Organ and Cellular Localization of Asparagine Synthetase in Rice Plants

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DNA gel blot analysis suggested that asparagine synthetase (AS; EC 6.3.5.4) occurred as a single gene in rice. A fusion protein consisting of 17 kDa tagged-region from pET32a(+) expression plasmid and 42 kDa N-terminal region of rice AS was first expressed in Escherichia coli. The resulting polypeptide was purified and a mono-specific antibody for rice AS was prepared after affinity-purification with the antigen. Immunoblotting revealed a high content of AS protein in the leaf sheath at the second position from the fully expanded top leaf and in grains at the middle stage of ripening. Accumulation of mRNA for AS was also observed in these organs. During the ripening of the spikelets, the AS protein contents increased during the first 21 days after flowering, then declined rapidly. Immunolocalization analysis revealed signals for AS protein in the companion cells of vascular bundles of leaf sheath and phloem-parenchyma cells, nucellar projection, and nucellar epidermis of dorsal vascular bundles of grains.

Key words: Asparagine synthetase (EC 6.3.5.4) — Cellular localization — Nitrogen metabolism — Protein distribution — Rice (Oryza sativa L.)

Introduction

The major source of nitrogen for developing leaves and ears in rice plants is the nitrogen remobilized from senescing leaves (Mae and Ohira 1981). Glutamine and asparagine are major forms of nitrogen in phloem sap of rice plants (Hayashi and Chino 1990). We have pointed out that cytosolic glutamine synthetase (GS1; EC 6.3.1.2) is important in the synthesis of glutamine for the export from senescing leaves, whereas NADH-glutamate synthase (NADH-GOGAT; EC 1.4.1.14) is important in the re-utilization of the transported glutamine in developing organs (Yamaya et al. 1999). These indications are based on the results from localization studies of enzyme proteins (Hayakawa et al. 1994, Sakurai et al. 1996, Ishiyama et al. 1998), analyses of mRNA accumulation (Yamaya et al. 1995, Hirose et al. 1997), and transgenic studies expressing either antisense RNA (Yamaya et al. 1999, Ogasawara et al. unpublished results) or a reporter gene under control of NADH-GOGATpromoter (Goto et al. 1998, Kojima et al. 2000). Compared to the recent progress on glutamine metabolism, much less is known on asparagine metabolism in rice plants.

Asparagine is probably synthesized by the reaction of glutamine-dependent and/or ammonium-dependent asparagine synthetase (AS; EC 6.3.5.4) in plants (Lea et al. 1990, Sechley et al. 1992, Ireland and Lea 1999). Molecular approaches have been employed to study the structure and gene expression for AS in various plants, such as pea (Tsai and Coruzzi 1991), asparagus (Davis and King 1993), Arabidopsis (Lam et al. 1994), broccoli (Downs et al. 1995), corn (Chevalier et al. 1996), rice (Watanabe et al. 1996), Lotus japonicus (Waterhouse et al. 1996), alfalfa (Shi et al. 1997), soybean (Hughes et al. 1997), and radish (Nozawa et al. 1999). Because the purF-type amidotransfer domain was found in the deduced amino acid sequences from those cDNAs, AS could be a glutamine-dependent enzyme. Some of those plants, mainly dicot plants such as Arabidopsis (Lam et al. 1998), Lotus japonicus (Waterhouse et al. 1996), pea (Tsai and Coruzzi 1991), and soybean (Hughes et al. 1997) contain two or three genes distinct for AS. Light has been shown to repress the expression of the AS gene in pea, alfalfa, and Arabidopsis. This light repression is probably involved via phytochrome (Lam et al. 1994). In young cotyledons of radish plants, dark treatment stimulated the expression of AS gene (Nozawa et al. 1999). Supply of sugar represses the accumulation of mRNA for ASV1 (Lam et al. 1994) in intact Arabidopsis plants and in excised root tips of corn (Chevalier et al. 1996). In Lotus japonicus, on the other hand, light was not found to have an appreciable effect on AS gene expression (Waterhouse et al. 1996). Recently, Lam et al. (1998) revealed a reciprocal regulation of AS genes by light and metabolites, such as sugars and amino acids.

Compared to the molecular studies, immunochemical and standard biochemical approaches for the biosynthesis of asparagine have not been extensively studied, because of the instability of the AS, the presence of AS inhibitor in plant extracts, and so on (Lam et al. 1994). Here, we examined the organ and cellular localization of AS protein in rice plants. We show that AS is located in vascular tissues of leaf sheath and grains at the middle stage of ripening. The possible role AS plays in nitrogen metabolism is discussed.
Materials and Methods

Plant materials

Rice (Oryza sativa L. cv. Sasanishiki) plants were grown in hydroponic culture in a greenhouse until either the 12.5 leaf stage on the main stem or maturity of all spikelets, as described previously (Kamachi et al. 1991, Hayakawa et al. 1993). We harvested the leaves from 10 plants at the 12.5 leaf stage, at which leaf blades 8 (the lowest position) to 12 on the main stem were fully expanded, between 8:00 and 10:00 am and separated them into leaf blades and leaf sheaths. The 13th leaf was cut into three segments, i.e. expanding green blade outside of the 12th leaf sheath, non-green leaf blade inside of the 12th sheath, and non-green leaf sheath. Non-green 14th leaf inside of the 13th sheath was also harvested without separating the blade from the sheath, as described previously (Yamaya et al. 1992). The apical spikelets on the primary branches at the position from the first (top) to the fifth were harvested from the main stem during the ripening period from 0 to 35 d after flowering (DAF), as described previously (Hayakawa et al. 1993). The samples were weighed, frozen in liquid nitrogen, and stored at −80°C until they were extracted. Approximately 1-mm-thick cross-sections of various organs were freshly prepared and fixed with paraformaldehyde solution in sodium cacodylate buffer, as described previously (Hayakawa et al. 1994). These fixed sections were used for immunocytological experiments.

Preparation of extract

The frozen samples (0.60–2.54 g FW) were first ground to a fine powder in a mortar with a pestle in the presence of washed quartz sand [0.3 g (g FW)−1] with liquid nitrogen. The powder was homogenized in 100 mM MES-NaOH buffer, pH 6.8, [3 ml (g FW)−1] that contained 0.2% (v/v) 2-mercaptoethanol, 30 mM Asp, 10 mM MgCl2, 2 mM phenylmethylsulfonyl fluoride, 200 μM antipain, 200 μM leupeptin and 2 mM ethene glycol·(β-amoethanol ether) N,N,N’,N’-tetraacetic acid, according to the Egli et al. (1989) after minor modification. The homogenate was filtered through four layers of gauze and the filtrate was centrifuged at 27,000 × g for 20 min at 4°C. The supernatant fraction was used for immunoblotting analysis and quantitation of soluble protein. Total RNA was extracted from various organs of rice plants as described previously (Hirose et al. 1997).

Construction of an expression plasmid

An expression plasmid was constructed using pET32a(+) (Novagen, Madison, WI, U.S.A.) and AS cDNA (e635: GenBank/EMBL/DDJ, accession No. D83378) from rice callus which was kindly provided by Rice Genome Research Program, Tsukuba, Japan. The c635 was first digested with DraIII, blunt-ended with Klenow fragment, and further digested with NotI. pET32a(+) was also digested with BamHI, blunt-ended, and digested with NotI. The resulting pET32a(+) and c635 were ligated and this construct, pETAS5′-32a(+), was used to transform E. coli BL21 (DE3). The DNA insert was confirmed to contain 53% of AS cDNA from the translational start codon by sequencing with the dideoxynucleotide chain-termination methods using a FS Ready Reaction kit (Applied Biosystems, Foster, CA, USA.). pETAS5′-32a(+) potentially expressed a fusion protein that consisted of a 17 kDa tagged-region and 42 kDa N-terminal region of rice AS in E. coli.

Bacterial expression and purification of fusion protein

The recombinant protein was purified and expressed in E. coli as recommended by the manufacturer (Novagen). The tagged-region alone with a 24-amino acid tail from the vector was also expressed in E. coli. The expressed products as an inclusion body were first solubilized and purified with an affinity column. The protein obtained from a Chelate Cellulofine (Seikagaku-kogyo, Tokyo, Japan) was further purified with a preparative SDS-PAGE (Ishida et al. 1999). The resulting main band at 59 kDa was electro-eluted and concentrated by trichloroacetic acid precipitation. It was then dissolved in phosphate–buffered saline (PBS)–8 M urea, dialyzed sequentially in PBS containing either 4 M or 2 M urea for 4 h and in PBS alone for 8 h. The dialyzed protein was dissolved in PBS containing 50% (v/v) glycerol at 1 mg protein in 1 ml and stored at −30°C. Fifty μg of the recombinant protein was digested with 1 μg of lysyl-endopeptidase (Wako Pure Chemical, Osaka, Japan) for 12 h at 37°C and the resulting peptides were separated by HPLC (LC-10AI, Shimadzu, Kyoto, Japan). Amino acid sequences of the digested peptides were determined using protein sequencer (ABI 473A, Applied Biosystems) to confirm that the recombinant protein contains the amino acid sequence for rice AS. The recombinant protein was immunoblotted using antiserum raised against synthetic peptide for rice AS, which was kindly provided by Dr. K. Sueyoshi, Niigata University.

Preparation of anti-AS-antibody

A polyclonal antibody raised against rice AS was prepared, using the recombinant protein as an antigen, essentially as described previously (Hayakawa et al. 1992). After preparation of the immunoglobulin G (IgG) fraction, the anti-AS-IgG was affinity-purified with the antigen as described previously (Yamaya et al. 1992). The affinity-purified IgG was further immuno-precipitated with the 17 kDa tagged-peptide, which was also expressed in E. coli using pET-32a(+) alone.

Immunoblotting analysis and immuno-localization

Immunoblotting was performed with the affinity-purified AS specific IgG. The immunoreacted peptides were incubated with a goat anti-rabbit IgG hors eradish peroxidase conjugate and visualized with ECL PLUS Detection Kit (Amersham Pharmacia Biotech) on a X-ray film (X-OMAT, Kodak). The intensity for AS protein was quantitated densitometrically (GS-700 Imaging Densitometer, Bio-Rad), using the expressed fusion protein as the standard for the calibration. Immunoblotting was performed at least three times with independent extracts from rice plants in triplicate. AS protein was immunolocalized essentially as described previously by Hayakawa et al. (1994).

Gel blot analyses of DNA and RNA

Extraction, digestion, separation, and blotting for DNA gel blot analysis were performed as described previously (Goto et al. 1998). RNA gel blot was also performed as described previously (Hirose et al. 1997). A digoxigenin (DIG)-labeled DNA fragment, 491 bp corresponding to +597 to +1,088 of AS cDNA (e635) was used as a probe for DNA gel blot analysis. Three DIG-labeled RNA fragments, 542 bp, 491 bp, and 219 bp corresponding to +114 to +578, +459 to +1,088, and +1,550 to +1,763 of the AS cDNA, respectively, were prepared and the mixed fragments were used as probes for RNA gel blot analysis.

Results

Expression of AS polypeptide in E. coli

To obtain a mono-specific AS antibody, a fusion gene containing 53% of AS cDNA (Watanabe et al. 1996) from the translational start codon was over-expressed in E. coli. The recombinant protein consisted of a 17 kDa tagged-region and 42 kDa N-terminal region of rice AS. Because we failed to express the full length cDNA for AS, we determined the internal amino-acid sequences of the resulting protein to confirm the pres-
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Presence of corresponding sequences for both the tagged-region protein and the rice AS polypeptide, after the digestion with lysyl-endopeptidase (results not shown). The fusion protein was used as an antigen for preparation of the antibody against AS in rice. We successfully produced a mono-specific antibody for the rice AS by affinity-purification of IgG with the antigen and also the immuno-precipitation with the tagged-region protein, as described in the Materials and Methods. One band at 66 kDa, which is in good agreement in the size of AS protein estimated from the cDNA for rice AS (Watanabe et al. 1996), was detected after SDS-PAGE of the extract from leaf sheath or spikelets followed by immunoblotting with the affinity-purified rice AS IgG. The numbers at the left or right refer to the positions of protein molecular mass in kilodaltons. AS protein in the extract was detected at 66 kDa. (C) Ten μg of crude protein from the spikelets at 21 DAF were subjected to SDS-PAGE followed by immunoblotting with the AS IgG (lane 1) or the AS IgG pretreated with an excess amount of the fusion protein (lane 2).

DNA and RNA gel blot analyses for AS

To estimate the gene copy number of AS in rice, DNA gel blot analysis of genomic DNA was performed using low stringency hybridization conditions. Rice genomic DNA digested with BamHI, EcoRI, and HindIII gave one hybridization signal (Fig. 2). The absence of a cleavage site for these restriction enzymes within the DNA probes and the corresponding region of AS structural gene suggests the presence of a single AS gene in rice plants.

Steady-state levels of mRNA for AS were compared after separation of the total RNA prepared from leaf blades and leaf sheaths at various positions and from spikelets during ripening (Fig. 3). For the analysis with leaf blades and leaf sheath, rice plants at the 12.5-leaf stage on the main stem were examined. At this stage, the 8th leaf was the lowest position on the main stem and nearly half of the 13th leaf blade had emerged. A single major band of an mRNA which corresponded to the size of AS cDNA was detected in all extracts, but the relative abundance of the signal from the 13th and the 12th leaf sheath was higher than that from other blades and sheaths. When spikelets were analyzed during ripening, accumulation of AS mRNA was observed up to 21 DAF, and the signal intensity declined thereafter. The highest signal was seen at 7 DAF.

Distribution of AS protein in leaf sheath and blade at different leaf positions

The amounts of AS protein were estimated from rice leaves by immunoblotting, using the fusion protein as the standard (Fig. 4). The highest contents of AS protein were detected in the leaf sheath of the 11th leaf. The contents gradually decreased in leaf sheaths as a function of increasing age (lower leaf position). AS protein was hardly detected in fully expanded leaf blades from the 8th to the 12th position. Detectable amounts of the AS protein were only seen in the developing 13th leaf blade. The actual content in the 11th leaf sheath was approximately 0.3 μg AS protein per g FW. This value was one to two orders of magnitude less than the contents for NADH-GOGAT and ferredoxin (Fd)-GOGAT (EC 1.4.7.1).
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proteins in leaf blades, which were mainly distributed in leaf blades of rice plants as we reported previously (Yamaya et al. 1992). Although the contents of GS1 protein in the leaf sheath were equivalent to those in relatively young leaf blades, senescing lower positions of the rice leaf blade contained much more GS1 protein (Yamaya et al. 1992). Thus, the distribution of AS protein in rice leaves was not identical to that of NADH-GOGAT, Fd-GOGAT, or GS1.

Changes in the content of AS protein in rice spikelets during the ripening process

In addition to the leaf sheath, AS protein was detectable in the extract from spikelets. Changes in the content of AS protein were determined in the primary branches at the position from the first (top) to the fifth of panicle on the main stem during the ripening process for 35 DAF (Fig. 5). The same amounts of soluble protein from the spikelets during ripening were separated with SDS-PAGE followed by immunoblotting. Faint bands were detected below the main band of AS, which were probably broken products from the mature protein. We used only the main 66 kDa band to estimate the content of AS protein in the current study. The AS content increased in the early stage of ripening, reached the maximum at 21 DAF, then declined rapidly and by 28 to 35 DAF became nearly undetectable. As shown in Fig. 3, accumulation of AS mRNA was also seen during the first 21 DAF. Storage protein is accumulated during ripening and increase in the dry weight of spikelets is nearly completed by 28 DAF as described previously (Hayakawa et al. 1993). Thus, AS protein transiently accumulated at the middle stage of ripening. This profile was different from the changes in the contents of NADH-GOGAT protein which was accumulated at the early stage of ripening (Hayakawa et al. 1993).

Distribution of AS protein in 21 DAF spikelets was investigated with mechanically separated chaff and grains. Most of the AS protein was detected in the grains (Fig. 5B).

Cellular localization of AS protein in leaf sheath and grains of rice

Ten-micrometer cross-sections of the 11th leaf sheath were prepared from paraffin-embedded tissues, and AS protein was detected immunocytoologically (Fig. 6). Signals of AS pro-
tein were clearly detected in companion cells of vascular bundles. The signals were hardly detected in other cell types of vascular bundles and mesophyll cells. With the AS IgG, signals for AS protein were detected in phloem parenchyma cells, nucellar projection, and nucellar epidermis of dorsal vascular bundles from grains (8 DAF). Signals were also detected in parenchyma cells of lateral vascular bundles (results not shown).

Discussion

This is the first paper describing organ and cellular localization of AS protein in plants. Lack of this kind of information was largely caused by the difficulty of purification of the native enzyme from plants and hence production of a mono-specific antibody for AS. AS is an unstable enzyme in plant extracts (Stulen and Oaks 1977, Streeter 1977), and there are substances inhibitory for measurement of the activity (Streeter 1977). These biochemical properties have limited the progress of study on this enzyme. In the current study, we failed to express the protein using the full length AS cDNA, which may be a result of the instability even in E. coli. We were, however, successful in expressing a fusion protein containing the 42 kDa N-terminal region of rice AS.

Immunoblotting revealed one major band at 66 kDa, which was the size estimated from the deduced amino acid sequence of rice AS cDNA (Watanabe et al. 1996) in the extracts from leaf sheath and developing grains of rice. Genomic Southern blot suggested that AS occurred as a single gene in rice. We isolated three cDNA clones for AS from a cDNA library prepared using mRNA from rice roots. These clones contain exactly the same sequences in the coding region to the AS cDNA from rice calli (Watanabe et al. 1996), but heterogeneity was found in the poly (A)+ tail region (results not shown). These results indicate that actively translated AS could be one molecular species in rice plants. This is in contrast to the presence of plural genes in some dicot plant species.

AS protein was accumulated in the leaf sheath of the fully expanded leaves, but was hardly detectable in mature leaf blades, although accumulation of AS mRNA was detectable in these organs. Thus, the distribution of AS protein in the leaves at different leaf positions was not always identical to the profile of accumulation of AS mRNA, suggesting that the steady-state levels of AS mRNA do not determine those of AS protein in rice leaves. The reason for this discrepancy remains be elucidated. The leaf sheath of rice is known to accumulate asparagine (Fukumorita and Chino 1982). AS found in leaf sheath could contribute to this accumulation. As well as glutamine, asparagine is also a major form in phloem sap, suggesting that these are major forms of nitrogen exported from the senescing rice organs (Hayashi and Chino 1990). Immunolocalization studies showed that companion cells in the vascular bundles of the mature leaf sheath contain AS protein. Companion cells are important in the regulation of phloem loading (Van Bel 1993). We have proposed in our previous immunolocalization studies that GS1 protein in companion cells of vascular bundles of the senescing leaf blade of rice is important in the synthesis of
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Glutamine for export (Sakurai et al. 1996). Although the organ distribution of AS protein was different from that of GS1 protein, AS in companion cells of leaf sheath could be important in the synthesis of asparagine for long distance transport via phloem.

AS protein was also detected in grains at the middle stage of ripening. Cellular localization of AS in dorsal vascular bundle of developing grains was analogous to that of NADH-GOGAT (Hayakawa et al. 1994), suggesting that AS is also important in the synthesis of asparagine in developing grains, probably using glutamine exported from senescing organs via phloem. However, the role of AS in grains is not clear yet, because large amounts of asparagine are transported via phloem (Hayashi and Chino 1990). The maximum accumulation of AS protein was seen at 21 DAF, a week later than that of NADH-GOGAT protein (Hayakawa et al. 1993). Glutelin and prolamin, storage proteins in rice grain, appear by 15 DAF and actively accumulate thereafter (Hayakawa et al. 1993). AS may have a function related to the synthesis of asparagine destined to these storage proteins in rice plants.

In Arabidopsis thaliana, three AS genes, ASN1, ASN2, and ASN3, have been identified (Lam et al. 1998). Phylogenetic analysis indicated that ASN1 and other dicot AS genes (group I) are distinct from ASN2/3 and monocot AS genes (group II). Although AS protein has not been examined in other plants, accumulation of mRNA for ASN1 and dicot AS genes was regulated negatively by light or sugars and positively by darkness (Tsai and Coruzzi 1991, Shi et al. 1997, Lam et al. 1994, Lam et al. 1998, Nozawa et al. 1999). On the other hand, light had no influence on the expression of the AS gene in the monocot plants, Asparagus (Davis and King 1993) and maize (Chevalier et al. 1996). Since the leaf sheath and grains, which accumulated AS protein, are the organs which store carbohydrates in rice, sugars may affect the expression of rice AS gene. Further studies are required to understand the regulation of AS gene expression and function of AS in rice plants.

**Fig. 6** Cellular localization of AS protein in vascular bundles of leaf sheath (A, B) and in dorsal vascular bundle of young grains (C, D) of rice plants. Sections were stained with affinity purified AS IgG (B, D) or with the IgG pretreated with an excess amount of AS protein (A, C) as the primary antibody. cc, companion cell; dvb, dorsal vascular bundle; me, mesophyll cell; ne, nucellar epidermis; np, nucellar projection; pp, phloem parenchyma. Bars: 50 μm for A and B and 100 μm for C and D.
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Reference


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