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Exogenous glycine inhibits root elongation and reduces nitrate-N uptake in pak choi (*Brassica campestris* ssp. *Chinensis* L.)

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Abstract

Nitrogen (N) supply, including NO_3^--N and organic N in the form of amino acids can influence the morphological attributes of plants. For example, amino acids contribute to plant nutrition; however, the effects of exogenous amino acids on NO_3^--N uptake and root morphology have received little attention. In this study, we evaluated the effects of exogenous glycine (Gly) on root growth and NO_3^--N uptake in pak choi (*Brassica campestris* ssp. *Chinensis* L.). Addition of Gly to NO_3^--N agar medium or hydroponic solution significantly decreased pak choi seedling root length; these effects of Gly on root morphology were not attributed to the proportion of N supply derived from Gly. When pak choi seedlings were exposed to mixtures of Gly and NO_3^--N in hydroponic culture, Gly significantly reduced $^{15}NO_3^-$-N uptake but significantly increased the number of root tips per unit root length, root activity and $^{15}NO_3^-$-N uptake rate per unit root length. In addition, ^{15}N-Gly was taken up into the plants. In contrast to absorbed NO_3^--N, which was mostly transported to the shoots, a larger proportion of absorbed Gly was retained in the roots. Exogenous Gly enhanced root 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and oxidase (ACO) activities and ethylene production. The ethylene antagonists aminoethoxyvinylglycine (0.5 μM AVG) and silver nitrate (10 μM $AgNO_3$) partly reversed Gly-induced inhibition of primary root elongation on agar plates and increased the NO_3^--N uptake rate under hydroponic conditions, indicating exogenous Gly exerts these effects at least partly by enhancing ethylene production in roots. These findings suggest Gly substantially affects root morphology and N uptake and provide new information on the specific responses elicited by organic N sources.

Introduction

Plant growth and nitrate (NO_3^-) accumulation in green leafy vegetables are influenced by the sources of available nitrogen (N) [1]. Consumption of vegetables containing high concentrations of NO_3^- has been related to risks to human health [2]. Plants accumulate NO_3^- when the rate of NO_3^--N uptake through the roots exceeds the rate of NO_3^- assimilation in plant tissues. Given that the roots are the major sites that directly affect NO_3^--N uptake in plants,

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understanding the effects of different N sources on root morphology is necessary to devise strategies to reduce accumulation of NO_3^- in green leafy vegetables.

N is found in a variety of inorganic and organic forms in soil. NO_3^--N has been traditionally viewed as the main form of inorganic N taken up by plants. However, an increasing number of studies have shown that organic N also contributes to plant N nutrition [3–7]. Amino acids are ubiquitously found in the soil solution and may represent a significant source of N for plants in terrestrial ecosystems. In general, the concentrations of amino acids in soil solutions are very low (< 60 μM) [8, 9]. However, in cropping systems that rely on recycling and decomposition of organic N sources, amino acids may represent a significant N input and important plant-available N pool [10]. Glycine (Gly) is one of the most abundant amino acids and frequently employed as a model amino acid in plant uptake studies because of its low molecular weight [11, 12].

Apart from the direct nutritional effects of N sources, different forms of N function as signals and can control parameters of root morphology, such as root length, lateral root number and root surface area [13–16]. Low concentrations of NO_3^--N stimulate elongation of the lateral roots [17, 18] and lateral root initiation [13], whereas higher NO_3^--N concentrations inhibit root growth [18]. While many researchers have focused on the effects of inorganic N on root growth [13, 17, 18], only a small number of studies have investigated how amino acids regulate root growth [19–20]. For instance, *L*-glutamate (Glu) suppresses primary root length [21–23]. Moreover, while the effects of *L*-Glu on root morphology are relatively well understood; few studies have investigated the response of roots to Gly, a model amino acid in organic N studies [24, 25], and the regulatory interactions between Gly and inorganic N on root growth. Therefore, additional studies on the effects of amino acid N sources on the root morphology of leafy vegetables are necessary.

In addition to root morphology, NO_3^--N uptake can also be affected by the presence of different forms of N and the interactions between these N forms. For example, the presence of exogenous ammonia (NH_4^+)-N in nutrient solution reduced the NO_3^--N uptake capacity of crops [26, 27]. Only a small number of studies have investigated the effects of the interactions between amino acids and inorganic N sources on the uptake of NO_3^--N by roots, and most studies on the effects of exogenous Gly on the uptake of NO_3^--N by roots have focused on agricultural crops, such as perennial ryegrass [28] and wheat [9], with only one study on leafy vegetables (Chinese kale) [27].

Root morphology and NO_3^--N uptake can also be influenced by ethylene production [29, 30]. The gaseous hormone ethylene is synthesized from methionine (Met), which can be converted to S-adenosyl *L*-methionine (SAM) by SAM synthetase. SAM is then converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS), and subsequently to ethylene by ACC oxidase (ACO) [31]. Previous studies have demonstrated ethylene regulates root growth in response to different N sources [30, 32–35]. For example, a high concentration of NO_3^--N activated ethylene production, which inhibited lateral root growth in *Arabidopsis thaliana* [33]. Similarly, Li et al. (2013) reported that NH_4^+-N inhibited *Arabidopsis thaliana* lateral root formation by inducing production of ethylene [34]. Domínguez-May et al. (2013) suggested that ethylene also plays a role in the reduced root length induced by Gly in habanero pepper [24]. In addition, NO_3^--N uptake is also regulated by ethylene in the presence of N sources. Zheng et al. (2013) detected rapid production of ethylene under low NO_3^--N conditions, which decreased NO_3^--N uptake [29]. However, only a few studies have evaluated the role of ethylene in exogenous Gly-induced inhibition of root length [21] and reduction of NO_3^--N uptake.

The green leafy vegetable pak choi is commonly cultivated and widely consumed in southern China. However, pak choi tends to accumulate high concentrations of nitrate [36].

Consumption of high levels of nitrate may lead to carcinogenesis and formation of methemoglobin [2]; thus, it would be highly desirable to reduce the accumulation of NO_3^- in leafy vegetable. Therefore, the aim of this study was to investigate the influence of exogenous Gly on root length and NO_3^--N uptake in pak choi. Moreover, the involvement of the ethylene signaling pathway in the changes in root morphology and NO_3^--N uptake observed in response to exogenous Gly application were investigated. We hypothesized that exogenous Gly would reduce root length and decrease NO_3^--N uptake via a mechanism related to increased ethylene production.

Materials and methods

Agar plate culture

Seeds of the pak choi cv. 'Huawang' were surface sterilized with 70% ethanol for 1 min, rinsed three times in sterile deionized water, infiltrated by soaking in 10% H_2O_2 for 5 min, and then extensively rinsed five times with sterile deionized water. The disinfected seeds were placed in 12 cm-diameter sterile Petri dishes containing 50 mL of agar (0.8% *w/v*) culture medium supplemented with 3 mM $NaNO_3$. The basic nutrient media (pH 6.0 ± 0.2) was comprised of 1 mM $MgSO_4$, 0.5 mM KH_2PO_4, 1.25 mM K_2SO_4, 2.5 mM $CaCl_2$, 0.05 mM EDTA·2Na, 0.05 mM $FeSO_4$, 48.5 μM H_3PO_3, 10 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 2.1 μM $NaMoO_3$ and 4.8 μM KI. The Petri dishes were half sealed with adhesive tape and vertically oriented in a growth chamber maintained at 25˚C (day-time) and 18˚C (night-time) under a 16-h/8-h light/dark cycle with a light intensity of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ during the day. Seedlings with a 5–6 mm-long primary root were transferred to sterile treatment dishes (four seedlings per dish) filled with 50 mL of solidified treatment medium containing different sources and concentrations of N. The superior segment of the medium in each dish was removed to ensure that the seedling shoots were not in contact with the medium.

Hydroponic culture

Seeds of pak choi cv. 'Huawang' were surface sterilized as described above, then germinated in plastic trays containing autoclaved perlite. The substrate was supplied daily with basic nutrient solution (as described above) and 3 mM $NaNO_3$. After 15 d, uniformly sized seedlings were selected and transplanted to a foam board floating on 3 mM $NaNO_3$ solution in hydroponic plastic pots and pre-cultivated for 3 d prior to the experiments. The solutions were renewed every 3 d. All plants were grown in a greenhouse at 25˚C (day-time)/18˚C (night-time) under a 14-h/10-h light/dark cycle with natural sunlight with photosynthetically active radiation in the range of 300–800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ during the day.

Experiment 1: Effect of Gly concentration on pak choi root development in the presence of NO_3^--N

To determine the effect of Gly on root growth, on agar plates containing treatment medium with either 0.5 or 10 mM $NaNO_3$ were supplemented with a range of Gly concentrations (0, 0.5, 1, 2.5, 5, 10 mM). To make the treatment media, sterile-filtered $NaNO_3$ or Gly were added separately to autoclaved basic medium that had been cooled to 50–55˚C. The amount of sodium (Na) in the medium containing 0.5 mM $NaNO_3$ was adjusted to 10 mM by adding Na_2SO_4. After 5 d treatment, primary root growth was measured with a ruler and lateral root number was recorded. To investigate whether the effects of Gly on the primary roots were due to altered N supply, seeds were germinated in treatment medium containing 0 mM N, 2.5 mM Gly or $NaNO_3$ and a range of NO_3^--N concentrations (0, 0.5 and 10 mM) for 3 d. The amount

of sodium (Na) in the medium containing 0 and 0.5 mM $NaNO_3$ was adjusted to 10 mM by adding Na_2SO_4. The length of primary root elongation was recorded after 24 and 48 h. All agar plate experiments performed twice independently, using five Petri dishes containing four seedlings each for each treatment.

Experiment 2: Time course of the effects of exogenous Gly on root system parameters and ^{15}N uptake

The dynamic changes in root system parameters and NO_3^--N uptake induced by exogenous Gly were investigated in hydroponic culture using nutrient solution containing 10 mM $NaNO_3$ or 10 mM $NaNO_3$ + 2.5 mM Gly (exogenous Gly). On days 0, 4, 8, 12 and 16 of treatment, 50 seedlings were selected, the roots were washed thoroughly with purified water, patted dry with filter paper and the seedlings were placed individually in 50 mL centrifuge tubes containing pretreatment nutrient solution (10 mM $NaNO_3$ or 10 mM $NaNO_3$ + 2.5 mM Gly); the tubes were covered with black plastic film to avoid the effects of light on root growth. The seedlings in tubes were preincubated in controlled-environment chambers at 25˚C (day-time)/ 18˚C (night-time) under a 16-h/8-h light/dark cycle (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After 24 h, the seedlings were transferred to centrifuge tubes filled with $Na^{15}NO_3$, $Na^{15}NO_3$ + Gly or $^{15}NGly$ + $NaNO_3$ treatment solution. Labelled N was provided as 10 mM 4.95 atom% $Na^{15}NO_3$ or 2.5 mM 4.95 atom% $^{15}NGly$ (Shanghai Research Institute of Chemical Industry, China). Fifteen pak choi seedlings were subjected to each treatment (3 replicates, 5 seedlings per replicate). In addition, five 'blank' seedlings were treated with the same concentration of the unlabeled $NaNO_3$ + Gly mixture. To prevent degradation of amino acids by bacteria, ampicillin (10 $mg \cdot L^{-1}$) was added to all solutions [37]. After the 4 h uptake period, the plants were harvested, divided into shoots and roots, and the five seedlings from each replicate were pooled to form single root/shoot samples. The roots were washed with sterile water, then 0.5 mM $CaCl_2$, followed by several washes with purified water to remove ^{15}N from the root surface. The shoots and roots were dried at 60˚C for 72 h, weighed and ground to a fine powder. The N and ^{15}N contents of the roots and shoots were analyzed using a Vario EL III IRMS elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

In addition, after 1, 5, 9, 13 and 17 d of the $NaNO_3$ and $NaNO_3$ + Gly treatments, the roots were placed in deionized water and scanned using an Epson Perfection V850 Pro scan system (Nagano, Japan). Root morphological parameters were calculated using WinRhizo analysis software (Regent Instruments Inc., Quebec City, Canada). Root activity was determined using the triphenyltetrazolium chloride (TTC) reduction method according to Islam et al. (2007) [38].

Experiment 3: Effects of exogenous Gly on ethylene production and the activity of ethylene synthesis enzymes

Fifteen-day-old pak choi seedlings (cultured as described in the hydroponic culture section) were pre-cultivated in 3 mM N nutrient solution for 7 d, cultivated in treatment nutrient solution containing 10 mM $NaNO_3$ or 10 mM $NaNO_3$ + 2.5 mM Gly for 10 or 15 d, then the plant roots were harvested to determine ethylene production and assay the activity of ethylene synthesis enzymes. The roots were washed as described in Experiment 2 to remove $NaNO_3$ or Gly on the root surfaces. To minimize wounding effects, the excised roots were weighed and placed in 20 mL gas-tight vials containing 1 mL agar medium (0.7% *w/v*) for 30 min and the vials were sealed with a gas-tight stopper. After incubation at 25˚C for 2 h in the dark, 1 mL of headspace gas was collected from the vials and analyzed using a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) to measure the

concentration of ethylene. Root 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and oxidase (ACO) activity were measured according to Tian et al. (2009) [33] and Yu et al. (2016) [39].

Experiment 4: Effects of ethylene biosynthesis inhibitors on root length in the presence of exogenous Gly

The effects of ethylene biosynthesis inhibitors (AVG and $AgNO_3$) on root growth in the presence of exogenous Gly were investigated. According to the culture conditions described in Experiment 1, pak choi seedlings were grown for 5 d in treatment medium containing 2.5 mM Gly and 10 mM $NaNO_3$ supplemented with 0, 0.5, 1, 2.5, 5 or 10 μM AVG (06665, Sigma-Aldrich) or 0, 5, 10, 20, 30 or 50 μM $AgNO_3$. The sterile-filtered Gly, $NaNO_3$, AVG or $AgNO_3$ solutions were added to autoclaved basic medium cooled to 50–55˚C. Primary root length was measured using a ruler after 5 d.

Experiment 5: Effects of ethylene biosynthesis inhibitors on ^{15}N uptake in response to exogenous Gly

The effect of AVG and $AgNO_3$ on $^{15}NO_3^-$-N uptake in the presence of exogenous Gly was investigated. Following the culture conditions described for Experiment 2, 15-day-old pak choi seedlings were pre-cultivated for 7 d in nutrient solution containing 3 mM $NaNO_3$. The plants were transferred to 50 mL centrifuge tubes containing the following pretreatment solutions: (1) 10 mM $NaNO_3$, (2) 10 mM $NaNO_3$ plus 0.5 μM AVG, (3) 10 mM $NaNO_3$ plus 10 μM $AgNO_3$, (4) 10 mM $NaNO_3$ + 2.5 mM Gly, (5) 10 mM $NaNO_3$ + 2.5 mM Gly plus 0.5 μM AVG, and (6) 10 mM $NaNO_3$ + 2.5 mM Gly plus 10 μM $AgNO_3$. After 36 h, the plants were transferred to new centrifuge tubes filled with ^{15}N-labelled solutions for 4 h for the short-term uptake test. In each treatment, one of the N sources was labelled with ^{15}N (4.95 atom%) for the $NaNO_3$ + Gly mixtures, either $^{15}NO_3^-$-N (4.95 atom%) or ^{15}N-Gly (4.95 atom%) creating a total of 9 treatments (with 3 replicates and 3 plants per replicate). After the 4 h uptake test, sampling and analysis were performed as described in Experiment 2.

Calculations

NO_3^--N and Gly uptake were calculated as the ^{15}N content of treated pak choi seedlings compared to the ^{15}N content of "blank" seedlings cultured in unlabeled NO_3^--N and Gly, according to Eq (1) [5, 6].

$$^{15}N_{uptake} = DW \times N\% \times \frac{A_s - A_c}{A_{applied}} \quad (1)$$

Where $^{15}N_{uptake}$ is the amount of absorbed N source in the roots or shoots, DW is the dry weight of the roots or shoots, N% is the N content of the roots or shoots, A_s is the ^{15}N atom% in the roots or shoots of the treated seedlings, A_c is the ^{15}N atom% in "blank" seedlings provided with unlabeled N sources, and $A_{applied}$ is the ^{15}N atom% used in the experiment (4.95% $^{15}NO_3^-$-N and 4.95% ^{15}N-Gly).

The fraction of N derived from each N source in the mixtures of $NaNO_3$ + Gly was calculated according to Eq (2).

$$\text{The fraction of N derived from N source} = \frac{^{15}N_{uptake}}{^{15}N_{total\ uptake}} \times 100 \quad (2)$$

Where $^{15}N_{uptake}$ is the amount of absorbed NO_3^--N or Gly in the roots or shoots of plants

cultured in a mixture of N sources, and $^{15}N_{total\ uptake}$ is the total amount of absorbed NO_3^--N and Gly in the roots or shoots.

$^{15}N_{uptake\ rate}$ was calculated according to Eq (3).

$$^{15}N_{uptake\ rate} = \frac{^{15}N_{uptake}}{DW \times 15 \times t} \quad (3)$$

Where $^{15}N_{uptake\ rate}$ is the uptake rate for the N source for whole seedlings, $^{15}N_{uptake}$ is the total amount of absorbed N source in whole seedlings, *DW* is the dry weight of whole seedlings, 15 is the molecular weight of labelled N, *t* is the duration of the uptake experiment (4 h).

The $^{15}N_{uptake\ rate\ per\ unit\ root\ length}$ was calculated according to Eq (4).

$$^{15}N_{uptake\ rate\ per\ unit\ root\ length} = \frac{^{15}N_{uptake\ rate}}{TRL} \quad (4)$$

Where $^{15}N_{uptake\ rate\ per\ unit\ root\ length}$ is the N uptake rate per unit root length. The $^{15}N_{uptake\ rate}$ is calculated from the Eq (3), and *TRL* is the total root length.

Statistical analyses

A one-way experimental design was employed. All statistical analyses were performed using SAS software (SAS Institute, Cary, NC, USA). Differences between treatments were analyzed using the Student's *t*-test (two treatments) or least significant difference test (*LSD*, ≥ three treatments) at $P < 0.05$. Data are presented as the mean ± SE (standard error). Figures were generated using SigmaPlot 10.0 (Systat Software, Inc., Erkrath, Germany).

Results

Root morphology and growth

Pak choi seedlings were co-treated with 0.5 or 10 mM NO_3^--N and varied concentrations of Gly for 5 d on agar plates. In both NO_3^--N treatments, exogenous Gly significantly reduced the primary root length in a concentration-dependent manner compared with NO_3^--N as a single N source (Fig 1A and S1 Fig). The primary root length of pak choi seedlings exposed to 0.5 or 10 mM NO_3^--N supplemented with 2.5 mM Gly was 31.0% and 38.5% lower, respectively, than seedlings exposed to 0.5 and 10 mM NO_3^--N. Thus, 2.5 mM Gly was used in subsequent experiments as this concentration obviously reduced primary root length. The presence of low to medium concentrations of Gly (≤ 5 mM Gly) also significantly increased lateral root number compared to seedlings cultured in the absence of Gly, though the addition of 10 mM Gly did not affect lateral root number in the 10 mM NO_3^--N treatment (Fig 1B). Moreover, the primary root length and lateral root number of 0.5 mM NO_3^--N-treated seedlings were significantly higher than seedlings cultured in 10 mM NO_3^--N (Fig 1A and 1B). Similar results were observed in seedlings supplied with varying concentrations of NO_3^--N (S2 Fig).

To examine whether the response of the roots to Gly was the result of the altered N supply, we assessed the root elongation of seedlings supplied with 2.5 mM NO_3^--N and 2.5 mM Gly (i.e. the same millimolar concentrations of each N source) in the presence of 0, 0.5 or 10 mM NO_3^--N. Compared with equimolar concentrations of NO_3^--N (2.5, 3 or 12.5 mM), the elongated root lengths of seedlings exposed to 2.5 mM Gly were 4.4%, 13.6% and 23.4% shorter during the first 24 h of treatment (Fig 1C) and 27.8%, 15.7% and 19.3% shorter during the second 24 h of treatment than seedlings exposed to 2.5 mM plus 0, 0.5 or 10 mM NO_3^--N (Fig

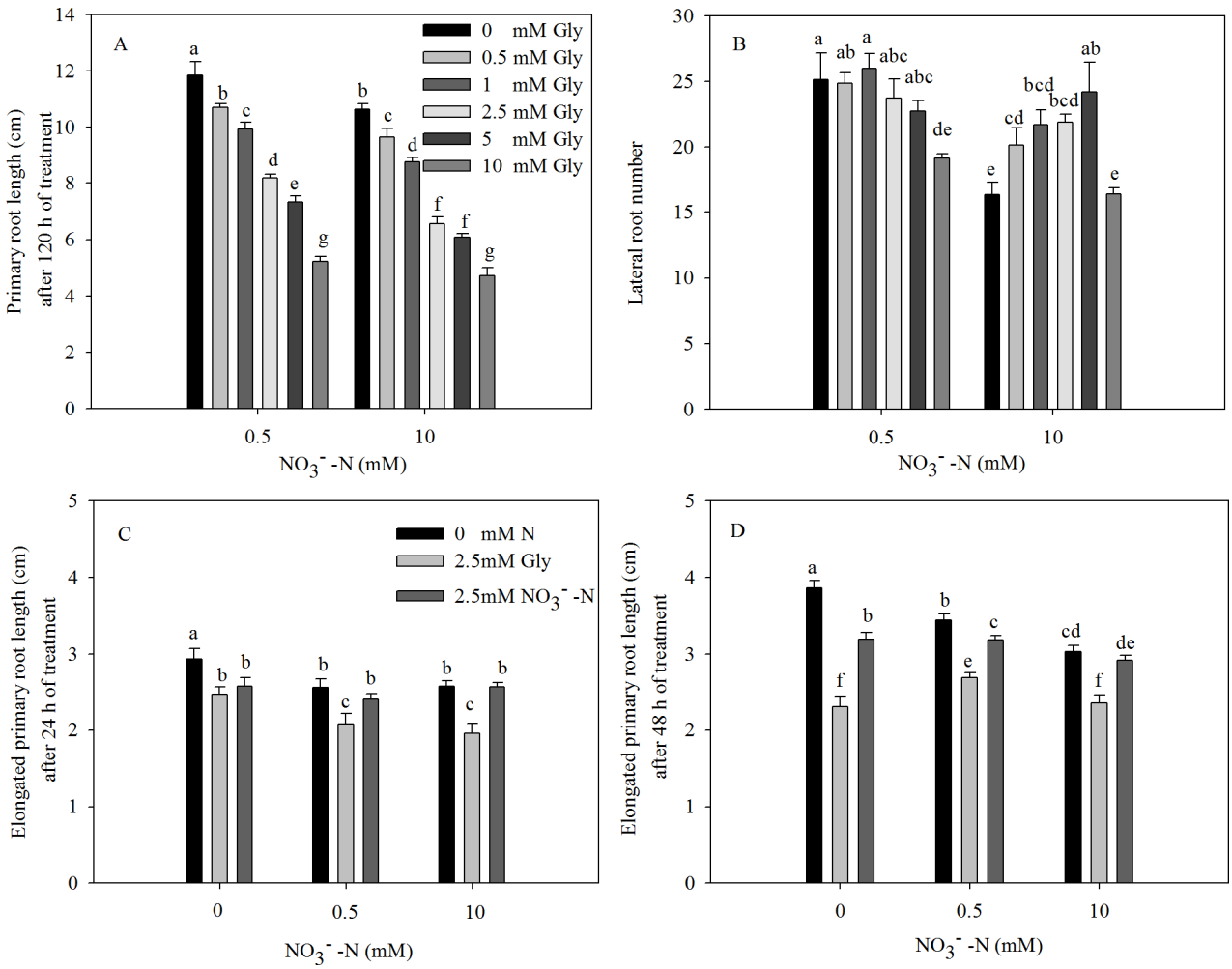

Fig 1. Effect of exogenous Gly and NO_3^--N on the primary root growth. (A) Primary root length and (B) lateral root number of pak choi seedlings grown on axenic agar medium containing 0.5 or 10 mM NO_3^--N and various concentrations of Gly (0–10 mM) for 5 d. (C) Primary root elongation after 24 h treatment and (D) after 48 h treatment under equivalent N concentrations (2.5 mM Gly or NO_3^--N in combination with 0, 0.5 or 10 mM NO_3^--N). The root tips of seedlings grown on agar medium were marked to determine the extent of root elongation every day. Daily differences in primary roots were obtained by measuring two successively dated root lengths. Data are mean ± SE (n = 5). Different letters indicate significant differences at $P < 0.05$, *LSD* test.

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1D). These results suggest the effects of Gly on root length were not due to a change in the concentration of N available to the roots.

Next, we performed a time course assessment of the changes in root morphological parameters and the fresh weight of pak choi seedlings induced by $NaNO_3$ + Gly under hydroponic conditions (Fig 2). Compared with $NaNO_3$ alone, $NaNO_3$ + Gly decreased the primary root length, total root length, number of root tips and root surface area (Fig 2A, 2B, 2C and 2E and S3 Fig), but increased the number of root tips per unit root length and root activity (Fig 2D and 2F) at 5 d. Moreover, $NaNO_3$ + Gly significantly decreased the root fresh weight and shoot fresh weight compared with $NaNO_3$ alone after 9 d (Fig 2G and 2H). After 17 d, the primary root length, total root length, number of root tips, root surface area, root and shoot fresh weights of seedlings exposed to $NaNO_3$ + Gly were 61.1%, 76.0%, 63.4%, 67.8%, 25.3% and 36.3% lower, and the number of root tips per unit root length and root activity were 57.0% and 95.0% higher than seedlings exposed to $NaNO_3$ (Fig 2 and S3 Fig).

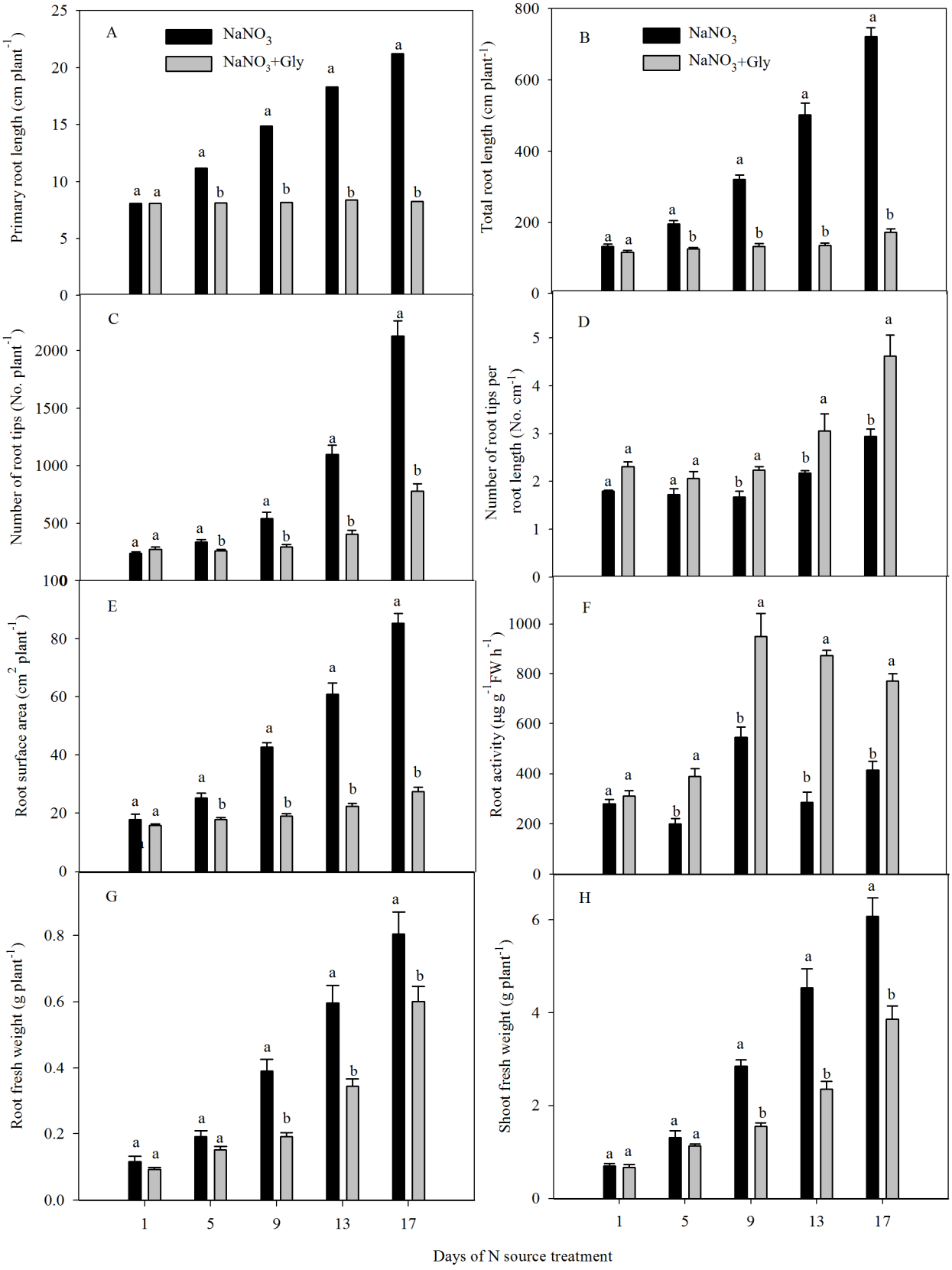
A
B
C
D
E
F
G
H
NaNO3
NaNO3+Gly
Primary root length (cm plant-1)
Total root length (cm plant-1)
Number of root tips (No. plant-1)
Number of root tips per root length (No. cm-1)
Root surface area (cm2 plant-1)
Root activity (μg g-1 FW h-1)
Root fresh weight (g plant-1)
Shoot fresh weight (g plant-1)
Days of N source treatment

Fig 2. Time course of changes in the root morphological parameters and growth of pak choi seedlings exposed to exogenous Gly. Eighteen-day-old pak choi seedlings were transferred to hydroponic nutrient solution containing 10 mM $NaNO_3$ with or without 2.5 mM Gly. (A) Primary root length, (B) total root length, (C) number of root tips, (D) number of roots per root length, (E) root surface area, (F) root activity, (G) root fresh weight and (H) shoot fresh weight of pak choi seedlings at various time points. Data are mean ± SE ($n = 9$). Different letters indicate significant differences between treatments at $P < 0.05$, Student's *t*-test.

https://doi.org/10.1371/journal.pone.0204488.g002

NO_3^--N uptake

Next, we examined the effects of exposure to $NaNO_3$ and Gly on the uptake of ^{15}N (by using $Na^{15}NO_3$ and ^{15}N-Gly) under hydroponic conditions (Fig 3). Compared to 10 mM $NaNO_3$, the addition of Gly significantly decreased $^{15}NO_3^-$-N uptake between days 1 to 17 (Fig 3A and 3C). Moreover, ^{15}N-Gly was detectable in seedlings exposed to $NaNO_3$ + Gly between days 1 to 17 of treatment. In the $NaNO_3$ + Gly treatment, the roots accumulated significantly less $^{15}NO_3^-$-N than ^{15}N-Gly, with $^{15}NO_3^-$-N accounting for 38.4–46.8% and ^{15}N-Gly accounting for 53.3–61.7% of root N (Fig 3B). In comparison, the shoots accumulated significantly more $^{15}NO_3^-$-N than ^{15}N-Gly between days 1 to 17, with $^{15}NO_3^-$-N accounting for 64.3–71.8% of shoot N (Fig 3D). Since shoots have a higher fresh weight than roots, the high fraction of N derived from NO_3^--N in the shoots led to an overall increase in the fraction of whole plant N

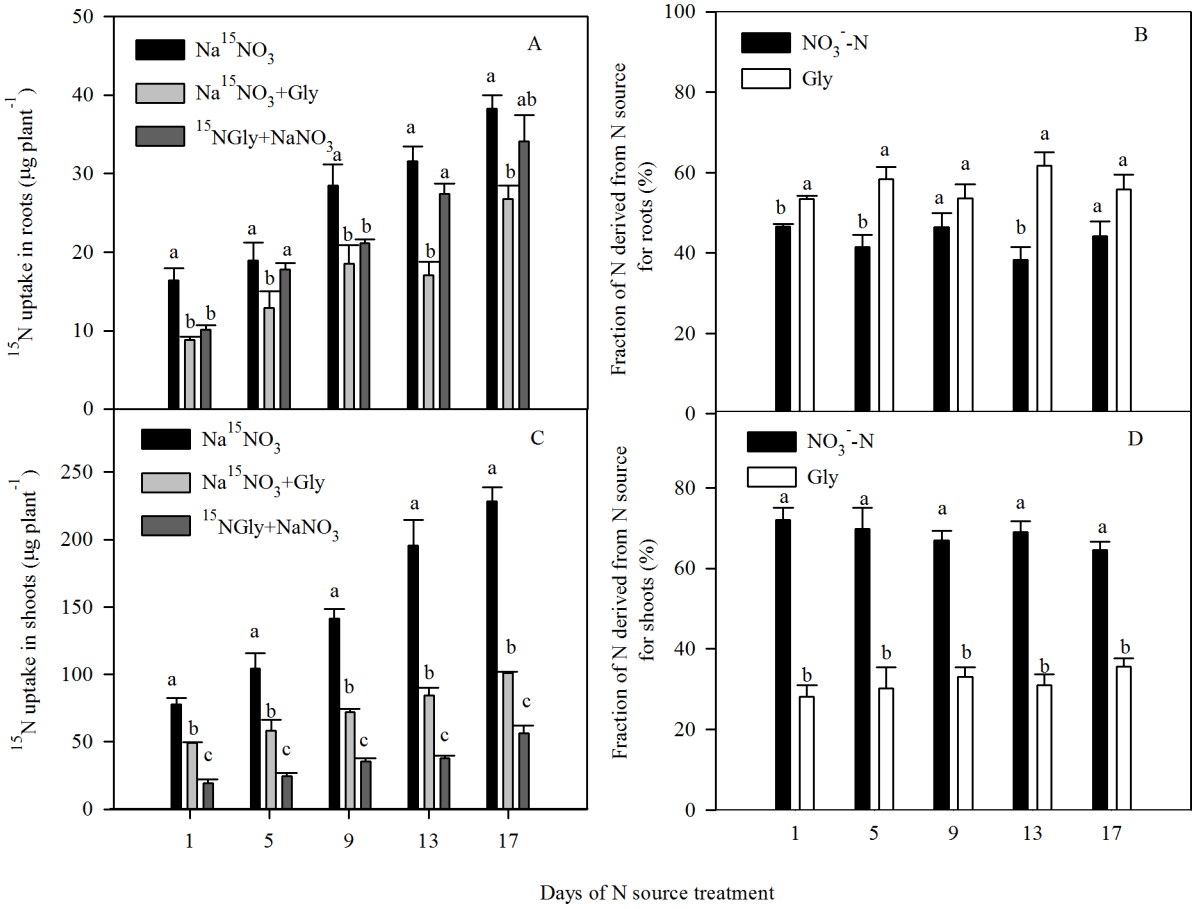

Fig 3. Time course of changes in ^{15}N uptake by pak choi seedlings exposed to $Na^{15}NO_3$, $Na^{15}NO_3$ + Gly or $^{15}NGly$ + $NaNO_3$. (A) Uptake of ^{15}N-labelled NO_3^--N and Gly in the roots, (B) fraction of N derived from each N source for the roots, (C) uptake of ^{15}N-labelled NO_3^--N and Gly in the shoots, and (D) fraction of N derived from each N source for the shoots. Data are mean ± SE ($n = 3$). Different letters indicate significant differences between treatments at $P < 0.05$, *LSD* test.

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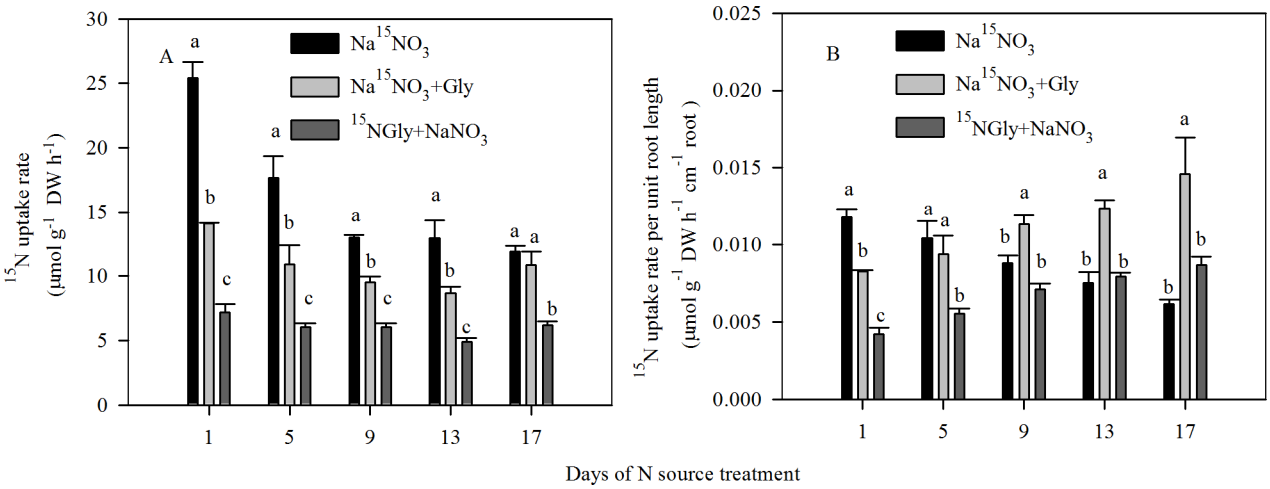

Fig 4. (A) ^{15}N uptake rate and (B) ^{15}N uptake rate per unit root length over time for pak choi seedlings exposed to $Na^{15}NO_3$, $Na^{15}NO_3$ + Gly or $^{15}NGly + NaNO_3$. Data are mean ± SE ($n = 3$). Different letters indicate significant differences between treatments at $P < 0.05$, *LSD* test.

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derived from NO_3^--N. In addition, the ratio of NO_3^--N translocated from the roots to the shoots was significantly higher in seedlings exposed to $Na^{15}NO_3$ and $Na^{15}NO_3$ + Gly than seedlings exposed to $^{15}NGly + NaNO_3$ (S4 Fig), suggesting N derived from NO_3^- tends to translocate to the shoots, whereas N derived from Gly tends to be retained in the roots.

The presence of exogenous Gly significantly decreased the $^{15}NO_3^-$-N uptake rate by 8.8–44.6% compared with seedlings exposed to solution containing $Na^{15}NO_3$ alone between days 1 to 17 of treatment. However, the reduction in the rate of $^{15}NO_3^-$-N uptake was compensated for by a significant increase in the ^{15}N-Gly uptake rate, thus, $NaNO_3$ + Gly-treated seedlings maintained similar or had even higher ^{15}N uptake rates than seedlings exposed to only $NaNO_3$ (Fig 4A). In addition, to allow a more complete characterization of the ^{15}N uptake in response to the total root length changes that were induced by exogenous Gly, the ^{15}N uptake rate per unit of root length was calculated (Fig 4B). After 1 d of treatment, the ^{15}N uptake rate per unit of root length was highest in $Na^{15}NO_3$-treated seedlings, followed by $Na^{15}NO_3$ + Gly and then $^{15}NGly + NaNO_3$-treated seedlings. However, during the course of the 17-day experiment, the ^{15}N uptake rate per unit root length gradually decreased in $Na^{15}NO_3$-treated seedlings, whereas the ^{15}N uptake rate per unit root length significantly increased in $Na^{15}NO_3$ + Gly and $^{15}NGly + NaNO_3$-treated seedlings over time (Fig 4B). At 17 d, the $^{15}NO_3^-$-N uptake rate per unit of root length was 1.4-fold higher in $Na^{15}NO_3$ + Gly-treated plants than $Na^{15}NO_3$-treated plants (Fig 4B).

Ethylene and activities of ethylene synthesis enzymes in the root

In seedlings exposed to $NaNO_3$ + Gly, uptake of Gly resulted in significant accumulation of free amino acids in the roots, including a 31.43% increase in the levels of the ethylene precursor methionine (Met) compared with the roots of $NaNO_3$-treated seedlings (S1 Table). This finding prompted us to examine whether exposure to $NaNO_3$ + Gly altered ethylene production. The roots of seedlings grown in $NaNO_3$ + Gly produced significantly higher levels of ethylene than seedlings grown in $NaNO_3$ (Table 1). Moreover, the roots of seedlings exposed to $NaNO_3$ + Gly had higher ACS and ACO activities compared with seedlings treated with $NaNO_3$ after 10 and 15 d treatment (Table 1).

Table 1. Effects of exogenous Gly on ethylene production and ACS and ACO activity in the roots of pak choi seedlings.

Treatment	10 days			15 days		
	Ethylene (nl $C_2H_4 \cdot g^{-1}$ $FW \cdot h^{-1}$)	ACS activity (nl $C_2H_4 \cdot g^{-1}$ $FW \cdot h^{-1}$)	ACO activity (nl $C_2H_4 \cdot g^{-1}$ $FW \cdot h^{-1}$)	Ethylene (nl $C_2H_4 \cdot g^{-1}$ $FW \cdot h^{-1}$)	ACS activity (nl $C_2H_4 \cdot g^{-1}$ $FW \cdot h^{-1}$)	ACO activity (nl $C_2H_4 \cdot g^{-1}$ $FW \cdot h^{-1}$)
$NaNO_3$	1.70±0.06b	0.80±0.04b	3.23±0.21b	n.d.	0.93±0.1b	1.08±0.07b
$NaNO_3$+Gly	2.75±0.26a	1.46±0.13a	9.28±0.36a	n.d.	1.73±0.16a	7.58±0.46a

n.d., not determined. Data are mean ± SE ($n = 4$). Different letters indicate significant differences within columns at $P < 0.05$, Student's *t*-test.

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The role of ethylene in exogenous Gly-induced changes in root elongation and $^{15}NO_3^-$-N uptake

To determine whether the inhibitory effect of exogenous Gly on root elongation were due to altered ethylene production, we investigated the effects of the ethylene inhibitor AVG and ethylene perception blocker $AgNO_3$ on the primary root length of pak choi seedlings grown in $NaNO_3$ + Gly agar medium. Exposure to $NaNO_3$ + Gly significantly reduced primary root length compared with NO_3^--N. However, AVG ($\leq$ 2.5 μM) markedly reversed the inhibition of primary root length induced by $NaNO_3$ + Gly (Fig 5A, S5A Fig); 0.5 μM AVG had the most significant effect. Moreover, 10 μM $AgNO_3$ led to a partial recovery of primary root length in seedlings exposed to $NaNO_3$ + Gly (Fig 5B, S5B Fig). Additionally, compared to treatment with $NaNO_3$ + Gly, the presence of 0.5 μM AVG or 10 μM $AgNO_3$ significantly increased the primary root length (by 41.9% and 21.7%, respectively).

To assess the effect of ethylene in the reduction in the $^{15}NO_3^-$-N uptake rate induced by exogenous Gly, we investigated the effects of 0.5 μM AVG and 10 μM $AgNO_3$ on the ^{15}N uptake rate in $NaNO_3$ + Gly-treated seedlings under hydroponic conditions (Fig 6). Compared with $Na^{15}NO_3$, addition of 2.5 mM Gly ($Na^{15}NO_3$ + Gly) or 2.5 mM $NaNO_3$ ($Na^{15}NO_3$ + $NaNO_3$) significantly decreased the $^{15}NO_3^-$-N uptake rate; however, there was an

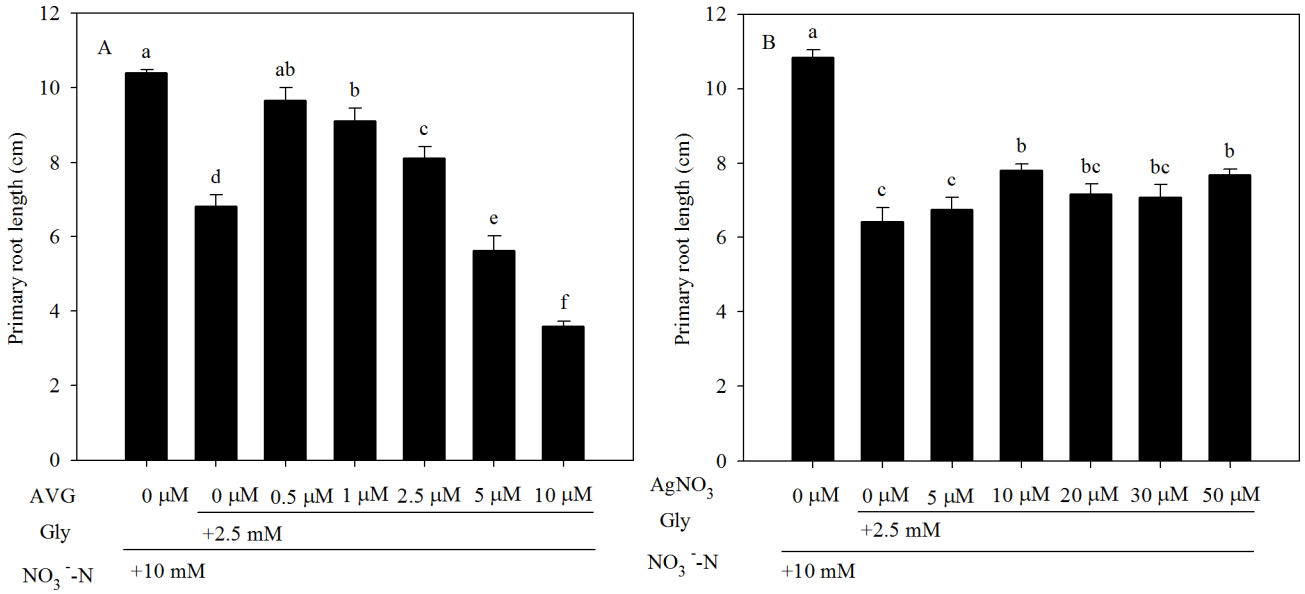

Fig 5. Effect of the ethylene synthesis inhibitors (A) AVG and (B) $AgNO_3$ on the inhibition of primary root length induced by exogenous Gly. Pak choi seedlings were grown on axenic agar medium containing 10 mM NO_3^--N with 2.5 mM Gly and various concentrations of AVG (0–10 μM) or $AgNO_3$ (0–50 μM) for 5 d. Data are mean ± SE ($n = 5$). Different letters indicate significant differences at $P < 0.05$, *LSD* test.

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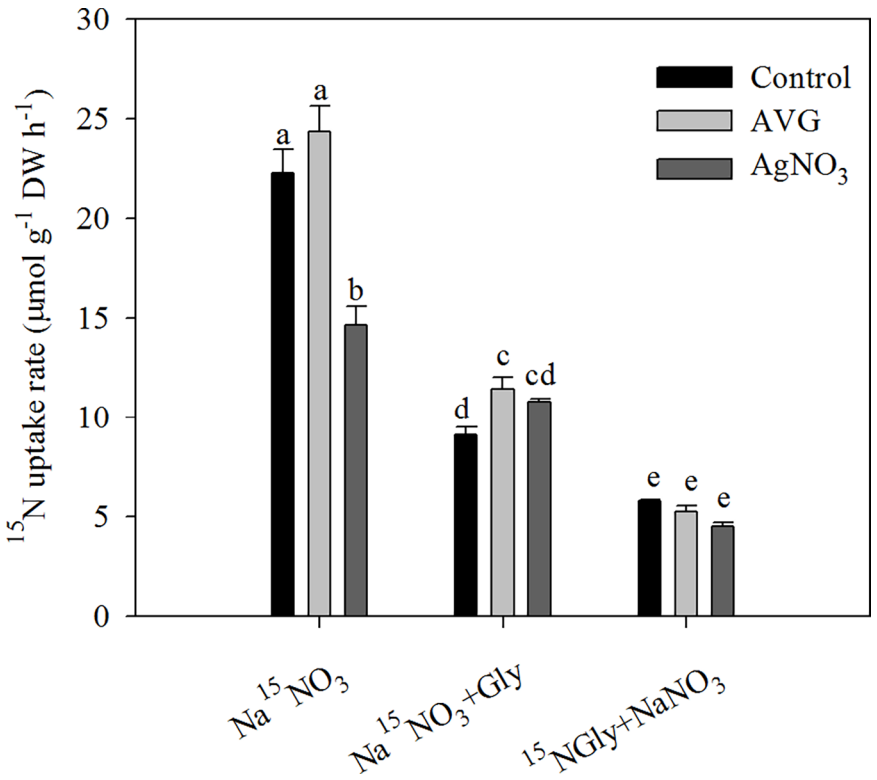

Fig 6. Effect of AVG or $AgNO_3$ and exogenous Gly on the ^{15}N uptake rate of pak choi seedlings. Twenty two-day-old pak choi seedlings were exposed to nutrient solution containing 10 mM NO_3^--N with or without 2.5 mM Gly in the presence or absence of 0.5 μM AVG or 10 μM $AgNO_3$ for 4 h. Data are mean ± SE (n = 3). Different letters indicate significant differences at $P < 0.05$, *LSD* test.

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approximately 1.2-fold difference in the $^{15}NO_3^-$-N uptake rate between the Na$^{15}NO_3$ + Gly and Na$^{15}NO_3$ + $NaNO_3$ treatments (Fig 6 and S6 Fig). In $NaNO_3$-treated plants, the $^{15}NO_3^-$-N uptake rate was slightly increased by 0.5 μM AVG, but significantly reduced by 10 μM $AgNO_3$; in $NaNO_3$ + Gly-treated plants, the $^{15}NO_3^-$-N uptake rate was significantly increased and the ^{15}N-Gly uptake rate was slightly decreased by 0.5 μM AVG or 10 μM $AgNO_3$. Moreover, significant differences in the fraction of N derived from the NO_3^--N source were observed in the shoots between treatments, but not in the roots (Fig 7). In the $NaNO_3$ + Gly-treated seedlings, 0.5 μM AVG or 10 μM $AgNO_3$ significantly increased the fraction of shoot N derived from the NO_3^--N source (Fig 7B).

Discussion

Studies typically quantify the effect of exogenous Gly on the NO_3^- content and other physiological responses of leafy vegetables grown in hydroponic culture [4, 5, 27, 40, 41]. Furthermore, hydroponic conditions has been employed to study root morphology and NO_3^--N uptake in Chinese cabbage [42], cucumber [43] and tomato [44], as well as the role of ethylene in the modulation of root length in wheat [40]. Therefore, we used a hydroponic system to assess the effect of exogenous Gly on the root morphology and NO_3^--N uptake and verify the role of ethylene in these processes in pak choi. However, to more easily observe the changes in root length (i.e. without large numbers of branching roots) after 24 or 120 h treatment, we used an agar plate system to investigate the effect of exogenous Gly and AVG/$AgNO_3$ on primary root elongation. We confirmed that Gly induced similar reductions in primary root

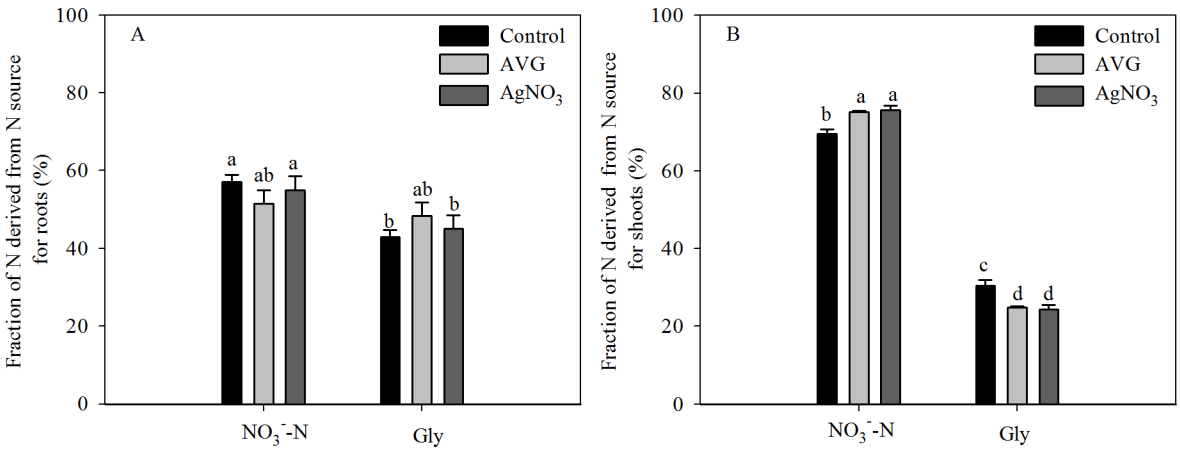

Fig 7. Effect of AVG or $AgNO_3$ on the fraction of N derived from each N source (NO_3^--N or Gly) in the (A) roots and (B) shoots. Twenty two-day-old pak choi seedlings were exposed to nutrient solution containing 10 mM NO_3^--N with 2.5 mM Gly in the presence or absence of 0.5 μM AVG or 10 μM $AgNO_3$ for 4 h. Data are mean ± SE ($n = 3$). Different letters indicate significant differences at $P < 0.05$, *LSD* test.

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length of pak choi in the agar plate system and hydroponic system (Figs 1 and 2). In hydroponic culture, exogenous Gly suppressed root length and reduced NO_3^--N uptake (Figs 3 and 4). Plant root morphology is an important variable required to ensure adequate access to NO_3^--N, which in turn influences accumulation of NO_3^- [42]. The inhibition of root length and reduction in NO_3^--N uptake induced by Gly in this study may explain how exogenous amino acids decrease the NO_3^- concentration in plants (S7 Fig). Additionally, exogenous Gly also enhanced production of ethylene in the roots of hydroponically grown pak choi (Table 1), and ethylene was at least partly involved in the changes in root development and NO_3^--N uptake induced by exogenous Gly.

Exogenous Gly inhibits root elongation

In both the agar plate and hydroponic systems, exogenous Gly significantly reduced primary root length (Fig 1A, 1C and 1D, Fig 2A and S1 Fig). This is similar to the effects of single amino acids, such as *L*-Glu and Gly [21, 24, 45] and mixtures of N sources supplied with NO_3^--N and *L*-Glu [19, 20] on plants grown on agar plates. Only Walch-Liu, Forde (2008) and Leblanc et al. (2013) have investigated the effect of Glu on root development in the presence of both NO_3^--N and Glu; the mixtures used in those studies more closely resemble soil conditions. Moreover, NO_3^--N partially antagonized that ability of *L*-Glu to inhibit root length in *Arabidopsis thaliana* [20]. However, unlike Walch-Liu and Forde (2008), we found low and high concentrations of NO_3^--N increased and inhibited, respectively, the primary root length of agar plate-grown pak choi in the presence of Gly (Fig 1A), indicating that NO_3^--N and Gly may exert partially antagonistic (at low NO_3^--N concentrations) or synergistic (at high NO_3^--N concentrations) effects on the growth of the primary roots. Bonner et al. (1996) reported that the inhibitory effect of Gly was probably related to the Glutamine-reversible phenomenon of 'general amino acid inhibition' [46]. In this study, the inhibitory effect was attributable to the impact that the exogenous Gly may have on plant metabolism. Low concentrations of NO_3^--N can stimulate the primary root growth directly [20]. Walch-Liu and Forde (2008) suggested that direct stimulation of primary root growth by NO_3^--N might be a manifestation of the same phenomenon as NO_3^--N antagonism of the inhibitory effect of amino acid on primary root growth. In this context, primary root growth may be negatively regulated by the

endogenous amino acids pool [20], and low concentrations of NO_3^--N would promote the primary root growth by alleviating this effect. Nevertheless, the high concentrations of NO_3^--N inhibited the primary root growth independently of the presence of exogenous Gly (S2 Fig). It has been suggested that the same NO_3^--N signaling pathway that operates in the lateral roots may also regulate primary root growth [20]. The development of lateral roots has been suggested to be inhibited by the accumulation of N metabolites in the roots [47]. The inhibition of primary root growth at high NO_3^--N concentrations would be negatively regulated by the accumulation of N metabolites. Therefore, we proposed that high concentrations of NO_3^--N further aggravated the inhibition of primary root growth induced by exogenous Gly. In addition, these findings showed that under agar plate growth conditions, exogenous Gly affected root morphology in a manner distinct to $NaNO_3$ (S2 Fig). When added at the same millimolar N concentrations, exogenous Gly inhibited primary root growth more severely than $NaNO_3$ (Fig 1C and 1D), demonstrating that the effect of Gly on primary root length are not directly attributed to the nutritional effects of N availability.

Amino acids such as Gly modulate root development when present in the growth medium at concentrations higher than 0.5 mM. However, all experiments in this study were completed under sterile conditions, thus eliminating microorganisms, which are considered to be more competitive for organic N than plants. Studies using sterile cultures do not always reflect the actual soil environment, including the forms and concentrations of N and turnover rates of organic N. While the concentrations of amino acids in soil solutions are low [28], concentrations in excess of the levels needed to affect root morphology are likely to occur within soils that absorb substantial quantities of amino acids [48] and decomposing organic matter, which contain millimolar levels of amino acids [10]. Nevertheless, knowledge of whether such high concentrations of amino acids exert unrecognized or negligible effects on root morphology in the field is still lacking. The findings presented in this study help to further understand the effect of organic N on plant root morphology in the presence of both NO_3^--N and organic N, which more closely reflects field conditions. Furthermore, the results of the present study contribute to the existing knowledge that has been generated using inorganic N or single organic N source test systems.

Exogenous Gly reduces NO_3^--N uptake but gradually increases NO_3^--N uptake rate per unit of root length

In addition to the inhibition of primary root length, exogenous Gly significantly reduced NO_3^--N uptake (rate) from day 1 to 17 of treatment (Figs 3, 4A and 6) under hydroponic conditions. These results are consistent with earlier studies showing that Glu reduced the uptake of NO_3^--N by plants grown on agar plates [19] and Gly reduced the uptake of NO_3^--N under hydroponic conditions [9]. The effects of amino acids may be due to accumulation of N metabolites such as assimilated amino acids. NO_3^--N uptake has been shown to be inhibited by an increase in the concentrations of the downstream products of N sources [49].

In contrast to the reductions in root length and NO_3^--N uptake, after 9 d of treatment, the NO_3^--N uptake rate per unit root length exhibited the opposite trend to that of NO_3^--N uptake (rate), with $NaNO_3$ + Gly-treated seedlings having smaller roots but a higher NO_3^--N uptake rate per unit root length, implying a compensatory mechanism related to NO_3^--N uptake by the roots (Fig 4B). Although this compensatory effect was observed, it was unable to restore shoot and root growth and ^{15}N uptake to the normal levels (Fig 3A and 3D). In addition, given that the root structure induced by N sources need long-term compensatory mechanisms [50] and that root activity reflects the capacity of the plant root system for nutrient uptake [51], the increased $^{15}NO_3^-$-N uptake rate per unit root length may be positively correlated with the

increased number of root tips per unit root length (Fig 2D) and higher root activity (Fig 2F). These results do not exclude the possibility that the increases in the number of root tips per unit root length, root activity and $^{15}NO_3^-$-N uptake rate per unit root length could reflect an important adaptive response to mixtures of Gly and NO_3^--N.

Role of Gly in pak choi N nutrition

Plants are able to take up amino acids as a source of N [3, 52], and we confirmed Gly was absorbed by pak choi seedlings (Figs 3, 4 and 6). For pak choi grown in mixtures of Gly and NO_3^--N, ^{15}N-Gly uptake accounted for 28.2–35.7% of shoot N and 53.3–61.7% of root N (Fig 3B and 3D). However, these fractions may be overestimated, as the plants were grown under sterile conditions in this study. Accurate methods of determining the quantitative contribution of amino acid N in the natural environment have not yet been devised, and are likely to be affected by competition with microorganisms; hence, the actual contribution of Gly to plant nutrition cannot be determined. In addition, Cambui et al. (2011) suggested that a significant share of absorbed amino acids resided, and was incorporated, at the site of primary assimilation [53]. Thus, we found N derived from Gly was more abundant in the roots than shoots (Fig 3 and S4 Fig), indicating that absorbed Gly may be preferentially metabolized in the roots, and slowly transported to the shoots [6].

Moreover, our data showed that the inhibition of NO_3^--N uptake induced by Gly in pak choi seedlings under hydroponic conditions was compensated for by an increase in ^{15}N-Gly uptake in order to maintain a similar total N uptake rate (Figs 4 and 6). However, despite N being taken up at a similar rate, the root length and shoot growth of exogenous Gly-treated seedlings were significantly impaired by Gly (Fig 2 and S3 Fig). Phytohormones have been demonstrated to be involved in growth inhibition [24, 54]. In this study, the growth retardation induced by Gly may be related to altered phytohormone levels.

Ethylene may be involved in the metabolism of absorbed Gly and participate in the regulation of root development and NO_3^--N uptake induced by exogenous Gly

Ethylene can be induced in response to different N sources [34, 55–58]. Our results suggest that exogenous Gly treatment enhanced ethylene production in the roots of pak choi (Table 1). Enhancement of ethylene production may be due to increased synthesis of amino acids (S1 Table) as explained by Kaack and Pedersen (2014) [59]. Moreover, Gly in the roots is incorporated into serine (Ser) and then converted to other amino acids via transamination [6]. The coupled increases in the Gly and Ser contents observed in the root tissues of hydroponically grown pak choi co-treated with $NaNO_3$ + Gly (S1 Table) indicate intact Gly can be absorbed by the plants [7, 52]. A previous study suggested absorbed Gly would likely be metabolized to Met [25], the precursor of ethylene [60]. Indeed, we observed an increase in the content of Met (S1 Table). Additionally, the activities of ACS and ACO, two key enzymes responsible for ethylene synthesis in plants, were significantly higher in plants treated with $NaNO_3$ + Gly than plants treated with only inorganic N sources (Table 1). In the ethylene synthesis pathway, SAM is the intermediate product [60] and an important methyl donor, while SAM synthase is a vital enzyme that directs the flux of SAM and directly participates in the metabolism of Gly [4]. In a previous study, we reported that SAM synthase was upregulated by Gly [4]. Thereby, these results indicate ethylene may be involved in the pathways by which absorbed Gly is metabolized in plants. These findings provide further evidence that nutritional and hormonal cues collectively regulate the growth of plants.

Ethylene can inhibit root growth and regulate NO_3^--N uptake [29, 61]. In the present study, the inhibition of root growth and NO_3^--N uptake observed in response to mixtures of Gly and NO_3^--N were related to increased ethylene production (Table 1). The ethylene inhibitors AVG and $AgNO_3$ attenuated the effects of ethylene under both agar plate and hydroponic conditions [39, 62]. One important finding of this study is that application of an ethylene biosynthesis inhibitor (0.5 or 1 μM AVG) or perception blocker (10 μM $AgNO_3$) to $NaNO_3$ + Gly-treated pak choi markedly alleviated the inhibition of primary root length under agar plate conditions (Fig 5 and S5 Fig). These findings are in agreement with Domínguez-May et al. (2013), who suggested that ethylene played a regulatory role in the inhibitory effects of Gly in habanero pepper [24]. Moreover, we also showed that exogenous application of 0.5 μM AVG and 10 μM $AgNO_3$ could markedly increase the $^{15}NO_3^-$-N uptake rate in hydroponically grown pak choi seedlings in response to $NaNO_3$ + Gly (Figs 6 and 7). Our results are consistent with Zheng et al. (2013), who reported ethylene negatively affected the $^{15}NO_3^-$-N uptake rate [29]. However, previous studies adopted both pharmacological and transgenic approaches to investigate the roles of ethylene in root development [33] and NO_3^--N uptake [29]. Therefore, application of transgenic lines will be required in future studies to further elucidate the mechanisms by which Gly induces inhibitory effects on root morphology and reduces NO_3^--N uptake.

Conclusion

The results presented here clearly show that the root morphological responses of pak choi to Gly and nitrate-N were different to those of seedlings exposed to a single nitrate-N source. Compared to the nitrate-N supply, addition of Gly inhibited the root elongation of pak choi seedlings, and this inhibitory effect was attributed to the specific N forms, rather than the total N concentration. When treated with mixed N sources, pak choi seedlings took up N in the form of nitrate-N and Gly. Furthermore, nitrate-N uptake was reduced by application of Gly. The inhibition of root growth and reduction in nitrate-N uptake induced by Gly was probably mediated by phytohormones, as the roots of pak choi supplied with Gly and nitrate-N showed enhanced production of ethylene. Further investigation also confirmed that the inhibition of root growth and reduction in nitrate-N uptake observed in the presence of Gly were partly related to an increase in ethylene levels. However, the mechanism underlying this phenomenon is not yet fully understood and will remain the focus of further investigations.

Supporting information

S1 Fig. Root growth of pak choi seedlings grown on axenic agar medium containing (A) 0.5 mM or (B) 10 mM NO_3^--N and a range of concentrations of Gly for 5 d.
(TIF)

S2 Fig. Effect of NO_3^--N supply on the (A) primary root length and (B) lateral root number of pak choi seedlings cultured for 4 d on agar plates. Data are mean ± SE (n = 5). Different letters indicate significant differences at $P < 0.05$, *LSD* test.
(TIF)

S3 Fig. Phenotypes of pak choi seedlings exposed to $NaNO_3$ or $NaNO_3$ + Gly. Eighteen-day-old pak choi seedlings were transferred to nutrient solution containing 10 mM $NaNO_3$ with or without 2.5 mM Gly and harvested after 17 d.
(TIF)

S4 Fig. Time course of percentages of each form of ^{15}N translocated from roots to shoots by pak choi seedlings exposed to $Na^{15}NO_3$, $Na^{15}NO_3$ + Gly or $^{15}NGly$ + $NaNO_3$. Data are

mean ± SE (n = 3). Different letters indicate significant differences between treatments at $P < 0.05$, *LSD* test.
(TIF)

S5 Fig. Effect of (A) 1 μM AVG and (B) 10 μM $AgNO_3$ in the presence of 10 mM $NaNO_3$ with or without 2.5 mM Gly on the primary root length elongation of pak choi seedlings on agar plates in the first 24 h. Data are mean ± SE (n = 5). Different letters indicate significant differences at $P < 0.05$, *LSD* test.
(TIF)

S6 Fig. Effect of exogenous Gly on the $^{15}NO_3^-$-N uptake rate of pak choi seedlings. Twenty two-day-old pak choi seedlings were exposed to nutrient solution containing 10 mM $Na^{15}NO_3$, 10 mM $Na^{15}NO_3$ + 2.5 mM NO_3^--N, or 10 mM $Na^{15}NO_3$ + 2.5 mM Gly for 4 h. Data are mean ± SE (n = 3). Different letters indicate significant differences at $P < 0.05$, *LSD* test.
(TIF)

S7 Fig. Effect of exogenous Gly on the nitrate contents of the shoots and roots of pak choi seedlings. Eighteen-day-old pak choi seedlings were transferred to nutrient solution containing 10 mM NO_3^--N with or without 2.5 mM Gly for 5 d. Data are mean ± SE (n = 4). Different letters indicate significant differences between treatments at $P < 0.05$, Student's t-test.
(TIF)

S1 Table. Effects of exogenous Gly on the concentrations of amino acids (μg·g^{-1} FW) in the roots of pak choi seedlings after 5 d treatment under hydroponic culture conditions.
(DOT)

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