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ASN2 is a key enzyme in asparagine biosynthesis under ammonium sufficient conditions

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Abstract The modification and enhancement of plant amino acid accumulation is potentially beneficial in terms of the production of suitable crop plants for food or feeds, and also with respect to possible environmental improvement via the effective use of nitrogen nutrition. It is also expected that modified plants will produce beneficial nitrogenous compounds, such as proteins, secondary metabolites, or nucleic acids. The size of amino acid pools in plants is strictly controlled by certain environmental factors such as light and nutrition. In order to gain an understanding of the mechanisms that control amino acid biosynthesis and metabolism, we investigated the diurnal changes in amino acid contents and also how the amino acid contents change with the progression of growth. We observed that almost all the amino acids in plant leaves undergo diurnal changes and that the pattern of these changes was different in young and old stages. We focused on correlation of the asparagine content and the expression of *ASN2* asparagine synthetase, since under our experimental conditions their fluctuating patterns were found to be similar. The asparagine contents of *ASN2*-overexpressing and -underexpressing plants were increased and decreased, respectively, when they were grown under normal light and nutrient conditions. These changes in asparagine content were marked when the plants were grown under conditions where ammonium was the sole nitrogen source. It has been previously reported that *ASN2* expression and ammonium metabolism are correlated. Our findings and these previous observations suggest that *ASN2* functions as a regulator of asparagine biosynthesis and metabolism and that it mediates the effective use of nitrogen under ammonium-sufficient conditions.

Key words: Asparagine synthetase, diurnal change.

Amino acids are the primary compounds of nitrogen assimilation and are used as nitrogen donors for the synthesis of various nitrogenous compounds. Plant amino acid biosynthesis is closely linked to photosynthesis and is controlled by certain environmental signals such as light, water, inorganic nutrients, and organic metabolites. The artificial modification of amino acid contents in plant leaves, fruits, and seeds could lead to an enhancement of plant nitrogen availability and the breeding of commercially beneficial crop plants. However, the artificial control of amino acid content is difficult since almost all amino acids function as nitrogen donors for the production of other nitrogenous compounds. In addition, there is a certain degree of redundancy among many of the genes encoding plant amino acid metabolic enzymes (Arabidopsis Genome Initiative 2000). In order to develop a technology targeted for artificial modification of amino acid accumulation, clarification of the function of these multicopy genes will be critical.

Previous studies have reported on the production trials of transgenic plant that accumulate essential amino acid such as lysine, methionine, and tryptophan, since plants containing elevated levels of these amino acids could serve as important sources of animal food (Huang et al. 2005; Shen et al. 2002; Tozawa et al. 2001). We have indicated that enhancement of the photorespiratory pathway via overexpression of *glutamate*: *glyoxylate aminotransferase* (*GGAT*) gene leads to a hyperaccumulation of serine and glycine (Igarashi et al. 2006). However, focusing on the primary reactions of nitrogen metabolism, and on an increase in the content of amino acids as the donors of nitrogen, is important for the production of beneficial plants.

Asparagine has a high nitrogen to carbon ratio. This amino acid accumulates when plants are grown in light with high levels of exogenous ammonium (Prianischnikow 1922). Under high light intensity, photorespiration occurs in C3 plants and this leads to an

Abbreviations: AGI, arabidopsis genome initiative; CaMV, cauliflower mosaic virus; RT-PCR, reverse transcription polymerase chain reaction; DAG, days after germination.

This article can be found at http://www.jspcmb.jp/

increased production of ammonium (Kozaki and Takeba 1996; Wingler et al. 2000). Under such conditions, asparagine can account for up to 7% of the total nitrogen (Ta et al. 1984; Sieciechowicz et al. 1988). Asparagine synthetase (ASN; EC 6.3.5.4) catalyzes the reaction of glutamine and aspartate to yield asparagine and glutamate. Each of the substrates and products of ASN-catalyzed reactions—that is, glutamate, glutamine, aspartate, and asparagine—are major nitrogen carriers in plant metabolism that function as nitrogen transporters.

ASN is believed to play an important role in regulating the flow of nitrogen into the organic nitrogen pool (Lam et al. 1994). In *Arabidopsis*, ASN is encoded by a small gene family (*ASN1*, *ASN2*, and *ASN3*). Phylogenetic analysis has indicated that *ASN1* is clustered with all dicot *ASN* genes and that *ASN2* and *ASN3* are related to monocot *ASN* genes. Organic forms of nitrogen treatment have been shown to positively regulate the levels of *ASN1* mRNA (Lam et al. 1994; Gutiérrez et al. 2008), whereas *ASN2* gene expression appears to be responsive to inorganic nitrogen sources but not to a downstream metabolite (Gutiérrez et al. 2008). In wild-type plants, the expression of *ASN1* and asparagine contents increase in dark-adapted plants, consistent with the idea that asparagine is an important nitrogen carrier, particularly under carbon-limiting conditions (Lam et al. 1998). In *ASN1*-overexpressing plants, asparagine contents increase in both source and sink tissues (Lam et al. 2003). These findings suggest that ASN1 plays a major role in the regulation of asparagine content.

The expression of *ASN2* is reciprocal to that of *ASN1* (Lam et al. 1998). *ASN2* gene expression is induced by light in an ammonium-dependent manner (Wong et al. 2004). Ammonium content and *ASN2* expression have been demonstrated to be correlated under NaCl and cold stress conditions (Wong et al. 2004). Further, *ASN2*-overexpressing plants accumulate less endogenous ammonium compared with wild-type plants. These results suggested that *ASN2* is involved in ammonium metabolism. In the study of Wong et al., the analysis of *ASN* gene expression and asparagine content were determined under conditions of long-term light and dark treatment. However, sometimes gene expression patterns are different in plants grown under normal light/dark conditions and those grown under long-term treatments. For instance, microarray analysis has indicated that *ASN1* expression is increased under light conditions (Harmer et al. 2000).

The objective of this study was to identify the key genes that regulate the amino acid contents in leaves. Analysis of the profiles of gene expression and amino acid contents diurnally and at different growth stages indicated that *ASN2* could be a key factor in asparagine biosynthesis due to the correlation of these profiles. Furthermore, plants overexpressing *ASN2* accumulated asparagine, suggesting that *ASN2* is significant in the control of asparagine content.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana L. (Col-0) plants were grown at a photosynthetically active radiation of 100 μmol m^{-2} s^{-1}, a photoperiod of 14 : 10-h L : D, a temperature of 23°C and a relative humidity of 60%, unless otherwise indicated. Plants were grown on rockwool moistened with nutrient solution containing 2.5 mM potassium phosphate (pH 5.7), 2.5 mM NH_4NO_3, 2 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 50 μM Na–Fe–EDTA, 10 μM NaCl, 70 μM H_3BO_3, 14 μM $MnCl_2$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 10 nM $CoCl_2$, and 0.2 μM $NaMoO_4$. The nutrient solution was renewed once a week. For the analysis of the amino acid contents of *ASN2*-overexpressing plants under different nitrogen conditions, plants were grown on agar in Petri dishes where $Ca(NO_3)_2$ and NH_4NO_3 were substituted with 2 mM $CaCl_2$ and various concentrations of KNO_3, NH_4NO_3 or ammonium succinate, respectively. For the analysis of *ASN2*-underexpressing plants, plants were grown on agar in Petri dishes where 2.5 mM NH_4NO_3 was substituted 5 mM KNO_3.

Construction of transgenic plants

In order to generate *ASN2*-overexpressing/underexpressing plants, a pBIDAVL-GWR1 vector containing a CaMV 35S promoter, a Gateway conversion cassette (Invitrogen), and a kanamycin gene was used as described previously by Nakazawa et al. (2003). The *ASN2* (At5g65010) cDNA was amplified by RT-PCR using gene-specific primers (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGTGTGGGATTCTCGCTGTTCTTG-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTTATGTTCCATTCTTAGGAAGAGG-3′). A polyA tail was added to the fragment and this was then cloned into a pCRII-TOPO vector (Invitrogen). After sequence confirmation, the *ASN2* cDNA was cloned into pDONR207 (Invitrogen) and pBIDAVL-GWR1 by means of a Gateway system (Invitrogen). An *ASN2* expression cassette was introduced into *Arabidopsis* by means of *Agrobacterium tumefaciens*-mediated transformation. The transgenic plants were grown on half strength MS agar medium containing 100 mg L^{-1} kanamycin for screening.

Quantitative RT-PCR

For RT-PCR, total RNA was isolated from the leaves using an RNeasy plant mini kit (Qiagen). First-strand cDNA synthesis was conducted with an oligo(dT)12–18 primer (GE Healthcare) and reverse transcriptase (SuperScript II; Invitrogen). Real-time PCR reactions were performed using an Applied Biosystems Prism 7500 sequence detector. The primers used for real-time PCR were as follows: *ASN2* (5′-CAACACTACAGGCATTGAGGA-3′ and 5′-TCCAGCTAGAAATGGACGAA-3′), and *ACT2* (5′-GGTAACATTGTGCTCAGTGGTGG-3′ and 5′-GGTGCAACGACCTTAATCTTCAT-3′). Real-time RT-PCR reactions were performed in duplicate with 0.9 μM of each primer and 1×SYBR green PCR master mix (Applied Biosystems) in a 50-μL volume. Relative differences were

determined by the ΔΔCt method described by the manufacturer.

Labeling of RNA probes and hybridization to an Arabidopsis GeneChip

Labeling and hybridization of RNA were performed in accordance with the manufacturer's instructions. Briefly, ATH1 *Arabidopsis* GeneChips (Affymetrix) were used to measure changes in gene expression levels. Total RNA was converted into cDNA, which was in turn used to synthesize biotinylated cRNA. The cRNA was fragmented into smaller pieces and then hybridized to the GeneChips. Following hybridization, the chips were automatically washed and stained with streptavidin phycoerythrin using an Affymetrix Fluidics Station. Microarray data were extracted from the scanned GeneChip images and analyzed using Microarray Suite (MAS) version 5.0 (Affymetrix). When necessary, the data produced by Microarray Suite were exported into Microsoft Excel and further analyzed.

Measurement of amino acid content

Amino acids were extracted from leaves or seedlings in 80% ethanol at 80°C. After evaporation, dried samples were dissolved in 20 mM HCl. Amino acid content was determined using an L-8800 amino acid analyzer (Hitachi). Briefly, amino acids separated by cation-exchange chromatography were detected spectrophotometrically after post-column reaction with ninhydrin reagent (Noguchi et al. 2006).

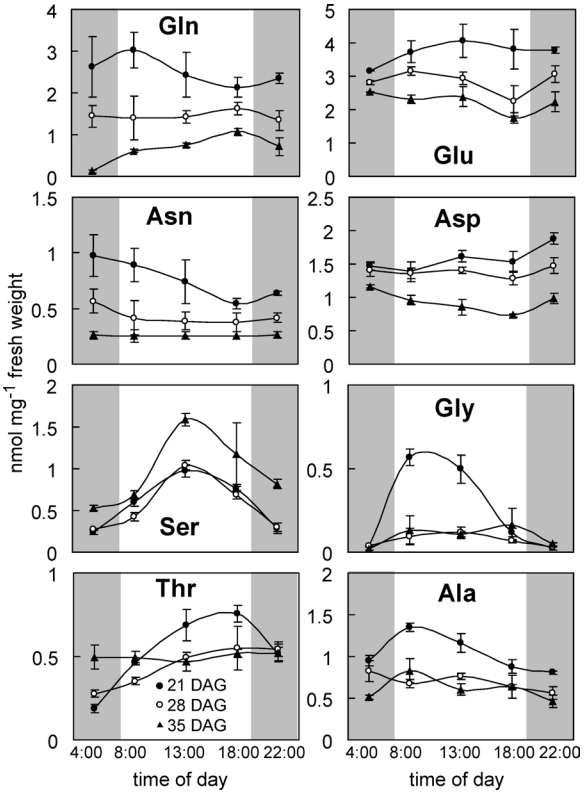

Figure 1. Diurnal changes in major amino acid contents in leaves at various developmental stages. Wild-type (Col-0) plants were grown for 21, 28, and 35 days. Freshly expanded rosette leaves (2 leaves per plant) were harvested at 5 time points and their amino acid contents were determined. From 6:00 to 20:00 is the light period. The values presented are the means±SD of 3 plants.

Results and discussion

Diurnal determinations of amino acid contents at different growth stages

We determined the diurnal changes in amino acid contents at 3 developmental stages: 21, 28, and 35 DAG. At the 21-DAG stage, rosette leaves were expanded, although bolting had yet to be initiated. At 28 DAG, bolting had commenced and approximately 10 flowers were opened. At 35 DAG, some primary siliques had started to turn yellow. Fresh fully expanded leaves were used for the analysis. The contents of amino acids other than serine were decreased during the stage progression (Figure 1). In addition, diurnal changes in glycine, asparagine, and threonine contents were changed between 21 DAG and 35 DAG (Figure 1). Glycine and threonine were accumulated after exposure to light at 21 DAG, whereas they had disappeared in plants at 28 and 35 DAG. These changes in the amino acid profiles could be the important indices in analyzing the regulation of amino acid biosynthesis and accumulation in leaves. Glycine is an amino acid that is mainly synthesized via the reaction of glutamate glyoxylate aminotransferase (GGAT) in photorespiration (Igarashi et al. 2003; Igarashi et al. 2006). Given that at 21 DAG, photosynthesis and photorespiration are vigorous compared to the later growth stages, it is possible that glycine accumulates during the daytime in the leaves of younger plants. Asparagine content was decreased during the daytime. Previous studies have indicated that asparagine is accumulated following dark adaptation (Lam et al. 1998; Lam et al. 2003) and during non-photosynthetic periods. However, in these studies the plants were exposed to long-term dark treatment. These conditions are probably inappropriate for examining the natural environmental regulation of amino acid metabolism.

In our study, we selected photosynthesis-dependent growing conditions; that is, plants were grown on rockwool under conditions of normal light and darkness. Furthermore, diurnal changes in asparagine contents were no longer observed at the older (35 DAG) stage. This could be a result of a reduction in nitrogen assimilation and activities of nitrogen metabolism or an increase in the transport of asparagine or related amino acids from source to sink tissues.

Gene expression levels of major amino acid metabolic enzymes during the diurnal cycle at different growth stages

On the basis of the observed marked changes in amino acid content during the diurnal cycle and at different growth stages, we analyzed the gene expression profiles of 21 DAG and 35 DAG plant leaves in order to identify the key genes that regulate the amino acid content under normal light/dark condition. The gene expression profiles of 21 DAG and 35 DAG samples were obtained by Affymetrix ATH1 GeneChip arrays, and genes for the major amino acid metabolic enzymes (Table 1) were selected for further analysis. Figure 2 depicts the transcript levels of these metabolic genes during the diurnal cycle at different growth stages. In addition to the profiles of amino acid contents (Figure 1), gene expression of the related metabolic enzymes (Figure 2) was also changed significantly under our experimental conditions. The profile of asparagine content, which exhibited a decrease following light exposure and an increase during the night (Figure 1), was well correlated with the profile of *ASN2* gene expression (Figure 2).

It has been reported that the expression of *ASN1* increases in dark-adapted plants, that the expression of *ASN2* increases in light-adapted plants in the presence of ammonium, and that asparagine content is correlated with *ASN1* gene expression (Lam et al. 1998). It is notable that our results conflict with these previous data;

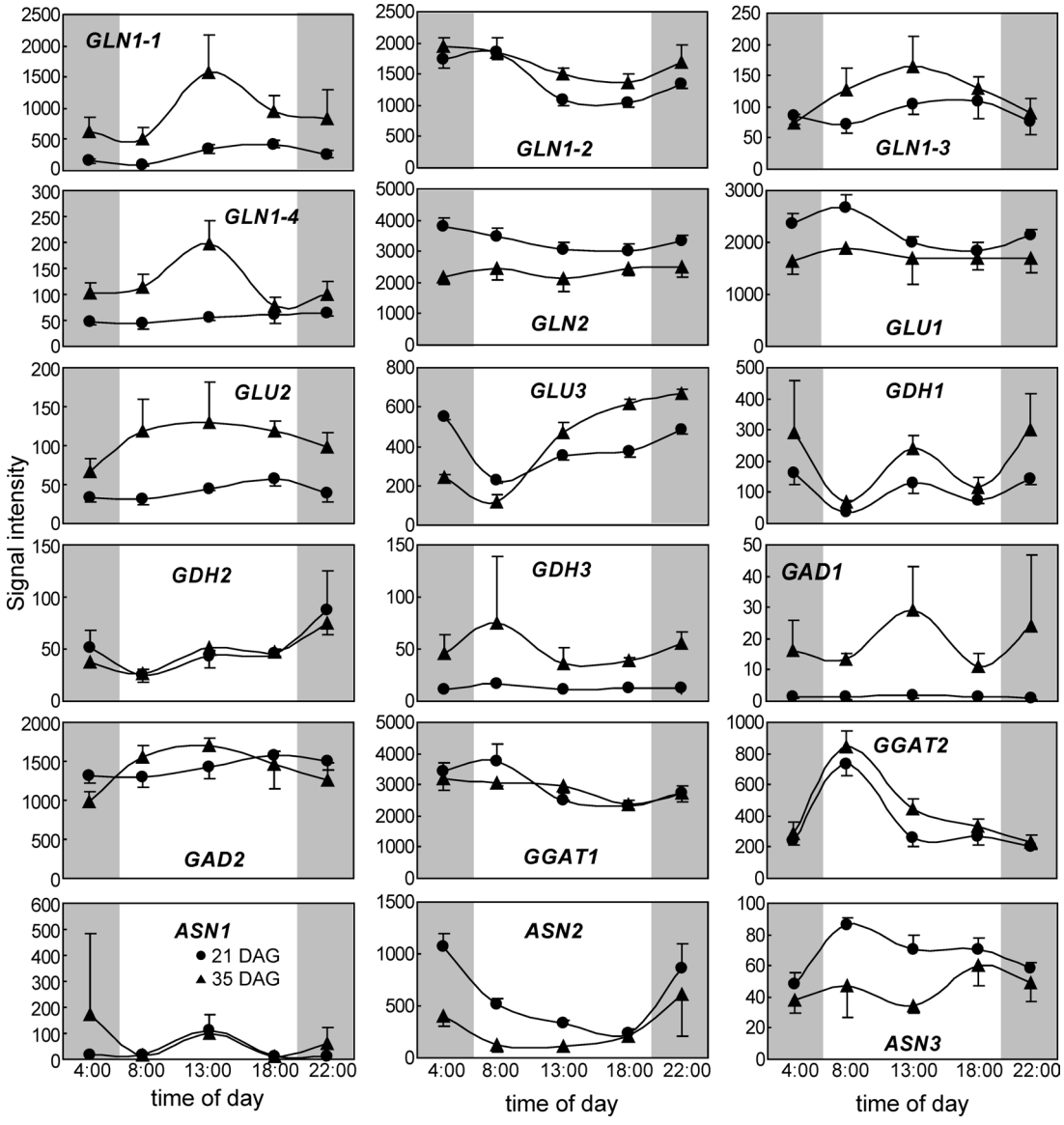

Figure 2. Diurnal changes in the expression of genes encoding major glutamate and glutamine metabolic enzymes. Wild-type (Col-0) plants were grown for 21 and 35 days. Freshly expanded rosette leaves (2 leaves per plant) were harvested at 5 time points and their gene expressions were determined by GeneChip analysis. From 6:00 to 20:00 is the light period. Information on each gene is listed in Table 1. The values presented are the means±SD of 3 plants.

Table 1. List of glutamine and glutamate metabolic genes.

Gene name	AGI code	GeneChip ID	Function
GLN1-1	At5g37600	249581_at	glutamine synthetase
GLN1-2	At1g66200	256524_at	glutamine synthetase
GLN1-3	At3g17820	258160_at	glutamine synthetase
GLN1-4	At5g16570	250100_at	glutamine synthetase
GLN2	At5g35630	249710_at	glutamine synthetase
GLU1	At5g04140	245701_at	ferredoxin-dependent glutamate synthase
GLU2	At2g41220	266365_at	ferredoxin-dependent glutamate synthase
GLU3	At5g53460	248267_at	NADH-dependent glutamate synthase
GDH1	At5g18170	250032_at	glutamate dehydrogenase
GDH2	At5g07440	250580_at	glutamate dehydrogenase
GDH3	At3g03910	259346_at	glutamate dehydrogenase
GAD1	At5g17330	250090_at	glutamate decarboxylase
GAD2	At1g65960	261970_at	glutamate decarboxylase
GGAT1	At1g23310	262988_at	glutamate glyoxylate aminotransferase
GGAT2	At1g70580	260309_at	glutamate glyoxylate aminotransferase
ASN1	At3g47340	252415_at	asparagine synthetase
ASN2	At5g65010	247218_at	asparagine synthetase
ASN3	At5g10240	250484_at	asparagine synthetase

however Lam et al. (1998) used somewhat artificial conditions of long-term dark and light exposure. The observed correlation between asparagine content and *ASN2* gene expression could therefore be a consequence of growing plants under normal light/dark conditions. Furthermore, gene expression analysis of circadian changes using microarrays indicated that *ASN1* expression increases during the daytime (Harmer et al. 2000). The results of our experiments led us to propose that *ASN2* rather than *ASN1* is the main regulator of asparagine content. We therefore selected *ASN2* as a target for the modification of amino acid contents and investigated the effect of modified *ASN2* gene expression using transgenic technology.

Generation of ASN2-over/underexpressing plants

We generated *ASN2*-overexpressing *Arabidopsis* plants using a standard method. We isolated *ASN2* cDNA from *Arabidopsis* by RT-PCR and cloned this into a binary vector (pBIDAVL-GWR1) that is used for the expression of target genes under the control of a CaMV 35S promoter (Nakazawa et al. 2003). Single locus insertions were identified by a 3 : 1 ratio (marker resistant versus sensitive) among the T2 progeny. We succeeded in isolating 4 *ASN2*-overexpressing plants (Figure 3A) and 4 *ASN2*-underexpressing plants (Figure 3B). The underexpression could be a result of overexpression-mediated gene silencing. Homozygous of T3 or T4 progenies were used for subsequent assays. Phenotypic observation indicated that *ASN2*-overexpressing plants grew normally, whereas the growth of *ASN2*-underexpressing plants was suppressed and the shape of

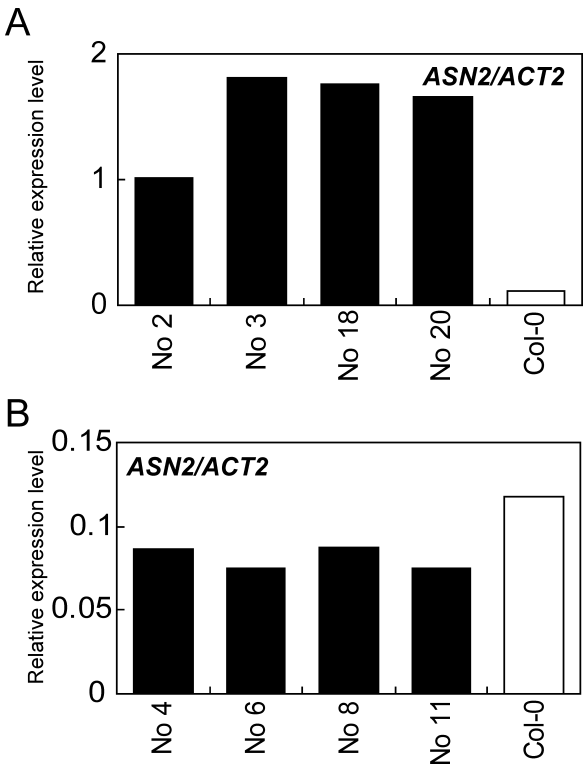

Figure 3. Levels of *ASN2* mRNA in *ASN2*-over- and underexpressing plants. Four independent T2 transgenic plants and the wild-type (Col-0) were grown on agar plates for 2 weeks. Aboveground tissues were harvested at midday and *ASN2* and *ACT2* mRNA levels were measured by means of real-time PCR. The gene expression levels were normalized by the expression of the actin gene (*ACT2*; At3g18780). A, overexpressing plants; B, underexpressing plants.

their leaves was slightly narrower compared to the wild-type plants (data not shown). Consequently, the role of *ASN2* in amino acid metabolism was analyzed by using *ASN2*-over/underexpressing plants.

Determination of amino acid contents in ASN2 gene-modified plants

ASN2-overexpressing plants were grown on agar plates under conditions of modified nitrogen nutrition—nitrate and/or ammonium as nitrogen sources at different concentrations. It has been suggested that asparagine synthetase functions in the detoxification of high concentrations of ammonium and asparagine accumulated under conditions of ammonium excess (Givan 1979; Stewart 1979; Wong et al. 2004). Furthermore, *ASN2* gene expression is induced by ammonium and is correlated with ammonium concentration, suggesting that *ASN2* functions in the control of ammonium metabolism (Wong et al. 2004). In the present study, *ASN2*-overexpressing plants accumulated asparagine under all growth conditions (Figure 4). When grown on medium containing ammonium succinate, there was a marked accumulation of asparagine in the *ASN2*-overexpressing plants. Under

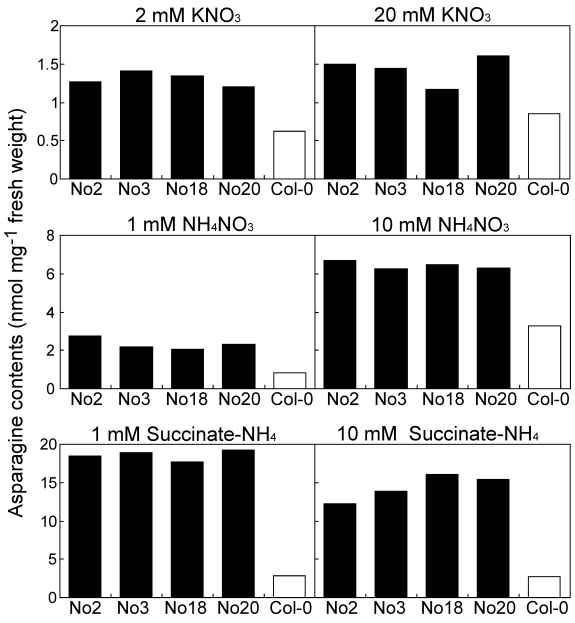

Figure 4. Asparagine content in the *ASN2*-overexpressing line of *Arabidopsis*. Four independent *ASN2*-overexpressing lines (T3) and the wild-type were grown for 2 weeks under modified (different forms/concentrations) nitrogen conditions. Aboveground tissues were harvested at midday and amino acid contents were determined. Each sample was a mix of 6 individual seedlings.

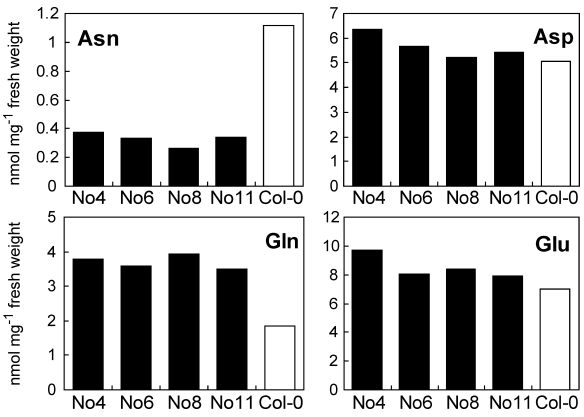

Figure 5. Amino acid content in the *ASN2*-underexpressing line of *Arabidopsis*. Four independent *ASN2*-underexpression lines (T3) and the wild-type were grown for 2 weeks. Aboveground tissues were harvested at midday and amino acid contents were determined. Each sample was a mix of 6 individual seedlings.

these growth conditions, the asparagine content in the wild-type plant was also higher than that of plants grown on other nitrogen-containing medium. Other amino acid contents were not, however, modified by *ASN2* overexpression under the various growth conditions we investigated (data not shown). *ASN2*-underexpressing plants were grown on normal agar plates and the amino acid contents in leaves were determined. As shown in Figure 5, the content of glutamine, which is a substrate of ASN, was increased, whereas asparagine content was decreased. The contents of glutamate and aspartate were also increased, suggesting that the accumulated glutamine was metabolized to these amino acids. These results suggest that *ASN2* is a factor that is closely related to asparagine content under normal day/night conditions. Previous studies have indicated that *ASN1* rather than *ASN2* controls the asparagine content and that *ASN2* is related to the metabolism of ammonium that is generated by the degradation of protein under stress conditions (Lam et al. 1998; Lam et al. 2003). Indeed, *ASN1* expression is regulated by ammonium, whereas it was not detected in the presence of methionine sulfoximine, which is an inhibitor of glutamine synthetase. On the other hand, *ASN2* gene expression is regulated by inorganic nitrogen (Gutiérrez et al. 2008). *ASN2*-overexpressing plants accumulate amino acids under conditions in which ammonium is the sole nitrogen source, suggesting that *ASN2* functions in ammonium detoxification and effective nitrogen assimilation under ammonium-sufficient conditions. Asparagine content was decreased in *ASN2*-underexpressing plants, indicating that *ASN2* could be a key factor in asparagine biosynthesis and ammonium metabolism.

In this study, we attempted to identify the key factor that regulates major amino acid metabolisms. As a result, it was indicated that *ASN2*, which is one of the ASN isoenzymes (*ASN1*, *ASN2*, and *ASN3*), is related to asparagine synthesis and ammonium assimilation. This relationship was suggested from the correlation of amino acid and gene expression profiles during the diurnal cycle, and was confirmed by analyzing the *ASN2*-overexpressing and -underexpressing plants. Amino acids function as nitrogen donors for many nitrogenous compounds and are metabolized actively during the synthesis of proteins, nucleic acids, and related nitrogenous compounds. This suggests that it will be difficult to isolate the key factor solely by correlation analysis of metabolite contents and related gene expressions. This study has provided a functional methodology for investigating the relationships between diurnally regulated metabolites and related enzymes.

In future, we will attempt to determine the factors that affect the regulation of total amino acid content and to enhance amino acid accumulation.

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