

Transcriptional signatures of nitrogen and amino acid metabolism under excess ammonium stress in *Nicotiana tabacum* leaves

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Research Article

Keywords: Ammonium toxicity, nitrogen assimilation, glutamate dehydrogenase, aspartate amino transferase, leaf transcriptome, inferred gene regulatory network, transcription factor, *Nicotiana tabacum*

Posted Date: July 3rd, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10012305/v1>

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Additional Declarations: No competing interests reported.

Abstract

High concentrations of soil ammonium are toxic to most plants, limiting their ability to use nitrogen efficiently. This study aims to identify candidate transcription factors that respond to excess ammonium stress in *Nicotiana tabacum*, a sensitive species. Seedlings at the seven-leaf stage were grown hydroponically for three weeks with 10 mM NH_4^+ , 10 mM NO_3^- , or a mixture of 5 mM each as their sole nitrogen source. Growth parameters and physiological traits, including enzymatic activities, free amino acid pools, and the transcriptome, were determined. The high NH_4^+ treatment significantly reduced plant growth, as well as the accumulation of C, N, P and K, causing a premature senescence. The free amino acid concentrations increased by around 30%, which featuring the increased accumulation of free ammonium, hydroxyproline, threonine and valine, while the decrease in glutamate, aspartate, GABA and homoalanine. Ammonium stress was found to upregulate genes involved in signaling, transcriptional regulation, mitochondrial activity and transporters, while downregulating genes in photosynthesis, the thylakoid membrane, responses to chemicals and cellular homeostasis. Notably, four genes encoding glutamate dehydrogenase and three encoding aspartate amino transferases were exclusively upregulated in stressed leaves. An inferred gene regulatory network (iGRN) identified six transcription factors as candidate regulators that are highly associated with the response to excess ammonium stress conditions. High ammonium levels lead to intricate interactions between nitrogen nutrition and other cellular processes. Our results pave the way for further deciphering of the molecular mechanisms underlying ammonium sensitivity and tolerance.

Introduction

Ammonium and nitrate in the soil are the main sources of inorganic nitrogen for plant growth (Jackson et al., 2008). As a reduced form of nitrate, ammonium ($\text{NH}_4^+ + \text{NH}_3^-$) can be immediately assimilated and transformed into amino acids by the plant cell upon uptake, thus bypassing the nitrate reduction process, which occurs partially in the plastid/chloroplast. In agriculture, groundwater pollution caused by nitrate leaching from fertilizer is a common problem. In this regard, ammonium is preferred to nitrate solely because it is more stable in soil solutions (Coskun et al., 2017; Xu et al., 2012; Yang et al., 2025). However, most plants are sensitive to high concentrations of external ammonium (> 5 mM) and exhibit growth inhibition and yellowing of the leaves in the presence of excess ammonium (Britto and Kronzucker, 2002; Gerendás et al., 1997; van der Eerden, 1982). Tolerance levels vary depending on the species (Britto and Kronzucker, 2002; De la Peña et al., 2024; Katz et al., 2022; Sarasketa et al., 2014; Xie et al., 2025). Improving plant tolerance to and assimilation of available ammonium would greatly benefit modern agriculture (Marino and Moran, 2019).

The symptoms of ammonium toxicity in sensitive plants are clear: reduced growth and root/shoot ratio, decreased photosynthesis, leaf chlorosis, carbon starvation, and imbalances in ions and osmosis, as well as oxidative stress conditions (Esteban et al., 2016; Gerendás et al., 1997; Sun et al., 2020). Despite many years of extensive study, the exact mechanisms underlying ammonium toxicity remain inconclusive. Several biochemical and cellular mechanisms have been proposed as potential causes.

These include futile ammonium cycling, extracellular acidification, defective cation homeostasis and chloroplast function, as well as depleted energy and carbon skeletons (Britto and Kronzucker, 2002; Britto et al., 2001; Coskun et al., 2013; Esteban et al., 2016; Hachiya et al., 2021; Kong et al., 2022; Kronzucker et al., 2001; Li et al., 2019; Li et al., 2025; Poucet et al., 2022; Xie et al., 2023).

This sophistication stems from the fact that ammonium is both the entry substrate for nitrogen assimilation and the cycling product for numerous nitrogen metabolism pathways. Large quantities of ammonium are recycled internally through the turnover of various nitrogen pools. Ammonium is also produced as a by-product of photorespiration and the shikimic acid pathway (Feng et al., 1998). These sources of ammonium are more abundant in the shoot than in the root (Husted et al., 2002; Mattsson and Schjoerring, 2002), meaning that plant shoots are more prone to accumulation of excess ammonium.

Movement of ammonium across the membrane is facilitated by proteins from the ammonium transporter (AMT) family and specific members of the aquaglyceroporin (AQP) family. Plant AMTs conduct electrogenic ammonium transport via unclear mechanisms and play a key role in ammonium uptake and retrieval in plants (Ludewig et al., 2007; Neuhauser et al., 2014; Ninnemann et al., 1994; von Wirén, 2000). Meanwhile, plant AQP channels, also known as major intrinsic proteins (MIPs), mediate the passage of the neutral ammonia NH_3 in a concentration-gradient-dependent manner (Holm et al., 2005; Jahn et al., 2004; Loqué et al., 2005; Wu and Beitz, 2007). As NH_3 is a weak base and a proton acceptor with a pKa of 9.25, and its intracellular concentration depends on the pH of the, which are tightly controlled to safeguard proper membrane potential and maintain proton homeostasis in metabolism (Casey et al., 2010; Shavrukov and Hirai, 2016). Consequently, it can easily become protonated at physiological pH levels, becoming trapped in an acidic compartment in the process. For example, the pH of the apoplast or vacuole is typically 1–2 units lower than that of the cytoplasm. This creates a concentration difference of 10–20-fold for NH_3 , thereby enhancing the diffusion of ammonia to the two acidic compartments.

From a biochemical perspective, incorporating NH_4^+ into one glutamate molecule yields four H^+ ions, whereas assimilating NO_3^- produces only two (Britto and Kronzucker, 2002). Indeed, adding nitrate to a mixture containing ammonium for plant nutrition has been shown to counteract the toxicity of the latter, even at very low concentrations (Du et al., 2021; Hachiya and Sakakibara, 2016; Hachiya et al., 2012; Li et al., 2023; Péliissier et al., 2024; Roosta and Schjoerring, 2008; Yan et al., 2023; Yang et al., 2025). A recent study by Hachiya et al. confirms that acidic stress caused by a plastid glutamine synthetase assimilation of ammonium induces toxic symptoms in *Arabidopsis thaliana* (Hachiya et al., 2021). Similarly, Poucet et al. reported that high ammonium stress provokes vacuole acidification, which coincides with glycolysis inhibition and organic acid depletion in tomato leaves. The authors argued that the higher energy cost of vacuole expansion resulting from pH alteration accounts for growth inhibition (Poucet et al., 2022). The effect of adding NO_3^- to alleviate NH_4^+ toxicity have also been suggested to be a result of upregulation of cytokinin biosynthesis (Britto and Kronzucker, 2002; Walch-Liu et al., 2000).

Nevertheless, little has been reported on the transcriptional regulation of sensitivity and resistance to ammonium toxicity, or on the key regulators that coordinate metabolic tolerance to excess ammonium. In the present study, we characterized the transcriptional response relevant to ammonium assimilation and amino acid metabolism in *Nicotiana tabacum*, a species sensitive to ammonium, which was grown hydroponically in a high-ammonium environment (10 mM ammonium as the sole nitrogen source) or in 10 mM nitrate or 5 mM each of ammonium and nitrate as controls. An inferred gene regulatory network module predicted several transcription factors (TFs) to be key candidates for regulating the response to high ammonium stress.

Materials and Methods

Plant material and growth conditions

Tobacco plant (*Nicotiana tabacum* L. cv. CB-1) seeds were sown on plastic trays and grown in a growth chamber for approximately one month, until the seedlings reached the seven-leaf stage. The plants were then transferred to 1 L hydroponic culture boxes and incubated for one week to adapt to the growth conditions of a 12 h day/12 h night light regime and a day/night temperature of 25°C/20°C. The pre-culture was prepared using a modified Hoagland formula containing the following: 1.79 mM KNO₃, 2.68 mM(NH₄)₂SO₄, 1.79 mM Ca(NO₃)₂•4H₂O, 0.89 mM K₂SO₄, 1.79 mM CaCl₂•2H₂O, 0.71 mM KH₂PO₄, 2 mM MgSO₄•7H₂O, 18 µM FeSO₄•7H₂O, 20 µM DTA-2Na, 36 µM H₃BO₄, 9.10 µM MnCl₂•4H₂O, 0.86 µM ZnSO₄ 7H₂O, 0.31 µM CuSO₄•5H₂O, and 0.11 µM Na₂MoO₄•2H₂O. The nutrient solution was added every three days and completely renewed every six days. The pH was adjusted to 6.0 by the addition of HCl or NaOH.

These plants were then divided into three treatment groups. Before being transferred to new boxes containing three different nitrogen-source solutions, the roots were rinsed three times with distilled water. All treatments contained 10 mM of nitrogen in different forms. The NH₄⁺-fed treatment solution was supplemented with 5.36 mM (NH₄)₂SO₄, 1.79 mM K₂SO₄, 3.57 mM CaCl₂•2H₂O; the NO₃⁻-fed treatment solution with 3.57 mM KNO₃, 3.57 mM Ca(NO₃)₂•4H₂O; and the Mix-fed treatment solution with 1.79 mM KNO₃, 2.68 mM(NH₄)₂SO₄, 1.79 mM Ca(NO₃)₂•4H₂O, 0.89 mM K₂SO₄, 1.79 mM CaCl₂•2H₂O. The plants were then subjected to three weeks of growth in the same growth chambers before sampling and analysis. The experiment was repeated twice, with each treatment group comprising 40 plants. Physiological trait, biochemical and transcriptomic analyses were conducted three weeks after treatment.

Root activity and tissue elemental analysis

The dry weight of the roots and shoots of three plants was determined three weeks after treatment. Samples of dried tissue were stored for later use in elemental analysis. Fresh roots were collected from

three plants in order to determine the root volume. This is done using an Epson Expression 12000XL scanner connected to WinRHIZO Pro 2010b software (Regent Instruments Canada Inc., Québec City) for image processing. Root vitality was estimated using a 2,3,5-triphenyltetrazolium chloride (TTC) reduction assay kit (Beijing Boxbio Science & Technology Co., Ltd., Beijing, cat.no. AKPL029M), following the manufacturer's protocol. For this assay, ca. approximately 0.3 g of fresh root tissue was used for each determination, and the final product (triphenyl formazan) was quantified using a spectrophotometer at a wavelength of 485 nm. Root electrolyte leakage was measured according to a previously published protocol (Radoglou et al., 2007).

The total content of phosphate and potassium in the roots and the stems was determined using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) on an iCAP 7000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). To measure total C and N, samples of dried, finely ground tissue were weighed into small foil capsules (80 mg) and combusted in an a SKALAR Primacs SCN100-1C automated carbon/nitrogen analyzers (SKALAR, Breda, The Netherlands), which measures the amount of CO₂ and N₂ released by gas chromatography equipped with a thermal conductivity detector for CO₂ and a non-dispersive infrared detector for N₂. Final values are expressed as the percentage of carbon or nitrogen in the dry stem tissue.

Determinations of sugars and free amino acid composition

The stems of three plants were collected, dried and ground into a fine powder. The soluble sugars and starch in the samples were quantified using commercial ELISA colorimetric assay kits (Youxuan Biotechnology Co., Ltd., Shanghai, catalogue numbers YX-W-B502 and YX-W-C400, respectively), in accordance with the manufacturers' protocols. To quantify the amino acid composition, 50 mg of the fine powder was suspended in 4 ml of 5% dilute hydrochloric acid and ultrasonically treated for 30 minutes. The solution was then filtered using a Millipore® 0.45 µm filter membrane, after which the supernatant was collected for amino acid analysis using a Biochrom 30 + AA analyser (Biochrom Ltd., Cambridge).

Enzyme activity assays

Three weeks after treatment, the fully expanded leaves (number 3 from the bottom) were collected, punched into 1 cm diameter discs and immediately ground into a fine powder in liquid nitrogen. This powder was then used immediately for the enzyme activity assay. The protocols used for the enzyme activity assay have been described previously for Nitrate reductase (NR) [EC 1.7.1.1-3] (Hofmann et al., 2013), glutamine synthetase (GS) [EC 6.3.1.2] using NH₂OH as an artificial substrate to measure the formation of γ-glutamyl hydroxamate (γ-GHM) (Vega-Mas et al., 2015), and glutamate synthase (NADH-GOGAT) [EC 1.4.1.14] by measuring the NADH consumption (Ma et al., 2016).

The activity of sucrose synthase (SuS) [EC 2.4.1.13] and sucrose-phosphate synthase (SPS) [EC 2.4.1.14] was measured using commercial ELISA kits (Youxuan Biotechnology Co., Ltd., Shanghai, catalogue numbers YX-C-B504 and YX-C-B505, respectively).

The assay for the activity of acid invertase (A-INV) [EC 3.2.1.26] and alkaline/neutral invertase (N-INV) [EC 3.2.1.26] was conducted using liquid nitrogen-frozen leaves homogenate in extraction buffer consisting of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.5% TritonX-100. The A-INV assay buffer consisted of 0.125 M sodium acetate with an adjusted pH of 4.9, and the N-INV assay buffer consisted of 0.125 M sodium acetate with an adjusted pH of 7.5. Measurements were taken at 37°C in the respective assay buffer using an invertase activity colorimetric assay kit (Youxuan Biotechnology Co., Ltd., Shanghai), following the manufacturer's protocol. The protein concentration of the extracts was determined spectrophotometrically using Bradford's method.

RNA-sequencing and transcriptome analysis

For RNA extraction, the fully expanded leaves (the third from the bottom) were collected and ground into a fine powder using liquid nitrogen. Total RNA was extracted from the powder of using a TRIzol reagent (Thermo Fisher Scientific, USA), and treated with RNase-free DNase I (Thermo Fisher Scientific, USA) to eliminate genomic DNA contamination. RNA sequencing was conducted by the company Novogene (Beijing, China). The clean reads obtained were mapped to the tobacco reference genome (https://solgenomics.net/organism/Nicotiana_tabacum/genome) (Edwards, 2017) using the Hisat2 v2.0.5 program (Kim, 2015). Differential expression analysis was performed using the DESeq2 program (Love, 2014). Genes with a $\log_2$ fold change ($\log_2FC$) ≥ 1 and a false discovery rate (FDR) ≤ 0.05 were considered as significantly differentially expressed.

Functional enrichment analysis was conducted based on Gene Ontology GO (terms) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) using the genome annotation files with the TBTools-II program (Chen et al., 2023).

Construction of the inferred gene regulatory network (iGRN)

This was achieved using the BioNERO R package in R version 4.5.2, implemented in the RStudio IDE (version 2026.04.0 Build 526). This involved calculating the average ranks of all edges using three different algorithms to obtain a consensus and high-confidence edges. The top edges leading to the best scale-free topology (SFT) fit were then selected (Almeida-Silva and Venancio, 2022). Transcription factors in the DEGs, as predicted via PlantTFDB 4.0 (Jin et al., 2017), were used as the input regulators in the global iGRN construction. The global iGRN was then screened with three sets of DEGs enriched in metabolism, amino acid and derivative biosynthesis, and signaling and transport processes to identify TFs linked to these targets. Subsequently, three sub-networks were reconstructed using these three sets

of TFs as input regulators. The hubs from these three sub-networks are overlaid to identify the candidates of interest.

Statistical analysis

The statistical analysis was performed on quantitative data obtained through three replications, using the appropriate R package and SPSS software.

N. tabacum gene ID conversion

The gene ID in the results was converted to the chromosome-ordered locus ID using the newly assembled genome version (Zan et al., 2025) and the TBTools-II program (Chen et al., 2023).

Result

Excess ammonium stress impairs carbon-nitrogen metabolism and reduces the growth of young N. tabacum plants.

Hydroponically grown seedlings that are given nitrate (10 mM) or a 1:1 mixture of nitrate (5 mM) and ammonium (5 mM) as their sole source of nitrogen display healthy growth and normal green leaves. However, seedlings grown with 10 mM ammonium exhibit weak growth and yellowish leaves, which is indicative of ammonium toxicity (Fig. 1A). Ammonium toxicity reduces root growth by a factor of $\sim 3-4$ and the root/shoot biomass ratio by a factor of $\sim 1.8-2.1$ compared to the other two treatments (Fig. 1B). Under ammonium toxicity, root vigour, as measured by the TTC reduction index, decreased by a factor of ~ 2.7 to 3.2 , while the root electrolyte leakage index increased by approximately a factor of two to three, suggesting cell membrane damage (Fig. 1B).

Furthermore, the potassium content of the roots and above-ground parts of plants supplied with toxic levels of NH_4^+ was significantly lower than that of plants supplied with NO_3^- alone or in combination with NH_4^+ at half the concentration. This suggests that ammonium toxicity may hinder the absorption of phosphate and, to a greater extent, potassium (Fig. 2A). Additionally, the total carbon and nitrogen content of the above-ground parts of plants exposed to ammonium toxicity was nearly halved compared to healthy plants (Fig. 2B).

Interestingly, plants experiencing ammonium toxicity have a much higher C/N ratio (Fig. 2C), which may indicate an imbalance in their carbon-nitrogen metabolism. Under ammonium toxicity, the glucose content decreased by $\sim 7\%$ in the leaves and by $\sim 10\%$ in the stems, compared to the other two treatments (Table 1). These appeared to be less affected by ammonium toxicity. However, the fructose, sucrose and starch content increased in plants subjected to ammonium toxicity. Fructose levels increased the most, by 4.0- to 5.6-fold, compared to healthy plants in the other two treatments. The sucrose and starch content increased by 1.7- and 1.4-fold, respectively, in plants fed solely with a high level of NH_4^+ compared to those fed with a non-toxic level of NH_4^+ and NO_3^- (Table 1).

Table 1
Soluble sugar and starch content in leaves and stem

Glucose ($\mu\text{mol/g DW}$)	10 mM NH_4^+	(5 + 5) mM Mix	10 mM NO_3^-
	177.37 $\pm$ 4.75 b	190.87 $\pm$ 1.33 a b	196.46 $\pm$ 2.27 a
Fructose ($\mu\text{mol/g DW}$)	391.75 $\pm$ 5.64 a	99.37 $\pm$ 7.96 b	69.79 $\pm$ 8.4 b
Sucrose ($\mu\text{mol/g DW}$)	72.42 $\pm$ 4.16 a	41.44 $\pm$ 0.67 b	30.66 $\pm$ 1.11 b
Starch ($\mu\text{mol glucose/g DW}$)	1.89 $\pm$ 0.07 a	1.36 $\pm$ 0.03 b	1.09 $\pm$ 0.04 c
Note: Data are presented as the mean $\pm$ standard error (n = 3). Different letters indicate significant differences between means within each row, as determined by a one-way ANOVA followed by a Tukey–HSD test.			

The apparent enzyme activity of nitrate reductase (NR) in the crude leaf extract increased by over 2.6-fold under ammonium toxicity. BY contrast, NADH-dependent glutamate synthase (GOGAT) activity decreased by up to a fifth compared to normal conditions (Fig. 3, upper panel). However, glutamine synthetase (GS) activity showed little difference under these conditions, as did the activities of the sugar metabolism enzymes sucrose phosphate synthase (SPS) and cytosolic alkaline/neutral invertase (CINV) (Fig. 3, lower panel). Conversely, the apparent activities of sucrose synthase (SuS) and acid invertase (AINV) increased and decreased, respectively, in plant leaves challenged by toxic levels of ammonium (Fig. 3, lower panel). The increase in SuS activity is consistent with a higher fructose accumulation of in the plant leaves under toxic ammonium conditions.

Excess ammonium stress alters the free amino acid pool in *Nicotiana* leaves

The concentration of free amino acids (FAAs) in the leaves of plants subjected to ammonium toxicity is approximately 30% higher than in plants grown under normal conditions, and the composition of the FAAs differs significantly (Fig. 4). Ammonium ($\text{NH}_3 + \text{NH}_4^+$) was detected in all sample groups, but the accumulation of ammonium in the leaves of plants treated with toxic levels of NH_4^+ was approximately three times higher.

Specifically, ammonium toxicity decreases the concentration of aspartate (Asp), glutamate (Glu), alpha-aminobutyric acid (homoalanine), gamma-aminobutyric acid (GABA), arginine (Arg) and leucine (Leu), while inducing greater accumulation of threonine (Thr), hydroxyproline (Hyp), valine (Val), serine (Ser) and alanine (Ala), as well as beta-alanine, cystine, tryptophan (Trp) and homocysteine (Fig. 4). Since Asp and Glu are two key entry points in plant amino acid metabolism, their deficiency in tobacco plants exposed to excess ammonium suggests that the biosynthesis of amino acid and N-containing metabolite biosynthesis is being reprogrammed.

Global gene expression highlights ammonium toxicity in *Nicotiana* leaves

The abnormally high accumulation of free ammonium in the leaves may have impaired photosynthesis and other cellular metabolic processes. Thus, RNA sequencing (RNA-seq) was performed on leaf samples to monitor global gene expression. Transcriptomic data from the leaves of the three experimental plant groups revealed moderate number of differentially expressed genes (DEGs) between plants supplied with a mixture of nitrogen sources and those supplied with nitrate alone. However, a large number of DEGs were found between plants supplied with NH_4^+ and those supplied with a mixture of nitrogen sources or nitrate alone at the same N level (Figures S1). As the DEGs between NH_4^+ -fed plants and both mix-fed and NO_3^- -fed plants largely overlapped in terms of up-regulation or down-regulation, these sets of DEGs were merged, totaling 14,202 genes (Figure S2A). Functional enrichment analysis was then performed on these genes based on Gene Ontology (GO). We found that genes involved in photosynthesis, thylakoid- and membrane-related genes, and genes with catalytic, transferase, and kinase activities were the most significantly affected compared to plants grown normally and supplied with either nitrate or a mixture of nitrate and ammonium in equal nitrogen amounts (Figure S2B).

The functional enrichment of the up- and down-regulated gene sets is clearly distinct (Fig. 5). Both sets of genes are enriched for GO annotations of kinase, transferase and catalytic activity, as well as the extracellular region, under conditions of ammonium toxicity. However, down-regulated genes are specifically enriched for photosynthesis, the thylakoid membrane, hydrolase activity, the response to endogenous stimuli, metabolites and energy precursors, and carbohydrate metabolic processes, among others. Conversely, the up-regulated genes are more significantly associated with transcription factors, transcription regulators, transporters, mitochondria, signaling and cell communication (Fig. 5A). The downregulation of photosynthesis and thylakoid genes, and the upregulation of mitochondrial genes in response to excess ammonium, are consistent with the previous conclusion that NH_4^+ toxicity impairs photosynthetic capacity and increases mitochondrial oxidative reactions (Esteban et al., 2016).

Further analysis of metabolic pathway enrichment in the DEGs based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database revealed that the upregulation of genes involved in the nitrogen and amino acid pathways, and in fatty acid degradation, flavonoid biosynthesis and oxidative phosphorylation, was more significant (Fig. 5B). Once again, genes in the photosynthesis and carbon metabolism pathway were markedly enriched in the down-regulated gene set, which also included genes for sulfur metabolism and conjugated sugar metabolism (Fig. 5B). The upregulation of several amino acid pathways likely contributes to the accumulation of free amino acids observed in the leaves in response to excess ammonium (Fig. 4). However, the marker genes in these pathways that are responsible for ammonium toxicity still need to be verified.

Expression profiles of genes involved in the primary ammonium assimilation and membrane transport under conditions of excess ammonium stress

The *N. tabacum* genome contains 13 homologous glutamate dehydrogenase (FAD- and NADH-GDH) genes, four glutamate synthase (GOGAT) genes, 22 glutamine synthetase (GS) genes, 20 aspartate aminotransferase (AAT) genes and 11 asparagine synthetase (ASN) genes. However, only four GDH genes, one GOGAT gene, three GS genes, three AAT genes and three ASN genes are present in the DEG between ammonium-stressed and non-stressed leaves (Fig. 6A). Both the GS and ASN genes have been up- and down-regulated, whereas the GDH and AAT genes have been exclusively up-regulated and the GOGAT gene has been down-regulated (Fig. 6A). This suggests that, under stressed conditions, the GDH route dominates the GS/GOGAT pathway in assimilating NH_4^+ , resulting in low NH_4^+ incorporation efficiency, as illustrated in Fig. 6B.

Ammonium transporters (AMTs) (Ninnemann et al., 1994) and specific types of aquaporins (AQPs) (Holm et al., 2005; Wu and Beitz, 2007) are two distinct families of proteins that are known to facilitate the transport of ammonium/ammonia across membranes. Of the tobacco *AMT* family (Figure S3), four *AMT* genes have been found to be up-regulated, while two *AMT1;3* homologues are down-regulated under high ammonium stress (Fig. 7A). Additionally, of the large number of differentially expressed *AQP* genes (Figure S4), the tonoplast *AQPs* (TIPs) are found to be down-regulated, while the endoplasmic reticulum-resident *AQPs* (*SIPs*) are exclusively up-regulated under stress (Fig. 7A). As TIPs were the first *AQPs* to be verified for NH_3 permeability (Jahn et al., 2004), the downregulation of these genes suggests that the NH_3 channels between the cytoplasm and the vacuole are blocked under conditions of excessive ammonium accumulation.

Since the pH-dependent equilibrium between uncharged NH_3 and charged NH_4^+ ($\text{pK}_a = 9.25$) may affect proton homeostasis in cellular compartments, we examined the expression patterns of proton-transporting ATPases. While the expression of cytosolic and chloroplast/plastid form-encoding ATPase genes increases or decreases, several mitochondrial, nuclear and vacuolar isoform genes have been shown to be exclusively upregulated (Fig. 7B), indicating the existence of a potential stress signal that regulates ion homeostasis in these compartments.

Furthermore, the abnormal accumulation of free amino acids under ammonium stress (Fig. 4) has prompted us to examine the expression patterns of the amino acid transporter genes. These genes belong to at least five distinct families with diverse functions, which could be clustered into a superfamily known as the amino acid, polyamine and organocation (APC) clan (Pratelli and Pilot, 2014). By comparing normal growth plants that were fed with mix nitrogen or sole NO_3^- , we found that four GABA transporter genes (GAT subfamily) were up-regulated under excess ammonium stress (Figure S5), despite their functionality not yet being fully established.

An inferred gene regulatory network (iGRN) provides candidate transcription factors that respond to ammonium toxicity

The up-regulated DEGs are enriched in the GO terms 'DNA binding', 'transcription factor activity' and 'transcription regulator activity', as well as 'signaling receptor activity' (Fig. 5). To identify the key transcription factors (TFs) involved in the response to and signaling of ammonium toxicity, we constructed a gene regulatory network (GRN) using the 'BioNERO' R package (Almeida-Silva and Venancio, 2022), which employs the "wisdom of the crowds" principle for GRN inference by implementing three popular algorithms: GENIE3(Huynh-Thu et al., 2010); ARACNE(Margolin et al., 2006); and CLR (Faith et al., 2007).

To this end, we first identified a set of 716 TFs in the total differentially expressed genes via PlantTFDB 4.0 prediction (Jin et al., 2017). We then used these as 'regulator' input to generate a global inferred GRN. This iGRN predicts 348 hubs connecting to 3,062 targets, with 422 TFs in the network (Fig. 8A, Supplementary Data 1). To refine the prediction, we mapped the DEG sequences to the KEGG orthology database to compile a set of genes with functional annotations related to amino acid and derivative metabolism (AA-related gene set, 302 genes). We also collected the genes enriched in the metabolism pathway, transporters and signaling pathway as the metabolic gene set (2017 genes) and transport-signaling gene set (503 genes), respectively. We then screened the iGRN using these three gene sets as 'targets' to extract the sub-network, which yielded three reconstructed sub-iGRNs annotated as 'AA and derivatives-related sub-GRN', 'metabolic sub-GRN' and 'transport-signaling sub-GRN', respectively (Fig. 8A, Supplementary Data 2–4). Each subnetwork has a unique structure, and the regulators are predicted to be linked to functional annotations of their targets. We expected the overlapping hubs to be important transcription factors that potentially regulate amino acid metabolism, transport, and signaling in response to excess ammonium stress (Fig. 8B).

Twelve transcription factors were ultimately identified as candidate hubs, belonging to the MYB, SBP (or SPL), ARF, bHLH, C2H2 zinc finger, ERF/AP2, GATA, NAC, Nin-like and WRKY families, and interacting with 129 unique target genes (Fig. 9A, Supplementary Data 5). Of these candidates, six genes with the highest expression levels and significance were found to be either upregulated (*Nt21G01826*, *Nt05G00384* and *Nt05G02228*) or downregulated (*Nt15G01675*, *Nt07G00272* and *Nt08G00335*) in response to excess ammonium stress (Fig. 9B).

Discussion

Metabolic constraints on carbon-nitrogen and amino acid pathways under high ammonium stress in *N. tabacum*

Tobacco plants are sensitive to a high concentration of ammonium as the sole nitrogen source. Our results show growth retardation of approximately 38% and 48% in plants fed with 10 mM ammonium compared to those provided with nitrate alone or the same amount of nitrogen in the form of ammonium and nitrate together, respectively. Root growth and activity are remarkably inhibited (Fig. 1), resulting in a significant reduction in the accumulation of potassium and nitrogen in the shoot (Fig. 2). The best growth was observed in plants supplied with a nitrate-ammonium mixture, which achieved the highest

carbon-nitrogen assimilation (Fig. 2B-C). Ammonium toxicity causes a visual symptom of premature leaf senescence in the shoot, indicating a decline in photosynthetic pigmentation (Fig. 1A). Under stress conditions, enzyme activities of NR and SuS increase, while those of GOGAT-NADH and acid invertase decrease (Fig. 3). This is probably a response to a shortage of nitrogen and carbon. Similar results were observed in earlier experiments on tobacco plants (Masclaux et al., 2000; Tercé-Laforgue et al., 2004b; Walch-Liu et al., 2000).

Although ammonium concentration in the leaves was significantly higher under stressful conditions than under non-stressful conditions, it was not readily assimilated. Instead, it may have become trapped in acidic compartments, such as the vacuole or the apoplast, when toxic levels accumulated in the cytoplasm (Fig. 4, (Britto et al., 2002; Tercé-Laforgue et al., 2004b; Walch-Liu et al., 2000)). Furthermore, the transcriptome data revealed the exclusive upregulation of four GDH isozyme and three AAT transcripts (Fig. 6). It has been demonstrated that GDH is highly induced in tobacco leaves when plants are grown using ammonium as the sole nitrogen source. In this case, GDH mainly functions in the deamination of glutamate to provide 2-oxoglutarate and ammonium (Masclaux-Daubresse et al., 2006; Tercé-Laforgue et al., 2004a; Tercé-Laforgue et al., 2004b). Skopelitis et al. demonstrated that ROS signaling was responsible for GDH induction under salt or ammonium stress conditions, or during senescence. The authors proposed that GDH might act as an anti-stress enzyme involved in ammonia detoxification and the production of glutamate (Glu) for proline (Pro) synthesis (Skopelitis et al., 2006). Other possible sources of free ammonium accumulation in leaf tissue may include photorespiration, amino acid degradation and the inhibition of biosynthesis due to limited carbon skeletons, as well as the blockage of N-containing metabolite biosynthesis due to an altered pH environment. For instance, a paper reporting on the impact of chloride nutrition on tobacco plants revealed that increased photorespiration activity in the leaves of Cl^- -treated plants led to a decreased glycine/serine ratio and greater ammonium accumulation (Peinado-Torrubia et al., 2022). This is similar to the situation in the leaves of plants stressed by excess ammonium.

Another interesting alteration is that of the free amino acid pools (Fig. 6). The total concentration of amino acids increases by around a third, with significant accumulations of hydroxyproline (Hyp), threonine (Thr), valine (Val) and serine (Ser), as well as beta-alanine. There are also notable declines in the concentrations of glutamate (Glu), aspartate (Asp), gamma-aminobutyric acid (GABA) and homoalanine. Since Hyp, Thr and Ser are typically found in cell wall proteins that are modified by the attachment of O-glycans at these residues (Xu et al., 2023), the higher concentrations of these amino acids in the total pools may indicate an impairment in the synthesis pathway of cell wall proteins, resulting in the accumulation of substrates. Conversely, GABA is known to be a signaling molecule involved in the regulation of multiple stress resistances in plants (El-Beltagi et al., 2026; Xu et al., 2026), including acidic stress (Shavrukov and Hirai, 2016) and ammonium toxicity (Ma et al., 2016). Thus, a decrease in GABA concentration in the leaves may indicate defective GABA-signaling pathways under excess ammonium stress. However, the key genes that regulate amino acid metabolism in relation to ammonium tolerance and C-N balances still need to be identified. One such gene is argininosuccinate lyase (ASL), which has been functionally demonstrated in rice to convert excessive glutamine to arginine

in the roots, thereby enhancing auxin accumulation in the root meristem and stimulating root elongation under high ammonium conditions (Xie et al., 2023).

Therefore, in hypersensitive tobacco plants at least, excess ammonium inhibits the expression of the chloroplast GS/GOGAT genes but enhances the expression of the cytoplasmic GDH and GS genes. This leads to low assimilation of the cytotoxic ammonium. Concomitantly, the flux into amino acid pathways, which are heavily dependent on functional chloroplasts, is compromised, leading to turmoil in AA compositions and protein biosynthesis.

Identifying candidate transcription factors associated with high levels of ammonium stress

Transcriptome data provides insight into the gene expression patterns in tobacco leaves conditions of excess ammonium. In addition to genes involved in nitrogen assimilation, pathways related to photosynthesis, carbohydrate metabolism, responses to chemicals, and thylakoid, membrane and extracellular structures were among the most affected. Metabolism, kinase and transporter genes were also among the most highly enriched DEGs (Fig. 5 and S2). Using an integrated implementation of algorithms based on the transcriptome data, we constructed an iGRN associated with excess ammonium stress conditions. This iGRN predicts significant hubs for 348 TF genes within a complex network of 422 regulators that link to 3,062 targets. After extracting the global iGRN with target genes enriched in pathways related to metabolism, amino acid and derivative biosynthesis, and transport and signaling activities, three sub-networks were reconstructed using obtained TFs as regulator inputs. Hubs in the three sub-networks overlapped with 12 transcription factors, six of which are significant candidates that respond to high ammonium stress (Figs. 8 and 9).

Currently, there is limited functional evidence regarding the specific roles played by the candidate TFs in tobacco plants, but hints from other studies exist. Three candidate regulator genes that encode homologues of ERF/AP2 (*Nt21G01826*), NAC (*Nt05G00384*) and Nin-like (*Nt05G02228*) were found to be upregulated in stressed leaves. The Arabidopsis NAC protein that is most closely related to *Nt05G00384* is ANAC036 (*At2G17040*). Previous studies have shown that overexpressing ANAC036 induces a dwarf phenotype (Kato et al., 2010). However, another Arabidopsis NAC protein, ANAC017, which is encoded by the *At1g34190* gene, has been shown to regulate mitochondrial retrograde signaling in response to nitrogen sources. Loss-of-function mutations in *ANAC017* enhance the ability of Arabidopsis plants to utilize NH_4^+ effectively (Ren et al., 2025). Furthermore, *Nt05G02228* encodes a homologous of nodule inception protein-like (Nin-like or NLP) that is most closely related to Arabidopsis NLP8 and NLP9. In Arabidopsis, NLP8, NLP9, NLP6 and NLP7 have well-demonstrated roles in nitrate signaling and in regulation of nitrogen nutrition, whereby these proteins bind to the nitrate-responsive cis-element to activate nitrate-responsive transcription (Armijo-Godoy et al., 2026; Cheng et al., 2023; Huang et al., 2025; Konishi et al., 2021; Konishi and Yanagisawa, 2013; Liu et al., 2017; Wang et al., 2022; Yan et al., 2016). The other three candidate TFs that are down-regulated in stressed leaves are SBP (*Nt15G01675*),

bHLH (*Nt07G00272*) and MYB (*Nt08G00335*). *Nt15G01675* encodes a SBP (SQUAMOSA PROMOTER BINDING PROTEIN) homologous that is closely related to Arabidopsis SPL10, SPL11 and SPL2. These are known to regulate the development of lateral organs in association with shoot maturation (Shikata et al., 2009). *Nt07G00272*, on the other hand, is an orthologue of *AtbHLH63* or *AtCIB1* (*CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX 1*), which is a floral initiation regulator responsive to blue-light (Liu et al., 2008). Nevertheless, verification of their specific molecular mechanisms is needed, for example through further experiments with tobacco mutants of these candidate genes generated using state-of-the-art genome editing approaches. This would provide further insight into how their functions are linked to ammonium tolerance and nitrogen use efficiency in the crop.

In conclusion, the results of this recent study pave the way for further deciphering of the molecular mechanisms underlying ammonium sensitivity and tolerance, and for improving nitrogen utilization efficiency in future crop studies.

Declarations

Competing Interests

The authors (JG, QC, HW, GZ, CW and WL) were employed by the Fujian Tobacco Science and Research Institute. The remaining authors declare that they have no competing financial interests or personal relationships that could have influenced the work reported in this paper.

Funding

This study was partly funded by a China Tobacco Corporation Science and Technology Project (number 110201902002) and a Fujian Branch Tobacco Corporation Key Project (number 2019350000240009). The funders were not involved in the design of the study, the collection or analysis of the data, the decision to publish, or the preparation of the manuscript.

Author Contribution

JG, WL and YM conceived the idea for this research. JG and RX planned and initiated the experiments. RX, QC, HW, GZ, CW conducted the analysis. JG wrote the original draft. WL and YM reviewed and edited the manuscript. WL and YM read and edited the final version of the manuscript. All authors read and approved the final draft.

Acknowledgement

The authors would like to thank Professor Binghua Wu for his valuable comments and suggestions regarding this study, as well as for his assistance in preparing the manuscript. Professor Zhujun Zhu was thanked for his critical reading of the manuscript.

Data availability

Data are available from the authors upon request.

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Figures

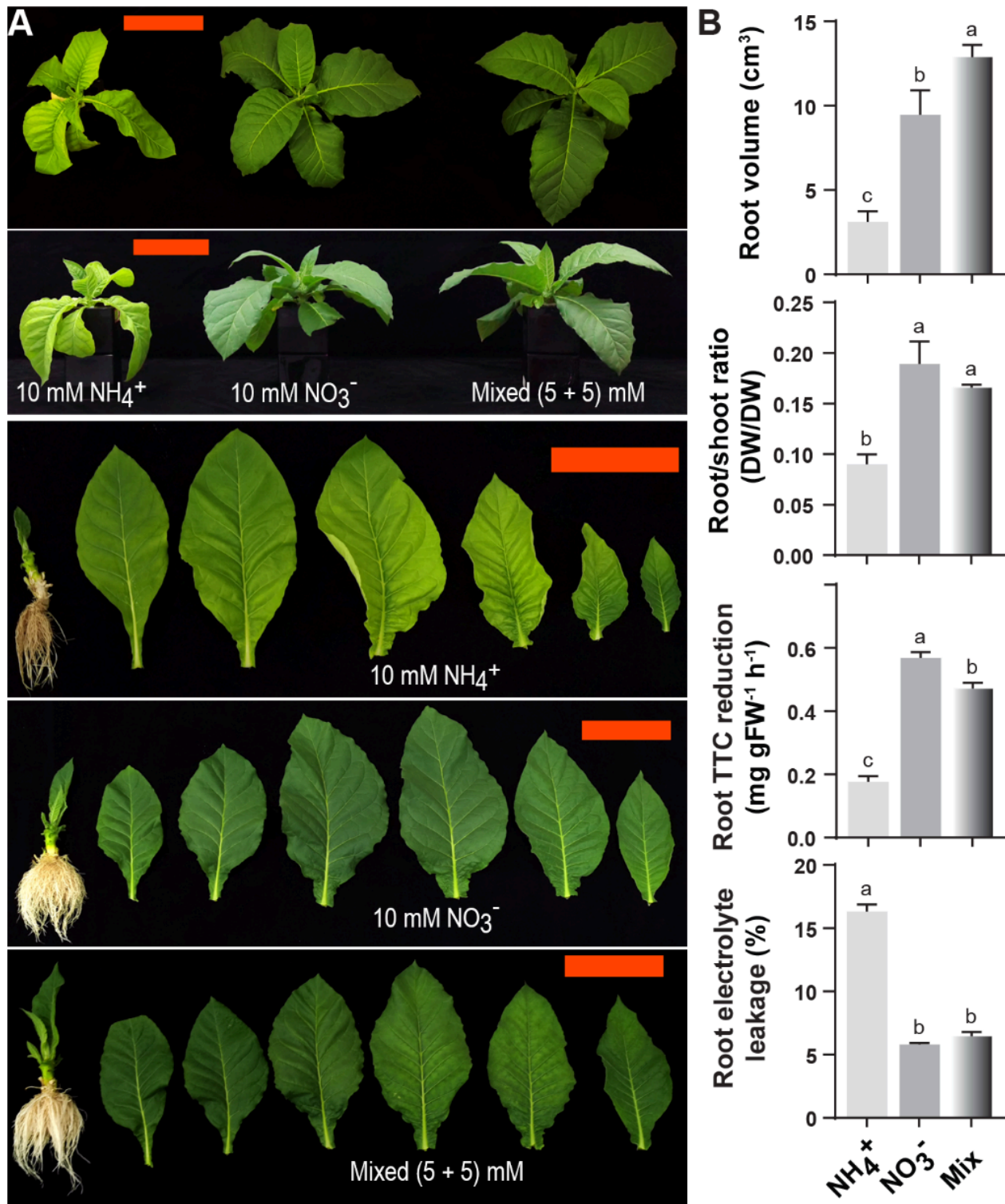


Figure 1

A high concentration of NH_4^+ (10 mM) causes toxic symptoms in young tobacco plants. (A) Seedlings at the seven-leaf stage were subjected to three weeks of hydroponic culture supplemented with different nitrogen sources, as indicated. Scale bar = 20 cm. (B) Root traits were characterised at three weeks post treatment. Different letters above the bars indicate significant differences in the means (adjusted $p < 0.05$, $n = 3$).

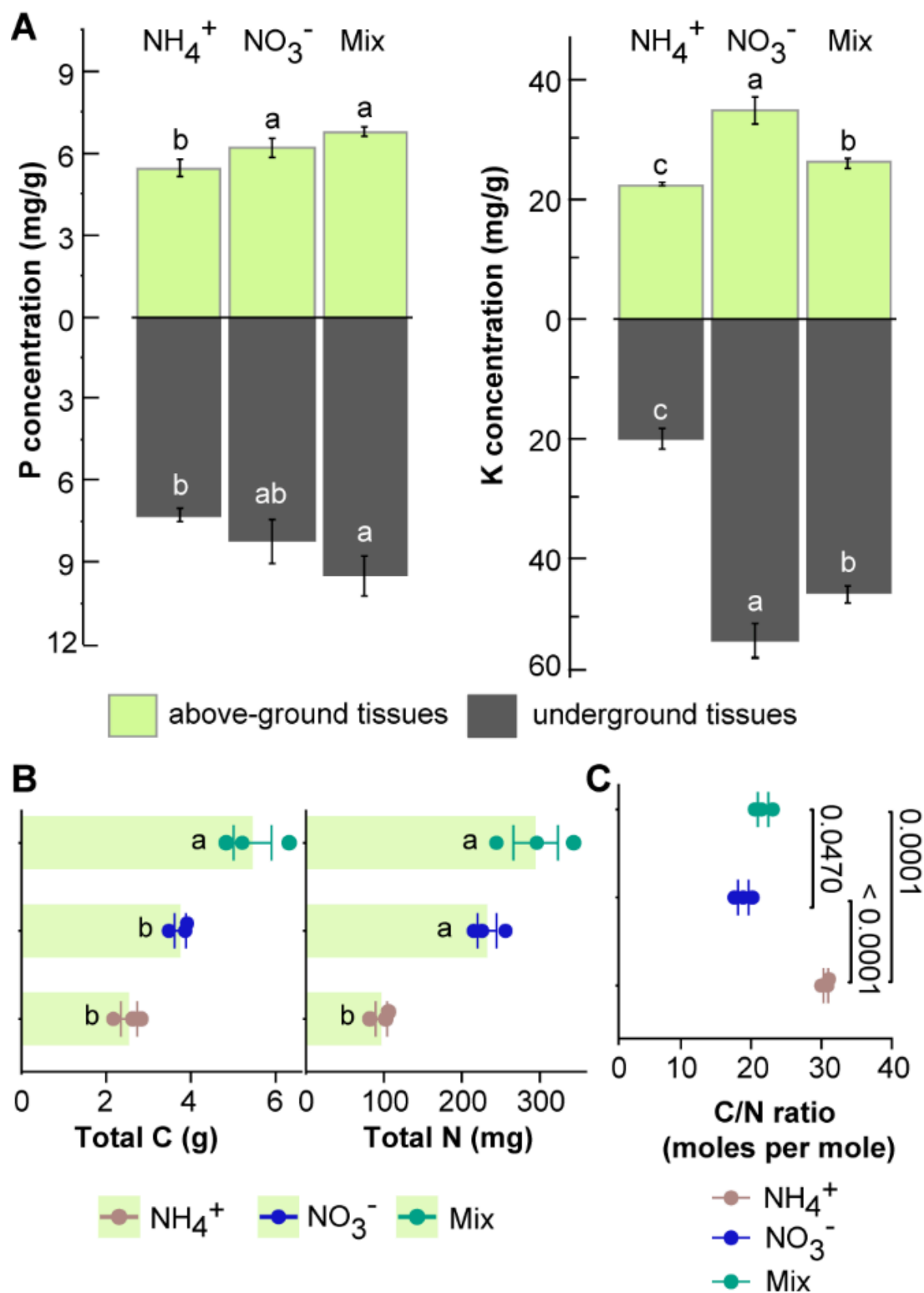


Figure 2

Reduction in the accumulation of P, K, C and N in tobacco plants fed with a toxic level of NH_4^+ . (A) Content of phosphorus (P) and potassium (K) nutrients in whole plant tissues. (B) Total carbon (Total C) and total nitrogen (Total N) in the above-ground parts of the plants. Different letters indicate significant differences between means (adjusted $p < 0.05$, $n = 3$).

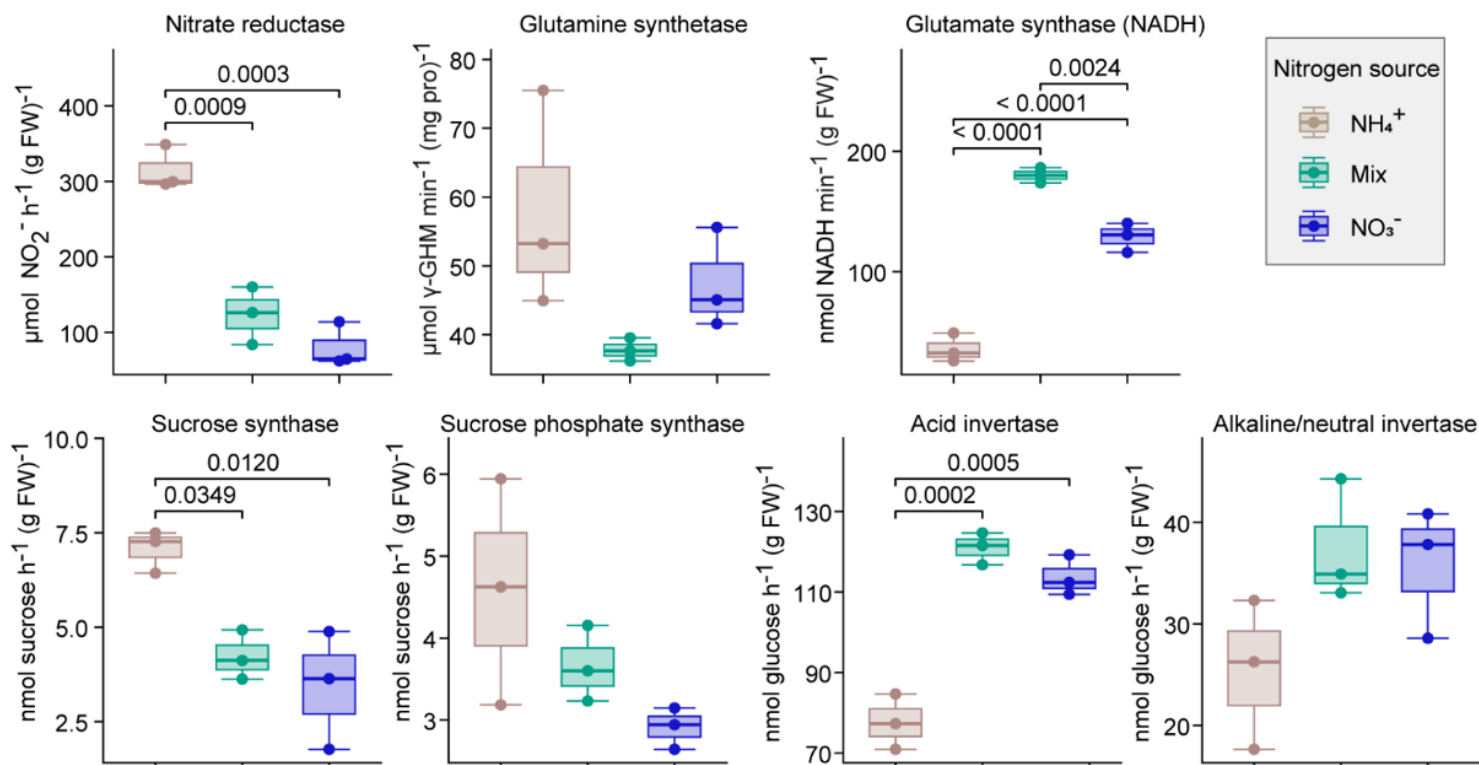


Figure 3

Enzyme activities in plant leaves following a three-week exposure to nitrogen sources. Leaf crude extracts were used to determine the apparent activity of the enzymes. Statistical significance was assessed using a one-way ANOVA and Tukey's post hoc HSD test ($n = 3$). Abbreviations: γ -GHM, γ -glutamylmonohydroxamate.

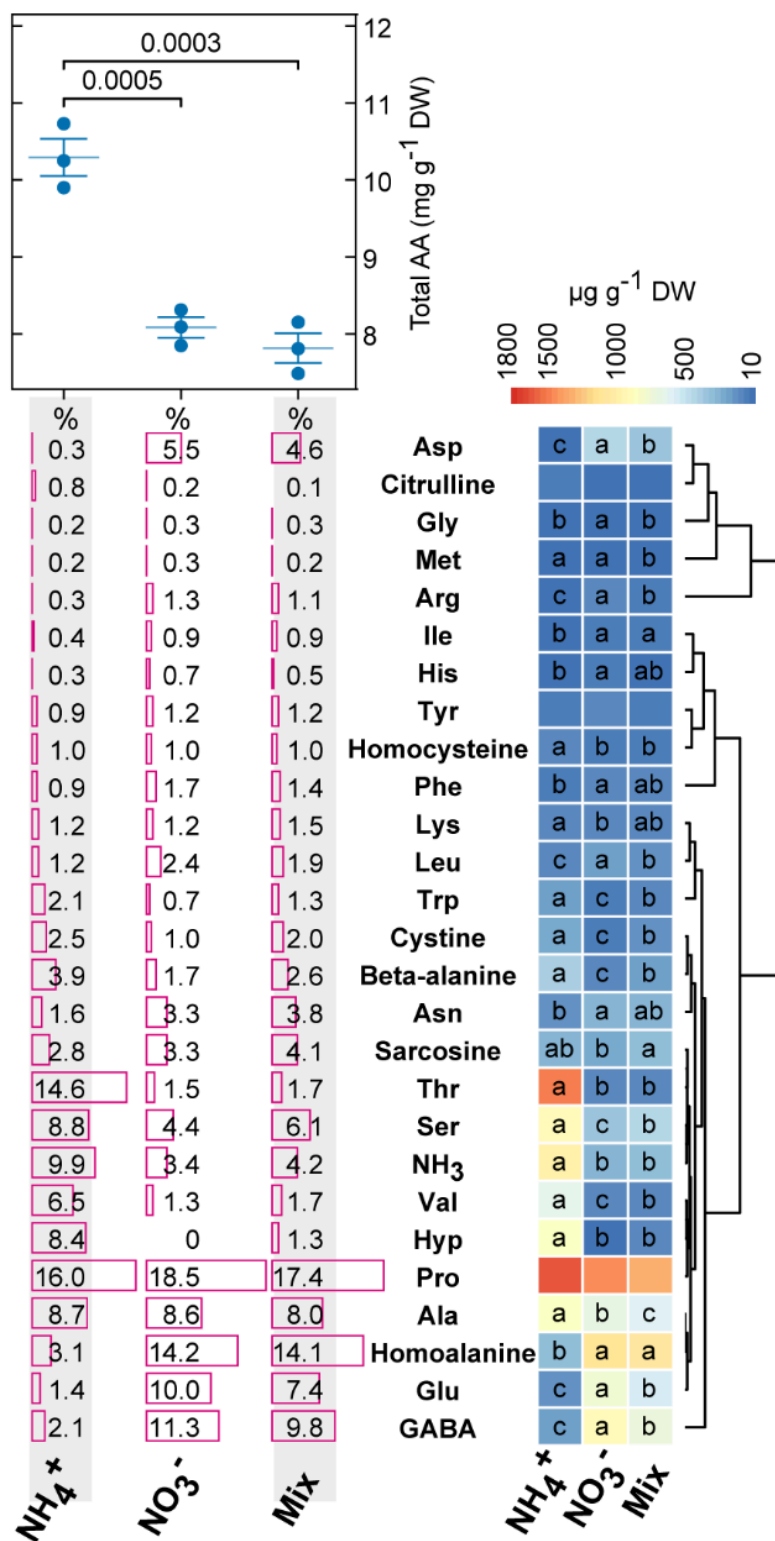


Figure 4

Abnormal accumulation of free amino acids in leaves caused by the toxic level of NH_4^+ supplementation. The total amino acid (AA) content and the percentage of individual AAs are shown on the left, while the heatmap on the right displays their absolute value. The mean of three determinations was compared using one-way ANOVA with Tukey's post hoc HSD test. Different letters on the heatmap denote significant differences (adjusted p-value <0.05, n=3) between groups.

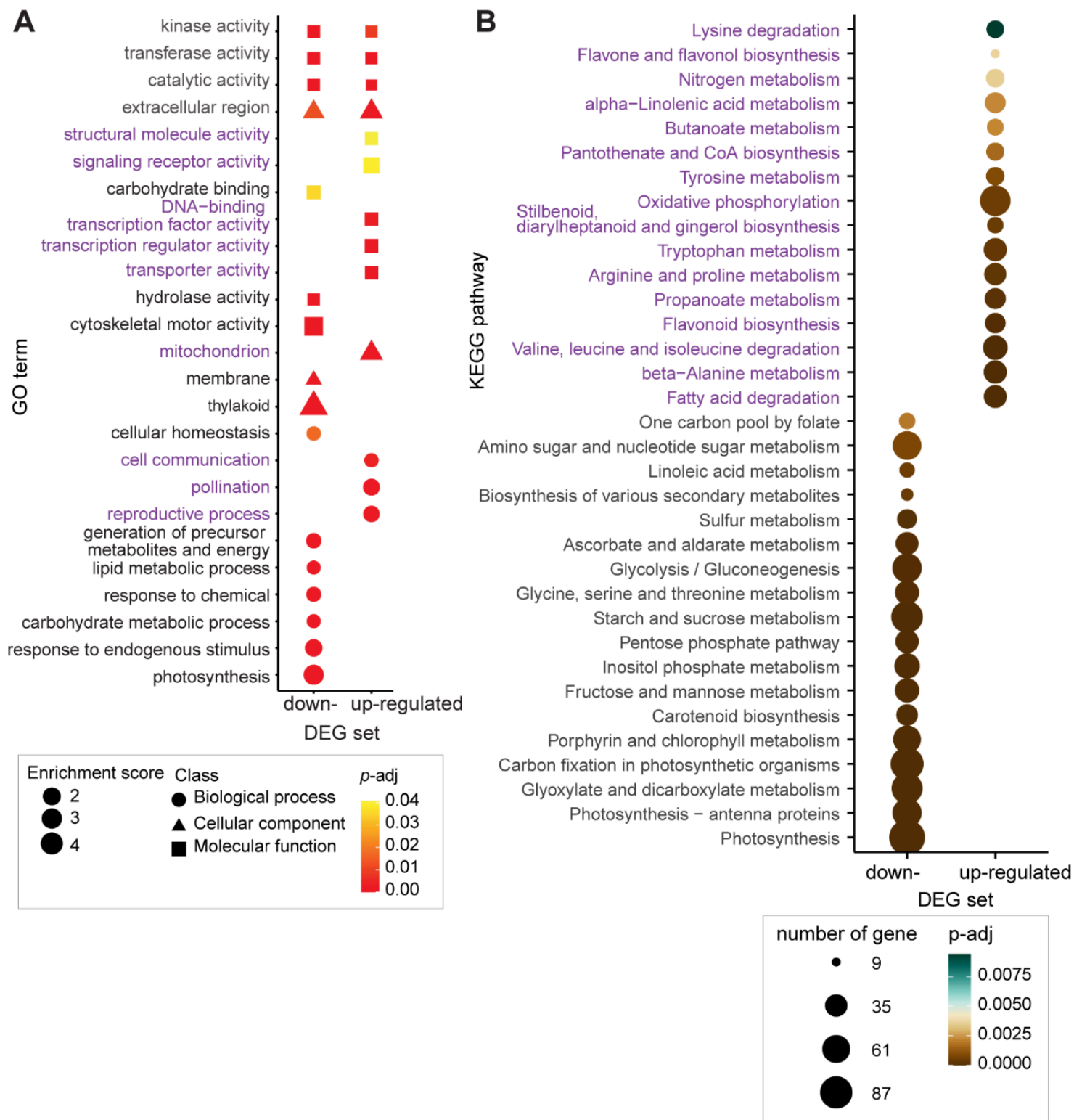


Figure 5

Highly represented GO functional annotations (A) and KEGG metabolic pathways (B) enriched in the up- or down-regulated DEG sets under excess ammonium stress. (Supplemented by Figure S1, S2 and Table S)

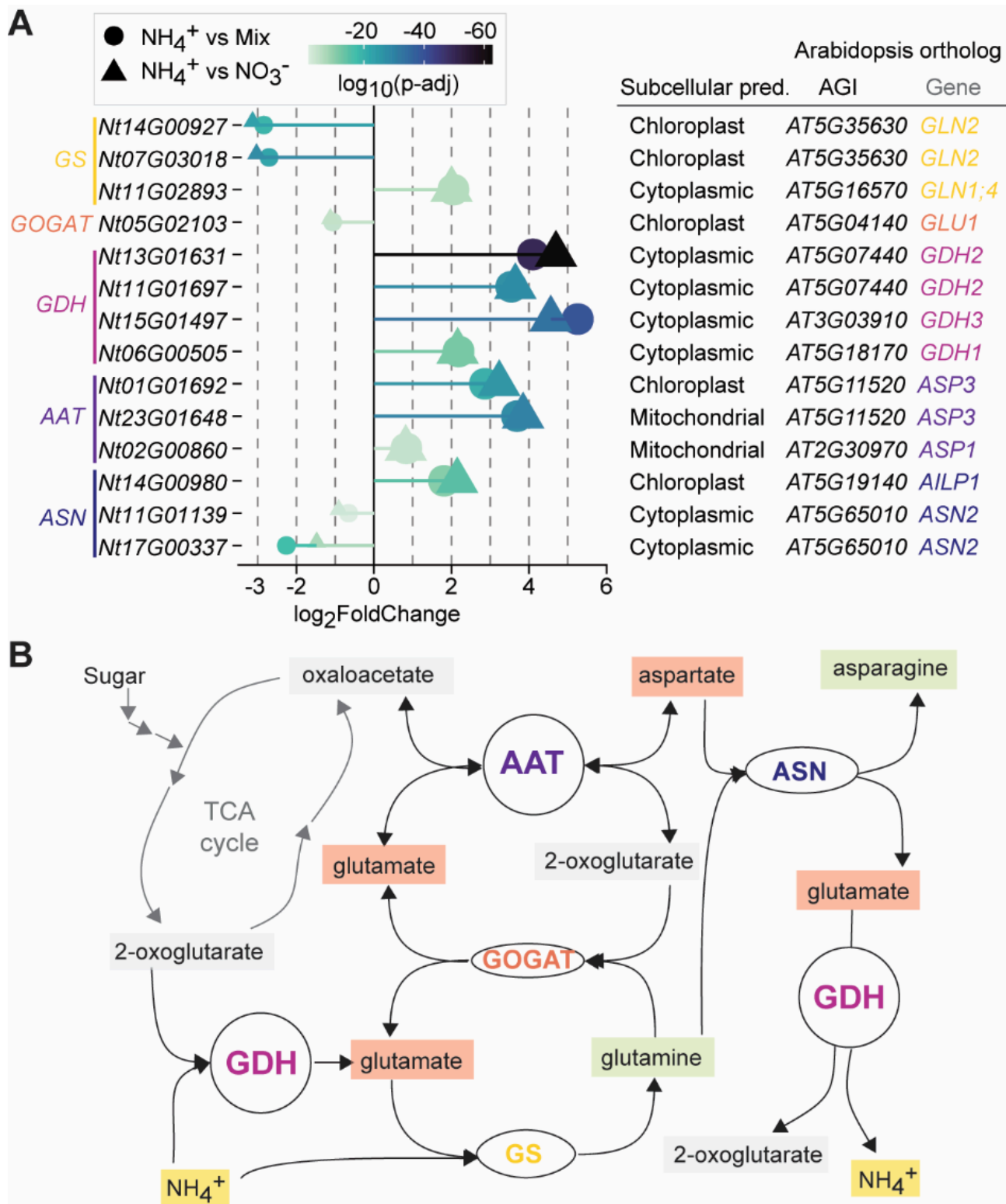


Figure 6

Differential gene expression in primary nitrogen assimilation pathways under conditions of excess ammonium stress. **A.** The DEGs in the treatment group and their closed homologues/orthologues from *A. thaliana*. **B.** A simplified illustration depicting the enzyme genes involving in the primary nitrogen assimilation pathways. Enzymes with genes that are all up-regulated are shown in circular brackets, while others are shown in ovals. GS: glutamine synthetase [EC. 6.3.1.2], GOGAT: glutamate synthase [EC.

1.4.7.1 or EC. 1.4.1.14], GDH: glutamate dehydrogenase [EC. 1.4.1.2-4], AAT: aspartate aminotransferase [EC. 2.6.1.1], ASN: asparagine synthetase [EC. 6.3.5.4].

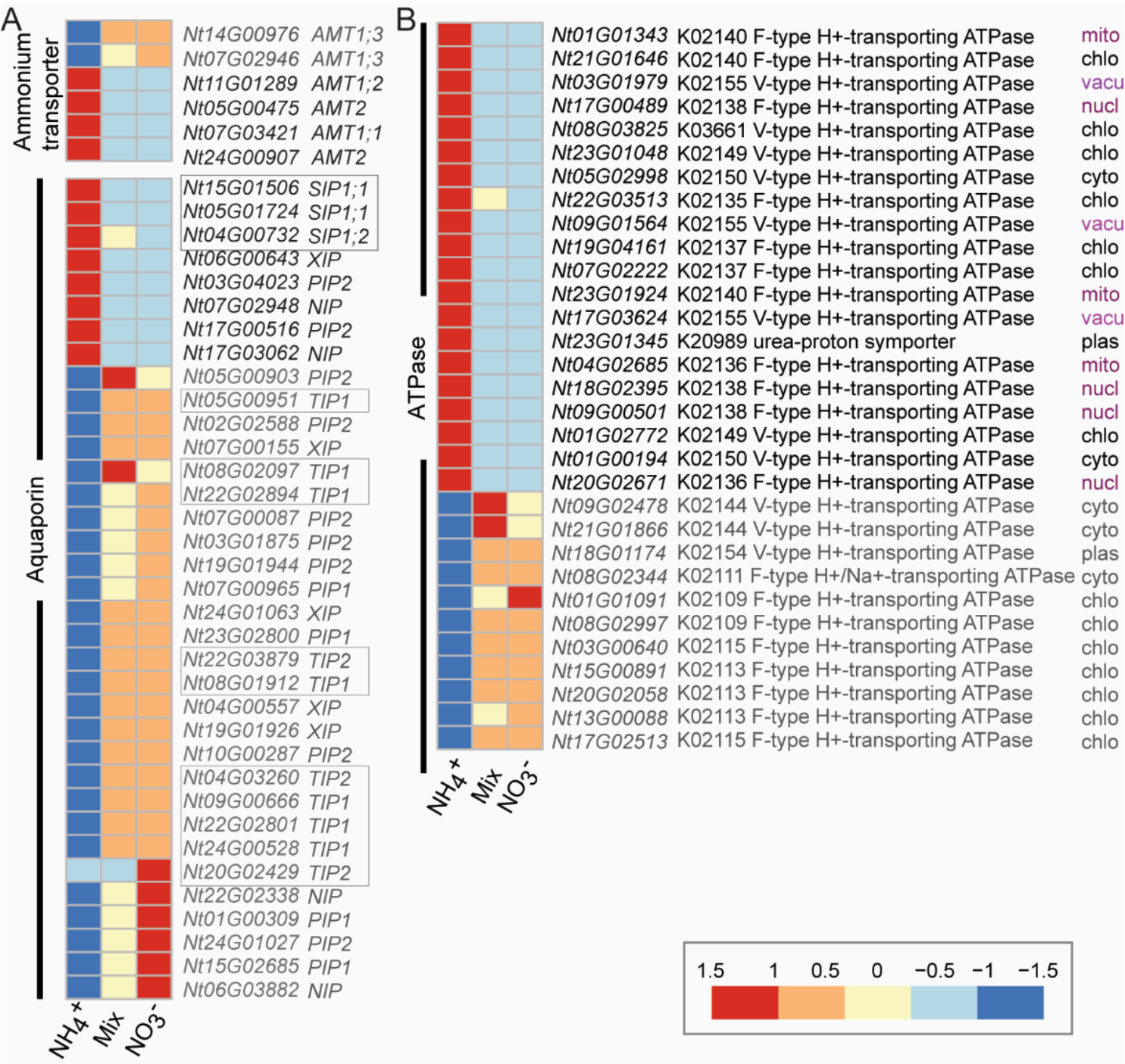


Figure 7

Heatmap showing differentially expressed genes involved in ammonium/ammonia or proton transporting proteins in the sample groups. **A**. The ammonium transporter family and the MIP aquaporin family. **B**. The proton-transporting ATPase families. The predicted subcellular localization (via WoLF PSORT web, <https://wolfpsort.hgc.jp/>) of the ATPase gene products are showed. Abbreviation, mito: mitochondria, chlo: chloroplast, vacu: vacuolar, nucl: nucleus, cyto: cytosolic, plas: plasma membran.

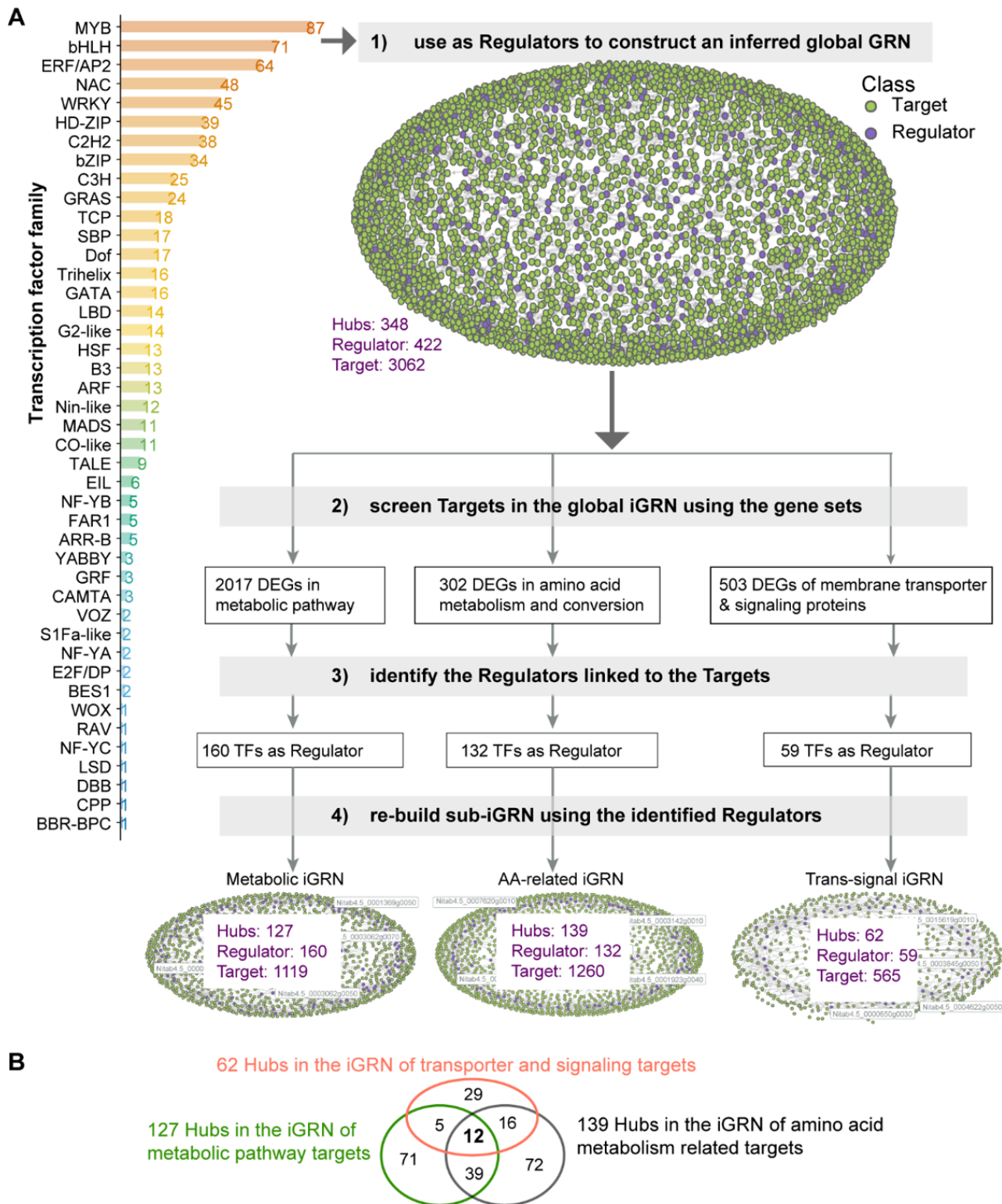


Figure 8

Construction of a co-expression-based inferred gene regulatory network (iGRN) and identification of 12 putative hub transcription factors (TFs) under excess ammonium stress conditions. **A.** A set of 716 TF genes were identified using the PlantTFDB (v5.0) prediction tool in the differentially expressed genes (DEGs) between NH_4^+ -fed plants and the other two groups of plants. These genes were used as 'regulators' to construct the global iGRN. Subsequently, screening the iGRN with metabolism-enriched

DEGs, amino acid metabolism- and conversion-related genes, and transporter- and signalling-enriched DEGs yielded three sub-GRNs. **B.** The overlapping hubs in the three sub-GRNs are represented by 12 TF genes.

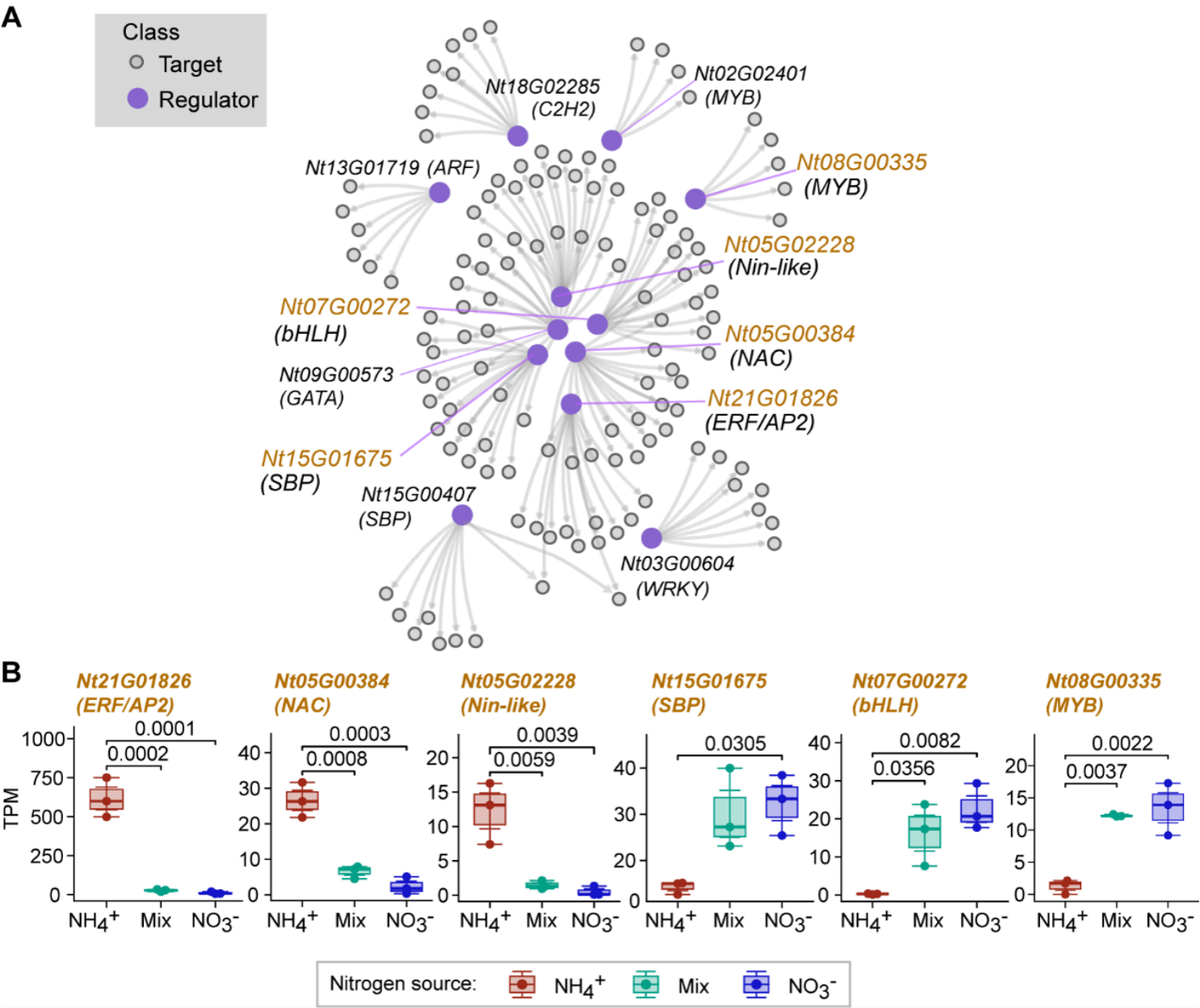


Figure 9

The twelve candidate transcription factors and the top six respond to excess ammonium stress in *N. tabacum* leaves. **A.** The network of regulator-target interactions. **B.** The top six TFs and the expression profiles. (See supplemental Table xx for more details of the interactions).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementarytableS1.theglobaliGRN.xlsx](#)
- [SupplementarytableS2.theAArelatedsubGRN.xlsx](#)
- [SupplementarytableS3.licsubGRN.xlsx](#)
- [SupplementarytableS4.thetransportsignalingsubGRN.xlsx](#)
- [SupplementarytableS5.theoverlappinghubstargets.xlsx](#)
- [Supplementaryfigures.docx](#)