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Iron-induced nitric oxide leads to an increase in the expression of ferritin during the senescence of *Lotus japonicus* nodules

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ABSTRACT

Iron is an essential nutrient for legume-rhizobium symbiosis and accumulates abundantly in the nodules. However, the concentration of free iron in the cells is strictly controlled to avoid toxicity. It is known that ferritin accumulates in the cells as an iron storage protein. During nodule senescence, the expression of the ferritin gene, *Ljfer1*, was induced in *Lotus japonicus*. We investigated a signal transduction pathway leading to the increase of *Ljfer1* in the nodule. The *Ljfer1* promoter of *L. japonicus* contains a conserved Iron-Dependent Regulatory Sequence (IDRS). The expression of *Ljfer1* was induced by the application of iron or sodium nitroprusside, which is a nitric oxide (NO) donor. The application of iron to the nodule increased the level of NO. These data strongly suggest that iron-induced NO leads to increased expression of *Ljfer1* during the senescence of *L. japonicus* nodules.

1. Introduction

Iron is one of the essential micronutrients for all plants. Legume nodules require large amounts of iron for biosynthesis of nodule-specific and iron-containing proteins such as leghemoglobin and nitrogenase (Guerinot, 1991; O'Hara, 2001; Peters and Szilagyi, 2006). The concentration of iron in soybean nodules is high compared with the leaves and roots (Burton et al., 1998); however, the concentration begins to decrease during nodule senescence (Santos et al., 2015). It is thought that the many iron molecules produced from proteolysis are reconstructed and transported to the seeds (Burton et al., 1998; Lucas et al., 1998). These iron molecules form the redox state and chelate to avoid iron toxicity (Kobayashi and Nishizawa, 2012). One of the proteins required in these processes is ferritin, which accumulates in cells as an iron storage protein (Briat et al., 2010a). Ferritin can store free iron safely as an inorganic complex because the mineral core is capable of keeping up to 4500 iron atoms inside its internal cavity (Briat et al., 2010a). The molecular structure of ferritin consists of 24 subunits assembled into a spherical shell and is highly conserved among plants, animals and bacteria. (Harrison and Arosio, 1996). It has been reported that ferritin accumulates in the nodules of soybean and lupin during nodule aging (Lucas et al., 1998; Matamoros et al., 1999). Therefore, accumulation of ferritin maintains iron homeostasis and protects against iron-mediated oxidative stress and abiotic stress (Zok et al., 2010; Ravet et al., 2009; Galatro et al., 2012). In *Arabidopsis thaliana*, ferritins are encoded by a multigene family (Petit et al., 2001). Among the ferritins, the expression of the *AtFer1* gene increases in response to application of iron (Briat et al., 2010b; Petit et al., 2001). The promoter of *AtFer1* has been characterized and a 22-bp cis-element, named the Iron-Dependent Regulatory Sequence (IDRS), is conserved (Strozycki et al., 2010). Ferritin promoter fused to GUS genes showed that the IDRS motif controlled ferritin expression in *Arabidopsis* and maize (Petit et al., 2001). Despite a number of physiological reports, little is known about the regulatory mechanism for the expression of ferritin in the nodule.

The expression of ferritin in *Arabidopsis* is mediated by nitric oxide (NO) (Arnaud et al., 2006). NO is known to be an important endogenous signaling molecule, which mediates a number of developmental and physiological processes in plants. It is involved in plant responses to abiotic stress such as osmotic stress, salinity, high temperature and plant defense against pathogens (Gould et al., 2003; Romero-Puertas et al., 2004). In legume nodules, excess

Abbreviations: cPTIO, 2-(4 carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM, diaminofluorescein-FM; DFOM, deferoxamine mesylate; GSNO, *N*-(*N*-L-γ-glutamyl-*S*-nitroso-L-cysteinyl) glycine; ICP-MS, inductively coupled plasma mass spectrometry; IDRS, iron dependent regulatory sequence; NO, nitric oxide; RFU, relative fluorescence units; SNP, sodium nitroprusside; wpi, weeks post inoculation.

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accumulation of NO inhibits nitrogen fixation and triggers nodule senescence (Cam et al., 2012; Hichri et al., 2015). However, NO has a regulatory role in nitrogen metabolism to maintain the energy status required for nitrogen fixation under hypoxic conditions (Boscari et al., 2013; Hichri et al., 2015).

Our previous experiments showed that one of the ferritin genes, *Ljfer1,* was induced by nodule senescence in *Lotus japonicus* (Chungopast et al., 2014). The promoter region of *Ljfer1* in *L. japonicus* includes the IDRS sequence, suggesting that *Ljfer1* might be regulated by iron. In this study, we investigated the regulatory role of ferritin in terms of nodule senescence.

2. Materials and methods

2.1. Bacterial strains and medium

Mesorhizobium loti MAFF303099, a gram-negative nitrogen-fixing bacterium (Kaneko et al., 2000), was used throughout the experiments. A tryptone-yeast extract (TY) medium was used for cultivation (Beringer, 1974).

2.2. Plant growth conditions

Seeds of *L. japonicus* B129 Gifu (Handberg and Stougaard, 1992) were surface-sterilized and germinated on sterile vermiculite with 0.5 × B&D medium (Broughton and Dilworth, 1971) in double Magenta jars, followed by inoculation with the *Mesorhizobium loti* 7 days later. The plants were grown in a growth cabinet (EYELA FLI-2000; Tokyo Rikakikai Co., LTD., Japan) controlled at 24 °C and exposed to 16 h light and 8 h dark (200 μmol m^{-2} s^{-1}).

To analyze the expression of ferritin by sodium nitroprusside (SNP), plants at 4 weeks post inoculation (wpi) were put in various concentrations of SNP or GSNO (0.1 mM, 0.5 mM, and 1 mM). To confirm the effect of 2-(4 carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) as a NO scavenger, plants were soaked in the cPTIO solution (1 mM, 10 mM) for 2 h and then transferred to medium containing cPTIO (1 mM, 10 mM) and 0.5 mM SNP or cPTIO (1 mM, 10 mM) and 300 μM iron citrate for 3 h. Each RNA was isolated from the nodules. Three independent biological experiments were performed with three replications.

For the experiments with iron citrate, plants at 4 wpi were soaked in a solution of 300 μM iron citrate or 5 mM DFOM (deferoxamine mesylate) or distilled water for 3 h. A stock solution of 50 mM iron citrate was prepared by mixing equal volumes of 100 mM $FeSO_4$ and 200 mM sodium citrate. To confirm the effect of DFOM, plants were soaked in the 5 mM DFOM solution for 3 h and then transferred to the medium containing 5 mM DFOM and iron citrate (300 μM or 3 mM) for 3 h. Each RNA was isolated from the nodules. Three independent biological experiments were performed with three replications.

2.3. Histochemical analysis of the promoter-GUS transformant

The 1.0 kb of sequence upstream from the start codon of *Ljfer1* was amplified from genomic DNA of *L. japonicus* GIFU using PrimeSTAR HS DNA polymerase (Takara, Japan) with the following specific primers: forward primer, 5′-AAATCTAGAATATACTTTGGAATGGAGGG-3′; reverse primer, 5′-TTTCCCGGGTAGAGCGAAATCTCAAAATG-3′. The PCR fragment was digested with *Xba*I and *Sma*I. The resulting fragment was ligated into pCAMBIA fused to the GUS gene (Mai et al., 2006). The construct was then introduced into *L. japonicus* using hairy root transformation mediated by *Agrobacterium rhizogenes* LBA1334, as previously reported (Kumagai and Kouchi, 2003). The transformed plants were inoculated with *M. loti* and grown in a growth cabinet. Nodules and roots were sampled at 2, 3 and 7 wpi, and GUS staining was performed as described previously (Mai et al., 2006).

2.4. NO detection by fluorescence microscopy

Fluorimetric quantification of NO production in the nodules was observed using diaminofluorescein-FM (DAF-FM). Nodules at 4 wpi were infiltrated with 1 mM SNP, 3 mM iron citrate or 3 mM potassium citrate under vacuum for 30 min and then transferred between filter papers moistened with 5 nM DAF-FM for 30 min. Nitric oxide accumulation was observed by fluorescence microscopy (SZX9; Olympus, Japan). The fluorescence intensity of the images was measured and the integrated density of each image was determined with a free software program (Image J; https://imagej.nih.gov/ij/), which calculated total cell fluorescence (CTCF) using the following the formula: CTCF = Integrated Density – (Area of selected cell × Mean fluorescence of background readings). Three independent biological experiments were performed with three replications.

2.5. Quantitation of NO by a fluorescence spectrophotometer

Quantitation of NO with a fluorescence spectrophotometer was performed as described previously (Nagata et al., 2008). All nodules at 4 and 7 wpi were soaked in the 7 nM DAF-FM solution for 10 min. The relative fluorescence units (RFU) of the DAF-FM solution was measured using a NanoDrop ND-3300 fluorospectrometer (NanoDrop Technologies Inc., USA) using a wavelength of 515 nm. Twelve independent biological experiments were performed

2.6. Iron measurement

The root nodules of *L. japonicus* were collected for iron analysis at 2, 4, and 8 wpi. The collected nodules were washed with 0.5 mM $CaCl_2$ three times, then centrifuged in an ultra-free-MC centrifugal filter (Millipore) at 3000 × *g* for 10 min. After taking out the cell sap, the nodule residue was washed three times with 70% ethanol, then dried. The residue of the nodule was resuspended in concentrated H_2SO_4. The iron concentration of both the cell sap and the nodule residue was determined using inductively coupled plasma mass spectrometry (ICP-MS, 7700×; Agilent Technologies). Three independent biological experiments were performed

2.7. Quantitative real time PCR

Total RNA from the nodules was extracted using an RNeasy plant mini kit (Qiagen, CA, USA). Each 500 ng of total RNA was converted to cDNA using Prime Script RT Master mix (Takara, Japan). After the reverse transcriptase reaction, target genes were amplified by PCR using the SYBR premix Ex Taq II kit (Takara, Japan) for 40 cycles (95 °C for 5 s and 60 °C for 30 s). Primer sequences were as follows: Leghemoglobin Forward (Lb Fw), agttggggacaa atggagtg, Lb Reverse (Rv), cataggctactccccaagca; *Ljfer1* Fw, aaagagggcatgcagagaaa, *Ljfer1* Rv, ccttccaccacgaatgttct; Ubiquitin (Ubi) Fw, atgcagatcttcgtcaagaccttg, Ubi Rv, acctcccctcagacgaag. The quantitative real-time PCRs were performed using the Thermal Cycler Dice Real-time System II (Takara, Japan). Three independent biological experiments were performed with three replications.

3. Results

3.1. Expression of ferritin gene in the L. japonicus nodules

We previously identified senescence-induced genes in *L. japonicus* nodules using microarray analysis (Chungopast et al., 2014). From the data, the *Ljfer1* gene (chr3.CM0116.300.r2.m) was induced