



# Appropriate Ammonium/Nitrate Mitigates Low Light Stress in *Brassica pekinensis* by Regulating the Nitrogen Metabolism and Expression Levels of Key Proteins

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## Abstract

Low light intensity is always the important factor that influences the growth and development of *Brassica pekinensis* during winter seasons. We have found out that appropriate ammonium/nitrate ratio mitigated low light intensity in the previous study. In the present study, the nitrogen metabolism and proteomic responses of *Brassica pekinensis* to light intensities and ammonium/nitrate were further investigated to find out the mitigation mechanism. The activities of nitrate reductase (NR) and glutamine synthetase (GS) and the relative expression of *GSI* as well as the content of nitric oxide (NO) were significantly higher in the  $\text{NH}_4^+:\text{NO}_3^-$  (15:85) treatment than in the  $\text{NH}_4^+:\text{NO}_3^-$  (0:100) treatment under normal light intensity. However, under low light intensity condition, the activities of NR and GS and relative expression of *NR* as well as the NO level in seedlings fertilized with  $\text{NH}_4^+:\text{NO}_3^-$  (10:90) were significantly higher compared with those fertilized with  $\text{NH}_4^+:\text{NO}_3^-$  (0:100). In addition, we found thirty-six protein spots, which exhibited significant changes in abundance using similarity searches across the uniprot database of *Brassica*. The proteins identified were classified into ten functional groups including photosynthesis, carbon and energy metabolism, stress/defense, protein folding, and modification and degradation response. We concluded that appropriate ammonium nitrate ratio improves the tolerance of mini Chinese cabbage seedlings to low light intensity by regulating the nitrogen metabolism and expression levels of some key proteins.

**Keywords** Nitrogen metabolism · Proteomic · Light intensity · Ammonium: nitrate · Nitric oxide · *Brassica pekinensis*

## Introduction

Solar radiation, particularly photosynthetically active radiation, is an important factor that influences the amount of dry matter produced by crops especially under field conditions (Campillo et al. 2012). Adequate solar radiation improves photosynthesis even under conditions of limited nitrogen (Abbate et al. 2009). Globally, average solar radiation is declining at  $0.51 \pm 0.05 \text{ W m}^{-2}$  per year (Stanhill and Cohen 2001). Moreover, under field conditions, solar radiation, can easily fluctuate and negatively affect photosynthesis, growth and yield of crops (Slattery et al. 2018). The development of crop varieties that are tolerant to low light intensity requires the understanding of the physiological mechanisms and genetics of the contributing traits of plants. Andersson et al. (2003) reported that the expression level of *Ohp2* gene was induced by light stress and that the *Ohp2* transcript and protein accumulation depended on light intensity. Until recently, several researchers investigated the

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key proteins induced by high light intensity stress (Choudhury et al. 2018; Hey and Grimm 2018; Lima et al. 2018). Chinese cabbage is one of the important vegetable crops widely cultivated and consumed in many Asian countries including China. It has been reported that insufficient irradiation greatly retards the growth of Chinese cabbage especially during the early and middle growth stages under field conditions. Moreover, low irradiation also negatively affects head formation in Chinese cabbage (Son et al. 2018). *Brassica pekinensis* and other members of the family generally require moderate light intensity (AVRDC, 1990), but this crop has been tested with a wide range of light intensity ( $100\text{--}1000\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ ) under greenhouse conditions (Hu et al. 2015; Son et al. 2018). Whereas, Son et al. (2018) reported the effect of  $200\text{--}1000\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$  on the growth and yield of mature *B. pekinensis*, Hu et al. (2015) reported the effect of  $\text{NH}_4^+/\text{NO}_3^-$  ratios on the physiological response *B. pekinensis* seedlings under  $100$  and  $200\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ . Although a moderate light intensity crop, increasing light intensity significantly increased photosynthesis, fresh and dry weights as well as the quality of Chinese cabbage (Son et al. 2018). However, little is known about proteins that are induced in plants subjected to low light intensity. Thus, understanding the physiological and proteomic response to different light intensities in plants is an important research area.

The availability of nitrogen in the form of nitrate ( $\text{NO}_3^-$ ) and ammonium ( $\text{NH}_4^+$ ) is a major factor affecting plant growth and development (Piwpuan et al. 2013). Until now, many researchers focused on the main inorganic nitrogen sources, and found that plants exhibited intricate responses at both morphological and physiological levels (Engelsberger and Schulze 2012; Forde 2014; Hachiya and Sakakibara 2017; Hu et al. 2017). Co-provision of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  is beneficial for plant growth regardless of the presence of stress or no stress as compared to the application of  $\text{NO}_3^-$  alone or  $\text{NH}_4^+$  alone, and the optimal  $\text{NH}_4^+/\text{NO}_3^-$  ratio depend on both species and the environment (Britto and Kronzucker 2002; Hu et al. 2015; Liu et al. 2017). For instance, Liu et al. (2017) reported that the optimal  $\text{NH}_4^+/\text{NO}_3^-$  ratio for tomato seedling appeared to be 25:75 for unstressed plants and 50:50 for plants exposed to low temperature stress. But in our previous study, we found the optimal  $\text{NH}_4^+/\text{NO}_3^-$  ratio for mini Chinese cabbage seedling to be 15: 85 for plants under normal light intensity and 10:90 for plants under low light intensity stress (Hu et al. 2015). Results of our previous study suggest that different  $\text{NH}_4^+/\text{NO}_3^-$  ratios changed the nitrogen metabolism pathway. The nitrogen assimilation in plants mainly contains  $\text{NO}_3^-$  and  $\text{NH}_4^+$  assimilation. There are two alternative assimilation fates of  $\text{NO}_3^-$  following its absorption from the soil. One involves immediate nitrate reduction to ammonium by nitrate reductase (NR) and nitrite reductase

(NiR) under aerated condition, and then ammonium is synthesized to amino acid mainly by glutamine synthetase/glutamate synthetase (GS/GOGAT) (Lea and Mifflin 2018). The other involves nitrate reduction to nitric oxide (NO) by NR/NiR at low pH or through the action of the mitochondrial electron transport chain under anaerobic conditions (Astier et al. 2018). Based on this hypothesis, we also explored the possible relationship between nitrogen assimilation and NO production in the present study.

Ding et al. (2018) reported that exposure of rice (*Oryza sativa*) plants to high-nitrogen levels resulted in larger leaves. They found that two proteins related to cell division, FtsZ and ERBB3 binding protein, increased in the developing leaves with the application of nitrogen. Recently, many researchers centered on proteomic analysis of plants response to nitrogen deprivation (Park et al. 2015; Tiffert et al. 2011) or different nitrogen levels (Bahrman et al. 2004; Ding et al. 2018). Little is known about proteomic analysis of plants response to different nitrogen forms, especially  $\text{NH}_4^+/\text{NO}_3^-$  ratio under different light intensities. In our previous study, we demonstrated that the supply of appropriate  $\text{NH}_4^+/\text{NO}_3^-$  ratio to mini Chinese cabbage seedling improves their tolerance of to low light intensity stress by improving root architecture, photosynthesis, carbohydrate and nitrogen content, some photosynthesis—related enzyme activities and the related-genes transcript levels (Hu et al. 2015, 2017). However, the influence of  $\text{NH}_4^+/\text{NO}_3^-$  ratio on protein expression in mini Chinese cabbage seedlings under different light intensities has not been reported. The current study was conducted with the hypothesis that appropriate  $\text{NH}_4^+/\text{NO}_3^-$  ratio can enhance nitrogen metabolism and regulate the expressions of proteins to improve the tolerance of mini Chinese cabbage seedlings to low light. As we all known, good seedlings are prerequisite for increased yield and quality of harvested Chinese cabbage. The first two weeks of growth of the seedlings is very critical and the survival of the seedlings within this period gives an indication of the possibility of producing good seedlings for transplanting. Moreover, in our previous experiments, morphological and physiological parameters of Chinese cabbage seedlings indicated significant differences at 14 days after seedlings treatment (Hu et al. 2015, 2017). Therefore, in this experiment, we investigated the genes related to nitrogen metabolism and conducted proteomic analysis (2-DE and MALDI TOF/TOF methods) of 14-old mini Chinese cabbage seedlings treated with different ammonium: nitrate ratios and grown under normal and low light intensities, with the aim of: (1) determining the activities of enzymes and gene expression level related to nitrogen metabolism (2) identifying the key proteins that will be expressed differentially and their related functions. The results of our experiment reveals appropriate  $\text{NH}_4^+/\text{NO}_3^-$  ratio can improve the tolerance of mini Chinese cabbage seedlings to low light

by enhancing the activities of enzymes involved in nitrogen metabolism and modulating key proteins.

## Materials and Methods

### Plant Growth and Treatments

The seed of mini Chinese cabbage (*Brassica pekinensis* cv. “Jinwa no. 2”) purchased from the Gansu Academy of Agricultural Sciences, was used for the experiment. The seeds were germinated within 16 h on moist filter paper under dark condition at 25 °C. The pre-germinated seeds were then sown in clean quartz sand and the seedlings were raised in a greenhouse (Photoperiod of 12 h, day/night temperature of  $25 \pm 2 / 18 \pm 2$  °C, and light intensity about  $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). At the second leaf stage, the seedlings were exposed to the different  $\text{NH}_4^+/\text{NO}_3^-$  (0:100, 10:90, 15:85 and 25:75) and light treatments ( $200 \mu\text{mol m}^{-2} \text{s}^{-1}$  and  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 14 d. The light source used in the experiment was provided with the LED lamps, whose correlated color temperature was about 3037 K. And the light spectra (shown in Fig. S1) of LED lamps was measured directly above the plants using a Plant Light Analyzer (PLA-30, Hangzhou Yuanfang Optoelectronic Information Co., Ltd.), showing the relative spectral intensity within the visible range 350–800 nm. The nutrient solution was prepared and used as described by Hu et al. (2017). The experiment was designed as  $2 \times 4$  factorial incompletely randomized designs with three replications. Each experimental unit consisted of 20 seedlings.

### Determination of Nitrate Reductase (NR) and Glutamine Synthetase (GS) Activity in Leaves

We determined the NR activity as the rate of nitrite production spectrophotometrically as described by Frungillo et al. (2014) with some modifications. The extracted protein samples were kept under dark condition. A total of 0.4 mL of clear supernatant was added into 1.4 mL of buffer containing 100 mM phosphate buffer (pH 7.5), 100 mM  $\text{KNO}_3$  and 0.25 mM NADH. The mixed solution was reacted at 25 °C for 30 min, and then a 1:1 mixture of 1% (w/v) sulfanilamide in 3 M HCl and 0.02% (w/v) *N*-(1-naphthyl)-ethylenediamine was added to the solution to stop the reaction and start coloration. After 15 min of incubation at room temperature, the amount of nitrite produced was determined at 540 nm.

The GS activity was measured with some modification of O’Neal and Joy (1973) method and the process was conducted at 4 °C. We incubated 700  $\mu\text{L}$  of protein extract with 1,600  $\mu\text{L}$  of reaction buffer (100 mM Tris–HCl, 80 mM  $\text{MgSO}_4$ , 20 mM Sodium glutamate, 20 mM L-cysteine, 2 mM EGTA, 80 mM hydroxylammonium chloride, 18 mM

ATP, pH 7.4) at 37 °C for 0.5 h. Reaction was stopped with stopping solution [200 mM trichloroacetic acid (TCA), 370 mM  $\text{FeCl}_3$  and 600 mM HCl] after 15 min. Centrifugation of samples was done at  $10,000 \times g$  for 10 min. The amount of  $\gamma$ -glutamyl hydroxamate in the supernatant was determined photometrically at 540 nm against an immediately stopped parallel sample.

### Total RNA Extraction and Analysis of Gene Expression Levels

We extracted total RNA using RNAiso Plus (TaKaRaD9108A) by following the instruction of the supplier. The relative mRNA expression levels of *NR* and *GS* genes in the leaves were analyzed by real time quantitative RT-PCR using a SYBR® Green QPCR MIX QPS-201 T (TOYOBO), following the manufacturer’s instructions. We used the *actin* gene of mini Chinese cabbage (Gen-Bank Accession No. JN120480.1) as an internal control and the primers were designed and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). On the basis of nucleotide, the primers for *NR* and *GS* (Gen-Bank Accession No. EU662272 and AY773089) and *actin* genes were designed and used for amplification. The primers were provided as following:

*NR* F: 5’-ATCCAAGATTCCCCAACGG-3’; R: 5’-GCC TCGGTGATAAACCTGT-3’

*GS* F: 5’-CGGGTGAACCAATCCCTACG-3’; R: 5’-CGACCTGGAAGTCCCACTGAC-3’

*actin* F: 5’-CCAGGAATCGCTGACCGTAT-3’; R: 5’-CTGTTGGAAAGTGCTGAGGGA-3’

### NO Content in Leave and NO-Dependent Fluorescence in Root Analysis

The NO content in leaves was measured using the Greiss reagent method as described by Zhu et al. (2016) with some modifications. Leaf samples (0.5 g each) of mini Chinese cabbage were frozen in liquid nitrogen, then ground in a mortar in 3 mL of 50 mM ice-cold acetic acid buffer (pH 3.6, containing 4% zinc diacetate). The homogenates were centrifuged at  $1000 \times g$  for 15 min at 4 °C, and the supernatants were collected. We added 1 mL of the above buffer was to the residue and repeated the process before we mixed the supernatants together. For each sample, we added 0.1 g activated charcoal and after mixing and filtration of vortex, the filtrate was leached and collected. We mixed 2 mL of the filtrate and 2 mL of Greiss reagent together and incubated these at room temperature for 30 min to convert nitrite into a purple azo-dye. The absorbance was assayed at 540 nm and the content of NO was calculated by comparison to a standard curve of  $\text{NaNO}_2$ .

The NO-dependent fluorescence in roots was determined using the fluorescence indicator 4, 5-diaminofluorescein diacetate (DAF-2 DA; Sigma) which can permeate into the cell membrane and emit fluorescence upon binding to NO (Fernandez-Marcos et al. 2011). The in situ measurement of NO was carried out following the procedure of Wang et al. (2013). We incubated the root samples for 2 h under dark condition with 20  $\mu$ M DAF-2 DA in 50  $\mu$ M Tris-HCl buffer (pH 7.5), and washed three times in fresh buffer. These were then visualized under a fluorescence microscope (Leica  $\times$  400, Planapo, Wetzlar, Germany) with excitation and emission wavelengths of 485 and 538 nm, respectively.

### Protein Extraction from the Leaves

We determined total proteins from leaves of seedlings exposed to different ammonium: nitrate for different light intensities (0:100 and 15:85 for normal light intensity, 0:100 and 10:90 for low light intensity) following the procedures of Wang et al. (2015) with slight modifications. Frozen leaf tissue (2 g) was ground in liquid nitrogen and incubated in ice-cold extraction buffer [10% (w/v) tricarboxylic acid (TCA) in acetone with 0.07% (v/v)  $\beta$ -mercaptoethanol ( $\beta$ -ME)] overnight at  $-20^{\circ}\text{C}$ . Homogenates were centrifuged at 20,000 $\times$ g for 40 min at  $4^{\circ}\text{C}$ . After centrifugation, the supernatant was discarded. We washed the residues with cold acetone which contained 0.07%  $\beta$ -ME, incubated at  $-20^{\circ}\text{C}$  for 1 h and centrifuged at 20,000 $\times$ g for 30 min at  $4^{\circ}\text{C}$ . This process was repeated once. In the following, the residues were washed twice with ice-cold 80% acetone which contained 0.07%  $\beta$ -ME, incubated at  $-20^{\circ}\text{C}$  for 30 min and centrifuged as described above until both the supernatant and residues became colorless, and then the residues were dried by vacuum freeze dryer. The dry residues were dissolved in a solubilization buffer [7 M urea, 2 M thiourea, 65 mM dithiothreitol (DTT), 4% (w/v) CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate)] at room temperature for 2 h and then centrifuged at 20,000 g for 30 min at  $4^{\circ}\text{C}$ . We then determined the protein content in each residue following to the method of Bradford (1976). We used Bovine serum albumin (BSA) as a standard.

### Two-Dimensional Gel Electrophoresis (2-DE), Gel Staining and Image Analysis

The 2-DE was carried out according to the method of Wang et al. (2015) with minor modification. For the first isoelectric focusing (IEF), 1 mg protein sample was dissolved in 400  $\mu$ L rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 65 mM DTT, 0.2% (w/v) ampholytes (pH 3–10) (Bio-Lyte; Bio-Rad, Hercules, CA, USA) and 0.001% (w/v)

bromophenol blue], then was loaded onto the IPG strip (pH 4–7, 17 cm; Bio-Rad) and rehydrated at 50 V for 14 h at  $20^{\circ}\text{C}$ . IEF was performed via a Protean IEF cell system (Bio-Rad, USA) using the following four-step processes at  $20^{\circ}\text{C}$ : 250 V for 1.5 h with a linear ramp; 1000 V for 2.5 h with a rapid ramp; 9000 V for 5 h with a linear ramp; and 9,000 V for 100,000 V-h with a rapid ramp. After the first IEF dimension, the strips were incubated in equilibration buffer I [6 M urea, 2% sodium dodecyl sulphate (SDS), 20% glycerol, 0.375 M Tris-HCl (pH 8.8) and 130 mM DTT] for 15 min, and then incubated in equilibration buffer II [6 M urea, 2% SDS, 20% glycerol, 0.375 M Tris-HCl (pH 8.8) and 135 mM iodoacetamide] for another 15 min.

The second dimension for separating proteins was conducted on a 12% polyacrylamide SDS gel using the PROTEAN II xi Cell system (Bio-Rad, USA). Protein spots in 2-DE gels were stained with Coomassie Brilliant Blue (CBB) G-250 and de-staining with double-distilled water. Three independent biological replications were performed for each sample. Each gel was scanned with a UMAX PowerLook 2100XL scanner (UMAX Systems GmbH, Willich, Germany). Image analysis of 12 2-DE gels was performed by using PDQuest software (version 8.0.1; Bio-Rad Laboratories, Hercules, CA). CBB-stained protein spots were selected for profile analysis. The Student's *t* test and a significance level of 95% ( $P < 0.05$ ) were selected to analyze spots with more than a two-fold change and indicate significant changes in abundance.

### The Identification of Protein and Database Searching

Protein spots that displayed reproducible change patterns were manually excised from the gels and digested according to the protocol described by Liu et al. (2015). MS and tandem mass spectrometry (MS/MS) data for identifying protein were obtained by using 4800 Plus MALDI TOF/TOFMS Analyzer (Applied Biosystems, USA). The MS/MS spectra search was performed using the software GPS Explorer (Applied Biosystems) and MASCOT version 2.2 (Matrix Science, London, UK) to search the uniprot database of *Brassica* (released data Dec. 5, 2018; containing 137,772,056 sequences) with the following parameters: trypsin cleavage (one missed cleavage allowed), 100 ppm precursor tolerance, carbamidomethylation set as fixed modification, oxidation of methionine allowed as a dynamical modification and MS/MS fragment tolerance set to 0.4 Da. The reliable results for the MALDI-TOF/TOF MS were the hits with high protein scores, similar molecular mass (*Mr*) and isoelectric point (pI) as experimental *Mr* and pI, and protein score confidence interval (C. I.%) of above 95%.

## Classification of Protein and Hierarchical Cluster Analysis

The function of the identified proteins was extracted from Blast searches against the UniProt (<https://www.uniprot.org>) and NCBI (<https://www.ncbi.nlm.nih.gov/protein>) databases. Combined with these findings, proteins were classified into different categories based on their biochemical functions, according to the convention used by Jiang et al. (2007). The WoLF PSORT (<https://psort.hgc.jp/>) software prediction programs were used to obtain the sub-cellular location information of each protein. The Cluster software (version 3.0; <https://rana.lbl.gov/EisenSoftware>) was used to perform a self-organizing tree algorithm of hierarchical clustering of protein relative abundance profiles based on the log-transformed fold change values of protein spots.

## Statistical Analysis

One-way analysis of variance and Tukey's test were used to determine significant differences among treatment means at  $P < 0.05$  level using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). All the results are represented as mean  $\pm$  standard error (SE) of at least three independent replications.

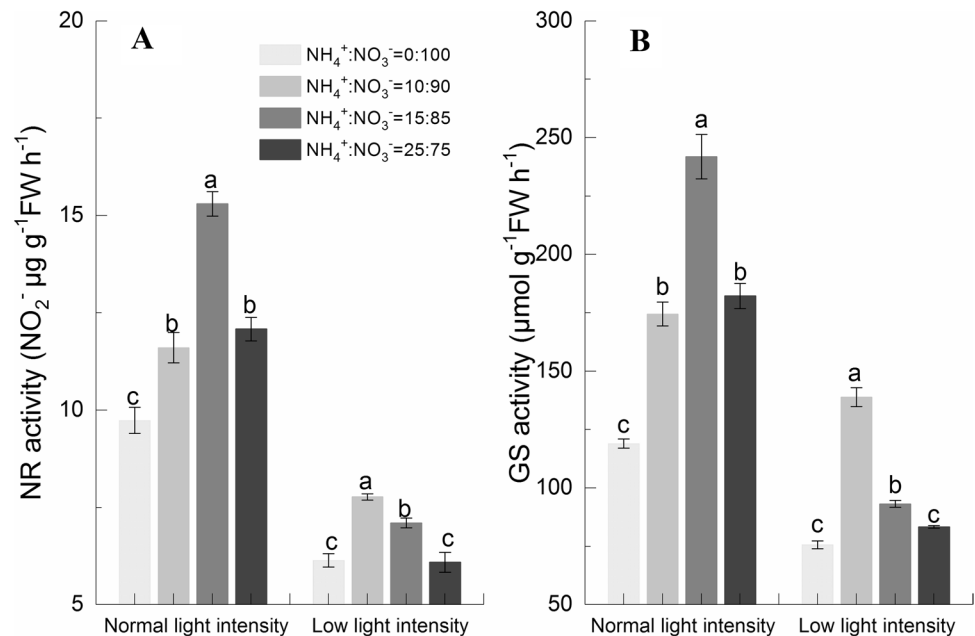
## Results

### The Activities of NR and GS in Mini Chinese Cabbage Seedlings

Nitrate reductase is one of the key enzymes involved in nitrogen metabolism which is related to the utilization of nitrogen and its activity influences the plant growth. Figure 1a shows that light intensity and  $\text{NH}_4^+/\text{NO}_3^-$  ratios significantly affected the activity of NR in mini Chinese cabbage seedlings. The NR activity initially increased and then decrease with increases in ammonium concentration under normal light intensity. The seedlings treated with  $\text{NH}_4^+/\text{NO}_3^-$  (15:85) had the highest NR activity, which significantly increased by 57.7% compared with the seedlings treated with  $\text{NH}_4^+/\text{NO}_3^-$  (0:100). Low light intensity stress obviously decreased the NR activity, however, the  $\text{NH}_4^+/\text{NO}_3^-$  (10:90) treatment could alleviate the inhibitory effect of low light intensity on NR activity compared with the other  $\text{NH}_4^+/\text{NO}_3^-$  ratios.

The variations in GS activities in the seedlings were similar. From Fig. 1b, we observed that the GS activity in seedlings treated with  $\text{NH}_4^+/\text{NO}_3^-$  (15:85) exposed normal light intensity was significantly the highest and it was 103.4% greater than the control treatment ( $\text{NH}_4^+/\text{NO}_3^- = 0:100$ ).

**Fig. 1** Effect of  $\text{NH}_4^+:\text{NO}_3^-$  ratios and light intensity on activities of nitrate reductase (a) and glutamine synthetase (b) in mini Chinese cabbage. Values given are means  $\pm$  SE ( $n=3$ ) and means followed by different letters are significantly different at  $P \leq 0.05$ , in each of the two light intensities



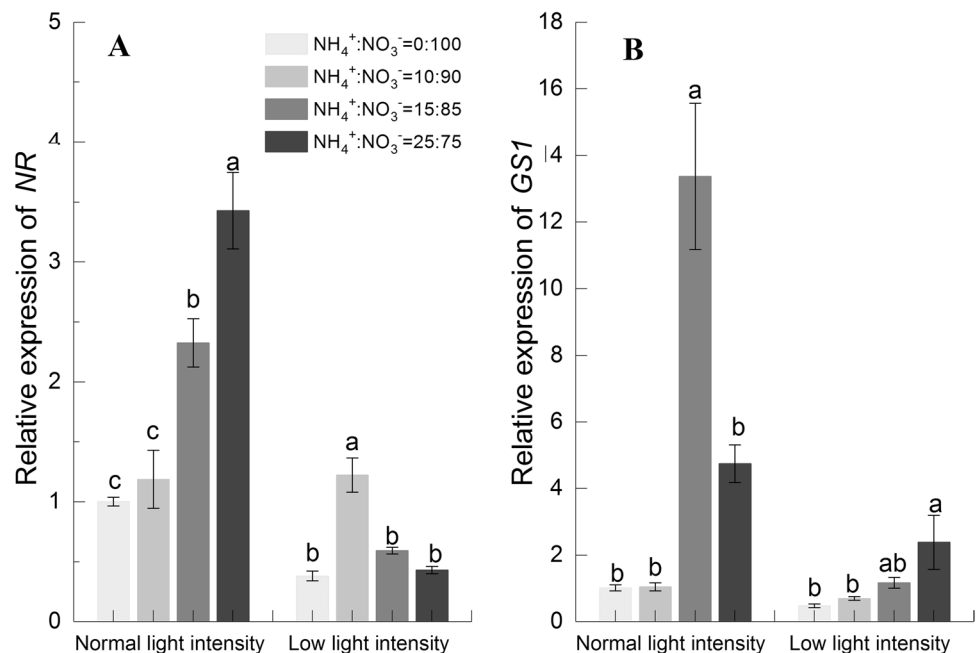
Low light intensity also decreased the GS activity but the seedlings treated with  $\text{NH}_4^+:\text{NO}_3^-$  (10:90) were the least affected. Thus, the moderate  $\text{NH}_4^+/\text{NO}_3^-$  ratio improved the activities of NR and GS of the seedlings exposed to different light intensity conditions.

### The Relative Expression Level of NR and GS1

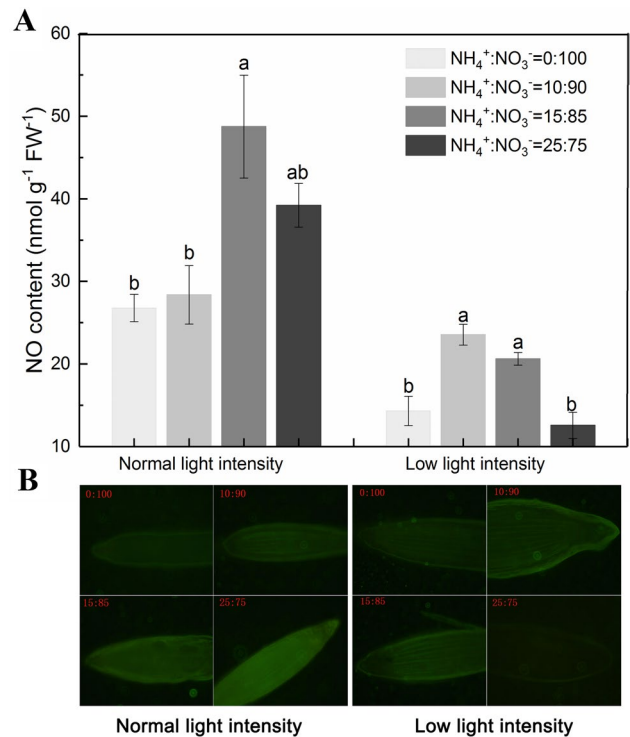
Our results showed that increasing the ammonium concentration in the nutrient solution led to up-regulation of the relative expression level of NR in the plants exposed to normal light intensity (Fig. 2a). The relative expression level of NR in the ammonium: nitrate (25:75) treated leaves was 3.4 times more as compared to the nitrate alone treated leaves. In plants under low light intensity, the relative expression level of NR in the plants which were treated with only  $\text{NH}_4^+/\text{NO}_3^-$  (10:90) was up-regulated by 1.22 times compared with the same  $\text{NH}_4^+/\text{NO}_3^-$  ratio under normal light intensity. However, the relative expression levels of NR in plants treated with other  $\text{NH}_4^+:\text{NO}_3^-$  ratios were all down-regulated under low light intensity.

From Fig. 2b, the relative expression level of GS1 in seedlings treated with  $\text{NH}_4^+:\text{NO}_3^-$  (15:85) was upregulated by 13.4 times more than that in plants with  $\text{NH}_4^+:\text{NO}_3^-$  (0:100) and significantly higher than other treatments under normal light intensity condition. In plants under low light intensity, the relative expression level of GS1 increased with increase in the ammonium concentration. The plant treated with  $\text{NH}_4^+:\text{NO}_3^-$  (25:75) reached the maximum relative expression level, which was 2.4 times more than that in plants supplied with  $\text{NH}_4^+:\text{NO}_3^-$  (0:100) and exposed to normal light intensity.

**Fig. 2** Effect of  $\text{NH}_4^+:\text{NO}_3^-$  ratios and light intensity on relative expression levels of NR (a) and GS1 (b) genes in mini Chinese cabbage. Values given are means  $\pm$  SE ( $n=3$ ) and means followed by different letters are significantly different at  $P \leq 0.05$ , in each of the two light intensities



### NO Level in Leaves and Image of NO-Dependent Fluorescence in Roots



**Fig. 3** Effects of  $\text{NH}_4^+:\text{NO}_3^-$  ratios and light intensity on NO content in leaves (a) and images of NO-dependent fluorescence in root (b) of mini Chinese cabbage. Values given are means  $\pm$  SE ( $n=3$ ) and means followed by different letters are significantly different at  $P \leq 0.05$ , in each of the two light intensities

To investigate the relationship between  $\text{NH}_4^+/\text{NO}_3^-$  ratio and NO production under different light intensity, we studied the NO level in leaves and observed the images of NO-dependent fluorescence in roots. In Fig. 3a, the NO level in the leaves initially increased and then decreased with increases in ammonium concentration in the nutrient solution. Under normal light intensity, the highest NO content was found in seedlings supplied with  $\text{NH}_4^+/\text{NO}_3^-$  (15:85) and this was 81.7% and 72.1% more than  $\text{NH}_4^+/\text{NO}_3^-$  (0:100) and  $\text{NH}_4^+/\text{NO}_3^-$  (10:90), respectively. Similarly, in the seedlings exposed to low light intensity, the NO level decreased across the treatments, but the highest NO level in the leaves was found in  $\text{NH}_4^+/\text{NO}_3^-$  (10:90) treatment. This was 64.3% and 87.3% more than  $\text{NH}_4^+/\text{NO}_3^-$  (0:100) and  $\text{NH}_4^+/\text{NO}_3^-$  (25:75), respectively. We also observed the NO-dependent fluorescence in the roots. The results showed that both light intensity and ammonium: nitrate ratio influenced the NO-dependent fluorescence intensity in root. We observed increasing the ammonium concentration in the solution of seedlings exposed to normal light intensity enhanced the NO-dependent fluorescence (Fig. 3b). However, the NO-dependent fluorescence intensity under low light intensity condition was initially enhanced and then later decreased with increases in ammonium concentration. Moreover, the NO-dependent fluorescence intensity in  $\text{NH}_4^+/\text{NO}_3^-$  (25:75) treatment was very low. Therefore, the application of moderate  $\text{NH}_4^+/\text{NO}_3^-$  ratio to the seedlings under low light intensity was beneficial for NO production.

### Light Intensity and $\text{NH}_4^+/\text{NO}_3^-$ Ratio Treatments Affect the Expressions of Key Proteins

To study the variations in the proteome profiles of mini Chinese cabbage seedlings leaves subjected to different light intensities and  $\text{NH}_4^+/\text{NO}_3^-$  ratios, we used the 2-DE gel to investigate the abundance patterns of proteins response to light intensity and nitrogen forms. We found most of the protein spots on the acidic area of the gel (pH 3–10, 17 cm). Hence, the narrow pH-range strips (pH 4–7, 17 cm) were applied in this experiment and approximately 1600 CBB-stained protein spots were detected (Figs. 4 and S2). Image analysis revealed that 51 reproducibly detected protein spots showed more than two-fold difference ( $P < 0.05$ ). These protein spots were subjected to in-gel digestion and submitted for protein identification. Out of the 51 protein spots analyzed by MALDI TOF/TOF MS/MS, 36 were assuredly assigned to protein sequences in the uniprot database of *Brassica*, while 15 spots did not search a significant match when the spectra were retrieved against the database (Table S1). In this study, Ribulose-bisphosphate carboxylase large chain (spots 24 and 29), Nucleic acid binding (spot 1, 3 and 6), Peroxiredoxin

(alkyl hydroperoxide reductase subunit C) (spot 7 and 11) were identified in more than one spot on the same gel (Table 1). The possible reason for the phenomenon was the presence of protein isoforms, post-translational modification or degradation (Gao et al. 2009).

The proteins were classified into ten groups based on their biochemical functions, which include photosynthesis, carbon and energy metabolism, translation and transcription, amino acid metabolism, protein folding, modification and degradation, stress and defense response, Signal transduction, cell growth/division, metabolism and some unknown functions (Table 1). The most common functions were photosynthesis (19.44%), translation and transcription (19.44%), protein folding, modification and degradation (16.67%), carbon metabolism and energy metabolism (13.89%), stress and defense responses (11.11%) (Fig. 5).

Figure 6 shows the results of a hierarchical clustering analysis of 36 proteins. Two main clusters were formed and cluster I included 7 protein spots (19.4%) which mainly showed decreased relative abundance under low light intensity and ammonium conditions. Cluster II included 29 protein spots (80.6%) which mainly showed increased relative abundance under low light intensity and ammonium conditions. The number of upregulated proteins induced by light intensity and  $\text{NH}_4^+/\text{NO}_3^-$  ratio treatments was significantly more than the down-regulated proteins. The protein spots in cluster I related to translation and transcription (spots 3 and 4), protein folding, modification and degradation (spots 10 and 22), stress and defense response (spot 33) and signal transduction (spot 35). The proteins in cluster II involved were related to photosynthesis (spots 5, 8, 14, 19, 24, 29 and 32), carbon metabolism and energy metabolism (spots 12, 13, 15, 25 and 28), stress and defense response (spots 7, 11 and 21), translation and transcription (spots 1, 2, 6, 16 and 27), protein folding, modification and degradation (spots 17, 18, 30 and 34), amino acid metabolism (spot 23), signal transduction (spot 26) and cell growth/division (spot 9). Our results showed that the abundance of proteins related to photosynthesis (spots 8, 14, 19, 24, 32 and 29), stress and defence (spots 7, 11 and 21), carbohydrate and energy metabolism (spots 13, 15 and 25), and translation and transcription (spots 6 and 16) were substantially increased under low light intensity and ammonium-induced conditions. Interestingly, the abundance of proteins related to photosynthesis (spot 5), which is Fructose-1,6-bisphosphatase I, was decreased under low light intensity and increased under ammonium-induced conditions regardless of the light treatment applied. These results, therefore, suggest that moderate  $\text{NH}_4^+/\text{NO}_3^-$  ratio induced changes in the biological processes of tolerance to low light intensity in mini Chinese cabbage seedlings.

**Table 1** Identification and analysis of proteins that were up-regulated and down-regulated in response to light intensity and ammonium: nitrate in leaves of mini Chinese cabbage

Functional category (n) Spot No. <sup>a</sup>	Protein name <sup>b</sup>	Plant species <sup>c</sup>	Accession No. <sup>d</sup>	Thr.MW/ pI <sup>e</sup> (kDa) pI	Protein score <sup>f</sup>	Coverage (%) <sup>g</sup>	Peptide match <sup>h</sup>	Cellular location <sup>i</sup>	Fold-change (Ratio ± SE) <sup>j</sup>		
									NR <sub>15,85</sub>	LR <sub>0,100</sub>	LR <sub>10,90</sub>
<i>Photosynthesis (7)</i>											
5	Fructose-1,6-bi- sphosphatase I (FBP)	<i>Brassica napus</i> (Rape)	tr A0A078J9M5	45.3 5.52	205	25	9	chlo	1.78 ± 0.02	0.41 ± 0.09	1.69 ± 0.02
8	Light-harvesting complex II chlorophyll a/b binding protein 3 (LHCB3)	<i>Brassica napus</i> (Rape)	tr Q39339	21.9 4.66	71	12	5	chlo	4.30 ± 0.13	1.98 ± 0.07	3.03 ± 0.13
14	Phosphoglycerate kinase (PGK)	<i>Brassica napus</i> (Rape)	tr A0A078CFK8	50.6 6.69	219	27	12	chlo	3.09 ± 0.04	2.04 ± 0.08	3.11 ± 0.05
19	Ferredoxin- NADP+ reductase (FNR)	<i>Brassica napus</i> (Rape)	tr A0A078D250	41.1 7.99	135	39	16	chlo	2.90 ± 0.07	2.35 ± 0.10	3.48 ± 0.05
24	Ribulose-bisphos- phate carboxylase large chain (rbcL)	<i>Brassica oleracea</i> (Wild cabbage)	tr A0A075M562	52.0 6.04	238	26	16	cyto	2.79 ± 0.06	3.06 ± 0.11	3.12 ± 0.05
29	Ribulose-bisphos- phate carboxylase large chain (rbcL)	<i>Brassica napus</i> (Rape)	tr G0WXXZ6	50.6 5.95	93	25	11	cyto	0.99 ± 0	1.32 ± 0.19	2.47 ± 0.09
32	Light-harvesting complex II chlorophyll a/b binding protein 1 (LHCB1)	<i>Brassica napus</i> (Rape)	tr A0A078EFM8	28.3 5.69	94	23	7	chlo	3.00 ± 0.03	2.05 ± 1.00	3.06 ± 0.04
<i>Carbon metabolism and energy metabolism (5)</i>											
12	Starch biosynthetic process	<i>Brassica rapa</i> subsp. <i>pekinensis</i> (Chinese cab- bage) ( <i>Brassica</i> <i>pekinensis</i> )	tr A0A078J107	49.1 5.2	78	23	12	chlo	2.00 ± 0.10	1.13 ± 0.12	2.15 ± 0.09
13	V-type H+-trans- porting ATPase subunit B (ATPeV1B)	<i>Brassica rapa</i> subsp. <i>pekinensis</i> (Chinese cab- bage) ( <i>Brassica</i> <i>pekinensis</i> )	tr M4D6W3	52.8 5.15	390	45	21	vacu	2.79 ± 0.12	2.17 ± 0.10	3.64 ± 0.13



Table 1 (continued)

Functional category (n) Spot No. <sup>a</sup>	Protein name <sup>b</sup>	Plant species <sup>c</sup>	Accession No. <sup>d</sup>	Thr.MW/ pI <sup>e</sup> (kDa) pI	Protein score <sup>f</sup>	Coverage (%) <sup>g</sup>	Peptide match <sup>h</sup>	Cellular location <sup>i</sup>	Fold-change (Ratio ± SE) <sup>j</sup>	
									NR <sub>15,85</sub>	LR <sub>0,100</sub>
15	V-type H +-trans- porting ATPase subunit A (ATPeV1A)	<i>Brassica rapa</i> <i>subsp. pekinensis</i> (Chinese cab- bage) ( <i>Brassica</i> <i>pekinensis</i> )	tr M4F1X7	69.0 5.12 304	35	21	cyto	2.13 ± 0.10	1.93 ± 0.06	3.17 ± 0.06
25	Adenylosuccinate synthase (purA)	<i>Brassica rapa</i> <i>subsp. pekinensis</i> (Chinese cab- bage) ( <i>Brassica</i> <i>pekinensis</i> )	tr M4CT00	53.7 6.48 261	24	16	chlo	2.33 ± 0.09	2.61 ± 0.09	2.76 ± 0.10
28	Enolase (ENO)	<i>Brassica napus</i> (Rape)	tr A0A078D646	46.3 5.39 147	28	13	cyto	1.22 ± 0.13	2.93 ± 0.15	1.77 ± 0.15
<i>Translation and transcription (7)</i>										
1	Nucleic acid bind- ing	<i>Brassica napus</i> (Rape)	tr A0A078DTQ4	32.9 4.78 162	28	12	chlo	1.71 ± 0.03	0.56 ± 0.03	1.53 ± 0.03
2	Small subunit ribo- somal protein S6 (RP-S6)	<i>Brassica napus</i> (Rape)	tr A0A078ID71	23.1 8.34 82	16	4	chlo	2.05 ± 0.10	0.40 ± 0.09	1.27 ± 0.06
3	Nucleic acid bind- ing	<i>Brassica oleracea</i> <i>var. oleracea</i>	tr A0A0D3DFX5	33.8 4.68 126	23	10	chlo	0.41 ± 0.02	0.07 ± 0.01	0.43 ± 0.0
4	RNA ligase (ATP) activity	<i>Brassica napus</i> (Rape)	tr A0A078GCD3	126.7 8.76 68	18	29	chlo	0.47 ± 0.02	0.54 ± 0.02	0.25 ± 0.01
6	Nucleic acid bind- ing	<i>Brassica rapa</i> <i>subsp. pekinensis</i> (Chinese cab- bage) ( <i>Brassica</i> <i>pekinensis</i> )	tr M4DRR7	33.4 4.86 74	20	10	chlo	3.68 ± 0.11	2.42 ± 0.08	3.66 ± 0.00
16	Elongation factor G (fusA)	<i>Brassica napus</i> (Rape)	tr A0A078I9U2	85.6 5.65 272	31	27	chlo	2.72 ± 0.06	1.72 ± 0.02	3.10 ± 0.09
27	Elongation factor Tu (tuf)	<i>Brassica oleracea</i> <i>var. oleracea</i>	tr A0A078D687	30.9 6.23 69	32	10	nucl	0.82 ± 0.00	2.94 ± 0.14	1.76 ± 0.10
<i>Amino acid metabolism (1)</i>										
23	Glutamate–cysteine ligase (gshA)	<i>Brassica rapa</i> <i>subsp. chinensis</i> (Pak-choi)	tr D0V0B1	57.9 5.79 94	19	13	chlo	2.24 ± 0.09	2.41 ± 0.07	2.48 ± 0.04

Table 1 (continued)

Spot No. <sup>a</sup>	Functional category (n)	Protein name <sup>b</sup>	Plant species <sup>c</sup>	Accession No. <sup>d</sup>	Thr.MW/ pI <sup>e</sup> (kDa) pI	Protein score <sup>f</sup>	Coverage (%) <sup>g</sup>	Peptide match <sup>h</sup>	Cellular location <sup>i</sup>	Fold-change (Ratio ± SE) <sup>j</sup>	
										NR <sub>15:85</sub>	LR <sub>0:100</sub>
Protein folding, modification and degradation (6)											
17		N-terminal protein myristoylation	<i>Brassica rapa</i> subsp. <i>pekinensis</i> (Chinese cabbage) ( <i>Brassica pekinensis</i> )	tr M4EPV1	76.6 5.19 78	22	2.49 ± 0.11	1.33 ± 0.14	chlo	2.49 ± 0.11	2.82 ± 0.11
18		ATP-dependent Clp protease ATP-binding subunit ClpC (clpC)	<i>Brassica napus</i> (Rape)	tr A0A078DC67	92.2 5.56 202	34	2.80 ± 0.10	1.93 ± 0.13	cyto	2.80 ± 0.10	2.76 ± 0.14
22		Peptidyl-prolyl cis-trans isomerase activity	<i>Brassica napus</i> (Rape)	tr A0A078HL04	47.4 5.07 122	24	1.10 ± 0.03	0.46 ± 0.04	chlo	1.10 ± 0.03	0.53 ± 0.02
10		Thioredoxin 1 (trxA)	<i>Brassica napus</i> (Rape)	tr A0A078IED5	16.6 9.06 71	60	0.38 ± 0.08	0.32 ± 0.05	chlo	0.38 ± 0.08	0.91 ± 0.06
30		ATP binding	<i>Brassica rapa</i> subsp. <i>pekinensis</i> (Chinese cabbage) ( <i>Brassica pekinensis</i> )	tr M4D677	61.9 4.99 761	40	2.95 ± 0.07	1.05 ± 0.09	chlo	2.95 ± 0.07	2.45 ± 0.05
34		Hydrolase activity	<i>Brassica napus</i> (Rape)	tr A0A078HPS2	34.9 8.3 148	41	1.20 ± 0.03	1.90 ± 0.04	chlo	1.20 ± 0.03	1.87 ± 0.04
Stress and defence response (4)											
7		Peroxioredoxin (alkyl hydroperoxide reductase subunit C) (PRDX)	<i>Brassica napus</i> (Rape)	tr A0A078F9Q1	28.9 5.97 89	38	4.88 ± 0.14	1.76 ± 0.07	chlo	4.88 ± 0.14	2.81 ± 0.15
11		Peroxioredoxin (alkyl hydroperoxide reductase subunit C) (PRDX)	<i>Brassica napus</i> (Rape)	tr A0A078EIW9	28.7 5.7 98	37	4.75 ± 0.05	1.85 ± 0.17	chlo	4.75 ± 0.05	3.11 ± 0.10
21		Peroxioredoxin (alkyl hydroperoxide reductase subunit C) (PRDX)	<i>Brassica napus</i> (Rape)	tr Q9FUC5	29.7 5.81 231	39	2.65 ± 0.07	1.75 ± 0.05	chlo	2.65 ± 0.07	1.78 ± 0.08
33		Cathepsin H (CTSH)	<i>Brassica napus</i> (Rape)	tr Q3HM22	40.0 5.56 348	22	0.68 ± 0.02	0.54 ± 0.06	vacu	0.68 ± 0.02	0.69 ± 0.04

Table 1 (continued)

Functional category (n) Spot No. <sup>a</sup>	Protein name <sup>b</sup>	Plant species <sup>c</sup>	Accession No. <sup>d</sup>	Thr.MW/ pI <sup>e</sup> (kDa) pI	Protein score <sup>f</sup>	Coverage (%) <sup>g</sup>	Peptide match <sup>h</sup>	Cellular location <sup>i</sup>	Fold-change (Ratio ± SE) <sup>j</sup>		
									NR <sub>15:85</sub>	LR <sub>0:100</sub> LR <sub>10:90</sub>	
<i>Signal transduction (2)</i>											
35	Auxin responsive GH3 gene family (GH3)	<i>Brassica napus</i> (Rape)	tr A0A078DWJ6	65.7 5.18 75	26	26	18	nucl	1.08 ± 0.08	1.14 ± 0.09	0.53 ± 0.06
36	Protein kinase activity	<i>Brassica rapa</i> subsp. <i>pekinensis</i> (Chinese cabbage) ( <i>Brassica pekinensis</i> )	tr M4DV34	46.9 6.2 72	23	23	15	vacu	3.13 ± 0.01	1.00 ± 0.04	1.12 ± 0.04
<i>Cell growth/division (1)</i>											
9	Ras-related protein Rab-8A (RAB8A)	<i>Brassica napus</i> (Rape)	tr A0A078JRP8	17.5 5.29 71	53	53	11	cysk	2.80 ± 0.10	1.28 ± 0.12	2.37 ± 0.17
<i>Metabolism (1)</i>											
20	Plastid lipid-associated protein 3, chloroplastic	<i>Brassica rapa</i> subsp. <i>pekinensis</i> (Chinese cabbage) ( <i>Brassica pekinensis</i> )	tr M4CM99	39.3 4.55 561	35	35	16	chlo	2.43 ± 0.04	2.05 ± 0.07	2.20 ± 0.08
<i>Unkown (2)</i>											
26	Uncharacterized protein	<i>Brassica oleracea</i> var. <i>oleracea</i>	tr A0A0D3CEU1	53.5 8.15 73	30	30	19	nucl	1.84 ± 0.19	1.91 ± 0.22	2.12 ± 0.18
31	BnaA05g10730D protein	<i>Brassica napus</i> (Rape)	tr A0A078IE55	137.9 4.8 72	17	17	30	golg	0.43 ± 0.04	0.74 ± 0.05	1.55 ± 0.05

<sup>a</sup>Assigned spot number as indicated in Fig. 4

<sup>b</sup>The name and functional categories of the proteins identified by matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF MS/MS). The MALDI-TOF/TOF MS/MS results are presented, if available

<sup>c</sup>The plant species that matched the peptides

<sup>d</sup>Database accession numbers from the Uniprot database

<sup>e</sup>Theoretical mass (kDa) and pI of identified proteins

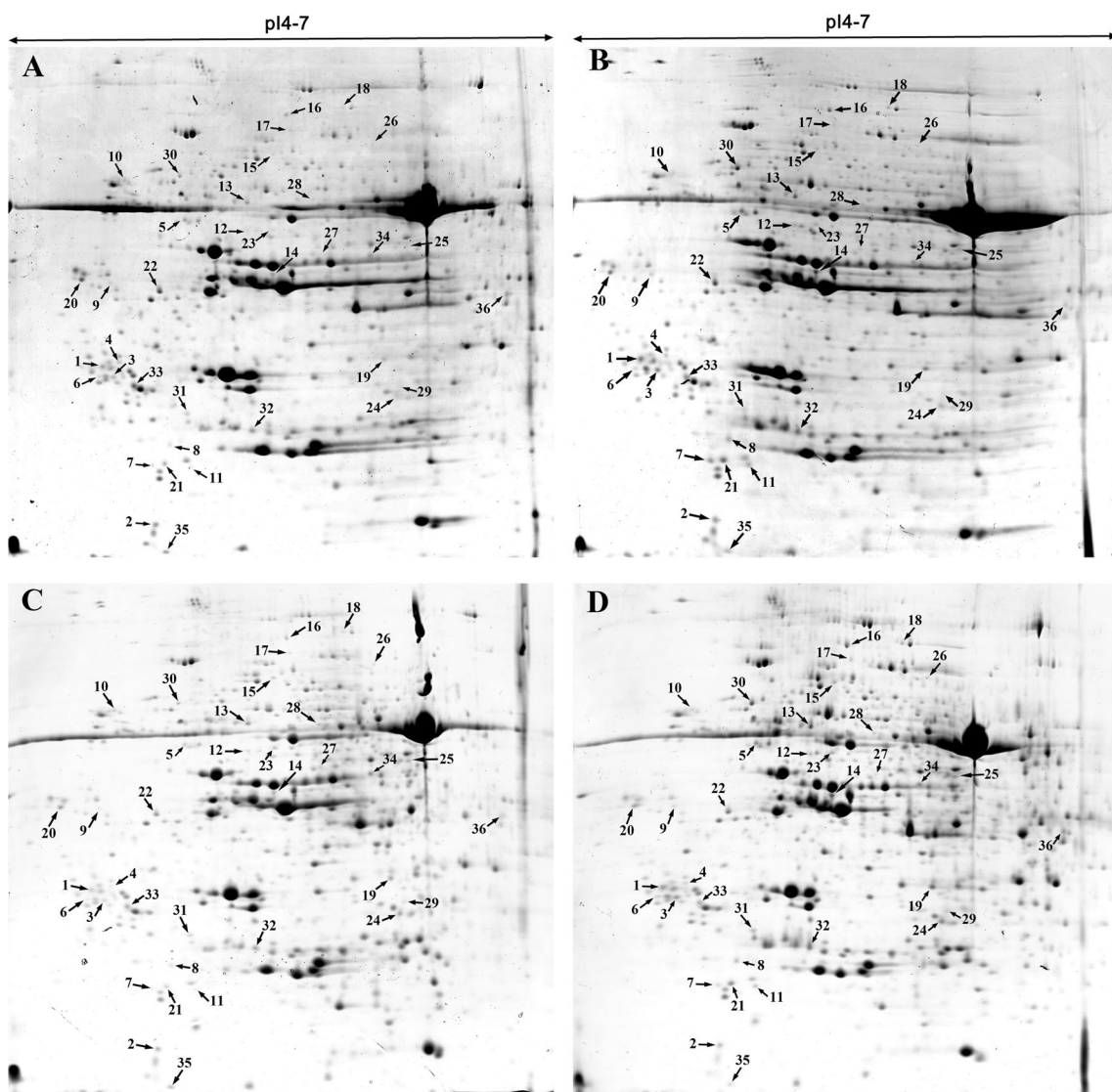
<sup>f</sup>The Mascot score obtained after searching against the Uniprot database

<sup>g</sup>The amino acid sequence coverage for the identified proteins

<sup>h</sup>The number of unique peptides identified for each protein

<sup>i</sup>The cellular location of identified proteins as predicted by Target P (<https://www.cbs.dtu.dk/services/TargetP>) and/or PSORT (<https://psort.hgc.jp/>). *chl* chloroplast; *cyto* cytoplasm; *vacu* vacuole; *nucl* nucleus; *cysk* cytoskeletal; *golg* golgin

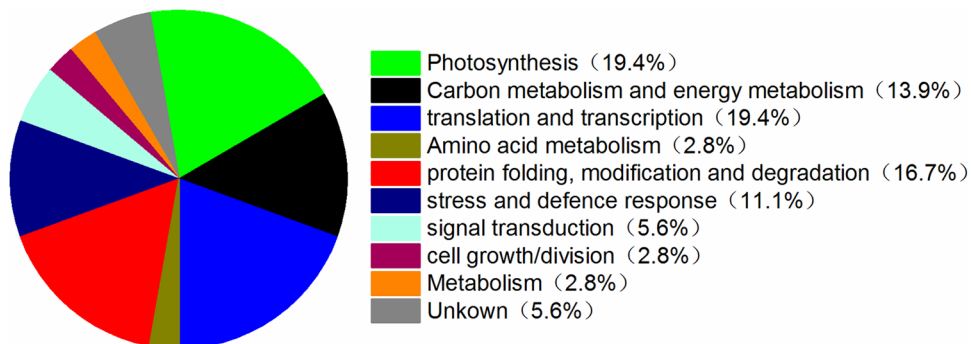
<sup>j</sup>Fold change is expressed as the ratio of the vol% between treatments (NH<sub>4</sub><sup>+</sup>:NO<sub>3</sub><sup>-</sup> = 15:85 in normal light intensity; NH<sub>4</sub><sup>+</sup>:NO<sub>3</sub><sup>-</sup> = 0:100 and NH<sub>4</sub><sup>+</sup>:NO<sub>3</sub><sup>-</sup> = 10:90 in low light intensity) and control (NH<sub>4</sub><sup>+</sup>:NO<sub>3</sub><sup>-</sup> = 0:100 in normal light intensity). Values are presented as means ± SE of triplicates (n = 3)

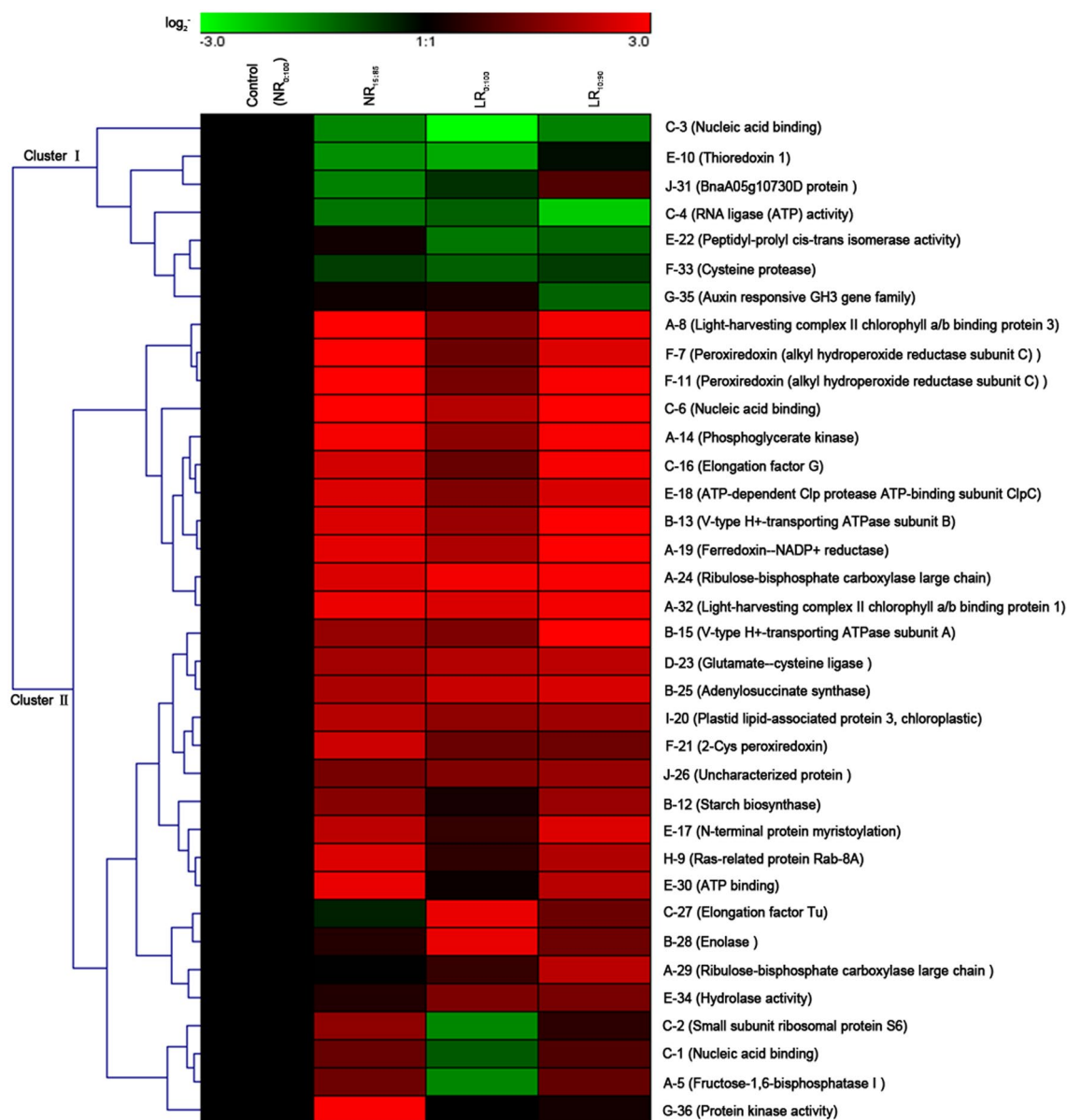


**Fig. 4** Representative two-dimensional gel electrophoresis (2-DE) gels of proteins extracted from leaves of mini Chinese cabbage treated with  $\text{NH}_4^+:\text{NO}_3^-$  0:100 (a) and 15:85 (b) under normal light

intensity,  $\text{NH}_4^+:\text{NO}_3^-$  0:100 (c) and 10:90 (d) under low light intensity for 14 days. Arrows indicate the 36 protein spots that were positively identified

**Fig. 5** Functional classification of the 36 identified differentially expressed proteins





**Fig. 6** Hierarchical clustering analysis of the expression profiles of the 36 identified proteins. The hierarchical cluster analysis was conducted using the Genesis 1.7.6 procedure and the  $\log_2^-$ -transformed values of fold-change ratios. The protein cluster is on the left side, and the treatment cluster is on the top. The four columns represent four treatments; columns 1, 2, 3 and 4 represent treatment for NR0:100 (Control), NR15:85, LR0:100 and LR10:90, respectively. The rows represent individual proteins. On the right, the letters before the spot numbers represent various functional categories of the pro-

teins: A Photosynthesis; B Carbon metabolism and energy metabolism; C Translation and transcription; D Amino acid metabolism; E Protein folding, modification and degradation; F Stress and defense response; G Signal transduction; H Cell growth/division; I Metabolism; J Unknown. The protein names in brackets are listed following spot numbers. The up and downregulated proteins are indicated in red and green, respectively. The intensity of colors increases with increasing expression differences, as shown in the legend

## Discussion

Ammonium and nitrate are the two forms of inorganic nitrogen essential for physiological and biochemical processes in higher plants. The degree of effectiveness of these two forms on plant growth and nutrient uptake is dependent on plant species and  $\text{NH}_4^+:\text{NO}_3^-$  ratio. Zhang et al. (2019)

have reported that the application of  $\text{NH}_4^+:\text{NO}_3^-$  at 25:75 stimulated root development, promote enzyme activities, and enhance the productivity and fruit quality in substrate cultivated pepper. Shang and Shen (2018) have reported that economic losses of pak choi (*Brassica chinensis* L.) caused by low light intensity and water deficit can be minimized by a proper adjustment of the ammonium/nitrate ratio. Similarly,

in our present study, the optimal ratio for mini Chinese cabbage growth under normal light intensity is 15:85, whereas the optimal ratio is 10:90 when mini Chinese cabbage grown under low light intensity. Therefore, it can be seen from above results that appropriate ammonium: nitrate ratio could mitigate low light stress in mini Chinese cabbage seedlings.

## Nitrogen Metabolism Affected by Light Intensity and $\text{NH}_4^+/\text{NO}_3^-$ Ratio

Nitrate absorbed by plants needs to be reduced to ammonium before it can be used by plants. The process requires the participation of NR and nitrite reductase (NiR), where NR is the key rate-limiting enzyme and a substrate-inducing enzyme. And NR is regulated at both the transcriptional and post-transcriptional levels by various endogenous and exogenous factors such as nitrate,  $\text{CO}_2$ , light, hormones, temperature, carbon and nitrogen metabolites (Sun et al. 2008). This process utilizes a lot of energy from the plant (Busch et al. 2018). The present results showed that with increases in  $\text{NH}_4^+$  concentration, the NR activity increased first and then decreased. In the seedlings grown under normal light intensity, the highest NR activity was attained when  $\text{NH}_4^+/\text{NO}_3^-$  was applied at 15:85 ratio. However, under low light intensity, the highest NR activity was attained when the seedlings were supplied  $\text{NH}_4^+/\text{NO}_3^-$  at 10:90 ratio. This is in line with the findings of Liu et al. (2017), who also found that the supply of moderate ammonium increases the NR activity in tomato plants compared to single nitrate under chilling temperature. However, his study also showed that NR activity decreased when the content of ammonium in the nutrient solution was increased under normal temperature. Huber et al. (1992) reported that light dephosphorylates nitrate reductase to increase its activity, while Champigny (1995) deemed that light changes the permeability of the cell membrane for  $\text{NO}_3^-$  to increase the NR activity. These opinions could explain the co-regulated effects of light intensity and nitrogen forms on NR activity. To further explore the effect of light intensity and nitrogen forms on NR activity, NR mRNA relative expression abundance in mini Chinese cabbage leaves was investigated subsequently. It has been reported that two kinds of NR in higher plants, NADH: NR (EC 1.6.6.1) and NAD(P)H: NR (EC 1.6.6.2). Among them, NADH: NR mainly exists in leaf tissue, while NAD(P)H: NR nearly distributes in all plant organism, especially in root (Sun et al. 2008). Therefore, in the present study, we chose the NR gene encoding NADH: NR to analyze the transcript abundance in mini Chinese cabbage leaves. Many research studies have demonstrated that external factors (such as substrate nitrate, nitrite, glutamine, carbon metabolites and hormones) and abiotic factors (such as light and salt stress) regulate the relative expression of NR

gene (Abouelsaad et al. 2016; Becker et al. 1992; Galan-gau et al. 1988; Vincentz et al. 1993). The results of our study indicate that low light intensity inhibited the expression of the NR gene, but the application of  $\text{NH}_4^+/\text{NO}_3^-$  ratio (10:90) significantly improved the NR expression under low light intensity. Similarly, Sun et al. (2008) reported that the lower nitrate supplies (0–30 mM) nitrate has positive effect on *BcNR* expression, while the *BcNR* transcript level reached a plateau value at the higher nitrate supplies (40 mM). They have predicted that *BcNR* expression is controlled by either the N-flux or plant N-status. In addition, the results also showed that the change in relative expression of NR gene and NR activity were not completely consistent. The possible reason for this phenomenon may be the regulation of post-transcriptional level (Chandok and Sopory 1996).

Glutamine synthetase (GS; EC 6.3.1.2) is a key enzyme of nitrogen assimilation, catalysing the synthesis of glutamine from ammonium and glutamate. The present study demonstrated that the GS activity initially increased and then decreased when the  $\text{NH}_4^+$  concentration increased, and it reached the maximum at ammonium: nitrate (15:85) under normal light intensity. Low light significantly reduced GS activity but ammonium: nitrate (10:90) significantly increased the GS activity. Horchani et al. (2010) demonstrated that the GS activity in tomato root was enhanced by  $\text{NH}_4^+$  concentrations up to 5 mM but did not change when  $\text{NH}_4^+$  concentrations was above 5 mM. However, the GS activity in tomato leaves did not change with  $\text{NH}_4^+$  content regardless of the concentration. Previous studies have shown that plant species with higher GS activities achieve higher tolerance to  $\text{NH}_4^+$  stress (Cruz et al. 2006; Fei et al. 2006; Glevarec et al. 2004). This study has also shown that the GS activity of the plants treated with  $\text{NH}_4^+$  was significantly higher than that of the plants which were supplied with only  $\text{NO}_3^-$ -fertilizer regardless of the light conditions. This observation was probably because the ammonium in the nutrient solution required higher GS activity to be assimilated such that it could promote nitrate metabolism under the action of NR. Regulation of GS begins with the GS genes. Thus, the GS mRNA abundance was then investigated to further study the effect of light intensity and nitrogen forms on GS activity. Plants have two types of GS isoenzyme that are localized in different compartments: one in the cytosol (GS1) and the other in the chloroplast (GS2). GS1 is the major form of GS in plant and directly converts ammonium taken up by plant roots to glutamine (Sun et al. 2010). They have also reported that *BcGS1* is a novel member of the GS family in plants. *BcGS1* was significantly related to N assimilation in Chinese cabbage, demonstrating that this gene plays an important role in plant growth and development (Sun et al. 2010). Our results showed that the relative expression of *GS1* gene initially increased and then decreased when the  $\text{NH}_4^+$  concentration was increased, and it reached the

maximum when  $\text{NH}_4^+/\text{NO}_3^-$  ratio was 15:85 under normal light. Low light significantly downregulated the relative expression of *GS1* gene, but its relative expression level gradually increased with increases in  $\text{NH}_4^+$  concentration in the nutrient solution. Light stimulated *GS1* mRNA accumulation during plant development, the levels of *GS1* transcripts increased in dark-grown seedlings when moved to light and decreased in dark-adapted seedlings (Zhang et al. 2006). It has been reviewed that the abundance of mRNA, protein and activity of *GS2* in senescing leaves of many species were declined, coinciding with an increase in abundance of cytosolic *GS* (*GS1*), indicating that cytosolic *GS* plays a role in the nitrogen assimilation and recycling (Bernard and Habash 2009). In rice, the homozygous lines of three *OsGS1;1*-knockout mutants showed a decline in cytosolic *GS* enzyme activity (Tabuchi et al. 2005). In our study, the variation in *GS* activity was not completely consistent with the relative expression of *GS1* gene, which may be due to the regulatory mechanism of post-transcriptional levels. To sum up, the optimum ratios of  $\text{NH}_4^+/\text{NO}_3^-$  under different light conditions improved the efficiency of nitrogen metabolism in the plants by increasing the activities of key enzymes and the relative expression level related to nitrogen metabolism.

As a signal molecule, NO plays a wide range of physiological functions in including growth of root and pollen tube tip, stomatal closure, flowering, senescence, as well as in plants response to various abiotic stresses (Yu et al. 2014). It is widely accepted that nitrite is a main substrate for NO synthesis through enzymatic and non-enzymatic routes. The enzymatic pathway involves mainly NR which might work together with NOFNiR (NO-forming nitrite reductase) as found in *Chlamydomonas reinhardtii* (Chamizo-Ampudia et al. 2016). Some scientists suggest that other processes rely on nitrite reduction at acidic pH or through the mitochondrial electron transport chain under anaerobic conditions (Astier et al. 2018; Gupta et al. 2011). Previous studies have shown that NO could increase leaf chlorophyll content and regulate stomatal behavior in plants (Desikan et al. 2004; Garcia-Mata and Lamattina 2001), and some studies have also shown that chloroplasts are organs that synthesize NO in plants, and their functions are crucial for maintaining NO content in soybean cotyledons (Galatro et al. 2013). In addition, studies have shown that a certain concentration of NO could promote the occurrence of adventitious roots in plants (Liao et al. 2012). The results showed that the appropriate  $\text{NH}_4^+/\text{NO}_3^-$  ratio increased the NO content in the leaves and increased the NO content in the root tip of the plant. It is preliminarily judged that NO might be involved in the regulation progress of moderate  $\text{NH}_4^+/\text{NO}_3^-$  ratio in mini Chinese cabbage seedlings exposed to low light intensity.

## Key Proteins Expressed Under Different Light Intensities and $\text{NH}_4^+/\text{NO}_3^-$ Ratios

Plant response to various stresses is a complicated process which depends on various types of proteins. In the present study, 36 proteins which were markedly altered were identified in the leaves of mini Chinese cabbage grown under normal and low light intensities and supplied with different  $\text{NH}_4^+/\text{NO}_3^-$  ratios. The 36 identified proteins were categorized into 10 according to their functions. Some of the major functions include photosynthesis-related proteins, carbon metabolism- and energy metabolism-related proteins, stress- and defense-related proteins, and folding-, modification-, and degradation-related proteins response to light intensity and  $\text{NH}_4^+/\text{NO}_3^-$  ratio are discussed.

### Proteins Related to Photosynthesis

Photosynthesis is one of the key factors determining biomass accumulation and yield. It consists of two phases: photoreaction and dark reaction. Our study identified seven proteins related to photosynthesis and three out of the seven were involved in the photoreaction phase, namely PSII light-harvesting complex chlorophyll a/b binding protein 3 (LHCB3), ferredoxin-NADP+ reductase (FNR) and PSII light-harvesting complex chlorophyll a/b binding protein 1 (LHCB1), respectively (Carrillo and Ceccarelli 2003; Crepin and Caffarri 2018). Those protein expression levels were up-regulated in different degrees under low light and ammonium conditions (Table 1, Fig. 6), indicating that the supply of appropriate  $\text{NH}_4^+$  to plants under low light enhanced the expression levels of LHCB and FNR. Thus, this improved the efficiency of light energy absorption and the transport rate of photosynthetic electron chain, which probably provided sufficient reducing power and ATP for the dark reaction in photosynthesis. The present result is consistent with our previous study on electron transport rate (ETR) in mini Chinese cabbage under different light intensities and  $\text{NH}_4^+:\text{NO}_3^-$  ratios (Hu et al. 2017). In addition, another four proteins which play roles in the Calvin cycle were identified. These were the ribulose 1, 5-diphosphate large subunit (rbcL), fructose-1,6-bisphosphatase (FBPase) and phosphoglycerate kinase (PGK), respectively. These proteins were involved in the carboxylation, reduction and regeneration phases of the Calvin cycle, respectively. Our study showed that low light intensity downregulated the abundance of FBPase but the application of  $\text{NH}_4^+/\text{NO}_3^-$  (10:90) upregulated the abundance of FBPase protein in the leaves. This observation is consistent with our earlier results which showed that the relative expression levels of *FBPase* gene and FBPase activity were upregulated with the application of  $\text{NH}_4^+/\text{NO}_3^-$  at 10:90 ratio under low light condition (Hu et al. 2017). Ohashi et al. (2018) indicated that the *OscFBP2*

expression was upregulated in the shoot basal portions of the wild type by supplying  $\text{NH}_4^+$  but not in those of the *gs1;2* mutants. Moreover, our study showed that the expression of *rbcL* and PGK protein was upregulated under low light and ammonium treatments (Table 1, Fig. 6). In our previous study, the activity of Rubisco enzyme decreased under low light, but the  $\text{NH}_4^+/\text{NO}_3^-$  (10:90) enhanced its activity (Hu et al. 2017). It is observed that upregulated expression of protein does not necessarily enhance the activity of the enzyme. Since the activity of Rubisco is regulated by light, the enzyme activity is also related to other environmental factors to some extent (Bloom and Kameritsch 2017; Gao et al. 2018). Yerramsetty et al. (2017) reported that colocalization occurred only in photosynthetic tissues, and the nuclear-encoded *rbcL* RNA S1 binding domain protein (RLSB) and Rubisco were simultaneously induced by light at post-transcriptional levels. Guo et al. (2007) revealed that water stress influenced the Rubisco activity than stomatal limitation, and the effects could be regulated by N forms, which the sole  $\text{NH}_4^+$  supply significantly increased Rubisco content and activity in newly expanded leaves. Thus, our results suggest that moderate  $\text{NH}_4^+/\text{NO}_3^-$  ratio enhance the expression levels of photosynthesis-related proteins in plants exposed to low light intensity stress.

### Proteins Related to Carbon and Energy Metabolism

A total of 5 proteins (Spot 12, 13, 15, 25, and 28) involved in carbon metabolism and energy metabolism was identified. Among these, protein 12 was identified as starch synthase (SSBase), which is involved in the metabolic pathway of starch synthesis. It is an important way to store polysaccharides in plants. Its expression level was up-regulated (Table 1, Fig. 6), indicating that ammonium: nitrate ratio enhanced the expression level of SSBase and increased the photosynthetic products of the plants. The results of our experiment agrees with the findings of Hu et al. (2017), who reported that the starch content reached maximum in  $\text{NH}_4^+/\text{NO}_3^-$  (15:85) treated plants under normal light intensity and in  $\text{NH}_4^+/\text{NO}_3^-$  (10:90) treated plants under low light intensity. Proteins 13 and 15 were identified as the B subunit and the A subunit on the V-type ATPase V1 subunit, respectively. V-ATPase is a heteromultimer,  $\text{H}^+$  transporter that is ubiquitous in eukaryotes. Most of the proteins are located on the plasma membrane and provide energy for different transport processes. A remarkable feature of V-ATPase is the regulation of enzyme activity and ion transport activity by a reversible hydrolysis reaction. V-type  $\text{H}^+$ -ATPase consists of two parts, V1 and V0. V1 is mainly responsible for the ATP hydrolysis, while V0 is mainly responsible for the transport of protons. The A subunit of V1 is a highly conserved subunit with an active site that catalyzes the ATP hydrolysis. The B subunit of V-type  $\text{H}^+$ -ATPase V1 is also a

fairly conserved subunit, which contains regulatory sites and plays vital role in the formation of catalytic sites (Huss et al. 2011; Zhao et al. 2015). The two proteins were upregulated, indicating that ammonium: nitrate ratio induced the expression of V-type ATPase V1 subtype and enhanced the ability of ATP hydrolysis, thus providing sufficient energy for various physiological processes. Protein 25 was identified as adenine succinate synthase, which regulates the adenine nucleotide biosynthesis pathway and catalyzes the formation of adenylate succinate, GDP,  $3\text{H}^+$  and phosphate by L-aspartate-binding GTP under the action of aspartate ligase, whose expression level was up-regulated under low light intensity and ammonium: nitrate ratio treatments. Protein 28 was identified as enolase (ENO), involved in the glycolysis process, catalyzing 2-phosphoglycerate form the high-energy phosphate molecule phosphoenolpyruvate. Zhu et al. (2018) reported that nitrogen deprivation reduced enolase protein abundance. We speculate that the sufficient energy plants need for various physiological metabolic processes were mainly acquired through ATP hydrolysis and glycolysis pathway.

### Proteins Related to Stress and Defense

In this study, we found a total of four differentially expressed proteins (Spots 7, 11, 21, and 33) which are associated with stress and defense responses in plants. The proteins included peroxidase reductase (alkyl hydroperoxide reductase C) (AhpC, Spot 7 and 11), a peroxide reductase with two conserved cysteine residues (2-Cys Prx, Spot 21) and a cysteine protease (CysP, Spot 33). Among these proteins, the CysP protein was downregulated, and the other three proteins were upregulated (Table 1, Fig. 6). AhpC is a peroxidase superfamily that is widely found in prokaryotic and eukaryotic organisms. It can remove ROS from plants to regulate the concentration of ROS in plants, protecting chloroplasts and other organelles from oxidative damage. It scavenges reactive oxygen species, stimulates cell proliferation and participates in cell signal transduction (Cha et al. 2015). Since Chae et al. (1994) first proposed the concept of peroxiredoxin (Prx), it had been widely used and it is still a significant area for study. In this study, the 2-Cys Prx was up-regulated, indicating that an antioxidant response to low light stress was induced. In line with our results, Kim et al. (2010) demonstrated that high levels of 2-Cys Prx in transgenic plants protect leaves exposed to heat stress from oxidative damage. Kim et al. (2009) revealed that the protein structure of 2-Cys Prx in Chinese cabbage changed in response to heat shock and oxidative stress. Therefore, the 2-Cys Prx, which is a stress-defense-related protein, plays a significant role in the plants response to abiotic stress. Cysteine protease (CysP), as a proteolytic enzyme, is mainly involved in the degradation of denatured or stressed proteins,



thereby providing free amino acids or peptides for the synthesis of new proteins. It is mainly found in the chloroplasts and vacuoles of plants. CysP in chloroplasts can participate in the degradation of Rubisco large subunits (Grudkowska and Zagdanska 2004), and it is also involved in the programmed cell death in plants (Singh et al. 2003; Solomon et al. 1999). The CysP identified in our experiment was down-regulated in the vacuole, indicating that the degree of hydrolysis of stored proteins in the leaves under low light and  $\text{NH}_4^+/\text{NO}_3^-$  treatment was reduced. Thus, the soluble protein content in the leaves increased, enhancing the penetration adjustability of the plants. Therefore, our study indicated that the low light tolerance of the plant increased in optimum  $\text{NH}_4^+/\text{NO}_3^-$ -treated plants, to a certain degree because low light intensity probably triggered the upregulation of AhpC and 2-Cys Prx, and induced the downregulation of cysteine protease, which improved the ROS scavenging ability and increased the soluble protein content.

### Proteins Related to Folding, Modification, and Degradation

We identified a total of six proteins associated with protein folding, modification and degradation (Spot 17, 18, 22, 10, 30 and 34) in this study. Protein 10 was identified as thioredoxin 1 (TrxA), which is mainly involved in the post-transcriptional protein modification process and regulates the disulfide exchange reaction (Bessette et al. 1999). It is a kind of multifunctional acidic protein widely distributed in organisms with a molecular weight of about 12 kDa. It is involved in a series of physiological and biochemical reactions of cells, including the regulation of transcription factors and enzyme activity (Balmer et al. 2003; Buchanan et al. 1994). It is also an important enzyme activity-regulating protein whose function is largely dependent on the reduction of disulfide bonds in target proteins by thioredoxin (Cheng et al. 2017). Protein 17 was identified as a protein N-terminal myristoylation, mainly involved in the modification of the N-terminal amino acid residue of the protein and covalently linked to the tetradecanoyl group at the N-terminal amino acid residue of the protein. Protein 18 was identified as ATP-dependent ClpC protease, and ATP-binding subunit clpC and it is a regulatory subunit of Clp protease. Clp protease plays an important regulatory role, which eliminates some intracellular potentially toxic irreversible damage proteins and maintains cell physiological functions in normal levels (Frees et al. 2004, 2014). Protein 22 was identified as a peptidyl-prolyl *cis*–*trans* isomerase involved in the protein folding process, facilitating the assembly of covalent or non-covalent single-chain polypeptides or multi-subunit proteins into the correct tertiary structure, and catalyzing the *cis*–*trans* isomerization of peptidyl-proline,  $\Omega$  changes from 180 to 0 after the reaction (Fanghanel and Fischer

2004). Our results showed that the abundance of the protein was down-regulated in low light intensity regardless of the nitrogen source supplied to the plants. The result agrees with the findings of Rahman et al. (2015), who indicated that the abundance of peptidyl prolyl *cis*–*trans* isomerase (PPIase) was decreased in *TERF1* overexpressed sugarcane under PEG-induced drought stress. Protein 30 was identified as an ATP-binding protein that binds selectively and non-covalently to ATP (adenosine triphosphate) and is a ubiquitous coenzyme and enzyme modulator. Protein 34 was identified as a hydrolase, which catalyzes various bonds of hydrolysis, such as cutting off C–O, C–N, C–C, and phosphoric anhydride bonds. Hydrolases are the systematic names of all enzymes belonging to EC3, which are a class of enzymes that catalyze the hydrolysis of substrates to form two compounds. This protein was up-regulated and the degree of up-regulation was greater in nitrate-treated plants than in  $\text{NH}_4^+/\text{NO}_3^-$ -treated plants (Table 1, Fig. 6). In summary, the abundance of TrxA was downregulated under low light and  $\text{NH}_4^+/\text{NO}_3^-$  ratio, while the expression levels of N-terminal myristoylation, ATP-dependent ClpC protease, ATP-binding protein were up-regulated suggesting the possibility of post-transcriptional changes in the response of protein to low light.

### Conclusion

The results of our experiment revealed that supply of  $\text{NH}_4^+/\text{NO}_3^-$  (10:90) to mini Chinese cabbage seedlings improved the seedlings tolerance to low light intensity by enhancing nitrogen metabolism and increasing the NO level in the seedlings. Moreover, 36 differentially expressed proteins were classified into ten functional groups. The main functions of these proteins were related to photosynthesis, carbohydrate and energy metabolism, stress and defense and protein folding and degradation responses. Hierarchical clustering results showed that the proteins related to photosynthesis, energy production and reactive oxygen species scavenging promoted low light intensity tolerance of mini Chinese cabbage, especially when the plants were fertilized with moderate  $\text{NH}_4^+/\text{NO}_3^-$ . Future research should focus on the identification of key  $\text{NH}_4^+/\text{NO}_3^-$ -responsive proteins in mini Chinese cabbage that underline the plants tolerance to low light intensity.

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## Compliance with Ethical Standards

**Conflict of interest** On behalf of all authors, the corresponding author states that there is no conflict of interest.

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