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Up-regulation of *CmNRTs* and *CmANR1* genes expression contribute to root configuration changes for efficient capturing NO_3^- in the roots of chrysanthemum

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ABSTRACT

Nitrogen is very important to the yield and quality of chrysanthemum. However, excessive application not only negatively affects the yield and quality of chrysanthemum, but also can not be timely absorbed and utilized for chrysanthemum, so causes a lot of waste of fertilizer resources and serious pollution of groundwater environment. Nitrate nitrogen is the main form of nitrogen uptake by the roots of chrysanthemum. The uptake of NO_3^- by plants is mostly achieved by NO_3^- transporters (NRTs) and lateral root formation. However, the morphological structure and molecular mechanism of the response to nitrate signaling in the roots of chrysanthemum are poorly understood. In this study, we investigated that the response to NO_3^- signaling from the viewpoint of roots morphology and anatomical structure, roots NO_3^- contents, endogenous hormones contents and expression of related genes in the roots of chrysanthemum. A hydroponic experiment was performed using the rooted cuttings of chrysanthemum, with KNO_3 treatments (control for Hogland nutrient solution without N element) for seven times at 0, 1, 2, 3, 4, 5, 6 days, respectively. The results showed that the roots morphological indexes increased significantly in the KNO_3 treatments compared to those of the controls at 6 days after treatments, which were associated with the high expression of *CmNRT1.1*, *CmNRT2.1*, *CmNAR2.1* and *CmANR1*, which related to NO_3^- transport genes and lateral root development gene, the contents of NO_3^-, indole-3-acetic acid (IAA) and cytokinin (CTK) in the roots and the improved root microscopic structure in KNO_3 treatment. The promoting effect of KNO_3 shows an obvious time-effect. The results from correlation analysis indicated that the responses of chrysanthemum root to NO_3^- signaling could be related with the induction of NO_3^- signaling, the gene expression of NO_3^- transporter genes and lateral root development gene due to regulating the IAA, CTK levels and adjusting the root architecture of the root system.

1. Introduction

In aerobic soils NO_3^- is the major source of nitrogen for most plant growth and development, so NO_3^- plays a very important role in plant productivity and crop yield (Forde, 2014). An appropriate increase in the amount of nitrogenous fertilizer is one of the measures necessary to obtain a high crop yield (Gruber et al., 2013). However, excessive application of nitrogen fertilizer not only reduce crop nitrogen uptake and utilization efficiency, but also result in reduced crop production efficiency, a waste of resources, environmental pollution and other issues (Krouk et al., 2011). Establishing the mechanism by which external NO_3^- is able to elicit the root morphological response is not only of ecophysiological interest but also is important for a more general understanding of root development and for future attempts to develop crop plants with improved root architecture for more efficient capture of nutrients and water (Forde and Lorenzo, 2002; Forde, 2014).

Many studies with *Arabidopsis* (*A. thaliana* L.) have shown that the localized increase in the growth rate of the lateral roots is triggered by the external presence of the NO_3^- ion acting as a signal to trigger increased meristematic activity in the lateral root tips (Zhang and Forde, 1998; Zhang et al., 1999; Zhang and Forde, 2000). The *ANR1* gene was first identified as a component of the signaling pathway that links external NO_3^- to an increased rate of lateral root growth (Zhang and Forde, 1998) and subsequently the NRT1.1 and NRT2.1 nitrate transporter was identified as another component of the same pathway (Remans et al., 2006a,b). Since then, further evidence has accumulated

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to support the idea that NRT1.1 acts as an NO_3^- transporter, performing the dual roles of NO_3^- transporter and NO_3^- sensor (Walch-Liu et al., 2005; Xu et al., 2016). Also, the protein interaction between NAR2.1 with NRT2.1 and NRT1.1 was identified by split-ubiquitin system and bimolecular fluorescence complementation system (Liu et al., 2014). In addition, a role for NRT1.1 as an NO_3^- regulated facilitator of auxin transport within the lateral root primordium has been proposed as a mechanism to explain the NO_3^- sensitivity of lateral root emergence (Krouk et al., 2010). Krusell et al. (2006) reported that nitrate-inducible CTK is also a key determinant of nitrate-dependent CTK biosynthesis (Krusell et al., 2002).

Chrysanthemum (*Chrysanthemum morifolium*) is one of the most well-known traditional flowers in China and it is known as one of the fertilizer-sensitive plants (Fan et al., 2014). Improper fertilization can aggravate the contradiction of vegetative and reproductive growth, resulting in a decline in growth and ornamental quality (Kocamaz et al., 2006). So studies on how to reduce the amount of nitrogen fertilizer and increase chrysanthemum nitrogen utilization efficiency are very important for the sustainable development of chrysanthemum industry. However, little information is reference on the expression of NO_3^- transporter genes (*CmNRTs*) and lateral root development gene (*CmANR1*) contribute to roots configuration changes for efficient capturing NO_3^- in the roots of chrysanthemum. Here, the isolation and the mode of expression of two NO_3^- transporter gene fragments of *CmNRT1.1* and *CmNRT2.1* and interacting protein gene of *CmNAR2.1*, and lateral root development related gene *CmANR1* of the roots of chrysanthemum are described. And the levels of IAA and CTK and NO_3^- contents in the roots and leaves of chrysanthemum are described. The purpose is to reveal the molecular mechanism of response to NO_3^- signaling in the roots of chrysanthemum.

2. Material and methods

2.1. Plant material and culture conditions

The experiment was conducted in an artificial climate chamber at Horticulture Experimental Center of Shandong Agricultural University (Shandong, China). The rooted cuttings of chrysanthemum seedlings (*Chrysanthemum morifolium* cv. 'Jinba') of similar height and diameter were transferred to a hydroponic growth system. Seedlings were transplanted into plots at black plastic plots(48 cm × 29 cm × 12 cm) of approximately 30 plants per pot(total 20 pots)on May 25th, 2016. The hydroponic growth system uses Hoagland nutrient solution without N element. Inside the artificial climate chamber temperatures ranged between 18 and 25 °C, relative humidity (RH) was 65%–75%, light cycle is set to day 14 h and night 10 h.

2.2. Treatments and experimental design

The nitrate treatment was initiated in a hydroponic growth system on May 27th (2 days after revive seedling). Chrysanthemum seedlings were divided into two groups for treatment. The two groups (10 pots in each group) of plants were treated 6 days with various concentrations of KNO_3 (0 mmol L^{-1} KNO_3 as a control, 5 mmol L^{-1} KNO_3) in 4 L Hoagland nutrient solution. The concentration of the nitrate we used was the results of the pre-experiment. The experiment was replicated three times in a randomized complete block design.

2.3. Analysis of real-time PCR

The relative expression levels of *CmNRT1.1*¡¢*CmNRT2.1*¡¢*CmNAR2.1*¡¢*CmANR1* in plant roots was analyzed by RT-PCR following treatment with various nitrate concentrations. RNA was isolated from the roots at 0 (before treatment), 1, 2, 3, 4, 5, 6 DAT (days after treatment) respectively. The chrysanthemum *actin* gene was used as internal reference gene. The primer pairs used for RT-PCR were designed with Primer 5.0 program listed as follows: *CmNRT1.1* forward, 5′-AACAGGGACCGTCAAGAGAA-3′, and reverse, 5′-AGCAATCAAGAACAGCGAAA-3′; *CmNRT2.1* forward, 5′-GTAACACCTCCGAGCAACAC-3′, and reverse, 5′-TTTCACAATGCAATAGCATG-3′; *CmNAR2.1* forward, 5′-AATGGCTGAAAACTCAAGAA-3′, and reverse, 5′-AATCATGCAATGGCATAAAC-3′; *CmANR1* forward, 5′-TTCTCAAAGAGGAGGAGTGGAT-3′, and reverse, 5′-CCATATTGCAGCTAGTATTCGAG-3′; and *Actin* forward, 5′-ACAACTGCTGAACGGGAAAT-3′, and reverse, 5′-TCATAGACGGCTGGAAAAGG-3′. The primers were pre-tested by general PCR amplification and 1.2% agarose gel electrophoresis to avoid disturbance. The PCR reactions were performed in 96 wells reaction plates scaled with optically clear film in the Light Cycler 480 system (Roche Diagnostics). PCR protocols are as follows: initial denaturation at 94 °C for 10 min, followed by 40 cycles with a denaturing time of 20 s at 94 °C, an annealing time of 30 s at 55 °C, and 2 cycles as above to analysis the melt curves. The formula used for calculating the relative expression level of the target gene was $2^{-\Delta\Delta CT}$. The data are presented as mean ± SD of 3 replicates.

2.4. Measurement of endogenous hormone levels

The extraction and determination of endogenous IAA and CTK were assayed at 0 (before treatment), 1, 2, 3, 4, 5, 6 DAT (days after treatment) respectively on the leaves and roots were as described by Lin et al. (2008). Around each 5 g root and leaf samples were grinded in 80% (v/v) iced methanol to homogenate and then extracted for 24 h at 4 °C in the dark with 80% methanol. Particulates were removed by centrifugation at 10,000*g* (4 °C) for 15 min, and the supernatant was then passed through a C18 Sep-Pak cartridge (Waters Corp., USA). The concentrated sample was dissolved by mobile phase and constant volume at 4 mL for HPLC. After purification, the methanolic extracts were injected in a Waters 201 HPLC system (C18 Nova-Par, chromatogram column, 150 mm × 5.0 mm, 0.45 um). The mobile phase was 20% methanol, 20% acetonitrile and 60% water (pH = 3.5). The IAA and CTK concentration was measured at wavelength 254 nm. Hormone contents (ng per gram fresh weight FW) were calculated from the mean of three replicates.

2.5. Measurements of NO_3^- contents

NO_3^- contents in the leaves and roots of Chrysanthemum were determined at 0, 1, 2, 3, 4, 5, 6 DAT by Salicylic Acid Colorimetry. The NO_3^- contents measurement on the roots and leaves was as described by (Wei et al., 2004). Take roots and leaves of each 2 g, the fresh samples were ground with 10 mL ddH_2O and were transferred into 15 mL EP tubes. Then the samples were removed by centrifugation at 3500 r·min^{-1} for 10 min after boiling for 30 min, and the supernatant was then transferred into a 25 mL volumetric flask. Absorb 0.1 mL sample solution, add 0.4 mL salicylic acid of 5%, mix and place at room temperature for 20 min, and then add 9.5 mL NaOH of 8% into the sample solution. The absorbance was read at 410 nm by UV–vis spectrophotometer (UV-2450, Shimando, Japan) when it has cooled. Each sample was repeated 3 times. The content of nitrate nitrogen (mg per gram fresh weight FW) was calculated according to the equation: C·V/W (Wei et al., 2004).

2.6. Observation of root vascular bundle

The root vascular bundle was measured at 6 DAT. The transverse section of 1st, 2nd and 3rd roots that distance from the rhizome about 1 cm were excised and placed in drops of water on a glass slide. With the help of a biological microscope with scale, the root vascular bundle of temporary pack pieces was observed at magnifications of 100× .