Asparagine Synthetase1, but not Asparagine Synthetase2, is Responsible for the Biosynthesis of Asparagine Following the Supply of Ammonium to Rice Roots

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Abstract

Asparagine is synthesized from glutamine by the reaction of asparagine synthetase (AS) and is the major nitrogen form in both xylem and phloem sap in rice (Oryza sativa L.). There are two genes encoding AS, OsAS1 and OsAS2, in rice, but the functions of individual AS isoenzymes are largely unknown. Cell type- and NH$_4^+$-inducible expression of OsAS1 as well as analyses of knockout mutants were carried out in this study to characterize AS1. OsAS1 was mainly expressed in the roots, with in situ hybridization showing that the corresponding mRNA was specifically accumulated in the three cell layers of the root surface (epidermis, exodermis and sclerenchyma) in an NH$_4^+$-dependent manner. Conversely, OsAS2 mRNA was abundant in leaf blades and sheathes of rice. Although OsAS2 mRNA was detectable in the roots, its content decreased when NH$_4^+$ was supplied. Retrotransposon-mediated knockout mutants lacking AS1 showed slight stimulation of shoot length and slight reduction in root length at the seedling stage. On the other hand, the mutation caused an approximately 80–90% reduction in free asparagine content in both roots and xylem sap. These results suggest that AS1 is responsible for the synthesis of asparagine in rice roots following the supply of NH$_4^+$.

Keywords: Ammonium assimilation • Asparagine synthetase • Cytosolic glutamine synthetase • Knockout mutant • NADH-glutamate synthase • Rice root.

Abbreviations: AMT, ammonium transporter; AS, asparagine synthetase; DIG, digoxigenin; GOGAT, glutamate synthase; GS, glutamine synthetase.

Introduction

Glutamine and asparagine play crucial roles in plant growth and development, as the primary compounds in long-distance transport of nitrogen within various plants (Urquhart and Joy 1981, Ireland and Lea 1999, Lea et al. 2007, Gaufichon et al. 2010). In rice plants, these amides are major nitrogen forms in both phloem sap (Hayashi and Chino 1990) and xylem sap (Fukumotora and Chino 1982, Funayama et al. 2013) and also major free amino acids in the roots of rice seedlings grown in the presence of 1 mM NH$_4$Cl (Funayama et al. 2013). Thus, both amides are important in the primary assimilation of NH$_4^+$ taken up by rice roots and responsible for normal growth and productivity.

According to our previous work, cytosolic glutamine synthetase1;2 (GS1;2) and NADH-glutamate synthase1 (NADH-GOGAT1) are important in the primary assimilation of NH$_4^+$ in rice roots (Funayama et al. 2013, Yamaya and Kusano 2014). GS1;2 is also important in the development of active tillers through the reassimilation of NH$_4^+$ which is generated during lignin synthesis (Ohashi et al. 2015). Asparagine is synthesized by the transfer of the glutamine-amide group to aspartate by asparagine synthetase (AS) (Lea et al. 2007, Gaufichon et al. 2010). A small gene family has been identified that encodes AS in various plants, and recent phylogenetic analysis showed two distinct groups corresponding to ‘Class I’ and ‘Class II’ (Gaufichon et al. 2010). AS enzymes in monocots and dicots form subgroups in either class. From the phylogenetic analysis, we named OsASN (CI197925: Os06g0265000) in Class I as OsAS1, and OsASN (D83378: Os06g0265000) in Class II as OsAS2.

Classical studies showed that expression of AS genes in various plants changes depending upon light irradiation, various stresses and post-harvest (Tsai and Coruzzi 1990, Tsai and Coruzzi 1991, Davis and King 1993, Lam et al. 1994, Chevalier et al. 1996, Lam et al. 1998). However, the function of each

Results

Comparison of the deduced amino acid sequences for AS1 and AS2

According to the previous phylogenetic analysis by Gaufichon et al. (2010), the presence of two genes encoding AS (Os03g0291500 and Os06g0265000) in the rice genome was confirmed by the Rice Annotation Project Database (RAP-DB; http://rapdb.dna.afrc.go.jp/) (Sakai et al. 2013). Reverse transcription–PCR (RT–PCR) strategies were used for cloning two AS cDNAs with specific oligonucleotide primers, using nucleotide sequence information of rice (cultivar Nipponbare) from the RAP-DB (Supplementary Table S1). Two cDNA clones derived from Os03g0291500 and Os06g0265000 sequences were named OsAS1 and OsAS2, respectively. The OsAS1 and the OsAS2 genes encode polypeptides of 67.3 kDa (604 amino acids) and 66.2 kDa (591 amino acids), respectively (Supplementary Fig. S1). These genes show 70% identity and 92% similarity at the deduced amino acid level, respectively. Based on a comparison of the peptide sequences of AS1 and AS2 with the crystal structure of AS-B in Escherichia coli (Larsen et al. 1999), a putative glutamine-binding site was positioned at Arg50, Asn75, Glu76 in both rice enzymes, and Asp98 for OsAS1 and Asp99 for AS2, respectively. According to the analyses of AS-B in E. coli, these polypeptides in rice contained a putative aspartate-binding site at Thr317–Thr318–Arg320–Cys324 for AS1 and Thr316–Thr317–Arg319–Cys323 for AS2, and a putative AMP-anchoring site at Leu232–Val268–Ser342–Gly343 for AS1 and Leu231–Ile267–Ser341–Gly342 for AS2 (Boehlein et al. 1997a, Boehlein et al. 1997b). It is assumed that the Cys324 residue for AS1 and the Cys323 residue for AS2 are probably essential in the reaction with aspartate and ATP (Boehlein et al. 1997b).

Expression profile of two OsAS genes in root and shoot of rice seedlings

Quantitative real-time PCR showed that OsAS1 mRNA was the major species in rice roots when the seedlings were hydroponically grown for 18 d after germination with water (pH 5.5 adjusted with 5 mM MES-NaOH) alone (Fig. 1a). Endogenous starch and proteins in rice seeds are fully consumed during this period, and autotrophic growth is started thereafter. In contrast, OsAS2 was the major species in leaf blades and leaf sheaths (Fig. 1b) under the same growth conditions. To test whether expression of the OsAS1 gene is stimulated by the supply of exogenous NH$_4^+$, as in the case of OsGS1;2 and OsNADH-GOGAT1 (Tabuchi et al. 2007), 18-day-old seedlings were transferred into fresh water in the presence of 1 mM NH$_4^+$ (pH 5.5 adjusted with 5 mM MES-NaOH) and then harvested over the following 72 h. OsAS1 mRNA accumulated rapidly after the addition of NH$_4^+$, reached a maximum at 8 h, then gradually decreased until 72 h; this increase was not observed in the absence of NH$_4^+$ (Fig. 1c). Only low levels of OsAS2 mRNA were detected in the roots grown in the presence or absence of 1 mM NH$_4^+$ (Fig. 1d). In addition, there was slight fluctuation in the accumulation of actin mRNA used as a control in the presence or absence of NH$_4^+$ (Fig. 1e). Under the same conditions, contents of some free amino acids, such as asparagine, glutamate and glutamine, and free NH$_4^+$ in the root extracts significantly increased following the supply of NH$_4^+$ (Supplementary Fig. S2).

Tissue localization of OsAS1 mRNA in roots of rice seedlings

Preliminary immunoblotting using an anti-AS2 antibody (Nakano et al. 2000) showed that AS protein content on a fresh weight basis was the highest in the root tip section (0–30 mm from the tip; Supplementary Fig. S3), although our antibody could not distinguish between AS isoenzymes. Older regions of the roots exhibited reduced AS protein levels. Therefore, root tip sections were tested to localize OsAS1 and OsAS2 mRNAs in situ, using specific probes (Supplementary Table S1).

Accumulation of OsAS1 mRNA was detected in the three cell layers (epidermis, exodermis and sclerenchyma) of the root
Significant differences between blade (2LB), third leaf blade (3LB), third leaf sheath (3LS), fourth ex-

Fig. 1 Quantitative real-time PCR detection of mRNAs for OsAS1 (black column) and OsAS2 (gray column) in roots (a) and leaf blade (LB) and leaf sheath (LS) at different leaf positions (b) in rice. Time course studies on the detection of mRNA for OsAS1 (c) and for OsAS2 (d) in rice roots were conducted in the presence or absence of 1 mM NH$_4^+$.”. The inset is a magnified figure taken in the first 24 h. Changes in actin mRNA are shown as a control. Rice plants (cv. Nipponbare) were grown hydroponically for 18 d in water (pH 5.5 adjusted with 5 mM MES-NaOH) and each organ was harvested; whole roots, second leaf blade (2LB), third leaf blade (3LB), third leaf sheath (3LS), fourth expanding leaf blade (4LB) and root. For the time course study, the 18-day-old seedlings were grown further for 72 h. Quantitative real-time PCR was performed using gene-specific primers for OsAS1, OsAS2 and actin, respectively. Means of four independent samples and SD values (n = 4) are indicated. The ‘n.d.’ in (b) indicates ‘not detected’. Significant differences between OsAS1 and OsAS2 identified by Student’s t-test are marked with asterisks: *P < 0.05; **P < 0.01; ***P < 0.001.

Characteristics of mutants lacking OsAS1

Lines of OsAS1 knockout mutants were screened by searching the flanking sequence database (Miyao et al. 2003) of the mutant panel (https://tos.nias.aflrc.go.jp/~miyao/pub/tos17/) of the Project for Rice Genome Research, where mutant lines, produced by the random insertion of the endogenous retrotransposon Tos17 into the rice (Oryza sativa L. cv Nipponbare) genome (Hirochika et al. 1996), were collected. In lines NF7811 (as1-m1) and NF4467 (as1-m2), Tos17 was inserted into intron 5 and intron 2 of the OsAS1 gene from +1,701 to +1,697 and from +867 to +863, respectively, where the translation start is +1 (Fig. 4a). The apparent abnormal order of nucleotide numbers was also shown in our previous studies of OsGS1:1 (Tabuchi et al. 2005), OsGS1:2 (Funayama et al. 2013), OsNADH-GOGAT1 (Tamura et al. 2010) and OsNADH-GOGAT2 (Tamura et al. 2011) mutants. Quantitative real-time PCR showed that the homozygous NF7811 and NF4467 lines transcribed almost no OsAS1 mRNA in the roots when the seedlings were grown for 18 d in water and then for 8 h in the presence of 1 mM NH$_4^+$, pH 5.5 (Fig. 4b). Quantitative real-time PCR indicated that the Tos17 homozygously inserted OsAS1 mutants expressed statistically identical amounts of mRNAs for OsAS2, OsGS1:1 and OsGS1:2 in roots, when the OsAS1 mutants and wild type rice were grown hydroponically for 18 d in water and then for 8 h in the presence or absence of 1 mM NH$_4^+$ (Fig. 4c–g). The OsNADH-GOGAT1 mRNA level was slightly changed in roots grown without NH$_4^+$, but it was statistically identical in the presence of NH$_4^+$ (Fig. 4f). Accumulation of actin mRNA, used as a control, was also identical in the roots of the wild type and OsAS1 mutants in the presence or absence of 1 mM NH$_4^+$ (Fig. 4g). Thus, we succeeded in obtaining a knockout mutants lacking OsAS1.

At the seedling stage, shoot length and shoot fresh weight were slightly but significantly stimulated and root length was slightly reduced in the OsAS1 knockout mutant (Fig. 5). Total nitrogen content in the shoots was slightly decreased (Fig. 6b: +NH$_4^+$) while total carbon was slightly increased in the shoots of the mutants on a dry weight basis (Fig. 6d: +NH$_4^+$), when rice seedlings were treated with 1 mM NH$_4^+$ for 24 h following the growth in water for 18 d. The roots of mutants contained wild-type levels of total nitrogen and carbon in the presence of 1 mM NH$_4^+$ (Fig. 6a, c). In the absence of NH$_4^+$, total carbon contents increased slightly in the roots of mutants (Fig. 6c). In contrast
Fig. 2  In situ detection of OsAS1 mRNA in rice root tips. Rice plants (cv. Nipponbare) were grown hydroponically until the seventh leaf stage in basal nutrient solution containing 1 mM NH$_4^+$ . The plants were then grown for 3 d in water (pH 5.5 adjusted with 5 mM MES-NaOH), then transferred into fresh water in the presence or absence of 1 mM NH$_4^+$ (pH 5.5 adjusted with 5 mM MES-NaOH). Root tissues were harvested at 6 h after the transfer and separated into three sections (meristem region, elongation region and mature region). The sense probe for OsAS1 mRNA was hybridized with the longitudinal sections from the NH$_4^+$ -fed plants as a negative control (a–d). The antisense probe for OsAS1 mRNA was hybridized with the sections from rice roots grown in the absence (e–h) or presence of 1 mM NH$_4^+$ (i–l). The staining reaction with or without NH$_4^+$ supply was performed for 3 h. Abbreviations: cc, central cylinder; c, cortex cells; ex, exodermis; ep, epidermis; and sc, sclerenchyma. Scale bars in (a), (e) and (i) are 200 μm, those in (b), (c), (f), (g), (j) and (k) are 100 μm, whereas those for (d), (h) and (l) are 25 μm.

Fig. 3  In situ detection of the OsAS2 mRNA in rice root tips. Rice plants (cv. Nipponbare) were grown as described in the legend of Fig. 2. The sense probe for OsAS2 mRNA was hybridized with the longitudinal sections from the NH$_4^+$ -fed plants as a negative control (a–d). The antisense probe for OsAS2 mRNA was hybridized with the sections from rice roots grown in the absence (e–h) or presence of 1 mM NH$_4^+$ (i–l). The staining reaction with or without NH$_4^+$ supply was performed for 16 h. Abbreviations: cc, central cylinder; c, cortex cells; ex, exodermis; ep, epidermis; and sc, sclerenchyma. Bars in (a), (e) and (i) are 200 μm, those in (b), (c), (f), (g), (j) and (k) are 100 μm, and those in (d), (h) and (l) are 25 μm.
to those slight changes, an approximately 80–90% reduction in free asparagine content was observed in two mutant lines, when plants were treated with 1 mM NH$_4^+$ following the growth in water for 18 d, even though total amino acid contents were identical between the mutants and wild type (Fig. 7a–c). In those mutants, glutamine contents increased approximately 30% in roots. When the amino acid contents of the xylem sap were determined, an approximately 80–90% reduction in asparagine contents and an approximately 70% increase in glutamine contents were observed (Fig. 7d–f). It should be noted, however, that any xylem sap was not collected with no NH$_4^+$ supply in the culture solution. When the whole root samples were equally divided into three regions (tip, middle and base), the free asparagine content was also the highest in the tip.
sections of wild-type roots (2.59 ± 0.26 μmol g⁻¹ FW), as in the case of AS protein determination (Supplementary Fig. S3). The asparagine content decreased in the middle and base region of the roots: it was 0.49 ± 0.02 and 1.50 ± 0.19 μmol g⁻¹ FW, respectively. On the other hand, 67–88% reduction in free asparagine contents in the root tip regions was observed in the OsAS1 knockout mutants. Reduction of free asparagine content was also observed in the middle (72–83%) and basal (83–90%) regions in the roots of mutants.
Discussion

Tissue localization (Figs. 2, 3) and NH₄⁺-responsive expression of OsAS1 and OsAS2 (Fig. 1) suggest distinct functions of the two AS isoenzymes in rice. The characteristics of OsAS1 expression in the three cell layers of the root surface in an NH₄⁺-dependent manner was identical to that of OsGS1;2 and OsNADH-GOGAT1 (Ishiyama et al. 2003, Ishiyama et al. 2004, Tabuchi et al. 2007, Yamaya and Kusano 2014). Given that GS1;2 and NADH-GOGAT1 in rice roots are important in the primary assimilation of NH₄⁺ taken up by roots, As1 in rice roots may also be related to primary nitrogen assimilation. In rice, there is a Casparian strip between the second and third cell layers of the root surface (Morita et al. 1996) in addition to the Casparian strip in the endodermis. This additional Casparian strip probably protects against radial oxygen loss from the roots of rice plants grown in paddy fields (Watanabe et al. 2014). The assimilated nitrogen, as glutamine, glutamate and asparagine, could be transported cellurally through the Casparian strip into the cortex and central cylinder. Mutants lacking OsAS1 exhibited reduced free asparagine contents in the roots, suggesting that AS1 is responsible for asparagine synthesis. OsAS1 and OsAS2, as well as OsGS1;1 and OsGS1;2 (Ishiyama et al. 2004), were constitutively expressed in the central cylinder, independently of the presence of NH₄⁺ (Figs. 2, 3). This suggests that asparagine is also generated within this tissue for root to shoot transport via the xylem.

Following the supply of NH₄⁺ into the roots, accumulated OsAS2 transcript under the no NH₄⁺ treatment decreased in the root surface (Fig. 3). This phenomenon is identical to the decrease in OsGS1;1 mRNA contents in rice roots (Tabuchi et al. 2007). OsAS2 mRNA mainly accumulated in the leaf blades and leaf sheaves (Fig. 1; Nakano et al. 2000) while the AS2 protein was detected in phloem companion and parenchyma cells (Nakano et al. 2000), along with the GS1;1 protein (Sakurai et al. 1996, Tabuchi et al. 2007). These results suggest that AS2 in rice leaves is probably important in the long-distance transport of asparagine from rice leaves during natural senescence.

In sunflower, the expression of AS isogenes, HAS1 and HAS1.1, was dependent on the presence of a nitrogen source (Herrera-Rodrı́guez et al. 2002, Herrera-Rodrı́guez et al. 2004, Herrera-Rodrı́guez et al. 2006). The accumulation of ASN2 mRNA in Arabidopsis in the presence of NH₄⁺ also required light (Wong et al. 2004). Expression of mRNA for maize NH₄⁺ transporters, ZmAMT1;1a and ZmAMT1;3 was stimulated in the presence of NH₄⁺ (Gu et al. 2013). As discussed earlier (Tabuchi et al. 2007), some genes related to nitrogen uptake and metabolism are regulated by glutamine, but not directly by NH₄⁺. Genes up-regulated by glutamine or related metabolites in rice are: OsAMT1;2 (Sonoda et al. 2003), OsNADH-GOGAT1 (Hirose et al. 1997) and genes for adenosine phosphate-isopen-tenytransferase (Kamada-Nobusada et al. 2013). In other plants, glutamine also regulates gene expression, i.e.
AtAMT1;1 (Rawat et al. 1999), AtAS (Lam et al. 1998), AtGS1 (Oliveira and Coruzzi 1999) and nitrate reductase in tobacco leaves (Vincentz et al. 1993). However, little is known about the molecular mechanisms of glutamine signaling, in contrast to nitrate signaling in plants (Sakakibara et al. 2006). Our three examples of OsAS1, OsGS1;2 and OsNADH-GDAGT1 in rice roots could be good tools in the future to understand molecular mechanisms of the regulation of gene expression controlled by NH$_4^+$/glutamine.

### Materials and Methods

#### Plant materials

Rice (cv. Nipponbare) seedlings were grown hydroponically for 18 d in water (pH 5.5 adjusted with 5 mM MES-NaOH) in order to deplete seed reserves. They were grown in a greenhouse controlled at 26°C with supplemental light from 05:30 to 18:30 h (13 h light/11 h dark), as described previously (Funayama et al. 2013). When the effect of NH$_4^+$ on the expression of the OsAS1 gene was tested, the 18-day-old seedlings were transferred into fresh water in the presence or absence of 1 mM NH$_4^+$, pH 5.5 adjusted with 5 mM MES-NaOH at 10:00 h. Roots and shoots of wild-type plants were then harvested at 4, 8, 24, 48 and 72 h after the treatment for quantitative real-time PCR experiments. For the in situ hybridization, rice plants were grown hydroponically in a basal nutrient solution (Kamachi et al. 1992) containing 1 mM NH$_4^+$ until the seventh leaf stage and then pre-treated with no nitrogen for 3 d. The seedlings were then transferred into a culture solution in the presence or absence of 1 mM NH$_4^+$ for 8 h.

The Project for Rice Genome Research, the Ministry of Agriculture, Forestry and Fisheries of Japan (mutant panel: https://tos.nias.afr.co.jp/~miyao/pub/tosts17/) provided 20 seed each from two lines (NF7811 and NF4467) of rice (Oryza sativa L. cv Nipponbare) in which the retrotransposon Tos17 could potentially have been inserted into the OsAS1 gene. For the isolation of knockout mutants, wild-type (O. sativa L. cv. Nipponbare) and Tos17-inserted lines of rice seeds were soaked in distilled water at 30°C for 2 d and were then transferred to nylon nets, floating on quarter-strength nutrient solution adjusted to pH 5.5 (Kamachi et al. 1992), in an 8 liter plastic container. Seedlings were grown for 2 weeks in a greenhouse. Genomic DNA was extracted from the two OsAS1 knockout candidates of Tos17 insertion lines, and the insertion point in the gene of NF7811 and NH4467 was determined by PCR using specific primer pairs against OsAS1 and Tos17 sequences, respectively (Supplementary Table S1) and fully sequenced according to our previous experiment (Funayama et al. 2013). Lines NF7811 and NF4467 were identified as insertion mutants for OsAS1 and named as1-m1 and as1-m2, respectively. The as1-m1 and as1-m2 were self-fertilized, and segregated homozygote plants for Tos17 were used as OsAS1 knockout mutants. Phenotypic characteristics were observed after the growth of both wild-type and mutant lines in water for 18 d. When the effect of NH$_4^+$ on expression of OsAS1 was determined, the seedlings were further treated for 8 h in the presence or absence of 1 mM NH$_4^+$ following the growth for 18 d in water, and whole roots were used for quantitative real-time PCR experiments. When contents of amino acids as well as total nitrogen and carbon were determined, wild-type and mutant lines were treated for 24 h.

#### Cloning of rice AS CDNAS

Molecular biological experiments were carried out according to our previous protocol (Ishiyama et al. 2004, Tamura et al. 2010, Funayama et al. 2013). The coding sequences of CDNAS encoding AS were isolated by RT–PCR for first-strand DNA synthesis using specific primer pairs for OsAS1 and OsAS2, respectively (Supplementary Table S1), designed according to the nucleotide sequence of rice. The locus number for the OsAS1 gene is Os03g0219500 and for the OsAS2 gene is Os06g0265000 (the Rice Annotation Project: http://rapdb.dna.affrc.go.jp/index.html). Total RNA was extracted using the RNeasy plant kit (Qiagen). Reverse transcription was carried out using a PrimeScript® RT reagent Kit with gDNA Eraser (TAKARA BIO INC.), and then PCR was carried with KOD Fx NEO DNA polymerase (Toyobo). The amplified PCR products were cloned into pCR-BluntII-TOPO (Technologies Corporation) and fully sequenced.

#### Quantitative PCR analysis

Quantitative PCR analysis was performed according to Konishi et al. (2014). Gene-specific primers of OsAS1, OsAS2 and actin1 (accession No. KC140126) for quantitative PCR analysis are shown in Supplementary Table S1. The PCR products were detected and quantified using Light Cycler® 480 (Roche Diagnostics K.K.) according to the following program: 10 s at 95°C, followed by 50 cycles of 95°C for 5 s, 60°C for 34 s. The mRNA contents were quantitatively determined using a purified cDNA clone as a standard for its calibration.

#### Extraction of AS protein

The protein extraction procedure was based on the method of Kawachi et al. (2002). Frozen tissues were homogenized with 5 vols. of extraction buffer containing 0.1 M Tris–HCl, pH 8.5, 0.1 mM EDTA, 10 mM MgCl$_2$, 2 mM aspartate, 50 mM 2-mercaptoethanol, 25% (v/v) glycerol, 1 mM EGTA, 1 mM leupeptin and 0.1 mM ATP. After centrifugation of the homogenate at 16,000 × g for 10 min, the supernatant was used for immunoblotting analysis of AS protein, as described previously (Nakano et al. 2000).

#### In situ hybridization of OsAS1 and OsAS2 mRNAs

Longitudinal sections of root tip approximately 5 mm in length, containing meristem, elongation and mature zones, and the center part of the root containing the mature region prepared from wild-type plants were first placed in FAA solution [1.85% (v/v) formaldehyde, 5% (v/v) acetic acid and 63% (v/v) ethanol], as described previously (Ishiyama et al. 2003, Ishiyama et al. 2004). The fixed sections were embedded in paraffin (Sigma), and stored at 4°C until required. The embedded sections were cut into 8 µm slices using a microtome (Yamato Kohki Industrial Co., Ltd.).

RNA probes were prepared as follows. The 200 bp fragments from the 3′-untranslated regions of OsAS1 and OsAS2 were amplified by PCR using specific primer pairs for transcript sequences (Supplementary Table S1). The amplified PCR products were cloned into pCR-4Blunt-TOPO (Life Technologies). After digestion with restriction enzyme, digoxigenin (DIG)-labeled sense and antisense probes were synthesized using T3 and T7 polymerases (Promega Corporation) and a DIG RNA labeling kit (Roche Diagnostics) according to Ishiyama et al. (2004). In situ hybridization was performed according to the procedures previously described with slight modification (Ishiyama et al. 2004, Luo et al. 2012). OsAS1 and OsAS2 mRNAs in situ were hybridized in Hybridization Solution (Sigma) containing 25 ng ml$^{-1}$ DIG-labeled RNA probe. Hybridization was carried out for 16 h at 50°C. After the hybridization, excess RNA probes on slides were removed by electrophoresis for 30 min at 100 V in 1 × TAE buffer using Mupid2 plus (ADVANCE). After electrophoresis, the final wash was performed with 4 × SSC for 1 h at 60°C and 0.1 × SSC containing 0.1% (w/v) SDS for 1 h at 55°C. The products of in situ hybridization were detected with alkaline phosphatase-conjugated anti-DIG antibody, nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Roche Diagnostics), according to the methods described by Ishiyama et al. (2004). Detection of OsAS1 mRNA was accomplished by 3h staining, whereas that of OsAS2 mRNA required 16 h. After the staining, sections of roots were observed by optical microscopy (Leica DMRB, Leica Microsystems) with a CCD camera (Leica DFC 500), visualized and photographed by image handling software (Leica IM50 image manager, Leica Microsystems).

#### Free amino acid measurement in rice roots and xylem sap

Extraction, derivatization and determination of free amino acids and NH$_4^+$ were performed according to methods described previously (Funayama et al. 2013, Konishi et al. 2014). For the collection of xylem sap, wild-type and OsAS1 knockout mutants were grown in water (pH 5.5 adjusted with 5 mM MES-NaOH) for 18 d. These seedlings were further grown for 24 h in fresh water in the presence of 1 mM NH$_4$Cl. Xylem sap was then collected as described previously (Funayama et al. 2013).
Determination of total carbon and nitrogen content

Wild-type rice and OsAS1 knockout mutants were cut into roots and shoots, air-dried at 80°C for a week and weighed. The samples were powdered with a Multi-Bead Shocker MB601U (Yasui Co. Ltd.) and stored in a desiccator.

Determination of total carbon and nitrogen contents was carried out as described previously (Tamura et al. 2011).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References


Asparagine synthetase in rice root


