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LIGHT REGULATION OF NITRATE UPTAKE, ASSIMILATION AND METABOLISM

Cathrine Lillo

INTRODUCTION

Evolving from a germinating seedling, dependent on a stored restricted energy supply, into an active photosynthetic plant capturing the energy from sunlight is obviously a vulnerable time period in the life of a plant. Expression of genes involved in nitrogen reduction and assimilation during this time span is strongly influenced by low intensity red and blue light acting through special light receptors, phytochromes and cryptochromes. After greening and establishing of the photosynthetic apparatus, light acts on nitrogen metabolism through photosynthesis and the products thereof, sugars and certain carbon compounds. The ratio between products of nitrogen and carbon metabolism regulates transcription of genes in the nitrogen pathway and acts post-transcriptionally on these gene products. Later in a plant's life, day length, measured with the help of the red and blue-light absorbing receptors and circadian rhythms, are important for transition into different developmental stages, like reproduction or dormancy. Abundant nitrogen supply is known to sometimes counteract transition into flowering or dormancy, while on the other hand scarce nitrogen supply may induce flowering or dormancy. These interactions between nitrogen metabolism and development in the mature plant are well known but poorly understood and will not be covered in this review. The subject of this review is the influence of light on transcriptional and post-transcriptional regulation of genes involved in nitrate uptake, reduction and incorporation into organic compounds during greening and the vegetative stage.

Photomorphogenesis

There are two general means by which light modifies plant growth. Long-term growth requires a sufficient supply of energy via photosynthesis, which provides essential raw material, i.e. organic carbon, as well as energy for all biochemical reactions and growth. Light also influences growth via special photomorphogenetic pigments. These include the red/far-red light absorbing phytochromes and blue/UV light absorbing pigments. These pigments detect one or more conditions in the environment such as quality, quantity, direction and duration of light (Cosgrove 1994).

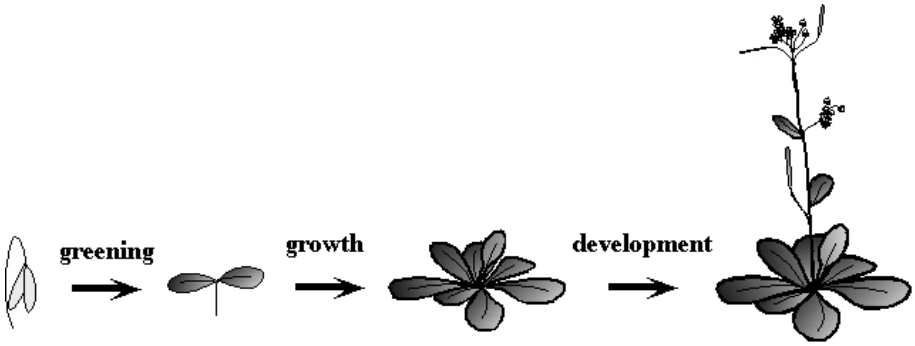


Figure 1. **Deetiolation, growth and development**
Greening: Low intensity red and blue light, in combination with nitrate induces expression of genes involved in nitrogen assimilation during deetiolation and greening.
Growth: Photosynthetic active light sustains further growth. Nitrate and products of both photosynthesis and nitrogen assimilation regulate expression of genes involved in nitrogen metabolism.
Development: Transformation into different developmental stages like flowering or dormancy can be induced by day length perceived by red and blue-light receptors. Nitrogen limitation may promote flowering and dormancy.

In the dark, plants develop in a special manner, stems become long and thin, leaves remain folded and small and the apical part of the stem often forms a hook (in dicotyledons). Furthermore, chloroplast development is suppressed and pigmentation reduced. Such appearance is referred to as etiolated and is a useful form of development for bringing a seedling or other propagules buried in the soil to the surface as rapidly as possible and without damage (Cosgrove 1994). On reaching the surface and light exposure, the developmental pattern changes, with leaves becoming green and photosynthetically active.

Efficiency in the greening process is obviously important for survival since the plant needs to capture energy and carbon through photosynthesis in order to replace the supply rapidly consumed from stored resources in seeds

and propagules. This critical transformation in the plant's life is much studied and reveals changes in metabolism preparing the plant not only for photosynthesis, but also for assimilation of nitrogen. The red and blue-light absorbing pigments are crucial for this transformation from etiolated into green, photosynthetically active plants.

The same groups of pigments are important for induction of flowering and dormancy, shading response etc.. Promotion of flowering by nitrogen limitation in otherwise day-length-influenced plants is well known (Tanaka 1986, Ishioka et al.1991). However, the interaction with nitrogen metabolism in processes like flowering induction or dormancy is not well understood at the molecular level.

Plants react differently to light during development. For example exposure to light often alters the sensitivity to further treatments of light exposure. The concentration of different light receptors changes as a result of illumination. There are certainly also species differences as well as tissue differences in response to light treatments. Action spectra related to a special process, for instance hypocotyl elongation show some variations concerning effects of red and blue light among species (Cosgrove 1994). Similarly, effects from red and blue light on expression of enzymes in the nitrogen pathway also vary among species.

Photoreceptors

Three sensory photoreceptor families are known: the phytochromes, cryptochromes, and phototropins (Quail 2002). In *Arabidopsis* 5 different phytochrome genes *PHYA-PHYE* have been identified, and the overall picture is that PHYB has a role at all stages of the life cycle, whereas PHYA, PHYD and PHYE exert their principal functions at selected stages (Smith 2000). Phytochromes have a tetrapyrrole chromophore, absorb light in the red and far-red spectrum, and undergo light-induced interconversion from a biologically inactive form into an active form. The phytochromes are cytosolically localised, but translocate into the nucleus upon irradiation where they may directly interact with transcription factors (Smith 2000). PHYB is activated by red light and inactivated by far-red light and is the form present in green tissue. PHYA is the abundant form in dark-grown tissue and responds to very low-fluence red light and high irradiance far-red light. Phytochromes also absorb in the blue-light region, which therefore results in overlapping action spectra of phytochromes and blue-light receptors (Casal 2002). The second class of photoreceptors, the cryptochromes, are blue-light receptors carrying two different chromophores, a flavine type and a pterin type. Two cryptochrome genes are found in *Arabidopsis*, *CRY1* and *CRY2*. Both cryptochromes are localised to the nucleus. The photochemical signal capture of CRY is not known, but is

likely to involve a redox reaction (Quail 2002). The third class of photoreceptors, phototropins, are also flavoproteins and hence blue-light receptors. Phytochromes and cryptochromes are generally found to be involved in morphogenetic processes, for example germination, de-etiolation and flowering, whereas the phototropins are involved in movements, like bending of the stem towards light or movements of chloroplasts in the cell and opening/closing of stomata (Briggs and Christie 2002). Phytochromes and cryptochromes are generally found to be involved in regulation of the nitrogen pathway in plants.

The picture may still not be complete concerning photoreceptors, another UV-B receptor with spectral properties different from cryptochromes and phototropins, appears to be present in plants as well (Briggs and Christie 2002).

The signal transduction chains connecting light perception by the various photoreceptors to regulation of gene expression is far from clear. Phosphorylation steps have been suggested to be involved, and a handful of proteins interacting with phytochromes or cryptochromes have been identified. COP1 is a protein found to interact directly with blue-light receptors (CRY and PHY) by protein-protein contact. COP1 has a role in mediating the regulated proteolysis of another protein, HY5, which is a bZIP transcription factor that promotes the expression of light-induced genes (Wang et al. 2001). PHY and CRY interact with COP1 and direct the migration of COP1 to the cytoplasm thereby reducing the activity of COP1. Mutations in *COP1* and *HY5* influence expression of nitrate reductase (see later paragraph; NITRATE REDUCTASE) as well as genes involved in photosynthesis. Another example of a transcription factor interacting with light is PIF3 (phytochrome interacting factor) which is constitutively expressed and member of the basic helix-loop-helix class of transcription factors. PIF3 binds to a G-box motif present in promoters of various light-regulated genes. It has been suggested that the active form of PHY translocate into the nucleus and bind to already promoter-bound PIF3 and thereby facilitate transcription (Quail 2002)

Light responsive elements (LRE)

When studying photosynthesis-associated nuclear genes a number of cis-acting elements involved in the control of transcription by light were identified (Arg ello-Astorga and Herrera-Estrella 1998). These elements were classified into different types, according to their nucleotide sequence and binding of transcription factors. Put simply, certain core sequence units are recognised in the DNA motifs, although not strictly identical in different plants or promoters. Examples of such motifs are: G-box (core unit ACGT), GTI-box (core unit GGTTAA), GATA-box (core unit GATA) and a special

GATA motif, I-box (core unit GATAAGA/G). A single motif can not function as a light responsive element (LRE), at least two different motifs have been found to be involved (Mart nez-Hern ndez et al. 2002). A minimal LRE containing an I-box (GATAAGA) and G-box (CACGTGGC) linked to a reporter gene was studied in *Arabidopsis* (Martinez-Hern ndez et al. 2002). Such an LRE resulted in reporter gene activation by both PHY and CRY, and red, blue or white light were all active in inducing a response. The minimal LRE also confined tissue specificity in adult plants, and expression of the reporter gene was only found in cells containing chloroplasts, like mesophyll cells of leaves, stems and petioles as well as stomata, but not in epidermis, vascular tissue or roots. The reporter gene was strongly expressed in *cop1* mutants, as expected, since COP1 mediates proteolysis of a factor (HY5) involved in transcription of light activated genes. Decreased expression was found in *hy5* mutants as predicted. However, 50% activity was retained in the *hy5* background showing that some other factor could partially replace HY5 (Mart nez-Hern ndez et al. 2002). Although putative light-responsive motifs are present upstream of many of the genes involved in the nitrogen pathway, very few elements have been examined for their function and shown to be involved in light-activation of the nitrogen pathway. Much, almost everything remains to be clarified concerning light reactive elements in promoters and enhancer regions of genes in the nitrogen pathway of plants.

Light activation by thioredoxins

Thioredoxins are small, ubiquitous, proteins found in both prokaryotes and eukaryotes. The highly conserved active site, Trp-Cys-Gly-Pro-Cys, contains a disulphide group that undergoes reversible redox changes between S-S and 2SH (Ruelland and Miginiac-Maslow 1999, Sch rmann and Jacquot 2000). Thioredoxins serve as electron donors in a variety of cellular redox reactions. Plants contain multiple forms, two in the chloroplasts (m and f), one in the cytosol and one in the nucleus. In chloroplasts and cyanobacteria, reduction of thioredoxins are coupled to light-driven photosynthetic electron transport and mediated through reduced ferredoxin. It is known for long that several Calvin cycle enzymes are activated by the ferredoxin-thioredoxin system. In addition to these enzymes related to CO_2-fixation, also other chloroplastic enzymes are regulated by thioredoxins, such as the coupling factor CF1 providing ATP for biosynthetic reactions, and NADPH-malate dehydrogenase which is important for the export of reducing equivalents (malate) to the cytosol. Also some chloroplast-located enzymes involved in nitrogen metabolism are activated by thioredoxins, i.e. glutamine synthetase, ferredoxin-dependent glutamate synthase, and the first enzyme in the

pathway of aromatic amino acids (Ruelland and Miginiac-Maslow 1999, Sch rmann and Jacquot 2000).

PII

PII signal transduction proteins are widespread, being present in eukaryotes, bacteria and archaea and are essential for regulation of nitrogen metabolism. In bacteria and archaea, PII proteins serve as the central processing unit for the integration of signals of carbon and nitrogen status (Ninfa and Atkinson 2000). A homologous gene has been identified in plants, and there is evidence that the same metabolites as in bacteria, 2-ketoglutarate and glutamate, are crucial for regulation of nitrogen metabolism in plants. This points to the possibility that PII may have a similar function in plants as in prokaryotes.

In *Escherichia coli* the PII protein is covalently modified by uridylylation of a conserved tyrosyl residue (gives PII-UMP). High concentration of glutamine triggers this uridylylation of PII. The carbon-status indicator molecule α-ketoglutarate binds directly to PII. Overall, in *E. coli* PII is converted into different forms in response to the concentration of nitrogen and carbon compounds, and acts post-translationally upon the activation and inactivation of an enzyme in the nitrogen assimilation pathways, i.e. glutamine synthetase (Ninfa and Atkinson 2000). PII also affects regulation on the level of transcription, and does so by influencing the phosphorylation status of a protein necessary for transcription of the glutamine synthetase gene in *E.coli*.

In Cyanobacteria (*Synechococcus*), PII regulates uptake of nitrate and nitrite, possibly by direct interaction with a component of the uptake system. Modification of PII in cyanobacteria is via phosphorylation of a conserved seryl residue. When PII is unphosphorylated the uptake processes are inhibited. This seryl residue is conserved also in plant PII proteins, whereas the tyrosyl residue, which is uridylylated in *E. coli*, is replaced by phenylalanine in plants (Hsieh et al. 1998). PII like proteins have so far been identified in *Arabidopsis thaliana* and *Ricinus communis* (Hsieh et al. 1998). The plant PII is a nuclear-encoded chloroplast protein. Intriguingly, expression of PII is influenced by the same factors that are supposed to be sensed by the PII protein itself. PII mRNA was induced by light in dark-adapted green *Arabidopsis* leaves, and sucrose stimulated mRNA accumulation in both darkness and light, whereas asparagine, glutamine and glutamate partly reversed the positive effect of light or sucrose. Red light did not lead to the accumulation of PII mRNA, hence phytochrome did not appear to be involved in PII expression. Ferrario-M ry and co-workers (2000) presented clear evidence with transgenic tobacco plants that the balance between the important modulators of PII proteins, glutamine and α-

ketoglutarate, are essential for regulation of nitrogen metabolism also in plants. The *Arabidopsis* PII has recently been cloned and overexpressed in *E.coli*, and polyclonal antibodies have been produced towards the *Arabidopsis* PII (Smith et al. 2002). Expression patterns, post-translational modifications and a role of PII in control of nitrogen metabolism is expected soon to be revealed also for plants.

Fungi

Although PII is found in bacteria, archaea, algae and plants and may have a crucial role in regulation of nitrogen metabolism in these organisms, there are no reports or Blast hits found for fungi or yeast when searching for this protein. However, another common principle concerning regulation of nitrogen metabolism is widespread among fungi and yeast. In these organisms compounds like ammonia, glutamine, glutamate, and asparagine are preferentially used as nitrogen source. As long as these compounds are present, expression of genes for nitrate uptake and reduction are repressed. However, when these favoured nitrogen sources are not available, other sources, for instance nitrate can be used. The necessary genes are then transcribed (Marzluf 1997). Positive acting, global regulatory genes are found in yeast and fungi, *Nit-2* in *Neurospora*, *AreA* in *Aspergillus*, and *Gln-3* in *Saccharomyces*. These genes all code for GATA-type zinc finger transcription factors. These global factors bind to promoters of various genes regulated by nitrogen. For example the promoter region of the *Neurospora* nitrate reductase gene has three binding sites for NIT2 all containing GATA elements. When glutamine and other preferable nitrogen sources are scarce NIT2 becomes active, binds to the promoter region of nitrate reductase and, together with a pathway specific transcription factor, NIT4, activate transcription. Glutamine is a critical metabolite, which exerts catabolite repression in *Neurospora*; however, a still unknown feature is the identity of the element, or signal pathway that senses the presence of repressing levels of glutamine (Marzluf 1997). The repression of gene expression by glutamine and other reduced nitrogen compounds has a parallel in plants since glutamine and NH_4^+ are known to repress transcription of several genes in the nitrogen pathway also in plants. This raised the question of whether the regulatory mechanism is conserved between fungi and plants, and attempts have been made to identify a similar system in plants as in fungi. The NIT2 protein of *Neurospora* was found to bind upstream of the NR structural gene from tomato in vitro (Jarai et al. 1992), and a gene homologous to the *NIT2* gene was identified in tobacco. This gene was called *NTL1* (for nit-2-like) and the amino acid sequence of the Zn-finger domain showed 60% identity to the NIT2 protein. It still remains to be shown if the NTL1 protein is involved in regulation of NR or other genes in

plants (Daniel-Vedele and Caboche 1993). GATA motifs are present in several of the genes involved in nitrogen uptake and metabolism in plants, hence putative targets for a NIT2 like transcription factor are present.

A complementation approach in yeast was undertaken, using a *Saccharomyces* mutant deficient in the global regulatory protein GLN3. Three *Arabidopis* cDNAs were found that restored growth of the yeast mutant on certain nitrogen sources, and led to the isolation of *RGA* and *GAI* cDNA. Surprisingly, these were the same genes already known to be involved in the response to gibberellins in plants. Loss of function (*rga2*, *gai-t6*) and gain of function (*gai-1*) mutants were also studied. These studies did not, however, identify genes involved in nitrogen metabolism or regulation thereof in plants (Bouton et al. 2002).

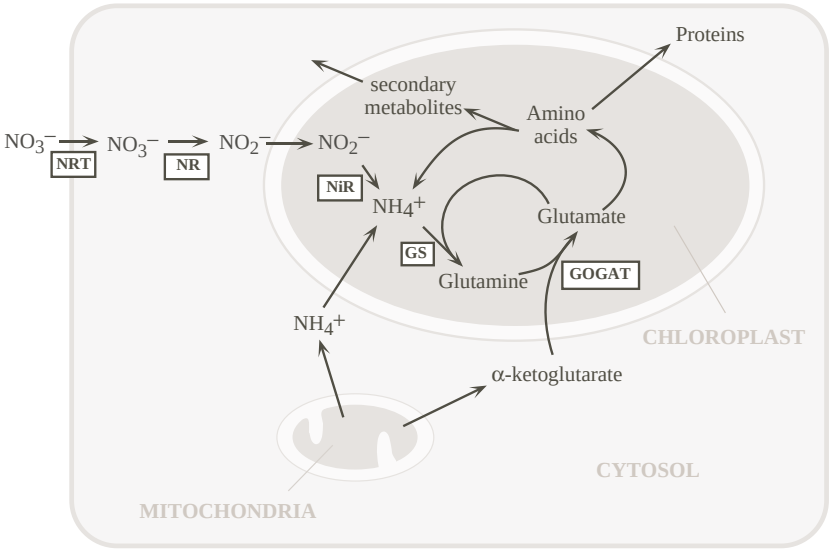


Figure 2. **Overview of the nitrogen assimilation pathway in (green) cells.** Nitrate is transported into the cell by the help of special nitrate transporters (NRT). Nitrate is then reduced in the cytosol by help of NAD(P)H and nitrate reductase (NR) to nitrite, which is further reduced in the chloroplast by reduced ferredoxin and nitrite reductase (NiR). Ammonium is then incorporated into amino acids by glutamine synthetase (GS) which converts glutamate and NH_4^+ to glutamine, and this reaction requires ATP. Glutamine and α-ketoglutarate are converted to two molecules of glutamate by glutamate synthase (GOGAT). Several amino acids are formed from glutamate and keto acids by transaminases, and form the basis also for further synthesis of other amino acids. Amino acids are used for building proteins, and different nitrogen compounds as well as being the start point for synthesis of secondary compounds. All the enzymes shown in brackets are known to be up-regulated by light in most plants tested.

NITRATE TRANSPORTERS

Recently genes from two different families of nitrate transporters named *NRT1* and *NRT2*, were cloned from a wide range of higher plants (Forde 2000). In *Arabidopsis* eight *NRT1* genes or closely related genes and seven different *NRT2* genes have been reported (*Arabidopsis* genome project, Orsel et al. 2002). Physiologically different types of nitrate transporters have been identified (Chapter 1). Some transporters have low affinity for nitrate, LATS (Low Affinity Nitrate Transporters), while others have high affinity for nitrate, HATS (High Affinity Nitrate Transporters). Generally, low affinity transporters were thought to be related to the *NRT1* family, and high affinity transporters were related to the *NRT2* family. However, the picture is more complex. For instance the *Arabidopsis NRT1.1* transporter contributes to high as well as low affinity transport (Touraine et al. 2001). Some *NRT* genes are induced by NO_3^-, whereas others are constitutively expressed, although also for these latter genes, expression may still be enhanced by NO_3- availability. Ammonium and various amino acids, especially gluatamine, are recognised as compounds decreasing expression of *NRT* genes. Carbohydrates are found to stimulate expression of the genes. Furthermore, expression of the various genes are tissue specific, many being preferably expressed in the roots (Forde 2000, Touraine et al. 2001). There is also evidence for post-transcriptional control being involved in regulation of nitrate transport (Vidmar et al. 2000, and references therein).

Nitrate transporters and phytochrome

There is some evidence that red light may influence uptake of nitrate in algae. Red and blue light pulses for 5 min stimulated nitrate uptake in the red alga *Corallina elongata*, and the light effects were reversed by far-red light in accordance with phytochrome being involved (Figueroa 1993). Also in a green alga, *Ulva rigida*, positive effects of red and blue light pulses on nitrate uptake reversed by far-red light were found (Lopez-Figueroa and Ruediger 1991). *Spirodela polyrhiza* (*Lemnaceae*), a moncot water-plant, has been much used for studying nitrogen metabolism in relation to phytochrome; however, no influence on nitrate uptake by the phytochrome system was found (Appenroth et al. 1992). The literature gives very little information on phytochrome effects on nitrate uptake in higher plants, and when examined generally no or very small effects on nitrate uptake as influenced by the phytochrome system have been found (Sasakawa and Yamamoto 1979).

Interestingly, oligonucleotide arrays revealed a nitrate transporter gene regulated by PHYA in *Arabidopsis*. One hour of far-red light induced the expression of this nitrate transporter gene *NTP3* (CAB38706) (Tepperman et al. 2001). The *NTP3* gene codes for a protein predicted to contain the 12 putative trans-membrane helices found in other transporters of the *NRT1* family; however, there is as yet no information on the transport function of the NTP3 protein. Four other nitrate transporters present on the Affymetrix oligonucleotide arrays were not identified as targets of PHYA signalling. It should be noted, however, that plants were grown on half-strength MS-medium, and the high concentration of ammonium and nitrate experienced by the plants for several weeks may inhibit expression of nitrate uptake genes. In addition to nitrate transporters providing uptake of nitrate into the roots, other nitrate transporters are involved in allocation of nitrate to the vacuole, or loading of nitrate into the xylem. Since phytochrome effects on nitrate uptake into the roots is not generally found, possibly the NTP3 transporter may be involved in nitrate translocation inside the cell, or loading of nitrate into the xylem to provide nitrate to other parts of the plant. The effect of far-red light on a nitrate transporter gene actualises results obtained several years ago showing that non-photosynthetic light reactions were important for intracellular nitrate movement in etiolated pea and barley leaves (Jones and Sheard 1979).

Nitrate transporters and blue light

In various algae, blue light was often found to have a positive effect on nitrate uptake. Low irradiance blue light has been found to stimulate nitrate uptake in the green algae *Monoraphidium braunii* (Aparicio and Quinones 1991, Ullrich et al. 1998), and *Ulva rigida* (Lopez-Figueroa and Ruediger 1991). Also in *Chlorella* blue light stimulated nitrate uptake independent of photosynthesis (Maetschke et al. 1997). The blue-light effect appears to act through a signal transduction chain involving phosphorylation since the blue-light effect was sensitive to protein kinase inhibitors (Tischner 2000). In higher plants, i.e. rice seedling, different light qualities, including blue light, were tested, but neither 5 min nor 6 h illumination with blue light stimulated nitrate uptake (Sasakawa and Yamamoto 1979).

Nitrate transporters and white, photosynthetic active light

Photosynthesis and carbon metabolism were recognised as important for nitrogen assimilation already in the early days of research on nitrogen assimilation back in the 50's using unicellular algae as the experimental system (reviewed by Huppe and Turpin 1994). An early observation was that nitrogen-sufficient algae required light to assimilate nitrogen, but nitrogen-limited cells rapidly assimilated nitrogen in the dark. Intracellular

carbohydrate stores decreased during dark assimilation and assimilation ceased when these stores were depleted (Huppe and Turpin 1994). From experiments on photosynthesis and uptake of $^{15}NO_3^-$ in maize seedlings, Pace and co-workers (1990) concluded that nitrate uptake and reduction were regulated by the supply of energy and carbon skeletons required to support these processes. Also in ryegrass nitrate uptake was closely related to photosynthesis with a time lag of about 5 h between photosynthesis and uptake (Scaife 1989). Furthermore diurnal regulation of nitrate uptake was found in several plants (Delhon et al.1995, Cardenas-Navarro et al. 1998). In *Arabidopsis* roots, the transcript levels of both *AtNRT1.1* and *AtNRT2.1* were found to undergo diurnal variations, with transcript levels in the light reaching more than 5-fold of those in the dark (Lejay et al.1999). Onset of darkness was accompanied by a rapid decrease in mRNA transcript levels as well as a decrease in $^{15}NO_3^-$ influx. Supply of 1% sucrose at the time of light to dark transition hindered the decrease in transcript levels and nitrate influx, supporting the view that the positive effect of light is due to products of photosynthesis being translocated to the roots. Nitrogen uptake genes are well known to be regulated by nitrogen status in the plant, a demand for nitrogen will enhance expression of these genes and increase uptake of nitrate. The effect exerted through products of photosynthesis or nitrogen deficiency appear to be differently mediated because *AtNRT1.1* and *AtNRT1.2* are affected in the same way by light and sucrose supply, but are differently affected by nitrogen deficiency (Lejay et al. 1999). The *Arabidopsis* genome project reveals a range of potential cis-regulating elements upstream of nitrate transporter genes, like GATA, ACGT (G-box core), AG/CTCA (nitrate element) and for some also GATAAGA/G (I-box). However, the significance for regulation of transcription by these elements remains to be examined. The mechanism of signal transduction from carbohydrates and nitrogen compounds leading to changes in expression are still unknown, as are the transcription factors and cis-elements actively involved.

Tissue specific expression

Since the nitrate transporter genes are differentially expressed in leaves and roots, specific tissue factors, as well as interaction with light are expected to be involved in achieving the specific expression of these genes. One of the *AtNRT2* genes, i.e. *AtNRT2.7* was expressed in leaves but not in roots (Orsel et al. 2002). A minimal promoter with an I-box and a G-box which confined light responsiveness to a reporter gene, and at the same time restricted expression to cells containing chloroplasts had previously been described (see INTRODUCTION, Light responsive elements (LRE)). However, the mechanism for tissue specificity of *AtNRT2.7* is not known,

and the I-box is not present in the promoter area of this gene. Interestingly *AtNRT2.7* is the only nitrate transporter (-like) gene known to code for a putative chloroplast targeted peptide, and therefore this transporter is a candidate for being located in the chloroplast membranes (*Arabidopsis* genome project, MIPS database). *NRT2.3* is the other *NRT2* gene strongly expressed in leaves. The gene was expressed in the shoots of young plants, but at the reproductive stage stronger expression was found in the roots (Orsel et al. 2002). The function of all the *NRT* genes is not known, and some may be involved in transportation of other anions than nitrate, for instance ions that need to be transported into chloroplasts. Most nitrate transporters are preferably expressed in roots, and generally, not much is known about root-specific expression. A member of the *NRT1* family, *NRT1.1*, was expressed in young leaves as well as in roots, and auxin, was found to be important for targeting *NRT1.1* expression to nascent organs (Guo et al. 2002). Except for auxin no signals for tissue specificity or elements involved in tissue specific regulation have yet been revealed for the nitrate transporters.

NITRATE REDUCTASE (NR)

$$NO_3^- + NAD(P)H \rightarrow NO_2^- + NAD(P)^+ + 0H^-$$

NR catalyses the reaction where electrons from NAD(P)H reduce nitrate to nitrite. In *Arabidopsis* two different genes coding for NADH:NR have been identified *NIA1* and *NIA2*. Most higher plants examined have been shown to possess two or more genes coding for NR; however, *Solanaceae* species such as *Nicotiana* spp. and tomato have only one *NIA* gene per haploid genome (Rouz and Caboche 1992). NR using NADH as an electron donor (EC 1.6.1.1) is the dominant form in higher plants. A NADH/NADPH bispecific form (EC 1.6.1.2) is found in addition to the NADH specific form in N_2 fixing plants like soybean, and also in monocot species including maize, rice and barley (Miyazaki et al. 1991). In birch only a bispecific form is present (Friemann et al. 1991). Monospecific NADPH:NR (EC 1.6.1.3) occurs in mosses and fungi but is not found in higher plants. Only small changes in the structure of NR are required to change the enzyme from a NADH specific to a NADPH (bi)specific form. In birch, altering only one amino acid changed the enzyme from a bispecific to a NADH specific enzyme (Schondorf and Hachtel 1995). The reason why plants possess NRs with different specificities for NADH and NADPH is not clear. Those plants that have both forms tend to express the bispecific form in the roots.

NR and phytochrome

Light has been known to influence nitrate reductase expression for several decades. NR induction mediated by phytochrome was first reported in etiolated peas in 1972 (Jones and Sheard). Since then the involvement of phytochrome in NR induction was established for a large number of dicot and monocot plants (Sopory and Sharma 1990, Lillo and Appenroth 2001). Light absorbed by phytochrome generally leads to increased levels of NR mRNA, protein and enzyme activity in etiolated plants. Often, the light effect was found to be dependent on availability of nitrate (Appenroth and Oelm ller 1995, Li and Oaks 1995, Migge et al.1997), although this was not always the case (Sopory and Sharma 1990).

Interestingly, different *NIA* genes within a species may show different responses to irradiation as demonstrated for *Arabidospis NIA1* and *NIA*2 in the wild type (Cheng et al. 1991), and the *cr88* mutant (Lin and Cheng 1997). *Cr88* is a chlorate resistant mutant of *Arabidopsis* that exhibits the etiolated phenotype not only in darkness but also in continuous red light; with long hypocotyls and closed, or partially expanded cotyledons, and delayed greening. At the seedling stage NR activity was low in *cr88*, and *NIA2* was not induced by light as in wild type seedlings. In mature *cr88* plants light effects were as for wild type, thus demonstrating that light effects in mature plants are mediated very differently from light effects in etiolated seedlings. Stimulation of NR expression by sucrose was similar in *cr88* and wild type plants, and is consistent with light effect in mature plants being mediated through products of photosynthesis such as sucrose or other carbohydrates. Although light absorbed by phytochrome increased *NIA* expression in a vast number of species, *NIA* expression and NR activity generally continued to increase to a much higher level when plants were placed under conditions allowing photosynthesis, or when given sucrose. For instance in etiolated barley and wheat, NR activity increased approximately 2-fold in response to red light pulses, although, the activity was still only 5 and 35%, respectively, of the activity found in plants grown in photosynthetic active light (Lillo and Henriksen 1984, Melzer et al. 1989). In tomato cotyledons, NR protein after red or far-red light treatments was 20 to 30% of NR protein in white light grown seedlings. The NR mRNA level was, however, nearly the same in response to red light or photosynthetic active light. White light apparently acts post-transcriptionaly by stabilising, or increasing synthesis of the NR protein in tomato (Migge et al. 1997).

NR and photosynthesis

Products of photosynthesis, i.e. various carbohydrates are well known to stimulate *NIA* expression and NR activation. The products of nitrogen

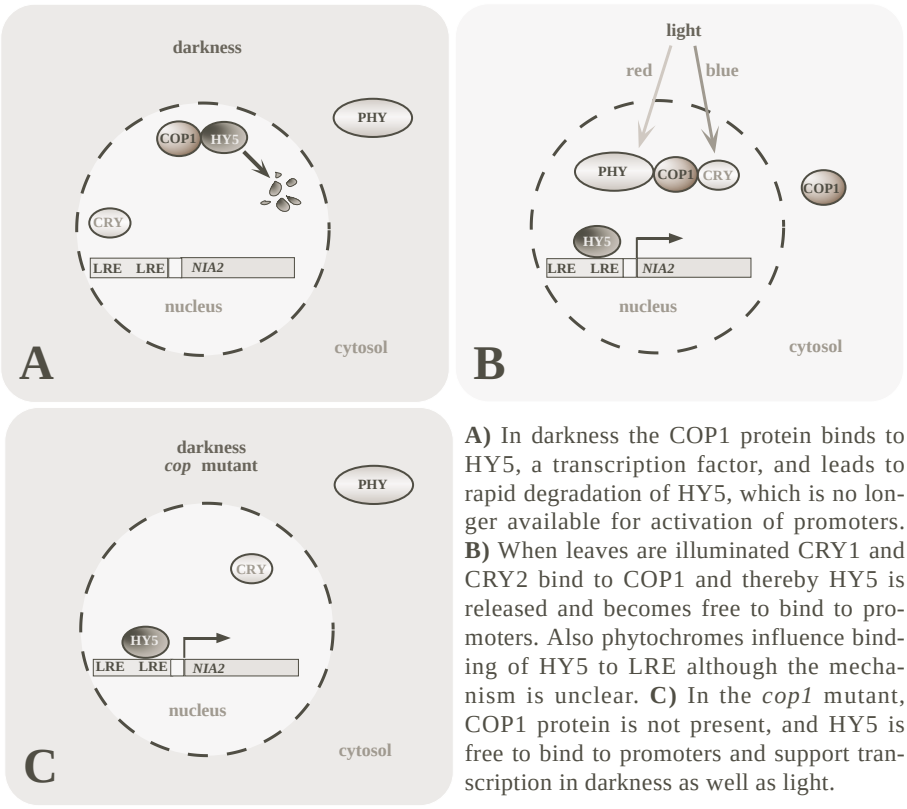
assimilation on the other hand, and especially glutamine, are known to exert a negative feedback on *NIA* expression (Sivasankar and Oaks 1996, Coruzzi and Bush 2001). In leaf discs of wild type tobacco plants the NR mRNA level was low when discs were fed with glutamine, whereas feeding sucrose or α-ketoglutarate resulted in a high NR mRNA level. In transgenic plants expressing antisense GOGAT, and thereby possessing only 10% of wild type GOGAT activity, the NR mRNA level was high in spite of high in situ concentrations of glutamine (Ferrario-M ry et al. 2001). However, in these transgenic plants the concentration of α-ketoglutarate was also high. It appears that the expression of NR depends mostly on the ratio between glutamine and α-ketoglutarate, and the inhibiting effect of glutamine on transcription is overcome by high concentration of α-ketoglutarate. The experiments of Ferrario-M ry and co-workers are strongly supportive of PII (see previous discussion) being involved in regulation of nitrogen metabolism in plants, and PII may be a "missing link" between photosynthesis and regulation of NR expression. However, sucrose, which increased the NR mRNA level, did not lead to increased α-ketoglutarate concentration in situ, as would have been expected if α-ketoglutarate were a component in the regulatory system. It still remains to be established that PII really is a sensor for products of photosynthesis and reduced nitrogen in plants. In *E. coli*, PII influences the phosphorylation status of a protein that interacts with transcription factors. In plants this would necessarily be more complicated since PII is a plastidic protein and hence separated from active transcription factors in the nucleus. Depending on carbon/nitrogen status PII may influence translocation of certain factors across chloroplast membranes and subsequently influence components in the nucleus, which then influence transcription. Such a signal transduction pathway from PII to the transcription level remains to be revealed.

NR promoters and light regulation

Deletion analysis of the 1.5-kb 5'-flanking regions of the *Arabidopsis NIA1* and *NIA2* genes showed that 238 and 188 bp, respectively, were important for nitrate induction of a reporter gene (*CAT*) in transgenic tobacco (Lin et al. 1994). A 12 bp conserved sequence with a core consensus AG/CTCA, a "nitrate element", was necessary for nitrate dependent transcription (Hwang et al. 1997). Sequences involved in light regulation of *Arabidopsis* NR have not been reported investigated so far.

In bean (*Phaseolus vulgaris*) two NADH:NR genes were identified and their promoters studied in transgenic tobacco with the help of a GUS reporter gene. The one *NIA* promoter studied in more details showed that a 900 bp region proximal to the transcription initiation site was necessary for

high expression in leaves and roots. Surprisingly it was not possible to find any significant effect of nitrate on the expression of GUS. Circadian rhythms of the GUS mRNA was retained, and this may indicate that light responsive elements were present within the construct since light responsive and circadian elements may be inseparable (Lillo et al. 2001). Many light and circadian regulated genes contain GATA motifs in their promoters (Teakle and Kay 1995). Five GATA motifs were found within the 700 bp upstream of the transcription start site, but their role in regulation of transcription of the bean *NIA* genes has not been clarified (Jensen et al. 1996). Deletion analysis of the 1.6-kb 5'-flanking sequence of the birch *NIA* gene were performed by



A) In darkness the COP1 protein binds to HY5, a transcription factor, and leads to rapid degradation of HY5, which is no longer available for activation of promoters. **B)** When leaves are illuminated CRY1 and CRY2 bind to COP1 and thereby HY5 is released and becomes free to bind to promoters. Also phytochromes influence binding of HY5 to LRE although the mechanism is unclear. **C)** In the *cop1* mutant, COP1 protein is not present, and HY5 is free to bind to promoters and support transcription in darkness as well as light.

Figure 3. **Working model for blue and red light effects on *NIA* transcription**

fusing promoter sequences with the GUS reporter gene and introducing them into *Nicotiana plumbaginifolia* (Strater and Hachtel 2000). Interestingly, the promoter length conferring maximal light stimulation was different in roots

and leaves. Light responsiveness was retained within 237 bp and 320 bp proximal of the transcription start site for roots and leaves, respectively. Only high intensity, white light was tested. Nitrate responsiveness was found for the 643 bp promoter fragment, but not for the 535 bp, indicating that important sequences for nitrate responsiveness were between 535 bp and 643 bp. As for bean, a leaf specific, negative cis-element was found around 1000 bp upstream from the transcription start site. Both the nitrate motif AG/CTCA and GATA motifs are present in the birch NR promoter, but their function has not been further evaluated. When *NIA* promoters from birch, bean or *Nicotiana* linked to a reporter gene or *NIA* structural gene were introduced into the *N. plumbaginifolia* genome only a small number of transgenic plants were obtained expressing that gene. Furthermore, expression was often very low (Strater and Hachtel 2000, Warning and Hachtel 2000). This points to the importance of location of the *NIA* gene in the genome for expression, a factor not yet fully understood.

Specific transcription factors or proteins mediating light effects on transcription of *NIA* genes are largely unknown. The HY5 protein appears to be the only example so far of an identified transcription factor likely to be involved in regulation of *NIA* expression. The *HY5* locus encodes a bZIP transcription factor known to be important for deetiolation and expression of several genes related to photosynthesis. In *Arabidopsis* the HY5 protein binds to Light Responsive Elements (LRE) in promoters, for instance a G-box (Chattopadhyay et al. 1998). HY5 degradation is stimulated by another protein, COP1. In darkness COP1 binds to HY5 and this leads to rapid degradation of HY5, hence HY5 is not available for activation of promoters (Fig. 2A). When leaves are illuminated, cryptochromes bind to COP1 and thereby releasing HY5, which becomes free to bind to promoters. Phytochromes influence binding of HY5 to LRE as well although the mechanism is still unclear (Quail 2002) (Fig. 2B). In the *cop1* mutant, seedlings deetiolate in total darkness as if they had perceived a light signal. Genes involved in photosynthesis are induced in darkness, as is also *NIA2* in this mutant (Deng et al. 1991). Since COP1 protein is not present, HY5 is free to bind to the promoters of these genes and support initiation of transcription (Fig. 2C). Other, still unknown transcription factors and components are likely also to be involved in *NIA* expression.

Light and post-transcriptional regulation of NR

Post-translational regulation of NR is also strongly influenced by light (Lillo 1994, Lillo and Appenroth 2001). Most of what is known about post-translational NR regulation, with few exceptions (Appenroth et al. 2000), has been deduced from experiments using green leaves in which mainly effects of photosynthetic active light have been studied. NR in higher plants is

phosphorylated at a special serine residue in the hinge between the molybdenum co-factor binding domain and the heme binding domain (serine 534 in *Arabidopspis*). Generally NR is inactivated in darkness by phosphorylation, and activated by dephosphorylation in the light (Kaiser and Spill 1991, MacKintosh 1992). However, the system is more complex than simply phosphorylation and dephosphorylation because members of the 14-3-3 protein family also bind to phosphorylated NR (reviewed by Kaiser and Huber 2001, Lillo and Appenroth 2001, MacKintosh and Meek 2001). 14-3-3 proteins belong to a highly conserved protein family with regulatory roles in plant, fungal and mammalian cells (MacKintosh and Meek 2001). It is after the binding of these 14-3-3 proteins that phosphorylated NR is actually inhibited, and inhibition is only observed in the presence of cations. The most important cations for this inhibition are Mg^{2+} and polyamines (Provan et al. 2000).

In etiolated barley leaves the inactive (phosphorylated) form of NR was dominant, and red light absorbed by phytochrome did not influence the activation state of NR. The potential for activation of NR was present in etiolated leaves since acid loading, a well known treatment to activate NR, is efficient for NR activation also in etiolated leaves (Appenroth et al. 2000). Post-translational activation of NR is triggered by photosynthesis, and mediated through the balance of kinases and phosphatases acting on NR. Photosynthesis may lead to a decrease in Ca^{2+} in the cytosol and an increase in phosphorylated sugar compounds, with both changes leading to decreased NR kinase activity and hence activation of NR. The significance of these factors for regulating NR in situ is not clarified. Phosphatases are involved directly, by dephosphorylating NR, and also indirectly by dephosphorylating the NR kinases, and thereby inactivating these kinases. Still, little is known about regulation of phosphatases in plants. In prokaryotes PII is involved in transcriptional as well as post-translational regulation of enzymes in nitrogen metabolism (see INTRODUCTION, PII). In plants this has not yet been deeply studied, but so far no indications of involvement of PII in post-translational regulation were found. In fact, high concentration in situ of the signal component, α-ketoglutarate, had no effect on the phosphorylation state of NR (Ferrario-M ry et al. 2001).

Light activates transcription of NR, and light activates NR post-translationaly. Light acts on yet another level, which became clear when examining expression of the NR gene linked to the 35S CaMV promoter (Vincentz et al. 1993). As expected, NR was constitutively expressed and NR mRNA levels were high in darkness (after 56 h) as well as in the light. Accumulation of NR protein and activity were, surprisingly, still promoted by light. This could be explained by increased rate of NR synthesis or decreased rate of degradation in the light, or both. Since light favours

dephosphorylation of NR it is tempting to assume that the non-phosphorylated form, which dominates in the light is more stable than the phosphorylated form. Post-translational modification of proteins has often been found to be a signal for degradation (Callis 1995). Experiments with spinach did indeed support this as phosphorylated NR was shown to be more rapidly degraded than non-phosphorylated NR (Kaiser and Huber 2001). This did not however, appear to be the case in *Arabidopsis* cell cultures (Cotelle et al. 2000) or in *Nicotiana* species (Lillo et al. 2003). The findings for *Nicotiana* species are in agreement with light having a positive effect on the translation process.

Toxic products and by-products of NR: nitrite, peroxynitrite and nitric oxide

The very complex means of regulation of NR may have evolved not only to optimise NR activity in response to nitrate and energy (light) supply, but evolution may also have been driven by the importance of down-regulating NR activity under certain conditions. For instance in darkness, further assimilation of the product from NR, NO_2^- (nitrite), is slow, because this step is usually closely linked to photosynthesis. Unless NR activity is rapidly down-regulated, nitrite may accumulate in darkness. Nitrite is mutagenic and accumulation of NO_2^- may be detrimental to the plant in the long run. Furthermore, NR reduces not only NO_3^- to NO_2^- (Ruoff and Lillo 1990), but under certain conditions reduces also O_2 to form O_2^- (superoxide anion) and NO_2^- to form NO (nitric oxide) (Yamasaki 2000, Rockel et al. 2002). Nitric oxide may have both detrimental and beneficial effects in plants, and the subject is far from fully understood. Peroxynitrite ($ONOO^-$), a highly reactive and very toxic compound, is made non-enzymatically from NO and O_2^-. A strict regulation of NR may, therefore, be necessary to avoid by-products causing oxidative damage and mutations in the plants. These reactions of NR are discussed in chapter 6 of this book (Kaiser et al.)

Nitric oxide also plays a key role in atmospheric chemistry and is important for atmospheric radical balance and for generation of photooxidants (Wildt et al. 1997). Previously, vegetation was not taken into account as a source of NO in the atmosphere, probably because NO emission from plants was firstly only measured under certain conditions. More recently, NO emission from a variety of nitrate nourished plant species was observed under normal growth conditions. During daytime the NO emission was closely correlated to CO_2 uptake. Furthermore, when nitrate content of the nutrient solution was enhanced, NO emission was observed also in darkness. On a global basis NO emission from plants was calculated to be 1-5%, only, compared to the NO evolved from soil due to microbial activity.

However, over areas with dense plant cover the emission from plants is not negligible (Wildt et al. 1997).

NITRITE REDUCTASE (NiR)

$$NO_2^- + 6\ \text{ferredoxin}_{red} + 8H+ \rightarrow NH_4^+ + 6\ \text{ferredoxin}_{ox} + 2\ H_20$$

After reduction of nitrate to nitrite in the cytosol, nitrite is translocated into the chloroplasts/plastids where further reduction takes place with the help of nitrite reductase (NiR, EC 1.7.7.1). Plant NiR uses reduced ferredoxin formed in photosynthesis as electron source. In darkness or in non-green tissue, ferredoxin or a ferredoxin-like protein can be reduced by NADPH from the oxidative pentose phosphate pathway. NiR is a nuclear-encoded protein with an N-terminal signal peptide that directs it to the chloroplasts (Meyer and Stitt 2001). Some higher plants contain only a single *NII* gene (gene encoding NiR) per haploid genome, whereas other plant species contain two or more copies (Wray 1993). The amphidiploid *Nicotiana tabacum* contains four genes, two from each ancestor. Because nitrite is toxic, cells must possess enough NiR to reduce all the nitrite produced by NR. As for *NIA* genes, nitrate and light are the two basic factors necessary for strong expression, and *NII* and *NIA* genes are generally regulated in co-ordination. In etiolated plants with no nitrate source, expression of *NII* genes is often very low, or not detectable. Variations among species and different organs and tissues are, however, clearly seen.

NiR and light effects in various plants

There are not many reports concerned with light effects on NiR in *Arabidopsis*, but *NII* expression was examined in the chlorate-resistant *cr88* mutant. At the seedling stage *cr88* was altered in the regulation of *NIA2* expression compared to wild type plants. *NIA2* expression, was not induced following 8 h (white) light exposure, nor were the photosynthetic genes coding for RBCS and CAB, but the seedlings still expressed *NII* as in the wild type. The work with *cr88* therefore showed that the signal transduction chains leading to activation of *NIA*2 and *NII* expression are different in *Arabidopsis* (Lin and Cheng 1997).

A phytochrome deficient tomato mutant, the *aurea* mutant, was used to study expression of both *NIA* and *NII* genes. This mutant has less than 5% of spectrophotometrically active phytochrome compared with wild type tomato. Both *NIA* and *NII* expression was impaired in the mutant when testing etiolated seedlings (Becker et al. 1992). This clearly showed that

phytochrome was important for inducing both *NIA* and *NII* expression in young etiolated seedlings. Analysis of wild type tomato plants revealed the presence of two *NII* genes. One gene being mainly expressed in etiolated leaves, whereas the other gene was expressed in green leaves (Migge et al.1998). Red, blue or UVA irradiation induced *NII* expression, consistent with the involvement of phytochrome and possibly a blue-light receptor (Goud and Sharma 1994, Migge et al. 1998). Blue light especially stimulated accumulation of one of the NiR proteins (NiR2). As pointed out by Migge et al. (1998), a UVA effect does not necessarily imply involvement of a specific blue/UV-light receptor because phytochrome may also be converted, to some extent, into its active form by UVA light. In fact Goud and Sharma (1994) found that the positive effect of blue light on NiR in wild type tomato disappeared in the *aurea* mutant, indicating that phytochrome was indeed the important photoreceptor. In mature, green leaves of the aurea mutant activities of NR and NiR were identical with activities in wild type on a per mg chlorophyll basis. Levels of NR and NiR mRNA and their diurnal variations were also very similar in leaves of the mature green aurea mutant and wild type plants (Becker et al. 1992). This indicates that different signal transduction chains from light to expression of *NIA* and *NII* are operating in seedlings contra mature plants. An interesting difference observed for tomato in comparison with other species was a strong, positive light effect on expression also when nitrate is removed from the growth medium and replaced with an alternative nitrogen source, such as glutamine.

Effects of different light sources on *NII* expression have also been studied in *Nicotiana tabacum* and *Nicotinana plumbaginifolia*. Both red and blue light enhanced *NII* expression in etiolated tobacco cotyledons, and light effects were only seen when nitrate was present in the growth medium. Light intensity was also important, and increasing light intensity had a positive effect on expression (Neininger et al. 1992). Four *NII* genes are present per haploid *Nicotiana* genome. The two genes more thoroughly tested, were found to be differently expressed in roots and leaves; one gene mainly in leaves and the other mainly in roots. Expression of both genes was induced by nitrate, and diurnal variation followed the same pattern in leaves and roots (Kronenberger et al. 1993). *NIA* and *NII* genes were similarly regulated in *Nicotiana* with respect to inhibition by glutamine and glutamate. An interesting difference was that in green leaves glucose apparently could replace light for the enhancement of *NIA* expression, while a positive effect on *NII* expression was much less pronounced (Vincentz et al. 1993).

In barley, where only one *NII* gene is present, nitrate was strictly necessary for detection of NiR activity and protein. In leaves, induction also depended on light, whereas in the roots expression was just as strongly induced in darkness (by nitrate). This showed that the gene was derepressed

in the roots by nitrate only, whereas some factor in the leaves apparently hindered its expression (Duncanson et al. 1992). In soybean at least three different *NII* genes were present, and one of these genes were constitutively expressed in the cotyledons, the others were induced by nitrate as in other plants (Kim et al. 2001).

NII promoters

As for *NIA* genes, nitrate and light are the most important factors known to enhance transcription of *NII* genes. Promoter analysis of bean (Sander et al. 1995), spinach (Rastogi 1993, Neininger et al. 1994, Sivasankar et al 1998) tobacco (Dorbe et al. 1998) and birch (Warning and Hachtel 2000) have revealed cis-acting elements involved in nitrate induction of *NII* transcription. Promoter analysis was performed by linking the promoters, or promoter fragments, to a reporter gene, and introducing this into tobacco, or in one case (Dorbe et al. 1998) *Arabidopsis*. For the *NIA* genes, reporter genes linked to the promoter very often showed only low expression in transgenic plants. For the *NII* promoter no such problems with gene extinction was encountered (Stitt and Meyer 2001). For all promoters tested, cis-acting elements providing nitrate induction of *NII* transcription were found to be present within 0.7 kb upstream of the transcription start site. In the fungus *Neurospora crassa* special GATA elements in the promoter have been shown to bind a regulatory protein, NIT2, resulting in induction of transcription by nitrate (Marzluf 1997). GATA elements were identified in the spinach, tobacco and birch *NII* promoters. However, apparently the GATA elements were not strictly necessary for nitrate induced transcription, because transcription was induced by nitrate also when these elements were deleted.

Neininger and co-workers (1994) found that the -200 to +131 bp (relative to transcription start) provided phytochrome mediated induction of transcription of the spinach *NII* gene. Warning and Hachtel (2000) identified light sensitive regions in the birch promoter between -155 and -267. Analysis of fragments of the birch *NII* promoter indicated that an inhibitor would bind to this region in the dark and thereby prevent transcription. When testing various promoter fragments linked to the GUS reporter gene it was also clear that a specific promoter fragment resulted in unlike activities in roots and leaves, possibly due to different concentrations of transcription factors present in the tissues.

Arg ello-Astorga and Herrera-Estrella (1998) found that a single motif cannot function as an LRE (light responsive element). Since the presence of nitrate was often a prerequisite for obtaining light effects on *NII* expression, possibly also one motif involved in nitrate activation could act in co-operation with one motif for light activation. Certainly both cis and trans-

acting elements still need to be explored for fully understand light regulation of *NII* genes.

GLUTAMINE SYNTHETASE (GS)

$$\text{glutamate} + NH_4^+ + ATP \rightarrow \text{glutamine} + ADP + P_i$$

In primary nitrogen metabolism the product of the nitrite reductase reaction, ammonium, is further assimilated into amino acids by glutamine synthetase (GS, EC 6.3.1.2). Importantly, the nitrite reductase reaction is certainly not the only source of ammonium in plants. Ammonium is released from amino acids by deamination during nitrogen remobilization. Ammonium is also released in special metabolic pathways as in the link between aromatic amino acid synthesis and secondary metabolism catalysed by phenylalanine ammonia lyase. Ammonium is formed in the glycine decarboxylase step during photorespiration, and this photorespiratory release of ammonium may exceed primary nitrogen assimilation 10-fold. Thus over the life span of a plant, nitrogen is released as ammonium and refixed several times (Miflin and Habash, 2002).

Several isoenzymes of GS are found, and these isoenzymes are encoded by one gene family in all plant species examined to date. An isoenzyme is located in the chloroplasts and generally only one gene per haploid genome is present for the chloroplastic GS in all plants (Lam et al. 1996). The chloroplastic form of GS is usually called GS2. It has a signal peptide and is cleaved as soon as it enters the chloroplast. It is generally considered that the role of GS2 is to assimilate ammonium in primary nitrogen assimilation as well as during photorespiration. Mutations of the GS2 gene in barley was lethal in normal atmosphere and it became evident that GS2 was necessary for reassimilation of photorespiratory ammonium. Several isoenzymes are found in the cytosol, and are referred to as GS1. For example three GS1 were identified in *Arabidopsis*, five in *Phaseolus vulgaris* (Lam et al.1996), seven in maize (Brugi re et al. 2001). In C3 plants GS1 isoenzymes are present at low concentrations in leaves and higher concentrations in roots and phloem. In C4 plants almost equal proportions of GS2 and GS1 are detected in leaves (Cren and Hirel 1999). A positive effect of light and ammonium and /or nitrate on expression of GS genes has been found in many plants.

Red and blue-light effects on GS gene expression

Regulation of GS mediated by phytochrome has been found in a wide range of higher plants; Scots pine (Elmlinger and Mohr 1991), *Spirodela* (Teller and Appenroth 1994), lettuce (Sakamoto 1990), mustard (Weber et al. 1990), pea (Edwards and Coruzzi 1989), tomato (Migge et al. 1998) and *Arabidopsis* (Oliveira and Coruzzi 1999). Generally GS2 is strongly induced by light, but not GS1 which is more developmentally regulated. Following illumination of etiolated leaves, an increase in both GS2 transcript and GS protein have been observed in a majority of plant species, and both red and blue-light receptors were involved (Cren and Hirel 1999).

Effects of sugars and amino acids on GS gene expression

As for other enzymes in the nitrogen assimilation pathways strong positive effects of carbohydrates are found. Light-induced changes not fully accounted for by phytochrome, were observed in pea (Edwards and Coruzzi 1989). A study performed with *Arabidopsis* showed that in addition to phytochrome-mediated red light effects which enhance expression of the GS2 gene, also different sugars stimulated accumulation of GS2 mRNA. Sucrose, fructose and glucose all mimicked the effect of light and enhanced GS2 gene expression when added to the growth medium in the dark. A moderate stimulation by sugars on GS1 gene expression was evident. Several amino acids, aspartate, asparagine, glutamate, glutamine added to the growth medium showed an antagonistic effect to sugar (Oliveira and Coruzzi 1999). The effects of sugars and amino acids on *Arabidopsis* GS are thus reminiscent of the regulatory mechanism found in *E. coli* where the PII protein is known to be a key component in regulation of GS (see INTRODUCTION/PII).

Promoters

Analysis of the GS gene promoters of soybean, *Phaseolus*, rice and tobacco revealed promoter fragments responsible for organ specific and developmental expression (Hirel and Lea 2001, Morey et al. 2002). Light responsive GS2 promoters were demonstrated for *Phaseolus vulgaris* (Cock et al. 1992) and pea (Tjaden et al. 1995). The GS2 pea promoter contains an AT-rich 33 bp region at 807 bp upstream of the transcription start site, and when this AT-rich region was deleted, expression was reduced 10-fold (Tjaden and Coruzzi 1994). Similarity to AT-rich elements in light-regulated photosynthetic genes in various species were pointed out. A trans-acting factor binding to this AT-rich sequence was identified, and found to be similar to AT-binding factors for other plant promoters. Interestingly, a phosphorylation step inhibited binding of the trans-acting factor to the AT-rich region. Important elements for light responsiveness were confined to a short promoter fragment 323 bp upstream from the transcriptional start site,

and sequences already known to be important for light responsiveness in other genes, like I-box and GT-1, were identified.

The upstream region of the *Arabidopsis* GS2 (At5G35630) also has I-boxes, but these are about 2000 and 2400 bp upstream from the transcription start site. Two nitrate elements within an AT-rich region are also present between -1645 and -1607 bp upstream of the transcription start site in *Arabidopsis* GS2. But no detailed promoter analysis or interacting trans-acting factors have been reported for *Arabidopsis*.

Post-translational regulation of GS

Cytosolic and chloroplastic GS from cauliflower were found to bind 14-3-3 proteins. Phosphorylation of GS may be important for its stability as found in *Brassica napus* (Finnemann and Schjoerring 2000). However, activity of GS was not influenced by 14-3-3 binding (Moorhead et al 1999, Riedel et al. 2001). As for NR, 14-3-3 proteins may be important for the regulation of GS; however, this point is still not clarified.

A redox change was found to modulate GS2 activity in *Canavalia lineata* and two cystein residues necessary for this activation was identified. These cystein residues were present in GS2, but not in GS1 in various plants. GS2 was activated by the sulfhydryl-reducing agent DTT like enzymes activated by photosynthetic electron transport by the ferredoxin-thioredoxin system (Choi et al. 1999). Regulation of GS2 by a redox change was further confirmed by identification of *Arabidopsis* GS as a binding target for thioredoxin (Motohashi et al. 2001).

GLUTAMATE SYNTHASE (GOGAT)

$$\text{glutamine} + \alpha\text{-ketoglutarate} + \text{Fd}_{\text{red}}\ (\text{NADH} + \text{H}^+) \rightarrow 2\ \text{glutamate} + \text{Fd}_{\text{ox}}\ (\text{NAD}^+)$$

Glutamate synthase (GOGAT) catalyses the conversion of glutamine and α-ketoglutarate to two molecules of glutamate. The enzyme is important in primary nitrogen assimilation as well as re-assimilation of NH_4^+ released in photorespiration. There are two forms of glutamate synthase; NADH-GOGAT (EC 1.4.1.14) primarily located in plastids of non-photosynthetic tissue, although the plastid localisation of NADH-GOGAT is controversial (Brugi re et al. 2001), and Fd-GOGAT (EC 1.4.7.1) primarily in leaf chloroplasts (Lam et al. 1996). Some Fd-GOGAT is also localised to roots.

NADH-GOGAT is involved in the ammonium assimilation in nodules of legumes. In *Arabidopsis*, expression of GS1 and NADH-GOGAT appears to be co-ordinated and may function together in primary nitrogen assimilation in roots (Lam et al.1996).

Fd-GOGAT is uniquely found in photosynthetic organisms. For *Arabidopsis* and tobacco two genes for Fd-GOGAT were found, whereas in other plants one gene has generally been found (Hirel and Lea 2001). In a number of plant species, synthesis of Fd-GOGAT does not strongly depend on nitrogen source, whereas in others nitrogen is an important factor controlling the final enzyme activity (Hirel and Lea 2001). When studying different mutants and transgenic plants, the importance of Fd-GOGAT in re-fixation of nitrogen released in photorespiration was striking. Fd-GOGAT deficient *Arabidopis*, barley, pea and tobacco were chlorotic and eventually died when grown in conditions allowing photorespiration (normal air). The plants recovered when grown under conditions suppressing photorespiration (Lam et al. 1996, Ferrario-M ry et al. 2000)). Apparently the NADH-GOGAT, or in *Arabidopsis* a second Fd-GOGAT coded for by another gene, were sufficient for primary nitrogen assimilation, although measured activity constituted only a few per cent of wild type total GOGAT activity (Temple et al. 1998). Recently, experiments with tobacco plants containing antisense Fd-GOGAT, and therefore only 10% Fd-GOGAT activity compared with wild type, were shown to have higher NAD(H)-glutamate dehydrogenase (GDH) activity especially when measured in the aminating direction. GDH and asparagine synthase were both considered as alternative routes for NH_4^+ reassimilation in the tobacco plants (Ferrario-M ry et al. 2002)

Light and sugar effects on GOGAT in various species

Light acting through phytochrome has been shown to enhance expression of Fd-GOGAT in several plants whereas the NADH-GOGAT activity generally was not influenced by light. *Arabidopis* has two genes coding for Fd-GOGAT (*GLU1* and *GLU2*). *GLU1* was expressed at the higher level in leaves, and its mRNA level was specifically enhanced by light (only high intensity white light was tested). Sucrose could partly replace light in inducing expression of *GLU1* when light-grown seedlings were dark-adapted for 3 days before testing effects of light and sucrose (Coschigano et al.1998). In contrast, G*LU2* was expressed at a lower, constitutive, level and accumulated primarily in roots (Coschigano et al. 1998).

Fd-GOGAT was expressed in etiolated tomato cotyledons, but expression increased by exposure to light. Phytochrome was involved in light perception. UV-A light also increased Fd-GOGAT expression, and the effect of UV-A was mediated through a special blue-light receptor or phytochrome

(Becker et al. 1993, Migge et al. 1998). As for *Arabidopsis*, no light effects were seen on NADH-GOGAT expression.

When etiolated barley seedlings were exposed to light (white, high intensity) for 48 h a 3-fold increase in Fd-GOGAT was observed. Although light did have a positive effect on Fd-GOGAT expression in barley a substantial level of Fd-GOGAT activity was present in the absence of light and also in the absence of nitrate. Early work on Fd-GOGAT activity in barley showed no effects of daily day/night shifts on Fd-GOGAT activity, whereas NR activity varied during the same period (Lillo 1983, 1984). In contrast to the situation in *Arabidopis*, light could not be replaced by sucrose or glucose added to the nutrient solution. No positive effect of sugar was found for Fd-GOGAT, although NR activity increased 2-fold in the same experiment. Hence in barley, Fd-GOGAT and NR did not react in parallel in response to the sugar treatment or circadian control (Lillo 1984, Pajuelo et al.1997).

In maize, exposure of etiolated seedlings to white light led to a 3-4-fold increase in Fd-GOGAT activity and mRNA levels. Red light also stimulated Fd-GOGAT expression, and far-red reversibility confirmed that phytochrome was involved. NADH-GOGAT activity was not affected in these experiments. Again a relatively high Fd-GOGAT level was found also in etiolated seedlings confirming that light was not strictly necessary for Fd-GOGAT expression (Suzuki et al. 1987, 2001)

Phytochrome was important for appearance of Fd-GOGAT activity in etiolated pine seedlings. Apparently also a blue-light/UV-A receptor like cryptochrome was involved. Blue or red light had no effect on NADH-GOGAT (Elmlinger and Mohr 1991)

Fd-GOGAT was induced by red light and blue light in etiolated turions of spirodela, and apparently both phytochrome and a blue-light receptor were involved. NADH-GOGAT was not influenced by red or blue light. Again a relatively high background activity of Fd-GOGAT was seen also in darkness (Teller et al. 1996).

Promoter analysis has been carried out for alfalfa NADH-GOGAT indicating four regulatory elements involved in the expression within the nodules (Trepp et al. 1999). Promoter analysis is otherwise scarce for NADH and Fd-GOGAT. The *GLU1* promoter of *Arabidopsis* has two AGTCA within AT-rich stretches (nitrate elements) in the 1200 bp region upstream of the transcription site, but involvement of these elements in regulation remains to be analysed.

LIGHT INFLUENCED AMINO ACID SYNTHESIS

The pathways proposed for amino acids synthesis in plants are largely derived from those known in *Escherichia coli* and yeast, but show more complexity and additional ways of regulation. Amino acids are usually divided into families related to a common precursor compound, and amino acids of one family share a common pathway in their scheme of synthesis.

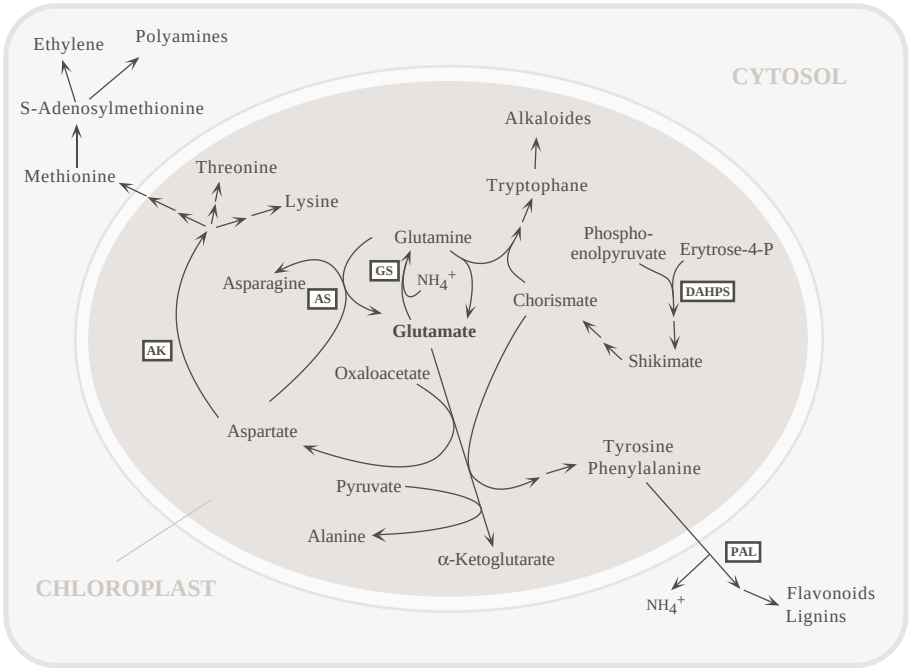


Figure 4. **Overview of metabolism from the amino acid glutamate, and pathways leading to secondary metabolites**. Glutamate and glutamine serve as nitrogen donors in important metabolic pathways. Glutamate is an amino donor in transaminase reactions generating for instance alanine, and aspartate from keto acids. Glutamate or glutamine also donate an amino group to chorismate in the pathways leading to tyrosine/phenylalanine or tryptophane, respectively. The first enzyme in the pathway for synthesis of amino acids in the aspartate family, aspartate kinase (AK) is stimulated by light. Asparagine synthetase (AS) activity is generally decreased in light, and is the main route for synthesis of asparagine, using aspartate and glutamine as substrates. Phosphoenolpyruvate and erythrose-4-phosphate are condensed by the 3-deoxy-arabinoheptulosonate-7-phosphate synthase (DAHPS) enzyme which is stimulated by light in many plants, and is the first step in the shikimate pathway leading to chorismate, and then aromatic amino acids. The first enzyme in the pathway converting aromatic amino acids to flavonoids and lignin is phenylalanine ammonia-lyase (PAL). PAL is

influenced by many environmental factors, also light. The PAL reaction is a main point for release of NH_4^+.

The majority of the amino acids can be synthesised within the chloroplasts; hence synthesis can be expected to be positively influenced by photosynthetic light. It has been known for a long time that the concentration of free amino acids in plants show diurnal variations and varies in response to light and darkness (Matt 1998, Coruzzi and Last 2000).

Asparagine

Asparagine is often present in very high concentrations in both xylem and phloem sap, and is used to carry nitrogen away from source tissue. Asparagine is more soluble, less reactive and has a higher nitrogen to carbon ratio than glutamine, all of which makes it a better transport and storage compound. Asparagine synthetase (AS: EC 6.3.5.4) is considered as the major route for asparagine biosynthesis in plants. In an ATP-dependent reaction AS catalyses the transfer of the amino group from glutamine to aspartate generating glutamate and asparagine (Coruzzi and Last 2000):

glutamine + aspartate + ATP $\rightarrow$ glutamate + asparagine + AMP + PP_i

Three AS genes (*ASN*) have been identified in *Arabidopis* (Lam et al. 1996, 1998). The expression of *ASN1* was repressed in leaves by light or presence of sucrose. The repression was reversed by asparagine, glutamine and glutamate. The two additional *ASN* genes were regulated in a reciprocal manner to *ASN1*, i.e., their mRNA levels were increased by light or sucrose. These latter genes were, however, expressed at much lower levels, and belonged to a different class of *ASN* genes. These genes may be involved in NH_4^+-detoxification (Lam et al. 1998). In pea leaves, two *ASN* genes were identified and the expression of both was stimulated by darkness. *ASN* genes from various plants have been cloned and in the majority of cases light and /or carbohydrates were shown to repress gene expression (Hirel and Lea 2001). Light up-regulates genes which are involved in assimilation of ammonium into glutamine and glutamate, and phytochrome is involved in this process. In contrast, light generally down-regulates *ASN* genes. Interestingly, this negative effect of light is also mediated, at least partially, by phytochrome. The negative effect of sucrose on *ASN* expression can be antagonised by amino acids. A model has been proposed where GS2 is enhanced by light, or when carbon skeletons are abundant. Thus in the light, nitrogen is assimilated into and transported as glutamine, which is a substrate for numerous reactions. In darkness, when no photosynthesis can

take place, *ASN* expression increases, and nitrogen is directed into the ideal storage and transport compound, asparagine.

ASN promoter and light repression

Using complementary loss and gain of function experiments, a 17 bp cis-element with a core unit TGGG (reverse) was identified at -43 upstream of the transcriptional start site in the *ASN1* pea promoter. This element was both necessary and sufficient for down-regulation by light. Other light depressed genes, like *PHYA* from several species, and *NPR* genes from Lemna also possess the TGGG element (Ngai et al. 1997).

The tomato *aurea* mutant, that has only 20% of wild-type levels of PHYA, was used to study the signal transduction chain leading from light to change in transcription. The results were in agreement with PHYA being the light receptor, and the *ASN1* gene was light repressed, rather than dark activated (Neuhaus et al. 1997).

Aspartate-derived amino acids

The amino acids lysine, methionine, threonine and S-adenosylmethionine belong to the aspartate family of amino acids. Important regulatory compounds like ethylene and polyamines are derived from S-adenosylmethionine. S-adenosylmethionine itself is the donor of virtually all the methyl groups in biosynthetic reactions. The aspartate-derived amino acids are of special interest, and their synthesis has been much studied, because these amino acids may be present in too low concentrations in food to support optimal growth and health of domestic animals, or humans confined to imbalanced diets.

Experiments with ^{14}C-labelled aspartate have shown that chloroplasts are capable of synthesising lysine, threonine, isoleucine and homocysteine in light-driven reactions, and all the enzymes required are present in the chloroplast. Comparison of the DNA sequences also confirms that plastid-target sequences are present within the N-terminal extensions of these enzymes. The final methylation step involved in the conversion of homocysteine to methionine has been shown to take place in the cytosol. It has long been known that the pathway is regulated by feedback mechanisms on the protein level. The different end products regulate enzymes at the branch point and beginning of the pathway (Coruzzi and Last 2000). For instance, aspartate kinase (AK), the committing enzyme in the pathway for synthesis of aspartate-derived amino acids, is regulated by threonine, lysine,

and S-adenosylmethionine (Aarnes and Rognes 1974). More recently, examination of AK expression has revealed that this key-enzyme is also light-regulated at the transcriptional level. The threonine-sensitive AK isoenzyme is a bifunctional protein that also contains homoserine dehydrogenase activity (HSD). In *Arabidopis*, a single AK/HSD gene was detected, and the promoter was linked to the GUS reporter gene and introduced into tobacco. Light stimulated expression of this reporter gene in cotyledons upon germination (Zhu-Shimoni et al. 1997). Red light acting through the phytochrome system was found to increase the AK mRNA level in etiolated chickpea (Dey and Guha-Mukherjee 1999) and barley (Rao et al. 1999). Expression of another key-enzyme, cystathionine gamma-synthase, at the branchpoint leading to methionine, was also stimulated by light (Hughes et al. 1999).

Aromatic amino acids and phenylpropanoids

The shikimate pathway is the first part of aromatic amino acid synthesis and comprises 7 enzymatic reactions whose endproduct is chorismate. After chorismate the pathway divides for synthesis of tryptophane, or tyrosine and phenylalanine (Fig 4). At this branch-point, feedback inhibition by the aromatic amino acids regulates the flow into the different branches. The shikimate pathway is found only in microorganism and plants, not in animals. The pathway is, therefore, an important target for herbicides and antibiotics. A well known example is the herbicide glyphosate, which inhibits the second last enzyme of the pathway. In bacteria, the pathway is almost entirely used for making aromatic amino acids for protein synthesis. In higher plants these amino acids are not only important constituents of proteins, but are also precursors of many important secondary compounds like; lignin, pigments, defensive phytoalexins, and alkaloids. Certainly this additional function of the pathway demands special regulation and capacity. Twenty per cent of the carbon fixed by plants may flow through the aromatic amino acid pathway (Coruzzi and Last 2000).

The shikimate pathway is strongly influenced by environmental stimuli such as light, pathogens and wounding. The first reaction of the pathway is the condensation between phophoenolpyruvate and erythrose-4-phosphate catalysed by the 3-deoxy-arabinoheptulosonate-7-phosphate synthase (DAHPS) enzyme. Activity of DAHPS is increased following wounding and correlates with an increase in secondary metabolites. In bacteria DAHPS is feedback inhibited by the aromatic amino acids, but this is not the case in plants. Early work revealed that aromatic amino acid biosynthesis can occur in isolated spinach chloroplasts. Molecular analysis also shows that all enzymes involved have N-terminal extensions characteristic of chloroplast transit sequences. The localisation to chloroplasts make light and

photosynthesis plausible candidates for regulation of the pathway, and in plants light may be the main regulator of the DAHPS. Light has been shown to upregulate DAHPS at the transcriptional level in parsley cell cultures (Henstrand 1992). However, although the shikimate pathway is located in the plastids, non-green tissue, like roots and flowers, contain the greatest amount of mRNA for the inducible isoenzymes of the pathway (Weaver and Herrmann 1997). *Arabidopsis* DAHPS has recently been shown to be activated by the ferredoxin/thioredoxin system (Entus et al. 2002). *Arabidopsis* DAHPS was expressed in *E. coli* and purified. Reduced thioredoxin, which could also be replaced to some extent by the reducing agent DDT, was necessary for activity of the enzyme. This indicates a likely way of linking carbon fixation and aromatic amino acid synthesis, since DAHPS and enzymes in the Calvin cycle are sharing reduced ferredoxin as an activator of the pathways (Entus et al. 2002).

Phenylalanine is the startpoint for synthesis of phenylpropanoids, which include, lignins, anthocyanins, flavonoids, coumarines, and small phenolic molecules like salicylic acid. These compounds have multiple functions in structural support, pigmentation, defence, and signalling. Taking into account the function of these compounds, it is likely that the evolution of the phenylpropanoid pathways played a key role in the ability of plants to colonise land (Douglas 1996). Manipulation of the phenylpropanoid pathway has important implications for instance with respect to pulping and forage digestibility. Since these factors are of great economical importance the subject continues to be an active area of research. All phenylpropanoids are derived from cinnamic acid, which is made from phenylalanine by the action of the key-enzyme phenylalanine ammonia-lyase (PAL). PAL represents the branchpoint between primary (shikimate pathway) and secondary (phenylpropanoid) metabolism (Dixon and Palva 1995) (Fig 4). PAL is a cytosolic enzyme, however, it may be attached to membranes in response to phosphorylation. Many of the subsequent enzymes in phenylpropanoid synthesis are localised to he endoplasmic reticulum. The amino group cleaved from phenylalanine by PAL is released as ammonium and recaptured by glutamine synthetase. PAL is encoded by a single gene or a multigene family, depending on the species, and is subjected to a number of control mechanism. For example, transcription of the PAL gene is enhanced by light, plant growth regulators and different types of stress. In tomato leaves, PAL activity is induced by wounding (excision) of the leaves, and reaches maximum activity 24 h after excision. In response to stressful light conditions, for example high UV irradiation, PAL expression and concentrations of phenylpropanoids like anthocyanins and flavonoids increase (Dixon and Palva 1995). These UV- absorbing compounds then act as a sunscreen and protect the plant against UV damage (Hirner et al. 2001).

Glycine and serine

Glycine and serine are two interconvertible amino acids present in many different compartments of the cell. There are two major pathways leading to serine and glycine synthesis. The first pathway is linked to glycolysis and leads to serine formation from 3-phosphoglycerate. The second route is linked to the C2 cycle (photorespiratory cycle). As part of the C2 cycle glycolate produced in the chloroplast by the oxygenase activity of Rubisco is exported to the peroxisomes where it is converted to glycine by transamination. Glycine is further transferred to the mitochondria where GDC (glycine decarboxylase) catalyses the oxidative decarboxylation of glycine to produce CO_2, NH_3, NADH and methylenetetrahydrofolate. Serine hydroxymethyltransferase then synthesises serine from the methylenetetrahydrofolate and a second molecule of glycine. It is assumed that (part of) the photorespiratory pathway is the major route for synthesis of glycine and serine in photosynthetic active tissue. It is still not clear if this pathway is active in all tissue or during non-photosynthetic conditions (Morot-Gaudry et al. 2001).

GDC is a complex enzyme that may constitute as much as 40% of the protein in the mitochondria, hence this is one of the most abundant proteins in green leaves, and a major metabolic route is directed through GDC. The cycle liberates large amounts of NH_3 that needs to be refixed. Leaves in the dark have low activity of the GDC enzyme complex, but expression is stimulated upon exposure to light and development of the photosynthetic apparatus (Douce et al. 2001).

CONCLUSION

Light acts on nitrogen uptake and metabolism through special red and blue-light receptors. This has been well documented at the seedling stage and for dark-grown tissue. The red and blue-light receptors are important also in mature plants. Low light irradiance absorbed through the special receptors may trigger for instance flowering, but not much is known concerning the interaction between these signals and nitrogen metabolism in the mature plant. Generally, many of the genes involved in nitrogen uptake, and metabolism are activated by light. A certain enzyme or transporter protein is often coded for by several genes, which are differently regulated by light. This diversity assures rational expression in different tissues, cell

compartments or developmental stages. In the green plant light acting through photosynthesis stimulates nitrogen uptake and metabolism. There has been considerable progress in defining components in the signal transduction chains leading from light to regulation of transcription or post-translational modifications. However, complete comprehension and identification of all components in any such chain is still lacking. The *Arabidopsis* genome project gives a powerful tool to identify putative regulatory elements, but characterisation of specific transcription factors and interacting cis-acting elements involved in nitrogen metabolism is still scarce.

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