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Isolation and molecular characterization of nitrite reductase (*RsNiR*) gene under nitrate treatments in radish

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ABSTRACT

Nitrite reductase (NiR, EC1.7.7.1) is a key enzyme that catalyzes the second step of nitrite reduction to ammonium in the process of nitrate assimilation in plants. To study the molecular mechanism underlying nitrate accumulation during plant development, the molecular characterization of the *NiR* gene (*RsNiR*) and its expression patterns under different nitrate concentrations and induction periods of nitrate treatments in leaf, root, petiole, stem and lateral root of radish (*Raphanus sativus* L.) were investigated in this study. Based on our radish transcriptome database, the genomic DNA sequence of *RsNiR* was isolated with four exons and three introns. The cDNA length was 1756 bp containing an open reading frame (ORF) of 1524 bp. The deduced protein consists of 506 amino acids, and showed highest identity with NiRs from *Arabidopsis*. Moreover, using the genomic DNA walking approach, the 843-bp 5'-flanking region upstream of *RsNiR* was isolated, which contained several basic *cis*-regulatory elements including TATA-box, CAAT-box and nitrate-responsive *cis*-element (NRE). With the increasing nitrate concentration, the NR activity (NRA), nitrate contents and *RsNiR* expression levels exhibited increasing trends in leaf and root, reached a maximum at 30 mM nitrate treatment and then decreased. In contrast, there was no obvious relationship among *RsNiR* expression level, NRA and nitrate content under different induction periods at 30 mM nitrate. These results could provide fundamental insight into clarification of the molecular regulation mechanism underlying nitrate assimilation in radish.

1. Introduction

Nitrogen (N) is the fundamental and essential mineral element in higher plants, and N availability is a key limiting factor for plant growth and crop production. Plants can utilize different forms of N, including gaseous ammonia (NH_3), N oxides (NO_x), mineral N (NO_3^- and NH_4^+) and other organic N forms (von Wirén et al., 1997). For plants growing in aerobic soils, the most important N source during growth is nitrate, which can cross the plasma membrane of epidermal and root cortical cells to be absorbed by plants (Gojon et al., 2009). Once absorbed by higher plants in root cell cytoplasm, nitrate may be stored in the vacuole for later use, transported into the xylem, or translocated to the shoot for assimilation or storage. Also, it can be converted into nitrite and ammonium, which are catalyzed by two kinds of enzyme catalysts, nitrate reductase (NR, EC 1.6.6.1) and nitrite reductase (NiR, EC 1.7.7.1), in the cytosol and chloroplast, respectively (Sun et al., 2008). Then ammonium is assimilated into different amino acids by the combined action of glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase (GOGAT, EC 1.4.7.1) in a cyclic way (Lam et al., 1996).

In addition to the role of an essential N nutritional component, nitrate is also used as a signaling molecule which can regulate root architecture, stimulate shoot growth, regulate ABA-independent stomata opening and relieve seed dormancy (Gupta and Beevers, 1987; Walch-Liu et al., 2005). Through its extensive influence on a variety of cellular functions, nitrate is considered to be one of the key environmental regulatory factors during plant development and growth (Wang et al., 2003).

The NR-based catalytic reduction of nitrate to nitrite is believed to be the rate-limiting step, and NiR, which promotes the reduction

Abbreviations: GOGAT, glutamine oxoglutarate aminotransferase (glutamate synthase); GS, glutamine synthetase; NiR, nitrite reductase; NNPP, neural network promoter prediction; NR, nitrate reductase; NRE, nitrate-responsive *cis*-element; ORF, open reading frame; qRT-PCR, quantitative reverse transcription-PCR; TSS, transcription start site.

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Table 1
The primers used in this study.

Primer name	Primer sequence(5′-3′)	Purpose
RsNiRF1	CGCTCCTCCGGCAGAATC	cDNA Isolation
RsNiRR1	TGGGCACAAAACACAAAGTATCAA	cDNA Isolation
DRsNiRF1	ACTAAGAGATGGGTTTTTCA	Genomic DNA isolation
DRsNiRR1	CGAGTCGCTTCCTATCC	Genomic DNA isolation
RTRsNiRF1	CGCTCCTCCGGCAGAATC	Semi-quantitative RT-PCR
RTRsNiRR1	CCAAGCATCAAGGGGAATAGC	Semi-quantitative RT-PCR
qRsNiRF1	TTAGGAAGTATGGCGAAGA	qRT-PCR
qRsNiRR1	GACATCAGGCAAGACAAC	qRT-PCR
β-Actin2/7F	GCATCACACTTTCTACAAC	qRT-PCR
β-Actin2/7R	CCTGGATAGCAACATACAT	qRT-PCR
Adaptors1	GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT	Promoter cloning
Adaptors2	PO_4-ACCAGCCC-NH_2	Promoter cloning
AP1	GTAATACGACTCACTATAGGGC-NH_2	Promoter cloning
AP2	ACTATAGGGCACGCGTGGT	Promoter cloning
PRsNiRR1	CATCTTCCCCATACTTCCTAATC	Promoter cloning
PRsNiRR2	CTCAGGCACATCAGGCAACA	Promoter cloning

of nitrite to ammonium, is considered to be a second key enzyme throughout the course of nitrate assimilation in plants. It shifts the six electrons from reduced ferredoxin to nitrite to form ammonium (Gupta and Beevers, 1987). In addition, NiR is mainly found in the chloroplasts of C_3 plants, mesophyll cells of C_4 plants and some plastids of non-green tissues (Tobin and Bowsher, 2005). The NiR protein is a monomer (60–70 kD) with two redox centers: a siroheme–Fe center for nitrite reactivity and an iron–sulfur center, which acts as the initial electron acceptor. The NiR protein is composed of three domains, a nitrite/sulfite reductase ferredoxin-like domain, iron–sulfur/siroheme-binding site and 4Fe-4S cluster. The NiR protein forms a complex with the electron donor (ferredoxin) and enzyme substrate (nitrite). Genomic DNA and cDNA of the *NiR* gene have been isolated from barley (*Hordeum vulgare*), bean (*Phaseolus vulgaris*), maize (*Zea mays*) and rice (*Oryza sativa*), and the nitrate-induced expression of the *NiR* gene has also been studied in these plants (Aslam and Huffaker, 1989; Kramer et al., 1989; Nishimura et al., 2005; Sander et al., 1995). However, neither genomic nor cDNA of the *NiR* gene from radish (*RsNiR*) have been reported until now. Moreover, excess nitrate levels and the subsequent changes in *NiR* expression levels, nitrate contents and NR activity levels have rarely been studied, particularly in radish.

The typical nitrate-inducible *NR* and *NiR* genes are directly induced by the nitrate supply, and their expressions are transcriptionally regulated. Moreover, this nitrate-inducible expression occurs without *de novo* protein synthesis, suggesting that *NR* and *NiR* genes are regulated by pre-existing components in the cell (Price et al., 2004). The previous analyses of the *NiR* gene promoters from various dicotyledonous species have shown that the proximal regions of these promoters are responsible for nitrate induction (Konishi and Yanagisawa, 2010). Furthermore, in the proximal region of the *NIR1* promoter in *Arabidopsis thaliana*, a 43-bp sequence containing a conserved sequence, 5′-tGACcCTTN$_{10}$AAGagtcc-3′, was identified as an authentic nitrate-responsive *cis*-element (NRE), which is both necessary and sufficient for the nitrate-inducible response (Konishi and Yanagisawa, 2010; Konishi and Yanagisawa, 2011).

Part of the nitrate in food is reduced to nitrite in human body. Nitrite is harmful to humans and can cause infant methemoglobinemia and produce harmful carcinogenic nitrosamine. Vegetables and drinking water are primary nitrate sources for humans. Some root vegetables, including radish ($2n = 2x = 18$), an annual or biennial herb and important root vegetable crop of the Brassicaceae family, may have high nitrate contents (Zhou et al., 2000). Although many nitrate-induced genes involved in nitrate metabolism have been studied, only a small number of genes were directly induced by nitrate, such as genes encoding nitrate transporter (NRT), nitrate/nitrite reductase (NR/NiR), glutamine synthetase (GS) and glutamate synthase (GOGAT) (Luo et al., 2013). Although many studies have been performed in plants in relation to N metabolism that focused on several model and crop species, the molecular mechanisms underlying the regulatory network of *NiR* genes associated with nitrate metabolism in radish remain to be clarified. The aim of this study is to characterize exon-intron structure and 5′-flanking region upstream of *RsNiR* gene involved in nitrate assimilation, and to investigate the *RsNiR* gene regulating nitrate accumulation and NR activity (NRA) in radish, with the ultimate goal of developing elite cultivars with low nitrate contents. For this purpose, genomic DNA and cDNA sequences of *RsNiR* from radish were isolated and analyzed. The expression patterns of the *RsNiR* gene under different nitrate treatments in different tissues were profiled using semi-quantitative RT-PCR and qRT-PCR, and the relationships among *RsNiR* gene expression, nitrate content and NRA under different nitrate treatments were also investigated in this study.

2. Materials and methods

2.1. Plant materials

Seeds of multi-resistant advanced radish inbred line 'NAU-QTSH' were germinated on moist filter paper in an incubator at 25 °C for 2 days and sown in 10 × 10-cm plastic dishes containing nursery substrate (soil: turf: vermiculite = 1:2:1) in the greenhouse at Nanjing Agricultural University. Seedlings were grown with 14 h light at 25 °C and 10 h dark at 18 °C for 30 days with one plant per plastic dish and later transplanted to 20-L plastic tanks filled with modified half-strength Hoagland's nutrient solution (Wang et al., 2013).

When seedlings reached the four-true-leaf stage, those with consistent growth were transplanted into the hydroponic tanks. Two experiments were conducted: (I) Potassium nitrate was added to the nutrient solution to make nitrate concentrations of 0, 5, 20, 30 and 50 mM, and the samples were harvested after 24 h of nitrate treatment. (II) Potassium nitrate was added to the nutrient solution to form a final nitrate concentration of 30 mM. The samples, including the leaf, root, petiole, stem and lateral root were harvested after 0, 4, 8, 12 and 24 h of nitrate treatment, respectively. All samples were immediately frozen in liquid nitrogen and stored at −80 °C for further use.

2.2. Isolation of DNA and RNA

Total genomic DNA was extracted from one-month-old radish leaf with a modified CTAB-chloroform-isoamyl alcohol procedure (Liu et al., 2008). Total RNA was extracted from different tissues