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 NO3- 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 NO3- uptake and root morphology have received little attention. In this study, we evaluated the effects of exogenous glycine (Gly) on root growth and NO3- uptake in pak choi (Brassica campestris ssp. Chinensis L.). Addition of Gly to NO3- 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 NO3- in hydroponic culture, Gly significantly reduced 15NO3- uptake but significantly increased the number of root tips per unit root length, root activity and 15NO3- uptake rate per unit root length. In addition, 15N-Gly was taken up into the plants. In contrast to absorbed NO3-, 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 AgNO3) partly reversed Gly-induced inhibition of primary root elongation on agar plates and increased the NO3- 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 (NO3-) accumulation in green leafy vegetables are influenced by the sources of available nitrogen (N) [1]. Consumption of vegetables containing high concentrations of NO3- has been related to risks to human health [2]. Plants accumulate NO3- when the rate of NO3- uptake through the roots exceeds the rate of NO3- assimilation in plant tissues. Given that the roots are the major sites that directly affect NO3- uptake in plants,
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].

Competing interests: The authors have declared that no competing interests exist.
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$_2$O$_2$ 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$_2$PO$_4$, 1.25 mM K$_2$SO$_4$, 2.5 mM CaCl$_2$, 0.05 mM EDTA 2Na, 0.05 mM FeSO$_4$, 48.5 μM H$_3$PO$_4$, 10 μM MnSO$_4$, 0.8 μM ZnSO$_4$, 0.2 μM CuSO$_4$, 2.1 μM NaMoO$_4$ 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 μmol·m$^{-2}$·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 μmol·m$^{-2}$·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$_2$SO$_4$. 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$_2$SO$_4$. 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 $^{14}$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].

$$^{15}\text{N}_{\text{uptake}} = DW \times N\% \times \frac{A_i - A_c}{A_{\text{applied}}}$$ (1)

Where $^{15}\text{N}_{\text{uptake}}$ 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, $A_i$ 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 $A_{\text{applied}}$ 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).

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

Where $^{15}\text{N}_{\text{uptake}}$ 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 $^{15}N_{\text{total uptake}}$ is the total amount of absorbed NO$_3$-N and Gly in the roots or shoots.

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

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

Where $^{15}N_{\text{uptake rate}}$ is the uptake rate for the N source for whole seedlings, $^{15}N_{\text{uptake}}$ 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 $^{15}N_{\text{uptake rate per unit root length}}$ was calculated according to Eq (4).

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

Where $^{15}N_{\text{uptake rate per unit root length}}$ is the N uptake rate per unit root length. The $^{15}N_{\text{uptake rate}}$ 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
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).
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.
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).
The role of ethylene in exogenous Gly-induced changes in root elongation and $^{15}\text{NO}_3^-$-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 (≤ 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 $^{15}\text{NO}_3^-$-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 $^{15}\text{NO}_3^-$-N uptake rate; however, there was an

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$_2$H$_4$ g$^{-1}$ FW h$^{-1}$) | ACS activity (nl C$_2$H$_4$ g$^{-1}$ FW h$^{-1}$) | ACO activity (nl C$_2$H$_4$ g$^{-1}$ FW h$^{-1}$) | Ethylene (nl C$_2$H$_4$ g$^{-1}$ FW h$^{-1}$) | ACS activity (nl C$_2$H$_4$ g$^{-1}$ FW h$^{-1}$) | ACO activity (nl C$_2$H$_4$ g$^{-1}$ FW 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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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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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$_3$-N 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
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 $^{15}\text{NO}_3^{-}$-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\textsubscript{3}^-N uptake [29, 61]. In the present study, the inhibition of root growth and NO\textsubscript{3}^-N uptake observed in response to mixtures of Gly and NO\textsubscript{3}^-N were related to increased ethylene production (Table 1). The ethylene inhibitors AVG and AgNO\textsubscript{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\textsubscript{3}) to NaNO\textsubscript{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 Dominguez-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\textsubscript{3} could markedly increase the $^{15}$NO\textsubscript{3}^-N uptake rate in hydroponically grown pak choi seedlings in response to NaNO\textsubscript{3} + Gly (Figs 6 and 7). Our results are consistent with Zheng et al. (2013), who reported ethylene negatively affected the $^{15}$NO\textsubscript{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\textsubscript{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\textsubscript{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 future 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\textsubscript{3}^-N and a range of concentrations of Gly for 5 d. (TIF)

S2 Fig. Effect of NO\textsubscript{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\textsubscript{3} or NaNO\textsubscript{3} + Gly. Eighteen-day-old pak choi seedlings were transferred to nutrient solution containing 10 mM NaNO\textsubscript{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\textsubscript{3}, Na$^{15}$NO\textsubscript{3} + Gly or $^{15}$NGly + NaNO\textsubscript{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 AgNO₃ in the presence of 10 mM NaNO₃
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 signifi-
cant differences at P < 0.05, LSD test.

(TIF)

S6 Fig. Effect of exogenous Gly on the ¹⁵NO₃⁻-N uptake rate of pak choi seedlings. Twenty
two-day-old pak choi seedlings were exposed to nutrient solution containing 10 mM
Na¹⁵NO₃, 10 mM Na¹⁵NO₃ + 2.5 mM NO₃⁻-N, or 10 mM Na¹⁵NO₃ + 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 contain-
ing 10 mM NO₃⁻-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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Root elongation and nitrate-N uptake as affected by glycine

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