Plant Science 263 (2017) 219–225

Contents lists available at ScienceDirect

Plant Science

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

NSR1/MYR2 is a negative regulator of *ASN1* expression and its possible involvement in regulation of nitrogen reutilization in Arabidopsis

Yoshimi Nakano[1], Yuki Naito[1], Toshitsugu Nakano[2], Namie Ohtsuki[3], Kaoru Suzuki*

National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki 305-8566, Japan

ARTICLE INFO

Keywords:
Asparagine synthetase
GARP-type transcription factor
Nitrogen
Remobilization
Reallocation

ABSTRACT

Nitrogen (N) is a major macronutrient that is essential for plant growth. It is important for us to understand the key genes that are involved in the regulation of N utilization. In this study, we focused on a GARP-type transcription factor known as *NSR1/MYR2*, which has been reported to be induced under N-deficient conditions. Our results demonstrated that NSR1/MYR2 has a transcriptional repression activity and is specifically expressed in vascular tissues, especially in phloem throughout the plant under daily light-dark cycle regulation. The overexpression of NSR1/MYR2 delays nutrient starvation- and dark-triggered senescence in the mature leaves of excised whole aerial parts of Arabidopsis plants. Furthermore, the expression of *asparagine synthetase 1* (*ASN1*), which plays an important role in N remobilization and reallocation, i.e. N reutilization, in Arabidopsis, is negatively regulated by *NSR1/MYR2*, since the expressions of *NSR1/MYR2* and *ASN1* were reciprocally regulated during the light-dark cycle and *ASN1* expression was down-regulated in overexpressors of *NSR1/MYR2* and up-regulated in T-DNA insertion mutants of *NSR1/MYR2*. Therefore, the present results suggest that *NSR1/MYR2* plays a role in N reutilization as a negative regulator through controlling *ASN1* expression.

1. Introduction

Nitrogen (N) is a major macronutrient that is essential for plant growth. Since nitrogen is a major limiting factor for crop productivity, large quantities of N fertilizers are applied to fields around the world to promote high crop yield. Excess N from fertilizer, however, has little effect on yield and can cause N pollution [1]. The production of synthetic N fertilizers consumes a great deal of energy and leads to greenhouse gas emissions, and their purchase represents a considerable financial cost to farmers. A means of significantly improving the efficiency with which crops use the N in fertilizer (N use efficiency or NUE) would provide important benefits including increased crop production to meet the food demands of the increasing world population as well as reduced environmental and economic costs.

To improve NUE, we must understand the key genes that are involved in the steps related to N utilization by plants, including uptake, translocation, assimilation, allocation, remobilization, reallocation, and reassimilation, then establish biotechnological methods by which to manipulate them. The biochemical mechanisms involved in N uptake, assimilation, and remobilization have been extensively studied and the key genes associated with these aspects of NUE have been identified [2,3]. At the initial step in N utilization, N compounds in their inorganic and organic forms are absorbed by plant roots from the soil and distributed to the whole plant. Nitrate is one of the major N sources and nitrate transporters have been shown to have important roles in the N uptake and transport systems in plants [4,5]. Nitrate is reduced to ammonium in a sequential reaction with nitrate and nitrite reductases [6], and ammonium is then assimilated into Gln and Glu by glutamine synthetase/glutamate synthase [7]. Assimilated N, which is allocated to mesophyll cells, is primarily stored in chloroplastic proteins as ribulose-1, 5-biphosphate carboxylase/oxigenase, phospho*enol*pyruvate carboxylase, and glutamine synthetase 2. These proteins are degraded into amino acids to enable remobilization of N from source to sink. The amino acids are transported in the phloem to developing sink organs such as young leaves and seeds [2,8]. Therefore, source to sink translocation of amino acids is an important process in the control of sink

Abbreviations: ASN, asparagine synthetase; *CaMV35S*, cauliflower mosaic virus 35S; GAL4-BS, GAL4-binding site; GAL4-DB, GAL4-DNA binding domain; *NSR1/MYR2*ox, *NSR1/MYR2* overexpression; NUE, nitrogen use efficiency; RT-PCR, reverse transcription-PCR

* Corresponding author at: National Institute of Advanced Industrial Science and Technology, 1-1-1 Higashi, Tsukuba, Ibaraki, 305-8566, Japan.
E-mail address: kaoru-suzuki@aist.go.jp (K. Suzuki).
[1] These authors contributed equally to this work.
[2] Present address: EARTHNOTE Co. Ltd., Nago, Okinawa 905-1152, Japan.
[3] Present address: Plant Genome Engineering Research Unit, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-8602, Japan.

http://dx.doi.org/10.1016/j.plantsci.2017.07.013
Received 25 January 2017; Received in revised form 29 May 2017; Accepted 15 July 2017
Available online 20 July 2017

development and seed yield [9–12]. Gln and Glu are converted to Asn and Asp by two amino transferase enzymes, asparagine synthetase (ASN) and aspartate aminotransferase, respectively [13]. Gln and Asn are the major N carriers translocated in the phloem of many plants, and Asn is preferentially accumulated in source tissues for remobilization [14]. Therefore, ASN may play an important role in N remobilization and reallocation within the plant in both source and sink tissues.

Many studies have attempted to improve NUE through quantitative genetics and transgenic approaches, but their success has been limited because of the complex nature of the traits [2,3,15–19]. In the case of transgenic approaches, metabolic engineering might be also limited by the interconnectedness of many metabolic processes including various feedback and trade-off mechanisms, not only at the single-cell level but also at the whole-plant level.

Utilization of transcription factors is a promising method that may enable us to overcome the obstacles to genetic improvement of NUE, because transcription factors can coordinately modulate the expression of a set of genes. Many transcription factor genes involved in nitrate response have been reported [20]. ZmDof1 [21] and CGA1/GNL [22] were reported to modulate N assimilation. Recently, it was reported that overexpression of NLP7 improved plant growth by enhancing nitrogen and carbon assimilation [23]. Overall, however, our knowledge of the transcription factors involved in N utilization is quite limited. In the present study, we focused on the Arabidopsis gene *NSR1/MYR2*, a member of the GARP family of transcription factors [24] (Supplemental Fig. 2), as a potential regulator of N utilization, because it has been reported that transcripts of *NSR1/MYR2* is found in phloem cambium [23] and increased in response to N starvation [26]. In the present study, we demonstrated that *NSR1/MYR2* had transcriptional repression activity in transient expression assay and that its expression was restricted in phloem tissue and increased in the daytime. In addition, overexpression of *NSR1/MYR2* resulted in delay of the senescence induced by treatment of nutrient starvation and darkness. The expression of *ASN1* was negatively regulated by *NSR1/MYR2*. Therefore, our results suggest that *NSR1/MYR2* might have a role in N remobilization and reallocation, i.e. N reutilization, as a negative regulator.

2. Materials and methods

2.1. Plant materials and growth conditions

All wild-type (WT) plants used in this study were *Arabidopsis thaliana* accession *Col*-0. The T-DNA mutants, *myr2-1* (SALK_069046) and *myr2-2* (SALK_087886), were backcrossed twice to WT, self-pollinated, and then homozygotes for each allele were selected from the progeny by genomic PCR. Plants were grown on half-strength MS medium containing 0.5% sucrose and 1% agar or in soil at 22 °C under long-day conditions (16L-8D).

2.2. Vector construction and transgenic plant generation

For constitutive overexpression, the coding sequence of At3g04030.3 for *NSR1/MYR2* was amplified with a primer set (Supplemental Fig. 1) and the cDNA fragment was cloned into pENTR/D-TOPO cloning vector (Thermo Fisher Scientific). The fragment was then transferred into the Gateway-based binary vector pK2GW7 [27], which was provided by Plant Systems Biology (University of Ghent, Ghent, Belgium; http://www.psb.ugent.be/gateway/index.php), using a Gateway LR Clonase Enzyme mix (Thermo Fisher Scientific). In this vector, *NSR1/MYR2* cDNA was inserted downstream of the cauliflower mosaic virus 35S (*CaMV35S*) promoter. On the other hand, a genomic DNA fragment of about 3 kb upstream of the translation start site of At3g04030.3 and the first 18 bases of its coding sequence was fused with the reporter gene GUS, the fragment was then transferred into the binary vector pBCKK derived from pBIG-KAN [28] using a Gateway LR Clonase Enzyme mix (Thermo Fisher Scientific). Arabidopsis plants were transformed via *Agrobacterium* according to the floral dip method, and transgenic seeds (T1 generation) were screened on the MS medium containing 25 mg L^{-1} kanamycin and 250 mg L^{-1} vancomycin or 50 mg L^{-1} kanamycin and 100 mg L^{-1} carbenicillin. The resistant plants were transferred and grown on antibiotic-free medium to prevent influence on plant growth.

2.3. GUS histochemical analysis

Transgenic plants harboring *NSR1/MYR2* pro:GUS were pre-fixed with cold 90% acetone at −20 °C and samples were washed with 50 mM sodium phosphate buffer (pH7.0). The samples were then incubated in X-Gluc solution which contained 1 mM X-Gluc, 0.1% Triton X-100, 1 mM ferricyanide, and 1 mM ferrocyanide in 50 mM sodium phosphate buffer (pH7.0) at 37 °C for several hours, with duration varying among the samples. After the X-Gluc solution was removed, the samples were washed with 50 mM sodium phosphate buffer (pH7.0), and post-fixed with 70% ethanol at 4 °C. Seedling samples were mounted on slide glasses with a chloral solution of chloralhydrate/glycerol/water (w:w:w = 8:1:2) and observed under a BZ-9000 fluorescence microscope (KEYENCE). Flower or silique samples were observed under an MZ16FA stereo microscope (Leica) and photographed with a DFC300FX CCD camera (Leica) and Leica Application Suite software (Leica). Stem cross-sections were stained with 1% phloroglucinol in 20% HCl, then observed with an Axioscope 2 plus microscope (Zeiss) and photographed with an AxioCam HRc CCD camera (Zeiss) and Axio Imaging software (Zeiss).

2.4. RNA extraction, reverse transcription, and quantitative real-time PCR analysis

Total RNA was isolated with a Plant RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. It was then treated with recombinant DNase I (TAKARA) to degrade genomic DNA. One μg of the total RNA and 2.5 μM oligo dT primer were used for reverse transcription with PrimeScript® RT reagent Kit (TAKARA). cDNA was appropriately diluted and used for semi-quantitative reverse transcription PCR (RT-PCR) analysis with Ex Taq HS (TAKARA) and quantitative RT-PCR with Power SYBR® Green PCR Master Mix and Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific). Primer sets for each gene are listed in Supplemental Fig. 1.

2.5. Induction of senescence through nutrient starvation and darkness

Whole aerial sections were excised from 21-day-old plants and put onto filter paper dampened with distilled water in 90 mm plastic dishes. The dishes were sealed and put into the growth chamber at 22 °C under conditions of darkness for up to four days. After the treatment, plant samples were weighed and subsequently powdered under liquid nitrogen using a Shake Master Neo device (BMS). These samples were stored at −80 °C for further experiments.

2.6. Measurement of chlorophyll contents

1 ml of 80% acetone (v/v, 4 °C) was added to each sample tube and mixed immediately. After overnight incubation at 4 °C, plant residues were centrifuged and supernatants were measured using a spectrophotometer (Pharmacia Biotech Ultraspec 3000). Chlorophyll contents (μg/mg FW) were calculated according to the formula in Porra et al. [29].