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THE FIRST CO-TREATMENT (AMMONIUM-SALT) EFFECTS ON *GLN* GENE EXPRESSION AND AMMONIUM ASSIMILATION IN AN *Arabidopsis thaliana* WILD-TYPE (Col-0) AND AN ASN2 T-DNA KNOCKOUT MUTANT

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ABSTRACT

Wild-type *Arabidopsis thaliana* Col-0 and a knockout T-DNA mutant for the *ASN2* gene encoding Asparagine synthetase (AS, EC 6.3.5.4) (*asn2-1*) was used, to investigate the primary effects of ammonium under salt stress. Plants were grown for one month in hydroponic culture and then exposed to either 2 mM NH_4 or 2 mM NH_4+ and 100 mM NaCl, for 6 hours.

In the wild-type, the ammonium-salt co-treatment increased the activities of both glutamine synthetase (GS, EC 6.3.1.2) and the aminating glutamate dehydrogenase (GDH) (NADH-GDH, EC 1.4.1.2), thus can be linked to ammonium accumulation. A slight increase in GS and GDH activities were noted in the *asn2-1* lines, despite there being more endogenous ammonium accumulation. These results suggest that in *asn2-1* lines, GS and the aminating GDH activity increase may be mediated by other metabolites when the *ASN2* gene is lacking. The co-treatment elevated too, the expression of the genes *GLN1.1* and *GLN1.2* encoding for GS1 in both lines. The results suggest that the co-treatment led to the upregulation of

ammonium assimilating enzymes, GS and GDH. The slight enzyme activity increase in the mutant lines may suggest a potential role for Asparaginein response to salt stress.

Keywords: Ammonium assimilation, Arabidopsis thaliana, asparagine synthetase enzyme, glutamine synthetase, salt stress

INTRODUCTION

Soil salinity is a serious environmental problem that may lead to changes in physiological and molecular processes in plants. The presence of high amounts of salt in the soil causes a reduction in the water potential and limits the uptake of nutrients that are essential for normal plant growth and development **[1]**. Nitrogen is one of the nutrients whose uptake and assimilation is affected by soil salinity and is integral for plant health, as it is a main element of amino and nuclear acids **[2]**. As a result, nitrogen regulation plays a key role in the life cycle of the plants. Most plants source nitrogen from their soils in forms such asnitrate (NO_3) and ammonium (NH_4) **[2]**, and therefore, saline environments cause competition between salt and nitrogen uptake **[3]**. This in turn leads to several metabolic changes in plants. The relationship between nitrogen and salinity is complex, affecting almost all processes, including nutrient uptake and metabolism, and ion accumulation. NaCl has been shown to be the dominant salt in soils **[1]**. Therefore, the NaCl ions that compete with N uptake, may lead to a decline in N accumulation in plants. Such affects have been reported in many plant species, including *Gossypium hirsutum* L. **[4]** *Oryza sativa* L. **[5]** *Sesbania karadge* L. **[6]**, and*Ipomoea batatas* L. **[7]**. Additionally, excessive amounts of salt ions in the soil may lead to inactivation of NO_3-transporters, which causes lower N assimilation and synthesis of amino acids **[8]**. In addition to the salinity effects on N uptake from the soil, several nitrogen assimilation enzymes are also affected, such as nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), and glutamate synthase (GOGAT) **[9]**.

Besides the deleterious effect of soil salinity, the excessive availability of ammonium can be problematic to plant growth and development. It was reported that increased availability of ammonium in growing media can induce toxicity symptoms in plants, hence, several hypotheses were advanced to explain ammonium toxicity. First, as ammonium absorption is coupled with the H + release in the root medium, and this is also associated with the ammonium incorporation in proteins **[10]**, it was often

suggested that ammonium leads to root medium acidification **[11]** and\or the disturbance of the intracellular pH **[12, 13]**. In addition, other works suggested that the sugar limitation in shoots can also contribute to this toxicity syndrome. This hypothesis is based on the lack of ammonium transfer to shoots in most plants **[14]**. All carbon skeletons that are necessary for ammonium assimilation are supplied in roots and result in local sugar deficiencies in the shoots **[15]**. Further hypotheses suggested that the external contribution of 2-oxoglutarate, a key carbon source for nitrogen assimilation, limits the toxicity symptoms **[16]**. However, in the other cases, the contribution of 2-oxoglutarate failed to improve the ammonium assimilation **[17]**, which strongly suggests that there are other factors that influence ammonium assimilation. Furthermore, it was supposed for a long time that ammonium toxicity results from the decoupling of photophosphorylation in chloroplasts **[10]**. This hypothesis could be a culprit, as large ammonium concentrations do not affect this process in intact chloroplasts **[18]**. Finally, the decrease of essential element content as K+, Mg2+ and Ca2+ in plant tissues exposed to ammonium for a long time was widely informed **[19-21]**. Whereas this decrease may be one of the effects of the toxicity syndrome, as it is clearly an effect resulting from the nutrient imbalance **[12, 22]**.

The ammonium toxicity could also be due to the hydric balance disturbance **[23]**. The plant sensibility to ammoniacal toxicity varies according to species. Several studies indicated plant sensitivity to the presence of ammonium, in particular in barley **[24]** and pea **[25]**. Other species have been found to be more tolerant to ammoniacal nutrition, such as rice **[26]**.

Ammonium assimilation into glutamine and glutamate via GS and GOGAT are considered to be vital for plant growth as these two precursors are essential for the majority of nitrogenous compounds. Understanding the relationship between ammonium accumulation and the enzymes involved in its assimilation, such as asparagine synthetases, glutamine synthetases, and glutamate dehydrogenases, is important for improving crop yield and stress resistance. The enzyme asparagine synthetase is used in catalyzing asparagine synthesis, and it plays a role in ammonium assimilation in the presence of salt stress **[27]**. In Arabidopsis, three asparagine synthetase genes were identified, *ASN1*, *ASN3,* and *ASN2*. It was shown that the expression of *ASN1* in the leaves of Arabidopsis plants was up-regulated with salt treatments **[27]**. On the other hand, the enzyme glutamate dehydrogenase (GDH) is

also speculated to be involved in ammonium assimilation in response to environmental stress **[27]**. Maaroufi *et al*. (2011) **[27]** showed that the expression of chloroplastic glutamine synthetase 2 (*GLN2*) was inhibited in response to 100 mM NaCl stress for a short time, from 6 to 24 h.

The relationship among ammonium assimilation enzymes (GS and GDH) and the levels of NH4 in the presence of both NH_4 and salt (NaCl) in wild-type Arabidopsis Col-0 and knockout mutants with T-DNA insertions in the*ASN2* gene encoding asparagine synthetase (AS, EC 6.3.5.4) (*asn2-1*) were investigated. This relationship was assessed by growing the plants for four weeks in basic nutrient medium (leaves nitrate (LN): 12mM NO3-), and then transferring them to three different sets of treatments: a basic nutrient medium, basic nutrient medium with 2mM NH4+ (leaves ammonium (LA)), and a co-treatment which includes a basic nutrient medium with 2mM NH4+ and 100mM NaCl, for a short time (6 hours). The soluble protein and ammonium content were evaluated in each treatment and ammonium-assimilating enzyme activities, namely GS and GDH enzymes, were investigated.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana wild-type Col-0 and the *ASN2* T-DNA mutants were used for the experiments. *Arabidopsis thaliana* (Col-0) with T-DNA insertions into intron 3 were isolated, and the homozygous knockout line (*asn2-1* mutant) was characterized **[28]**. Seeds were sterilized and stratified at 4°C for 4 days. Plants were grown under hydroponic culture in a growth chamber with an 8-h-light/16-h-dark cycle and 80 % relative humidity. The plants were supplied with the basic nutrient medium containing 8 mM KNO_3, 1 mM $MgSO_4$, 1mM KH_2PO_4, 2mM $CaNO_3$, 5µM $MnSO_4$, 30µM H_3BO_3, 1µM $ZnSO_4$, 1µM $CuSO_4$, and 30µM K-iron-EDTA. Plants were grown for 4 weeks and were then divided into 3 groups. The first was maintained on the same media (control). The second and third groups were transferred to basic nutrient medium containing 2 mM NH_4+, and both 2 mM NH_4+ and 100mM NaCl, respectively. The treatments were applied for 6 hours.

Ammonium content

Ammonium was extracted from the leaves at 4°C with 0.3 mM H_2SO_4 and 0.5 % (w/v) polyclar AT. The ammonium content was quantified according to the reaction of Berthelot modified by Weatherburn (1967) **[29]**.

Protein content

Soluble protein contents in both lines and all treatments were quantified using

Coomassie Brilliant Blue (Bradford 1976) **[30]** with bovine serum albumin as a protein standard.

RNA isolation, cDNA synthesis, and quantitative real-time PCR analysis

Total RNA was extracted from leaves using Trizol reagent. Genomic DNA was removed using DNAse (Ambion Europe Ltd, Huntingdon, UK), then RNA quality was determined by agarose gel electrophoresis and spectrophotometer. One microgram of total RNA was used to prepare cDNA by reverse transcription with M-MuLV reverse transcriptase (Biolab) and oligo (dT) 18 primers, according to the manufacturer's protocol. Transcript levels were estimated by quantitative real-time PCR (MyiQ real-time PCR detection system, Bio-Rad) using the gene-specific primers. PCR was carried out with iQ SYBR Green supermix (Bio-Rad) following the manufacturer's instructions.

Enzyme assays

Glutamine synthetase

The enzyme essay was carried out on each line and each treatment. Frozen samples were homogenized in a cold mortar and pestle with grinding medium containing 25mM Tris-HCl buffer (pH 7.6), 1mM $MgCl_2$, 1mM EDTA, 14mM 2-mercaptoethanol and 1 % (w/v) polyvinyl-pyrrolidone (PVP). The homogenate was centrifuged at 25000 g for 30 min at 4°C. GS activity was determined using hydroxylamine as a substrate, and the formation of γ-glutamylhydroxamate (γ-GHM) was quantified with acidified ferric chloride **[31]**.

Glutamate dehydrogenase

The enzyme essay was carried on each line and on each treatment. GDH extractions were performed according to the method described by Magalhaes and Huber (1991) **[32]**. Frozen samples were homogenized in a cold mortar and pestle with 100mM Tris-HCl (pH 7.5), 14mM 2-mercaptoethanol, and 1 % (w/v) PVP. The extract was centrifuged at 12000 g for 15 min at 4°C. GDH activity was determined by following the absorbance changes at 340 nm.

Western blot analysis

Proteins were extracted from frozen leaf material in cold extraction buffer containing 50mM Tris-HCl (pH 7.5), 1mM EDTA, 1mM MgCl2, 0.5 % (w/v) PVP, 0.1 % (v/v) 2-mercaptoethanol, and 4mM leupeptin and separated by SDS-polyacrylamide gel electrophoresis (PAGE) **[33]**. Equal amounts of protein (40µg) were loaded in each gel track. The percentage of polyacrylamide in the running gels was 12 %. Proteins were electrophoretically transferred to nitrocellulose membranes for western blot analysis. Polypeptide detection was done using polyclonal antiserum raised

against a synthetic polypeptide common to GS1 and GS2 of *Arabidopsis thaliana*.

Detection of GDH activity on the gel

Leaf and root soluble proteins were extracted from frozen material in cold extraction buffer containing 100mM Tricine, 1mM EDTA, 40mM $CaCl_2$, 0.5 % (w/v) PVP, 0.1 % (v/v) 2-mercaptoethanol, and 1mM AEBSF (4-(2-AminoEthyl)-benzenesulfonylfluoride). The protein separation was carried out in a 1 mm-thick non-denaturing gel, as described previously. Equal amounts of protein (40µg for roots and 60µg for leaves) were loaded into each gel track. Native PAGE of the partially purified GDH extracts was performed by the method of Davis **[34]** on 5 % running gel with a 4 % stacking gel. The buffer system was 100mM Tris-Glycine adjusted to pH 8 with HCl. Running was at 4°C, 120V, for about 2 h, and bands containing GDH activity were visualized with the tetrazolium system. The staining solution contained 150mM Tris-HCl (pH 8.8), 50mM glutamate, 0.5mM NAD^+, 0.5mM NBT(Nitro Blue Tetrazolium chloride), and phenazine.

Statistical analysis

The data presented in the figures is the average of at least six replicates per exposure treatment and means ± confidence limits at alpha = 0.05level. Each experiment was conducted in duplicate.

RESULTS

Soluble protein content

The results showed that when the wild type was transferred to 2 mM NH4 + medium for 6 hours, the leaf soluble protein (SP) content increased by 21 %, compared to the control. However, this increase was reduced by half to 10 % when salt was added to the ammonium medium. In *asn2-1*, the ammonium treatment did not cause a notable change (2 %) to the SP contents in the leaves refer to control. However, the ammonium-salt co-treatment increased the leaf SP contents by 20 % refer to control **(Figure 1)**.

Ammonium content

In the WT leaves, the plants transferred to the 2mM NH4+ medium or the ammonium-salt medium, induced an endogenous ammonium accumulation of about 30 %, compared with the control. In the *asn2-1* leaves, the transfer to the ammonium medium lead to an endogenous ammonium accumulation of about 30 %,in comparison with the control. The addition of salt to the ammonium medium increased the endogenous ammonium contents by 47 %, in comparison with the control **(Figure 2)**.

Salt effects on ammonium-assimilating enzymes

Glutamine synthetase activity and enzyme protein level

In the WT leaves, the plant transfer to the ammonium medium was associated with increased GS activity of about 60 %. The salt treatment in the ammonium medium further increased the GS activity, to approximately twice that of the control **(Figure 3A)**. In the *asn2-1* leaves, both the transfer to the ammonium medium andthe ammonium- salt medium, stimulated GS activity in the leaves by about 18 %, in comparison with the control **(Figure 3A)**. The western Blot analysis showed that plastidic GS2 was the major isoenzyme and predominated cytosolic GS1 in the wild-type and *asn2-1* lines. The plant transfer to the ammonium medium induced only a small increase in GS2 protein levels in the wild type, in spite of important increases of GS activity. In the*asn2-1*, the ammonium treatment led to an increase in GS1 protein levels **(Figure 3B)**. The increase of GS1 protein levels was linked to GS activity stimulation. The ammonium-salt co-treatment further increased the GS1 and GS2 protein levels in the wild-type and *asn2-1* lines. In both lines, the salt treatment increased the GS1 protein levels more than GS2 protein levels.

Glutamate dehydrogenase activity and GDH subunit patterns

The plant transfer to ammonium medium was associated with an important stimulation of foliar GDH activity by 70 % and 40 % in the wild type and *asn2-1* lines respectively **(Figure 4A)**. The ammonium-salt co-treatment also increased the GDH activity stimulation in leaves of the wild-type and *asn2-1* lines. In the wild type, the GDH activity more than doubled, in comparison with the control. In the *asn2-1* leaves, the GDH activity increased by about 25 %, in comparison with the control **(Figure 4A)**. The GDH activity on the polyacrylamide gels showed the presence of 7 GDH isoform patterns, formed by the association of six of the two α and β-subunits **(Figure 4B)**. The apical GDH band was formed by six β-subunits, while the basal band was formed by six α-subunits. The detection of GDH activity on the gel and the protein quantification revealed a higher staining intensity at the apical bands of the wild-type leaves. The results showed that ammonium induced β-enriched isoforms. Likewise, for the co treatment with ammonium-salt, the β-enriched isoform activity was increased. In *asn2-1* leaves, the GDH activity on the gel revealed a slight increase with the ammonium and ammonium and salt treatments.

GLN2, GLN1.1, and GLN1.2 expression

The effects of ammonium and the ammonium-salt co- treatment were examined at the transcript level for *GLN2*, *GLN1.1,* and *GLN1.2*; the major genes

encoding glutamine synthetase in the leaves **(Figure 5)**. A chloroplastic GS2 encoded by a single *GLN2* gene is the major GS isoform in the vegetative stage of Arabidopsis leaves. We measured *GLN2* mRNA abundance in response to the ammonium and ammonium-salt treatments. The plant transfer in the ammonium medium induced a decrease in *GLN2* transcript levels by about 20 % and 15 % in the wild-type and *asn2-1* lines, respectively. The ammonium-salt treatment induced a larger decrease in *GLN2* transcript levels in both lines.

The cytosolic GS1 is encoded by several genes, but only *GLN1.1* and *GLN1.2* were investigated. The plant transfer to the ammonium medium induced a slight decrease in *GLN1.1* transcript level in both lines. The ammonium-salt treatment induced an increase in *GLN1.1* transcript level by about 20 % and 40 % in the wild-type and *asn2-1* lines, respectively. After 6 h of plant exposure to the ammonium medium, the *GLN1.2* transcript levels increased by about 8 % in both the wild-type and *asn2-1* lines. The co-treatment increased the *GLN1.2* transcript levels more in the*asn2-1* leaves, by about 18 %, in comparison to the control.

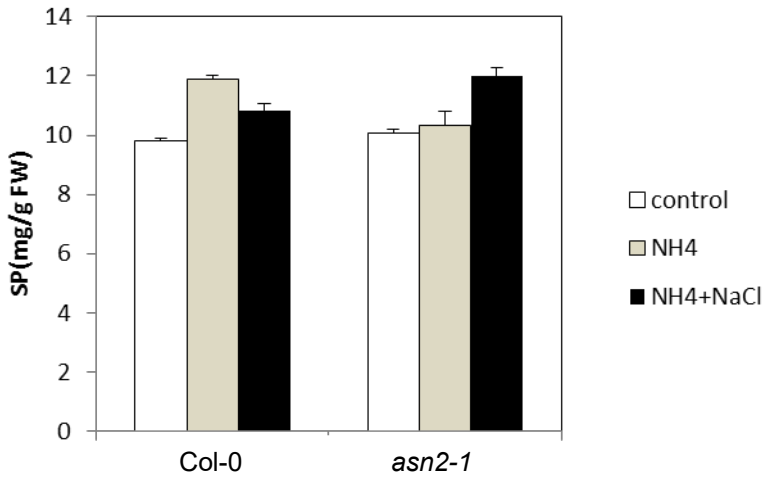

Figure 1: Effect of NH4+ and NaCl on soluble protein content (SP: mg/g FW) in leaves of wild-type (Col-0') and *asn2-1* lines of *Arabidopsis thaliana*. Plants were grown for four weeks with a basic nutrient medium (12 mM NO3-). The plants were then grown under the basic medium (control) either with 2 mM NH4+ (NH4) or both 2 mM NH4+ and 100 mMNaCl (NH4+ NaCl), for 6 hours. Data are means of six replicates ± CL at a significance level of 0.05.

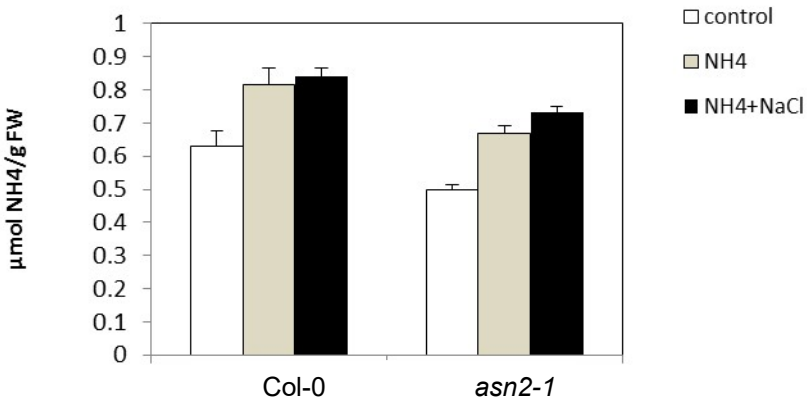

Figure 2: Effect of NH4+ and NaCl on endogenous ammonium content (µmol NH4/g FW) in leaves of wild-type (Col-0) and *asn2-1* lines of *Arabidopsis thaliana*. Plants were grown for four weeks with a basic nutrient medium (12 mM NO3-). The plants were then grown under the basic medium (control) either with 2 mM NH4+ (NH4) or both 2 mM NH4+ and 100 mMNaCl (NH4+NaCl), for 6 hours. Data are means of six replicates ± CL at a significance level of 0.05.

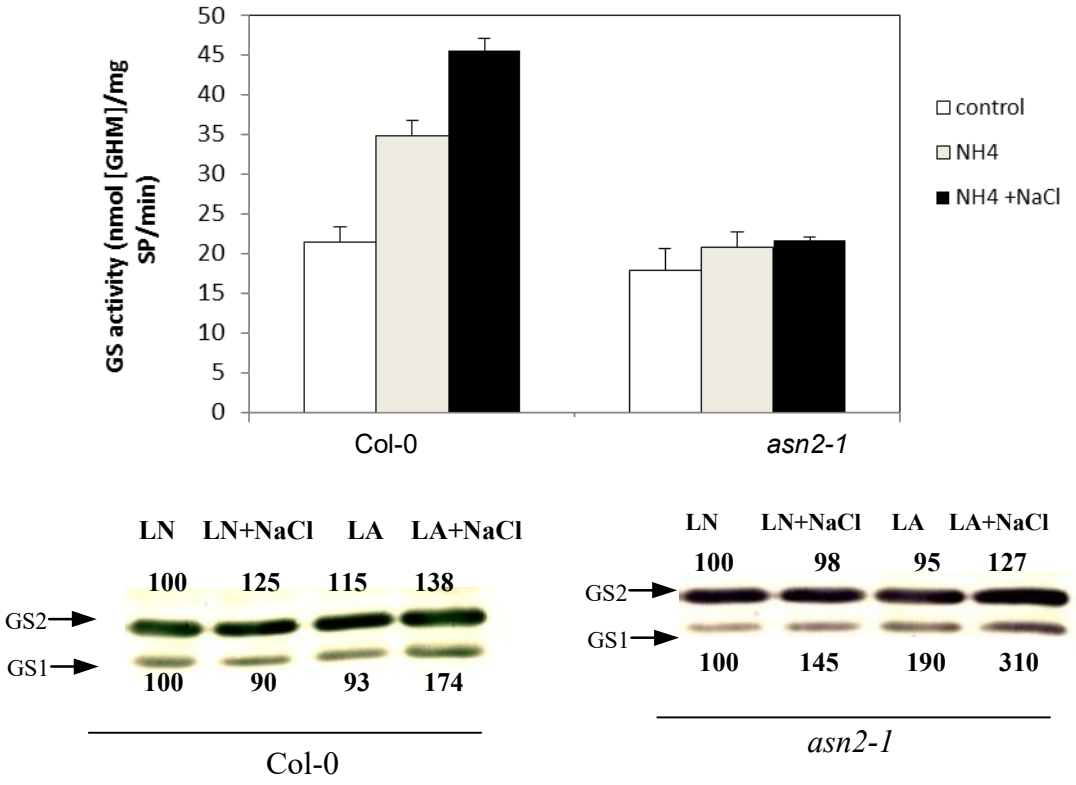

Figure 3: Effects of NH4+ and NaCl on (A) Glutamine synthetase activity (µmol [GHM]/mg PS/min) and GS protein content in leaves of wild-type (Col-0) and *asn2-1* lines of *Arabidopsis thaliana*. Plants were grown for four weeks with a basic nutrient medium (12 mM NO3-). The plants were then grown under the basic medium (control) either with 2 mM NH4+ (NH4) or both 2 mM NH4+ and 100 mMNaCl (NH4 +NaCl), for 6 hours. Data are means of six replicates ± CL at a significance level of 0.05. (B) GS1 and GS2 protein amounts are expressed as % relative to the corresponding protein amount prior to NH4+ and NaCl treatments

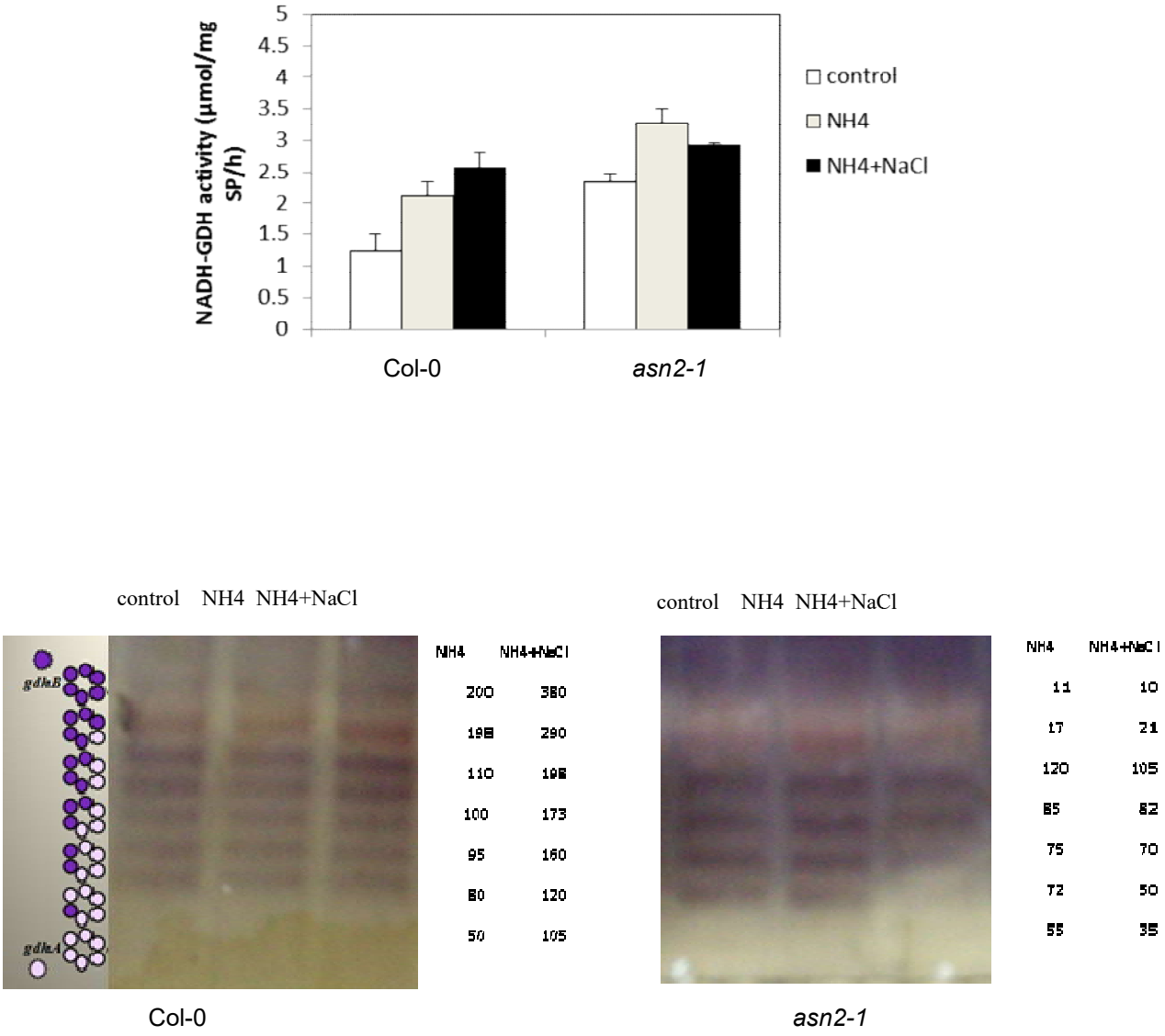

Figure 4: Effect of NH4+ and NaCl on (A) NADH-glutamate dehydrogenase activity (µmol NADH oxidized /mg SP/h) and (B) NADH-GDH proteinsin the leaves of wild-type (WT) and *asn2-1* lines of *Arabidopsis thaliana*. Plants were grown for four weeks with a basic nutrient medium (12 mM NO3-). The plants were then grown under the basic medium (control) either with 2 mM NH4+ (NH4) or both 2 mM NH4+ and 100 mM NaCl (NH4 +NaCl), for 6 hours. Data are means of six replicates ± CL at a significance level of 0.05.
(B) The amount of each GDH protein is expressed as % relative to the corresponding protein amount prior to NH4+ and NaCl treatments.

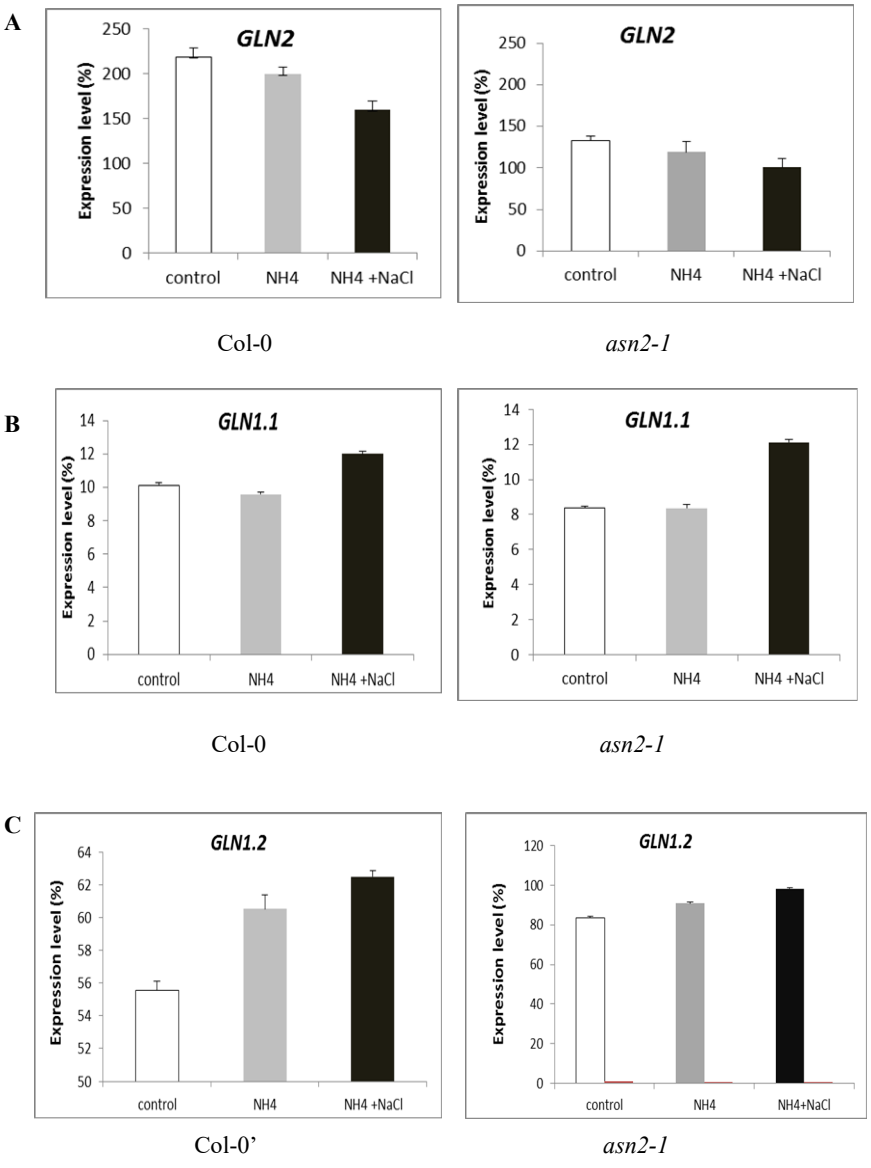

Figure 5: Effect of NaCl and NH4+ on the transcript levels of (A) *GLN2*, (B) *GLN1.1*, and (C) *GLN1.2* for chloroplastic and cytosolic glutamine synthetase in leaves of wild-type (WT) and *asn2-1* lines of *Arabidopsis thaliana*. Plants were grown for four weeks with a basic nutrient medium (leaves nitrate (LN): 12 mM NO3-). The plants were then grown under the basic medium either with 2 mM NH4+ (leaves ammonium (LA)) or both 2 mM NH4+ and 100 mM NaCl, for 6 hours. Transcript levels are expressed as % *EF1α* transcript levels. Data are means of three replicates ± CL at a significance level of 0.05.

DISCUSSION

As enzymes play a key role in re-assimilating ammonium into asparagine under environmental stresses, and it is difficult to use biochemical approaches to determine AS activity, we have used wild-type *Arabidopsis thaliana* Col-0 and knockout T-DNA mutants inserted in the *ASN2* gene encoding Asn (AS, EC 6.3.5.4) (*asn2-1*), to study the effect of NaCl stress in the presence of ammonium. The main aim was to investigate how ammonium assimilation would be influenced in the presence and partial absence of AS enzyme. Thus, plants were grown for four weeks with a basic nutrient medium, then the plants were transferred to a different medium containing ammonium (2mM) or 2 mM ammonium and 100 mM NaCl and left on these media for only 6 hours.

The presence of NaCl with the ammonium treatment increased the soluble protein and endogenous ammonium content, mainly in the *asn2-1* lines. The ammonium-salt co-treatment increased activities of both GS and GDHin the wild-type and this can be linked to ammonium accumulation. However, a slight increase in GS and GDH activities were observed in the*asn2-1* lines, despite more endogenous ammonium accumulation. These results may indicate that other metabolites control GS and aminating GDH activity when the*ASN2* gene is lacking.

The GS protein (GS1 and GS2) content increased with the co-treatment both in the wild-type and *asn2-1* lines with a notable increase in GS1 content, compared either to the control (no treatment) or to the plants grown on ammonium medium only. The expressions of genes encoding GS1; *GLN1.1,* and *GLN1.2*were observed to be upregulated with the co-treatment. The results indicate that the partial absence of the AS enzyme in the mutant lines may account for the slight increase observed in the activities of ammonium-assimilating enzymes, which could reveal a potential role of Asnin plant responses to salt stress and in activating such enzymes.

Soluble protein (SP) and endogenous ammonium content was measured in the leaves of wild type and *asn2-1* lines. The results illustrated in figure 1 show that soluble protein (SP) content was higher in the plants transferred to media containing 2mM ammonium, in the leaves of wild-type and *asn2-1* lines, compared to plants grown in basic nutrient media with no ammonium. In the*asn2-1* leaves, the co-treatment (2mM ammonium + 100 NaCl) led to a more

important SP accumulation than that in the wild-type.

In both lines, the ammonium accumulation with the co-treatment of ammonium and salt was greater than when ammonium and salt were applied separately. Under such conditions, ammonium accumulation in the*asn2-1* is more notable compared to the wild-type. These results support the notion that *ASN2* is closely correlated to ammonium metabolism in higher plants **[27, 35]**. In fact, salinity stress leads to higher ammonium accumulation, this stress also enhances cellular free Asn levels **[36]**.

The ammonium accumulation with the salt co-treatment was accompanied by SP accumulation **(Figure 1, 2)**. Thus, the accumulated ammonium was not necessarily connected to the protease activity induction that is generally stimulated under abiotic stress, but could be due to the stimulation of photorespiration or amino acid degradation under salt stress **[37]** and\or the ammonium absorption of culture medium in the case of the ammonium-salt co-treatment.

Salt effects on ammonium-assimilating enzymes, including GS and GDH, were evaluated. The glutamine synthetase (GS) had a major role in ammonium assimilation. The GS enzyme is involved in assimilation of ammonium formed by nitrate reducing, photorespiration and the protease activity **[38]**. The plant transfer in ammonium medium and salt treatment on the same medium induced an increase in GS activity in wild-type and *asn2-1* lines. The GS activity increase is more apparent in the wild-type, despite more ammonium accumulation in the*asn2-1* line. The salt treatment in the ammonium medium increased GS activity in both lines.

Western blot analysis was used to evaluate the level of the two different isoenzymes for the GS enzyme, namely GS1 and GS2. In wild type plants, the GS2 level was slightly higher in plants transferred to media with ammonium or those supplied with salt than that observed in the control. However, the GS1 level was a bit lower compared to the control (no treatments). Thus, these results cannot explain the increase in the GS activity in the wild type plants. However, in term of mutant plants, the increase of GS activity can be attributed to the elevated level of GS1 protein. It was reported that the GS activity increase was correlated to the GS1 protein level increase in tomato under cadmium treatment [39]. Other studies have shown that GS is subject to post-translational regulation by stress. Indeed, Motohashi et al. (2001) **[40]** showed that GS2 activity is modulated by redox changes of two cysteine residues involved in the activation of the enzyme.

The plant transfer to the ammonium medium and salt treatments decreased the*GLN2* transcript level in wild-type as well as the *asn2-1* mutant line **(Figure 5)**. A number of studies have shown that salt stress down-regulated *GLN2* mRNA level within 6 h in the wild-type **[27]**.

The decrease inthe *GLN2* transcript level is linked to a slight induction of the GS2 protein level referring to GS1 protein level increase. Of the five *GLN1* genes for cytosolic GS1, we studied two. *GLN1.2* transcript levels were more notable than those of *GLN1.1* **(Figure 5)**. The plant transfer to the ammonium with salt treatment induced an increase in *GLN1.1* and *GLN1.2* transcript levels. The increase in the expression of *GLN1.1* and *GLN1.2* may explain the increased GS1 protein levels. Zilli *et al*. (2008) **[41]** reported that *GLN1* mRNA levels in soybean roots were induced by salt stress together with the GS1 protein. In both lines, there is a close relationship between ammonium accumulation and *GLN1* expression increase. It has been suggested that aminating GDH activity catalyzes an alternative ammonium assimilating pathway when GS activity is impaired by abiotic stress **[42, 43]**. Expression of two genes encoding the two subunits α and β of the NADH-GDH is regulated by metabolites and environmental stress **[44, 45]**.

NADH-GDH is formed by seven bands following PAGE **[46]**. The 7 NADH-GDH bands were detected in grapevine shoot callus **[47]**, while in triticale seedlings NAD-GDH activity revealed on the gel showed only one band **[48]**. In the wild-type leaves, β-enriched NADH-GDH bands accumulated with ammonium and salt treatments **(Figure 4B)**. This may be associated with an increase in aminating GDH activity in the wild type under saline conditions **(Figure 4A)**. The stimulation of aminating GDH activity is thought to improve salt resistance **[42]**. However, GDH bands from the *asn2-1* leaves remained stable, indicating that there was no linked contribution of GDH aminating activity in the *asn2-1* mutant.

In the wildtype, after 6 h in the ammonium medium, the endogenous ammonium content increased. This may be the origin of the GS activity and the aminating GDH activity increase. The salt treatment in the same medium induced more endogenous ammonium content and a greater increase in GS and aminating GDH activities in young barley plants and the endogenous ammonium content induced GS activity **[49]**.

In *asn2-1*, after 6 h in the ammonium medium, the GS and aminating GDH

activities showed a lower increase compared with the wild-type and despite greater endogenous ammonium accumulation. In these lines, the salt treatment in the ammonium medium induced more endogenous ammonium content. This increase was associated to same GS activity increase referring to the ammonium treatment. In addition, the aminating GDH activity was noticed to be decreased in the salt co-treatment when compared with the ammonium treatment. This suggests that GS and GDH activity control is not linked to ammonium accumulation in the*asn2-1* line. In the absence of *ASN2* expression, GS and GDH activity control may be related to other metabolites such us asparagine and proline**.** 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 **[50]**. The possible contribution of Asnin response to environmental stress such as soil salinity may be a key feature for future genetic manipulations in order to increase crop yields in such conditions. This study focused on the short-term effect of the treatment (after 6 hours); hence, it would be interesting to study if the effect remains the same with longer exposure times.

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Author contribution

All the practical work has been prepared by Dr. Houda Maaroufi Dguimi.

Dr. Fatima Omari Alzahrani participated in the data analysis as well as the writing and the linguistic correction of the manuscript

Additional information

The authors have no competing interests as defined by Nature Research, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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