Correlation of *ASN2* Gene Expression with Ammonium Metabolism in Arabidopsis[1]

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In Arabidopsis, asparagine (Asn) synthetase is encoded by a small gene family (*ASN1*, *ASN2*, and *ASN3*). It has been shown that *ASN1* and *ASN2* exhibit reciprocal gene expression patterns toward light and metabolites. Moreover, changes in total free Asn levels parallel the expression of *ASN1*, but not *ASN2*. In this study, we show that *ASN2* expression correlates with ammonium metabolism. We demonstrate that the light induction of *ASN2* is ammonium dependent. The addition and removal of ammonium exerted fast and reciprocal effects on the levels of *ASN2* mRNA, specifically under light-grown conditions. NaCl and cold stress increased cellular free ammonium and *ASN2* mRNA levels in a coordinated manner, suggesting that the effects of stress on *ASN2* expression may be mediated via accumulation of ammonium. The correlation between *ASN2* and cellular ammonium metabolism was further demonstrated by analysis of *ASN2* transgenic plants. When plants were grown on Murashige and Skoog medium containing 50 mM ammonium, *ASN2* overexpressors accumulated less endogenous ammonium compared with the wild-type Colombia-0 and *ASN2* underexpressors. When plants were subjected to high-light irradiance, ammonium levels built up. Under such conditions, *ASN2* underexpressors accumulated more endogenous ammonium than the wild-type Colombia-0 and *ASN2* overexpressors. These results support the notion that *ASN2* is closely correlated to ammonium metabolism in higher plants.

To systematically study the differential physiological roles of different members in the asparagines synthetase (AS; the enzyme catalyzing the biosynthesis of asparagines) gene family, we previously cloned all the members of AS gene in the model plant Arabidopsis (*ASN1*, *ASN2*, and *ASN3*). Phylogenetic analysis revealed that although *ASN1* clustered with all dicot AS genes, *ASN2* and *ASN3* are more closely related to monocot AS genes (Lam et al., 1998). *ASN1* and *ASN2* were shown to be reciprocally regulated by light and metabolites in Arabidopsis (Lam et al., 1998). Based on the phylogenetic data and gene expression data, it was suggested that *ASN1* and *ASN2* may play very different physiological roles in plant nitrogen metabolism (Lam et al., 1998).

The expression level of *ASN1* is tightly correlated with free Asn levels (Lam et al., 1994, 1998, 2003). In wild-type Arabidopsis, *ASN1* and free Asn increase in dark-adapted plants, consistent with the idea that Asn is an important nitrogen carrier, especially under carbon-limiting conditions. In transgenic plants overexpressing the *ASN1* gene, free Asn levels in source and sink tissues as well as in the phloem increase accordingly. Total amino acid pools in seeds of the

ASN1 overexpressors are enhanced quantitatively and qualitatively (Lam et al., 2003). These findings suggest that *ASN1* plays a major role in nitrogen assimilation, regulating nitrogen transport and storage during seed development. These data are also consistent with the notion that free Asn is an important nitrogen carrier for long-range transport and storage in higher plants (Lea and Miflin, 1980; Sieciechowicz et al., 1988; Lea et al., 1990).

By contrast, studies have shown that although expressed at lower levels, the expression of *ASN2* is reciprocal to that of *ASN1*. The discovery of a light-induced *ASN2* gene was initially paradoxical because free Asn does not accumulate to a high level in light-grown plants (Lam et al., 1998). It was proposed that *ASN2* may play a role other than in primary nitrogen assimilation.

Previous physiological studies suggest that there may be some correlations between Asn accumulation and ammonium metabolism, especially under light conditions. For instance, it was shown in 1922 that when plants were grown in light with high levels of exogenous ammonium, levels of Asn accumulated (Prianischnikow, 1922). On the other hand, under high-light intensity, photorespiration occurs in C3 plants and leads to an increased production of ammonium (Magalhaes and Wilcox, 1984; Givan et al., 1988; Kozaki and Takeba, 1996; Wingler et al., 2000). Under such circumstances, Asn can account for up to 7% of the total nitrogen (Ta et al., 1984; Sieciechowicz et al., 1988). It has been proposed that Asn may play a role in ammonium detoxification (Kanamori and

Matsumoto, 1974; Givan, 1979; Stewart, 1979) and may act as an alternative nitrogen donor during photorespiration (Ta et al., 1984, 1985; Ta and Joy, 1986).

Ammonium accumulation may also occur when plants are under abiotic and biotic stresses. For instance, an increase of ammonium level was observed in tomato (*Lyocopersicon esculentum*) plants subjected to water and salinity stresses (Feng and Barker, 1993). On the other hand, when tomato was infected with root-knot nematode, foliar accumulation of ammonium was observed (Barker, 1999a). In fact, ammonium accumulation can be generally viewed as an index of stress in plants (Barker, 1999b).

Interestingly, although water and salinity stresses lead to ammonium accumulation, these stresses also enhance cellular free Asn levels. For example, when soybean (*Glycine max*) was subjected to severe water stress, 54% loss of leaf protein was balanced by a gain in the free amino acids in which 41% accumulated as Pro and Asn (Fukutoku and Yamada, 2002). Prolonged salt treatment also resulted in an increase of Asn and other amino acids in source tissues of *Coleus blumei*. The accumulation of Asn was at least partially due to de novo synthesis (Gilbert et al., 1998). It was also reported that genes encoding Asn synthetase in maize (*Zea mays*) are up-regulated by salt and heavy metal stresses (Chevalier et al., 1996).

The above findings suggest a correlation between Asn biosynthesis, ammonium accumulation, and stress conditions in higher plants. However, the possible regulation of AS genes in relation to ammonium metabolism remains unexplored. Because multiple AS isozymes (ASN1, ASN2, and ASN3) exist in the plant and AS activities are proven to be difficult to assay in vitro (Kern and Chrispeels, 1978; Joy et al., 1983; Huber and Streeter, 1985; Sieciechowicz et al., 1988), we addressed the question using molecular and transgenic approaches. In this report, we provided in vivo evidence showing that *ASN2* gene expression correlates with changes in ammonium metabolism in Arabidopsis.

RESULTS

### Ammonium Is Required for Light Induction of *ASN2*

It was shown that supplementation of exogenous ammonium leads to Asn accumulation in plants (Prianischnikow, 1922). Therefore, we examined the regulation of *ASN* gene expression in Arabidopsis in response to light and ammonium treatment. In previous studies, we demonstrated that light induces *ASN2* expression and represses *ASN1* expression in plants grown in Murashige and Skoog media (Lam et al., 1998). Here, we show that the light regulation of *ASN2*, but not *ASN1* is dependent on the presence of ammonium in the media (Fig. 1). When the plants were grown on regular Murashige and Skoog medium (20 mM ammonium/40 mM nitrate) for 48 h under continuous light (Fig. 1, lane 1), *ASN2* mRNA
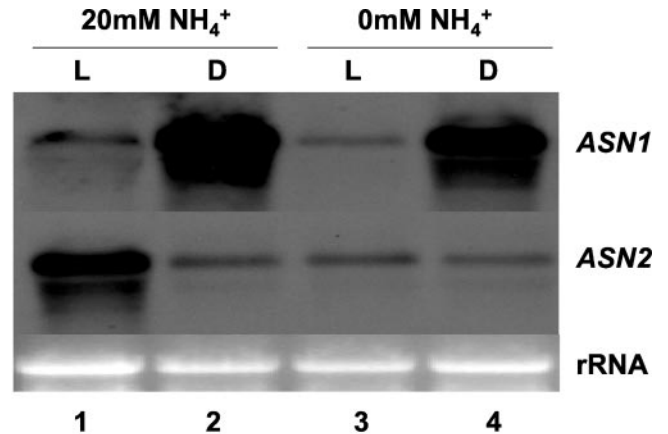
Figure 1. Ammonium controls the light induction of *ASN2*. Twelve-day-old seedlings grown on Murashige and Skoog agar plates under a regular day/light cycle (16 h of light and 8 h of dark) were transferred to new Murashige and Skoog medium containing 20 mM (Lanes 1 & 2) or no (Lanes 3 & 4) ammonium. NaNO$_3$ was added to ammonium-free medium to maintain an equal molar concentration of nitrogen resources. Plants were subsequently treated under continuous light (L) or continuous dark (D) conditions for 48 h. Total RNA was extracted from the seedlings as described in "Materials and Methods." An aliquot of 15 μg of total RNA from each line was loaded onto each lane. Northern-blot analysis was performed as described in "Materials and Methods."

level was increased compared with plants that had been dark adapted for 48 h (Fig. 1, compare lanes 1 and 2). This result is consistent with previous findings on light induction of *ASN2* gene expression (Lam et al., 1998). However, if ammonium was removed from the growth medium, no light induction of *ASN2* could be observed (Fig. 1, compare lanes 3 and 4). By contrast, the presence of ammonium has no effect on the light repression of *ASN1* gene expression (Fig. 1).

We further investigated the kinetics with which ammonium effects on *ASN2* mRNA accumulation. For this, plants were first grown on ammonium-free Murashige and Skoog medium for 14 d before being transferred to growth medium containing 20 mM ammonium (Fig. 2A). Induction of *ASN2* mRNA levels was first observed 4 h after the switch to the ammonium-containing media (Fig. 2A, lane 3). Maximal induction of *ASN2* mRNA was observed after 24 h (Fig. 2A, lane 7). A reciprocal experiment was performed by first growing the plants on regular Murashige and Skoog medium containing 20 mM ammonium before transferring the plants to ammonium-free Murashige and Skoog medium (Fig. 2B). An initial decrease of *ASN2* mRNA was observed 2 h after the transfer (Fig. 2B, lane 2), and maximal repression was observed after 16 h (Fig. 2B, lane 7). The reduction of *ASN2* mRNA in ammonium-free medium was not due to nitrogen deprivation because additional nitrate was added to the ammonium-free medium to maintain an equal nitrogen supply. The above data further supports
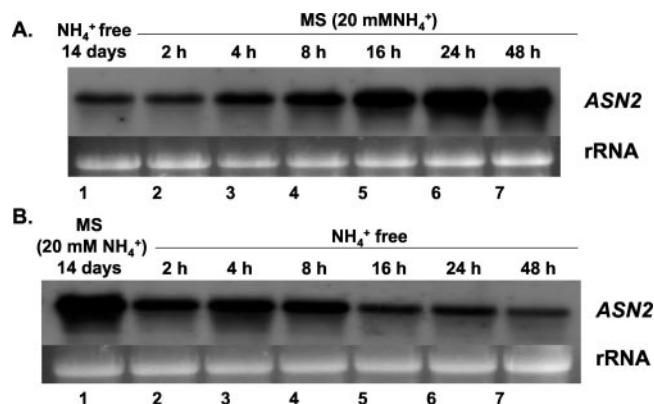
Figure 2. Ammonium controls the light induction of *ASN2*. Seedlings were germinated and grown under a regular day/light cycle on Murashige and Skoog agar plates containing no (A) or 20 mM (B) ammonium for 14 d before being transferred to Murashige and Skoog agar plates containing 20 mM (A) and no (B) ammonium respectively, and grown under continuous light. NaNO₃ was added to ammonium-free medium to maintain an equal molar concentration of nitrogen resource. Samples were collected at time 0, 2, 4, 8, 16, 24, and 48 h (lanes 1–7, respectively) after treatments. Total RNA was extracted from the seedlings as described in "Materials and Methods." An aliquot of 15 μg of total RNA from each line was loaded onto each lane. Northern-blot analysis was performed as described in "Materials and Methods."

that *ASN2* gene expression is strongly dependent on the presence of ammonium, especially under light-grown conditions.

Stresses Increased Steady-State mRNA Level of *ASN2* and Cellular Ammonium Contents

The *ASN2* gene of Arabidopsis has been shown to group phylogenetically with the maize AS gene (Lam et al., 1998). Because the maize AS gene was found to be induced by stresses such as NaCl (Chevalier et al., 1996), we tested the effects of NaCl and cold stress on the levels of *ASN2* mRNA in Arabidopsis. Plants were first grown on regular Murashige and Skoog medium before being subjected to NaCl and cold treatments (Fig. 3). Leaf samples were collected at 4, 8, and 16 h after each treatment. Northern-blot analysis using total RNA extracted from the leaf samples showed that *ASN2* mRNA levels were increased at time points 8 and 16 h for NaCl (Fig. 3A, lanes 5 and 6) and cold (Fig. 3B, lanes 5 and 6) treatments. Ammonium content in the same batch of leaf samples was also determined (Fig. 4). A significant increase of ammonium content was observed at time points 8 and 16 h for NaCl (Fig. 4, lanes 5 and 8) and cold (Fig. 4, lanes 6 and 9) treatments, matching the induction of the *ASN2* mRNA levels (Fig. 3). In control plants without stress treatments, no increase of *ASN2* mRNA (Fig. 3, A, lanes 1–3, and B, lanes 1–3) or ammonium content (Fig. 4, lanes 1, 4, and 7) was observed. These findings suggest that the induction of *ASN2* expression by NaCl and cold stress may be
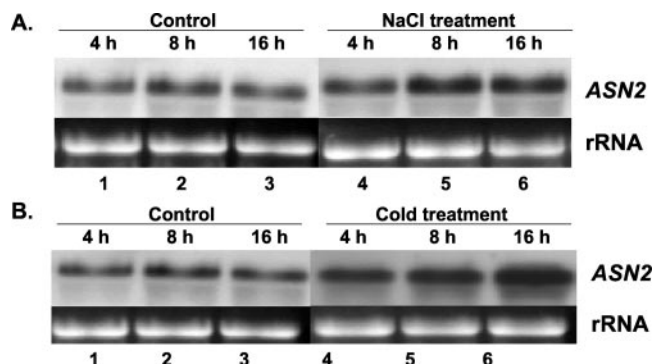

Figure 3. NaCl and cold stresses increase the levels of *ASN2* mRNA. The effects of NaCl (200 mM; A) and cold (10°C; B) on the levels of *ASN2* mRNA were tested. Twelve-day-old seedlings grown on Murashige and Skoog agar plates under a regular day/light cycle were transferred to new growth medium (A) or were subjected to new growth temperature (B) for 4 (lane 4), 8 (lane 5), and 16 h (lane 6). Samples of control plants without stress treatments were also collected at same time points (lanes 1–3). Total RNA was extracted from the seedlings as described in "Materials and Methods." An aliquot of 15 μg of total RNA from each line was loaded onto each lane. Northern-blot analysis was performed as described in "Materials and Methods."

mediated through accumulation of ammonium under these stress conditions. This hypothesis is further supported by our data that the *ASN2* gene is specifically responsive to ammonium treatments (Figs. 1 and 2).

## Overexpression or Underexpression of *ASN2* Alters Ammonium Content in Arabidopsis

To further investigate the possible relation between *ASN2* and ammonium metabolism, transgenic Arabi-
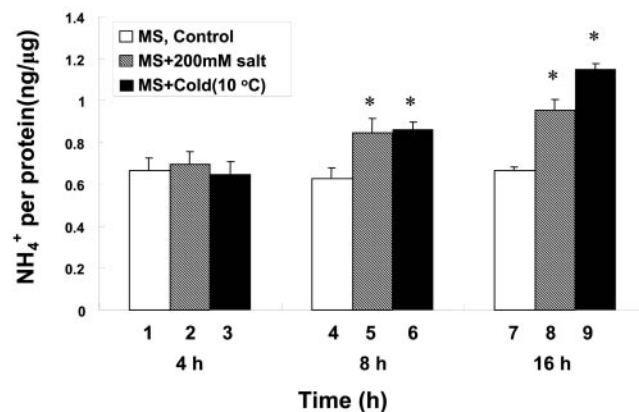
Figure 4. Endogenous free ammonium content increases under stress treatments. Samples were harvested as described in Figure 3. Free ammonium content was assayed as described in "Materials and Methods." Each bar represents an average of five samples. Error bars = SEs. The data was analyzed by one-way analysis of variance (ANOVA) followed by LSD test. An asterisk indicates significant difference when compared NaCl- (lanes 2, 5, and 8) and cold- (lanes 3, 6, and 9) treated samples to untreated controls (lanes 1, 4, and 7), with a *P* value less than 0.05.

dopsis lines overexpressing or underexpressing the *ASN2* gene were produced. In *ASN2* underexpressors, the coding region of *ASN2* was expressed in antisense orientation under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The level of *ASN2* mRNA was found to decrease, especially in light-grown conditions (Fig. 5, lane 3). In *ASN2* overexpressors, the coding region of *ASN2* was placed in sense orientation under the control of the CaMV 35S promoter. Native *ASN2* is expressed at higher levels in the light compared with dark (Fig. 5, lanes 1 and 2). By contrast, in *ASN2*-overexpressing transgenic plants, constitutive expression of *ASN2* was observed and the light control of *ASN2* was deregulated (Fig. 5, lanes 5 and 6). Figure 5 showed the results of one typical antisense (lanes 3 and 4) and one typical sense (lanes 5 and 6) lines. Other antisense and sense lines used in the research exhibited similar *ASN2* expression patterns (data not shown).

To test if the misexpression of *ASN2* in transgenic plants has effects on the level of endogenous ammonium, two sets of experiments were performed. When the ammonium concentration in the growth medium was increased from 20 mM (in regular Murashige and Skoog medium) to 50 mM, endogenous ammonium content started to increase accordingly in all lines (Fig. 6). However, the overexpressing lines exhibited lower endogenous ammonium contents compared with wild type when grown on Murashige and Skoog medium containing 50 mM ammonium (Fig. 6, lanes 9 and 10) compared with the wild-type (Colombia) Col-0 (Fig. 6, lane 6). One *ASN2*-overexpressing line also showed a slight decrease in ammonium content even at the lower concentration of ammonium (Fig. 6, lane 4) compared with wild type. On the other hand, no significant difference was observed in ammonium content when comparing the underexpressing lines and Col-0 grown on 50 mM ammonium (Fig, 6, lanes 7 and 8).
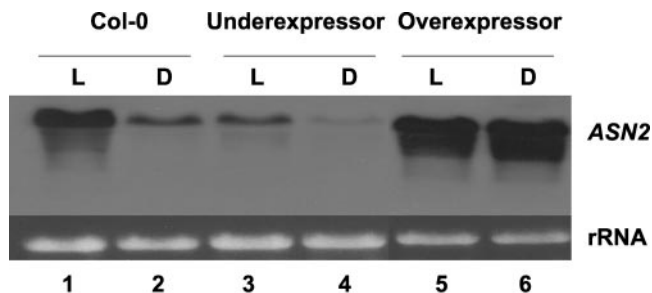
Figure 5. Northern-blot analysis of *ASN2* transgenic lines. Twelve-day-old seedlings of the wild-type Col-0 (lanes 1 and 2), the *ASN2*-underexpressing line 401-A4-c1 (lanes 3 and 4), and the *ASN2*-overexpressing line 402-A1-b1 (lanes 5 and 6) grown on Murashige and Skoog agar plates under a regular day/light cycle were subsequently treated under continuous light (L) or continuous dark (D) conditions for 48 h. Total RNA was extracted from the seedlings as described in "Materials and Methods." An aliquot of 15 μg of total RNA from each line was loaded onto each lane. Northern-blot analysis was performed as described in "Materials and Methods."
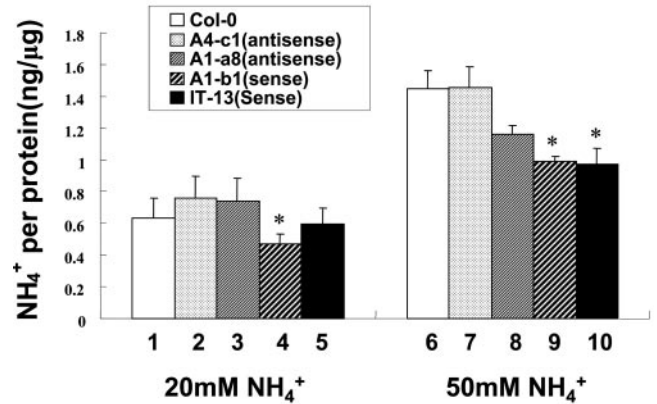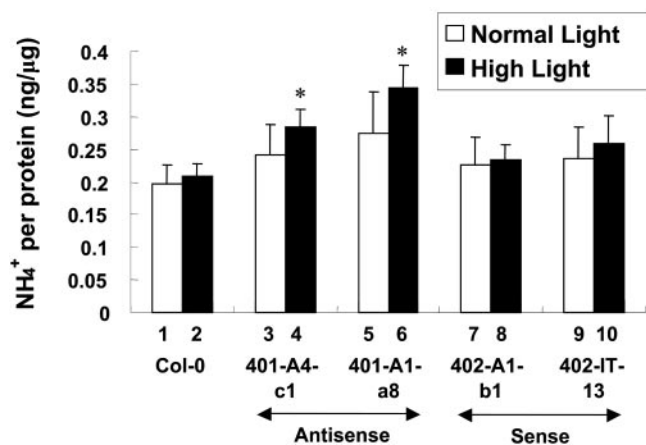
Figure 6. Endogenous free ammonium content decreases in *ASN2*-overexpressing transgenic lines grown on ammonium-containing medium. Ten-day-old seedlings of the wild-type Col-0 (lanes 1 and 6), *ASN2*-underexpressing lines 401-A4-c1 (lanes 2 and 7) and 401-A1-a8 (lanes 3 and 6), and *ASN2*-overexpressing lines 402-A1-b1 (lanes 4 and 9) and 402-IT-13 (lanes 5 and 10) grown under a regular day/light cycle on regular Murashige and Skoog (containing 20 mM ammonium) agar plates were transferred to Murashige and Skoog medium supplemented with 20 (lanes 1–5) or 50 mM (lanes 6–10) exogenous ammonium. Free ammonium content was assayed as described in "Materials and Methods." Each bar represents an average of five samples. Error bars = SEs. The data was analyzed by one-way ANOVA followed by LSD test. An asterisk indicates significant difference with a *P* value less than 0.05.

To directly increase the internal pool of ammonium without external supplements, the transgenic lines were subjected to high-light irradiance that may increase the levels of photorespiratory ammonium (Magalhaes and Wilcox, 1984; Givan et al., 1988; Kozaki and Takeba, 1996; Wingler et al., 2000). Under high-light conditions, *ASN2* underexpressors accumulated significantly higher levels of cellular ammonium (Fig. 7, lanes 4 and 6) when compared with Col-0 (Fig. 7, lane 2). Such differences were not significant when the comparison was made under low-light irradiance (Fig. 7, lanes 1, 3, and 5). No significant difference was found between the overexpressing lines (Fig. 7, lanes 7–9) and Col-0 (Fig. 7, lanes 1 and 2).

The loss-of-function and gain-of-function approaches using transgenic lines described above thus showed that *ASN2* overexpressors accumulated lower levels of ammonium, whereas the *ASN2* underexpressors accumulated higher levels of ammonium. This data suggests that *ASN2* gene expression levels correlate with changes in ammonium metabolism in Arabidopsis.

DISCUSSION

Drastic changes in the levels of free Asn under different physiological and environmental conditions have suggested that Asn may play an important role in various aspects of plant physiology and metabolism (Sieciechowicz et al., 1988). In general, AS en-

Figure 7. Endogenous free ammonium content increases in *ASN2* antisense transgenic lines grown under high-light irradiance. Twelve-day-old seedlings of the wild-type Col-0 (lanes 1 and 2), *ASN2*-underexpressing lines 401-A4-c1 (lanes 3 and 4) and 401-A1-a8 (lanes 5 and 6), and *ASN2*-overexpressing lines 402-A1-b1 (lanes 7 and 8) and 402-IT-13 (lanes 9 and 10) grown under a regular day/light cycle at low light irradiance (35 μE) on regular Murashige and Skoog agar plates were exposed to continuous light at low (35 μE; lanes 1, 3, 5, 7, and 9) or high (150 μE; lanes 2, 4, 6, 8, and 10) for 72 h. Free ammonium contents were assayed as described in "Materials and Methods." Each bar represents an average of five samples. Error bars = SEs. The data was analyzed by one-way ANOVA followed by LSD test. An asterisk indicates significant difference with a *P* value less than 0.05.

zymes were found to be encoded by a small gene family in various species of legume and nonlegume plants (for example, Davis and King, 1993; Waterhouse et al., 1996; Hughes et al., 1997; Lam et al., 1998). Expression of AS genes is under the control of light, metabolites, postharvesting conditions, and stress treatments (Tsai and Coruzzi, 1990, 1991; Davis and King, 1993; Lam et al., 1994, 1998; Chevalier et al., 1996). These complex modes of regulation of AS gene expression support the notion that AS genes may play different roles in various aspects of plant physiology.

In this study, we found that the expression of *ASN2* is correlated with ammonium metabolism. We showed that when plants were grown on Murashige and Skoog media, the light induction of *ASN2* is ammonium dependent. Time kinetics studies of *ASN2* expression revealed a rapid and reciprocal control of *ASN2* by ammonium supplementation or deprivation (Fig. 2). To our knowledge, it is the first report on ammonium induction of an AS gene. In addition to ammonium, we showed that stresses such as salinity and cold also increase *ASN2* mRNA levels, and that these stresses correlate with increases in internal ammonium (Figs. 3 and 4). The effect of stresses on the induction of AS genes was shown in maize previously, but the relationship to cellular ammonium accumulation was not addressed (Chevalier et al., 1996).

Interestingly, there is a close relationship between ammonium accumulation and stress conditions. For instance, ammonium accumulates under various biotic and abiotic stresses (Feng and Barker, 1993; Barker, 1999a, 1999b). Under stress conditions, the photosynthetic rate will drop significantly and hence the carbon skeleton reaching the sink tissues may become limiting (Farrar, 1981; Kerr et al., 1985; Baysdorfer et al., 1998). Nitrogen assimilation and protein synthesis will decrease and catabolism of amino acids will increase to provide the carbon skeleton to sustain respiration and other metabolic processes (Saglio and Pradet, 1980; Journet et al., 1986; Brouquisse et al., 1991; Dieuaide et al., 1992, 1993; Baysdorfer et al., 1998). Extensive protein degradation leads to a drastic increase of internal ammonium content (Feng and Barker, 1992). To save valuable nitrogen resources, it is important for plants to recapture the ammonium otherwise lost via protein degradation. Nitrogen resources in the form of ammonium will be mainly recaptured by the enzymes Gln synthetase-Glu oxoglutarate aminotransferase that function in a cycle. We have shown that *ASN2* is induced by ammonium treatments (Figs. 1 and 2) and by stress treatments that cause ammonium accumulation (Figs. 3 and 4). These findings suggest that the physiological role of *ASN2* may be related, directly or indirectly, to the recapturing of lost nitrogen resources under stress conditions. By contrast, the role of *ASN1* may be related to primary nitrogen assimilation and transport.

To further investigate the possible interaction between *ASN2* and ammonium metabolism, transgenic Arabidopsis lines overexpressing or underexpressing the *ASN2* gene were constructed (Fig. 5). Under high concentrations of exogenous ammonium supplies, endogenous ammonium accumulates in the treated plants (Fig. 6). However, *ASN2* overexpressors exhibit a significantly lower level of endogenous ammonium under such conditions compared with wild type. Although ammonium is an important intermediate in nitrogen assimilation, high ammonium concentration is toxic to plants (Vines and Wedding, 1960; Fangmeier et al., 1994; Gerendas et al., 1997). Previous studies showed that Asn accumulates under high levels of exogenous ammonium (Prianischnikow, 1922) and that Asn may play a role in ammonium detoxification (Kanamori and Matsumoto, 1974; Givan, 1979; Stewart, 1979). Together with our finding that overexpression of *ASN2* can reduce levels of endogenous ammonium, it reaffirms our hypothesis that *ASN2* plays an important role in ammonium metabolism in Arabidopsis.

Under high-light irradiance, photorespiration is enhanced and leads to an increase in the loss of fixed carbon and fixed nitrogen as carbon dioxide and ammonium, respectively (Magalhaes and Wilcox, 1984; Givan et al., 1988; Kozaki and Takeba, 1996; Wingler et al., 2000). In accordance with this, we

observed a significant increase of ammonium in wild-type plants grown under high-light irradiance compared with plants that received lower light irradiance (Fig. 7). However, *ASN2* underexpressors showed a significant increase in levels of ammonium, especially when grown under high-light irradiance conditions. The data of our loss-of-function experiment suggests a possible relationship between the level of *ASN2* gene expression and the level of ammonium loss via the photorespiratory pathway. It has been reported that Asn may act as an alternative nitrogen donor in the photorespiratory pathway in pea (*Pisum sativum*; Ta et al., 1984, 1985; Ta and Joy, 1986).

Whether *ASN2* directly or indirectly involved in ammonium metabolism is still unclear. Some studies suggested that monocot AS enzymes may use ammonium as the direct substrate (Oaks and Ross, 1984; Oaks and Hirel, 1985). The protein encoded by the Arabidopsis *ASN2* gene is actually more closely related to monocot AS proteins than to the ASN1 protein that clusters with dicot AS enzymes in a phylogeny tree (Lam et al., 1998). However, only a separate detailed enzyme kinetic studies of the purified ASN2 protein can give the ultimate answer. In planta studies of the AS activities in wild-type and transgenic plants has proven to be difficult due to instability of the enzyme, presence of inhibitors, and asparaginase activities (Kern and Chrispeels, 1978; Joy et al., 1983; Huber and Streeter, 1985; Sieciechowicz et al., 1988). As the transgenic results suggest that *ASN2* may be playing a special role in ammonia assimilation, we will attempt to validate this hypothesis in future biochemical studies of the ASN1 and ASN2 enzymes using a variety of heterologous expression systems.

MATERIALS AND METHODS

Plant Growth and Stress Treatments

Seeds were surface sterilized by bleach and washed with autoclaved distilled water before sowing on Murashige and Skoog (Murashige and Skoog, 1962) basal medium (Invitrogen, Carlsbad, CA; pH adjusted to 5.7 with KOH) containing 3% (w/v) Suc and 0.9% (w/v) bactoagar. After being imbibed at 4°C in dark for 2 d, the agar plates were transferred to environmentally controlled growth chambers with regular day/light cycle (16 h of light at 22°C and 8 h of dark at 20°C).

In experiments testing the ammonium dependence of light induction of *ASN2*, 12-d-old seedlings were transferred to new Murashige and Skoog medium containing 20 mM ammonium (concentration in regular Murashige and Skoog medium) or ammonium-free Murashige and Skoog medium that contained the same molar amount of nitrogen (supplied in form of NaNO$_3$). The plates were exposed to 48 h continuous light or continuous dark conditions before harvesting. In experiments showing the kinetics of ammonium effects on *ASN2* gene expression, 14-d-old seedlings originally grown in regular day/light cycle on Murashige and Skoog medium containing no or 20 mM ammonia were transferred to Murashige and Skoog medium containing 20 mM and no ammonia, respectively. The seedlings were allowed to grow under continuous light, and samples were harvested at 2, 4, 8, 16, 24, and 48 h after treatments.

In NaCl stress experiments, the seedlings were transferred to Murashige and Skoog medium supplemented with 200 mM NaCl. In cold stress experiments, the seedlings were transferred to regular Murashige and Skoog medium but were grown in a 10°C growth chamber. The samples were harvested at 4, 8, and 16 h after treatment. All treatments were performed during the regular day/light cycle.

To test the endogenous ammonium contents in *ASN2* transgenic lines when grown in medium supplemented with different concentrations of exogenous ammonium, 10-d-old seedlings were transferred to Murashige and Skoog medium containing 20 or 50 mM ammonium and were allowed to grow under regular day/light cycle for 5 d before harvesting.

To test the accumulation of ammonium under different light intensities, seeds were allowed to germinate on Murashige and Skoog medium containing 1% (w/v) Suc. Twelve-day-old seedlings grown under a regular day/light cycle (at in irradiance of 35 μE) were divided into two sets. The two sets of seedlings were exposed continuous low irradiance (35 μE) or high irradiance (150 μE) for 72 h before harvesting.

Construction of *ASN2* Transgenic Lines

The *ASN2* cDNA (Lam et al., 1998) was subcloned into a binary vector (Brears et al., 1993) and expressed under the control of the CaMV 35S promoter in sense or antisense orientation. Binary vector constructions were transformed into the disarmed *Agrobacterium tumefaciens* GV3101/pMP90 (Koncz and Schell, 1986) by electroporation. Vacuum infiltration in planta transformation (Bechtold and Pelletier, 1993) was performed using a modified protocol (Lam et al., 2003). The transformed plants were allowed to continue to grow and set seeds. Positive transformants were identified as green seedlings grown on Murashige and Skoog agar plates supplemented with 50 μg mL^{-1} kanamycin. Single-locus insertions were scored (using the chi-square test) by observing a 3:1 ratio (kanamycin resistant versus kanamycin sensitive) in T$_2$ progenies resulted from self-pollination of T$_1$ parents. Homologous T$_3$ progenies were used for subsequent assays.

RNA Analysis and Ammonium Assay

For RNA analysis, samples were freshly collected and frozen immediately in liquid nitrogen before grinding with a precooled mortar and pestle. Total RNA was extracted using a modified phenol extraction protocol (Jackson and Larkins, 1976). Digoxygenin-labeled RNA probes were generated by a commercial kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Northern-blot analysis was performed by hybridizing RNA blots with labeled probes at 68°C in 50% (v/v) formamide hybridization solution for at least 16 h. Posthybridization washing and chemiluminescent detection were carried out as described in the manufacturer's instructions. All northern analyses were conducted in biological replicates of two to three times. A sample northern blot was shown for each experimental design.

For free ammonium assays, plant tissues were harvested and frozen immediately in liquid nitrogen. Each sample was ground in 150 μL of protein grinding buffer (50 mM Tris-HCl, pH 8.0, 10 mM imidazole, and 0.5% [w/v] β-mercaptoethanol; Oliveira et al., 2002). The samples were vortexed vigorously and spun for 15 min at maximum speed in a microfuge. The upper layer was transferred to a new microtube, of which 50 μL was saved for the protein assay (Bradford, 1976). Free ammonium was assayed in the remaining sample using a commercial kit (Boehringer Mannheim, Mannheim, Germany).

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