

緑葉におけるアスパラギンとグルタミン
の合成に関与する遺伝子の発現と
その生理学的意義

2000

野澤 彰

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Studies on the physiological roles of asparagine synthetase and glutamine synthetase in green leaves

学位論文
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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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December, 1999

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Abbreviations

AK/HSD	[aspartate kinase-homoserine dehydrogenase]
ATP	[adenosine 5'-triphosphate]
AS	[asparagine synthetase]
BCKDH	[branched chain α -keto acid dehydrogenase]
bp	[base pair]
cDNA	[complementary DNA]
DCMU	[3-(3',4'-dichlorophenyl)-1,1-dimethylurea]
DNA	[deoxyribonucleic acid]
EDTA	[ethylenediaminetetraacetic acid]
FW	[fresh weight]
g	[gram]
<i>g</i>	[gravitational acceleration]
GS	[glutamine synthetase]
GS1	[cytosolic glutamine synthetase]
GS2	[chloroplastic glutamine synthetase]
HPLC	[high-performance liquid chromatography]
mRNA	[messenger RNA]
ORF	[open reading frame]
PCR	[polymerase chain reaction]
PFD	[photon flux density]
pI	[isoelectric point]
poly(A) ⁺ RNA	[polyadenylated RNA]
RACE	[rapid amplification of cDNA ends]
RNA	[ribonucleic acid]
rRNA	[ribosomal RNA]
RT-PCR	[reverse transcription-PCR]

S	[Svedberg unit]
SSC	[saline sodium citrate]
TCA	[tricarboxylic acid]
Tris	[tris(hydroxymethyl)aminomethane]
v	[volume]
w	[weight]

Chapter I

General Introduction

In nature, plants grow under the ever changing environment. Plants are non-motile and hence, they have evolved intricate mechanisms for adapting environmental variables. From germination to flowering and maturation of fruits, the growth of both vegetative and reproductive organs is critically influenced by many environmental factors, such as light, temperature, water status, and nutrient levels. To date, a tremendous amount of studies have been performed on the effects of those factors on the plant's growth (Lawlor 1991; Hall 1993). Among those factors, the effects of nutrients on the plant's growth have been most extensively studied because they are tightly related to the crop yield. Since most of the studies have been focused on agricultural application, especially attaining the maximal crop yield, a large bunch of information has been given on what materials are available to plants as nutrients, on how much amount of nutrients plants need and on how those nutrients are utilized by plants. However, it has not been well understood what enzymes play a key role in nutrient utilization and how the enzymes are controlled. Hence, a complete picture of the mechanism of nutrient utilization in a plant is still lacking at molecular level. Toward the understanding of the regulatory mechanism of nutrient utilization in plants at molecular level, I started my present study.

At the beginning of this thesis, I briefly summarize the current knowledge on nutrients and their utilization by plants.

[1] The essential nutrients for plants

Nutrient elements that are required for the growth and the development of plants are regarded as essential elements which are traditionally grouped into several categories as described below.

(a) Macronutrients

Currently, 17 essential elements in the periodic table are known to be required by all higher plants (Welch 1995). Of these, 9 are macronutrients, H, C, O, N, K, Ca, Mg, P and S, that are normally contained in plant tissues at concentrations of greater than 0.1% (w/dry weight). The macronutrients are largely involved in the structural molecules, which to some extent accounts for their need in large quantities.

(b) Micronutrients

Micronutrients are defined as essential elements that are contained in plant tissues at concentrations of less than 0.01% (w/dry weight). Eight micronutrients, Cl, B, Fe, Mn, Zn, Cu, Ni and Mo, are now recognized as essential for all higher plants. Micronutrients are required as the catalytic activity of an enzyme or its regulatory component(s).

(c) Beneficial elements

There are some elements that have not been shown to be essential to higher plants but have beneficial effects on the growth of certain plant species. They are referred to as beneficial elements. For example, Na is required by plants that use the C₄ photosynthetic pathway and those that under some conditions use the CAM (crassulacean acid metabolism) metabolic pathway (Brownell and Crossland 1972). Co is a beneficial

element for symbiotic nitrogen fixation by legumes, although the requirement can be traced to the needs of the nitrogen-fixing bacterium rather than the host plants. Si has been reported to have beneficial effects on a wide variety of higher plants, including improved resistance to fungal diseases and insect damage.

[2] Nitrogen as a limiting nutrient

The need of individual plants for any particular element is normally defined in terms of a critical concentration. It is a concentration that gives a 10% reduction in growth, although it is not well understood how the critical concentration limits the growth of plants. When a nutrient level becomes below the critical concentration, growth of a plant falls off sharply and several deficiency symptoms occur in the plant. In such circumstances, the nutrient limits growth of the plant. Plants are composed predominantly of H, C and O, and these elements are taken easily in the forms of CO_2 and H_2O . Nitrogen is the fourth most abundant nutrient element in plants. The bulk of the atmosphere consists of molecular nitrogen, but no organisms except for cyanobacteria and nitrogen-fixing bacteria can fix the nitrogen into a form available to organisms. Thus, nitrogen is often the most limiting nutrient for plants in natural ecosystems (Stewart 1991). With the exception of certain highly specialized plants such as insectivores and those engaging in symbiosis with nitrogen-fixing organisms, plants depend almost entirely on dissolved forms of inorganic nitrogen in the environment.

[3] Translocation system for effective utilization of nutrients

The amount of a nutrient in environment is always limited. Furthermore, plants can not absorb nutrients beyond the reach of their

root system. Thus, plants need to export several nutrients from one part to the other within the plant bodies to recycle the nutrients. This relocation of nutrients between organs is conducted through a translocation system termed the vascular system. With this system, nutrients absorbed or produced at a particular site of a plant are distributed to other sites, where they are utilized as materials to build up new cellular components (Pate 1973). Higher plants have evolved a highly organized vascular system that enables the efficient translocation of nutrients within a plant (Zimmermann 1960).

[4] Timing of translocation

While nutrients are translocated constitutively within the plant, massive relocation has to be activated especially during following developmental stages.

(a) Germination

During germination the seed rapidly changes from a quiescent state to a dynamic state. Metabolism required for energy production and the syntheses of protoplasm and structural components increase in rapidly growing parts. Lipids, proteins and carbohydrates are major storage reserves that serve as the substrates for this rapid metabolism (Comai *et al.* 1989; Tanaka *et al.* 1993; Lai *et al.* 1995). The largest amount of these storage reserves are usually stored in the endosperm of monocots or cotyledons of dicots. They are hydrolyzed and translocated to developing organs during germination.

The storage proteins in seeds are hydrolyzed to amino acids by proteolytic enzymes (Ryan 1973). The amino acids may remain in the storage tissue but are mostly translocated to the developing organs (Duke

et al. 1978). They are used as a source of nitrogen for various enzymes, structural proteins, and other nitrogen compounds (Ashton 1976). Some of the amino acids may also undergo deamination. The resultant products may then be used for the synthesis of non-nitrogen containing compounds or further metabolized to yield energy (Ashton 1976).

(b) Leaf senescence

Leaf senescence is a very important process in plant life from the viewpoint of nutrient recycling (Leopold 1961; Noodén and Guiamét 1996). Senescing leaves could be a very precious source of nutrients for the plant. During senescence, proteins, membranes and nucleic acids in the leaves are degraded, and resultant nutrients such as N and P are translocated to young growing tissues and storage organs of the plant (Thomas and Stoddart 1980; Huffaker 1990). Although senescence naturally occurs by aging, it can also be induced artificially by several treatments such as dark treatment, heat stress, cold stress, drought stress and mineral deficiency (Thomas and Stoddart 1980; Smart 1994; Noodén *et al.* 1997). However, it is under a strong debate to which extent natural senescence is identical to artificial senescence.

During leaf senescence, remobilization of nitrogen from the organ have been shown to be activated. It is estimated that roughly 90% of translocated nitrogen out of a senescing leaf comes from chloroplasts (Morita 1980), and Rubisco is thought to be the largest source of the mobilized nitrogen (Huffaker 1990). About 50% of the nitrogen stored in rice grains has been estimated to be translocated from senescing leaves (Mae and Ohira 1981).

[5] Translocation compounds of nitrogen

Among the mineral essential elements, N, P and S are known to be translocated (Hopkins 1997). Plants normally absorb these elements from soil in inorganic forms, which are to a less extent translocated as intact inorganic forms and have to be converted to other translocation compounds at a particular site. Such translocation compounds are found in a large quantity in phloem and xylem saps.

Major nitrogen compounds found in phloem and xylem saps are amino acids in many plant species, although ureides are also found in a large quantity in tropical legume species (Pate 1973; Mifflin and Lea 1977; Pate 1980). Recently, Bourgis *et al.* (1999) reported that S-methylmethionine play a major role in sulfur translocation in wheat. Although metallothionein could have a role in translocation of several metal ions (Butt *et al.* 1998), translocation forms of other elements have been less understood.

There are a number of candidates for translocation compounds of nitrogen as follows. However, there is some controversy as to which compounds contribute best to the translocation of nitrogen.

(a) Amide amino acids

In some species of legumes, such as pea, clover, and alfalfa which are those from temperate origin, amide amino acids, Asn and Gln, are the predominant forms of translocating nitrogen (Pate 1973; Schubert 1986). Asn and Gln have low carbon to nitrogen molar ratios as shown in Figure 1-1 and thus have economic structure with respect to carbon investment per mole of translocating nitrogen. Therefore, these amide amino acids have also been considered as major translocating nitrogen compounds in other plant species.

Asparagine (C:N=4:2)

Glutamine (C:N=5:2)

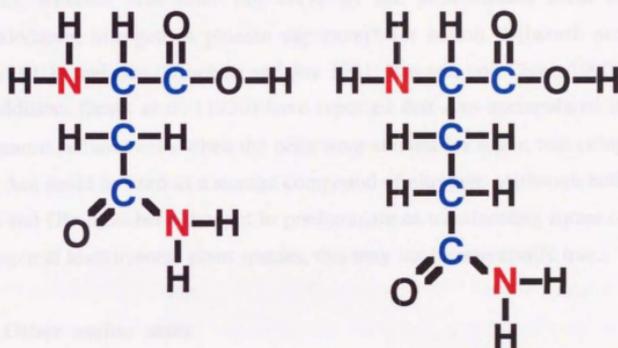


Figure I-1. Structures of asparagine and of glutamine.

However, an important point is that a vast majority of studies on nitrogen translocation have been done by the analysis of xylem sap in legumes, and there have not been many studies analyzing amino acid contents in phloem saps in both legume and non-legume plants. Gln is often found as a major amino acid in phloem sap even in non-legume plants (Hayashi and Chino 1990; Caputo and Barneix 1997; Lohaus *et al.* 1997), whereas Asn does not serve as the predominant form of translocating nitrogen in phloem sap except for cotton (Dilworth and Dure 1978) and rice (Urquhart and Joy 1981; Hayashi and Chino 1990). In addition, Genix *et al.* (1990) have reported that Asn accumulated in sycamore cultured cells when the cells were starved for sugar, indicating that Asn could be used as a storage compound of nitrogen. Although both Asn and Gln have been thought to predominate as translocating forms of nitrogen at least in some plant species, this may not be universally true.

(b) Other amino acids

Glu, Asp and Ser are also often found as predominant amino acids in phloem and xylem saps in many plant species such as *Salix* spp. (Mittler 1954), pea (Urquhart and Joy 1981), wheat (Hayashi and Chino 1986; Caputo and Barneix 1997), oats (Weibull *et al.* 1990), barley (Weibull *et al.* 1990), rice (Hayashi and Chino 1990), castor bean (Jeschke *et al.* 1997) and tomato (Valle *et al.* 1998). These amino acids are constitutively translocating in plant bodies.

Of the proteinaceous amino acids, Arg has the most economic structure with respect to carbon investment per mole of translocating nitrogen. Although Arg is likely to be used as long-distance translocation in apple (Tromp and Ovva 1973), other plant species seem to use Arg as a storage compound of nitrogen (Shargool *et al.* 1988; Galston and

Sawheny 1990).

One of tropical legume species, peanut (*Arachis hypogaea*), seem to translocate nitrogen as 4-methyleneglutamine. Winter *et al.* (1981) reported that 4-methyleneglutamine accounted for 90% of nitrogen in the root xylem sap. However, peanut seems to change the translocating form of nitrogen from 4-methyleneglutamine to Asn when nodule formation starts (Peoples *et al.* 1986).

(c) Other nitrogen compounds

Legumes of tropical origins, for example soybean, mungbean, and cowpea, appear to predominantly translocate urea derivatives, known as ureides (Pate 1973; Schubert 1986). Ureides are compounds such as allantoin and allantoic acid. In those plant species, allantoin and allantoic acid could be synthesized *de novo* by the oxidative catabolism of purines (Schubert 1986; Kim *et al.* 1995), but the synthesis of those ureides seems to be limited in active nodules containing nitrogen-fixing microorganisms (Reynolds *et al.* 1982). Although ureides use less organic carbon to translocate the same amount of nitrogen as do amides, translocation of nitrogen in the forms of ureides is not known except for some legumes of tropical origins (Schubert 1986).

[6] Syntheses of amide amino acids

As described above, plants seem to use a limited number of compounds for nitrogen translocation (Mifflin and Lea 1977; Pate 1980). Accordingly, the translocation of nitrogen has been thought to be regulated partly by the synthesis of these translocating compounds. I summarize here the synthesis of amide amino acids, which are possible major translocation compounds.

(a) Synthesis of asparagine

To date, three routes have been proposed to account for the synthesis of Asn. One involves the synthesis of Asn from 2-oxosuccinamic acid and suitable amino donor in a reaction catalyzed by asparagine aminotransferase (EC 2.6.1.14). This reaction has been demonstrated in rat liver extracts (Cooper 1977; Noguchi and Fujiwara 1988), but has been hardly detected in any plant, and no gene encoding asparagine aminotransferase has been isolated from plants.

A second pathway of Asn synthesis involves incorporation of cyanide and Cys into β -cyanoalanine which is then hydrolyzed to Asn by β -cyanoalanine hydrolase (EC 4.2.1.65). This β -cyanoalanine pathway is generally considered to be utilized only when detoxication of cyanide is required (Castric *et al.* 1972).

A third route leading to Asn synthesis is mediated by asparagine synthetase (AS; EC 6.3.5.4) which catalyzes an ATP-dependent amide-transfer reaction between Asp and Gln to form Asn and Glu (Huber and Streeter 1985) (Figure I-2). Biochemical properties of AS have not been characterized extensively due to several reasons such as its extreme instability *in vitro*, copurification of heat-stable inhibitors, and presence of contaminating asparaginase activity (Streeter 1977; Lea and Mifflin 1980; Joy *et al.* 1983). On the other hand, cDNA clones for AS have been isolated and characterized in several plant species. It is reported that the expression of AS genes leads to increase in the level of Asn in several plant species (Davies and King 1993; Lam *et al.* 1995; Waterhouse *et al.* 1996). From these results, Asn synthesis in plants is thought to be mediated mainly by the activity of AS. It is reported that transcripts from an AS genes accumulate in leaves during dark treatment in several plant species including pea (Tsai and Coruzzi 1990), *Arabidopsis* (Lam *et*

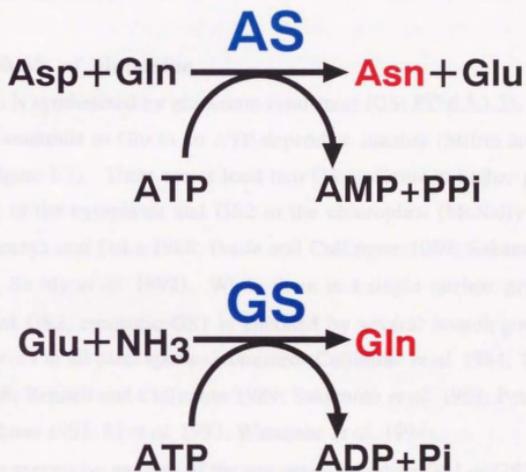


Figure I-2. Enzymatic reactions of asparagine synthetase and of glutamine synthetase.

al. 1994) and alfalfa (Shi *et al.* 1997). Although dark treatment accelerates leaf senescence, it is yet unknown whether Asn is actually related to the nitrogen translocation from dark-induced senescent leaves.

Alternatively, protein degradation is another route of generating Asn. The content of Asn residues in protein is known as about 4.4% (Voet and Voet 1990).

(b) Synthesis of glutamine

Gln is synthesized by glutamine synthetase (GS; EC 6.3.1.2), which transfers ammonia to Glu in an ATP-dependent manner (Mifflin and Lea 1977) (Figure I-2). There are at least two GS isoforms in higher plants, *i.e.*, GS1 in the cytoplasm and GS2 in the chloroplast (McNally *et al.* 1983; Yamaya and Oaks 1988; Forde and Cullimore 1989; Sakamoto *et al.* 1989; Sechly *et al.* 1992). While there is a single nuclear gene for chloroplast GS2, cytosolic GS1 is encoded by several homologous but distinct genes in all plant species examined (Cullimore *et al.* 1984; Tingey *et al.* 1988; Bennett and Cullimore 1989; Sakamoto *et al.* 1989; Peterman and Goodman 1991; Li *et al.* 1993; Watanabe *et al.* 1994).

The expression patterns of the pea genes encoding GS1 or GS2 have been analyzed with transgenic tobacco and alfalfa which contained promoter-GUS chimeric genes. A promoter of GS1 gene induced expression of GUS gene exclusively in the phloem, whereas that of GS2 gene was active in mesophyll cells where photorespiration occurs (Edwards *et al.* 1990; Brears *et al.* 1991). From these observations, GS1 is suggested to be responsible for producing Gln for translocation (Kawakami and Watanabe 1988; Edwards *et al.* 1990; Kamachi *et al.* 1991a, b; Carvalho *et al.* 1992; Sakurai *et al.* 1996), whereas GS2 is for detoxication of ammonia generated by photorespiration (Wallsgrave *et al.*

1987; Edwards and Coruzzi 1989, 1990; Brears *et al.* 1991). While it has been well established that the expression of GS2 genes is regulated by light and photorespiration (Edwards and Coruzzi 1989; Cock *et al.* 1991), little is known about a regulatory mechanism underlying the expression of GS1 genes. It has been reported that the transcripts from GS1 genes accumulates during senescence in radish cotyledons (Kawakami and Watanabe 1988; Watanabe *et al.* 1994), rice leaves (Kamachi *et al.* 1992), *Arabidopsis* leaves (Bernhard and Matile 1994) and carrot leaves (Higashi *et al.* 1998). However, there are some conflicting reports that the expression of GS1 genes is down-regulated by darkness, which induces senescence, in *Arabidopsis* leaves (Lam *et al.* 1994) and radish young cotyledons (Watanabe *et al.* 1994).

Gln is also generated by protein degradation. It has been reported that the content of Gln residues in protein is about 3.9% (Voet and Voet 1990).

As described earlier, among several nutrients, nitrogen is generally considered to be a major rate-limiting element in plant growth. However, it has not been well understood in many pathways of nitrogen utilization what enzymes play key roles and how the enzymes are regulated. Therefore, I will try to dissect mechanism of nitrogen utilization in plants, especially focusing on nitrogen recycling in this study, because it is one of the important processes for efficient utilization of nitrogen and is a unique process for plants as described above. Because nitrogen recycling by translocation is thought to be one of the limiting steps for the plant's growth and crop yield, the study on nitrogen translocation would also bring us valuable informations for agriculture. Since AS and GS1 have been suggested to be related to nitrogen translocation, I selected

genes for AS and GS1 out of many genes. However, there have been conflicting results about the involvement of AS and GS1 in nitrogen translocation. Hence, I first analyze whether AS and GS1 are really involved in translocation of nitrogen from senescing cotyledons (Chapter II). The results in Chapter II suggest that GS1 is actually involved in the translocation of nitrogen from senescing cotyledons but AS is not. However, I have shown in Chapter III that AS also contributes effective utilization of nitrogen, by another way, *i.e.*, the transient storage of nitrogen under photosynthesis limiting conditions. I have also shown in Chapter IV that the expression of *Asn1* is regulated in response to the intracellular balance between carbon to nitrogen. From these results, the importance of AS and GS1 in nitrogen utilization will be discussed.

In this study, I chose radish cotyledons as an experimental material, because it has been shown that yellowing of radish cotyledons progresses relatively synchronously during senescence (Kawakami and Watanabe 1988). In addition, radish cotyledons are relatively large, its feature is suitable for both physiological and biochemical experiments as well as molecular biological experiments.

Chapter II

Involvement of Asparagine Synthetase and Cytosolic Glutamine Synthetase in Nitrogen Translocation from Senescing Radish Cotyledons

Introduction

As described in Chapter I, nitrogen is often a limiting nutrient for the plant's growth. Accordingly, plants use the limited nitrogen very efficiently by translocating among separate organs. Nitrogen has been known to be translocated mainly in the form of amino acid. Amide amino acids, Asn and Gln, have been regarded as the major forms of nitrogen translocation, since these amino acids are found in both phloem sap and xylem sap as predominant organic nitrogen (Pate 1973; Urquhart and Joy 1981; Hayashi and Chino 1990). In plants, Asn is thought to be synthesized mainly by AS, and GS1 is thought to be responsible for producing Gln for translocation as described in Chapter I. If Asn and Gln are used as the major forms of nitrogen translocation, AS and GS1 would play key roles in the nitrogen translocation. However, there are some conflicting observations, as described in Chapter I, whether both Asn and Gln play major roles in nitrogen translocation. Therefore, I will re-evaluate the role of AS and GS1 in senescing radish cotyledons in this chapter.

Dark treatment has long been employed as an experimental cue to initiate or promote leaf senescence in plants (Azumi and Watanabe 1991; King *et al.* 1995; Oh *et al.* 1996; Kleber-Janke and Krupinska 1997; Nakashima *et al.* 1997; Park *et al.* 1998; Nakabayashi *et al.* 1999). At the

physiological level, natural and dark-induced senescence in leaves have many events in common, since both imply sucrose starvation and degradation of chlorophylls, proteins, lipids and nucleic acids, as well as other changes. However, Becker and Apel (1993) reported that there are differences in the gene expression between natural senescence and dark-induced senescence. Similarly, Watanabe *et al.* (1994) reported that transcripts from GS1 genes, which is induced by senescence, does not accumulated during dark treatment in young radish cotyledons. These observations may indicate that dark-induced senescence is not totally identical to the natural senescence at the molecular level.

In this chapter, I will analyze whether AS and GS1 are related to nitrogen translocation in radish cotyledons kept in the dark as well as in senescing cotyledons. However, there are some technical problems in the analysis. AS from all plant sources examined so far are very unstable *in vitro*, and it is difficult to separate GS1 from GS2 in radish by conventional ion-exchange column chromatography. Although one cannot directly extrapolate the amounts of transcripts to enzyme activity, I will analyze the accumulation levels of transcripts from genes for AS and GS1 which are most likely to reflect the activity of AS and GS1 in the tissue to circumvent these difficulties. The results obtained will be compared with the change in the amount of Asn and Gln in the tissue and the phloem exudates as well, using radish seedlings at various growth stages and with a different length of dark treatment. The results obtained suggest that GS1 is really involved to translocation of nitrogen from senescing cotyledons, whereas AS does not play a major role in translocation of nitrogen, but is important in storage *in situ* of nitrogen in the dark.

Materials and Methods

Plant Material

Radish (*Raphanus sativus* L. cv. Comet) seeds were sown on a vermiculite bed in a plastic tray (25 cm × 35 cm), and grown in a growth chamber regulated at 25°C under a relative humidity of 60% with a 16-h daylength at a photon flux rate of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were watered daily and were supplied with Hoagland's solution (Arnon and Hoagland 1940) on the 4th, 11th and 18th day after sowing. Cotyledons were harvested at the end of the light period. For experiments with dark treatment, radish plants were transferred to continuous darkness at the end of the light period, and cotyledons were harvested at 0, 24 and 72 h after the start of the dark treatment.

Measurement of chlorophyll content

Chlorophyll contents were measured with a chlorophyll analyzer (SPAD-502, Minolta Camera Co., Ltd., Tokyo, Japan), according to the manufacture's instruction.

Extraction and Analysis of RNA

RNA was extracted from radish cotyledons by the SDS-phenol method (Schmidt *et al.* 1981). RNA (20 μg per lane) was separated on a 1% agarose gel containing 2% formaldehyde and capillary blotted onto a nylon membrane (Biodyne A, Pall BioSupport Corp., NY). A hybridization probe for GS1 was prepared from a *Bam*HI-*Eco*RI fragment of *Gln1;1* containing a 3'-noncoding region (Watanabe *et al.* 1994), and that for AS from a cDNA fragment of *Arabidopsis din6* (Fujiki, Y. *et al.* submitted for publication), which is identical to the +844

- +1749 region of *ASN1* (Lam *et al.* 1994). Hybridization probes were labeled with [α - 32 P]dCTP using a Megaprime DNA Labelling System kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Hybridization for high stringent conditions was carried out at 42°C for 16 h with a radiolabeled probe in a hybridization buffer containing 5 \times SSC, 5 \times Denhardt's solution, 0.1% (w/v) SDS, 0.05 M sodium phosphate (pH 6.5), 0.25 mg/mL denatured salmon sperm DNA and 50% (v/v) formamide. Hybridization for low stringent conditions was carried out under the same conditions except that the concentration of formamide in the hybridization buffer was 15%. After hybridization, the membrane was rinsed once with 2 \times SSC containing 0.05% SDS, and washed three times with 5 mM sodium phosphate (pH 7.0) containing 1 mM EDTA and 0.2% SDS at room temperature for 15 min and three times with 5 mM sodium phosphate (pH 7.0) containing 0.1 \times SSC, 1 mM EDTA and 0.3% SDS at 55°C for 20 min. The membrane was then exposed to an Imaging Plate (Fuji Photofilm Co., Ltd., Tokyo, Japan) and the radioluminographic image was analyzed with a Bioimaging Analyzer (BAS2000, Fuji Photofilm).

Amino Acid Analysis

Cotyledons were harvested, weighed and quickly frozen in liquid nitrogen. Frozen samples were stored at -80°C until use. Samples were ground to a fine powder with a mortar and pestle, suspended and incubated in 70% (v/v) ethanol at 75°C for 1 h to extract free amino acids. Insoluble materials were removed as sediments by centrifugation at 700 g for 15 min. The supernatant was recovered and the solvent was evaporated to dryness *in vacuo*. The resultant residues were dissolved in 1 ml of 0.01 M HCl and used as cotyledon samples.

Phloem exudates were collected according to the method of King and Zeevaart (1974). Six cotyledons were excised with a razor blade at the base of petioles, and incubated for 2 h with the cut ends in 1 ml of 20 mM EDTA. Phloem exudates thus obtained in the EDTA solution were adjusted to pH 2.1 with 1 M HCl and centrifuged for 5 min at 15,000 g to remove insoluble materials. The supernatants were used as phloem exudate samples.

The samples were filtered through a Minisart SM16534 filter (Sartorius AG, Göttingen, Germany) and subjected to HPLC analysis for the constitution of amino acids as described elsewhere (Hayashi and Chino 1990).

Results

Accumulation of transcripts from AS and GS1 genes in naturally senescing cotyledons

Radish seeds sown on a vermiculite bed were grown for 7, 20 and 23 d at 25°C under a photoperiodic regime of 16 h light/8 h dark. They germinated 2 d after sowing, cotyledons fully expanded 7 d after sowing. They began to turn yellow after about 20 d of growth, and finally fell off about 1 week after the onset of yellowing. It has been shown that chlorophyll contents in cotyledons also start to decline at 20 d after sowing parallel with those observation (Figure II-1). Cotyledons from 7-d-old plants were used as the non-senescent (young) control, and those from 20- and 23-d-old plants as senescing cotyledons in this study.

Although radish has three GS1 genes (*Gln1;1*, *Gln1;2*, *Gln1;3*) (Watanabe *et al.* 1994), only the results with *Gln1;1* are presented in this study, because under all conditions examined, the transcripts from *Gln1;2* were hardly detectable, and the accumulation patterns of transcripts from *Gln1;3* were very similar to those of *Gln1;1* although the level of *Gln1;3* was lower than that of *Gln1;1* (data not shown). As shown in Figure II-2, the level of transcripts from *Gln1;1* was very low until the 14th day of sowing. Thereafter, it started to increase and reached a peak on the 20th day. The peak level was about 15-fold higher than that in the cotyledons of 7-d-old plants. Since AS is encoded by plural nuclear genes in many plant species, RNA gel blot analysis for AS gene was performed under high and low stringent conditions. In contrast with GS1, transcript from the AS gene was undetectable in cotyledons throughout the senescence stages examined under both high (Figure II-2) and low stringent conditions (data not shown).

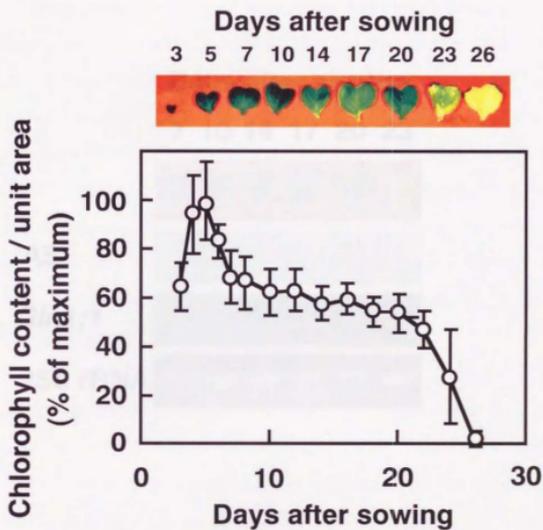


Figure II-1. Change in relative content of chlorophyll in radish cotyledons during natural senescence. Chlorophyll content per unit area was determined and shown as a percentage of maximum. Vertical bars represent standard deviations.

As a result of this experiment, the AS and Gln1;1 genes in the cotyledons of the radish

transcripts from AS and Gln1;1 genes were determined by the RNA gel blot analysis. In the cotyledons from 7 d of the dark, the concentration level of transcripts from AS gene increased about 25-fold, and this level was maintained for 3 w in the dark. In contrast, that of transcripts from Gln1;1 gene was



Figure II-2. RNA gel blot analysis of transcripts from AS gene and *Gln1;1* in radish cotyledons during natural senescence. RNA was extracted from cotyledons at various stages of growth and subjected to RNA gel blot analysis. Each lane contained 20 μg of RNA and blotted membrane was hybridized with a probe prepared from a cDNA clone for either AS or *Gln1;1*. Ethidium bromide-stained 25S rRNA bands are shown at the bottom.

Accumulation of transcripts from AS and GS1 genes in the cotyledons in the dark

Seven-d-old plants were incubated for 1 and 3 d in the dark, and the transcripts from AS and GS1 genes were determined by the RNA gel blot analysis. In the cotyledons kept for 1 d in the dark, the accumulation level of transcripts from AS gene increased about 15-fold, and this level was maintained for 3 d in the dark. In contrast, that of transcripts from *Gln1;1* did not change in the dark and remained at a very low level even after 3 d in the dark (Figure II-3A). In 20-d-old plants, transcripts from AS gene were hardly detected even after 3 d in the dark, whereas that of *Gln1;1*, which was abundant at the beginning, was approximately doubled after 1 d in the dark, and then slightly decreased during the subsequent 2 d in the dark (Figure II-3B). The hybridization patterns of AS gene examined under high and low stringent conditions were identical in these experiments (data not shown).

Free amino acids in naturally senescing cotyledons and in the phloem exudates

To examine which amino acids contribute to the translocation of nitrogen from naturally senescing cotyledons, I determined free amino acids in the cotyledons as well as in phloem exudates from the cotyledons in 7-, 20-, and 23-d-old plants.

In 7-d-old plants, the content of free amino acids in the cotyledons was 240.8 nmoles per cotyledon, and Asp, Glu, Gln, Ala, Ser, Ile, Thr, His, Asn and Val accounted for 23%, 23%, 15%, 10%, 7.5%, 6.3%, 4.2%, 2.7%, 2.0% and 1.6% of the total amino acids, respectively (Table II-1). In 20-d-old plants, it went down to 45% of that in 7-d-old plants, and Glu, Asp, Gln, Ser, Ala, Thr and Phe accounted for 44%, 15%,

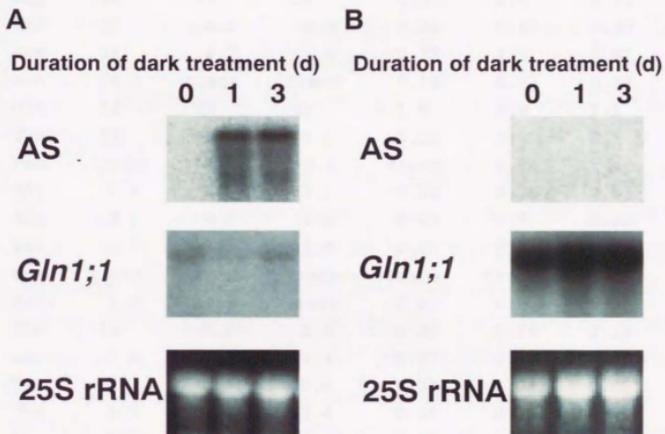


Figure II-3A, B. RNA gel blot analysis of transcripts from AS gene and *Gln1;1* in radish cotyledons during dark treatment. Plants were transferred to the dark 7 d (A) and 20 d (B) after sowing, then kept in continuous darkness for 1 and 3 d. RNA was extracted from cotyledons and subjected to RNA gel blot analysis. Each lane contained 20 μ g of RNA and blotted membrane was hybridized with a probe prepared from a cDNA clone for either AS or *Gln1;1*. Ethidium bromide-stained 25S rRNA bands are shown at the bottom.

Table II-1 Changes in the contents of free amino acids in naturally senescing cotyledons and in phloem exudates

	Whole cotyledons (nmol/cotyledon)			Phloem exudates (nmol/h cotyledon)		
	7-d-old	20-d-old	23-d-old	7-d-old	20-d-old	23-d-old
Asp	56	16	11	0.40	2.0	0.31
Thr	10	4.1	2.5	0.22	0.68	0.30
Ser	18	4.7	5.4	0.73	1.5	0.61
Asn	4.8	trace	trace	0.18	0.83	0.18
Glu	54	47	43	1.5	5.8	1.1
Gln	37	12	0.6	0.33	6.5	4.5
Pro	trace	2.9	2.2	trace	0.36	trace
Gly	1.4	2.6	3.3	0.80	0.76	0.53
Ala	24	4.4	1.4	0.40	1.7	0.42
Val	3.9	2.3	2.5	0.15	0.84	0.34
Cys	trace	trace	trace	trace	trace	trace
Met	1.0	trace	trace	0.07	0.01	0.03
Ile	15	2.8	1.6	0.09	0.38	0.19
Leu	1.4	1.5	1.4	0.07	0.10	0.08
Tyr	0.7	0.6	0.8	0.03	0.08	0.08
Phe	1.7	3.1	2.4	0.01	0.35	0.13
Lys	2.9	trace	1.4	0.05	0.10	0.01
His	6.5	2.4	2.4	0.10	0.13	0.08
Arg	2.5	0.8	trace	0.02	0.02	0.12
Total	240.8	107.2	81.9	5.15	22.14	9.01

Trace means that the content of amino acid was lower than 0.05 nmol/cotyledon in whole cotyledons, and 0.005 nmol/h cotyledon in phloem exudates.

11%, 4.4%, 4.1%, 3.8% and 2.9% of the total amino acids, respectively (Table II-1). The contents of all amino acids except for Pro, Gly, Phe and Leu was lower in the 20-d-old plants than in the 7-d-old plants (Table II-1). In the 23-d-old plants, the content in the cotyledons was only 34% of that in the 7-d-old plants, and Glu, Asp, Ser, Gly, Ala, Thr, His, Phe and Pro accounted for 53%, 13%, 6.6%, 4.0%, 3.1%, 3.1%, 2.9%, 2.9% and 2.7% of total amino acids, respectively (Table II-1). The Gln content in 23-d-old plants was 5% of that in the 20-d-old plants, whereas the contents of other amino acids did not differ significantly between 20- and 23-d-old plants (Table II-1).

To estimate the rate of export of amino acids from cotyledons into phloem sap, I collected phloem exudates from the detached cotyledons and amino acids in the exudate were determined. In the 7-d-old plants, the rate of export was calculated to be $5.15 \text{ nmoles h}^{-1}$ per cotyledon, and the contents of Glu, Gly, Ser, Ala, Asp, Gln, Thr, Asn, Val and His in the exudates accounted for 29%, 16%, 14%, 7.7%, 7.7%, 6.4%, 4.3%, 3.5%, 2.9% and 1.9% of the total amino acids, respectively (Table II-1). In the 20-d-old plants, it increased 4.3-fold relative to that in the 7-d-old plants, and Gln, Glu, Asp, Ala, Ser, Val, Asn, Gly and Thr in the exudates accounted for 29%, 26%, 9.0%, 7.7%, 6.8%, 3.8%, 3.7%, 3.4% and 3.1% of the total amino acids, respectively (Table II-1). The amounts of all amino acids except for Gly and Met were higher in 20-d-old plants than in 7-d-old plants: the content of Gln in the old cotyledons was 20-fold higher whereas that of other major amino acids including Asn was only 2.0 to 5.6-fold higher than that in the young cotyledons. In the 23-d-old plants, the rate of exudation of total amino acids decreased to 41% of that in the 20-d-old plants, and Gln, Glu, Ser, Gly, Ala, Val, Asp, Thr, Ile and Asn accounted for 50%, 12%, 6.8%, 5.9%, 4.7%, 3.8%, 3.4%,

3.3%, 2.1% and 2.0% of the total amino acids, respectively (Table II-1). In summary, the amount of total amino acids in the cotyledons decreased with progress of senescence, whereas the rate of export of amino acids, particularly Gln and Asn, into phloem sap increased.

Free amino acids in young cotyledons and in the phloem exudates in the dark

Seven-d-old plants were incubated for 1 and 3 d in the dark, and the content of free amino acids was determined in the cotyledons and the phloem exudates.

After the dark treatment for 1 d, the amount of free amino acids in the cotyledons was 2.1-fold higher than that before the dark treatment, and Glu, Asn, Arg, His, Asp, Gln, Ser, Val and Lys accounted for 36%, 16%, 14%, 4.4%, 4.0%, 4.0%, 3.8%, 3.8% and 3.6% of total amino acids, respectively (Table II-2). Among major amino acids, the contents of Asp, Gln, Ala and Ile were 0.36 to 0.73-fold of those in the cotyledons before the dark treatment, whereas those of Val, Glu, Thr and Ser increased 1.1 to 4.9-fold, respectively (Table II-2). In contrast, the content of Arg and of Asn increased 29- and 17-fold, respectively, during 1 d in the dark (Table II-2). In plants kept in the dark for 3 d, the content of free amino acids in the cotyledons was 4.1-fold higher than that at the onset of the dark treatment, and Asn, Arg, Glu, Gln, Phe, Lys, Ser, Val and Asp accounted for 49%, 12%, 8.9%, 6.5%, 4.6%, 4.0%, 4.0%, 3.1% and 2.4% of total amino acids, respectively (Table II-2). Among major amino acids detected in the cotyledons after 3 d in the dark, the content of Glu and of His were 0.48- and 0.64-fold, respectively, whereas those of Asn, Gln, Lys, Ser, Arg, Val and Asp were 1.2 to 5.9-fold of those after 1 d in the dark (Table II-2). In addition, the content of Asn

Table II-2 Changes in the contents of free amino acids in cotyledons and in phloem exudates in the dark

	Whole cotyledons (nmol/cotyledon)			Phloem exudates (nmol/h cotyledon)		
	0 d dark	1 d dark	3 d dark	0 d dark	1 d dark	3 d dark
Asp	56	20	24	0.40	2.3	1.5
Thr	10	13	8.4	0.22	0.24	0.13
Ser	18	19	39	0.73	0.71	0.47
Asn	4.8	81	480	0.18	3.0	4.9
Glu	54	180	87	1.5	1.9	1.2
Gln	37	20	64	0.33	1.5	1.4
Pro	trace	trace	trace	trace	trace	0.08
Gly	1.4	1.9	1.0	0.80	0.19	0.10
Ala	24	14	14	0.40	0.82	0.54
Val	3.9	19	30	0.15	0.46	0.43
Cys	trace	trace	trace	trace	0.02	trace
Met	1.0	0.9	0.8	0.07	0.04	0.03
Ile	15	11	10	0.09	0.34	0.18
Leu	1.4	5.3	3.0	0.07	0.01	0.05
Tyr	0.7	1.4	1.6	0.03	0.08	0.05
Phe	1.7	1.9	45	0.01	0.05	0.50
Lys	2.9	18	39	0.05	0.22	0.10
His	6.5	22	14	0.10	0.41	0.44
Arg	2.5	72	120	0.02	0.02	0.10
Total	240.8	500.4	980.8	5.15	12.29	12.2

Plants were transferred to darkness 7 d after sowing. Trace means that the content of amino acid was lower than 0.05 nmol/cotyledon in whole cotyledons, and 0.005 nmol/h cotyledon in phloem exudates.

and of Arg increased 100- and 48-fold, respectively, after 3 d in the dark (Table II-2).

In the plants kept in the dark for 1 d, the rate of export of total amino acids from cotyledons was 2.4-fold higher than that before the dark treatment, and Asn, Asp, Glu and Gln accounted for 24%, 16%, 15% and 12% of the amino acids in the exudates, respectively (Table II-2). The rates of export of Asn, Asp, and Gln from cotyledons increased 17-, 5.8-, and 4.5-fold, respectively, during the dark treatment whereas those of other amino acids did not change significantly (Table II-2). In the plants kept in the dark for 3 d, the rate of export was similar to that after 1 d in the dark, and Asn, Asp, Gln and Glu accounted for 40%, 12%, 11% and 9.8%, respectively, of the total amino acids in the exudates (Table II-2). The rate of export of Asn after 3 d in the dark was 1.6-fold higher than that after 1 d in the dark, which was the highest, whereas that of other amino acids including Gln was similar or slightly lower (Table II-2). It should be noted that the rate of export of Arg which accumulated noticeably in the cotyledons during 3 d in the dark was negligible throughout the dark period (Table II-2). In summary, Asn was the most abundant amino acid in the phloem exudates of cotyledons after incubation in the dark for 3 d. However, unlike senescing plants, Asn but not Gln increased in the cotyledons as well as in the phloem exudates.

Developmental change in the response to darkness of genes for AS and GS1

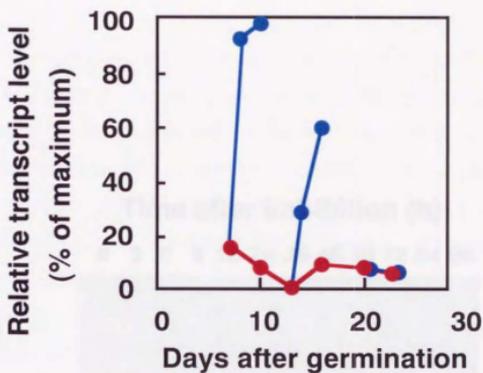
Since young and old cotyledons showed totally different responses to darkness in respect to the expression of genes for AS and GS1, I looked for the switching point of the response of the genes to darkness during the growth of the cotyledons. Several differently aged plants were incubated

for 1 and 3 d in the dark, and the transcript levels of AS and GS1 genes were determined by RNA gel blot analysis. The results were quantified by an Imaging Analyzer and shown in Figure II-4. The accumulation of transcripts from AS gene in the dark was observed until the 14th day after sowing, it did not occur on the 20th day after sowing. On the other hand, increase in the level of transcripts from *Gln1;1* by dark treatment was observed only on the 20th day after sowing. These results showed that there are a switching point of the response of these genes to darkness between the 14th day and the 20th day. This time point corresponds to that when the cotyledons are about to get into senescence stage in terms of chlorophyll content (Figure II-1).

Accumulation of transcripts from AS and GS1 genes in cotyledons during germination

Translocation of nitrogen is known to occur during germination as well as senescence in cotyledons. To examine the level of transcripts from AS and GS1 genes during germination, total RNA was isolated from cotyledons harvested at various times after imbibition, and was used for RNA gel blot analysis. The results were shown in Figure II-5. The level of transcripts from *Gln1;1* increased transiently in cotyledons during germination. The level of the transcripts reached maximum at 12 h after imbibition, and then decreased and became almost undetectable at 96 h after imbibition. In contrast, the level of transcripts from AS gene was not detected in the cotyledons of germinating seedlings during the experimental period (Figure II-5).

A



B

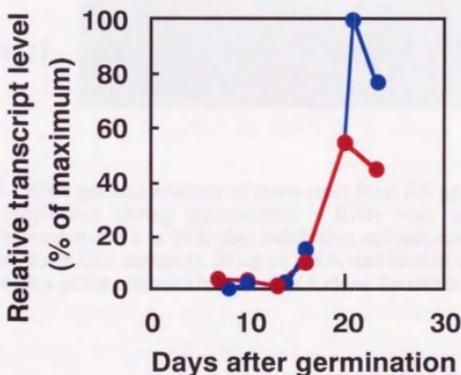


Figure II-4. Developmental change in the response to darkness of genes for AS and GS1. RNA was extracted from cotyledons and subjected to RNA gel blot analysis. Plants were transferred to the dark 7, 14, and 20 d after sowing, then kept in continuous darkness for 1 and 3 d. RNA (20 μ g) was separated by agarose gel electrophoresis, blotted onto membrane and hybridized with a probe prepared from a cDNA clone for either AS (A) or *Gln1;1* (B). Hybridized probes were quantitated with a Bioimaging Analyzer (BAS2000, Fuji Photofilm). Relative transcript level of AS and GS1 genes in the light is shown as ●, and that in the dark is shown as ●.

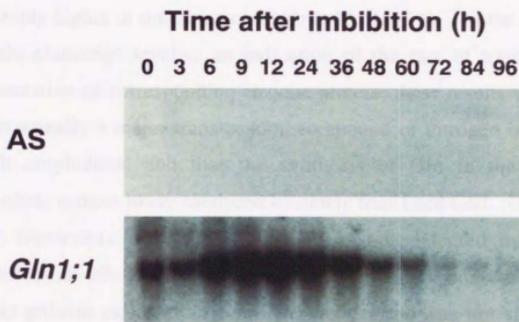


Figure II-5. RNA gel blot analysis of transcripts from AS gene and *Gln1;1* in radish cotyledons during germination. RNA was extracted from cotyledons harvested at 0 h to 96 h after imbibition and subjected to RNA gel blot analysis. Each lane contained 20 μ g of RNA, and blotted membrane was hybridized with a probe prepared from a cDNA clone for either AS or *Gln1;1*.

Discussion

We have shown that the level of transcripts from *Gln1;1* was markedly higher in the senescing cotyledons of 20- and 23-d-old radish plants than in young cotyledons of 7-d-old plants (Figure II-2). In parallel the rate of export of Gln from cotyledons to phloem saps was noticeably higher in senescing cotyledons (Table II-1). On the assumption that the transcript level is an indication of the rate of translation and accumulation of corresponding enzyme protein, these results suggest that Gln is actually a major translocation compound of nitrogen in senescing radish cotyledons, and that the synthesis of Gln in the senescing cotyledons is most likely catalyzed by newly translated GS1. On the other hand, transcripts from the AS gene was not detected in senescing cotyledons in 20- and 23-d-old plants (Figure II-2), and the content of Asn in phloem exudates in senescing cotyledons was not significantly higher than that in young cotyledons (Table II-1). These observations suggest that, at least in radish cotyledons, the activity of AS is not closely associated with the progress of senescence and that Asn is not a predominant compound for nitrogen translocation from senescing cotyledons.

It has been thought that nitrogen is translocated from cotyledons to developing organs during germination as well as senescence. During germination, storage proteins of cotyledons are hydrolyzed to amino acids by proteolytic enzymes (Ryan 1973), and the amino acids are translocated to developing organs (Ashton 1976). They are used for the synthesis of various enzymes and structural proteins in the developing organs (Ashton 1976). During germination, as shown in Figure II-5, the accumulation level of transcripts from *Gln1;1* increased transiently, but those from AS

gene was not detected in radish cotyledons. These results may also suggest that GS1 is related to translocation of nitrogen, but AS is not.

The accumulation level of transcripts from the AS gene in the young cotyledons increased in the dark (Figure II-3A), and this increase paralleled the increase in the contents of Asn in cotyledons and in phloem exudates (Table II-2). However, the ratio of the level of Asn (nmoles per cotyledon) to that in the phloem exudate (nmoles h^{-1} per cotyledon) increased from 27:1 to 98:1 during the 3-d incubation in the dark. These results imply that Asn in the dark is stored in the cotyledons rather than translocated and that Asn at this time is considered to be synthesized by the action of AS, on the assumption that the transcripts are translated and lead to accumulation of corresponding enzyme protein. The accumulation of free Asn has also been found in plant tissues that were stressed or limited in nutrients. Barley roots cultured in a sulphate-deprived medium contained about an 8-fold higher amount of free Asn compared with control roots (Karmoker *et al.* 1991). Similarly, deletion of phosphate from the culture medium led to an increase in the content of Asn in soybean stems and roots (Rufy *et al.* 1993). In addition, Genix *et al.* (1990) found that sucrose starvation in sycamore cell cultures caused the accumulation of free Asn. These observations and my results suggest that one of the physiological roles of Asn is the storage of extra nitrogen within the cell.

Increases in the amounts of Arg, Lys and His, which are amino acids with a low C/N ratio, were also observed in the whole cotyledons during the dark treatment (Table II-2). Especially, the content of Arg which is thought to be a storage compound for nitrogen in plants (Shargool *et al.* 1988), increased about 50-fold in the cotyledons, but remained constant in phloem exudates during the 3 d in the dark (Table

II-2). Young cotyledons transferred to the dark may accumulate nitrogen in the form of low C/N ratio compounds such as Asn, Arg, Lys and His. The accumulation of these amino acids was not observed during natural senescence. These results suggest that dark-induced senescence shares a large part of the metabolic and molecular process with natural senescence, but is not totally identical to the latter.

The dark treatment accelerated the accumulation of transcripts from *Gln1;1* in old cotyledons, but not in young cotyledons (Figure II-3A, 4). On the other hand, the increase in those from AS gene by the dark treatment was observed only in young cotyledons (Figure II-3A, 4). As shown in Figure II-4, the switching point of the response of these genes to darkness exists between the 14th day and the 20th day after sowing. During the period, the accumulation level of transcripts from *Gln1;1* started to increase even under normal light conditions (Figure II-2, 4). The induction of AS gene by dark treatment seemed to cease after the time when expression of *Gln1;1* started to increase (Figure II-4). If AS in the dark is associated with nitrogen storage and GS1 is associated with nitrogen translocation, the changes in the response of the genes to darkness may be related to the conversion of cotyledons from a sink to a source organ in respect to nitrogen utilization, as the organ enters into senescence stage. Although further work is needed to confirm this hypothesis, understanding of the regulatory mechanism of expression of genes for both AS and GS1 may provide us with information on the molecular mechanism of leaf senescence.

Chapter III

Expression of a Gene for Asparagine Synthetase in the Dark Is Related to Transient Storage of Nitrogen in Radish Cotyledons

Introduction

As described in Chapter II, I found that AS does not play a major role in the translocation of nitrogen in senescing cotyledons, but is related to accumulation of Asn in the cotyledons in the dark. In order to understand a physiological role of AS in the dark, I will examine the physiological significance of Asn accumulation in radish cotyledons in this chapter.

Asn is the first amino acid that was discovered in the plant kingdom (*Asparagus sativus*) (Vauquelin and Robiquet 1806). Thereafter, Asn has been detected in many plant species and is known as one of ubiquitous nitrogen compounds among all living organisms. In several higher plants, Asn is often observed as one of major free amino acids in specialized organs and its content has been reported to vary under various circumstances. For example, Asn is a predominant form of organic nitrogen in xylem saps from nodulated regume plants (Pate 1973; Urquhart and Joy 1981; Peoples *et al.* 1986). Accumulation of Asn was also observed in sugar-deprived cycamore suspension-cultured cells (Genix *et al.* 1990), harvested asparagus spears (King *et al.* 1990) and dark-treated maize leaves (Brouquisse *et al.* 1998). However, the physiological significance of such accumulation of Asn has been under discussion and the mechanism of Asn accumulation is also less

understood.

In Chapter II, I described that Asn is stored in the cotyledons rather than translocated from the cotyledons during short-term dark treatment. Although senescing symptoms such as yellowing were not observed in the young cotyledons during the short-term dark treatment, the cotyledons would senesce by far longer incubation in the dark. Therefore, I will examine whether accumulation of Asn can be observed in the cotyledons under the prolonged darkness in this chapter. In addition, to evaluate the physiological significance of Asn synthesis in the dark, I will describe several physiological changes in radish cotyledons submitted to the prolonged darkness.

Materials and Methods

Plant material

Radish (*Raphanus sativus* L. cv. Comet) seeds were sown on a vermiculite bed in a plastic tray (25 cm X 35 cm) and grown in a growth chamber regulated at 25°C under a relative humidity of 60% with a 16-h daylength at a photon flux rate of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were watered daily and were supplied with Hoagland's solution (Arnon and Hoagland 1940) on the 4th day after sowing. Cotyledons were harvested at the end of the light period. For experiments with dark treatment, radish plants were transferred to continuous darkness at the end of the light period.

PCR-based cloning of a cDNA for AS

A cDNA for AS was obtained by PCR-based cloning. Degenerate oligonucleotide primers were designed from highly conserved amino acid sequences among AS polypeptides published so far: 5'-TA(CT)TT(CT)-CA(CT)AA(AG)GC(N)CC(N)AA-3' and 5'-CT(CT)CG(N)AT(AG)AT-(AG)AT(AG)(TG)C(N)TAC-3' represented sense and antisense primers, respectively, where N represented all four deoxyribonucleotides and nucleotides in parentheses are degenerated. The first-strand cDNA was synthesized by reverse transcriptase (SuperScript Preamplification System kit, Gibco-BRL, Gaithersburg, MD) using 1 μg poly(A)+RNA from 1 d dark-treated cotyledons as a template. Following the reverse transcriptase reaction, a cDNA for AS was amplified by PCR with the degenerate oligonucleotide primers. PCR were carried out 30 cycles with a denaturing step at 94°C for 1 min, an annealing step at 45°C for 2 min and an extension step at 72°C for 2 min. The amplified fragment was

cloned into a pGEM-T Vector (Promega, Madison, WI) and its nucleotide sequence was determined. 5'-RACE and 3'-RACE were performed as described by Frohman *et al.* (1988).

DNA sequencing

The nucleotide sequence of cDNA was determined by a DNA sequencer (model LIC-4000, LI-COR Inc., Lincoln, NE) using a Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech) according to the manufacture's instruction. Both strands were entirely sequenced.

Extraction and analysis of RNA

Extraction of total RNA and RNA gel blot analysis were carried out as described in Chapter II.

Amino acid analysis

Amino acid analysis was carried out as described in Chapter II.

Measurement of Chlorophyll Content

Chlorophyll contents were measured as described in Chapter II.

Protein assay

Cotyledons were frozen in liquid nitrogen and ground to fine powder with a mortar and pestle. Those powders were suspended in 70% ethanol and boiled at 75°C for 30 min. After centrifugation at 15,000 g for 3 min, the resultant supernatant was used as free amino acids and the pellet was used as a total protein fraction, respectively. Nitrogen contents of the protein were determined by the method of Hikosaka *et al.* (1994).

Starch and Sucrose assay

Plant samples were weighed and immediately frozen in liquid nitrogen and stored at -80°C until measurements. Samples were ground in liquid nitrogen with a mortar and pestle and extracted twice with 80% ethanol for 30 min at 80°C in order to separate soluble sugars from starch. Ethanol-insoluble material was collected by centrifugation and the supernatants were pooled. Starch present in the ethanol-insoluble fraction was quantified by the method of Rufty and Huber (1983). The ethanol-soluble fraction including sucrose was evaporated to remove ethanol and redissolved in 0.4 ml of water. To remove chlorophyll, 0.4 ml chloroform was added and the chloroform-water mixture was centrifuged for 10 min at 15,000 g. The upper clear phase was used for sucrose analysis. Sucrose was determined by the method of Rao *et al.* (1990).

Results

Isolation and characterization of a cDNA clone for AS

In Chapter II, the expression of AS gene was examined by RNA gel blot analysis using a heterologous probe. To analyze expression of AS gene more precisely, I first isolated a cDNA clone for AS from radish in this chapter. A cDNA fragment for radish AS was amplified by RT-PCR, as described in Materials and Methods, using degenerate oligonucleotide primers which were designed from conserved amino acid sequences among AS from other plant species, and the 5' and 3' ends of the cDNA were further isolated by 5' RACE and 3' RACE, respectively. Although the longest cDNA clone lacked its in frame stop codon at the 5' region, the cDNA clone was shown to contain a single ORF of 1755 bp. The deduced amino acid sequence was similar to AS from other plant species and I designated a gene corresponding to the cDNA clone *Asn1*.

The radish *Asn1* contained an ORF encoding a polypeptide of 585 amino acid residues with a predicted molecular weight of 65,553 and a pI of 6.00. The deduced amino acid sequence of *Asn1* was the most similar to that of *Brassica oleracea* AS (Downs *et al.* 1995) among all plant AS proteins currently registered in the GenBank database. The predicted *Asn1* protein showed a 96% identity to *Brassica oleracea* AS. Figure III-1 represents alignments of deduced amino acid sequences between *Asn1* and other AS proteins. The amino acid sequence of the N-terminal part of radish *Asn1* is highly conserved among other plant AS proteins. This domain is characterized by the presence of three amino acid residues, *i.e.*, Cys-2, Asp-34 and His-104, which are conserved in a triad structure of a *purF*-type Gln-binding domain (Mei and Zalkin 1989).

<i>Raphanus sativus</i>	* MCGILAVLGCSDSQAKRVVLELSRRLRHRGPDNSGIYQNGNYLAHQRLAIDPASGD	60
<i>Brassica oleracea</i>F.....D.....	60
<i>Arabidopsis thaliana</i>L.....D.....V.....	60
<i>Medicago sativa</i>I.....K.....LH.H.D.....V.....	60
<i>Zea mays</i>VVEV.L.S.II.....LHCHEDC.....T.....	60
<i>Raphanus sativus</i>	QPLFNEDKSI VVTVNGE IYNHEELRKLKHKHFGTGDGVIAHLYEEHGFVMDLMDI	120
<i>Brassica oleracea</i>	120
<i>Arabidopsis thaliana</i>T.....R.....R.....E.....Y.VD.....	120
<i>Medicago sativa</i>I.....D.....Q.P.....R.QC.....Y.....	120
<i>Zea mays</i>Y.....TV.....KAK..T.E.Q.....E.....Y..E.....M	120
<i>Raphanus sativus</i>	FSFVLLDTRDSFMVARDVAVGVTSLYIGWGLDGSLVWSSEMKGLEDCEHFEAFPPGHLY	180
<i>Brassica oleracea</i>	180
<i>Arabidopsis thaliana</i>I.....V.I.....ND.....T.....E.....	180
<i>Medicago sativa</i>I.....I.....V.IA..L..NDE.....V.....	180
<i>Zea mays</i>K..IA.....I.ICP..M.....V.F.....A.SD.....R.IT.....	180
<i>Raphanus sativus</i>	SSKSGGGKQFYKPNPWFNEV-PSTPYEPLAIRRAFEDAVIKRLMTDVPFVGLSGGLDS	239
<i>Brassica oleracea</i>S.....	239
<i>Arabidopsis thaliana</i>L.....R.....N.....	238
<i>Medicago sativa</i>DRE.FR.....A.I.T.....D..VL.N..K.....	239
<i>Zea mays</i>T..LRR.....S.T.....NA.FL.E.M..K.....	238
<i>Raphanus sativus</i>	SLVASITARHLAGTKAAKRNGPQLHSFCVGLGSDPKAGKVAEYLTGVHHEFFVTQD	299
<i>Brassica oleracea</i>	299
<i>Arabidopsis thaliana</i>Q.....S.....	298
<i>Medicago sativa</i>AV..Y.....Q..AK.....K.A.....R..DF.....Q..I.....	299
<i>Zea mays</i>VAS.....NE..VDRQ..NK..T..I..K.....AR..D..S.....E	298
<i>Raphanus sativus</i>	GIDAIEDVIHVETVDVTTIRASTPMFLMSRKKLSLGVKMLSGEGSDIEFGYLYPHKA	359
<i>Brassica oleracea</i>	359
<i>Arabidopsis thaliana</i>T.....A.....S.....W.I.....	359
<i>Medicago sativa</i>L.E.....I.....	358
<i>Raphanus sativus</i>	PNKQEFHQETCRKIKALHKYDCLRANKATSFAFLEAVPFLDKEFINTAMSLDPEKMKI	419
<i>Brassica oleracea</i>	419
<i>Arabidopsis thaliana</i>K.....S.....D.....	418
<i>Medicago sativa</i>E.....R.....S.Y.W.....KV..DI..Y.....	419
<i>Zea mays</i>K..LE.....L.....W.V.....S..SV..DI.....WN.....	418
<i>Raphanus sativus</i>	PEEGRIEKVWLRRAFDEERPVLPKHILYRQKEQFSDGVGYSWIDGLKHAHAENVNDKMM	479
<i>Brassica oleracea</i>	479
<i>Arabidopsis thaliana</i>H.....I..K.....N.....G.....I.D..KH.T.R.....	478
<i>Medicago sativa</i>RDL.....M.K.....D.H.....N.....SPTQQ.T.E.....	478
<i>Raphanus sativus</i>	SNAAYIFPHNTPLTKEAYYRMI FERFFPONSARLTPVGGATVACSTAKAVENDASWSN	539
<i>Brassica oleracea</i>K..F.....	539
<i>Arabidopsis thaliana</i>GH.....N.....	538
<i>Medicago sativa</i>L..SI..F.....S.....E..I.....	539
<i>Zea mays</i>N.....QM..Y.....VN.....L..D..E..W.PSI.....PA.I..VEQ.KAS	538
<i>Raphanus sativus</i>	MDSGRAAIGVHLSAY-DGSKVALPLP--PKHAI DMPHMMGQEVVIQT	585
<i>Brassica oleracea</i>A.....I.....	586
<i>Arabidopsis thaliana</i>KN.....TI.....L.....N.....G.....	584
<i>Medicago sativa</i>L.....F.....N.....ENQ--NSVAKTVEPEK.IPKMEISNLG.A..S	586
<i>Zea mays</i>N.....S.S.D..AT.HTA.SRRK.TAAARPANGTVNGKDV.P.F.AV	586

Figure III-1. Alignments of amino acid sequences of AS among various plant species. The *Raphanus sativus* AS sequence was aligned with those from *Brassica oleracea* (GenBank accession number X84448; Downs *et al.* 1995), *Arabidopsis thaliana* (GenBank accession number L29083, Lam *et al.* 1994), *Medicago sativa* (GenBank accession number U89923, Shi *et al.* 1997) and *Zea mays* (GenBank accession number X82849, Chevalier *et al.* 1996). Identical amino acids are shown by dots. Dashes indicate gaps introduced to maximize sequence similarity. Numbers to the right of each sequence indicate the position of residues of diagram. The asterisks indicate the residues of the putative *purF*-type Gln-binding triad: boldface C, Cys-2; boldface D, Asp-34; boldface H, His-104.

Effect of prolonged darkness on the accumulation level of transcripts from Asn1 in the cotyledons

In Chapter II, I showed that the level of transcripts from AS gene(s) increased in radish cotyledons by dark treatment. When cotyledons from 7-d-old radish plants was placed in the dark for 3 d, a significant increase in AS transcripts was detected by RNA gel blot analysis using *Arabidopsis ASN1* cDNA fragments as a heterologous probe. Cotyledons had stayed green during the 3 d dark treatment. However, when 7-d-old plants were further incubation in the dark, the cotyledons turned yellow and fell off about a week after the onset of the dark treatment. Then, a question arose whether the accumulation level of transcripts from *Asn1* is modulated by the prolonged dark treatment. The transcripts from *Asn1* accumulated in the cotyledons to a detectable level at 16 h (0.6 d) after the onset of dark treatment and increased gradually during the dark period. The level of the transcripts reached 50-fold on the 7th day after the onset of dark treatment (Figure III-2). On the other hand, the level of transcripts from *Gln1;1*, which was induced by senescence as shown in Chapter II, started to increase on the third day and peaked on the 5th day after the onset of dark treatment (Figure III-2).

Free amino acids in the cotyledons and in the phloem exudates in the dark

In the plant species so far examined, it was not successful in demonstrating the AS activity *in vitro*. Therefore, I analyzed the change in the content of Asn to extrapolate the activity of AS. Seven-d-old plants were incubated for 1, 3, 5 and 7 d in the dark and the contents of free amino acids in the cotyledons and phloem exudates were determined.

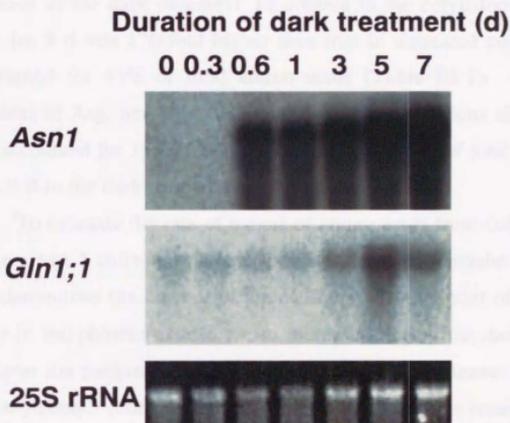


Figure III-2. RNA gel blot analysis of transcripts from *Asn1* and *Gln1;1* in radish cotyledons during prolonged dark treatment. Seven-d-old plants were transferred to the dark, then kept for 0.3, 0.6, 1, 3, 5, and 7 d. RNA was extracted from cotyledons and subjected to RNA gel blot analysis. Each lane contained 10 μ g of RNA and blotted membrane was hybridized with a probe prepared from a cDNA clone for either *Asn1* or *Gln1;1*. Ethidium bromide-stained 25S rRNA bands are shown at the bottom.

The amount of free amino acids in the cotyledons increased by dark treatment and peaked on the 5th day after the onset of dark treatment. The peak level was about 7-fold higher than that in the cotyledons before the dark treatment. Among all amino acids, Asn showed the largest increase by the dark treatment: Its content in the cotyledons kept in the dark for 5 d was 170-fold higher than that in untreated cotyledons and accounted for 45% of total amino acids (Table III-1). In addition, contents of Arg, Ser, Phe, Val and His in the cotyledons also increased and accounted for 13%, 7.8%, 6.1%, 3.1% and 2.6% of total amino acids after 5 d in the dark, respectively (Table III-1).

To estimate the rate of export of amino acids from cotyledons into phloem sap, I collected phloem exudates from the detached cotyledons and determined the content of amino acids. The amount of total amino acids in the phloem exudates also increased during the dark treatment, however the patterns of the increase was slightly different from that in the cotyledons. It doubled after 1 d in the dark and kept constant until the third day of the dark treatment. It reached a peak on the 5th day of dark treatment and then decreased. The level on the 5th day was about 10-fold higher than that from the cotyledons before the onset of dark treatment. The content of most of the amino acids increased when radish plants were incubated for 5 d in the dark: Gln, Asn, Val, Ser and Phe were the major amino acids in the exudates (Table III-2); Gln was the most abundant amino acid, and its content accounted for 37% of total amino acids (Table III-2). For the same dark-incubation period, the content of Asn in the phloem exudate was 54-fold higher than that before dark treatment and Asn accounted for 19% of total amino acids (Table III-2).

Table III-1 Changes in the contents of free amino acids in cotyledons in the dark

	Content (nmol/cotyledon)				
	0 d dark	1 d dark	3 d dark	5 d dark	7 d dark
Asp	56	20	24	72	30
Thr	10	13	8.4	23	20
Ser	18	19	39	140	210
Asn	4.8	81	480	820	300
Glu	54	180	87	190	59
Gln	37	20	64	7.9	34
Pro	trace	trace	trace	7.2	420
Gly	1.4	1.9	1.0	12	8.4
Ala	24	14	14	25	2.6
Val	3.9	19	30	56	73
Cys	trace	trace	trace	0.9	1.0
Met	1.0	0.9	0.8	3.2	6.2
Ile	15	11	10	13	19
Leu	1.4	5.3	3.0	5.5	0.5
Tyr	0.7	1.4	1.6	10	9.7
Phe	1.7	1.9	45	110	100
Lys	2.9	18	39	27	23
His	6.5	22	14	46	52
Arg	2.5	72	120	230	130
Total	240.8	500.4	980.8	1798.7	1498.4

Plants were transferred to darkness 7 d after sowing. Trace means that the content of amino acid was lower than 0.05 nmol/cotyledon.

Table III-2 Changes in the contents of free amino acids in phloem exudates in the dark

	Rate of exudation (nmol/h cotyledon)				
	0 d dark	1 d dark	3 d dark	5 d dark	7 d dark
Asp	0.40	2.3	1.5	1.2	0.76
Thr	0.22	0.24	0.13	0.68	0.08
Ser	0.73	0.71	0.47	4.1	1.1
Asn	0.18	3.0	4.9	9.9	6.8
Glu	1.5	1.9	1.2	2.1	1.7
Gln	0.33	1.5	1.4	19	2.5
Pro	trace	trace	0.08	0.22	trace
Gly	0.80	0.19	0.10	0.23	0.22
Ala	0.40	0.82	0.54	0.46	0.37
Val	0.15	0.46	0.43	4.5	1.2
Cys	trace	0.02	trace	trace	trace
Met	0.07	0.04	0.03	0.37	0.08
Ile	0.09	0.34	0.18	2.0	0.29
Leu	0.07	0.01	0.05	0.43	0.08
Tyr	0.03	0.08	0.05	0.33	0.05
Phe	0.01	0.05	0.50	3.9	1.5
Lys	0.05	0.22	0.10	0.65	0.08
His	0.10	0.41	0.44	1.5	1.1
Arg	0.02	0.02	0.10	0.86	0.33
Total	5.15	12.29	12.2	52.43	18.24

Plants were transferred to darkness 7 d after sowing. Trace means that the content of amino acid was lower than 0.005 nmol/h cotyledon.

Changes in the contents of chlorophyll, protein, starch, sucrose and free amino acids in the cotyledons during dark treatment

In order to estimate physiological changes occurring in radish cotyledons during the dark period, I measured such parameters as contents of chlorophyll, protein, starch, sucrose and free amino acids. These parameters were also used to evaluate the physiological role of AS. Seven-d-old plants were incubated for 1, 3, 5 and 7 d in the dark, and the parameters were determined. The contents of chlorophyll and protein in the cotyledons decreased almost linearly during the dark treatment and became about 16% and 20% of those in untreated cotyledons after 7 d in the dark, respectively (Figure III-3A, B). In a reciprocal relationship with the decrease in protein contents, the content of free amino acids increased until the 5th day after the onset of dark treatment and then decreased (Figure III-3E). In contrast, almost all starch and sucrose were consumed within a day, but only 20% of protein were degraded during the same period (Figure III-3B, C, D).

Effect of re-illumination on the accumulation level of transcripts from Asn1, and on the contents of starch, sucrose, protein and free Asn in the cotyledons

In the former section, I found that sucrose content was lowered most rapidly among other parameters examined in the dark. I was tempted to speculate that the level of free sugar is directly related to the regulation of the dark-induced expression of *Asn1*. To test this idea, I examined the effect of re-illumination on the level of transcripts from the gene. Seven-d-old plants were transferred to darkness and grown for 1, 3 or 5 d, and re-illuminated for an additional day. As shown in Figure III-4, the level of transcripts from *Asn1* increased continuously during

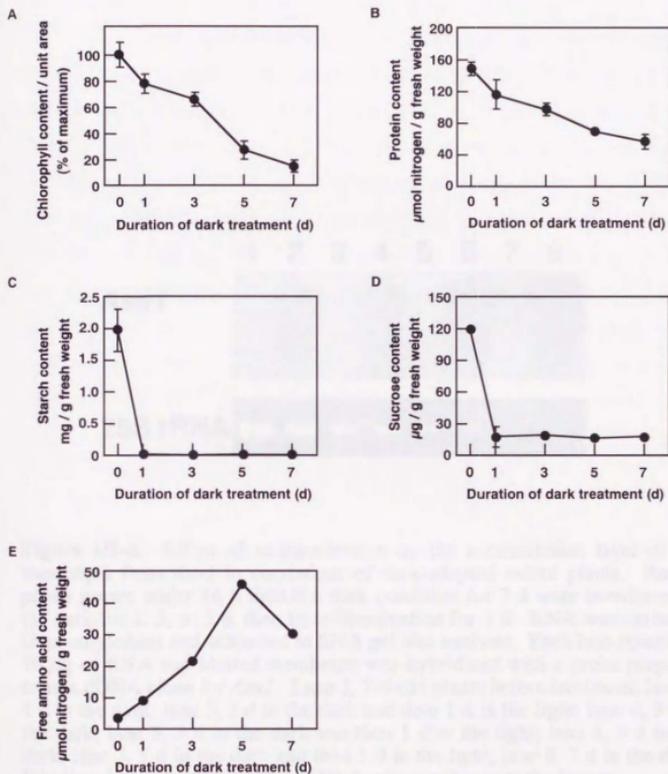


Figure III-3A, B, C, D, E. Changes in the contents of chlorophyll (A), protein (B), starch (C), sucrose (D) and free amino acid (E) in cotyledons during dark treatment. Seven-d-old plants were transferred to the dark, then kept in continuous darkness for 1, 3, 5, and 7 d. Data are means of results from triplicates. Vertical bars represent standard deviations. When vertical bars are not shown, it means that the standard deviation is too small to be seen.

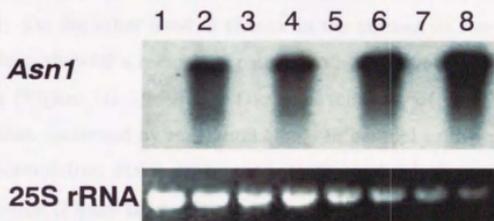


Figure III-4. Effect of re-illumination on the accumulation level of the transcripts from *Asn1* in cotyledons of dark-adapted radish plants. Radish plants grown under 16 h light/8 h dark condition for 7 d were transferred to the dark for 1, 3, or 5 d, then to re-illumination for 1 d. RNA was extracted from cotyledons and subjected to RNA gel blot analysis. Each lane contained 10 μg of RNA and blotted membrane was hybridized with a probe prepared from a cDNA clone for *Asn1*. Lane 1, 7-d-old plants before treatment; lane 2, 1 d in the dark; lane 3, 1 d in the dark and then 1 d in the light; lane 4, 3 d in the dark; lane 5, 3 d in the dark and then 1 d in the light; lane 6, 5 d in the dark; lane 7, 5 d in the dark and then 1 d in the light; lane 8, 7 d in the dark. Ethidium bromide-stained 25S rRNA bands are shown at the bottom.

dark period (Figure III-4, lane 2, 4, 6), and it decreased to the undetectable level after a day in the light (Figure III-4, lane 3, 5, 7).

I also examined the effect of re-illumination on the contents of starch, sucrose, protein and free Asn in the cotyledons. The contents of starch and sucrose which were at the very low levels in dark-adapted cotyledons came to increase, when plants were returned to the light condition (Figure III-5A, B). The content of protein in cotyledons also decreased during dark treatment, but increased by re-illumination (Figure III-5C). On the other hand, a change in the content of free Asn in the cotyledons showed a reciprocal pattern with that of starch, sucrose and protein (Figure III-5A, B, C, D). The content of free Asn in the cotyledons decreased by re-illumination. In parallel to this observation, the accumulation level of transcripts from *AsnI* decreased by re-illumination (Figure III-4, 5D).

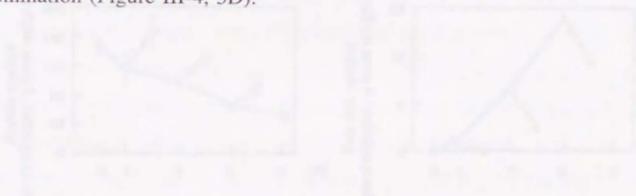


Figure III-5A, B. Effect of re-illumination on the contents of starch (A) and sucrose (B) in cotyledons of dark-adapted plants. Dark-adapted plants were treated for 2 days in the dark and then transferred to the light for 2, 4, or 6 days in re-illumination. The y-axis values were for the dark and re-illumination samples measured by HPLC. *M. longicaulis*.

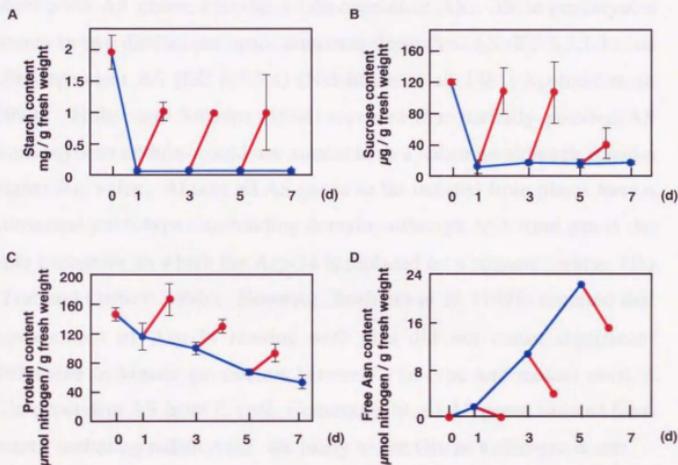


Figure III-5A, B, C, D. Effects of re-illumination on the contents of starch (A), sucrose (B), protein (C) and free Asn (D) in the cotyledons of dark-adapted radish plants. Radish plants grown under 16 h light/8 h dark condition for 7 d were transferred to the dark for 1, 3, or 5 d, then to re-illumination for 1 d. Each value taken for the dark and re-illumination samples is shown by ● and ●, respectively.

Discussion

An AS from *Raphanus sativus*, which is encoded by *Asn1*, shared a very strong similarity with AS from other plant species. The predicted amino acid sequence of the AS protein contained a complete *purF*-type Gln-binding domain (Mei and Zalkin 1989), indicating that *Asn1*, like other plant AS genes, encodes a Gln-dependent AS. AS in prokaryotes occurs in two distinct isoforms, ammonia-dependent AS (EC 6.3.1.1) and Gln-dependent AS (EC 6.3.5.4) (Nakamura et al. 1981; Scofield et al. 1990). Huber and Streeter (1984) reported that partially purified AS from soybean nodules could use ammonia as a substrate although it had a higher K_M value. Almost all AS genes so far isolated from plants have a conserved *purF*-type Gln-binding domain, although AS2 from pea is the only exception, in which the Asp-34 is replaced by a cognate residue, Glu (Tsai and Coruzzi 1990). However, Boehlein et al. (1994) reported that replacement of Asp-34 residue with Glu did not cause significant difference in kinetic parameters between wild-type and mutant *asnB*, a Gln-dependent AS from *E. coli*. Consequently, all AS genes isolated from plants, including radish *Asn1*, are likely to use Gln as a nitrogen donor.

In Chapter II, I demonstrated that in young radish plants the content of free Asn increases in the cotyledons rather than in the phloem exudates after 3 d in the dark. A similar tendency was also observed in this chapter by a prolonged dark treatment (Table III-1, 2). When the 7-d-old plants were kept in the dark for 5 d, the content of free Asn increased 170-fold in cotyledons relative to the initial level, whereas the content increased only 55-fold in phloem exudates (Table III-1, 2). On the other hand, the same treatment caused increase in the Gln level only in phloem exudates (Table III-1, 2), as was observed in naturally

senescing cotyledons (Chapter II). The content of Gln in phloem exudates increased 58-fold relative to that in the 7-d-old plants, whereas that in cotyledons decreased to 21%. These results suggest that a major nitrogen compound for translocation from cotyledons during dark-induced senescence could be Gln and that Asn may be used for storage of nitrogen within the cell rather than for translocation. On the other hand, free Asn declined its level in whole cotyledons from 5 d to 7 d, but became one of the most abundant amino acids in the phloem exudates 7 d after the onset of dark treatment (Table III-1, 2). These results might suggest that Asn stored in the cotyledons during dark treatment could also contribute to nitrogen translocation when senescence of cotyledons proceeds to the last stage after the prolonged darkness.

Results of RNA gel blot analysis showed that the transcripts from *Asn1* started to increase at 0.6 d after the onset of dark treatment and continued to increase, whereas the transcripts from *Gln1;1* increased at 5 d after the onset of dark treatment (Figure III-2). The kinetics of transcript accumulation for *Asn1* and *Gln1;1* was identical to that of increases in Asn and Gln in the cotyledons and in the phloem exudates, respectively, suggesting that, in radish plants incubated in the dark for prolonged periods, AS and GS1 play major roles in the syntheses of Asn and Gln, respectively.

Dark treatment totally limits the supply of carbohydrate because no photosynthesis is expected under this condition. As shown in Figure III-3B, carbohydrates were rapidly exhausted within a day in the dark. When carbohydrates were depleted, degradation of protein occurred subsequently in the dark. The resultant free amino acids are thought to be subjected to deamination, leaving toxic ammonia in the cell, whereas the carbon skeletons of the amino acids are catabolized and finally enter

the TCA cycle for respiration. In accordance with the latter view, Fujiki *et al.* (2000) reported that the expression of genes for branched chain α -keto acid dehydrogenase (BCKDH) complex was induced by dark treatment in *Arabidopsis thaliana*, which encoded subunits of BCKDH complex, a key enzyme in the catabolism of branched-chain amino acids. To avoid the accumulation of toxic ammonia, cells would first reassimilate ammonia into Gln by GS1, which in turn would be transferred to Asp by AS to form Asn which has a higher molar ratio of nitrogen to carbon than Gln. Taken together, AS in the dark can be regarded as being responsible for detoxication of ammonia.

In bacteria, yeast and animal cells Asn is thought to be used only for protein synthesis. The content of free Asn is indeed one of the lowest among free amino acids in those organisms (Tallan *et al.* 1954; Ramos and Wiame 1980). The accumulation of Asn is, to my best knowledge, a phenomenon observed only in plant cells. These differences between plants and other organisms might arise from that in the processes of ammonia detoxication. In animals, ammonia is detoxified by the ornithine cycle in liver and the resultant urea is excreted as a waste from the bodies. On the other hand, nitrogen is limited in plant bodies and thus ammonia released under carbon limited conditions would be detoxified and stored as Asn in plants. Asn is known as one of non-toxic inert compounds (Sieciechowicz *et al.* 1988a).

Asn accumulated in the cotyledons during dark treatment rapidly declined when plants were re-illuminated (Figure III-5D). Sieciechowicz *et al.* (1985, 1988b) have observed a diurnal variation in the asparaginase activity from pea leaves, *i.e.*, it increased in the light and decreased in the dark. These observations suggested that asparaginase might play a major role in degradation of Asn to Asp and ammonia, when the dark-adapted

plants are re-illuminated. When calculated on a basis of the nitrogen equivalent, decreased Asn could account for about 30% of proteins increased during re-illumination of the 5-d dark-treated radish plants (Figure III-5C, D), indicating that ammonia released from Asn by asparaginase may well be reassimilated for the synthesis of nitrogen compounds such as proteins. It has recently been reported that a promoter of a gene for *Arabidopsis* aspartate kinase-homoserine dehydrogenase (AK/HSD) was activated by light in transgenic tobacco (Zhu-Shimoni *et al.* 1997, 1998). Because AK/HSD is a bifunctional key enzyme in the Asp-family pathway through which Lys, Thr, Met and Ile are synthesized from Asp. There is the possibility that Asp produced from Asn in re-illuminated plants could be used as a substrate for syntheses of Asp-family amino acids. Asn might serve as nitrogen donor in re-illuminated plants as well as one of the major storage compound of nitrogen in the dark. Consequently, AS in plant is probably related to transient storage of nitrogen as well as detoxication of ammonia through the synthesis of non-toxic inert compound Asn. This physiological role of AS is endemic to the plant kingdom.

Chapter IV

The Gene for Asparagine Synthetase Can Also Be Expressed under Light of Low Intensities

Introduction

Expression of genes for AS has been examined in many plant species since a cDNA clone for AS was first isolated from pea in 1990 (Tsai and Coruzzi 1990). The accumulation of transcripts from AS genes is induced by dark treatment in the leaves of pea (Tsai and Coruzzi 1990), tobacco (Tsai and Coruzzi 1991), *Arabidopsis* (Lam *et al.* 1994), alfalfa (Shi *et al.* 1997) and soybean (Hughes *et al.* 1997). In *Arabidopsis*, the induction is repressed when dark-treated leaves were incubated with sucrose (Lam *et al.* 1994). In accordance with this observation, Chevalier *et al.* (1996) have isolated an AS gene from maize as one of genes induced by sugar-starvation and reported that expression of the AS gene in this plant was also repressed by sugar feeding. Thus, it is proposed that induction of AS gene expression is influenced by intracellular carbon status (Lam *et al.* 1994; Chevalier *et al.* 1996). On the other hand, sucrose repression of AS gene expression was weakened by addition of nitrogen in the form of amino acids to the incubation medium of *Arabidopsis* leaves (Lam *et al.* 1994) and maize root tips (Chevalier *et al.* 1996). From these observations, it has been suggested that alteration in the carbon to nitrogen ratio regulates the accumulation level of transcripts from AS genes (Lam *et al.* 1994; Chevalier *et al.* 1996). Therefore, it is tempting to hypothesize that the expression of radish *Asn1* in dark-treated cotyledons is regulated by sugar and nitrogen levels.

I demonstrated in the former chapter that the expression of *Asn1* was induced in radish cotyledons by a prolonged dark treatment. In addition, although the expression of AS genes has been investigated in various plant species under various conditions, most of which were rather artificial and stressful. The expression of AS genes has been observed in harvested spears of asparagus (King and Davies 1992), sugar-starved maize root tips (Chevalier *et al.* 1996), shoots of etiolated soybean (Yamagata *et al.* 1998) and in leaves of dark-adapted tobacco (Tsai and Coruzzi 1991), *Arabidopsis* (Lam *et al.* 1994), alfalfa (Shi *et al.* 1997) and soybean plants (Hughes *et al.* 1997). In some leguminous plants, the expression of AS genes has been exceptionally detected under natural conditions: Dark-induced expression of the AS gene was observed in pea leaves even in a normal day/night growth condition (Tsai and Coruzzi 1991); and the level of transcripts from AS genes increased in developing nodules in alfalfa (Shi *et al.* 1997) and *Elaeagnus umbellata* (Kim *et al.* 1999). However, neither induction nor repression of AS gene expression has ever been observed in nonleguminous plants growing under natural conditions. It has neither been shown that *Asn1* is expressed under naturally occurring conditions in radish.

In this chapter, I am going to address a question of whether expression of *Asn1* could occur under naturally occurring conditions in radish cotyledons, which is essential to understand the physiological role of AS in nature. To answer this question, I focus my study on the effects of sugar and nitrogen compounds on the accumulation level of transcripts from *Asn1*, and try to speculate if in naturally occurring conditions physiological concentrations of these metabolic compounds become what are required for expression of *Asn1*.

Materials and Methods

Plant Material

Radish (*Raphanus sativus* L. cv. Comet) seeds were sown on a vermiculite bed in a plastic tray (25 cm X 35 cm) and grown in a growth chamber regulated at 25°C under a relative humidity of 60% with a 16-h daylength at a photon flux density of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were watered daily and were supplied with Hoagland's solution (Arnon and Hoagland 1940) on the 4th day after sowing. Cotyledons were harvested at the end of the light period.

Extraction and Analysis of RNA

Extraction of total RNA and RNA gel blot analysis were carried out as described in Chapter II.

Measurement of Chlorophyll Content

Chlorophyll contents were measured as described in Chapter II.

Protein Assay

Protein contents in radish cotyledons were determined as described in Chapter III.

Starch and sucrose Assay

Starch and sucrose contents in cotyledons were quantitated as described in Chapter III.

Results

Effect of DCMU and exogenously supplied sucrose on the accumulation level of transcripts from Asn1 in the cotyledons

I have shown that the accumulation of transcripts from *Asn1* in cotyledons of dark-treated radish plants was canceled by re-illumination of the plants. To determine whether this cancellation was one of direct consequences of photosynthesis, 7-d-old plants were incubated for 24 h in the light with or without 10 μ M DCMU, an inhibitor of photosynthesis. As shown in Figure IV-1A, the accumulation level of the transcripts increased when the cotyledons were incubated with DCMU in the light.

To investigate whether a product of photosynthetic carbon assimilation, sucrose, inhibits the accumulation of transcripts from *Asn1*, detached cotyledons were incubated for 24 h in the dark with or without 100 mM sucrose, and RNA was isolated and subjected to RNA gel blot analysis. As shown in Figure IV-1A, the accumulation of the transcripts in the dark was abolished by the addition of sucrose. Mannitol had no such effect, indicating that the effect of sucrose was not due to an osmotic effect. In addition, sucrose also canceled the accumulation of the transcripts from *Asn1* in the DCMU-treated cotyledons (Figure IV-1A). In a control experiment, the level of transcripts from *Gln1;1*, which is regulated by a Gln/Glu ratio (Watanabe *et al.* 1997), was also monitored. *Gln1;1* showed a different pattern of expression from *Asn1*. The level of transcripts from *Gln1;1* was neither increased by DCMU treatment nor decreased by sucrose feeding (Figure IV-1A).

The effect of sucrose on the accumulation of transcripts from *Asn1* was further investigated. Detached cotyledons were incubated for 24 h in the dark at various concentrations of sucrose, and RNA was extracted and

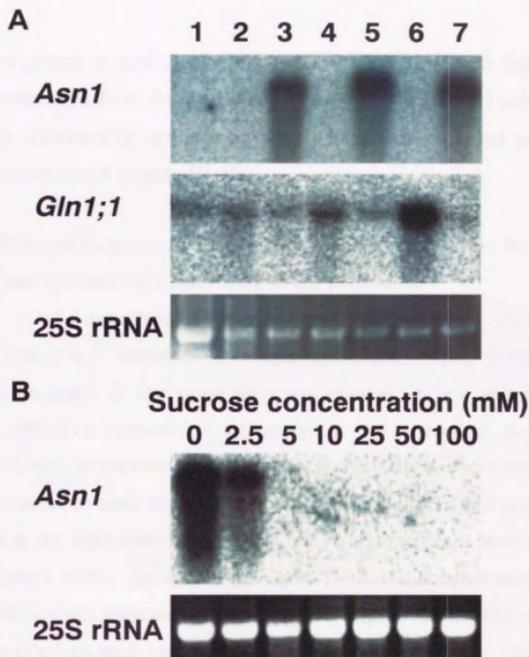


Figure IV-1A, B. A: Effect of DCMU and exogenously supplied sucrose on the accumulation level of transcripts from *Asn1* and *Gln1;1* in cotyledons. Detached cotyledons from 7-d-old radish plants were incubated for 24 h with or without 10 μ M DCMU and/or 100 mM sucrose. RNA was extracted from the cotyledons and subjected to RNA gel blot analysis. Each lane contained 10 μ g of RNA and blotted membrane was hybridized with a probe prepared from a cDNA clone for either *Asn1* or *Gln1;1*. Lane 1, 7-d-old plants before treatment; lane 2, in the light; lane 3, in the light with 10 μ M DCMU; lane 4, in the light with 10 μ M DCMU and 100 mM sucrose; lane 5, in the dark; lane 6, in the dark with 100 mM sucrose; lane 7, in the dark with 100 mM mannitol. Ethidium bromide-stained 25S rRNA bands are shown at the bottom.

B: Effect of sucrose at various concentrations on the accumulation level of transcripts from *Asn1* in cotyledons. Detached cotyledons were incubated for 24 h in the dark with various concentrations of sucrose (0, 2.5, 5, 10, 25, 50, 100 mM). RNA was extracted from the cotyledons and subjected to RNA gel blot analysis. Each lane contained 10 μ g of RNA and blotted membrane was hybridized with a probe prepared from a cDNA clone for *Asn1*. Ethidium bromide-stained 25S rRNA bands are shown at the bottom.

subjected to RNA gel blot analysis. It was shown that the level of transcripts from *Asn1* declined to 40% by feeding 2.5 mM sucrose, and to undetectable levels when cotyledons were supplied with sucrose of above 5 mM (Figure IV-1B).

Effect of exogenously supplied nitrogen compounds on the accumulation level of transcripts from Asn1 in the cotyledons

As described in Chapter II, the accumulation level of transcripts from *Asn1* increases in young cotyledons when subjected to dark treatment. At the same time, the content of free amino acids in the cotyledons increases significantly. It was, therefore, possible that the cellular concentration of free amino acids affects the expression of *Asn1*. In order to confirm this possibility I incubated detached cotyledons for 24 h in the light with or without 60 mM nitrate, 60 mM ammonia or 60 mM amino acids. As shown in Figure IV-2, the accumulation level of the transcripts increased to a detectable level under illumination when cotyledons were incubated with nitrate, ammonia, Gln, Glu, Asn or Asp. However, the increased level of transcripts from *Asn1* was not as high as that caused by dark treatment (Figure IV-2).

Time course of accumulation of transcripts from Asn1 in the dark

I examined the time course of the accumulation of transcripts from *Asn1* to find whether the induction could occur in a natural day/night cycle. Radish plants were grown for 7 d under a normal day/night cycle of 16 h light and 8 h dark. Then, a half portion of plants were grown under the same day/night cycle, whereas the remaining half were transferred to the dark for an extended period. As shown in Figure IV-3A, the accumulation of transcripts from *Asn1* was not observed under

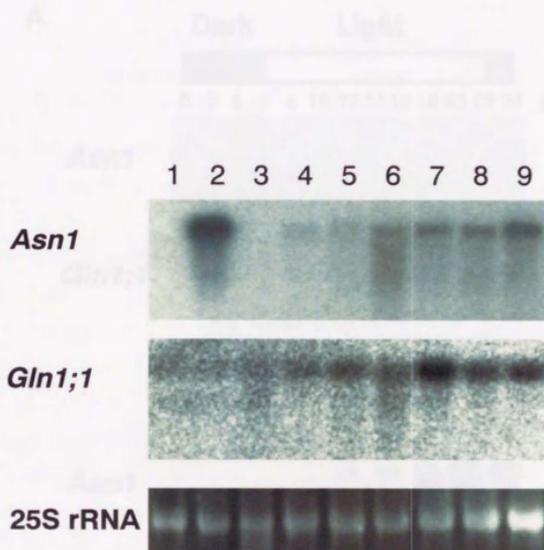


Figure IV-2. Effect of exogenously supplied nitrogen compounds on the accumulation level of transcripts from *Asn1* and *Gln1;1* in cotyledons. Detached cotyledons from 7-d-old radish plants were incubated for 24 h with or without nitrogen compounds. RNA was then extracted from the cotyledons and subjected to RNA gel blot analysis. Lane 1, 7-d-old plants before treatment; lane 2, in the dark; lane 3, in the light; lane 4, in the light with 60 mM KNO_3 ; lane 5, in the light with 60 mM NH_4Cl ; lane 6, in the light with 60 mM Gln; lane 7, in the light with 60 mM Asp; lane 8, in the light with 60 mM Glu; lane 9, in the light with 60 mM Asn. Each lane contained 10 μg of RNA and blotted membrane was hybridized with a probe prepared from a cDNA clone for either *Asn1* or *Gln1;1*. Ethidium bromide-stained 25S rRNA bands are shown at the bottom.

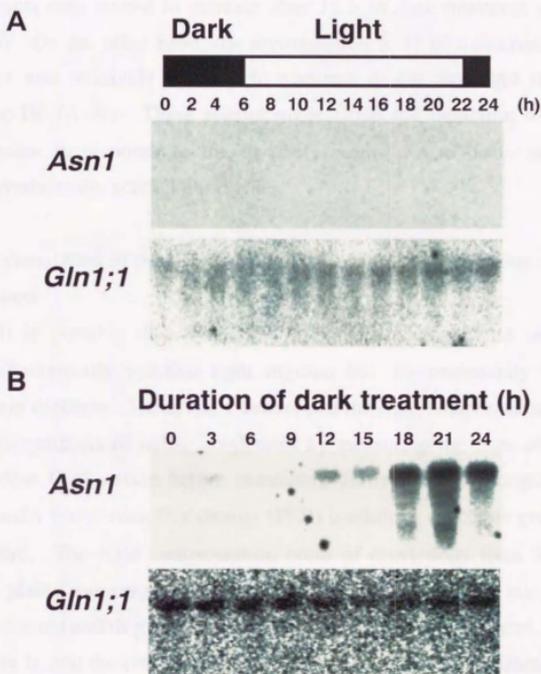


Figure IV-3A, B. RNA gel blot analysis of transcripts from *Asn1* and *Gln1;1* in radish cotyledons during short-term dark treatment. Plants were grown under a normal day/night cycle (16 h light and 8 h dark). Seven d after sowing, a group of plants were kept in the normal day/night cycle (A), and others were kept in the dark for 24 h (B). Cotyledons were collected at every 2 h (A) or 3 h (B), respectively. RNA was extracted from the cotyledons and subjected to RNA gel blot analysis. Each lane contained 10 μ g of RNA and blotted membrane was hybridized with a probe prepared from a cDNA clone for either *Asn1* or *Gln1;1*.

the normal day/night cycle condition, whereas the accumulation of the transcripts only started to increase after 12 h of dark treatment (Figure IV-3B). On the other hand, the accumulation level of transcripts from *Gln1;1* was relatively constant in response to the day/night regimes (Figure IV-3A, B). These results suggest that the induction of *Asn1* expression in response to the darkness seems not to occur under a photosynthetically active light regime.

The accumulation of transcripts from Asn1 under low photon flux density conditions

It is possible that transcripts from *Asn1* accumulate under a photosynthetically inactive light regime, but not necessarily in the complete darkness. Therefore, I determined the light compensation point of photosynthesis of radish cotyledons by measuring the rates of light-dependent CO₂ uptake before examining the level of transcripts from *Asn1* under low photon flux density (PFD) conditions which are prevalent in nature. The light compensation point of cotyledons from 7-d-old radish plants was ranged between 12 - 18 $\mu\text{mol m}^{-2} \text{s}^{-1}$ by my hands. Then, 7-d-old radish plants were grown at a PFD of 5 or 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 24 h, and the cotyledons were harvested. RNA was extracted and subjected to RNA gel blot analysis to examine the accumulation level of transcripts from *Asn1*. The level of the transcripts increased to a detectable level when plants were grown at a PFD of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure IV-4). The level of transcripts from *Asn1* was further increased by 5-fold when plants were grown at a PFD of 5 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure IV-4). These results suggest that expression of *Asn1* was induced when the light condition was unfavorable for photosynthesis.



Figure IV-4. RNA gel blot analysis of transcripts from *Asn1* in radish cotyledons under low PFD conditions. Seven-d-old plants were grown at a PFD of 100 (1), 10 (2) or 5 (3) $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 24 h. RNA was extracted from cotyledons and subjected to RNA gel blot analysis. Each lane contained 20 μg of RNA and blotted membrane was hybridized with a probe prepared from a cDNA clone for *Asn1*. The top panel shows the RNA gel blot analysis. Ethidium bromide-stained 25S rRNA bands are shown in the middle. In the bottom panel, quantification of hybridizing RNA is shown as percentage of the maximum value determined by radioluminography using BAS 2000 (Fuji Photofilm).

Changes in the levels of chlorophyll, protein, starch and sucrose under low photon flux density conditions

In this chapter, I have obtained results suggesting that the carbon to nitrogen balance may play a key role in regulation of AS gene expression. Therefore, I examined whether low PFD conditions would accompany the changes in the carbon to nitrogen balance in radish plants. Seven-d-old radish plants were grown under low PFD conditions for 24h and the contents of chlorophyll, protein and carbohydrates were determined in radish cotyledons. The level of chlorophyll in cotyledons was significantly unaffected under low PFD conditions examined (Figure IV-5A). The content of protein in cotyledons decreased with decreasing PFD: The content of protein in plants grown at a PFD of $5 \mu\text{mol m}^{-2} \text{s}^{-1}$ was about 20% lower than that in the plants grown at a PFD of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure IV-5B). Highest decrease rates were observed in carbohydrates: The contents of starch and sucrose decreased dramatically to about 10% under a PFD of $10 \mu\text{mol m}^{-2} \text{s}^{-1}$, and further decrease was observed under a PFD of $5 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure IV-5C, D).

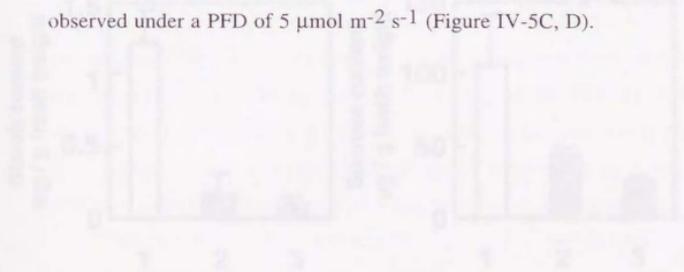


Figure IV-5A, B, C, D. The contents of chlorophyll (A), protein (B) and starch (C) in the cotyledons under low PFD conditions. Seven-d-old plants were grown at a PFD of 100 (1), 10 (2) or 5 ($\mu\text{mol m}^{-2} \text{s}^{-1}$) for 24 h. Data are means of three independent replicates. Vertical bars represent standard deviations.

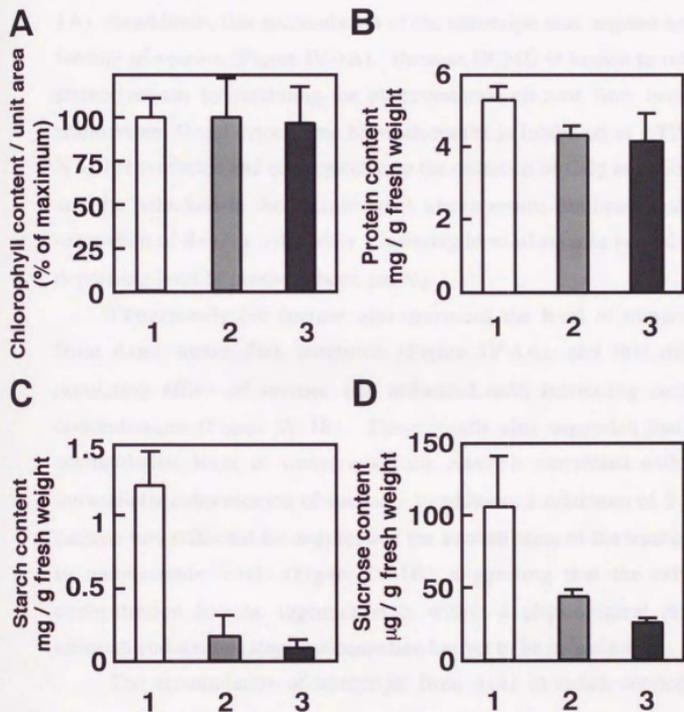


Figure IV-5A, B, C, D. The contents of chlorophyll (A), protein (B), starch (C) and sucrose (D) in the cotyledons under low PFD conditions. Seven-day-old plants were grown at a PFD of 100 (1), 10 (2) or 5 (3) $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 24 h. Data are means of results from triplicates. Vertical bars represent standard deviations.

Discussion

I have found that transcripts from *Asn1* accumulated in the cotyledons when DCMU was applied under light condition (Figure IV-1A). In addition, this accumulation of the transcripts was negated by the feeding of sucrose (Figure IV-1A). Because DCMU is known to inhibit photosynthesis by inhibiting the photosynthetic electron flow between photosystem II and cytochrome b, which results in inhibition of ATP and NADPH syntheses and consequently, in the cessation of CO₂ assimilation and the reduction in the sucrose level, above results implicate that the expression of *Asn1* is induced by a lowering level of sucrose caused by a depressing level of photosynthetic activity.

Exogenously fed sucrose also decreased the level of transcripts from *Asn1* under dark condition (Figure IV-1A), and this down-regulating effect of sucrose was enhanced with increasing sucrose concentrations (Figure IV-1B). These results also suggested that the accumulation level of transcripts from *Asn1* is correlated with the intracellular concentration of sucrose. In addition, a minimum of 5 mM sucrose was sufficient for suppressing the accumulation of the transcripts to undetectable levels (Figure IV-1B), suggesting that the critical concentration for the suppression is within a physiological range, although endogenous sugar concentration has yet to be investigated.

The accumulation of transcripts from *Asn1* in radish cotyledons increased to detectable levels by supplementing with nitrate, ammonia, Gln, Glu, Asn or Asp in the light (Figure IV-2), although the increased level of the transcripts was much lower than that caused by dark treatment. These results indicated that the expression of *Asn1* in radish is regulated by the level of nitrogen as well. Similar effects of these

nitrogen compounds have been reported for *Arabidopsis ASN1* and maize AS gene (Lam *et al.* 1994; Chevalier *et al.* 1996). In Chapter II, however, I described that the accumulation of transcripts from AS gene by dark treatment was not observed in senescing cotyledons, indicating a possibility that the expression of *Asn1* might be under developmental control as well as metabolic control. These possibilities, however, need to be examined by further experiments.

The feeding of Asn weakly enhanced the expression of *Asn1* (Figure IV-2). On the other hand, expression of *asnB* in *E. coli*, which encodes Gln-dependent AS, is inhibited in the presence of Asn (Scofield *et al.* 1990). In mammalian cells, the expression of AS gene was caused by starvation for not only Asn but also several other amino acids (Gong *et al.* 1991). Similarly, yeast AS genes were found to be more strongly expressed in the presence of the general control factor Gcn4p, which is responsible for the general response to amino acid starvation (Dang *et al.* 1996). These differences in regulation of AS gene expression between plants and other organisms suggest that physiological roles of AS are different among those organisms. I proposed in Chapter III that AS in plants may be related to the storage of nitrogen as well as detoxication of ammonia, whereas AS in *E. coli*, mammal and yeast could only serve as the maintenance of Asn levels in response to Asn deficiency. These differences might result from differences in the amount of nitrogen availability between plants and other organisms, *i.e.*, plants have a limited source of nitrogen in comparison with animals. AS in plants may contribute to the synthesis of Asn for short-term storage of precious nitrogen which is extravagantly produced by whatever the cause is.

Tsai and Coruzzi (1991) reported that dark-induced expression of *AS1*, which encodes pea AS, was detected in leaves even in a normal

day/night (16 h light/ 8 h dark) growth condition. On the other hand, the level of transcripts from radish *Asn1* did not increase in such a time frame (Figure IV-3A), suggesting that *Asn1* would not function during the night period in the normal day/night growth condition. However, I suspect from the results obtained in this chapter that expression of *Asn1* is induced under conditions limiting photosynthesis, *e.g.*, under the light of low PFD. In such conditions, the content of carbohydrates in plants decreases rapidly, then protein is degraded to amino acids which serve as substrates for respiration with concomitant release of toxic ammonia. As a result, the intracellular carbon to nitrogen ratio in the plants decreases. As expected, when radish plants were placed under the light below the light compensation point ($12 - 18 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 1 d, the contents of starch, sucrose and protein in the cotyledons decreased and the level of transcripts from *Asn1* increased (Figure IV-4, 5). To ascertain if there is anywhere such conditions occur in nature, I measured a PFD under various meteorologic conditions and found that on rainy days or in the shade of a tree on a cloudy day a PFD in the daytime actually become lower than light compensation points of radish cotyledons, *i.e.*, about $5 - 10 \mu\text{mol m}^{-2} \text{s}^{-1}$. These results suggested that the PFD of natural environment can switch the expression of *Asn1*. In addition to low PFD conditions, significant decreases in photosynthesis were observed in several conditions, such as shading, lower temperature and salt stress, and these conditions also lead to degradation of proteins (Kang and Titus 1989; Vierstra 1993; Hikosaka and Terashima 1996; Sonoike 1998). Taken together, radish *Asn1* is likely to function in nature for short-storage of excess nitrogen under various photosynthetically unfavorable conditions.

Chapter V

General Discussion

In this study, I have described my study on the roles of AS and GS1 in nitrogen utilization. I have found that AS and GS1 play differential roles in nitrogen utilization in radish cotyledons, both of which are associated with effective utilization of nitrogen in plants. Analysis of expression of genes for AS and GS1 revealed that the expression of these genes was affected by an intracellular carbon to nitrogen balance.

I first examined whether Gln and Asn are really major compounds for nitrogen translocation from senescing cotyledons. The results described in Chapter II suggested that Gln is involved in the translocation of nitrogen from senescing cotyledons and that Asn is stored in dark-treated cotyledons, and also that the syntheses of Asn and Gln are dependent on the expression of genes for AS and GS1, respectively. I also described in Chapter II that expression of genes for AS and GS1 during dark treatment changes according to the growth stage in radish cotyledons. The changes might be related to the conversion of cotyledons from a sink to a source organ in respect to nitrogen utilization.

The findings described in Chapter III are that the amount of free Asn in cotyledons increased during dark treatment and that the Asn accumulated in the dark was rapidly decreased when radish plants were re-illuminated. These findings suggested that the large amount of nitrogen released by degradation of cellular protein in the dark could be retrieved as Asn, and the Asn might be one of the major storage compounds of nitrogen under dark conditions. Although further work is needed to confirm the hypothesis for physiological role of AS, these

results suggest that Asn synthesized in the dark is very likely to relate to transient storage of nitrogen and detoxication of ammonia.

In Chapter IV, I described the effects of sugar and nitrogen compounds on the expression of *Asn1*, to understand how *Asn1* expression is regulated. The accumulation of transcripts from *Asn1* in the dark is canceled by exogenous sucrose and the feeding of exogenously supplied nitrogen compounds increased the level of the transcripts in the light. From these observations I suggest that the expression of *Asn1* is regulated in response to the intracellular balance between carbon to nitrogen. Moreover, the accumulation of transcripts from *Asn1* in the cotyledons was observed when radish plants placed under conditions of light intensity lower than the light compensation point, suggesting that AS in plants may function under low PFD conditions such as rainy days or the shade of a tree on a cloudy day in nature.

From these observations, I would like to propose following hypotheses about when and how AS and GS1 function for nitrogen utilization in nature.

Leaf senescence

During both natural and artificial leaf senescence, GS1 play a major role in the synthesis of Gln in companion cells of phloem and Gln is translocated from the senescing leaf to developing and/or storage organs. On the other hand, although Asn seems not to be a major translocation compound of nitrogen at least in radish plants in natural senescence, Asn seems to be translocated from cotyledons during dark-induced senescence. In addition, the level of transcripts from *din6*, which encodes AS, slightly increased in senescing *Arabidopsis* leaves (Nozawa *et al.* unpublished). However, the increased levels of the transcripts during senescence were

much lower than that caused by dark treatment. In addition, expression of GS1 genes, *atgsr1* and *atgsr2*, were induced extensively in those leaves (Nozawa *et al.* unpublished). Taken together, I propose that Asn might also be used as translocation compounds of nitrogen, but it may be of auxiliary importance.

Deterioration of weather

When light intensity is lower than light compensation point for a plant, as is anticipated on a rainy day or under a shade of a tree on a cloudy day, plants would be brought to starvation by limited photosynthesis. Under such conditions, plants would have rapidly consumed carbohydrates and start using proteins as substrates for respiration. At this time, leaves seem to respond to those conditions with age-dependent manner. In young leaves, Asn is synthesized by AS as a transient storage compound of nitrogen. I suggest that transient storage of nitrogen in the form of Asn is related to survival of the young leaves from the unfavorable light condition. This would be reasonable because photosynthetic capability of the young leaves is high. The stored Asn is used as a nitrogen source for protein synthesis and consequently could contribute rapid recovery of photosynthetic activity when light conditions returned favorable. In other words, AS is involved in intracellular recycling of nitrogen. On the other hand, old leaves in which photosynthetic activity has already decreased may not give benefits corresponding with costs to the whole plants, and senescence would progress in the old leaves and nitrogen translocation could have been accelerated through promotion of Gln synthesis by GS1 activity. Hence, GS1 would be related to interorgan recycling of nitrogen.

I found several novel properties of AS and GS1 in this study. The results obtained in this study suggest that both AS and GS1 are likely to be related to recycling of nitrogen in plant bodies, although physiological roles of them seem to be different. However, further studies are needed to confirm the hypothesis described above. To date, no AS and GS1 mutants have been isolated, possibly because there are more than one gene encoding AS and GS1. However, those mutants could be isolated from transposon or T-DNA tag lines recently introduced. Through analysis of those mutants, understandings of physiological importances of AS and GS1 will further progress. The localization of an enzyme often gives an important clue for understanding the physiological role of the enzyme. Immunocytochemical analysis also might be needed to evaluate physiological roles of those enzymes. I speculate from the results in this research that plants in nature might adapt flexibly to changes in growth environment by changing the pattern of metabolism through gene expression. I anticipate that further studies on AS and GS1 will provide significant informations on environmental adaptation of plants at molecular level as well as physiological roles of the enzymes.

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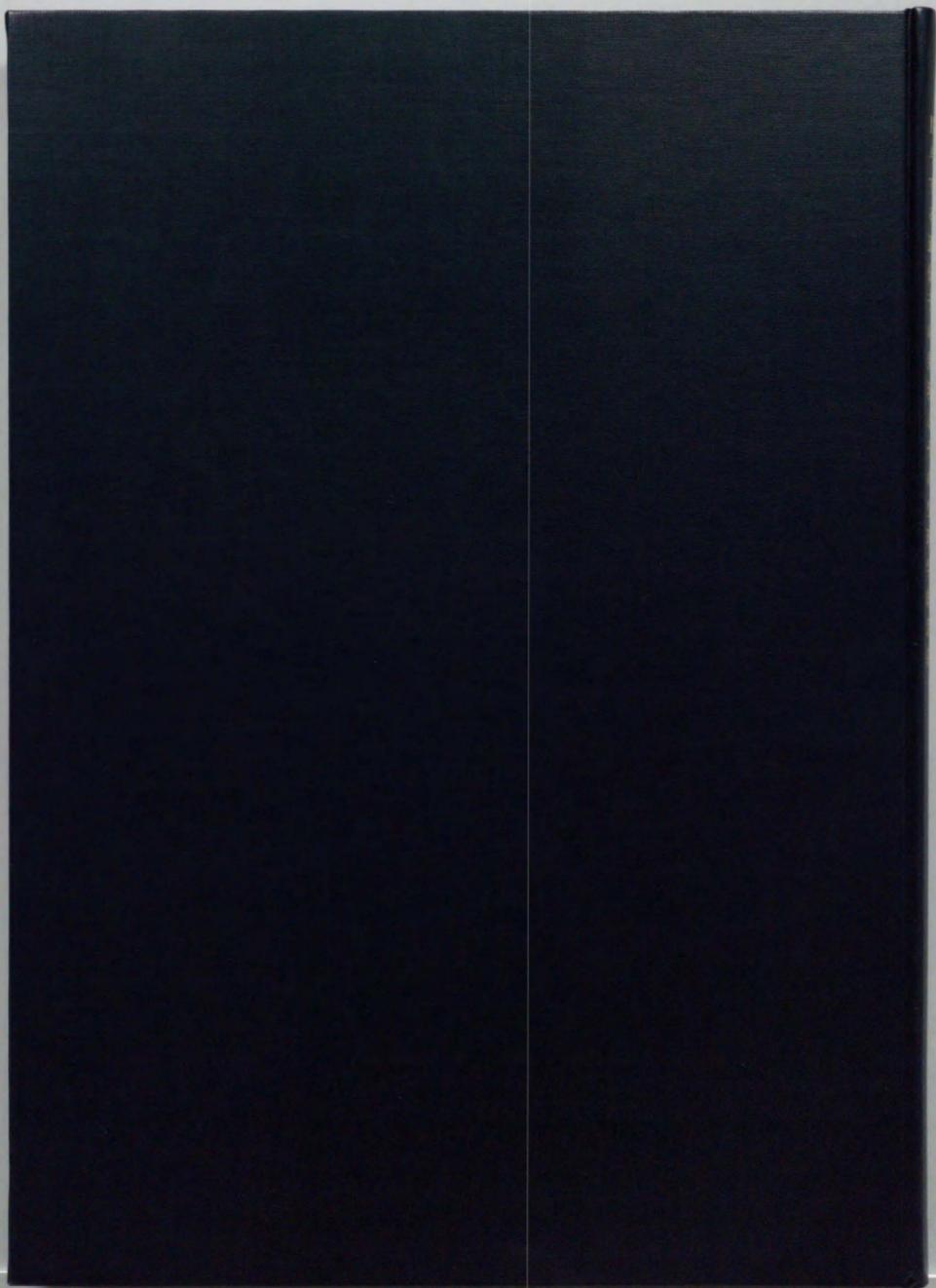
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