Plant Physiology and Biochemistry 70 (2013) 14–20

Contents lists available at SciVerse ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Genotypic differences in nitrate uptake, translocation and assimilation of two Chinese cabbage cultivars [*Brassica campestris* L. ssp. *Chinensis* (L.)]

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ARTICLE INFO

Article history:
Received 8 February 2013
Accepted 29 April 2013
Available online 17 May 2013

Keywords:
Chinese cabbage
Nitrate
Uptake
Translocation
Assimilation

ABSTRACT

A hydroponic trial was conducted to investigate genotypic differences in nitrate uptake, translocation and assimilation between low nitrate accumulator L18 and high accumulator H96 of Chinese cabbage [*Brassica campestris* L. ssp. *Chinensis* (L.)]. The results suggested that H96 could uptake more nitrate than L18 in the root but lower transport inside leaf cells and assimilation in the leaf. It was showed that root morphology parameters – length, surface area and volume of H96 were 18.0%, 31.6% and 46.5% higher than L18. Nitrate transporters *NRT1.1* and *NRT2.1* transcription levels were 41.6% and 269.6% higher than those of L18 respectively. *NRT1.1* and *NRT2.1* expression amount in leaf blade of two cultivars were opposite to in the root, L18 *NRT1.1* and *NRT2.1* expressions were 279.2% and 80.0% higher than H96. In addition, nitrate assimilation capacity of L18 was significantly higher than H96 in leaf. It was showed that Nitrate Reductase (NR; EC 1.7.1.1) activity and *NIA* expression level of L18 leaf were 234 0.4% and 105.4% higher than those of H96, Glutamine Synthetase (GS; EC 6.3.1.2) activity, *Gln1* and *Gln2* expression levels in the leaf blade of L18 were 43.9%, 331.5% and 124.8% higher than those of H96 respectively. Nitrate assimilation products-Glu, total amino acid, soluble protein content in the leaf of L18 were all significantly higher than those of H96. The results above suggested that nitrate accumulation differences were due to differential capacities to uptake, mechanisms for nitrate transport in leaves and assimilate nitrate. Comparing contribution of three aspects in nitrate accumulation, translocation and assimilation were contributed more in low nitrate concentration in the leaf blade.

1. Introduction

High nitrate accumulation in vegetable is harmful to human health because of the possibility of resulting in gastric cancer, thyroid cancer and other diseases [1,2]. Vegetables, the major source of daily nitrate intake by human beings, is responsible for 75–80% of the total intake [3]. Thus reducing nitrate content in Chinese cabbage can decrease a risk of human illness. Chinese cabbage [*Brassica campestris* L. ssp. *Chinensis* (L.)] was classified as high nitrate concentration vegetable with nitrate ranging from 1500 to 4000 mg N kg^{-1} [4]. Differences in nitrate accumulation among cultivars were due to their differential capacities in absorbing, reducing and assimilating nitrate [5–7].

Uptake of nitrate in plants are mediated by nitrate transporters such as *NRT1* (Low-affinity transport system, LATS) and *NRT2* (High-affinity transport system, HATS) families [8]. In *Arabidopsis*, low- and high-affinity transport systems are mediated primarily by *AtNRT1.1* and *AtNRT2.1* [9]. Expression of *AtNRT1.1* was induced by NO_3^- in the roots rapidly, and reached a maximum level that was 2.5 times higher than that of the 0 h. This high level of transcription was sustained from 12 h to 48 h [9]. Further studies indicated that *AtNRT1.1* was a dual-affinity nitrate transporter, its mode of action being switched by phosphorylation and dephosphorylation of threonine T101 [10,11]. In seven *NRT2* members, *AtNRT2.1* transcript abundance in the root was in significant correlation ($r^2 = 0.74$) with HATS activity [9]. Several mutants disrupted for both *AtNRT2.1* and *AtNRT2.2* genes have lost up to 75% of the high-affinity nitrate uptake activity [12], with *AtNRT2.2* expression pattern marching the HATS profile only during the first 3 h [9]. Thus, *AtNRT2.1* appears to be a more likely candidate for HATS influx. In Chinese cabbage,

Abbreviations: N, nitrogen; LATS, low-affinity transport system; HATS, high-affinity transport system; NR, nitrate reductase; NRA, nitrate reductase activity; NiR, nitrite reductase; GS, glutamine synthetase; GSA, glutamine synthetase activity; RT-PCR, real-time polymerase chain reaction; Glu, glutamic acid; GOGAT, glutamate synthase.

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http://dx.doi.org/10.1016/j.plaphy.2013.04.027

different expression patterns of *BnNRT1* and *BnNRT2* have been suggested to partly explain the different nitrate concentration between cultivars [13].

Once taken up by plants, NO_3^- is either stored in the vacuole or reduced to NO_2^- by Nitrate Reductase (NR; EC 1.7.1.1) in cytosol. NR as rate-limiting enzyme catalyzes the first step in nitrate reduction and it have been found to have a negative effect on nitrate concentration [14]. Besides that, its activity and gene expression levels also contribute to differences between high and low nitrate accumulation of Chinese cabbage [15]. Then NO_2^- enters into the chloroplast (or plastid) and reduces to NH_4^+ by Nitrite Reductase (NiR; EC 1.7.7.1). NH_4^+ can then be assimilated into carbon by the action of Glutamine Synthetase (GS; EC 6.3.1.2) [16]. Biochemical studies have shown that distinct isoenzymes of GS were located in cytosol (GS1) and chloroplast/plastid (GS2) of numerous plant species [17]. GS1 plays an important role in primary nitrogen assimilation in roots, and functions to generate Gln for transporting in phloem in the stem [18]. GS2 plays a crucial role in reassimilation of NH_4^+ released via photorespiration in plants [19]. GS (both GS1 and GS2) activity determined whether or not cultivar had high nitrogen use efficiency under low nitrogen condition [20].

In this study, we hypothesized that nitrate accumulation difference between two Chinese cabbage cultivars may be due to differences in nitrate uptake, translocation and assimilation. Relative expression levels of NRT, NR and GS genes in tissues were measured by RT-PCR. The NR, GS activity, time-course analysis of nitrate levels in nutrient solution and root morphological parameters were also measured, with the aim of exploring the main factors that contribute to nitrate accumulation in plants and finding the reasons for the different accumulation patterns between the two cultivars.

2. Materials and methods

2.1. Plant material

The experiments were conducted in greenhouse using high nitrate accumulator H96 and low accumulator L18 of Chinese cabbage [*B. campestris* L. ssp. *Chinensis* (L.)]. Seeds were germinated in distilled water at room temperature for 7 days, and then every eight seedlings were transferred to one-fourth strength Hoagland – Arnon nutrient solution (1.18 g L^{-1} $Ca(NO_3)_2{\cdot}4H_2O$; 0.14 g L^{-1} KH_2PO_4; 0.51 g L^{-1} KNO_3; 0.493 g L^{-1} $MgSO_4{\cdot}7H_2O$; 13 mg L^{-1} EDTA-Fe; 2.86 mg L^{-1} H_3BO_3; 1.81 mg L^{-1} $MnCl_2{\cdot}4H_2O$; 0.22 mg L^{-1} $ZnSO_4{\cdot}7H_2O$; 0.08 mg L^{-1} $CuSO_4{\cdot}5H_2O$; 0.016 mg L^{-1} $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$) in 4 L plastic box. Each cultivar was replicated four times. One-fourth and half-strength nutrient solution were used in the first and second five days, respectively, and then full-strength was used till seedlings were 30 days old. The solution was refreshed every five days.

2.2. Determination of root morphology

On the 15th day after transplanting, root morphological parameters of four seedlings per cultivar were estimated by a root scanner and the WINRhizo optical scanner-based image analysis system [21].

2.3. Measurement of nitrate uptake

Nitrate uptake was determined by the disappearance of NO_3^- from the nutrient solution and expressed as μmol NO_3^- fresh root weight. Nitrate uptake rate represents a net NO_3^- flux into the root and this influx was expressed as μmol NO_3^- fresh weight per hour. Four-week-old seedlings were transferred to 100 mL plastic pots (one seedling per pot) and grown for 2 d in Hoagland–Arnon nutrient solution. The nutrient solution was then replaced with a pretreatment solution containing 0.2 mM $CaSO_4{\cdot}2H_2O$. After 24 h pretreatment, the seedlings were transferred to basal Hoagland–Arnon nutrient solution. At each time interval (0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h), NO_3^- concentration in nutrient solution were determined [22].

2.4. Measurement of nitrate content

Nitrate content determination followed the method described by Li [23]. Plant samples were cut and separated into leaf blade, petiole and root. Approximately 1 g sample of certain tissue was suspended in 10 mL deionized water and kept for 10 min in boiling water bath. Then the sample was diluted to 50 mL and shaken thoroughly. The supernatant was used to determine NO_3^- concentration. The assay mixture (samples of 0.5 mL containing 0.4 mL 5% salicylic acid-sulfuric acid and 0.1 mL of the extract) was retained for 20 min at room temperature, then mixed with 9.5 mL 8% (w/v) NaOH and shaken thoroughly. After the mixture had cooled, its absorbance was measured at 410 nm.

2.5. Determination of enzymes activity and metabolite content

For assessment of NR activity, approximately 1 g of tissues sample was extracted by 4 mL cold 0.1 M phosphate buffer (pH 7.5). The extracts were then centrifuged at 4000 rpm at 4 °C for 15 min. The supernatant was used to determine NR activity using the method of Li [23].

The method of determining GS activity was described by O'Neal and Joy [24]. Samples containing approximately 1 g of frozen plant tissue each were homogenized in 3 mL cold 0.05 M Tris-HCl buffer solution (pH 8.0) contain 2 mM $MgSO_4$, 2 mM DTT and 0.4 mM sucrose. Homogenate was centrifuged at 15,000 g for 20 min at 0–4 °C. The reaction mixture (3 mL samples each containing 1.6 mL 0.1 M Tris-HCl buffer (pH 7.4) mixed with 80 mM $MgSO_4$, 20 mM glutamate, 20 mM cysteic acid, 2 mM EGTA and 80 mM hydroxylamine hydrochloride; 0.7 mL 40 mM ATP and 0.7 mL enzyme extract) was incubated for 30 min at 37 °C and a blank without hydroxylamine hydrochloride was also run simultaneously. The reaction was stopped by adding 1 mL $FeCl_3$ reagent (containing 0.2 M TCA, 0.37 M $FeCl_3$ and 0.6 M HCl). After 10 min, homogenate was centrifuged at 5000 g for 10 min, and the supernatant was measured at 540 nm.

Free NH_4^+ content in the supernatant was determined by the Berthelot color reaction method [25], the absorbance at 480 nm was determined, and NH_4^+ content were calculated from the standard curve of NH_4Cl. Soluble protein concentration was determined by coomassie brilliant blue method [23]. The samples used in determining NH_4^+ and soluble protein were the same to GS activity analysis. Concentration of Glu and total amino acid content in tissues were determined by automatic amino acid analyzer [26].

2.6. Determination of genes expression levels

Total RNA extracted from leaf blade, petiole and root were using TRIzol reagent (Invitrogen, USA). First-strand cDNA was synthesized from 1 μg of total RNA using M-MLV Reverse Transcriptase (Promega, USA) according to the manufacturer's instructions and the product was diluted to concentration of 1000 ng μL^{-1}. Quantitative PCR for detecting the expression of *NIA, NRT1.1, NRT2.1, Gln1, Gln2* and *Actin* gene were performed using the SYBR Green Real-Time PCR Master Mix Kit (TOYOBO, Japan) and the CFX96™ Real-Time PCR Detection System (Bio-Red, USA). A total volume of 20 μL solution containing 2 μL of the synthesized cDNA, 0.8 μL of each