Ammonium and nitrate impact petal color traits and amino acid profiles differently in *Petunia*

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Abstract

Nitrogen (N) is vital for ornamental plants to fulfill fundamental cellular functions. Effects of inorganic N, including nitrate and ammonium, on ornamental traits at the biochemical level are less reported. Given that the continuously increased concentration of CO_2 in the atmosphere has greater negative effects on nitrate reduction than ammonium utilization in C_3 plants, it is vital for us to dissect various effects of different inorganic N forms on flower development, which will lead to a better understanding of the metabolic regulation of ornamental traits. In this study, *Petunia × hybrida* cv 'Mitchell Diploid' (MD) plants at 6–8-leaf stage were treated hydroponically with the same and regular amount of inorganic N for one month but in three different forms, 1 mM NH_4NO_3 as the control (NH_4NO_3), 2 mM NH_4Cl alone (NH_4-N) and 2 mM $NaNO_3$ alone (NO_3-N). Results indicated that relative to the control, effects of NH_4-N and NO_3-N treatment alone on plant growth, N utilization and distribution, petal color traits and petal amino acid profiles were different by varying degrees. We have confirmed that NH_4-N could specifically increase ^{15}N distribution to floral tissues, change the amino acid profiles in petals which in turn bring changes to petal colors. Results with four commercially available *Petunia* cultivars with different flower colors also validated our findings. This study has been designed to specifically investigate effects of ammonium or nitrate as the sole source of inorganic N on the developmental and chemical features of floral tissues in *Petunia*.

INTRODUCTION

Production and care of ornamental plants usually receive less attention relative to other horticultural crops and therefore may suffer more from suboptimal growth conditions. It is vital for us to understand the development of ornamental plants at the biochemical level in response to environmental cues, which will lead to a healthier cultivation systems.

Growth of plants is inseparable from the supply of nitrogen (N)[1]. Nitrogen plays key roles in the formation of cells, tissues and organs as the primary constituent of proteins, nucleic acids, nucleoside phosphates, phospholipids, and more importantly, secondary metabolites[2]. Both inorganic and organic forms of N exist in plants and soils with ammonium and nitrate as the most important inorganic N for growth and development. Generally, ornamental plants prefer nitrate over ammonium[3]. Recently, several studies suggest that with the rise of atmospheric CO_2 concentrations, nitrate reduction in C_3 plants such as Arabidopsis and wheat is inhibited whereas ammonium utilization is unaffected[4]. This is problematic as this reduction in nitrate assimilation can lead to the decrease of N use efficiency and the accumulation of ammonium. And the latter will cause ammonium toxicity in plants *via* acidic stress[5] and impair shoot growth significantly which also affects the production of ornamental plants. Therefore, different forms of inorganic N should be assessed independently in horticultural crops including ornamental plants.

Plant roots can take up both ammonium and nitrate from soil[6]. The absorption of inorganic N is an active process that is controlled by plasma membrane-localized transporters in the root[7]. There are two nitrate uptake transporter families in higher plants, namely low-affinity transporter (LATS) NRT1/PTR and high-affinity transporter (HATS) NRT2[8,9], while ammonium uptake is mediated by the high affinity transporters of the AMT/MEP/Rh (AMT) subfamily[7]. In cells, the reduction of nitrate to ammonium requires two consecutive reactions. Firstly, NO_3^- is reduced to nitrite in the cytoplasm by nitrate reductase (NR)[10]. Secondly, nitrite is reduced by nitrite reductase (NiR) to form ammonium in the chloroplast[11]. Ammonium is then incorporated into organic molecules *via* the glutamine synthase (GS)-glutamate synthase (GOGAT) pathway in leaves or roots[12–14]. Sequentially a variety of amino acids are synthesized in both roots and shoots and participate in biosynthesis of diverse metabolites. On the other hand, cellular ammonium can also come directly from photorespiration and the cycling of amino acids. Nitrate assimilation and ammonium assimilation can both be regulated at the transcriptional and posttranslational level and is a core metabolic network in plant carbon-nitrogen interactions[15].

Generally, the assimilation of ammonium is far more energy efficient compared to nitrate assimilation[16]. However, nitrate is more available for plants due to its higher mobility[17]. Also, soil aeration status and soil pH value have effects on the availability of inorganic N. In well aerated agricultural soils, nitrate is the principal inorganic N source, whereas in undisturbed soils, ammonium is dominant. Plants grown in acidic soils take up more nitrate, while in soils with neutral pH values, they prefer taking up more ammonium. Meanwhile, different plant species will preferentially select specific N sources, thereby forming

different N utilization and distribution mechanisms, which are mainly related to the long-term adaptation of plants to N environments.

The floral organ is one of the main nitrogen pools and its formation requires the regulation of multiple metabolic pathways. However, roles of N metabolism in flower development and function fulfillment are still poorly understood. Amino acid metabolism, a part of plant N metabolism, is of great significance to the development and function of floral organs[18]. In addition, amino acids are also important precursors for the synthesis of secondary metabolites, such as phenolics, alkaloids and other nitrogen-containing secondary substances. Studies have shown that the expression of the gene encoding 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase in *Petunia* was associated with petal specificity, and the expression level was significantly increased during flower opening[19]. EPSP synthase is also a key enzyme in the shikimate pathway and is essential for the production of aromatic compounds that provide substances such as phenylpropanoid and indoleacetic acid for petal coloration and development[19,20]. Studies have shown that inhibition of EPSP synthase leads to a decrease in the level of aromatic amino acids, which in turn inhibits protein synthesis[21,22]. So far, besides studies related to amino acid transporters in various floral tissues[23,24], only a few studies have focused on amino acid metabolism in floral tissue[25]. The *de novo* synthesis of proline has been reported in floral tissues such as pollen grains[18]. Efforts should be made to dissect different forms of inorganic N on floral nitrogen metabolism, which will lead to a better understanding of the metabolic features in floral tissues.

In this study, we treated *Petunia* plants with three forms of inorganic N at the same concentration of total N including 1 mM NH_4NO_3 (the control), 2 mM NH_4Cl (NH_4-N) and 2 mM $NaNO_3$ (NO_3-N) from the vegetative to the reproductive stage. Plant growth, ^{15}N utilization and distribution in different tissues, petal color traits and petal amino acid profiles were thoroughly analyzed to compare effects of different inorganic N on floral development and establish unique roles of ammonium in floral tissues. Results are discussed in the context of establishing roles of different forms of inorganic N in the formation of ornamental traits including flower development and petal pigmentation.

MATERIALS AND METHODS

Plant material and growth conditions

Petunia × *hybrida* cv 'Mitchell Diploid' (MD) plants were used in this study. After approaching their 6–8 leaf stage grown in culture medium (Pindstrup Sphagnum peat moss: perlite: vermiculite = 3:1:1) under 16/8-h day/night cycles, temperature of 22/18 °C, light intensity of 150 μmol m^{-2} s^{-1}, and 50% relative humidity, MD plants were then hydroponically cultured with the same and regular amount of inorganic N for one month but in three different forms, 1 mM NH_4NO_3 as the control (NH_4NO_3), 2 mM NH_4Cl alone (NH_4-N) and 2 mM $NaNO_3$ alone (NO_3-N). This experimental set-up had been tested to make sure that after one month of treatment, MD plants could reach their reproductive stage and produce flowers for sampling. The total N content at 2 mM was chosen because it was the regular amount of N used in nutrient solution. Other than inorganic N, the nutrient solution was prepared as the

following formula: 0.67 mM NaH_2PO_4, 0.60 mM $MgC1_2·6H_2O$, 0.30mM K_2SO_4, and 0.30 mM $CaCl_2·2H_2O$. The pH value of nutrient solution was adjusted to 5.8. During the treatment, nutrient solutions were replaced every 10 d and clean water was supplemented as needed.

Measurement of *Petunia* developmental phenotypes

After 30 d of hydroponic treatment with different forms of inorganic N, the plant height, branch number, flower number, leaf area, corolla area, and the fresh and dry weight of both above- and under-ground tissues in MD plants were measured. Plant height, the natural height of the main stem, was analyzed with a tape measure. Stem thickness was the width of the main stem near the root of the plant, measured with a vernier caliper. Considering that the *Petunia* plant was short, the number of branches longer than 5 cm was counted. Petals and leaves were randomly collected from plants and measured for their area *via* Image J software (https://imagej.nih.gov/ij). Tissue weights were determined using ME820 balance. A total of 3–4 biological replicates were analyzed for each parameter.

^{15}N isotope labeling and related analysis

In the first and second weeks of hydroponic treatment, 0.3% ^{15}N-urea (Shanghai Research Institute, Shanghai, China), ^{15}N natural abundance is 10.16%; Natural ^{15}N abundance is 0.365%) solution was brushed on the same leaves. At the end of treatment, we collected roots, stems, leaves and flowers (three biological replicates) and dried them in ovens at 60 °C for 2 d to obtain the dry weight *via* a ME820 balance. The samples were ground with a mortar into fine powder and sieved with a 100-mesh sieve. Total N content in various tissues was measured *via* a Kjeldahl nitrogen determination apparatus. ^{15}N abundance in a specific tissue (%) was determined by DELTA V Advantage isotope mass spectrometer (Thermo Fisher, MAT-251). The calculations of ^{15}N absorption, ^{15}N utilization rate and ^{15}N distribution rate in each tissue were carried out according to the following equations:

N_{dff} (%) = (^{15}N abundance in a specific tissue − Natural ^{15}N abundance) / (^{15}N abundance in ^{15}N urea − Natural ^{15}N abundance) × 100;

^{15}N absorption (mg plant^{-1}) = N_{dff} × Total N in a specific tissue;

^{15}N utilization rate (%) = (N_{dff} × Total N in a specific tissue) / Total content of ^{15}N applied to plants × 100;

^{15}N distribution rate (%) = (^{15}N absorption of a specific tissue / Total ^{15}N absorption of 4 tissues) × 100.

In the equations, N_{dff} (%) is the percentage of N derived from ^{15}N urea; total content of ^{15}N applied to plants was 0.4893 g according to the amount of ^{15}N urea used in the label experiment.

Determination of amino acid content in *Petunia* petals

Petal tissues were collected at the early, middle and mature stages during the development of MD flowers and quenched with liquid nitrogen. Petal tissues (~100 mg in FW) were ground into a fine powder in liquid nitrogen and then extracted with 1.5 ml methanol/chloroform/water (4 °C). Norvaline was added to extracts as the internal standard. The extract was filtered with a 0.45 μm cellulose acetate filter and by aspiration with N_2. Amino acid content was determined according to the LC-MS/MS method[26] with slight modifications. After re-dissolution with ddH_2O, samples were injected into a Thermo Scientific TSQ Quantum Access MAX Triple Quadrupole Mass

Spectrometer coupled with a Thermo Fisher Hypersil GOLD™ C18 column (product# 25002-102130).

Determination of *Petunia* flower color

CIELab coordinates including perceptual lightness ($L*$), redness/greenness ($a*$), yellowness/blueness ($b*$), chroma ($C*$), and hue angle ($h°$) of *Petunia* petals were measured for each treatment *via* a portable colorimeter (CR-10). Four biological replicates were analyzed.

Data analysis

Data were recorded and analyzed in Excel, and statistical analyses were performed using Data Processing System (DPS) Software[27]. Duncan's Multiple Range Test was performed in DPS software to determine the statistical difference with P value less than 0.05 between treatments. Clustering analysis, correlation analysis and principal component analysis were performed in R Studio Version 1.1.456 (www.rstudio.com) with R version 3.5.2 using gplots and pheatmap (https://cran.r-project.org/web/packages/pheatmap/) packages, and facto-extra (http://www.cran.r-project.org/web/packages/factoextra/) packages, respectively.

RESULTS

Different forms of inorganic N had effects on plant growth of *Petunia*

In this study, MD plants were treated hydroponically with the same level of inorganic N for one month but in three different forms, 1 mM NH_4NO_3 as the control (NH_4NO_3), 2 mM NH_4Cl alone (NH_4-N) and 2 mM $NaNO_3$ alone (NO_3-N). The concentration of total N for each treatment was set up at 2 mM as this is the regular level of N used in nutrition solutions. After one month of treatment, MD plants approached their reproductive stages and could produce a couple of flowers (Fig. 1a). We measured the total content of N in above- and underground tissues and confirmed that *Petunia* plants treated with NH_4NO_3, NH_4-N and NO_3-N had very similar content of total N, which were 4.0 ± 0.8, 4.0 ± 0.5 and 4.4 ± 0.2 g plant⁻¹, respectively (Fig. 1a). Relative to the NH_4NO_3, NH_4-N treatment only enhanced the dry weight (DW) of above-ground tissues whereas NO_3-N treatment significantly increased the fresh weight (FW) and DW of above- and under-ground tissues (Fig. 1b, c). NH_4-N and NO_3-N treatments did not affect plant height (Fig. 1d) but increased the number of branches by 36.8% and 26.3%, respectively (Fig. 1e). NH_4-N and NO_3-N also significantly increased the number of flowers by 45.7% and 32.6%, respectively (Fig. 1f). However, NH_4-N and NO_3-N treatments greatly reduced the area of leaf and corolla (Fig. 1g, j) with NH_4-N had more negative effects on leaf area than NO_3-N treatment.

¹⁵N uptake and distribution in *Petunia* differed with treatments at the tissue-level

In order to determine the effects of different N forms on uptake and distribution of N in *Petunia* plants, we performed a labeling experiment with ¹⁵N-urea. At first, N content in each tissue was measured and the results indicated that leaves had the highest content of N relative to roots, stems and flowers. Different forms of N did not affect N conent on the level of individual tissues (Fig. 2a). The absorption, ulitization rate and distribution rate of ¹⁵N in each tissue were then calculated based on formulas described in the Materials and Methods

(Fig. 2b–d). Realtive to NH_4NO_3 treatment, NO_3-N did not have effects on the tissue-level ¹⁵N absorption, utilization and distribution. In both NH_4NO_3 and NO_3-N treatments, leaf-absorption of ¹⁵N was the highest among all tissues and roots, stems and flowers absorbed and utilized similar amounts of ¹⁵N. However, for the treatment of NH_4-N, flower-absorption of ¹⁵N was significantly higher than that of roots. Therefore, in NH_4-N, both ¹⁵N utilization and ¹⁵N distribution rate in flowers were significantly higher than those in roots. More importantly, among the three treatments, NH_4-N had the highest ¹⁵N distribution rate in flowers.

Different forms of inorganic N influence petal color traits in *Petunia*

Considering that NH_4-N dramatically changed ¹⁵N distribution into floral tissues compared to other forms of N, we measured the color traits of *Petunia* plants for each treatment *via* a portable colorimeter. Changes in CIELab coordinates including perceptual lightness ($L*$), redness/greenness ($a*$), yellowness/blueness ($b*$), chroma ($C*$), and hue angle ($h°$) of *Petunia* petals are presented in Fig. 3. Relative to NH_4NO_3 treatment, both NH_4-N and NO_3-N treatment decreased $L*$ and $h°$ and increased $b*$ and $C*$ (Fig. 3a). However, there was no significant difference between NH_4-N and NO_3-N treatments in terms of their effects on color traits of white petals. The principal component analysis (PCA) of color traits further confirmed our findings (Fig. 3b).

Different forms of inorganic N changed the petal amino acid profile in *Petunia*

Most pigments in *Petunia* petals (except yellow) are derived from flavonoid and anthocyanins and amino acids were their direct precursors. We then analyzed the effects of different N forms on the petal amino acid profile in *Petunia* plants at three developmental stages (Fig. 4a). *Via* LC-MS/MS, we had identified a total of 14 amino acids in petals including four glutamate (Glu) family amino acids, five aspartate (Asp) family amino acids, three aromatic amino acids and serine and leucine (Supplemental Table S1). We first compared effects of different N forms on total content of glutamate family, aspartate family and aromatic amino acids (Fig. 4b–d). Under treatment of NH_4NO_3, amino acid profiles displayed clear patterns of changes with the development of flowers. Content of Glu family and aromatic amino acids increased from S1 to S3 stage whereas Asp family decreased in NH_4NO_3 treated petals. NO_3-N treated plants lost this developmental pattern with similar content of amino acids accumulated in petals throughout the three stages. NH_4-N treated plants accumulated slightly more amino acids that NO_3-N treated plants. More importantly, Glu family amino acids in NH_4-N treated plants showed similar developmental patterns to those treated with NH_4NO_3, suggesting that NH_4-N treatment has more positive effects on the petal amino acid profile than NO_3-N treatment.

We then presented changes in individual amino acid content in detail (Fig. 5). As mentioned above, Glu family amino acids including Glu, glutamine (Gln), arginine (Arg) and proline (Pro) showed very similar trends in changes of their content in both NH_4NO_3 and NH_4-N treatments but not in NO_3-N treatment (Fig. 5a). Different forms of N did not affect the developmental changes of the content of threonine (Thr), isoleucine (Ile) and lysine (Lys) in NH_4-N and NO_3-N treatments relative to NH_4NO_3 (Fig. 5b). However, content of two major Asp family amino
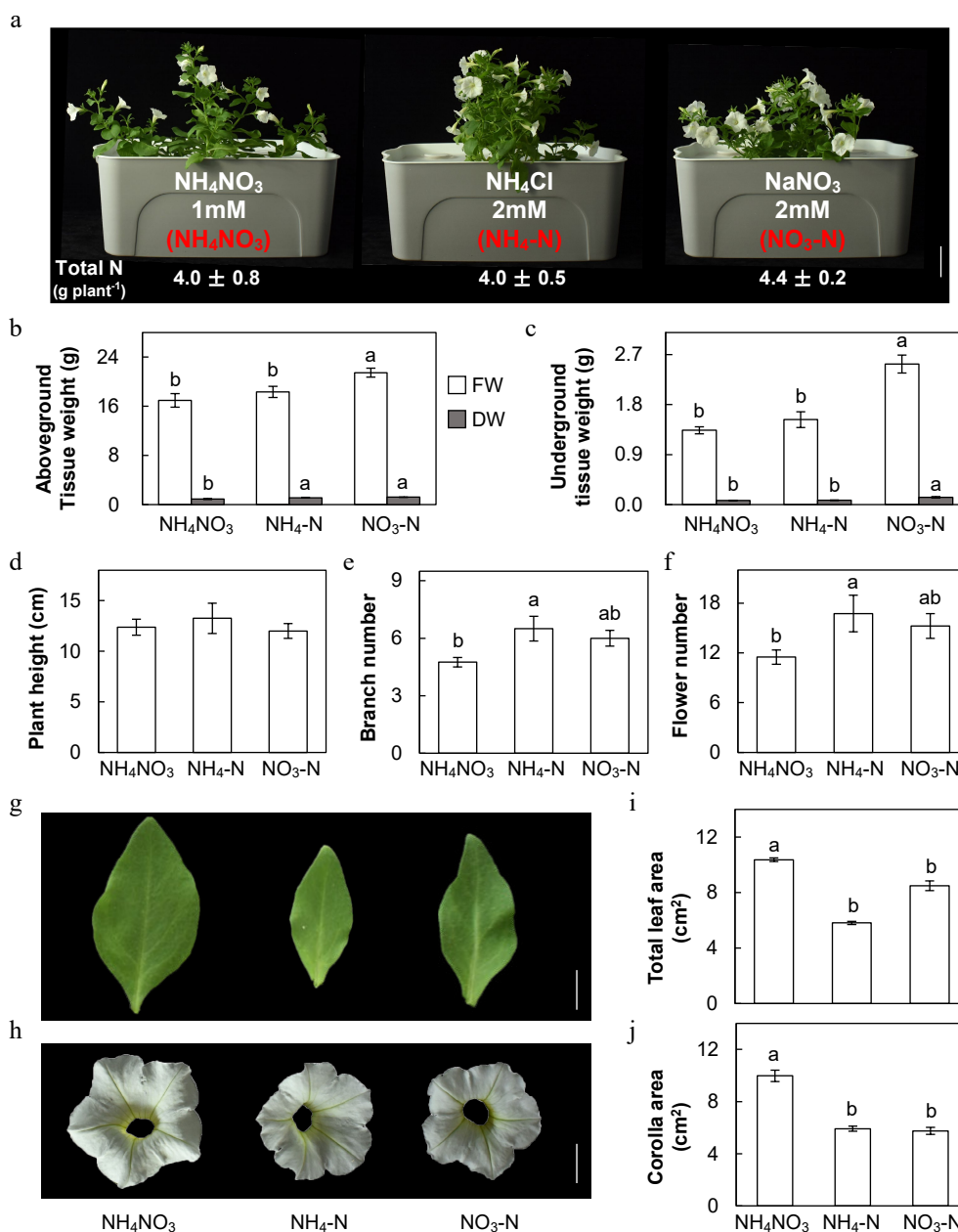
Fig. 1 Effects of ammonium-nitrate (NH$_4$NO$_3$), ammonium (NH$_4$-N), and nitrate (NO$_3$-N) on *Petunia* plants. (a) *Petunia* plants (MD) after treatment (scale bar = 5 cm). (b), (c) Fresh weight (FW) and dry weight (DW) of above- and under-ground tissues (SE, n = 4). (d)−(f) Plant height, number of branches and number of flowers measured for each treatment (SE, n = 4). (g), (h) Close-up view of the representative, fully expanded leaves and petals of fully-opened flowers of each treatment (scale bar = 1 cm). (i), (j) Total leaf area and corolla area measured for each treatment (SE, n = 4). Different letters above the bars indicate significant difference between treatments using one-way ANOVA test in SPSS software at $P < 0.05$.

acids, Asp and asparagine (Asn), were significantly influenced by treatments of NH$_4$-N and NO$_3$-N mainly at the S1 stage of flower development. Aromatic amino acids displayed significant changes under various N treatments. Content of tyrosine (Tyr), tryptophan (Trp) and phenylalanine (Phe) in NH$_4$NO$_3$ treated petals increased with flower development except at the S3 stage, content of Phe decreased (Fig. 5c). NH$_4$-N and NO$_3$-N treatments did not change content of Tyr, Trp and Phe significantly from the S1 to the S3 stage. To be noted, NH$_4$-N petals did have slightly higher content of Tyr than those treated with NO$_3$-N. Content of serine (Ser) did not significantly change

with various treatments and content of leucine (Leu) in NO$_3$-N was lower than those in the other two treatments.

Clustering analysis and principal component analysis of petal amino acid profiles highlighted differences between treatments

We carried out clustering analysis and PCA with amino acid data to better dissect the effects of different N forms on the petal amino acid profile (Fig. 6). First, *via* clustering analysis, amino acids could be categorized into two groups. Group one included Asp, Asn, Ile, Leu and Arg whose content were the
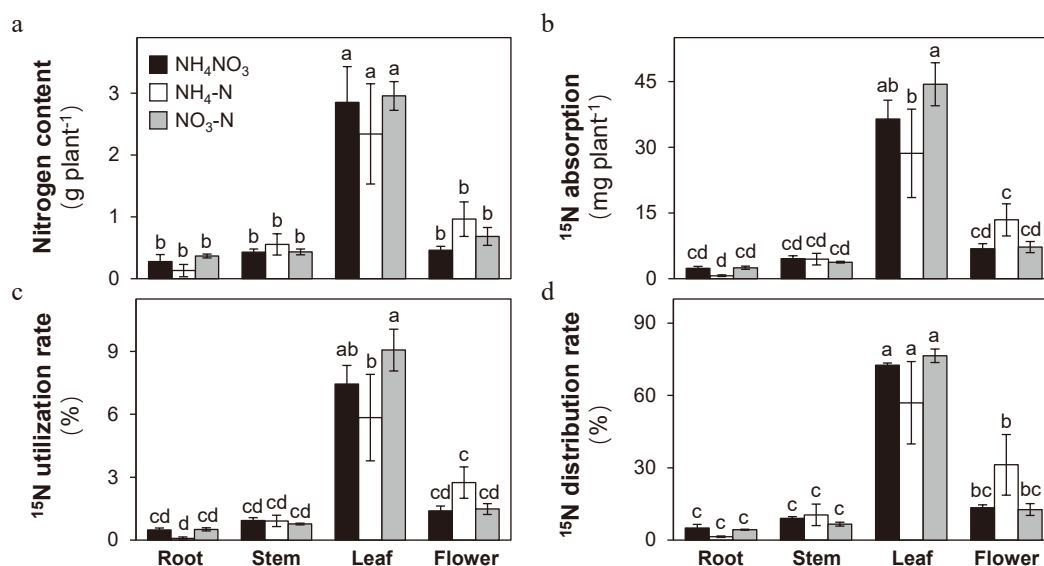
Fig. 2 Effects of ammonium-nitrate (NH_4NO_3), ammonium (NH_4-N), and nitrate (NO_3-N) on the tissue-level distribution of N within *Petunia* plants. (a) Nitrogen content , (b) ^{15}N absorption content, (c) ^{15}N utilization rate, and (d) ^{15}N distribution rate in roots, stems, leaves and flowers measured for each treatment (SE, n = 3). Different letters above the bars in each tissue indicate significant difference between treatments using one-way ANOVA test in SPSS software at $P < 0.05$.
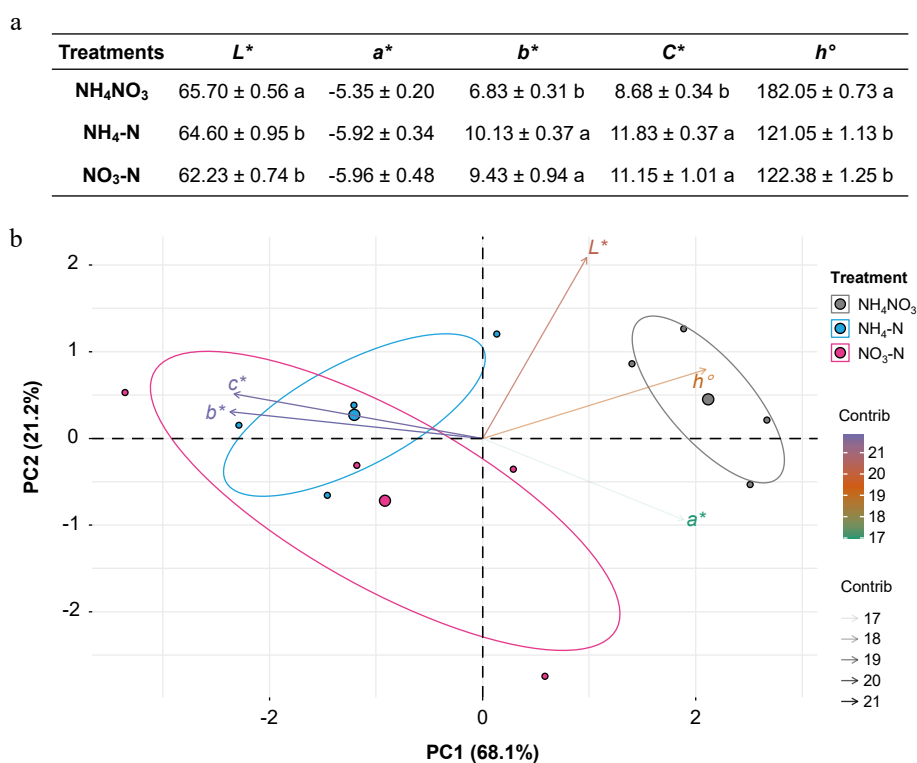
a

Treatments	L*	a*	b*	C*	h°
NH_4NO_3	65.70 ± 0.56 a	-5.35 ± 0.20	6.83 ± 0.31 b	8.68 ± 0.34 b	182.05 ± 0.73 a
NH_4-N	64.60 ± 0.95 b	-5.92 ± 0.34	10.13 ± 0.37 a	11.83 ± 0.37 a	121.05 ± 1.13 b
NO_3-N	62.23 ± 0.74 b	-5.96 ± 0.48	9.43 ± 0.94 a	11.15 ± 1.01 a	122.38 ± 1.25 b

b

Fig. 3 Effects of ammonium-nitrate (NH_4NO_3), ammonium (NH_4-N), and nitrate (NO_3-N) on petal color traits. (a) CIELab coordinates including perceptual lightness (L*), redness/greenness (a*), yellowness/blueness (b*), chroma (C*), and hue angle (h°) of *Petunia* petals measured for each treatment *via* a portable colorimeter (SE, n = 4). Colors (green to purple) and transparency of arrows toward each parameter indicate their contributions to the principal components. Different letters above the bars in each column indicate significant difference between treatments using one-way ANOVA test in SPSS software at $P < 0.05$. (b) The principal component analysis (PCA) of color traits in three treatments.

highest at the S1 stage with NH_4NO_3 treatment. Group two including Phe, Ser, Thr, Tyr, Trp, Glu, Gln, Pro and Lys, on the other hand, increased in their content at the S2 and S3 stages with NH_4NO_3 treatment. We then could clearly observe that both NH_4-N and NO_3-N treatment significantly influenced the petal amino acid profile relative to that with NH_4NO_3 treatment

(Fig. 6a). PCA with amino acid data provided more information (Fig. 6b). Except S2, the petal amino acid profile in NH_4-N was much closer to that in NH_4NO_3 at both the S1 and S3 stages. Taken together, different forms of N could greatly affect the amino acid profile in *Petunia* petals. However, relative to NO_3-N treatment, total content of amino acids and changes in content
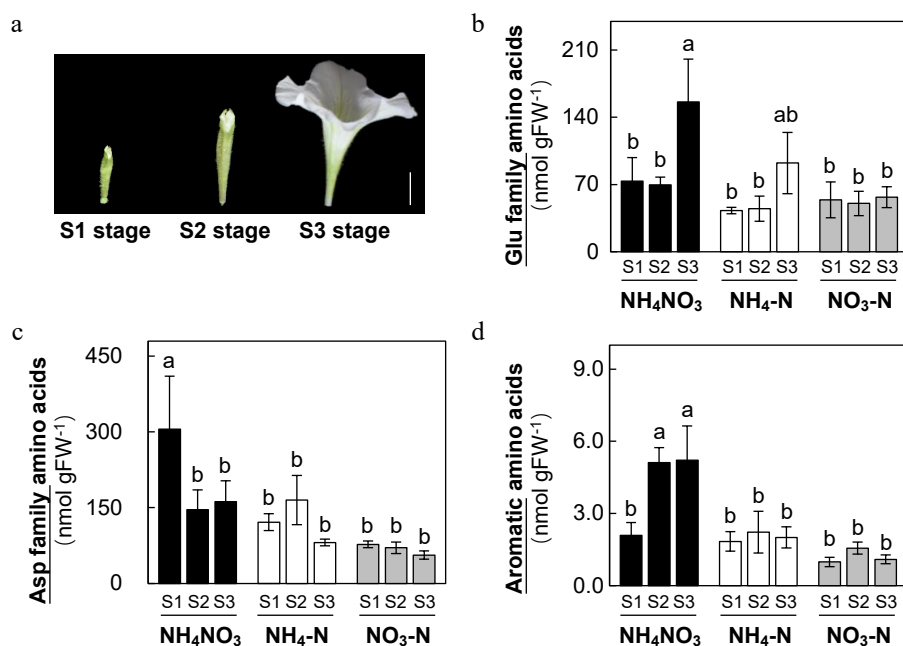
Fig. 4 Effects of ammonium-nitrate (NH_4NO_3), ammonium (NH_4-N), and nitrate (NO_3-N) on petal amino acid profiles in *Petunia* plants. Petal tissues were collected at S1, S2 and S3 stages indicated in (a) (scale bar = 1 cm) and total concentrations of glutamate family amino acids, aspartate amino acids and aromatic amino acids (b)–(d) (SE, n = 4). Different letters above the bars indicate significant difference between treatments using one-way ANOVA test in SPSS software at $P < 0.05$. Concentrations (nmol g FW^{-1}) of individual amino acids are reported Supplemental Table S1.
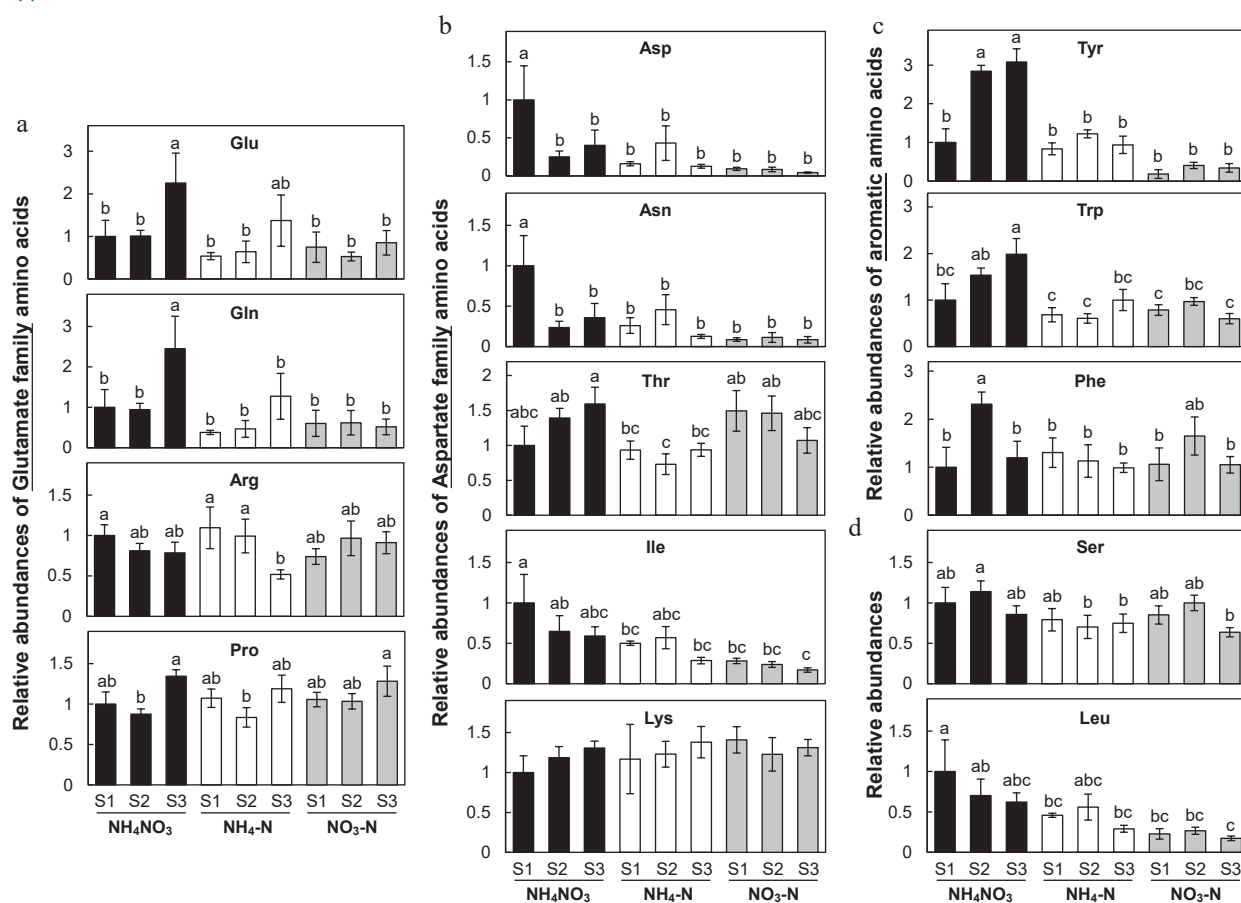

Fig. 5 Effects of ammonium-nitrate (NH_4NO_3), ammonium (NH_4-N), and nitrate (NO_3-N) on relative abundance of amino acids in *Petunia* petals. (a) Relative abundance of glutamate family amino acids, (b) aspartate family amino acids, (c) aromatic amino acids, and (d) Ser as well as Leu were displayed in column plots (SE, n = 4). Different letters above the bars indicate significant difference between treatments using one-way ANOVA test in SPSS software at $P < 0.05$. Concentrations (nmol g FW^{-1}) of individual amino acids are reported in Supplemental Table S1.
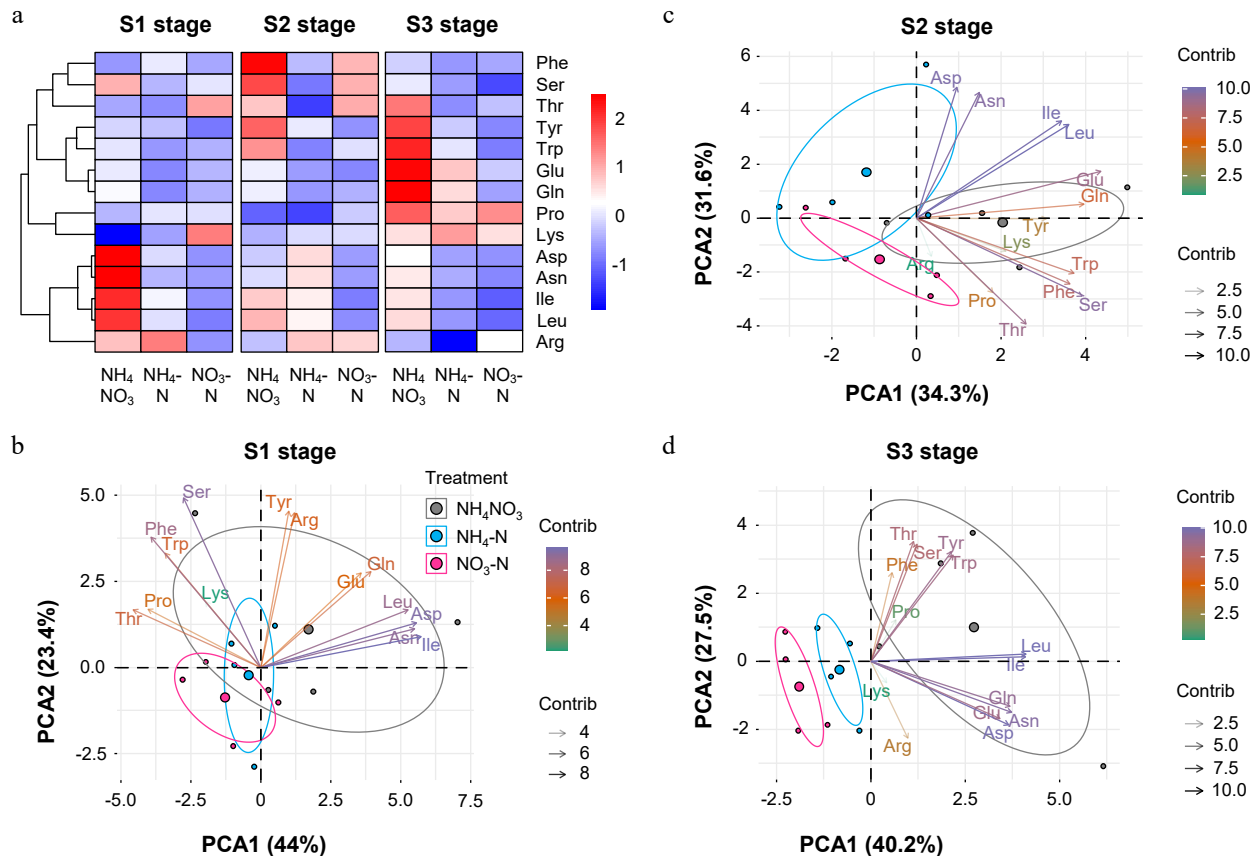
Fig. 6 Further analysis of amino acid data. Heatmap representation of concentrations of 14 amino acids at (a) S1−S3 stages and (b), (c), (d) PCA of amino acids at each developmental stage. Colors (green to purple) and transparency of arrows toward each parameter indicate their contributions to the principal components.

of individual amino acids in NH_4-N treated petals were closer to those with NH_4NO_3 treatment.

Amino acid profiles were correlated with petal color traits in response to different forms of N

Correlation analysis with petal color traits and amino acid profile data indicated that changes in the content of some amino acids were connected to petal color traits (Fig. 7). Thr, Tyr and Trp was positively correlated with the hue angle ($h°$) of *Petunia* petals. Thr and Leu were negatively correlated with the yellowness/blueness ($b*$) and chroma ($C*$) whereas Tyr was negatively correlated with $b*$. These data suggested that when the petal amino acid profile was modified by different forms of N, the color traits such as color and brightness of petals could change accordingly.

Considering we collected these data from a white-flower cultivar 'Mitchell Diploid', we purchased four commercially available cultivars of *Petunia* with four flower colors (Fig. 8a, b) and treated them in the same way to further analyze various forms of N on *Petunia* petals with colors. Petal color traits including $L*$, $a*$, $b*$, $C*$ and $h°$ of white-, pink-, dark pink- and red-flower *Petunia* plants treated with NH_4NO_3, NH_4-N, and NO_3-N were analyzed and presented in Supplemental Table S2. Interestingly, regardless of petal colors, relative to NH_4NO_3 treatment, both NH_4-N and NO_3-N treatment could influence $L*$. For pink-flower cultivar, NH_4-N and NO_3-N treatments slightly decreased $a*$, $b*$ and $C*$. And for dark pink- and red-flower cultivars, NH_4-N and NO_3-N treatments slightly increased

$a*$, $b*$ and $C*$ (Supplemental Fig. S1). PCA of petal color traits for each cultivar are shown in Fig. 8c–f and revealed more information about the different treatments. For the white-flower cultivar, similar to data we collected from 'Mitchell Diploid', NH_4-N and NO_3-N could be clearly separated from NH_4NO_3 with $a*$, $b*$ and $C*$ contributing the most to separation (Fig. 8c). But NH_4-N and NO_3-N could not be separated from each other. For the pink-flower cultivar, NH_4-N could be partially separated from NO_3-N in PC2 with $b*$ and $h°$ contributed the most (Fig. 8d). And for the dark pink-flower cultivar, NH_4-N could be completely separated from NO_3-N in PC2 with $a*$ and $C*$ contributed the most (Fig. 8e). For the red-flower cultivar, NH_4-N could be partially separated from NO_3-N in PC2 with $b*$ and $h°$ contributed the most. To be noted, NO_3-N could be completely separated from NH_4NO_3 in PC1 (Fig. 8f). Taken together, different forms of N could impact petal color traits. And for petals with colors, NH_4-N and NO_3-N could affect the redness/greenness and yellowness/blueness but the lightness of petals contributed less to differences between treatments.

DISCUSSION

Ornamental traits such as size of flowers and color of petals are fundamentally determined by the metabolic status on the whole-plant level. Extensively performed agricultural practice and continuously increased concentration of CO_2 in the atmosphere have both aggravated N conditions in soil. This
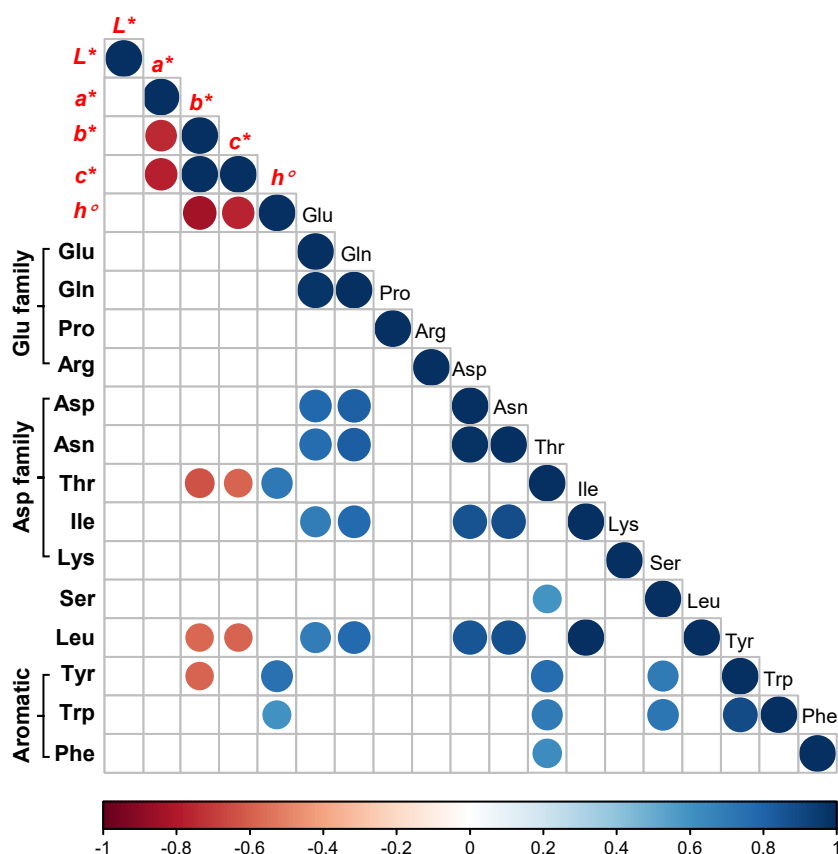
Fig. 7 Correlation analysis of L*, a*, b*, c*, h° and 14 amino acids. Data were processed *via* R programming. The intensity of blue and red color as well as the size of each dot are proportional to the calculated correlation coefficient (r).

study has been designed to specifically investigate the effects of ammonium or nitrate as the sole source of inorganic N on the developmental and chemical features of floral tissues in *Petunia*.

According to the total N content on the whole-plant level after each treatment (Fig. 1a), we think that the *Petunia* x *hybrida* cv 'Mitchell Diploid' plants we used in this study do not have preferences to a specific form of inorganic N. To be noted, given the same amount of inorganic N (2 mM), plants treated with NO$_3$-N alone accumulated more biomass. Previous studies[28] pointed out that responses of *Petunia* plants to different forms of inorganic N depended on the concentrations of N applied to plants and the medium used for plant growth. Ammonium is more effective than nitrate on the growth promotion in rice[29] and maize[30]. Complex effects of ammonium and nitrate on plant growth, CO$_2$ assimilation and activities of key enzymes involved in N metabolism have also been reported in blueberry, raspberry and strawberry[31]. Similarly, different ratios of nitrate to ammonium applied to sweet pepper at different developmental stages bring various effects on flowering, fruit set and yield[32]. It is then clear to us that effects of different forms of inorganic N on ornamental plants should be discussed case by case[17].

We found that in *Petunia* plants, NH$_4$-N and NO$_3$-N treatment could both enhance the number of branches and flowers at the cost of leaf and corolla area. Plants can generally benefit from the direct application of NH$_4$-N at an appropriate level since the assimilation of ammonium is far more energetically efficient relative to nitrate assimilation. Meanwhile, nitrate is more

mobile which can compensate this limitation. Therefore, applications of NH$_4$-N and NO$_3$-N alone could be superior to NH$_4$NO$_3$ treatment. N can shape the architecture of plants. Nitrate regulates shoot branching on the molecular level to control the outgrowth of axillary buds[33]. Nitrate can also control the transition from the vegetative to reproductive phase and therefore, is involved in flowering time regulation[34]. On the other hand, ammonium application usually leads to accumulation of proteins which is generally beneficial to crop growth. However, there are clear differences between the two treatments. Studies on tobacco have also shown that ammonium nitrogen treatment results in a reduction in leaf area[35]. ^{15}N labeling experiments confirmed that only NH$_4$-N changed the distribution of ^{15}N between various tissues with floral tissues absorbing and utilizing more ^{15}N. Meanwhile, relatively smaller amounts of ^{15}N was distributed into the roots and leaves in NH$_4$-N treated plants. Taken together, when ammonium is used as the sole source of inorganic N, *Petunia* plants can manage to import more N into floral organs and produce more flowers with smaller size. More importantly, both NH$_4$-N and NO$_3$-N treatment can modify the amino acid profile on the whole-plant level. And as the basic compounds for plant development and signaling molecules[36], amino acids may play direct roles in the formation of plant architecture *via* regulating the meristem tissues[37], which requires further study.

Here we need to clarify that in most cases, ammonium (NH$_3$ or NH$_4^+$) is a stress cue[38,39]. However, ammonium nutrition can also be advantageous as it can improve crop quality[40]. For example, when grown under ammonium nutrition, compared
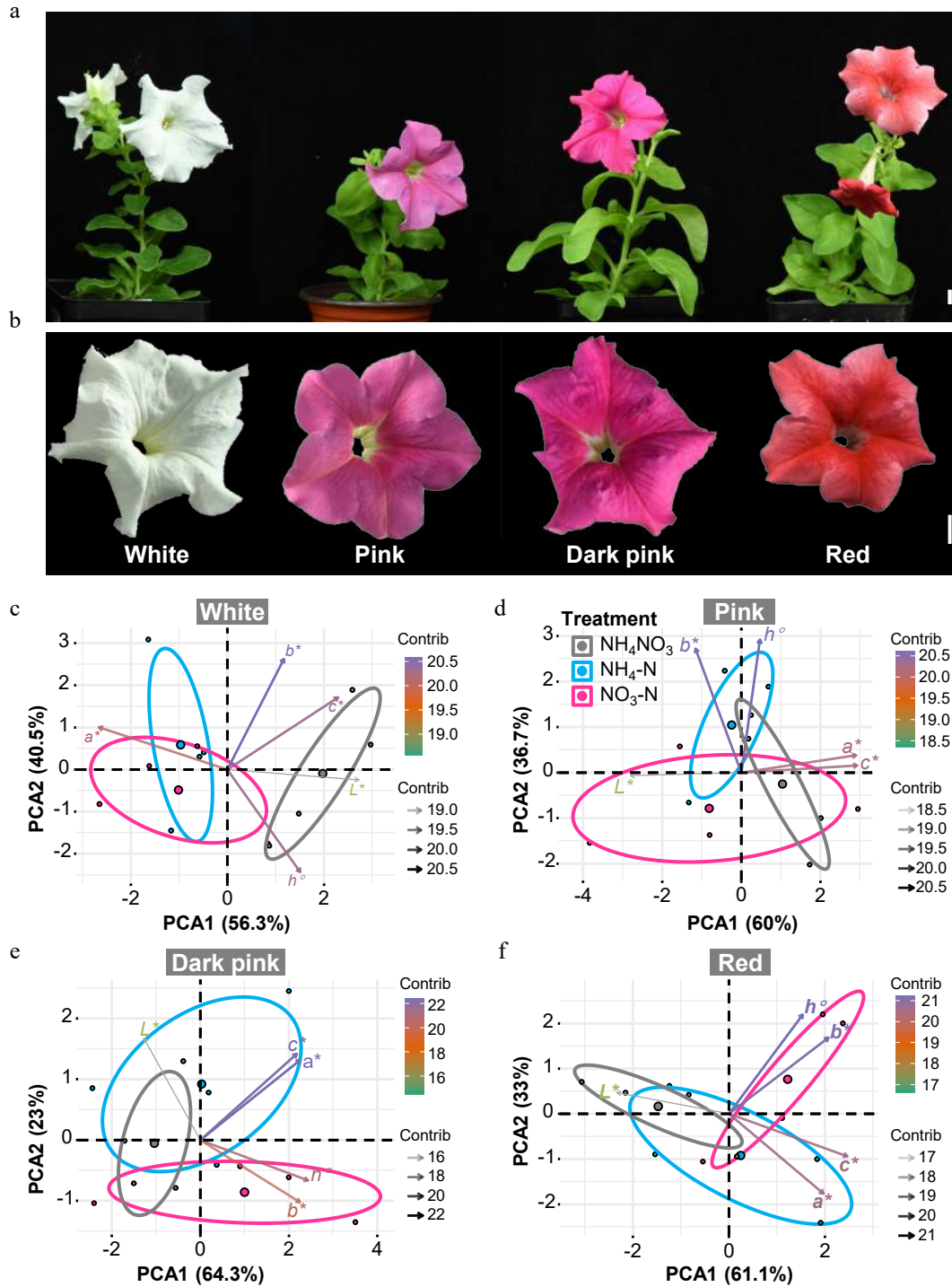
Fig. 8 Relations between amino acids and petal color traits of *Petunia* cultivars with different colors. (a), (b) Plant and petal appearance of four *Petunia* cultivars (Scale bar = 1 cm). (c)–(f) PCA of *L**, *a**, *b**, *C** and *h°* in each cultivar. Colors (green to purple) and transparency of arrows toward each parameter indicate their contributions to the principal components.

with nitrate, wheat grains can accumulate more proteins leading to an increased wheat bread-making quality[41]. This is somehow consistent with what we have found in *Petunia* plants. Petal color traits and amino acid profiles measured in this study are highly informative. For *Petunia x hybrida* 'Mitchell Diploid' with white flowers (Fig. 3), NH₄-N and NO₃-N treatments could significantly increase petal yellowness/blueness (*b**) and chroma (*C**) and decrease lightness (*L**) and hue angle

(*h°*). We got very similar data with the commercially available white-flower cultivar (Fig. 8c). And data collected for commercially available cultivars with pink-, dark pink- and red-flowers, further indicated that effects of NH₄-N and NO₃-N on petal pigmentation could be different when petal colors differed. Further studies should be carried out to determine an appropriate ratio of ammonium to nitrate which can be applied to ornamental plants to modify their petal colors.

For amino acids in petals, there are few studies available. Roles of amino acids in flower development and senescence have been briefly reported in *Eustoma grandiflorum*[42]. Although most local amino acids in floral tissues are imported from roots and leaves, it has been recently proposed that some amino acids can be synthesized in flowers[18]. For example, *de novo* synthesis of Asn may exist in flowers due to the fact that *asparagine synthetase1* (*ASN1*) is expressed abundantly in Arabidopsis flowers[43]. The biological roles of Asn in floral tissues can be important. On one hand, Asn carries more N than other amino acids and serves as a good N sink[44]. On the other hand, hydrolysis of Asn can release ammonium which in turn can be used to synthesize Gln[45,46]. More importantly, amino acids provide precursors and energy for petal color and scent. We think the metabolic roles of amino acids in regulating ornamental traits should be much more emphasized in this field. Accumulation of free amino acids is usually considered a sign for ammonium stress[47]. The fact that total content of 14 amino acids in NH_4-N petals were not higher than those in NH_4NO_3 indicates that the NH_4-N in our study was not a stress treatment. Given that the assimilation of inorganic N into amino acids primarily occur in roots, the ^{15}N label data and petal amino acid profiles collected in this study indicated that *Petunia* plants treated with NH_4-N alone loaded relatively more amino acids such as Glu, Gln, Asp and Asn to their flowers (Fig. 5a, b). We also found that changes in petal amino acid profiles were correlated with changes in their color traits (Fig. 7), which should be further investigated at the molecular level in ornamental plants.

CONCLUSIONS

In summary, we have analyzed effects of ammonium and nitrate alone on plant growth, the tissue-level N utilization and distribution, petal color traits and petal amino acid profiles in *Petunia* plants. We have confirmed that NH_4-N can increase ^{15}N distribution to floral tissues, modify the amino acid profiles in petals which in turn bring changes to petal colors. Mechanisms of governing flower development by different forms of N or other environmental cues require further investigation and will definitely lead to a better understanding of the metabolic regulation of ornamental traits.

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Conflict of interest

The authors declare that they have no conflict of interest.

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