimilates from the maternal plant or was correlated with the concentration of amino acids within the developing cotyledons. To do this, N-assimilate supply *in vivo* to developing seeds of two closely related genotypes, differing in protein content at maturity, was evaluated by analysing the profile and concentration of amino acids in the maternal tissues (seed coat and apoplast) and the filial soybean cotyledons at mid-maturation stage. The analyses show that high SPC at maturity is specifically associated with higher contents of Asn within the developing cotyledons. In a separate series of experiments, *in vitro*-cultured seeds were used (Saravitz and Raper, 1995) to examine the influence of the Gln concentration and light on the partitioning of radiolabel from [U-^{14}C]Suc between protein and oil.

Materials and methods

Plant growth and in vitro *seed culture*

Soybean plants [*Glycine max* (L.) Merr. cv. Ransom II or cv. NC103 and NC106] were grown in growth chambers at the Phytotron facility of North Carolina State University as previously described (Raper *et al.*, 1984). Ransom II protein and oil content has been established

as 41.9% and 19.8%, respectively, of total seed dry weight. Standard values for NC103 are 44.9% protein and 17.7% oil, and 50.4% protein and 15.2% oil for NC106 (Carter *et al.*, 1986). Developing soybean seeds were grown *in vitro* according to Obendorf *et al.* (1984). Between 15 and 20 d after flowering, immature soybean pods were harvested, surface-sterilized for 5 min with 0.5% bleach, washed five times for 5 min in sterile distilled water, and dissected to extract the seeds. Seeds were weighed and immediately transferred to test tubes containing culture medium (Obendorf *et al.*, 1983) over folded filter paper (static cultures). Rapid expansion of the cotyledons resulted in the rupture of the seed coat and release of the embryo within the first 24 h of culture. Glu was added to all treatments at 10% of the Gln concentration. Seed FW ranged from 50 to 150 mg, depending on the experiment. Cultures were placed in growth chambers at 26 °C under continuous light (85 μmol m^{-2} s^{-1} of cool and incandescent light) or continuous dark. At the end of each experiment, seeds were weighed, and immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. Seeds collected from plants growing in the field were harvested 21 d after flowering from the top of the canopy, peeled out of the pods *in situ*, rapidly frozen in liquid nitrogen, and stored at −80 °C until further analysis.

Growth conditions were optimized prior to using cultured seeds for metabolic studies. The optimal Suc concentration was found to be about 75 mM, which is equivalent to about 2.5% (w/v), and is a common moderate level often used in mixotrophic tissue cultures. A concentration of 60 mM Gln stimulated growth without undesired osmotic stress side-effects and was considered as the high-level nitrogen treatment. A concentration of 6 mM Gln was selected as the condition of low nitrogen supply, since 30 mM Gln still yielded optimal growth. In the present experiments, seeds grew well under continuous dark conditions for at least 48 h. Raper *et al*. (1984) reported no or very slow growth under these conditions, but only measured growth after 15 d.

Apoplastic seed coat exudates

Apoplast exudates produced by the seed coat were collected into Phytagel agar traps for 3 h, as described in Thorne and Rainbird (1983). To obtain apoplastic seed coat exudates, cotyledons were carefully removed from the seed, quickly frozen in liquid nitrogen and kept at −80 °C until further analyses. The remaining empty seed coat was filled with *c.* 100 μl of 4% Phytagel containing 25 mM EGTA. The Phytagel was kept warm in a water bath at about 40 °C, and solidified upon use inside the seed coat. To insert the agar trap, seed coats were cut and the cotyledons removed within 5 min or less. The seed coat was covered back with the pod wall and wrapped in Parafilm. The first surgery was performed after plants were exposed to 5 h of light in the growth chamber, with 12 seeds being handled in 30 min. The Phytagel traps were left for 3 h.

Free amino acid extraction and analysis

Frozen cotyledons, seeds, seed coats, or Phytagel traps were placed in a 2 ml tube containing ceramic beads (Q-Biogene, Carlsbad, CA), and 800 μl methanol:chloroform:water (12:5:3, v/v) as previously described (Paquin and Lechasseur, 1979; Hernández-Sebastià *et al.*, 2001). The tubes were placed in a FastPrep Instrument (Q-Biogene) to pulverize the material for 20 s. Samples were centrifuged 10 min at 17 530 *g*. The supernatant was transferred to a new tube, and 213 μl of water and 53 μl of chloroform were added to separate the phases. Samples were then centrifuged 10 min at 17 530 *g*, 4 °C. The upper phase was transferred to a fresh tube and lyophilized. Lyophilized extracts were dissolved in 100 μl of lithium buffer pH 2.2, containing 50 mM citric acid, 200 mM lithium hydroxide, 10 mM phenol, 2% (v/v) thiodiglycol, and 1.6% (v/v) hydrochloric acid. Sulphosalicylic acid (2.5 mg) was added to precipitate proteins. The solution was centrifuged for 5 min at 358 *g* and the supernatant filtered through a 0.2 μm nylon mesh before injection in HPLC. A sample of 20–80 μl of deproteinized supernatant was charged on a cation exchange column of 8 μm with an elution gradient of five citrate lithium buffers, as described in the manual of standard LKB Alpha plus 4151 amino acid analyser used with a Hewlett Packard 3392A integrator. Amino acid analyses were carried out by ion exchange chromatography on sulphonated polystyrene resin linked to divinyl benzene. The amino acid analyses were conducted at the Réseau de Médecine Génétique du Québec, Centre Universitaire de Santé de l'Estrie, Sherbrooke, Québec, Canada.

RNA isolation and northern blots

Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Ten micrograms of cytoplasmic RNA was subjected to electrophoresis in a 1.5% agarose–formaldehyde gel and transferred to nylon membranes (Schleicher & Schuell, Dassel, Germany). ^{32}P-Labelled random-primed DNA probes were generated using the High Prime DNA Labeling Kit (Roche Diagnostics, Indianapolis, IN) and purified using NucTrap Probe purification columns (Stratagene, La Jolla, CA). Hybridization was performed overnight at 65 °C in a buffer containing 10% dextran sulphate, 1× SSPE, 1% SDS, 100 μl salmon sperm DNA (from 10 mg ml^{-1} of stock), and 5× Denhardt's solution (Sambrook *et al*., 1989). The membranes were washed at 65 °C in 100 ml of 2× SSPE and 0.1% SDS for 20 min, followed by 1× SSPE and 0.1% SDS for 20 min, 0.5× SSPE and 0.1% SDS twice for 20 min, and a final wash of 0.1× SSPE and 0.1% SDS for 15 min.

Experimental design for radiolabelling experiments

Immature soybean seeds growing in culture were labelled with 0.5 μCi [U-^{14}C]Suc (NEN Life Science Products, Boston, MA) per 10 ml of medium. A factorial experiment in a Randomized Complete Block was designed with three replicates per treatment. Factors were Gln (high 60 mM and low 6 mM), time course (6, 24, and 48 h), and light conditions (continuous light versus continuous dark). Suc levels were kept at 75 mM in all treatments. Tubes were gently shaken once a day to avoid nutrient depletion around the seed.

Incorporation of [^{14}C]Suc in lipids and proteins

[U-^{14}C]Suc-labelled seeds were ground in a cold mortar with liquid nitrogen until a fine powder was obtained. Half of the powdered seed was transferred to a chilled 2 ml microcentrifuge tube for lipid extraction, and the other half was used to extract proteins. Total lipids were extracted in 1 ml of chloroform:methanol (2:1, v/v), mixed by vortexing, and incubated for 15 min at room temperature. Samples were centrifuged for 10 min at 22 900 *g*. The supernatant was collected in a new tube, and 0.75 ml water was added. The mixture was mixed vigorously, and centrifuged 10 min at 22 900 *g*. The total volume of the upper phase corresponding to the methanol/water fraction (about 1.3 ml) was used for counting. Half of the lower phase containing the chlorophyll pigments and corresponding to the lipid fraction (about 500 μl) was used for counting, and the remaining half was used for TLC. The pellet was resuspended directly into scintillation cocktail. Proteins were extracted in 50 mM TRIS–HCl pH 7.5, 10 mM dithiothreitol, 0.5 M Suc, 5 mM *p*-aminobenzamidine, and 5% (w/v) insoluble polyvinylpolypyrrolidone prepared in cold, sterile deionized water. Protein concentration was estimated using the Bradford assay (Bio-Rad, Hercules, CA) for microtitre plates, with bovine serum albumin as standard. To measure the disintegrations per minute (dpm) present in 50 μg protein, the corresponding volume was transferred to a new tube, and proteins were precipitated by adding trichloroacetic acid to a final concentration of 7.2%, followed by centrifugation at 17 530 *g* for 15 min at 4 °C. The pellet was washed with 500 μl of acetone to remove residual trichloroacetic acid, air-dried,

resuspended in 10 µl of 50 mM TRIS–HCl pH 7.5, and the dpm counted in a scintillation counter.

TLC of neutral lipids

Prior to loading, TLC plates of 60 Å silica gel (LK6D 20×20 cm, Whatman, Clifton, NJ) were baked 1 h at 110 °C, and cooled down to 30–40 °C in a chamber containing desiccant. Samples were evaporated under nitrogen at 35–40 °C, and resuspended in 50 µl chloroform:methanol (2:1, v/v), of which 25 µl were used for TLC. The solvent system consisted of petroleum ether:anhydrous ether: glacial acetic acid (80:20:1, by vol.). Phosphatidylcholine (10 µg μl^{-1}), tristearin (25 µg μl^{-1}), dipalmitin (25µg μl^{-1}), and branched chain fatty acid methyl ester (25 µg μl^{-1}) were used as standards, and 5 µl of each were loaded. TLC plates were developed by spraying with 0.2% (w/v) 2-7-dichlorofluorescein in 95% ethanol. Chromatograms were visualized in a UV lamp chamber. TLC plates were scanned to obtain counts per minute with a scanner (System 200, Bioscan Inc., Washington, DC), and then exposed to film for 3 weeks to confirm the radioactive profile obtained with the scanner.

Statistical analysis

Analysis of variance (ANOVA) and orthogonal contrasts were conducted using the SuperANOVA statistical program (Abacus Concepts, Berkeley, CA). To satisfy the assumptions of the ANOVA test, the homogeneity of the variances was visually inspected by residual graphic analysis. When variances were not homogeneous, the variables were transformed as log (x+1), or raised at the power of two (x^2), depending on the distribution of residuals. In cases where the homogeneity of the variances was not acceptable after mathematical transformation of the values, the equivalent of non-parametrical statistics were applied, in the form of the rank transformation of means in the parametric test ANOVA [Rank (x, Allrows) for the SuperANOVA program] (Conover, 1980). In this case, the assumption of the normal distribution of the errors is no longer required. In all the analyses, the independence of errors in the ANOVA test was satisfied by a randomized sampling for each treatment. Correlations between variables were analysed using Spearman's rank-order correlation. The test was carried out in Excel by ranking the data and performing a normal correlation as described in Dytham (1999).

Results

Amino acid supply in planta

To investigate the role of amino acids *in situ*, the free content of 32 amino acids in cotyledons, apoplast, and seed coat was analysed at different times of the day/night cycle. In these experiments, two closely related genotypes differing in SPC (NC103 with 44.9% considered low protein and NC106 having 50.4% high protein) (Carter *et al.*, 1986) were grown in chambers and developing pods were sampled about 15 d after flowering. The aim was to determine whether high SPC at maturity was associated with higher contents of specific amino acids during cotyledon development and, if so, at what position in the transport pathway.

Seed coat

Twenty-two different amino acids were detected in the seed coat tissues of NC103 and NC106 (Table 1). The major amino acid, Asn, accounted for about 38% of the free amino acid pool in the seed coat. No significant differences in Asn content were observed between cultivars or as a function of the photoperiod. This was the case for most of the amino acids measured with only a few exceptions. Four

Table 1. *Amino acid content (nmol mg^{-1} FW) in seed coats of soybean embryos harvested 15 d after flowering*

Seed coat was about 35 mg FW. Values in bold are statistically significant with the probabilities shown below.

Cultivar	Photoperiod	Asn	Gln	Glu	Arg	His	Citrulline	Asp	GABA	Ala	Pro	Gly	Thr	**Ser**
NC 103	9 h light	14.1	**6.4**	**3.0**	1.1	0.8	0.5	1.5	5.9	**1.9**	0.37	**0.74**	**0.83**	**1.40**
	3 h dark	12.7	**1.8**	**3.1**	1.2	0.3	0.4	1.6	3.8	**1.1**	0.27	**0.37**	**0.35**	**0.95**
NC 106	9 h light	13.9	**6.7**	**1.4**	1.1	0.8	0.5	1.3	8.1	**1.7**	0.40	**0.50**	**0.90**	**1.70**
	3 h dark	12.1	**2.4**	**1.1**	1.4	0.6	0.2	1.1	4.5	**0.9**	0.23	**0.26**	**0.51**	**1.09**
Source of variation	d.f.	ANOVA P values												
Cultivar (C)	1	n.s.	**0.005**	**0.03**	n.s.	n.s.	n.s.	n.s.	n.s.	**0.01**	n.s.	n.s.	n.s.	n.s.
Photoperiod (P)	1	n.s.	**0.0001**	n.s.	n.s.	n.s.	n.s.	n.s.	0.08	**0.0009**	n.s.	**0.03**	**0.03**	**0.01**
C×P	1	n.s.	0.06	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Error	5													

Cultivar	Photoperiod	Val	Met	Ileu	Leu	**Tyr**	Phe	Lys	Orn	ANBA	Total amino acids
NC 103	9 h light	0.49	**0.14**	0.20	0.23	**0.06**	0.20	0.31	0.06	0.31	**40.2**
	3 h dark	1.55	**0.00**	0.04	0.10	**0.00**	0.04	0.32	0.04	0.14	**28.8**
NC 106	9 h light	0.47	**0.17**	0.20	0.24	**0.07**	0.17	0.38	0.09	0.44	**41.3**
	3 h dark	0.34	**0.09**	0.17	0.26	**0.00**	0.11	0.46	0.09	0.26	**28.1**
Source of variation	d.f.	ANOVA P values									
Cultivar (C)	1	n.s.	**0.0001**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Photoperiod (P)	1	n.s.	**0.0001**	n.s.	n.s.	**0.03**	n.s.	n.s.	n.s.	n.s.	**0.01**
C×P	1	n.s.	**0.0001**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Error	5										

ANBA, amino *N*-butyric acid; n=2; d.f., degrees of freedom; n.s., not significant (P >0.05).

amino acids were slightly different between the mother tissues of cultivars NC103 (low-protein line) and NC106 (high-protein line): Gln, Glu, Ala, and Met. Gln varied as a function of the photoperiod, ranging from 16% of the total amino acids in the light to 7% in the dark, and in function of the cultivar (12% in NC103 and 13% in NC106; significantly different). Glu varied only in function of the cultivar (9% in NC103 and 4% of total amino acids in NC106). Ala content decreased at night and was slightly but significantly lower in NC106. Met was almost below detectable levels at night and slightly higher in NC106 than in NC103. In both cultivars, Gly, Thr, Ser, and Tyr were also sensitive to the photoperiod, and their contents were significantly reduced in the seed coat after 3 h of darkness. γ-Aminobutyric acid (GABA) accounted for 13–20% of the amino acid pool and had a tendency to decrease at night ($P \leqslant 0.08$, Table 1). GABA was previously detected in soybean seeds, and may enter the Krebs cycle after conversion to succinate, providing a potential link between amino acid metabolism and carbon flux (Tuin and Shelp, 1996). The total amino acid pool in seed coats, calculated by addition of all the measured amino acids, was similar between both cultivars, and fluctuated in a similar way with the photoperiod. After 3 h of darkness, it decreased in both cultivars from 41 to 29 nmol mg^{-1} FW.

Apoplast

Similar to the seed coats, Asn was the major amino acid found in the exudate (about 32% of the total measured) (Table 2). Since the technique involves some surgery steps on the seed coat, the levels of GABA were raised to similar or higher percentages than those observed for Asn (about 32%). It is well established that GABA accumulates in response to wounding (Shelp *et al.*, 1999). Only three amino acids were different in the exudates of both cultivars: Gln, His, and GABA. High-protein line NC106 had more Gln (10% versus 6% of the total amino acids) and His (2% versus 1%) and slightly less GABA (25% versus 33%) than low-protein NC103 at all time points. In both cultivars, several amino acids were significantly reduced in the exudates after 3 h in the dark: Gln, His, citrulline, Pro, Gly, Met, and Leu. However, the total amino acids released from the seed coat to the Phytagel were similar for the two cultivars (Table 2).

Phytagel traps contained 25 mM EGTA, which strongly enhances (up to 30-fold) the unloading kinetics of the seed coat (Thorne and Rainbird, 1983), perhaps increasing membrane permeability in the vascular tissues through chelation of membrane-bound divalent cations. EGTA has been proven to greatly stimulate the import and, thus, the sink strength of a seed without affecting the remaining seeds of the same pod. The release of assimilates has been

Table 2. *Amino acid content (nmol $seed^{-1}$) in the apoplast after a 3 h collection of assimilates released into phytagel traps*

Soybean embryos were surgically removed from their seed coats 15 d after flowering as described by Thorne and Rainbird (1983). Values in bold are statistically significant with the probabilities shown below.

Cultivar	Photoperiod	Asn	Gln	Glu	Arg	His	Citrulline	Asp	GABA	Ala	Pro	Gly	Thr	Ser
NC 103	3 h light	354	**98**	16	25	**18**	**8**	35	402	67	**24**	**21**	26	52
	3 h dark	449	**46**	10	30	**13**	**6**	39	431	91	**8**	**15**	23	49
NC 106	3 h light	388	**145**	16	27	**37**	**11**	34	355	69	**25**	**17**	28	60
	3 h dark	378	**96**	14	34	**19**	**1**	38	264	73	**13**	**13**	28	52
Phytagel blank		0	0	0	0	0	0	2	0	1	0	2	0	1
Sources of variation	d.f.	ANOVA *P* values												
Cultivar (C)	1	n.s.	**0.006**	n.s.	n.s.	**0.0001**	n.s.	n.s.	0.0594	n.s.	n.s.	n.s.	n.s.	n.s.
Photoperiod (P)	1	n.s.	**0.004**	0.07	n.s.	**0.0001**	**0.02**	n.s.	n.s.	n.s.	**0.0008**	**0.03**	n.s.	n.s.
C×P	1	n.s.	n.s.	n.s.	n.s.	**0.0010**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Error	23													

Cultivar	Photoperiod	Val	Met	Ileu	Leu	Tyr	Phe	Lys	Total amino acids
NC 103	3 h light	20	**6**	12	**18**	7	12	15	1236
		19	**4**	10	**16**	8	12	17	1296
NC 106	3 h light	22	**9**	13	**19**	7	12	17	1311
		21	**4**	12	**15**	8	13	18	1114
Phytagel blank		0	0	0	0	0	0	0	6
Sources of variation	d.f.	ANOVA *P* values							
Cultivar (C)	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Photoperiod (P)	1	n.s.	**0.002**	n.s.	**0.04**	n.s.	n.s.	n.s.	n.s.
C×P	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Error	23								

n=4; d.f., degrees of freedom; n.s., not significant ($P > 0.05$).

reported to be too slow without EGTA, therefore controls without EGTA were omitted (Thorne and Rainbird, 1983).

Cotyledon

Asn was the major amino acid in the cotyledons, with the high-protein line NC106 having overall more Asn (mean of all treatments 75 nmol mg^{-1} FW, 72% of average amino acid pool) than low-protein NC103 (35 nmol mg^{-1} FW, 52% of amino acid pool) (Table 3). Consequently, NC106 had higher total amino acid content (addition of all amino acids and mean for all treatments 103.5 nmol mg^{-1} FW) than NC103 (67.5 nmol mg^{-1} FW). Besides Asn, high-protein line NC106 had higher amounts of hydrophobic amino acids Ala, Val, Leu, Ile, and Met, as well as more Thr, Ser, and Phe than NC103.

Three major amino acids, Gln, Arg, and citrulline, differed significantly between cultivars in response to the photoperiod (C×P interactions, Table 3). This was also observed for the minor amino acids Val, Ile, Leu, Phe, and Orn. The most dramatic variations were observed for Gln. After 30 min of dark period, Gln levels were raised about 10-fold, from 0.8 to 10.6 nmol mg^{-1} FW in low-protein line NC103, whereas in the high-protein line NC106, they decreased about 3-fold, from 3.2 to 1.1 nmol mg^{-1} FW. Arg increased in the dark in NC103 but remained similar in NC106 throughout the experiment. Citrulline levels in the cotyledons also varied in opposing directions in each cultivar under dark conditions. In NC103, the citrulline content was raised about 2-fold after exposure to dark, whereas in NC106 it rapidly decreased by about 4-fold (Table 3). Orn content increased significantly in the dark in NC103, whereas it stayed relatively constant in NC106. By contrast, the content of the hydrophobic amino acids Val, Ile, Leu, Phe, and Met stayed higher during the dark period

Table 3. *Amino acid content (nmol mg^{-1} FW) in cotyledons of soybean embryos harvested 15 d after flowering*

Cotyledons were about 110 mg FW. Values in bold are statistically significant with the probabilities shown below.

Cultivar	Photoperiod	Time	Asn	Gln	Glu	Arg	His	Citrulline	Asp	GABA	**Ala**	Pro	Gly	**Thr**	**Ser**
NC 103	Light	6 h	**32**	**0.9**	4.7	**2.9**	2	**1.8**	1.0	0.7	**1.1**	0.064	0.213	**0.273**	**0.450**
		9 h	**40**	**0.8**	6.5	**3.5**	2.5	**1.9**	1.1	1.1	**1.7**	0.091	0.269	**0.318**	**0.573**
	Dark	0.5 h	**32**	**10.6**	10	**9.7**	2.2	**2.7**	1.3	1.0	**0.8**	0.095	0.173	**0.258**	**0.343**
		3 h	**38**	**8.4**	14	**16.4**	1.9	**4.0**	1.7	0.9	**0.5**	0.070	0.159	**0.283**	**0.413**
NC 106	Light	6 h	**62**	**5.9**	4.3	**4.9**	3.9	**1.8**	1.3	1.0	**4.0**	0.079	0.267	**0.613**	**1.117**
		9 h	**120**	**3.2**	4.9	**4.9**	2.9	**3.2**	1.7	1.0	**1.9**	0.118	0.236	**0.402**	**0.709**
	Dark	0.5 h	**66**	**1.1**	13.2	**4.1**	2.9	**0.5**	2.0	1.4	**4.6**	0.107	0.394	**0.495**	**0.965**
		3 h	**52**	**3.3**	3.5	**5.7**	3.0	**0.8**	2.2	0.7	**2.4**	0.107	0.200	**0.587**	**0.933**
Sources of variation		d.f.	ANOVA *P* values												
Cultivar (C)		1	**0.03**	n.s.	n.s.	n.s.	0.06	n.s.	n.s.	n.s.	**0.001**	n.s.	n.s.	**0.003**	**0.001**
Photoperiod (P)		1	n.s.	n.s.	n.s.	**0.01**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Time course (T)		1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
C×P		1	n.s.	**0.03**	n.s.	**0.01**	n.s.	**0.01**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
C×T		1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
L×T		1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**0.04**	n.s.	n.s.	n.s.	n.s.
C×L×T		1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Error		17													

Cultivar	Photoperiod	Time	Val	Met	Ileu	Leu	Tyr	Phe	Lys	Orn	Total amino acids
NC 103	Light	6 h	**0.241**	**0.055**	**0.064**	**0.127**	0.082	**0.105**	0.069	**0.023**	48.9
		9 h	**0.291**	**0.086**	**0.086**	**0.168**	0.109	**0.141**	0.100	**0.023**	61.4
	Dark	0.5 h	**0.192**	**0.030**	**0.062**	**0.088**	0.074	**0.099**	0.136	**0.072**	71.9
		3 h	**0.174**	**0.025**	**0.057**	**0.093**	0.067	**0.081**	0.179	**0.270**	87.7
NC 106	Light	6 h	**0.433**	**0.264**	**0.137**	**0.254**	0.125	**0.172**	0.294	**0.074**	92.9
		9 h	**0.354**	**0.094**	**0.094**	**0.213**	0.094	**0.165**	0.142	**0.094**	146.4
	Dark	0.5 h	**0.428**	**0.132**	**0.160**	**0.226**	0.073	**0.165**	0.155	**0.043**	99.1
		3 h	**0.493**	**0.133**	**0.213**	**0.307**	0.040	**0.187**	0.173	**0.067**	77.0
Sources of variation		d.f.	ANOVA *P* values								
Cultivar (C)		1	**0.002**	**0.007**	**0.001**	**0.0003**	n.s.	**0.03**	n.s.	n.s.	n.s.
Photoperiod (P)		1	n.s.	n.s.	n.s.	n.s.	0.06	n.s.	n.s.	n.s.	n.s.
Time course (T)		1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
C×P		1	**0.04**	0.06	**0.02**	**0.006**	n.s.	**0.01**	n.s.	**0.01**	n.s.
C×T		1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
L×T		1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
C×L×T		1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Error		17									

n=2; d.f., degrees of freedom; n.s., not significant ($P > 0.05$).

in NC106 than in NC103 (Table 3, significant interaction C×P; $P \leqslant 0.06$ for Met).

Developing seeds from field-grown cultivars

To validate the results obtained with the NC103 and NC106 cultivars grown under controlled environmental conditions, the free amino acid content was measured in seeds harvested at noon (during light conditions) from field-grown plants for five cultivars with SPC varying from 42% to 48.6% (Table 4). These analyses were repeated twice, in May and October 2003, using material harvested in September 2002, with essentially the same results. Results from the second analyses are presented. Asn was the most abundant amino acid, ranging from 33% to 49% of the free amino acid pool in the different cultivars. Together, the major amino acids Asn, Gln, and Glu accounted for about 65% of the total amino acids.

Higher levels of amide amino acids were measured in the high-protein cultivars. Asn content was higher in the high-protein cultivar Prolina as compared with all other cultivars, and had a tendency to be higher in NC106 than in the low-protein cultivar N94-552 ($P \leqslant 0.06$) (Table 4). There was a positive correlation between the values measured for Asn content and SPC at maturity (Spearman's rank order correlation, r_s=0.59, $P \leqslant 0.05$). High Gln content was also associated with the high-protein cultivars: it was significantly higher in NC106 than in PI146, and higher in Prolina as compared with PI146 and N94-552 (Table 4). Among minor amino acids, Ala content was higher in the two high-protein cultivars, Prolina and NC106, than in the three other cultivars, confirming the results obtained with cotyledons (Table 3). The other minor amino acids, Leu, Gly, Tyr, and Orn, were higher in Prolina than in other cultivars (Table 4). A different distribution was observed for the Glu-derived amino acids citrulline and GABA, which were higher in Prolina and Brim than in other cultivars (Table 4). This was also observed for the minor amino acid Phe (Table 4).

Transcript levels of seed storage protein and lipogenic genes in high- and low-protein lines

Since there is a negative relationship between the accumulation of protein and lipids in soybean seeds (Brim and Burton, 1979), the aim was to investigate whether this relationship was reflected in the transcript levels of seed storage protein and lipogenic genes. Transcript levels were analysed for the biotin carboxyl carrier protein (BCCP) of multi-subunit acetyl CoA carboxylase (MS-ACCase) (Reverdatto *et al.*, 1999), omega 6-desaturase (FAD2-1) (Heppard *et al.*, 1996), β-subunit of β-conglycinin (Harada *et al.*, 1989), and 24 kDa oleosin (Kalinski *et al.*, 1991). MS-ACCase was chosen as a marker, since it catalyses a rate-limiting step in fatty acid biosynthesis. FAD2-1 catalyses the first step in the main pathway leading to the production of polyunsaturated lipids. Northern analyses were performed for the cultivars NC103 and NC106, using seeds that were 100 mg FW. Seeds were harvested at four different time points during the day and night cycle, but because transcript levels for the different genes did not appear to vary significantly with the photoperiod, the signals were averaged over the four time points (Fig. 1). The high-protein line NC106 had higher transcript levels of the β-subunit of β-conglycinin (approximately 2-fold), 24 kDa oleosin, and FAD2-1 (approximately 3-fold) than NC103, but transcript levels for the BCCP subunit of MS-ACCase were reduced approximately 2-fold.

Metabolism in cultured seeds: Gln concentration and light affect partitioning of [^{14}C]Suc between protein and oil

To define the short-term patterns of carbon partitioning in response to nitrogen supply and light exposure, [^{14}C]Suc

Table 4. *Amino acid content (nmol mg^{-1} FW) in developing seeds of soybean grown in the field*

Seeds were about 200 mg FW.

Cultivar	Asn	Gln	Glu	Arg	His	Citrulline	Asp	GABA	Ala	Pro	Gly	Thr
NC106	13.2	2.7	5.2	2.8	2.8	0.25	0.8	0.4	2.9	0.060	0.37	0.37
Prolina	19.9	3.3	7.6	3.8	2.4	1.35	1.2	1.2	2.7	0.083	0.55	0.41
Brim	11.1	2.1	7.4	2.6	1.7	0.95	0.9	1.2	1.2	0.048	0.38	0.30
PI146	8.8	1.0	4.1	1.4	1.1	0.14	1.3	0.5	1.1	0.010	0.21	0.41
N94-552	7.5	1.7	5.5	1.7	1.2	0.10	0.9	0.6	1.0	0.031	0.26	0.23
LSD	6.1	1.3	2.8	1.8	1.8	0.60	0.8	0.4	1.4	0.067	0.20	0.38

Cultivar	Ser	Val	Met	Ile	Leu	Tyr	Phe	Lys	Orn	Total AAs	%SPC
NC106	0.95	0.86	0.150	0.066	0.070	0.052	0.100	0.17	0.024	34.8	48.6
Prolina	0.90	1.08	0.135	0.078	0.104	0.127	0.326	0.21	0.045	47.5	47.9
Brim	0.71	0.97	0.097	0.037	0.068	0.056	0.272	0.21	0.016	32.3	43.3
PI146	0.38	0.42	0.093	0.020	0.031	0.022	0.084	0.10	0.007	21.2	42.9
N94-552	0.48	0.74	0.071	0.029	0.048	0.042	0.119	0.13	0.012	22.4	42
LSD	0.49	0.49	0.094	0.050	0.044	0.064	0.136	0.12	0.020	15.4	

LSD, Fisher's protected least significant difference at $P \leqslant 0.05$.

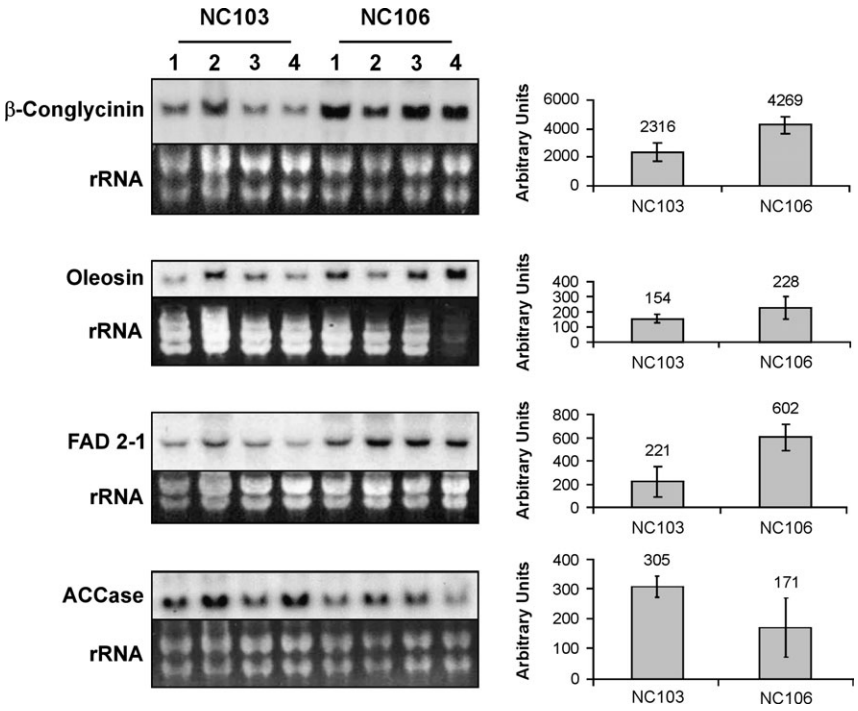

Fig. 1. Northern analyses of developing soybean seeds (*c*. 100 mg) of the low- and high-protein cultivars NC103 and NC106, respectively. Transcripts were detected using probes for the β-subunit of β-conglycinin, 24 kDa oleosin, FAD2-1, and the BCCP subunit of MS-ACCase. Time points were as follows: 1, 5 h light, 12.30 p.m.; 2, 8 h light, 3.30 p.m.; 3, 0.5 h dark, 4.30 p.m.; and 4, 3 h dark, 7.30 p.m. Histograms on the right indicate the mean of the four signals as determined by scanning densitometry. Bars represent standard deviations. rRNA of the last sample in the 24 kDa oleosin blot was not stained properly with ethidium bromide, but the amount of total RNA was assumed to be comparable to the other samples.

was added to the media of whole seeds at low and high Gln concentrations for 48 h. Although Asn is the major amino acid in seeds, previous studies have established that Gln is the best nitrogen source for *in vitro* growth (Haga and Sodek, 1987). Incorporation of ^{14}C label derived from Suc was measured in total lipids and soluble proteins. Incorporation into the TAG fraction, the main storage lipids in seeds, was determined by radiochromatogram scanning of chloroform extracts after separation by TLC in comparison with reference standards (Fig. 2). Table 5 shows all fractions where radiolabelled metabolites were measured. The methanol–water fraction represents the pool of all soluble metabolites not yet incorporated into oil or protein, including [^{14}C]Suc taken up by the seed, as well as metabolites derived from re-fixation of respired CO_2. The pellet fraction includes all insoluble polymers (starch, cell walls, and insoluble proteins) precipitated during lipid extraction. Incorporation into soluble protein and lipid fractions were both light stimulated (significant effect; Table 5; Fig. 3). This was not due to a reduced uptake of [^{14}C]Suc in the dark, because both light- and dark-grown seeds treated with 6 mM Gln had similar levels of incorporation in the methanol–water fraction (Table 5). In the light, there was no statistically significant effect of Gln on carbon flux into the protein or oil fraction (Table 5; Fig. 3). However, in the dark, high-Gln treatment resulted in a significant decrease in incorporation in all fractions between 24 h and 48 h, which was not observed under low Gln levels (Table 5). This translated into a reduced incorporation into protein, total lipids, and TAG as a fraction of total lipids after 48 h (Fig. 3; Table 5). These results indicate that under conditions of elevated nitrogen supply, light is required to sustain Suc uptake and anabolic processes.

Regulation of seed storage-protein genes by Gln and light

The effect of Gln and light on the expression of genes related to protein and lipid accumulation in developing seeds grown *in vitro* was investigated. Northern analyses of transcript levels were performed for selected seed storage-protein and lipogenic genes. High-Gln treatment (60 mM) resulted in higher transcript levels of β-conglycinin and 24 kDa oleosin, especially in the light (Fig. 4). However, there was no significant variation in transcript levels of the BCCP subunit of MS-ACCase and FAD2-1 in response to the treatments (data not shown).

Discussion

In this study, soybean lines with a similar genetic background were used to evaluate the role of seed nitrogen

supply in the regulation of SPC. Amino acid profiles were determined in the embryo and, in maternal tissues, the seed coat and the apoplast using the empty seed coat technique (Thorne and Rainbird, 1983). This technique is considered to provide a reliable measurement of assimilate release from maternal tissues (reviewed in Wolswinkel, 1992). Samples were analysed at several time points, providing a dynamic view of steady-state levels during the day and

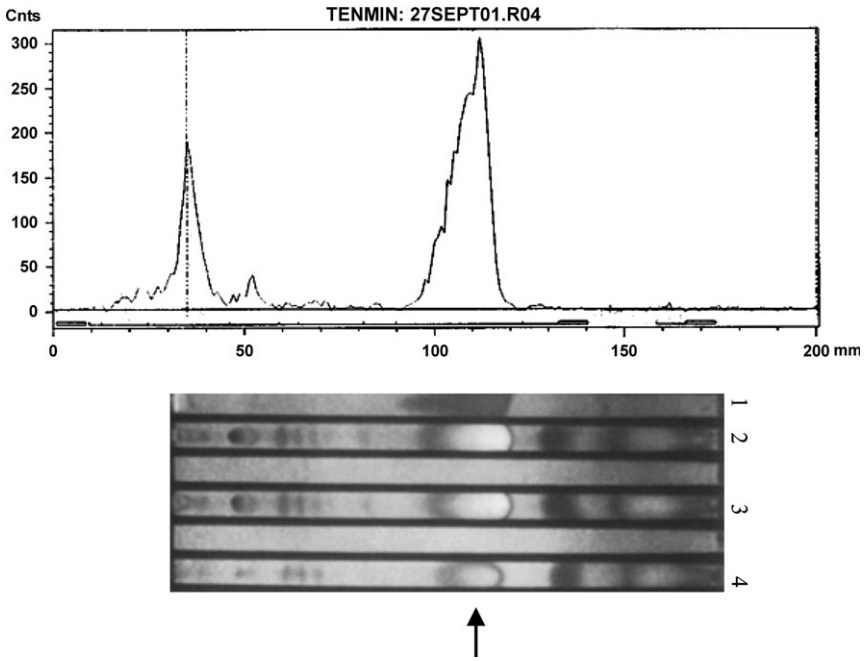

Fig. 2. Neutral lipids TLC of the ^{14}C-labelled chloroform fraction. The arrow refers to the TAG fraction containing the major labelled compounds, as shown on the ^{14}C scan of a representative lane on the left. Lane 1, TAG standard (tristearin); lanes 2, 3, and 4, chloroform fractions extracted from soybean seeds.

Table 5. *Time-course of [^{14}C]Suc incorporation in total chloroform fraction (total lipids), soluble protein, water-methanol fraction, and insoluble pellet (starch, cell walls, and insoluble protein) of soybean embryos (dpm mg^{-1} FW)*

Seeds were cultured in liquid Obendorf medium for 6, 24, and 48 h, under two different Gln concentrations. Illumination treatments consisted in continuous light versus continuous dark.

Photoperiod	[Gln]	Time	Total lipid	Total protein	Methanol–water	Pellet fraction	[Protein] (μg mg^{-1} FW)	Increment FW (%)
Light	6 mM	6 h	2.6	1.1	30	17	12.9	5.8
		24 h	7.7	5.4	36	38	6.4	40.4
		48 h	21.2	17.3	42	78	18.0	32.2
	60 mM	6 h	1.5	0.7	29	17	12.5	54.0
		24 h	9.9	8.3	52	52	11.8	67.7
		48 h	20.4	25.6	68	99	23.1	68.5
Dark	6 mM	6 h	0.6	0.6	14	6	16.9	26.4
		24 h	3.1	3.1	36	23	20.1	22.7
		48 h	8.0	4.4	48	47	19.9	50.7
	60 mM	6 h	1.2	0.6	34	15	14.6	40.0
		24 h	2.3	2.4	36	25	13.2	40.0
		48 h	1.0	1.1	23	10	14.9	41.6

Sources of variation	d.f.	ANOVA *P* values					
Light (L)	1	0.0003	0.0001	0.07	0.002	n.s.	n.s.
Gln (G)	1	n.s.	n.s.	n.s.	n.s.	n.s.	0.003
Time-course (T)	2	0.0001	0.0001	0.04	0.001	0.04	0.04
L×G	1	n.s.	n.s.	n.s	n.s.	n.s.	0.008
L×T	2	n.s.	n.s	n.s.	n.s.	n.s.	n.s.
G×T	2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
L×G×T	2	n.s.	n.s.	0.06	n.s.	n.s.	n.s.
Error	24						

d.f., degrees of freedom; n.s., not significant (P >0.05); n=3.

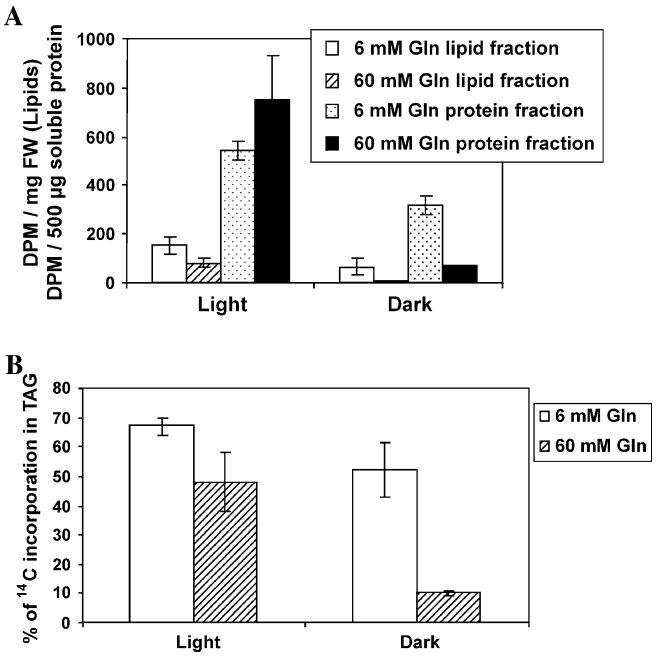

Fig. 3. (A) Partitioning of [^{14}C]Suc between total lipid and protein fractions after 48 h. (B) Percentage of ^{14}C label from the total lipid fraction incorporated in the TAG fraction after 48 h.

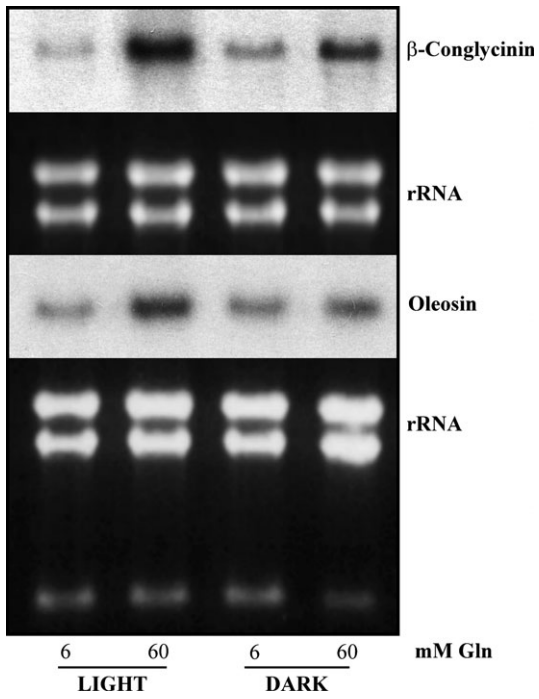

Fig. 4. Northern analyses of developing soybean seeds grown *in vitro* for 72 h. Transcripts were detected using probes for the soybean β-subunit of β-conglycinin and 24 kDa oleosin.

night cycle, because it is well known that amino acid composition varies diurnally in leaf and phloem sap (for example, see Riens *et al.*, 1991).

The high-protein line NC106 had a higher Asn content in cotyledons compared with low-protein NC103 (Table 3). By contrast, at the maternal level (i.e. seed coat and apoplast), the Asn content was similar between the two lines (Tables 1, 2). A positive correlation between Asn content in seed and SPC was confirmed in five unrelated cultivars grown in the field (Table 4), where high Gln levels in developing seeds were associated with high-protein content at maturity (Table 4). In cotyledons, Gln levels were higher in the high-protein line NC106 than in NC103 during the light period (average of two time points equals 4.6 nmol mg^{-1} FW versus 0.8 nmol mg^{-1} FW) (Table 4). NC106 also accumulated higher levels of several other amino acids in cotyledons, including Ala and other minor amino acids (Table 3). These results are consistent with the notion that nitrogen supply within the developing cotyledons may be a factor controlling seed composition.

A similar association between SPC and Asn content in seed has previously been observed in grasses, in recombinant inbred lines of rye (Dembinski and Bany, 1991), and in genetically related strains of maize (Dembinski *et al.*, 1991; Lohaus *et al.*, 1998). The positive correlation observed between Asn levels and SPC in soybean is also consistent with the recent observation that *Arabidopsis* transgenic lines over-expressing ASN1 constitutively have a higher level of free Asn and total amino acids in developing siliques, as well as an increased protein content in seeds (Lam *et al.*, 2003). A potential control point regulating free amino acid contents in the embryo is the uptake from the apoplasm. This process involves active transport, with the participation of amino acid/H$^+$ symporters of the amino acid permease family (Delrot *et al.*, 2001). Most amino acid permeases characterized to date have a broad specificity. Possible scenarios contributing to the higher Asn content in the high-protein line include a higher density of transporters at the plasma membrane, a different composition resulting in selective uptake, or a different regulation of transporter activity by amino acids. However, a higher transport rate into the cotyledon coupled with a higher rate of protein synthesis could, as well, result in a concentration of free Asn in the steady state that would be similar between high- and low-SPC genotypes. However, this was not observed, which suggests that the biosynthesis and catabolism of Asn are tightly regulated in developing seeds and that the concentration of Asn may be important, perhaps as a 'signal' of N-assimilate availability.

By contrast with amino acid profiles in the embryo, those of maternal tissues were generally similar between the high- and low-protein lines. The present findings support the conclusion that high SPC in soybean is determined largely by the capacity of the embryo for the uptake of nitrogen sources and the synthesis of storage proteins, as proposed previously by Hayati *et al.* (1996). In maize, Lohaus *et al.* (1998) found that a high-SPC line accumulated higher levels of free amino acids, especially Asn and Gln, in leaf phloem sap, suggesting a higher capacity of the mother

plant to deliver N-assimilates to the developing seed. In rape cultivars with high SPC, higher free amino acid levels were also measured in the leaf phloem sap, as well as an increased rate of N-translocation to the phloem (Lohaus and Moellers, 2000). The present results do not exclude a higher rate of delivery of N-assimilates to the seed apoplast in the high-protein line since steady-state levels were measured, and not flux rates.

The levels of accumulation of seed storage proteins are also likely to be influenced by differences in the rates of metabolism of nitrogen sources and their interconversion to other amino acids. The low-protein line NC103 accumulated increased levels of Gln in cotyledons in the dark period (approximately 10-fold), as well as the Glu-derived amino acid Arg and its precursors citrulline and Orn, whereas they were reduced or stayed approximately constant in cultivar NC106 (Table 3). This result is particularly striking since Gln levels are significantly reduced in the seed coat and apoplast of both lines under dark conditions (Tables 1, 2). The accumulation of Gln- and other Glu-derived amino acids in NC103 suggests a less efficient process of ammonia assimilation in the dark in this line, perhaps due to reduced levels or a more rapid depletion of organic acids required for amino acid interconversions. This hypothesis is supported by the fact that, in the dark, NC106 maintains higher levels of the pyruvate-derived amino acids, Val, Leu, and Ile, as well as the phosphoenolpyruvate-derived amino acid, Phe (Table 3). Phosphoenolpyruvate carboxylase activity (producing oxaloacetate) is correlated with SPC in soybean and *Vicia faba* (Sugimoto *et al.*, 1989; Golombek *et al.*, 2001), while pyruvate kinase activity (producing pyruvate) is correlated with both oil and storage-protein accumulation in developing soybean seeds (Platt and Bassham, 1978; Smith *et al.*, 1989). It would be interesting to compare these enzyme activities and the levels of associated metabolites in the high- and low-protein soybean lines during the day and night cycle. The hypothesis of an increased efficiency of amino acid interconversions in NC106 is further supported by the recent results obtained by constitutive over-expression of the maize transcription factor Dof1 in transgenic *Arabidopsis* (Yanagisawa *et al.*, 2004). Transgenic lines had increased transcript levels and catalytic activity of phosphoenolpyruvate carboxylase and pyruvate kinase, leading to an elevated amino acid and nitrogen content in whole plants. In addition, transgenic expression in potato of a phosphoenolpyruvate carboxylase insensitive to feedback inhibition by malate resulted in a shift of carbon flux from soluble carbohydrates and starch to organic acids and amino acids (Rademacher *et al.*, 2002).

The influence of nitrogen supply and light on carbon flux from Suc into storage products was examined using seeds grown *in vitro*. Light-stimulated incorporation of label from [^{14}C]Suc into both protein and oil (Fig. 3; Table 5). The combination of high Gln and continuous dark resulted in a decrease in the flux of label into all fractions analysed between 24 h and 48 h, which was not observed under low-nitrogen conditions (Table 5). High Gln also led to a reduction in incorporation into oil, protein, and TAG as a fraction of total lipids after 48 h (Fig. 3). Under continuous dark conditions, the refixation of respired CO_2 in the dark is probably lost, and the absence of photosynthesis may lead to higher rates of respiration. Gln provided at high concentration may be used as substrate for respiration (Saravitz and Raper, 1995), but the levels of organic acid acceptors may not be sufficiently high for reassimilation of the generated ammonia. Research on developing seeds in several species has revealed the role of photosynthesis in O_2 generation and energy maintenance, and the relationship between oxygen levels and storage product accumulation (Borisjuk *et al.*, 2003; Rolletschek *et al.*, 2003; Vigeolas *et al.*, 2003; van Dongen *et al.*, 2004). Recent studies in canola and soybean seed have highlighted the function of photosynthesis and Rubisco in refixation of CO_2 produced during fatty acid biosynthesis (Ruuska *et al.*, 2004; Schwender *et al.*, 2004). The present results emphasize the contribution of light, and possibly photosynthesis, in stimulating the accumulation of storage products and co-ordinating the metabolism of nitrogen and carbon in seeds.

The negative relationship between accumulation of protein and oil in soybean seeds was reflected in the transcript levels of storage proteins and the BCCP subunit of the lipogenic enzyme MS-ACCase in cultivars NC106 and NC103 (Fig. 1), indicating that partitioning into the different storage products may be regulated at the level of gene expression. Amide amino acids act as signal molecules controlling the expression of a variety of genes involved in nitrogen assimilation, such as Asn synthase (Lam *et al.*, 1994). Several genes coding for storage proteins are also regulated by the nitrogen status. The promoter of storage-protein patatin class I, is up-regulated by carbohydrates and Gln (Martin *et al.*, 1997), and this regulation persists when using the promoter in heterologous systems such as *Arabidopsis* (Hellmann *et al.*, 2000), indicating a conserved regulatory pathway for sink-specific, metabolite-dependent gene expression. High Gln concentration in soybean seed cultures has been shown to up-regulate transcripts for two different storage proteins, 24 kDa oleosin and β-subunit of β-conglycinin (Fig. 4). This up-regulation was enhanced by light, consistent with the stimulation of biosynthetic flux into soluble proteins (Fig. 3; Table 5). Ohtake *et al.* (2002) have shown previously that transcripts for the β-subunit of β-conglycinin are suppressed under conditions of nitrogen deficiency, and that they are inducible by Gln in cotyledons grown *in vitro*. The induction of both oleosin and β-conglycinin transcripts by high-Gln treatment suggests that nitrogen status may globally regulate gene expression of storage proteins. Using the *in vitro* system, no evidence was found that transcript

levels of the BCCP subunit of MS-ACCase are regulated by nitrogen levels. These results parallel those obtained for the effect of Gln levels on carbon flux into lipids.

In conclusion, it has been shown that the closely related NC103 and NC106 lines constitute a useful system for studying the co-ordinated regulation of amino acid uptake and biosynthesis, and storage-protein accumulation. The results presented here suggest a better co-ordination in the production of carbon skeletons used for amino acid synthesis in the high-protein line NC106. In addition, high SPC at maturity in soybean is associated with higher levels of Asn and several other amino acids in developing cotyledons, but not in maternal tissues or the apoplast. An enhanced capacity for uptake of nitrogen sources by cotyledons may therefore be a major factor responsible for increased protein accumulation. In addition, the biosynthesis and catabolism of Asn are probably tightly regulated in the embryo of high-protein lines. The present results suggest that the Asn content of developing cotyledons may be a determinant of storage-protein biosynthesis. If so, the Asn content of developing seeds could be useful as a physiological marker for high SPC, and perhaps as a target for manipulation, in order to influence SPC. The metabolism of nitrogen sources and their conversion to other amino acids represents another factor likely to influence the levels of storage-protein accumulation.

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