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L-cysteine desulfhydrase-related H_2S production is involved in OsSE5-promoted ammonium tolerance in roots of Oryza sativa

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ABSTRACT

Previous studies revealed that rice heme oxygenase PHOTOPERIOD SENSITIVITY 5 (OsSE5) is involved in the regulation of tolerance to excess ammonium by enhancing antioxidant defence. In this study, the relationship between OsSE5 and hydrogen sulfide (H_2S), a well-known signalling molecule was investigated. Results showed that NH_4Cl triggered the induction of $_L$-cysteine desulfhydrase ($_L$-DES)-related H_2S production in rice seedling roots. A H_2S donor, not only alleviated the excess ammonium-triggered inhibition of root growth, but also reduced endogenous ammonium, both of which were aggravated by the hypotaurine (HT, a H_2S scavenger) or $_{DL}$-propargylglycine (PAG, a $_L$-DES inhibitor). Nitrogen-metabolism related enzymes were activated by H_2S, thus resulting in induction of amino acid synthesis and total nitrogen content. Interestingly, activity of $_L$-DES, as well as the enzymes involved in nitrogen metabolism was significantly increased in OsSE5-overexpression line (35S:OsSE5), whereas impaired in OsSE5-knockdown mutant (OsSE5-RNAi). Application of HT/PAG or H_2S donor could differentially block or rescue NH_4Cl-hyposensitivity or hypersensitivity phenotypes in 35S:OsSE5-1 or OsSE5-RNAi-1 plants, with a concomitant modulation of nitrogen assimilation. Taken together, these results illustrated that H_2S function as an indispensable positive regulator participated in OsSE5-promoted ammonium tolerance, in which nitrogen metabolism was facilitated.

Key-words: Hydrogen sulfide; rice; OsSE5; excess ammonium; nitrogen assimilation

INTRODUCTION

Nitrogen is an essential macronutrient for plants and a primary limiting factor in plant biomass production. Ammonium (NH_4^+) and nitrate (NO_3^-) are available as major sources of inorganic nitrogen in most soils (Yuan et al. 2013). NH_4^+ is the predominant nitrogen source for many plant species at low concentrations (Von et al. 2000). However, when NH_4^+ is the sole nitrogen source, most plants exhibited toxic symptoms including the inhibition of root growth and biomass (Britto & Kronzucker 2002; Li et al. 2014; Esteban et al. 2016). In addition, glutamate (Glu) and aspartic (Asp) play a central signaling and metabolic role at the interface of nitrogen assimilatory pathways (Forde & Lea 2007; Labboun et al. 2009). The abundance of many free amino acids such as Glu and Asp was increased when NH_4^+ is excessively supplied, which is regarded as an important detoxification strategy with the channeling of excess ammonia into essential metabolic processes and defence compounds. (Tapia et al. 1996; Bialczyk et al. 2005).

It is well-known that the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle is the main way for ammonium assimilation in plants (Tabuchi et al. 2007; Lea & Miflin 2003, 2011). GS produces glutamine (Gln) from ammonium and Glu, and GOGAT transfers the amino group of Gln to 2-oxoglutarate to generate two molecules of Glu in the cycle (Ishiyama et al. 2004). Numerous studies suggested that plant species with higher GS activities achieve an elevated tolerance to NH_4^+ stress (Glevarec et al. 2004; Cruz et al. 2006; Fei et al. 2006). Another possible ammonium assimilation pathway is via the action of glutamate dehydrogenase (GDH), which

64 catalyze the reversible amination of 2-oxoglutarate with ammonium to form Glu
65 (Fontaine et al. 2012). GDH activity can be induced by higher levels of ammonia
66 (Cammaerts & Jacobs 1985; Tercé-Laforgue et al. 2004), and the positive effect of
67 GDH in response to stress also has been suggested (Balestrasse et al. 2003; Dubois et
68 al. 2003; Restivo 2004).

69 Heme oxygenase (HO; EC 1.14.99.3) catalyzes the oxidative conversion of haem
70 to carbon monoxide (CO), biliverdin (BV), and free iron (Fe^{2+}) (Shekhawat & Verma
71 2010). In rice, PHOTOPERIOD SENSITIVITY 5 (OsSE5) which may function in
72 phytochrome chromophore biosynthesis, was first assumed to encode HO with high
73 similarity to Arabidopsis HY1/HO1 (long hypocotyls mutant 1). The OsSE5 mutant
74 line exhibited a very early flowering phenotype and is completely deficient in
75 photoperiodic response (Izawa et al. 2000). Plant HO has recently been shown to have
76 a positive role in the plant responses to abiotic stresses (Noriega et al. 2004; Xie et al.
77 2012, 2013). Plants with knockdown of OsSE5 expression exhibited hypersensitive to
78 the herbicide methyl viologen (MV)-induced oxidative stress, whereas transgenic
79 Arabidopsis plants overexpressing OsSE5 showed tolerance to MV (Xu et al. 2012b).
80 OsSE5 was also involved in the improvement of plant tolerance to NH_4^+ stress in both
81 NH_4^+-tolerant (rice) and NH_4^+-sensitive species (Arabidopsis) by the activation of
82 antioxidant defense, thereby neutralizing excess reactive oxygen species produced by
83 excess NH_4^+ (Xie et al. 2015). Interestingly, up-regulation of soybean HO could
84 protect the soybean nodule nitrogen fixation and assimilation under salt stress (Zilli et
85 al. 2008). However, little molecular information is known about the relationship

between OsSE5 and nitrogen assimilation under NH_4^+ stress in rice.

Hydrogen sulfide (H_2S) is emerging as a signalling molecule in plants (Wilson et al. 1978; Winner et al. 1981; Rennenberg 1983). $_L$-cysteine desulfhydrase ($_L$-DES) is considered as the major enzyme for endogenous H_2S generation in plants, which degrades cysteine into H_2S, pyruvate, and ammonium, using pyridoxal 5'-phosphate as a cofactor (Álvarez et al.2010). The transcript abundance/total enzymatic activity of $_L$-DES was induced/increased by drought stress, salicylic acid, abscisic acid (Zhang et al. 2010a; Xie et al. 2013). Recently, the positive effects of H_2S/DES is being discovered in multiple physiological processes (Guo et al. 2016), such as seed germination (Zhang et al. 2010b), stomata movement (Scuffi et al. 2014), salt stress (Christou et al. 2013), and heavy-metal stress (Chen et al. 2013). Interestingly, H_2S could obviously promote accumulation of aspartic acid, glutamate and arginine in wheat seeds under Cu stress (Zhang et al. 2008), which were involved in nitrogen metabolism and may influenced by the activities of nitrogen assimilation enzymes. However, the integrated molecular mechanisms of H2S responses in plants remain to be further elucidated.

In this work, the relationship between DES/H_2S and OsSE5 in the modulation of NH_4^+ stress tolerance in rice seedlings was investigated. Our results showed that total activity of $_L$-DES was induced by NH_4^+ in rice seedling roots. NH_4^+-induced toxic symptoms were alleviated by the application of sodium hydrosulfide (NaHS, a well-know H_2S donor, whereas aggravated by the hypotaurine (HT, a scavenger of H_2S; Ortega et al. 2008) or $_{DL}$-propargylglycine (PAG, an inhibitor of $_L$-DES; Lisjak et

al. 2013). The protective effect of H_2S is associated with the improved ammonia assimilation and thus altered amino acid profiles. Our results further showed that compared with that of wild-type, $_L$-DES activity was significant increased in OsSE5-overexpression line (35S:OsSE5-1), while OsSE5-knockdown mutant exhibited lower $_L$-DES activity upon NH_4^+ stress. Importantly, NH_4^+-tolerant or sensitive phenotypes of 35S:OsSE5-1 or OsSE5-RNAi-1 line was blocked or rescued by the application of HT/PAG or NaHS, respectively, in parallel with the enhancement or impairment of nitrogen assimilation. Therefore, this work indicated that there exist a link between H_2S and OsSE5 responsible for the enhancement of NH_4^+ stress tolerance, and providing a hint for the role of the nitrogen assimilation.

MATERIALS AND METHODS

Plant materials, growth conditions

Rice (Oryza sativa L., Wuyunjing 7) was kindly provided by Jiangsu Academy of Agricultural Sciences, Jiangsu Province, China. The OsSE5 overexpression lines (35S:OsSE5-1 and 35S:OsSE5-2) and OsSE5-RNAi transgenic lines (OsSE5-RNAi-1) were previously generated (Xu et al. 2012b). OsSE5 overexpression lines were selected on solid 1/2 MS media supplemented with 30 mg/L hygromycin. Two independent lines of T2 plants (35S:SE5-1/2) were used for further analysis.

Wild-type, OsSE5 overexpression and transgenic seeds were surface-sterilized with 5% NaClO for 20 min, washed extensively with distilled water and then germinated in distilled water at 28 ^{o}C for 2 d . Germinated seeds were transferred into a growth chamber with 16/8 h (28/25 ^{o}C) day/night regimes at 150 μmol $m^{-2}s^{-1}$ irradiation and cultivated with half-strength ammonium-free Murashige and Skoog (MS) liquid medium for 14 d (nitrogen was supplied in form of $NaNO_3$, pH 5.8; Wong et al. 2004). The seedlings were then transferred into the half-strength ammonium-free MS solution with or without NaHS (concentrations shown in each figure legend; a H_2S donor), HT (2 mM; a scavenger of H_2S; Ortega et al. 2008) or PAG (2 mM; an inhibitor of $_{L}$-DES; Lisjak et al. 2013) for 6 h, and exposed with or without NH_4Cl (10 mM) for indicated times. Sample without chemical treatments was used as the control. The pH for both nutrient medium and treatment solutions was adjusted to 5.8 by using NaOH or HCl. Under our experimental conditions, the

pH of nutrient solution decreased to 4.37 after 24 h of NH_4^+ treatment, and turned into 4.03 after 7 d of NH_4^+ treatment. This result might be due to the deprotonation of ammonium during nitrogen assimilation process. After various treatments, the seedlings were sampled, then used immediately or frozen in liquid nitrogen, and stored at -80^{o}C for further analysis.

Phenotype analysis

For ammonium tolerance assay, 14-day-old rice seedlings of each genotype were transferred to 1/2 MS medium with or without indicated concentrations of NH4Cl in the presence or absence of various chemical pretreatments for the indicated times, respectively. After various treatments as indicated, corresponding phenotypes of rice, including root elongation and dry weight were determined at the indicated time points and corresponding photographs were taken. Meanwhile, different samples were immediately frozen in liquid nitrogen and stored an -80 ^{o}C until further analysis.

Determination of ammonium content

Ammonium was quantified by phenol–hypochlorite method (Weatherburn 1967). The reaction was performed with 0.5 ml of the extract, in addition to 3ml of reagent A (containing 1% phenol and 0.005% sodium nitroprusside in 100 ml of water) and 3ml of reagent B (containing 0.5% NaOH and 0.042% NaClO in 100 ml of water). The sample tubes were incubated at 37 ^{o}C for 20 min, and the

absorbance was read at 625 nm. A standard curve of ammonium was obtained by 4 different concentrations of ammonium solutions (2, 5, 10, 20, and 30mM).

Determination of activity of L-DES

Total L-DES activity was determined according to previous method with some modifications (Xie et al. 2013). Soluble proteins were extracted by adding 1 ml of 20mM Tris-HCl (pH 8.0) to 0.2 g of samples. Centrifuged at 12,000 × g for 15 min, the protein content of the supernatant was adjusted to 100 μg ml^{-1} to obtain an equal amount of protein in each assay sample. Total L-DES activity was determined by the release of H_2S from L-cysteine in the presence of dithiothreitol (DTT). The assay contained in a total volume of 1 ml: 0.8 mM L-cysteine, 2.5 mM DTT, 100 mM Tris-HCl (pH 9.0) and 10 μg protein solution. The reaction was intiated by the addition of L-cysteine. After incubated for 15 min at 37 ^oC, the reaction was terminated by adding 100 μl of 30 mM $FeCl_3$ dissolved in 1.2 N HCl and 100 μl of 20 mM N,N-dimethyl-p-phenylenediamine dihydrochloride dissolved in 7.2 N HCl. The formation of methylene blue was determined at 670 nm by a spectrophotometer. Blanks were prepared by the same procedures and known concentrations of Na_2S were used in a standard curve, protein was determined by the method of Bradford (Bradford 1976).

Determination of malondialdehyde (MDA) content

The lipid peroxidation level was determined in terms of malondialdehyde (MDA) content by the thiobarbituric acid (TBA) reaction as previously described (Xie et al. 2012). About 500 mg fresh tissue was ground in 0.2% 2-thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA) using a mortar and pestle. After heating at 95 ^{o}C for 30 min, the mixture was quickly cooled in an ice bath and centrifuged at 10,000 × g for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The blank was 0.25% TBA in 10% TCA. The concentration of lipid peroxides together with oxidatively-modified proteins of plants was thus quantified in terms of MDA level using an extinction coefficient of 155 mM^{-1} cm and expressed as nmol g^{-1} fresh weight.

Determination of the activities of GS, NADH-GOGAT and NADH-GDH

GS activity was measured according to O'Neal and Joy (1973) with some modifications. The synthetase activity of GS in extracts was determined in a reaction mixture containing Tris-HCl buffer. After the mixture was incubated at 37 ^{o}C for 30 min, the reaction was terminated by adding an acidic $FeCl_3$ solution (370 mM $FeCl_3$, 600 mM HCl, 200 mM trichloroacetic acid). Production of γ-glutamyl hydroxamate was measure with a spectrophotometer at 540 nm. One unit of GS activity was the enzyme catalyzing the formation of 1 μmol γ-glutamyl hydroxamate min^{-1} at 37 ^{o}C.

GOGAT was assayed by the method of Srivastava and Ormrod (1984). The assay mixture contained 0.4 ml 20 mM L-glutamine, 0.5 ml 20 mM 2-oxoglutarate, 0.1 ml 10 mM KCl, 0.2 ml 3 mM NADH and 0.3 ml of the enzyme extract in a final volume of 3 ml, made up with 25 mM Tris-HCl buffer (pH7.6). The reaction was started by adding L-glutamine immediately following the enzyme preparation. The decrease in absorbance was recorded for 3 min at 340 nm. One unit of enzyme activity is defined as a decrease of 1 OD_{340} per min.

GDH activity was measured according to Glevarec et al. (2004) with some modifications. The composition of the reaction mixtures were: 115 mM Tris-HCl buffer (pH8.0), 266 mM $(NH_4)_2SO_4$, 23 mM a-ketoglutarate, 30 mM $CaCl_2$, 6 mM NADH and 0.1 ml of the enzyme extract in a final volume of 3 ml. The assays were performed at 30 ^oC, the decrease in absorbance was recorded for 3 min at 340 nm. One unit of enzyme activity is defined as 1 nmol of NADH oxidised per minute.

Determination of Kjeldahl nitrogen and nitrate nitrogen

Kjeldahl nitrogen was measured by the method of Wada et al. (2015). Total kjeldahl nitrogen was determined by using a micro Kjeldahl procedure with sulphuric acid, digestion catalyst and conversion of organic nitrogen into ammonium form according to the Total Kjeldahl nitrogen method (2300 Kjeltec Analyzer Unit, Foss Tecator AB, Sweden). Nitrate nitrogen content was measured according to Patterson et al. (2010). For tissue analysis, 100 mg of fresh root tissue

was frozen in liquid nitrogen, pulverized, and added to 1 ml of deionized water. The suspension was incubated at 45 ^{o}C for 1 h and then centrifuged at 5000 × g for 15 min. The supernatant was utilized for nitrate quantization.

Measurement of free amino acids

For amino acid measurement, samples were prepared in Ultrasonic Cell Disruptor with 10 mmol/L HCl for 1.5 h, and free amino acids in roots were analyzed by a Hitachi L-8900 amino acid analyzer (Hitachi Ltd., Tokyo, Japan).

Statistical analysis

Data are means ± SE from three independent experiments with three replicated measurements. For statistical analysis, Duncan's multiple range test ($P<0.05$) or the t-test ($P<0.05$) was chosen.

Results

The Ammonium content and L-DES activity are induced by NH_4Cl

Exposure of plants to ammonium stress often causes root growth inhibition. To assess the toxicity of rice seedling upon NH_4^+ stress, root elongation and dry weight were determined after exposing seedlings to different NH_4^+ concentrations for 7 d. Our results showed that compared with the seedlings grown in nitrate-only medium (control, nitrogen was supplied in form of $NaNO_3$), NH_4^+ treatment led to significant shoot and root growth inhibition (Fig. 1a). Moreover, seedlings root elongation and dry weight was inhibited in a dose-dependent manner by increasing NH_4^+ concentrations (2.5-20 mM; Fig. 1b and c).

In order to investigate whether H_2S is involved in above-mentioned processes triggered by NH_4^+ exposure, changes of ammonium content and total activity of H_2S synthetic enzyme L-DES were further measured in rice seedling roots. As expected, levels of ammonium content and total L-DES activity were increased in a dose-dependent manner after NH_4Cl treatment ranging from 2.5 to 20 mM (Fig. 1d and e). For instance, compared with the control samples, a treatment of 10 mM NH_4^+ for 24 h increased ammonium content or total L-DES activity by 59 or 57%, respectively. Therefore, we used NH_4Cl at the concentration of 10 mM in the following study.

The time-course analysis of ammonium content and total L-DES activity upon NH_4Cl treatment were measured. As shown in Fig.1f, compared with the control samples, the ammonium content in rice roots was increased gradually over the whole

duration after the application of NH_4Cl. Total $_L$-DES activity was peaked at 24 h and remained higher levels within 72 h of NH_4Cl treatment (Fig. 1g). These results indicated a possible interrelationship between the inhibition of root growth and $_L$-DES-related H_2S production upon NH_4^+exposure.

NH_4Cl-triggerd toxic symptoms are mitigated by H_2S donor, whereas aggravated by H_2S scavenger/biosynthesis inhibitor

To verify the protective role of H_2S in rice plants upon NH_4^+ stress, sodium hydrosulfide (NaHS), a well-known H_2S donor, was used in the following experiment. It could be observed that compared with NH_4^+-stressed sample, pretreatment of NaHS with concentration ranging from 10-200 μM progressively alleviated the NH_4^+-induced lipid peroxidation, with a maximal response at 100 μM (Fig. 2a). By contrast, pretreatment with high NaHS level (1000 μM) led to a negative response.

Consequently, NaHS at the concentration of 100 μM was applied to investigate the protective role of H_2S in the following experiments. Three parameters, including the ammonium content, root dry weight and elongation were measured, respectively. As shown in Fig. 2b, time-course experiment revealed that the NH_4^+Cl-triggered induction of endogenous ammonium content was dramatically reduced by the pretreatment of NaHS. Meanwhile, our results confirmed that pretreatment with NaHS could significantly alleviate the NH_4^+-toxic symptoms in terms of root biomass and growth inhibition (Fig. 2c and d). For example, compared with those seedlings treated with NH_4^+ alone, the root growth inhibition was markedly alleviated by NaHS

pretreatment by 90%. Regarding to antioxidant enzymes, activities of SOD, APX and CAT were detected. upon NH_4Cl exposure, the total activities of SOD, APX and CAT were reduced respectively, which showed similar tendency as our previous results (Xie et al., 2015). Pretreatment of NaHS followed by NH_4Cl treatment showed alleviation in the decreases of total activities of SOD, APX and CAT (Supporting Information Fig. S1). These results supported the protective effect of H_2S in the process of the alleviation of NH_4^+ toxicity.

Pharmacological investigation by using hypotaurine (HT, a H_2S scavenger, Ortega et al. 2008) or $_{DL}$-propargylglycine (PAG, a $_{L}$-DES inhibitor，Lisjak et al. 2013) was also conducted. With respect to the alleviation of NH_4^+-triggered toxicity induced by NaHS (Fig. 2a), pretreated with HT or PAG could further aggravate the NH_4^+-induced toxicity symptoms. For instance, pretreatment with HT or PAG significantly increased NH_4^+-induced ammonium accumulation (Fig. 2e) and lipid peroxidation as evaluated by MDA content in rice seedling root (Fig. 2f). These results suggested that $_{L}$-DES-related endogenous H_2S homeostasis conferred the protection against NH_4^+-induced toxicity effect in rice roots.

H2S increases ammonia incorporation into aminio acids

It is now well established that GS/GOGAT cycle is the major route for NH_4^+ assimilation in plants. This pathway is able to ameliorate the toxic effect of excess ammonium (Tabuchi et al. 2007; Lea & Miflin 2003, 2011). Thus, the effect of NaHS on GS and NADH-GOGAT were determined in rice seedling roots. While the

maximal extractable GS and NADH-GOGAT activities were significantly increased compared to controls after 24 h of NH_4^+ treatment rice seedling roots, NaHS-pretreatment of the seedling roots led to a much greater increase in the activities of these enzymes, with for example NADH-GOGAT being 56% higher (Fig. 3a and b). NADH-GDH, another important nitrogen metabolism enzyme (Lea 1999) also displayed similar responses (Fig. 3c).

The NH_4^+ treatment also induced total nitrogen (Fig. 3d) and amino acid contents (Fig. 3e). These parameters were further increased as a result of NaHS pretreatment. Increases in Glu and Asp accumulated accompanied the increases in nitrogen assimilation enzymes, indicating that more ammonia was incorporated into these and other amino acids. Taken together, above results suggested that NaHS accelerate ammonium assimilation into primary amino acid in rice roots.

L-DES activity and nitrogen assimilation are regulated by OsSE5 in response to excess ammonium

Our previous study illustrated that rice heme oxygenase OsSE5 is involved in the improvement of plant tolerance to excess ammonium in both rice and Arabidopsis (Xie et al. 2015). Two independent OsSE5 overexpression lines of T2 plants (35S:OsSE5-1, 35S:OsSE5-2) were generated and validated by hygromycin selection and RT-PCR. Levels of OsSE5 was increased obviously in 35S:OsSE5-1 and 35S:OsSE5-2 roots, being 6.5 and 5.4 times higher than that of wild-type. (Supporting Information Fig. S2). We further observed that NH_4^+-triggered toxic symptoms was

329 significantly alleviated in 35S:OsSE5-1 and 35S:OsSE5-2 plants, further reinforcing
330 the proposition that OsSE5 could regulate rice tolerance to excess ammonium
331 (Supporting Information Fig. S3). Thus, the rice transgenic lines with overexpression
332 of OsSE5 (35S:OsSE5-1) or knockdown of OsSE5 (OsSE5-RNAi-1; Xu et al. 2012b)
333 were used to investigate the biological function of H_2S in rice upon NH_4^+ stress. As
334 expected, compared with wild-type, NH_4^+-induced inhibition of root growth was
335 significantly alleviated in 35S:OsSE5-1 plants, whilst aggravating in OsSE5-RNAi-1
336 plants, in terms of root dry weight, root elongation and MDA content (Fig. 4a and
337 Supporting Information Fig. S4). Subsequently, the time-course determination of
338 ammonium content and total $_L$-DES activities were measured for each genotypes upon
339 NH_4^+ stress, respectively. Ammonium content was increased gradually after the
340 application of NH_4Cl treatment in wild-type roots whereas significantly weakened or
341 strengthened in 35S:OsSE5-1 or OsSE5-RNAi-1 plants (Fig. 4b). Most importantly, as
342 shown in Fig. 4c, compared with wild-type, total activity of $_L$-DES was significantly
343 higher in NH_4^+-treated 35S:OsSE5-1 plants, whereas much lower in OsSE5-RNAi-1
344 plants. Interestingly, similar responses were also observed under control conditions,
345 indicating that overexpression or knockdown of OsSE5 could up- or down-regulated
346 $_L$-DES activities. These results indicated that OsSE5-regulated $_L$-DES activity might
347 be involved in the alleviation of NH_4^+-triggered toxic symptoms,

348 In order to assess whether nitrogen assimilation was influenced by OsSE5 when
349 rice plants were exposed to excess ammonium, the changes enzymatic activities
350 involved in primary ammonia assimilation were measured, respectively. Compared

with that of wild-type,

Maximal extractable GS, NADH-GOGAT and NADH-GDH activities were significantly increased in 35S:OsSE5-1 plants compared to the wild type following exposure to NH_4^+ stress (Fig. 5). In contrast, OsSE5-knockdown mutants exhibited much lower GS and NADH-GOGAT activities following NH_4^+ treatment (Fig. 5). Ammonium triggered a significant increase in the tissue nitrogen contents of the 35S:OsSE5-1 plants, as well as an increase in the abundance of amino acids, particularly Glu and Asp (Fig. 6). These results were not observed in the OsSE5-RNAi-1 plants suggesting that OsSE5 is important in ammonium-dependent activation of nitrogen assimilation in rice seedling roots (Fig.6)

NH_4^+-tolerant or sensitive phenotypes of the 35S:OsSE5-1 or OsSE5-RNAi-1 lines are blocked or rescued by the application of H_2S scavenger/biosynthesis inhibitor or donor

To further assess the functional link between OsSE5-regulated ammonium tolerance and H_2S homeostasis upon NH_4Cl stress in rice, we adopted a pharmacological investigation by using NaHS, HT or PAG, which could resulted in the alternation of endogenous H_2S homeostasis, separately. As expected, the pretreatment of HT or PAG could fully block the NH_4^+-tolerant phenotype of 35S:OsSE5-1 plants. NH_4^+-triggered inhibition of root growth were significantly aggravated by pretreamtent of HT or PAG in 35S:OsSE5-1 plants (Fig. 7a, b). Contrasting results were observed in OsSE5-RNAi-1 plants, showing that the pretreatment with NaHS could significantly

rescue the NH_4^+-sensitive symptoms of OsSE5-RNAi-1 plants. For example, the application of exogenous NaHS resulted in the increase of root elongation by 137% compared with stressed alone OsSE5-RNAi-1 plants.

Subsequently, ammonium and MDA contents were measured to evaluate the effects of H_2S production on OsSE5-regulated ammonium tolerance in each genotype. As shown, pretreated with HT or PAG brought a slight but significant increased in NH_4^+-induced accumulation of ammonium in 35S:OsSE5-1 plants (Fig. 7c). Interestingly, those pretreatments exacerbated the NH_4^+-triggered lipid peroxidation (Fig. 7d). On the other side, NH_4^+-induced ammonium accumulation was significantly reduced by NaHS in OsSE5-RNAi-1 plants as well as MDA content. Taken together, above results indicated that there exist a link between $_L$-DES-associated H_2S production and the OsSE5-mediated ammonium tolerance in rice upon NH_4^+ stress.

$_L$-DES-associated H_2S production in response to altered OsSE5 function

Maximal extractable GS, NADH-GOGAT, and NADH-GDH activities were determined 24 h that alter H_2S production (Fig. 8). NH_4^+-induced increases in GS, NADH-GOGAT, and NADH-GDH were prevented by treatment with either HT or PAG in 35S:OsSE5-1 plants. For example, pretreatment with either HT or PAG resulted in decreases of in NADH-GOGAT activities of up to 68%. In contrast, pretreatment with NaHS significantly increased the activities of all the nitrogen metabolism enzymes measured in OsSE5-RNAi-1 plants, particularly NADH-GOGAT. These findings suggest that the positive effect of OsSE5 in nitrogen assimilation is

regulated by $_{L}$-DES-associated H_2S production. Meanwhile, the enzymatic activities of SOD, APX and CAT exhibited approximately similar tendencies (Supporting Information Fig. S5).

DISCUSSION

It is well-known that high concentrations of NH_4^+ can cause serious root growth inhibition as well as other severe negative effects (Britto & Kronzucker 2002; Li et al. 2014; Esteban et al. 2016). H_2S, similar to nitric oxide (NO) and carbon monoxide (CO), functions as a gaseous signaling molecule in plant growth, development and multiple physiological processes (Guo et al. 2016; Zhang et al. 2010b; Scuffi et al. 2014; Christou et al. 2013; Zhang et al. 2008). However, whether H_2S can regulate plant NH_4^+ tolerance and its related molecular mechanism is not still unknown.

In the present study, we demonstrated that H_2S could enhance plant tolerance against NH_4^+ stress in rice. NH_4^+ exposure elicited approximately dose-dependent increase in ammonium accumulation as well as total activity of $_L$-DES in rice roots, a key enzyme in H_2S biosynthesis in plants (Fig. 1d and e; Álvarez et al. 2010). Subsequent time course results revealed that NH_4Cl exposure triggered a rapid increase of $_L$-DES activity at 24 h and then remained higher levels within 72 h of NH_4Cl treatment (Fig. 1g). Meanwhile, it was found that above endogenous $_L$-DES induction apparently preceded the inhibition of root elongation and dry weight upon NH_4^+ stress (Fig. 1a-c). Consistent with our results, it has also been reported that the total enzymatic activity of $_L$-DES was induced by salicylic acid (Li et al. 2015), drought (Ziogas et al. 2015), abscisic acid (Shi et al. 2015). Subsequently, the experiments investigated the beneficial effects of H_2S by using NaHS, which is a well-known H_2S donor (Lisjak et al. 2013), could mimic NH_4^+-triggered changes of endogenous H_2S homeostasis. Our study illustrated that NaHS could not only

421 decreased ammonium accumulation, but also significantly alleviate the NH_4^+-toxic
422 symptoms in terms of root growth inhibition (Fig. 2b-d). The changes of MDA
423 content were also in parallel with this notion (Fig. 2a). Such positive effect of NaHS
424 was also observed in barley, Arabidopsis and Medicago sativa under aluminum and
425 salt stress (Chen et al. 2013; Li et al. 2014; Lai et al. 2014). Meanwhile, we noticed
426 that pretreatment with HT, a scavenger of H_2S (Ortega et al. 2008), or PAG, an
427 efficient inhibitor of $_L$-DES (Lisjak et al. 2013), could aggravate NH_4^+-triggered
428 ammonium accumulation and MDA content (Fig. 2e, f). Taken together, above results
429 suggested that $_L$-DES-related endogenous H_2S homeostasis conferred the protection
430 against NH_4^+-induced toxicity effect in rice roots, which had been reported in maize
431 and Arabidopsis upon heat or salt stress (Li et al. 2013; Shi et al. 2015). Overall, these
432 work showed that H_2S could act as an indispensable endogenous modulator for plant
433 tolerance to multiple stresses.

434 In plants, it is well-established that GS/GOGAT-GDH cycle is the main way
435 for ammonium assimilation (Tabuchi et al. 2007; Lea & Miflin 2003, 2011). Here,
436 we found that H_2S was involved in ammonium assimilation. H_2S could
437 significantly strengthen the NH_4Cl-induced activities of GS, NADH-GOGAT and
438 NADH-GDH (Fig. 3a-c). Several studies had showed that plant species with higher
439 GS activities can achieve an elevated tolerance to excess NH_4^+ (Glevarec et al.
440 2004; Cruz et al. 2006; Fei et al. 2006). Cytosol GS1 and NADH-GOGAT have
441 been proposed to play the crucial role in ameliorating the toxic effect of excess
442 ammonium (Peterman & Goodman 1991; Ishiyama et al. 1998).Application of

443 inhibitor of GS, not only inhibited root growth, but also caused ammonium
444 accumulation in rice (Hirano et al. 2008). Accordingly, results from contents of
445 nitrogen and amino acids revealed that excess ammonia was incorporated into
446 amino acids (Fig. 3d and e). It was observed that H_2S can promote the
447 accumulation of free amino acids in wheat and Arabidopsis, including Asp,
448 glutamic acid and arginine, which were involved in nitrogen metabolism and may
449 influence GS/GOGAT cycle indirectly (Zhang et al. 2008; Shi et al. 2015).
450 Therefore, the protective effect of H_2S might be ascribed to the ability of H_2S to
451 facilitate ammonium assimilation.

452 Ample evidence has confirmed that the HO plays a crucial role in plant response
453 to multiple stresses, including heavy metal-induced oxidative damage (Noriega et al.
454 2004), drought (Liu et al. 2010), and salinity stress (Xie et al. 2011a; 2011b). In rice,
455 OsSE5 encoded a putative HO with high similarity to Arabidopsis HY1/HO1 (Xu et al.
456 2012b). The loss of OsSE5 function in RNAi transgenic plants increased sensitivity to
457 NH_4^+ stress with impaired antioxidant defence (Xie et al. 2015). This work extended
458 our previous observation. We found that overexpression of OsSE5 in rice resulted in
459 its NH_4^+-tolerant characteristics in terms of the alleviation of NH_4^+-triggered
460 inhibition of root growth, ammonium and MDA accumulation (Fig. 4a and 4b;
461 Supporting Information Fig. S3 and S4). Interestingly, further results showed that
462 NH_4^+-induced total $_L$-DES activity was significantly increased in 35S:OsSE5-1 plants,
463 whilst obvious decreased in OsSE5-RNAi-1 plants compared with that of wild-type
464 upon NH_4^+Cl stress (Fig. 4c). These results indicated that $_L$-DES activities is regulated

by OsSE5 and might be related to the OsSE5-regulated rice ammonium tolerance. Especially, a recent paper showed that HO functions as a downstream component in H_2S-induced adventitious root formation by the modulation of expression of DNAJ-1 and CDPK1/5 genes (Lin et al. 2012). Therefore, it is possible that the H_2S and HO might be on a linear signalling cascade in the process of plant adaptive responses against abiotic stresses. Moreover, our results further showed that NH_4^+-induced enzymatic activates involved in ammonium assimilation were significantly enhanced in 35S:OsSE5-1 plants, whereas were not obvious induced in OsSE5-RNAi-1 plants than in wild-type (Fig. 5). Together with the results from nitrogen content as well as the abundance of free amino acids, our results illustrated that OsSE5 could facilitate ammonium assimilation upon excess NH_4^+ in rice seedling roots, supporting the conclusion that OsSE5 acts as an essential positive regulator in adaptive signalling to NH_4^+ toxicity. In accordance with our results, up-regulation of HO under salt stress protected nitrogen metabolism in nodules of soybean by the modulation of GS and NADH-GOGAT (Zilli et al. 2008).

This study also provided evidence showing that nitrogen assimilation was modulated in OsSE5-trangenic plants, which was concomitant with the alternation of $_L$-DES activity, as well as the alleviation of NH_4^+-triggered toxic symptoms. Exogenously application of HT or PAG was able to aggravate the NH_4Cl-toxic symptoms, including the inhibition of root fresh weight and elongation in 35S:OsSE5-1 plants. By contrast, NH_4^+-triggered hypersensitivity phenotypes was significantly rescued by the addition of NaHS in OsSE5-RNAi-1 plants (Fig. 7a and

b). Consistently, a significant increase in NH_4^+-induced accumulation of ammonium or overproduction of MDA was observed by HT- or PAG-treated 35S:OsSE5-1 plants, whereas treatment of NaHS significantly decreased ammonium content or MDA content in OsSE5-RNAi-1 plants (Fig. 7c and d). HT or PAG pretreatment fully blocked the induction of the activities of involved in nitrogen assimilation, leading to a markedly decrease of total nitrogen content in 35S:OsSE5-1 plants, and vice versa in OsSE5-RNAi-1 plants (Fig. 8). These results provided a powerful hint for the role of ammonium assimilation in the OsSE5/H_2S-enhanced NH_4^+ stress tolerance. It has been reported that the carbon flux through the partial TCA and the anaplerotic pathway were increased upon such stressful conditions (Rollins et al. 2013). There might be an accompanying switch of carbon metabolism away from carbohydrate synthesis towards amino acid synthesis. Together with the activation of nitrogen assimilation, this carbon redirection could provide necessary carbon skeletons for channeling excess ammonia efficiently into essential metabolic processes and defence compounds. Considering that HO transcripts and its protein levels were significantly induced by H_2S in cucumber and wheat (Lin et al. 2012; Xie et al. 2014), future work should combine proteomic and metabolomic approaches to investigate the systematic molecular networks of OsSE5/$_L$-DES-modulated plant ammonium tolerance.

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REFERENCES

Álvarez C., Calo L., Romero L.C., García I. & Gotor C. (2010) An Oacetylserine(thiol)lyase homolog with L-cysteine desulfhydrase activity regulates cysteine homeostasis in Arabidopsis. Plant Physiology **152,** 656-669.

Balestrasse K.B. Benavides M.P., Gallego S.M. & Tomaro M.L. (2003) Effect of cadmium stress on nitrogen metabolism in nodules and roots of soybean plants. Functional Plant Biology **30,** 57-64.

Bialczyk J., Lechowski Z., Dziga D. & Molenda K. (2005) Carbohydrate and free amino acid contents in tomato plants grown in media with bicarbonate and nitrate or ammonium. Acta Physiologiae Plantarum **27,** 523-529.

Bradford M.M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry **72,** 248-254.

Britto D.T. & Kronzucker H.J. (2002) NH_4^+ toxicity in higher plants:a critical review. Journal of Plant Physiology **159,** 567-584.

Cammaerts D. **&** Jacobs M. (1985) A study of the role of glutamate dehydrogenase in the nitrogen metabolism of Arabidopsis thaliana. Planta **163,** 517-526.

Chen J., Wang W.H., Wu F.H., You C.Y., Liu T.W., Dong X.J., He J.X. & Zheng H.L. (2013) Hydrogen sulfide alleviates aluminum toxicity in barley seedlings. Plant and Soil **362,** 301-318.

Christou A., Manganaris G.A., Papadopoulos I. & Fotopouls V. (2013) Hydrogen sulfide induces systemic tolerance to salinity and nonionic osmotic stress in

535 strawberry plants through modification of reactive species biosynthesis and
536 transcriptional regulation of multiple defense pathways. Journal of Experimental
537 Botany **64,** 1953-1966.
538 Cruz C., Bio A.F., Domínguez-Valdivia M.D., Aparicio-Tejo P.M., Lamsfus C. &
539 Martins-Loução M.A. (2006) How does glutamine synthetase activity determine
540 plant tolerance to ammonium? Planta **223,** 1068-1080.
541 Dubois F., Terce´-Laforgue T., Gonzalez-Moro M.B., Estavillo M.B., Sangwan R.,
542 Gallais A. & Hirel B. (2003) Glutamate dehydrogenase in plants: is there a new
543 story for an old enzyme? Plant Physiology & Biochemistry **41,** 565-576.
544 Esteban R., Ariz I., Cruz C. & Moran J.F. (2016) Review: Mechanisms of ammonium
545 toxicity and the quest for tolerance. Plant Science **248,** 92-101.
546 Fei H., Chaillou S., Hirel B., Polowick P., Mahon J.D. & Vessey J.K. (2006) Effects
547 of the overexpression of a soybean cytosolic glutamine synthetase gene (GS15)
548 linked to organ-specific promoters on growth and nitrogen accumulation of pea
549 plants supplied with ammonium. Plant Physiology & Biochemistry **44,** 543-550.
550 Forde B.G. & Lea P.J. (2007) Glutamate in plants: metabolism, regulation, and
551 signalling. Journal of Experimental Botany **58,** 2339-2358.
552 Fontaine J.X., Tercé-Laforgue T., Armengaud P., Clément G., Renou J.P., Pelletier
553 S., Catterou M., Azzopardi M., Gibon Y., Lea P.J., Hirel B. & Dubois F. (2012)
554 Characterization of a NADH-Dependent Glutamate Dehydrogenase Mutant of
555 Arabidopsis Demonstrates the Key Role of this Enzyme in Root Carbon and
556 Nitrogen Metabolism. The Plant Cell **24,** 4044-4065.

557 Glevarec G., Bouton S., Jaspard E., Riou M.T., Cliquet J.B., Suzuki A. & Limami
558 A.M. (2004) Respective roles of glutamine synthetase/ glutamate synthase cycle
559 and glutamate dhydrogenase in ammonium and amino acid metabolism during
560 germination and post-germinative growth in the model legume Medicago
561 truncatula. Planta **219,** 286-297.
562 Guo H., Xiao T., Zhou H., Xie Y. & Shen W. (2016) Hydrogen sulfide: a versatile
563 regulator of environmental stress in plants. Acta Physiologiae Plantarum **38,** 1-13.
564 Hirano T., Satoh Y., Ohki A., Takada R., Arai T. & Michiyama H. (2008) Inhibition
565 of ammonium assimilation restores elongation of seminal rice roots repressed by
566 high levels of exogenous ammonium. Physiologia Plantarum **134,** 183-190.
567 Ishiyama K., Hayakawa T. & Yamaya T. (1998) Expression of NADH dependent
568 glutamate synthase protein in the epidermis and exodermis of rice roots in response
569 to the supply of ammonium ions. Planta **204,** 288-294.
570 Ishiyama K., Inoue E., Tabuchi M., Yamaya T. Takahashi H. (2004) Biochemical
571 Background and Compartmentalized Functions of Cytosolic Glutamine Synthetase
572 for Active Ammonium Assimilation in Rice Roots. Plant & Cell Physiology **45,**
573 1640-1647.
574 Izawa T, Oikawa T, Tokutomi S, Okuno K, Shimamoto K (2000) Phytochromes
575 confer the photoperiodic control of flowering in rice (a short-day plant). The Plant
576 Journal **22,** 391-399.
577 Labboun S., Tercé-Laforgue T., Roscher A., Bedu M., Restivo F.M., Velanis C.N.,
578 Skopelitis D.S., Moschou P.N., RoubelakisAngelakis K.A., Suzuki A. & Hirel B.

(2009) Resolving the role of plant glutamate dehydrogenase. I. In vivo real time nuclear magnetic resonance spectroscopy experiments. Plant & Cell Physiology **50,** 1761–1773.

Lai D., Mao Y., Zhou H., Li F., Wu M., Zhang J., He Z., Cui W. & Xie Y. (2014) Endogenous hydrogen sulfide enhances salt tolerance by coupling the reestablishment of redox homeostasis and preventing salt-induced K^+ loss in seedlings of Medicago sativa. Plant Science **225,** 117-129.

Lea P.J. & Miflin B.J. (2003) Glutamate synthase and the synthesis of glutamate in plants. Plant Physiology & Biochemistry **41,** 555-564.

Lea P.J. & Miflin B.J. (2011) Nitrogen assimilation and its relevance to crop improvement. In Nitrogen Metabolism in Plants in the Post-Genomic Era, C.H. Foyer and H. Zhang, eds (Chichester, UK:Wiley-Blackwell) pp. 1–40.

Li Z.G., Yang S.Z., Long W.B., Yang G.X. & Shen Z.Z. (2013) Hydrogen sulphide may be a novel downstream signal molecule in nitric oxide-induced heat tolerance of maize (Zea mays L.) seedlings. Plant, Cell & Environment **36,** 1564-1572.

Li B., Li G., Kronzucker H.J., Baluška F. & Shi W. (2014) Ammonium stress in Arabidopsis: signaling, genetic loci, and physiological targets. Trends in plant science **19,** 107-114.

Li J., Jia H., Wang J., Cao Q. & Wen Z. (2014) Hydrogen sulfide is involved in maintaining ion homeostasis via regulating plasma membrane Na^+/H^+ antiporter system in the hydrogen peroxidedependent manner in salt-stress Arabidopsis thaliana root. Protoplasma **251,** 899-912.

601 Li Z.G., Xie L.R. & Li X.J. (2015) Hydrogen sulfide acts as a downstream signal
602 molecule in salicylic acid-induced heat tolerance in maize (Zea mays L.) seedlings.
603 Journal of Plant Physiology **177,** 121-127.
604 Lin Y.T., Li M.Y., Cui W.T., Lu W. & Shen W.B. (2012) Haem Oxygenase-1 is
605 Involved in Hydrogen Sulfide-induced Cucumber Adventitious Root Formation.
606 Journal of Plant Growth Regulation **31,** 519-528.
607 Ling T., Zhang B., Cui W., Wu M., Lin J., Zhou W., Huang J. & Shen W. (2009)
608 Carbon monoxide mitigates salt-induced inhibition of root growth and suppresses
609 programmed cell death in wheat primary roots by inhibiting superoxide anion
610 overproduction. Plant Science **177,** 331-340.
611 Liu Y., Xu S., Ling T., Xu L. & Shen W. (2010) Heme oxygenase/carbon monoxide
612 system participates in regulating wheat seed germination under osmotic stress
613 involving the nitric oxide pathway. Journal of Plant Physiology **167,** 1371-1379.
614 Lisjak M., Teklic T., Wilson I.D., Whiteman M. & Hancock J.T. (2013) Hydrogen
615 sulfide: Environmental factor or signalling molecule. Plant, Cell & Environment
616 **36,** 1607-1616.
617 Noriega G.O., Balestrasse K.B., Batlle A. & Tomaro M.L. (2004) Heme oxygenase
618 exerts a protective role against oxidative stress in soybean leaves. Biochemical and
619 Biophysical Research Communications **323,** 1003-1008.
620 O'Neal D. & Joy K.W. (1973) Glutamine synthetase of pea leaves: I. Purification,
621 stabilization, and pH optima. Archives of Biochemistry and Biophysics 159,
622 113-122.

623 Ortega J.A., Ortega J.M. & Julian D. (2008) Hypotaurine and sulfhydryl-containing
624 antioxidants reduce H_2S toxicity in erythrocytes from a marine invertebrate.
625 Journal of Experimental Biology **211,** 3816-3825.
626 Patterson K., Cakmak T., Cooper A., Lager I., Rasmusson A.G. & Escobar M.A.
627 (2010) Distinct signalling pathways and transcriptome response signatures
628 differentiate ammonium-and nitrate-supplied plants. Plant, Cell & Environment **33,**
629 1486-1501
630 Peterman T.K. & Goodman H.M. (1991) The glutamine synthetase gene family of
631 Arabidopsis thaliana: light-regulation and differential expression in leaves, roots
632 and seeds. Molecular Genetics and Genomics **230,** 145-154.
Rollins J.A., Habte E., Templer S.E., Colby T., Schmidt J. & von Korff M. (2013)
Leaf proteome alterations in the context of physiological and morphological
responses to drought and heat stress in barley (Hordeum vulgare L.). Journal of
Exiperiment Botany **64**, 3201-3212.
633 Rennenberg H. (1983) Role of O-acetylserine in hydrogen sulfide emission from
634 pumpkin leaves in response to sulfate. Plant Physiology 73, 560-565.
635 Restivo F.M. (2004) Molecular cloning of glutamate dehydrogenase genes of
636 Nicotiana plumbaginifolia: structure analysis and regulation of their expression by
637 physiological and stress conditions. Plant science **166,** 971-982.
638 Scuffi D., Álvarez C., Laspina N., Gotor C., Lamattina L. & García-Mata C. (2014)
639 Hydrogen sulfide generated by L-cysteine desulfhydrase acts upstream of nitric

oxide to modulate abscisic acid-dependent stomatal closure. Plant Physiology **166,** 2065-2076.

Shekhawat G.S. & Verma K. (2010) Haem oxygenase (HO): an overlooked enzyme of plant metabolism and defence. Journal of Experimental Botany **61,** 2255-2270.

Shi H., Ye T., Han N., Bian H., Liu X. & Chan Z (2015) Hydrogen sulfide regulates abiotic stress tolerance and biotic stress resistance in Arabidopsis. Journal of Integrative Plant Biology 57, 628-640.

Srivastava H.S. & Ormrod D.P. (1984) Effects of nitrogen dioxide and nitrate nutrition on growth and nitrate assimilation in bean leaves. Plant Physiology **76,** 418-423.

Tabuchi M., Abiko T. & Yamaya T. (2007) Assimilation of ammonium ions and reutilization of nitrogen in rice (Oryza sativa L.). Journal of Experimental Botany **58,** 2319-2327.

Tapia M.I., Alda J.A.G.O., Llama M.J. & Serra J.L. (1996) Changes in intracellular amino acids and organic acids induced by nitrogen starvation and nitrate or ammonium resupply in the cyanobacterium Phormidium laminosum. Planta **198,** 526-531.

Tercé-Laforgue T., Dubois F., Ferrario-Méry S., de Crecenzo MA., Sangwan R. & Hirel B. (2004) Glutamate dehydrogenase of tobacco is mainly induced in the cytosol of phloem companion cells when ammonia is provided either externally or released during photorespiration. Plant Physiology **136,** 4308–4317

661 Von Wirén N., Gazzarrini S., Gojon A. & Frommer W.B. (2000) The molecular
662 physiology of ammonium uptake and retrieval. Current Opinion in Plant Biology **3,**
663 254-261.
664 Wada S., Hayashida Y., Izumi M., Kurusu T., Hanamata S., Kanno K., Kojima
665 S., Yamaya T., Kuchitsu K., Makino A. & Ishida H. (2015)
666 Autophagy supports biomass production and nitrogen use efficiency at the
667 vegetative stage in rice. Plant Physiology **168,** 60-73.
668 Weatherburn M.W. (1967) Phenol-Hypochlorite Reaction for Determination of
669 Ammonia. Analytical Chemistry **39,** 971-974.
670 Wilson L.G., Bressan R.A. & Filner P. (1978) Light-dependent emission of hydrogen
671 sulfide from plants. Plant Physiology 61, 184-189.
672 Winner W.E., Smith C.L., Koch G.W., Mooney H.A., Bewley J.D. & Krouse H.R.
673 (1981) Rates of emission of H_2S from plants and patterns of stable sulfur isotope
674 fractionation. Nature 289, 672-673.
675 Wong H.K., Chan H.K., Coruzzi G.M. & Lam H.M. (2004) Correlation of ASN2 gene
676 expression with ammonium metabolism in Arabidopsis. Plant Physiology **134,**
677 332-338.
678 Xie Y., Cui W., Yuan X., Shen W. & Yang Q. (2011a) Heme oxygenase-1 is
679 associated with wheat salinity acclimation by modulating reactive oxygen species
680 homeostasis. Journal of Integrative Plant Biology **53,** 653-670.
681 Xie Y.J., Xu S., Han B., Wu M.Z., Yuan X.X., Han Y., Gu Q., Xu D.K., Yang Q. &
682 Shen W.B. (2011b) Evidence of Arabidopsis salt acclimation induced by

683 up-regulation of HY1 and the regulatory role of RbohD-derived reactive oxygen
684 species synthesis. The Plant Journal **66,** 280-292.
685 Xie Y., Xu D., Cui W. & Shen W. (2012) Mutation of Arabidopsis HY1 causes UV-C
686 hypersensitivity by impairing carotenoid and flavonoid biosynthesis and the
687 down-regulation of antioxidant defence. Journal of Experimental Botany **63,**
688 3869-3883.
689 Xie Y., La D., Mao Y., Zhang W., Shen W. & Guan R. (2013) Molecular cloning,
690 characterization, and expression analysis of a novel gene encoding L-cysteine
691 desulfhydrase from Brassica napus. Molecular Biotechnology **54,** 737-746.
692 Xie Y., Zhang C., Lai D., Sun Y., Samma M.K., Zhang J. & Shen W. (2014)
693 Hydrogen sulfide delays GA-triggered programmed cell death in wheat aleurone
694 layer by the modulation of glutathione homeostasis and heme oxygenase-1
695 expression. Journal of Plant Physiology **171,** 53-62.
696 Xie Y., Mao Y., Xu S., Zhou H., Duan X., Cui W., Zhang J. & Xu G. (2015)
697 Heme-heme oxygenase 1 system is involved in ammonium tolerance by regulating
698 antioxidant defence in Oryza sativa. Plant, Cell & Environment **38,** 129-143.
699 Xu S., Wang L., Zhang B., Han B., Xie Y., Yang J. Zhong W., Chen H., Wang
700 R., Wang N., Cui W. & Shen W. (2012b) RNAi knockdown of rice SE5 gene is
701 sensitive to the herbicide methyl viologen by the down-regulation of antioxidant
702 defense. Plant Molecular Biology **80,** 219-235.

703 Yuan L., Gu R., Xuan Y., Smith-Valle E., Loqué D., Frommer W.B. & von Wirén N.
704 (2013) Allosteric regulation of transport activity by heterotrimerization of
705 Arabidopsis ammonium transporter complexes in vivo. The Plant Cell **25,** 974-984.
706 Zhang H., Hu L.Y., Hu K.D., He Y.D., Wang S.H. & Luo J.P. (2008) Hydrogen
707 sulfide promotes wheat seed germination and alleviates oxidative damage against
708 copper stress. Journal of Integrative Plant Biology **50,** 1518-1529.
709 Zhang H., Jiao H., Jiang C.X., Wang S.H., Wei Z.J., Luo J.P. & Jones R.L. (2010a)
710 Hydrogen sulfide protects soybean seedlings against drought-induced oxidative
711 stress. Acta Physiologiae Plantarum **32,** 849-857.
712 Zhang H., Wang M.J., Hu L.Y., Wang S.H., Hu K.D., Bao L.J. & Luo J.P. (2010b)
713 Hydrogen sulfide promotes wheat seed germination under osmotic stress. Russian
714 Journal Plant Physiology **57,** 532-539.
715 Zilli C.G., Balestrasse K.B., Yannarelli G.G., Polizio A.H., Santa-Cruz D.M. &
716 Tomaro M.L. (2008) Heme oxygenase up-regulation under salt stress protects
717 nitrogen metabolism in nodules of soybean plants. Environmental and
718 Experimental Botany **64,** 83-89.
719 Ziogas V., Tanou G., Belghazi M., Filippou P., Fotopoulos V., Grigorios D. &
720 Molassiotis A. (2015) Roles of sodium hydrosulfide and sodium nitroprusside as
721 priming molecules during drought acclimation in citrus plants. Plant Molecular
722 Biology **89,** 433-450.
723

FIGURE LEGENDS

Figure 1. Morphology, root elongation root dry weight, ammonium content and total $_L$-DES activity in rice seedling roots upon NH_4Cl stress. 14-day-old rice seedlings were exposed to 1/2 MS solution containing different concentrations of NH_4Cl. Photographs were taken after 7 d of treatment (a). Bar = 1 cm. Root elongation and dry weight were recorded (b, c). Ammonium content and total $_L$-DES activity in seedling roots were determined 24 h after various treatments (d, e) or at the indicated time points (f, g; 10 mM NH_4Cl). Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Figure 2. Effects of NaHS, HT and PAG on the MDA content, root dry weight, root elongation and ammonium content in rice seedling roots upon NH_4Cl stress. 14-day-old seedlings were pretreated with or without NaHS (different concentrations or 100 μM), HT (2 mM) or PAG (2 mM) for 6 h, and then shifted to 1/2 MS solution with or without NH_4Cl (10 mM) for 7 d. Afterwards, MDA content (a,f), time-course analysis of ammonium content (b) or at the indicated time points (e), root dry weight (c) and elongation (d) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Figure 3. Effects of NaHS on the total activities of glutamine synthetase (GS), NADH-glutamate synthase (NADH-GOGAT), NADH-glutamate dehydrogenase (NADH-GDH), nitrogen content and free amino acids content in rice seedling roots upon NH_4Cl stress. 14-day-old seedlings were pretreated with or without NaHS (100 μM) for 6 h, and then shifted to 1/2 MS solution with or without NH_4Cl (10 mM) for another 24 h. Afterwards, the total activities of GS (a), NADH-GOGAT (b), NADH-GDH (c), nitrogen content (d), free amino acids (e) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test. Particularly for amino acid profiles, the letters represent the significant differences for one amino acid between 4 different treatments.

Figure 4. Effect of NH_4Cl stress on the morphology, time-courses analysis of ammonium content and total $_L$-DES activity in wild-type, 35S:OsSE5-1, OsSE5-RNAi-1 seedling roots. 14-day-old seedlings were exposed to 1/2 MS solution with or without NH_4Cl (10 mM). Photographs were taken after 7 d of treatment (a). Bar = 1 cm. Time-course analysis of ammonium content (b) and total $_L$-DES activity (c) were determined, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Figure 5. Effect of NH_4Cl stress on the activities of glutamine synthetase (GS), NADH-glutamate synthase (NADH-GOGAT), NADH-glutamate dehydrogenase (NADH-GDH) in wild-type, 35S:OsSE5-1, OsSE5-RNAi-1 seedling roots. 14-day-old seedlings were exposed to 1/2 MS solution with or without NH_4Cl (10 mM) for another 24 h. Afterwards, the total activities of GS (a), NADH-GOGAT (b), NADH-GDH (c) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Figure 6. Effects of NH_4Cl on the nitrogen content and free amino acids content in wild-type, 35S:OsSE5-1, OsSE5-RNAi-1 seedling roots. 14-day-old seedlings were shifted to 1/2 MS solution with or without NH_4Cl (10 mM) for another 24 h. Afterwards, the nitrogen content (a), free amino acids content (b) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test. Particularly for amino acid profiles, the letters represent the significant differences for one amino acid between 4 different treatments.

Figure 7. Effects of NaHS, HT and PAG on the root dry weigh, root elongation, ammonium content and MDA content in the seedling roots of wild-type, 35S:OsSE5-1, OsSE5-RNAi-1

upon NH_4Cl stress. 14-day-old seedlings were pretreated with or without NaHS (100 μM), HT (2 mM) or PAG (2 mM) for 6 h, respectively, and then shifted to 1/2 MS solution with or without NH_4Cl (10 mM) for 7 d. Afterwards, root dry weight (a), root elongation (b), ammonium content (c) and MDA content (d) were determined, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Figure 8. Effects of NaHS, HT and PAG on the activities of glutamine synthetase (GS), NADH-glutamate synthase (NADH-GOGAT), NADH-glutamate dehydrogenase (NADH-GDH) and nitrogen content in wild-type, 35S:OsSE5-1, OsSE5-RNAi-1 seedling roots upon NH_4Cl stress. 14-day-old seedlings were pretreated with or without NaHS (100 μM), HT (2 mM) or PAG (2 mM) for 6 h, respectively, and then shifted to 1/2 MS solution with or without NH_4Cl (10 mM) for another 24 h. Afterwards, the total activities of GS (a), NADH-GOGAT (b), NADH-GDH (c) and nitrogen content (d) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

SUPPORTING INFORMATION

Table S1. Primers used for real-time RT-PCR analysis

Figure S1. Effects of NaHS on the total activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) in rice seedling roots upon NH_4Cl stress. 14-day-old seedlings were pretreated with or without NaHS (100 μM) for 6 h, and then shifted to 1/2 MS solution with or without NH_4Cl (10 mM) for another 24 h. Afterwards, the total activities of SOD (a), APX (b) and CAT (c) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Figure S2. Relative OsSE5 gene expression in wild-type and OsSE5 overexpressing lines under control conditions. 14-day-old seedlings were cultivated in 1/2 MS solution. Afterwards, the transcript levels of the OsSE5 was analyzed by real-time RT-PCR. Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Figure S3. Effect of NH_4Cl stress on the root dry weight, root elongation, ammonium content and MDA content in wild-type, 35S:OsSE5-1, 35S:OsSE5-2 seedling roots. 14-day-old seedlings were exposed to 1/2 MS solution with or without NH_4Cl (10 mM) for 7d. Afterwards, Root dry weight (a), root elongation (b), ammonium content (c) and MDA

content (d) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Figure S4. Effect of NH_4Cl stress on the root dry weight, root elongation and MDA content in wild-type, 35S:OsSE5-1, OsSE5-RNAi-1 seedling roots. 14-day-old seedlings were exposed to 1/2 MS solution with or without NH_4Cl (10 mM) for 7d. Afterwards, Root dry weight (a), root elongation (b) and MDA content (c) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Asterisks indicate significantly different between treatments at the same time points at $P < 0.05$ according to t-test.

Figure S5. Effects of NaHS, HT and PAG on the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) in wild-type, 35S:OsSE5-1, OsSE5-RNAi-1 seedling roots upon NH_4Cl stress. 14-day-old seedlings were pretreated with or without NaHS (100 μM), HT (2 mM) or PAG (2 mM) for 6 h, respectively, and then shifted to 1/2 MS solution with or without NH_4Cl (10 mM) for another 24 h. Afterwards, the total activities of SOD (a), APX (b) and CAT (c) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly

856 different at $P < 0.05$ according to Duncan's multiple range test.

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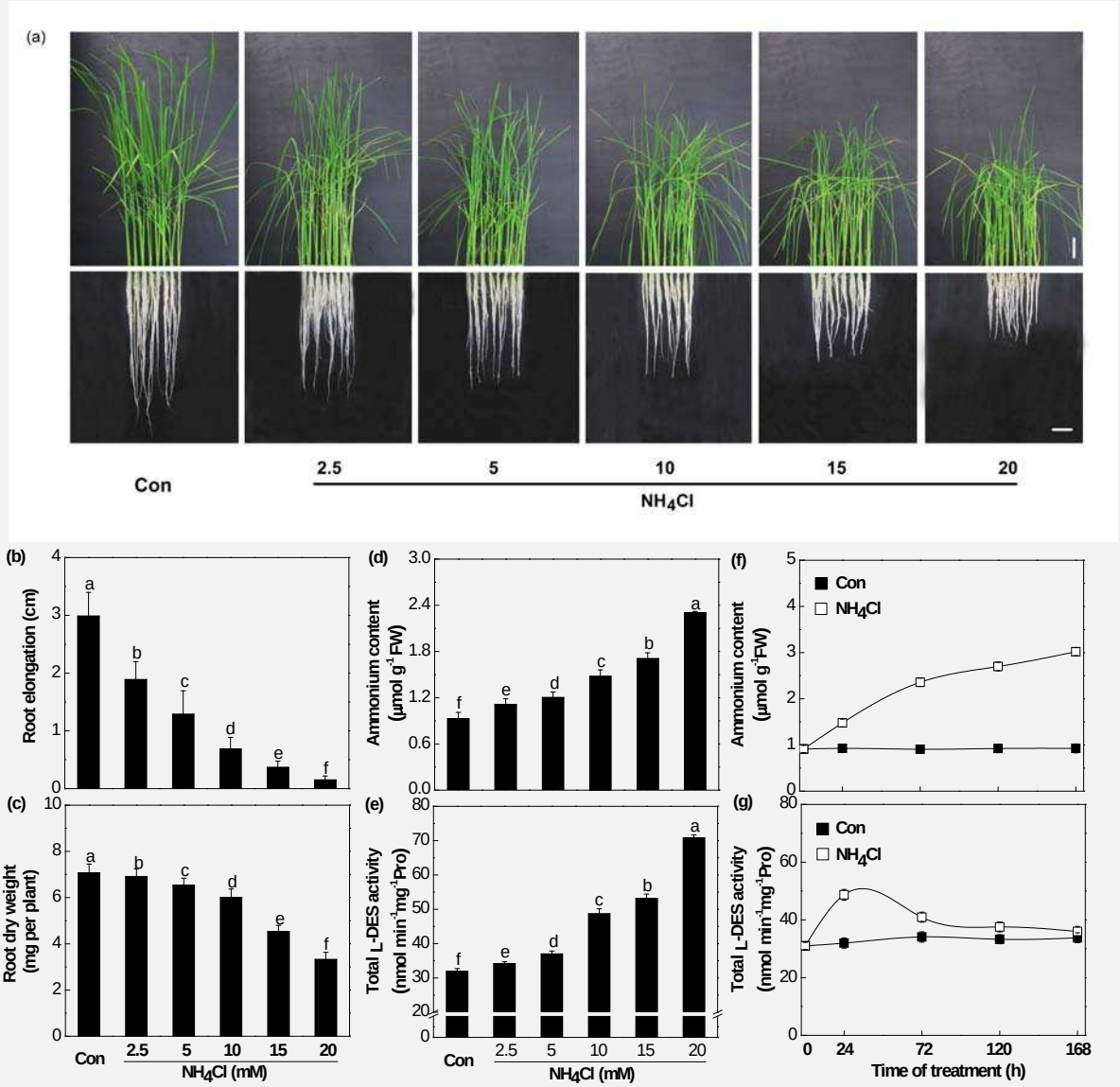

Figure 1. Morphology, root dry weight, root elongation, ammonium content and total $_{L}$-DES activity in rice seedling roots upon NH_4Cl stress. 14-day-old rice seedlings were exposed to 1/2 MS solution contained different concentrations of NH_4Cl. Photographs were taken after 7 d of treatment (a). Bar = 1 cm. Root dry weight and elongation were recorded (b, c). Ammonium content and total $_{L}$-DES activity in seedling roots were determined 24 h after various treatments (d, e) or at the indicated time points (f, g; 10 mM NH_4Cl). Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

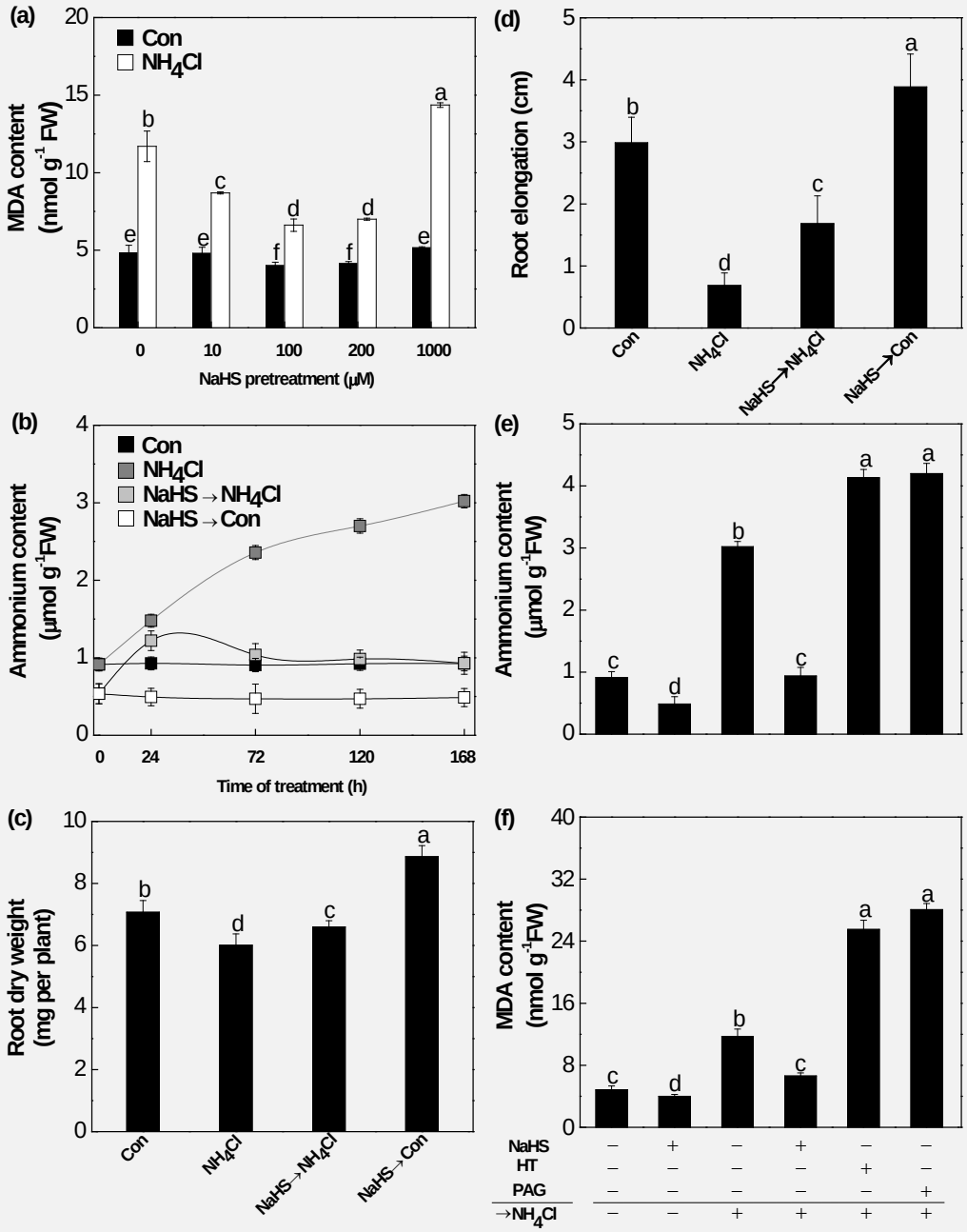

Figure 2. Effects of NaHS, HT and PAG on the MDA content, root dry weight, root elongation and ammonium content in rice seedling roots upon NH_4Cl stress. 14-day-old seedlings were pretreated with or without NaHS (different concentrations or 100 μM), HT (2 mM) or PAG (2 mM) for 6 h, and then shifted to 1/2 MS solution with or without NH_4Cl (10 mM) for 7 d. Afterwards, MDA content (a,f), time-course analysis of ammonium content (b) or at the indicated time points (e), root dry weight (c) and elongation (d) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

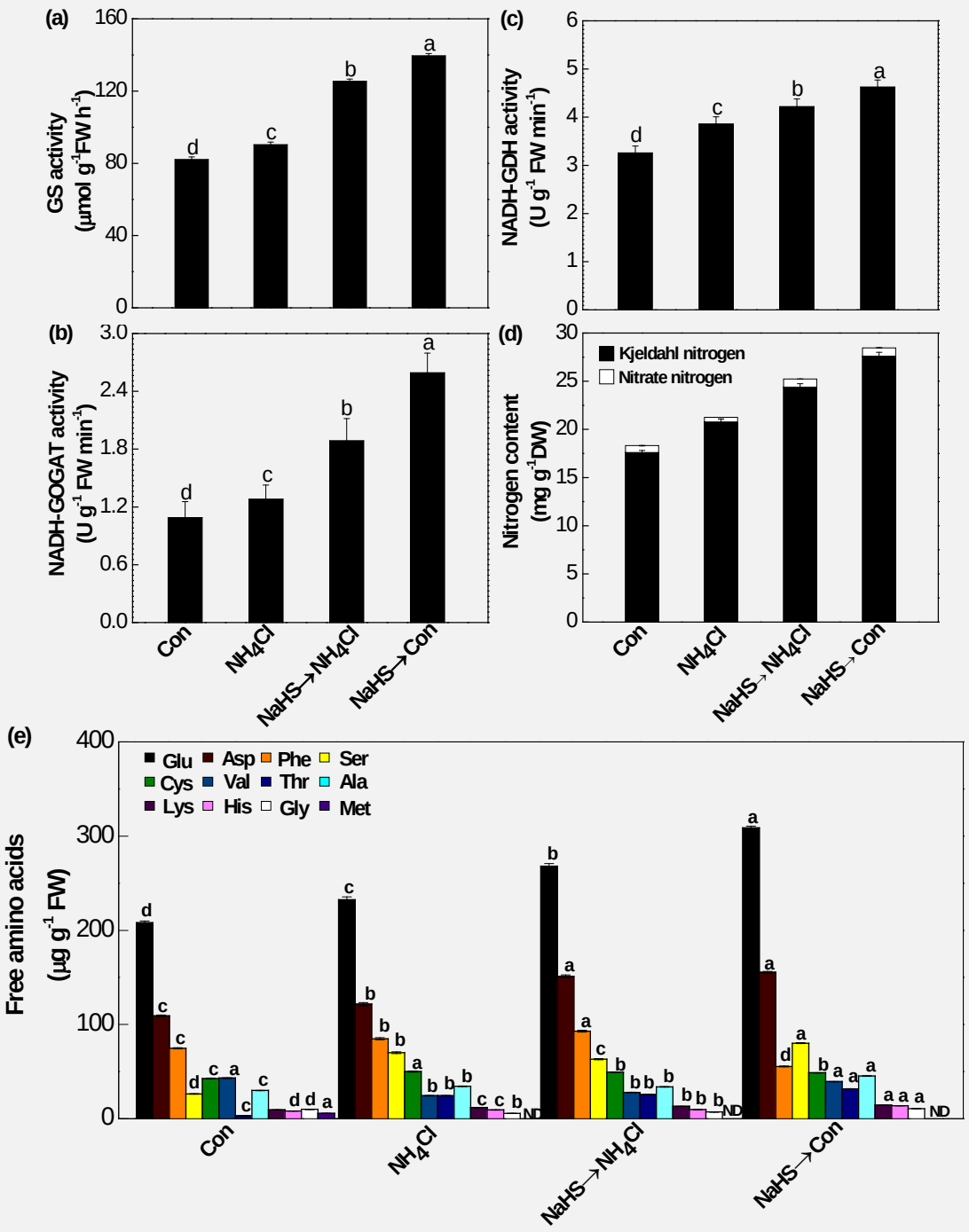

Figure 3. Effects of NaHS on the total activities of glutamine synthetase (GS), NADH-glutamate synthase (NADH-GOGAT), NADH-glutamate dehydrogenase (NADH-GDH), nitrogen content and free amino acids content in rice seedling roots upon NH_4Cl stress. 14-day-old seedlings were pretreated with or without NaHS (100 μM) for 6 h, and then shifted to 1/2 MS solution with or without NH_4Cl (10 mM) for another 24 h. Afterwards, the total activities of GS (a), NADH-GOGAT (b), NADH-GDH (c), nitrogen content (d), free amino acids (e) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

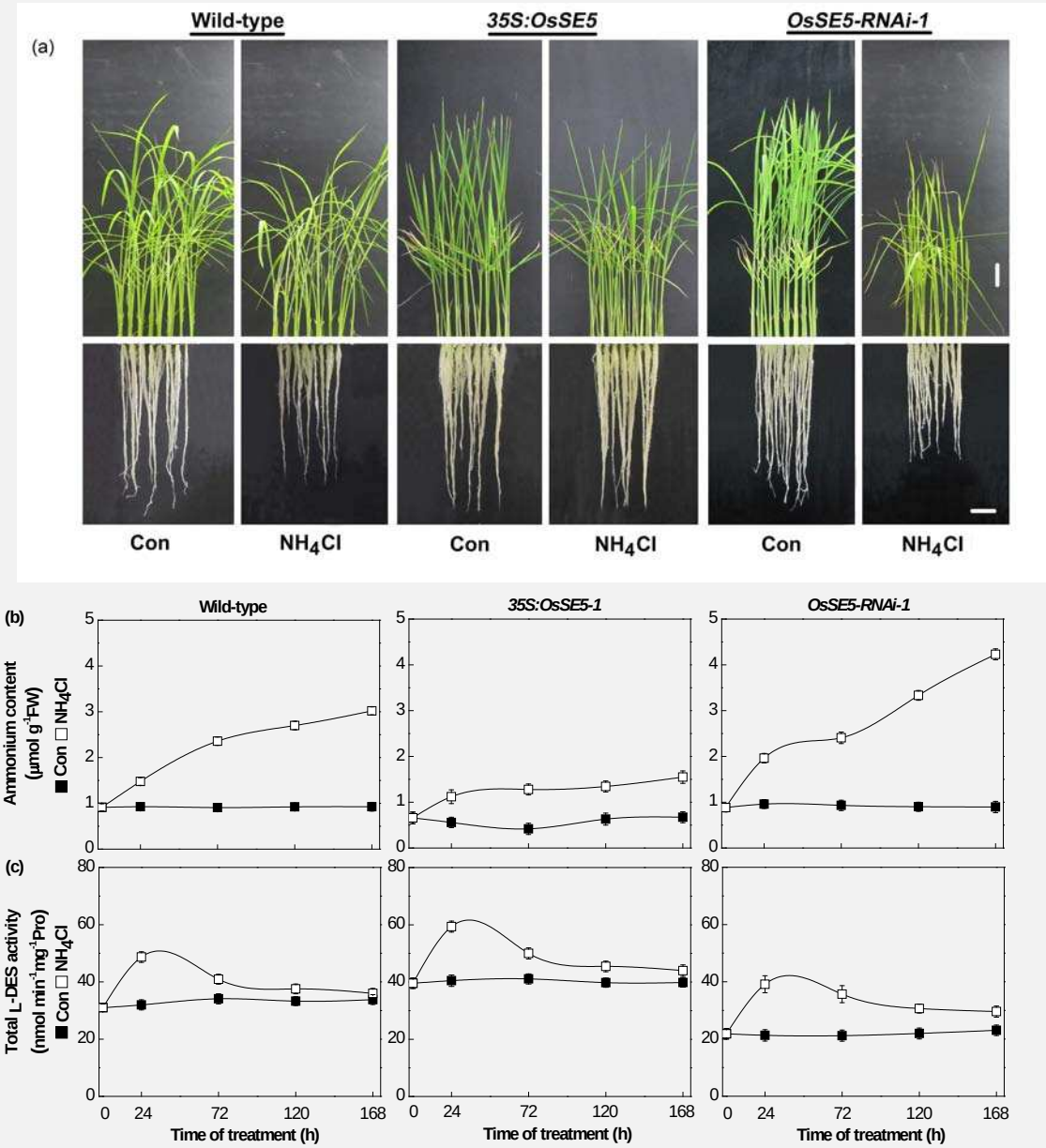

Figure 4. Effect of NH_4Cl stress on the morphology, time-courses analysis of ammonium content and total $_L$-DES activity in wild-type, *35S:OsSE5-1*, *OsSE5-RNAi-1* seedling roots. 14-day-old seedlings were exposed to 1/2 MS solution with or without NH_4Cl (10 mM). Photographs were taken after 7 d of treatment (a). Bar = 1 cm. Time-course analysis of ammonium content (b) and total $_L$-DES activity (c) were determined, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

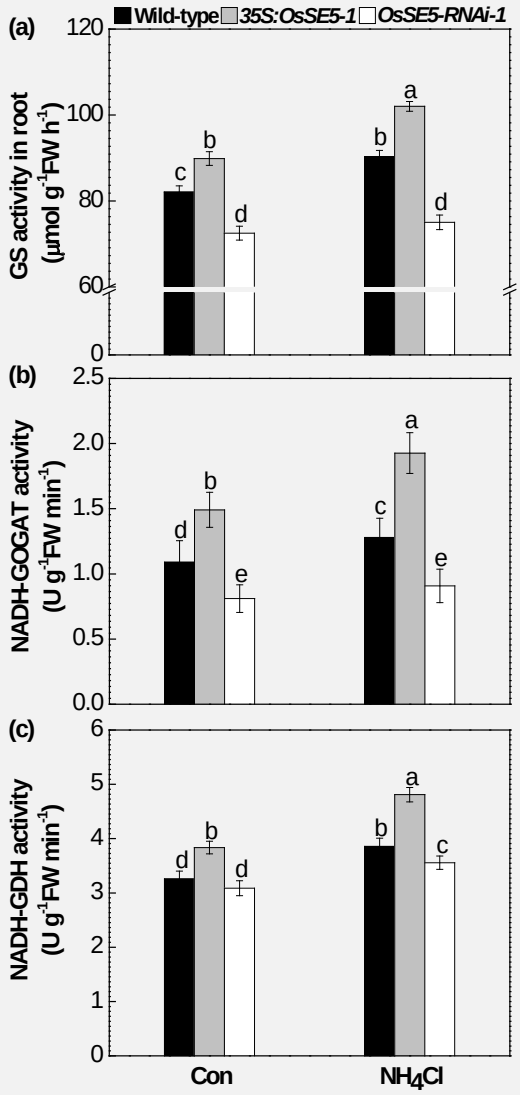

Figure 5. Effect of NH_4Cl stress on the activities of glutamine synthetase (GS), NADH-glutamate synthase (NADH-GOGAT), NADH-glutamate dehydrogenase (NADH-GDH) in wild-type, *35S:OsSE5-1*, *OsSE5-RNAi-1* seedling roots. 14-day-old seedlings were exposed to 1/2 MS solution with or without NH_4Cl (10 mM) for another 24 h. Afterwards, the total activities of GS (a), NADH-GOGAT (b), NADH-GDH (c) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

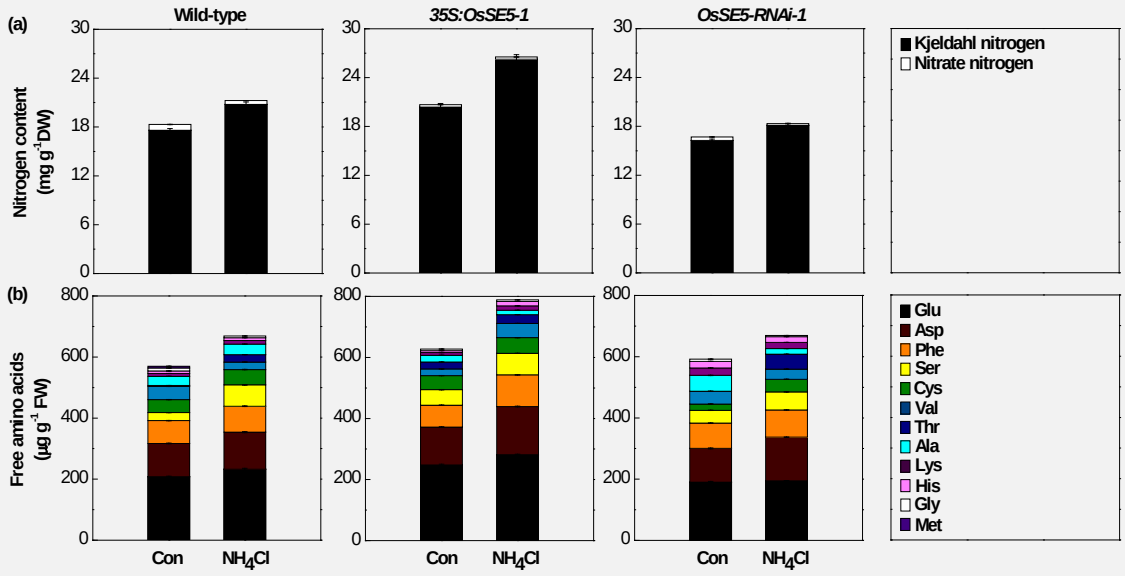

Figure 6. Effects of NH_4Cl on the nitrogen content and free amino acids content in wild-type, *35S:OsSE5-1*, *OsSE5-RNAi-1* seedling roots. 14-day-old seedlings were shifted to 1/2 MS solution with or without NH_4Cl (10 mM) for another 24 h. Afterwards, the nitrogen content (a), free amino acids content (b) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

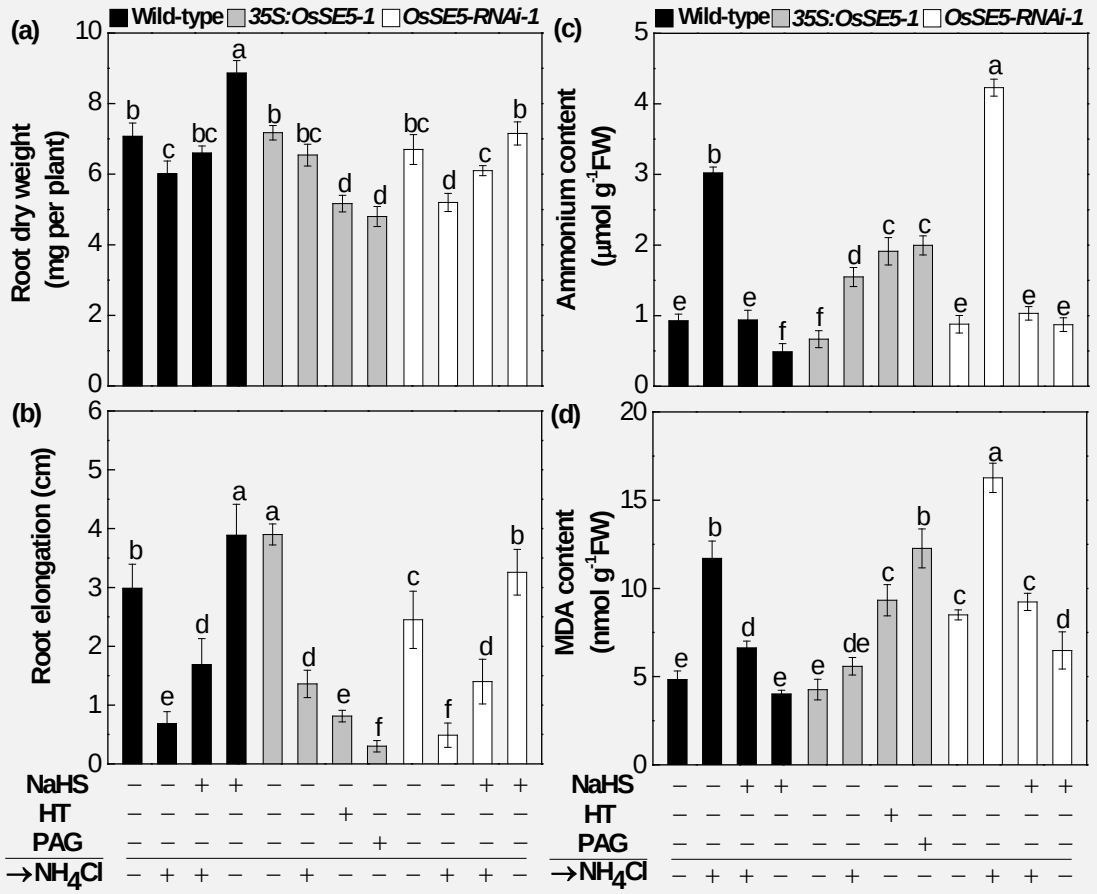

Figure 7. Effects of NaHS, HT and PAG on the root dry weigh, root elongation, ammonium content and MDA content in the seedling roots of wild-type, *35S:OsSE5-1*, *OsSE5-RNAi-1* upon NH_4Cl stress. 14-day-old seedlings were pretreated with or without NaHS (100 μM), HT (2 mM) or PAG (2 mM) for 6 h, respectively, and then shifted to 1/2 MS solution with or without NH_4Cl (10 mM) for 7 d. Afterwards, root dry weight (a), root elongation (b), ammonium content (c) and MDA content (d) were determined, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

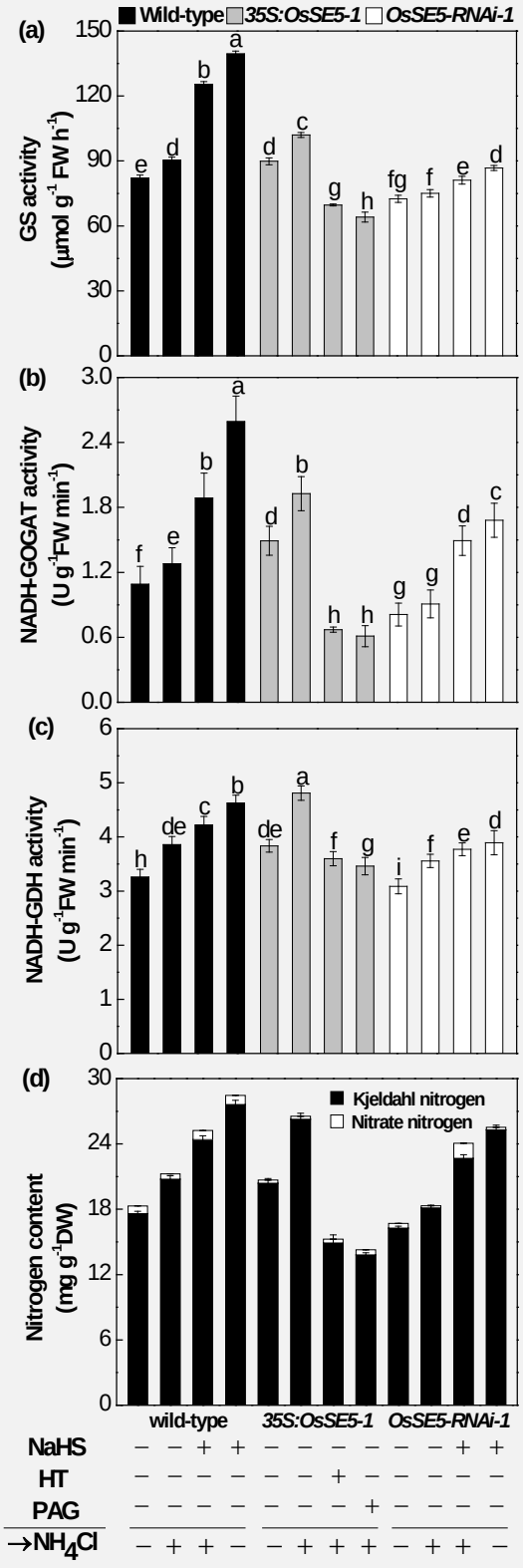
Wild-type
35S:OsSE5-1
OsSE5-RNAi-1
(a)
GS activity (μmol g-1 FW h-1)
(b)
NADH-GOGAT activity (U g-1FW min-1)
(c)
NADH-GDH activity (U g-1FW min-1)
(d)
Nitrogen content (mg g-1DW)
Kjeldahl nitrogen
Nitrate nitrogen
wild-type
35S:OsSE5-1
OsSE5-RNAi-1
NaHS
HT
PAG
→NH4Cl

Figure 8. Effects of NaHS, HT and PAG on the activities of glutamine synthetase (GS), NADH-glutamate synthase (NADH-GOGAT), NADH-glutamate dehydrogenase (NADH-GDH) and nitrogen content in wild-type, *35S:OsSE5-1*, *OsSE5-RNAi-1* seedling roots upon NH4Cl stress. 14-day-old seedlings were pretreated with or without NaHS (100 μM), HT (2 mM) or PAG (2 mM) for 6 h, respectively, and then shifted to 1/2 MS solution with or without NH_4Cl (10 mM) for another 24 h. Afterwards, the total activities of GS (a), NADH-GOGAT (b), NADH-GDH (c) and nitrogen content (d) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

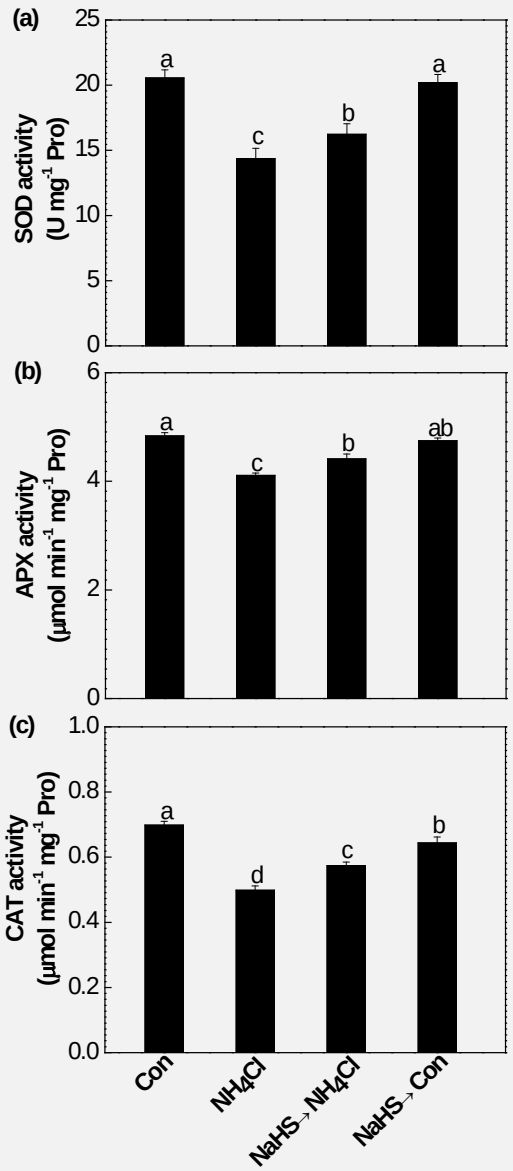

Figure S1. Effects of NaHS on the total activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) in rice seedling roots upon NH_4Cl stress. 14-day-old seedlings were pretreated with or without NaHS (100 μM) for 6 h, and then shifted to 1/2 MS solution with or without NH_4Cl (10 mM) for another 24 h. Afterwards, the total activities of SOD (a), APX (b) and CAT (c) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

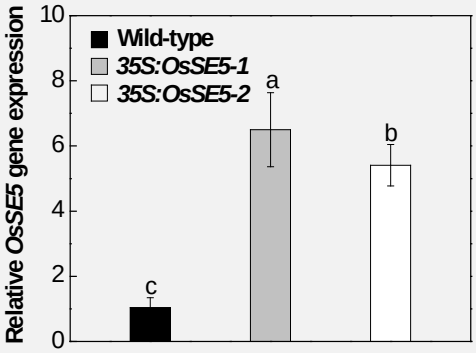

Figure S2. Relative *OsSE5* gene expression in wild-type and *OsSE5* overexpressing lines under control conditions. 14-day-old seedlings were cultivated in 1/2 MS solution. Afterwards, the transcript levels of the *OsSE5* was analyzed by real-time RT-PCR. Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

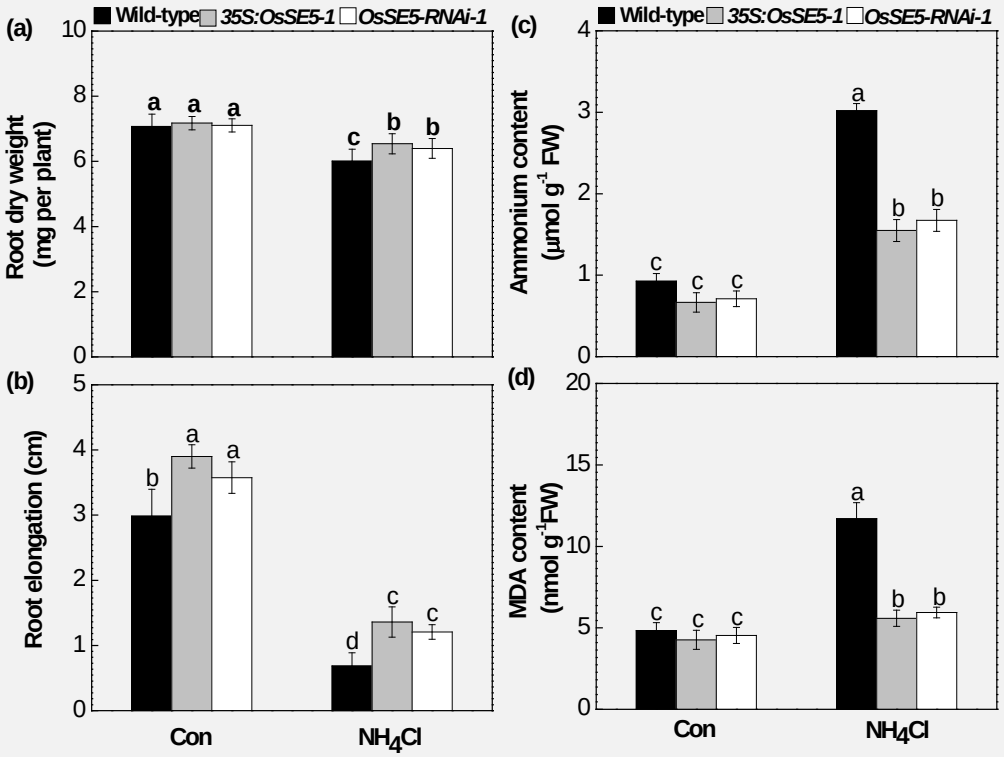

Figure S3. Effect of NH_4Cl stress on the root dry weight, root elongation, ammonium content and MDA content in wild-type, *35S:OsSE5-1*, *35S:OsSE5-2* seedling roots. 14-day-old seedlings were exposed to 1/2 MS solution with or without NH_4Cl (10 mM) for 7d. Afterwards, Root dry weight (a), root elongation (b), ammonium content (c) and MDA content (d) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

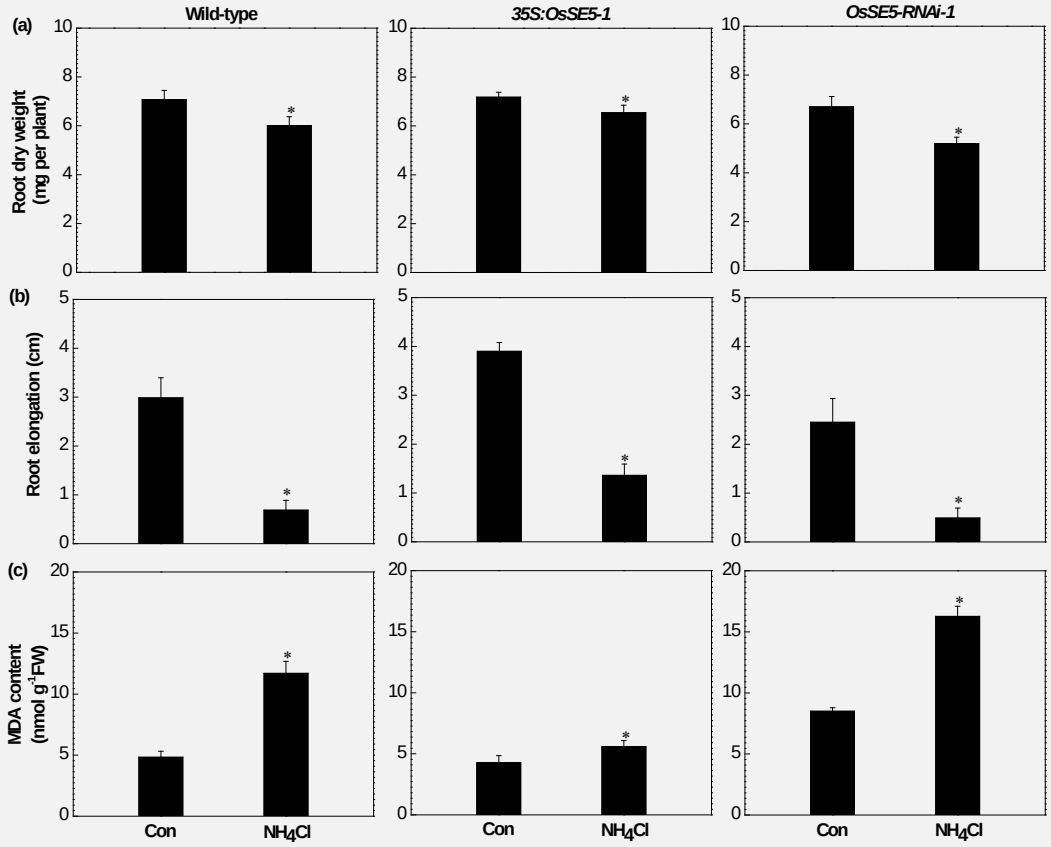

Figure S4. Effect of NH_4Cl stress on the root dry weight, root elongation and MDA content in wild-type, 35S:*OsSE5-1*, *OsSE5-RNAi-1* seedling roots. 14-day-old seedlings were exposed to 1/2 MS solution with or without NH_4Cl (10 mM) for 7d. Afterwards, Root dry weight (a), root elongation (b) and MDA content (c) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Asterisks indicate significantly different between treatments at the same time points at $P < 0.05$ according to *t*-test.

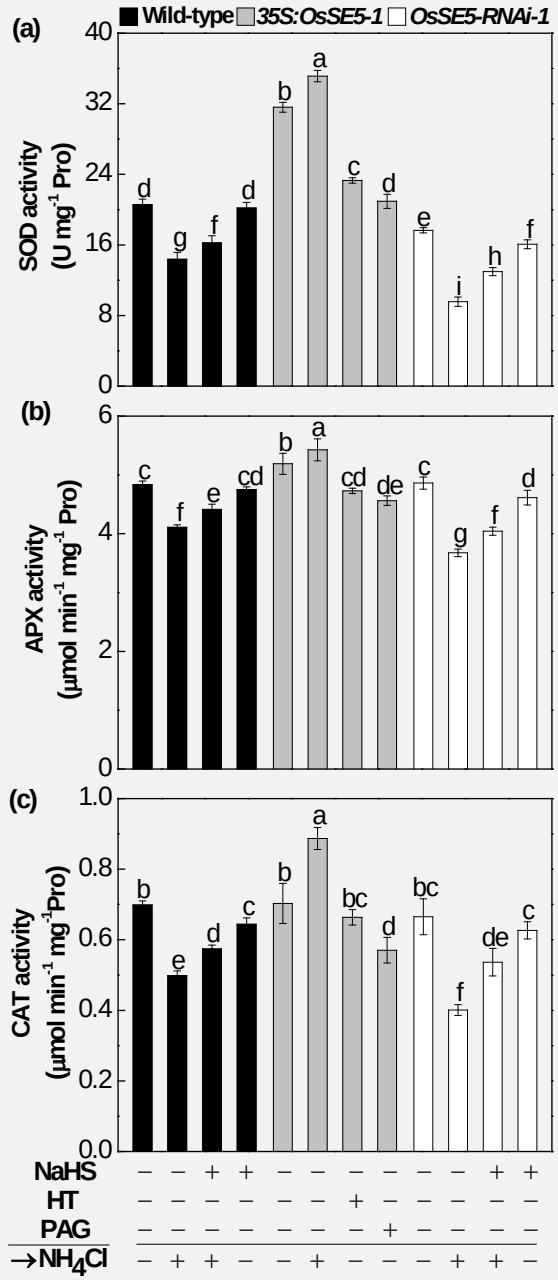

Figure S5. Effects of NaHS, HT and PAG on the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) in wild-type, *35S:OsSE5-1*, *OsSE5-RNAi-1* seedling roots upon NH4Cl stress. 14-day-old seedlings were pretreated with or without NaHS (100 μM), HT (2 mM) or PAG (2 mM) for 6 h, respectively, and then shifted to 1/2 MS solution with or without NH_4Cl (10 mM) for another 24 h. Afterwards, the total activities of SOD (a), APX (b) and CAT (c) were measured, respectively. Seedlings without chemical treatment were regarded as the control (Con). Values are means ± SE of three independent experiments with at least three replicates for each. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.