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Glutamine-synthetase isoforms appearing in sunflower cotyledons during germination

Effects of light and nitrate

P. de la Haba, P. Cabello, and J.M. Maldonado*

Departamento de Biología Vegetal y Ecología, División de Fisiología Vegetal, Facultad de Ciencias, Universidad de Córdoba, Avda. San Alberto Magno, E–14004 Córdoba, Spain

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Abstract. Ion-exchange chromatography has been used to separate the isoforms of glutamine synthetase (GS; EC 6.3.1.2) appearing in sunflower (*Helianthus annuus* L. cv. Peredovic) cotyledons during seedling growth under different light and nitrogen conditions. Both in dry and imbibed seeds, only a single form of GS (GS_S) was detected. Upon seed germination, the GS_S isoform was gradually replaced by cytosolic (GS_1) and plastidic (GS_2) isoforms. Light and nitrate decreased the levels of GS_1. In contrast, the appearance of GS_2 was greatly stimulated by light. Nitrate also had a positive effect, particularly in the light. Light and nitrate acted synergistically on the appearance of GS_2. The GS_2:GS_1 ratio in cotyledons of 9-d-old seedlings ranged from about 2, in darkness and nitrate-deprivation conditions, to 16 under light and nitrate application. The possible physiological roles of the distinct GS isoforms appearing in the epigeal cotyledons of sunflower during germination, and their differential regulation by light and nitrate, are discussed.

Key words: Ammonia/ammonium assimilation – Enzyme regulation – Glutamine synthetase – *Helianthus* (glutamine synthetase) – Seed germination

Introduction

Glutamine synthetase (GS; EC 6.3.1.2) is the major enzyme involved in higher plants in the primary assimilation of ammonia (Lea and Miflin 1979). In developing seedlings, the catabolism of amino acids coming from degradation of storage proteins also generates ammonia. This ammonia is reassimilated by GS and the resulting glutamine is transported from the cotyledons to the growing parts of the seedling (Lea and Joy 1983).

Higher-plant GS is an octameric enzyme with a molecular mass of 300–360 kDa (Lea et al. 1990). An active tetrameric GS has also been detected in the leaves of sugarbeet (Mäck and Tischner 1990). Distinct GS isoforms, differing in their subunit composition, are present in different tissues. Leaves of most plants possess two forms of GS, a cytosolic form (GS_1) and a plastidic form (GS_2), but in some other plants only a single isoform is present (McNally et al. 1983). Studies on barley mutants lacking GS_2 have demonstrated the essential role of this isoenzyme in the reassimilation of the photorespiratory ammonia (Wallsgrove et al. 1987).

The level of chloroplast GS_2 activity has been shown to increase during the light-induced greening of etiolated tissue (Hirel et al. 1982; Schmidt and Mohr 1989). The increase in GS_2 activity is mediated, at least in part, by phytochrome and involves de-novo synthesis of the isoenzyme (Tingey et al. 1988; Weber et al. 1990). The levels of total GS activity were also found to increase in germinating sunflower cotyledons when nitrate was supplied (de la Haba et al. 1988). Schmidt and Mohr (1989) observed that the appearance of GS_2 activity, the unique isoform detected in cotyledons of mustard *(Sinapis alba)*, was stimulated more by light than by nitrate. In green cotyledons of sunflower seedlings, both cytosolic and plastidic GS are present, GS_2 representing the major isoform (Cabello et al. 1991).

In the present study, we have investigated the appearance of GS isoforms in sunflower cotyledons during germination and seedling development. The response of each isoenzyme to light and nitrate nutrition has also been examined.

Material and methods

Growth conditions. Seeds of *Helianthus annuus* L. cv. Peredovic, kindly supplied by Eurosemillas (Córdoba, Spain), were surface-sterilized in a 1% (v/v) hypochlorite solution for 15 min. After rinsing in distilled water, seeds were imbibed for 5 h and then sown about 2 cm deep in plastic trays containing a 1:1 (v/v) mixture of perlite and vermiculite. Germinating seedlings were irrigated daily

* To whom correspondence should be addressed

Abbreviations: GS = glutamine synthetase; GS_1 = cytosolic GS; GS_2 = plastidic GS; GS_s = GS from seeds

with a nutrient solution either free from combined nitrogen or with 10 mM KNO_3 (Hewitt 1966).

Seedlings were grown either in darkness or with a daily 16-h light period and a day/night regime of 25/19° C temperature and 70/80% relative humidity. Light was supplied by 'cool white' fluorescent lamps supplemented with incandescent bulbs, giving a photon flux density of 200 μmol · m^{-2} · s^{-1} of photosynthetically active radiation at the top of the plants.

Enzyme extraction. At various times after sowing, seedlings were harvested within the first 2 to 3 h of the light period. Dark-grown seedlings were harvested at the same time. Cotyledons were excised, rinsed in distilled water and wiped on a paper towel. Cotyledons were immediately cut into small slices and 4 g were homogenized at 4° C with 16 ml of the extraction medium in a Waring-Blendor for 40 s at full speed. The extraction medium consisted of 100 mM Tris-HCl buffer (pH 7.6), 10 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μM leupeptin, 25 mg · ml^{-1} insoluble polyvinylpolypyrrolidone (PVPP), and 5 mg · ml^{-1} streptomycin sulphate. The homogenate was filtered through four layers of gauze and then centrifuged at 40 000 · *g* for 30 min at 4° C. The clear supernatants were desalted on PD-10 Sephadex G-25 minicolumns equilibrated with 10 mM Tris-HCl buffer (pH 7.6), 1 mM EDTA, and 1 mM $MgCl_2$.

Separation of GS isoenzymes. The desalted homogenate was loaded onto a diethylaminoethyl (DEAE)-Sephacel (Pharmacia, Uppsala) column (10 cm long, 1 cm i.d.) previously equilibrated with 10 mM Tris-HCl buffer (pH 7.6) and 1 mM $MgCl_2$. The column was washed with the same buffer until no protein was detectable in the eluate. Elution of adsorbed proteins was carried out with a linear gradient of 0–0.5 M NaCl dissolved in 100 ml of the equilibrating buffer. The flow rate was maintained at 20 ml · h^{-1} and 2-ml fractions were collected. The NaCl concentration in the eluted fractions was estimated by measurement of resistivity.

All the above procedures were carried out at 4° C.

Determination of GS activity. Glutamine-synthetase activity was measured by the transferase assay in a reaction mixture containing, in a final volume of 1 ml, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (pH 7.5), 30 mM L-glutamine, 60 mM NH_2OH, 0.4 mM ADP, 3 mM $MnCl_2$, 20 mM Na_2HASO_4, and an adequate amount of enzyme preparation. Control tubes without ADP and arsenate were run in parallel to correct non-specific formation of γ-glutamylhydroxamate. The mixtures were incubated at 30° C for 20 min, and the reactions terminated by addition of 2 ml of cold ferric-chloride reagent (120 mM $FeCl_3$, 78 mM HCl and 73 mM trichloroacetic acid). Before measurements of absorbance at 500 nm, the mixtures were centrifuged at 3000 · *g* for 10 min to remove turbidity.

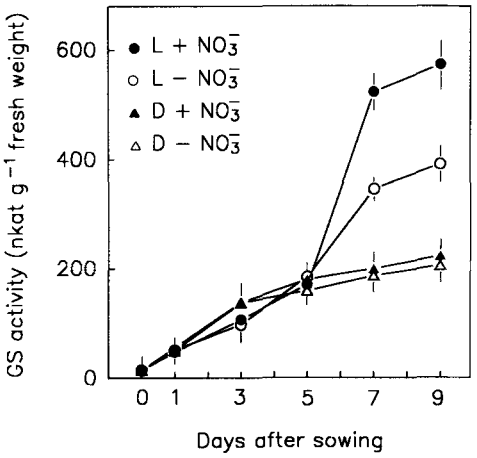


Fig. 1. Time courses of the appearance of GS activity in sunflower cotyledons during germination. Seedlings were grown either on a nitrogen-free medium or on 10 mM nitrate, and either kept in darkness (*D*) or in the light (*L*) from sowing onwards. The GS activity was assayed in extracts from cotyledons harvested at the indicated times after sowing. Values are means of three experiments (two determinations per experiment). *Vertical bars* represent SD

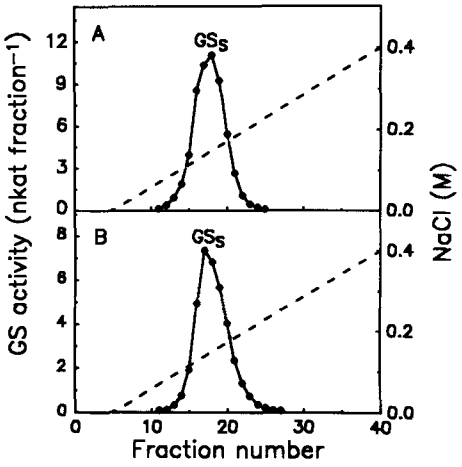


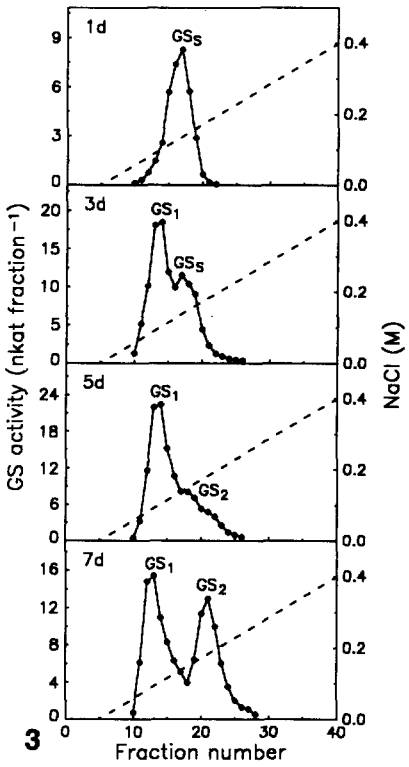
Fig. 2A, B. Elution profile of GS activity in extracts of dry (**A**), and imbibed (**B**) sunflower seeds. Separation was carried out by ion-exchange chromatography and further elution with an NaCl gradient. ●—●, GS activity; – – –, NaCl gradient

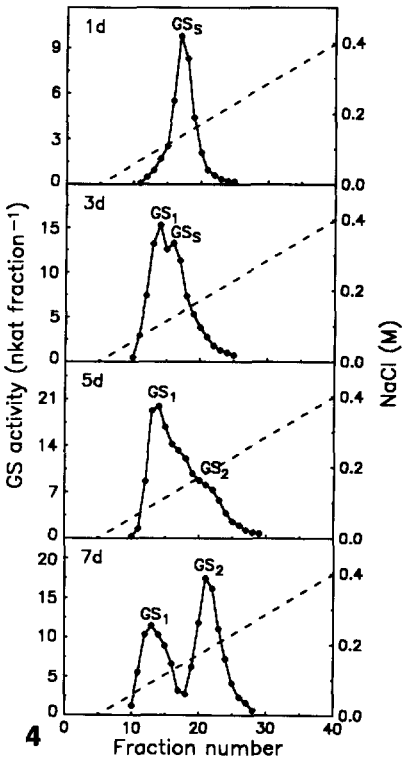
Results

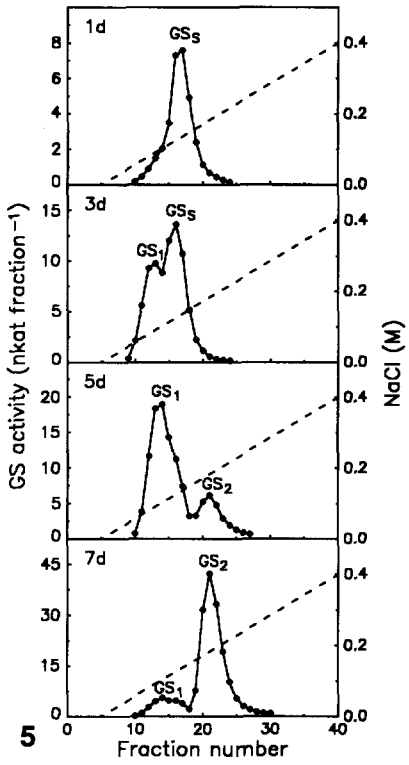
Time course of the appearance of GS activity in sunflower cotyledons. Figure 1 shows the appearance of total GS activity in cotyledons during germination and seedling growth as affected by light and nitrate. Glutamine-synthetase activity was detected in cotyledons at early stages of germination and it gradually increased during the subsequent days. After day 5, and coinciding with the emergence of cotyledons from the soil and their light-induced greening, a remarkable increase of GS activity occurred under light. Addition of nitrate enhanced the light-promoted appearance of GS activity. In darkness, nitrate had little effect.

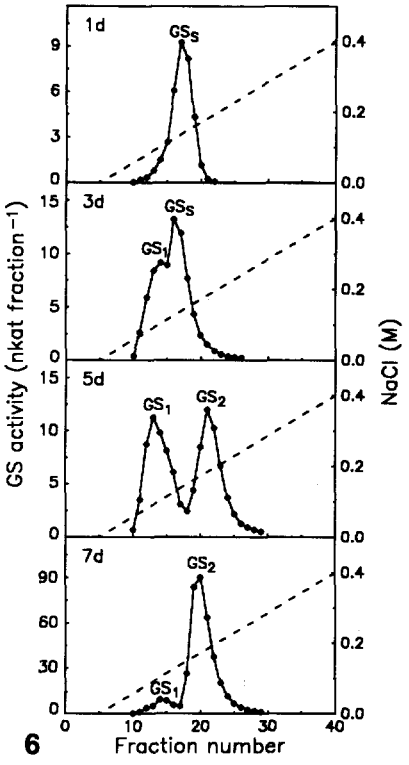
Glutamine-synthetase activity in the sunflower seed. Ion-exchange chromatography of an extract of dry seeds revealed a single peak of GS activity which eluted at an NaCl concentration of 130 mM (Fig. 2A). A similar peak was observed in extracts from imbibed seeds (Fig. 2B). As will be shown below, this peak of GS activity disappears as seed germination proceeds, hence it appears to correspond to a GS isoform specific to the seed, therefore designated GS_S.

Changes in GS isoforms in sunflower cotyledons during seedling development. Sunflower seeds were germinated and grown under the following conditions: (i) darkness without nitrate (Fig. 3); (ii) darkness with nitrate (Fig. 4); (iii) light without nitrate (Fig. 5); and (iiii) light with nitrate (Fig. 6). At various times after sowing, GS isoforms in extracts of cotyledons were separated by ion-exchange chromatography. Under our experimental









Figs. 3–6. Activities of GS isoforms in cotyledons of sunflower seedlings. Cotyledons were harvested 1 d, 3 d, 5 d and 7 d after sowing. Isoenzymes were separated by ion-exchange chromatography. ●—●, GS activity; – – –, NaCl gradient. **Fig. 3.** Seedlings grown without nitrate in darkness. **Fig. 4.** Seedlings grown on nitrate in darkness. **Fig. 5.** Seedlings grown without nitrate in the light. **Fig. 6.** Seedlings grown on nitrate in the light

conditions, seed germination occurred between days 1 and 2, and emergence of epigeal cotyledons between days 4 and 5 after sowing.

A single form of GS was always found in cotyledons at day 1 after sowing. It eluted at 130 mM NaCl, and therefore corresponded to the isoform present in the seed (GS_S). Three days after sowing, two peaks of GS activity were detected. The first peak, eluting at an NaCl concentration of 100 mM, corresponds to the GS_1 isoform (Cabello et al. 1991), whereas the second peak eluted at 130 mM NaCl and corresponds to the GS_S isoform. At day 5, the levels of GS_S dropped in cotyledons of dark-grown seedlings and were extremely low in light-grown seedlings. Simultaneously, a new GS peak, eluting at 180 mM NaCl and corresponding to the GS_2 isoform (Cabello et al. 1991), was visible, specially under light and nitrate. Only the GS_1 and GS_2 isoforms were detected in cotyledons of 7-d-old seedlings. Both light and nitrate stimulated the appearance of GS_2.

Comparative effects of light and nitrate on GS_1 and GS_2 levels in sunflower cotyledons. The influence of light and added nitrate on the levels of GS_1 and GS_2 was studied in cotyledons of 9-d-old seedlings (Table 1). Both light and nitrate had a negative effect on the appearance of the GS_1 isoform. In contrast, the appearance of GS_2 was greatly stimulated by light and, to a lesser extent, by nitrate. Nitrate supply increased the activity by 15% in darkness and by about 80% in the light. On the other hand, light increased the activity by about 100% in the absence of nitrate and by about 220% when nitrate was

Table 1. Effects of light and nitrate on the activities of GS_1 and GS_2 isoforms in cotyledons of 9-d-old sunflower seedlings. Seedlings were grown under the indicated conditions. The level of activity of each isoenzyme was estimated from the area of the corresponding elution profile after separation by ion-exchange chromatography. The relative proportion of each isoenzyme is expressed as a percentage of the total GS activity. Data are means ± SD of three experiments (two determinations per experiment)

Growth conditions	GS_1		GS_2		GS_2:GS_1 ratio
	nkat	%	nkat	%	
Darkness minus NO_3^-	54.6 ± 1.6	36	96 ± 3	64	1.8
Darkness plus NO_3^-	37.1 ± 1.3	25	111 ± 6	75	3.0
Light minus NO_3^-	26.6 ± 0.9	12	196 ± 11	88	7.4
Light plus NO_3^-	22.5 ± 1.1	6	358 ± 16	94	15.9

present. Light and nitrate acted synergistically on the appearance of GS_2, as inferred from the observation that the combined action of light and nitrate was greater than the sum of their separate, individual actions. The differential regulation of GS_1 and GS_2 by light and nitrate led to GS_2:GS_1 ratios ranging from about 2 in etiolated, nitrate-starved cotyledons to around 16 in green, nitrate-supplied cotyledons.

Discussion

In this paper we have shown that three forms of GS, which can be separated by ion-exchange chromatography, are expressed in sunflower cotyledons during germination and seedling development. A seed-specific GS isoform (GS_S) is present both in ungerminated and newly germinated seeds. As germination proceeds and cotyledons develop, the GS_S isoform is replaced by the GS_1 and GS_2 isoforms. The observed changes in the patterns of isoenzymes could be the result of a differential expression of GS genes in cotyledons during germination, as reported to occur in legumes during nodule development (Cullimore and Bennett 1988; Coruzzi 1991).

Only one peak of GS activity has also been detected in ungerminated seeds of barley (Mann et al. 1979) and lettuce (Sakamoto et al. 1990). However, two activity peaks, corresponding to two cytosolic GS isoenzymes, were identified in plumule tissue from *Phaseolus vulgaris* seeds, and an additional minor peak, representing a plastidic GS isoenzyme, appeared in 2-d-germinated seeds (Bennett and Cullimore 1989). In sunflower cotyledons, two peaks of GS activity (GS_S and GS_1) are detected during early germination; later, the GS_S peak disappears and a new peak (GS_2) emerges. Both GS_S and GS_1 isoforms are probably involved in the reassimilation of the ammonia released from the amino acids resulting from the proteolysis of storage proteins in the cotyledons.

In contrast to GS_1, the levels of plastidic GS_2 in cotyledons of germinating sunflower greatly increase under light. This increase begins from the day 5 after sowing, coinciding with the emergence of cotyledons and their greening upon light action. The role of the light-induced stimulation of GS_2 levels in the developing seedling is probably to ensure the reassimilation of the ammonia released in the photorespiration of the green cotyledons. Edwards and Coruzzi (1989) have found that the light-increased expression of GS_2 in pea leaves is mediated by phytochrome but is also dependent on those growth conditions that favour the production of photorespiratory ammonia.

The appearance of GS_2 in sunflower cotyledons is also enhanced by added nitrate, particularly in the light. Nitrate is another source of internal production of ammonia and it would therefore also act, either directly or through its derived ammonia, as a signal to stimulate the synthesis of GS_2. It is worth noting that light and nitrate also induce nitrate reductase and nitrite reductase in the epigeal cotyledons of mustard (Schuster et al. 1987) and sunflower (de la Haba et al. 1988). Moreover, the appearances of nitrite reductase and GS_2 in the presence of light and nitrate are strictly coordinated to avoid the accumulation of ammonia (Weber et al. 1990). Nevertheless, ammonia provided externally does not appreciably increase the level of GS_2 in sunflower (de la Haba et al. 1988) or in mustard (Schmidt and Mohr 1989). Thus it seems that nitrate, rather than its derived ammonia, enhances the synthesis of GS_2. Supporting this view is the observation that nitrate addition also efficiently stimulates the synthesis of GS in sunflower seedlings pretreated with tungsten, a metal which inhibits nitrate reductase activity and therefore prevents the production of ammonia from nitrate (Agüera et al. 1987).

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References

Agüera, E., de la Haba, P., Maldonado, J.M. (1987) Induction of nitrate reductase, nitrite reductase and glutamine synthetase by nitrate and/or nitrite in germinating sunflower cotyledons. Effect of plant hormones. Plant Physiol. (Life Sci. Adv.) **6**, 255–258

Bennett, M.J., Cullimore, J.V. (1989) Glutamine synthetase isoenzymes of *Phaseolus vulgaris* L.: subunit composition in developing root nodules and plumules. Planta **179**, 433–440

Cabello, P., de la Haba, P., Maldonado, J.M. (1991) Isoforms of glutamine synthetase in cotyledons, leaves and roots of sunflower plants. J. Plant Physiol. **137**, 378–380

Coruzzi, G.M. (1991) Molecular approaches to the study of amino acid biosynthesis in plants. Plant Sci. **74**, 145–155

Cullimore, J.V., Bennett, M.J. (1988) The molecular biology and biochemistry of plant glutamine synthetase from root nodules of *Phaseolus vulgaris* L. and other legumes. J. Plant Physiol. **132**, 387–393

de la Haba, P., Agüera, E., Maldonado, J.M. (1988) Development of nitrogen-assimilating enzymes in sunflower cotyledons during germination as affected by the exogenous nitrogen source. Planta **173**, 52–57

Edwards, J.W., Coruzzi, G.M. (1989) Photorespiration and light act in concert to regulate the expression of the nuclear gene for chloroplast glutamine synthetase. Plant Cell **1**, 241–248

Hewitt, E.J. (1966) Sand and water culture methods used in the study of plant nutrition. Commonwealth Bureau of Horticultural and Plantation Crops, East Malling Tech. Commun. No. 22

Hirel, B., Vidal, J., Gadal, P. (1982) Evidence for a cytosolic-dependent light induction of chloroplastic glutamine synthetase during greening of etiolated rice leaves. Planta **155**, 17–23

Lea, P.J., Miflin, B.J. (1979) Photosynthetic ammonia assimilation. In: Encyclopedia of plant physiology, N.S., vol. 6: Photosynthesis II, pp. 445–456, Gibbs, M., Latzko, E., eds. Springer, Berlin Heidelberg New York

Lea, P.J., Joy, K.W. (1983) Amino acid interconversion in germinating seeds. In: Recent advances in phytochemistry, vol. 17: Mobilization of reserves in germination, pp. 77–109, Nozzolillo, C., Lea, P.J., Loewus, F.A., eds. Plenum Press, New York London

Lea, P.J., Robinson, S.A., Stewart, G.R. (1990) The enzymology and metabolism of glutamine, glutamate, and asparagine. In: The biochemistry of plants, vol. 16: Intermediary nitrogen metabolism, pp. 121–159, Miflin, B.J., Lea, P.J., eds. Academic Press, San Diego London

Mäck, G., Tischner, R. (1990) Glutamine synthetase oligomers and isoforms in sugarbeet (*Beta vulgaris* L.). Planta **181**, 10–17

Mann, A.F., Fentem, P.A., Stewart, G.R. (1979) Identification of two forms of glutamine synthetase in barley (*Hordeum vulgare*). Biochem. Biophys. Res. Commun. **88**, 515–521

McNally, S.F., Hirel, B., Gadal, P., Mann, A.F., Stewart, G.R. (1983) Glutamine synthetases of higher plants. Evidence for a specific isoform content related to their physiological role and their compartmentation within the leaf. Plant Physiol. **72**, 22–25

Sakamoto, A., Takeba, G., Shibata, D., Tanaka, K. (1990) Phytochrome-mediated activation of the gene for cytosolic glutamine-synthetase (GS_1) during imbibition of photosensitive lettuce seeds. Plant Mol. Biol. **15**, 317–323

Schmidt, S., Mohr, H. (1989) Regulation of the appearance of glutamine synthetase in mustard (*Sinapis alba* L.) cotyledons by light, nitrate and ammonium. Planta **177**, 526–534

Schuster, C., Oelmüller, R., Mohr, H. (1987) Signal storage in phytochrome action on nitrate-mediated induction of nitrate and nitrite reductases in mustard seedling cotyledons. Planta **171**, 136–143

Tingey, S.V., Tsai, F.-Y., Edwards, J.W., Walker, E.L., Coruzzi, G.M. (1988) Chloroplast and cytosolic glutamine synthetase are encoded by homologous nuclear genes which are differentially expressed in vivo. J. Biol. Chem. **263**, 9651–9657

Wallsgrove, R.M., Turner, J.C., Hall, N.P., Kendally, A.C., Bright, S.W.J. (1987) Barley mutants lacking chloroplast glutamine synthetase. Biochemical and genetic analysis. Plant Physiol. **83**, 155–158

Weber, M., Schmidt, S., Schuster, C., Mohr, H. (1990) Factors involved in the coordinate appearance of nitrite reductase and glutamine synthetase in the mustard (*Sinapis alba* L.) seedling. Planta **180**, 429–434