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Determining the function of the *NPF1B* and *NPF1C* genes in root and nodule development in *Lotus japonicus*

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Introduction

Legumes form a symbiosis with a group of soil bacteria collectively known as *Rhizobium*. In this symbiosis, *Rhizobium* induce the legumes to form lateral root organs called nodules, which house the *Rhizobium*. The legumes provide sugars to their symbiotic partners, and in exchange, the *Rhizobium* convert atmospheric nitrogen to a form that their host plant can assimilate through a process known as nitrogen fixation (Haag et al., 2012).

The evolution of this symbiotic organ is interesting; one mainstream hypothesis is that lateral roots are the evolutionary origin of nodules. Nodules and lateral roots develop in similar ways. Both nodules and lateral roots form next to the xylem, develop a meristem at their tip, and emerge from the root (Brewin, 1991). The meristem is maintained in indeterminate nodules, like those formed by peas and the model legume *Medicago truncatula*. However, other legumes like soybean and the model legume *Lotus japonicus* have lost the ability to maintain a meristem in their nodules, and therefore form determinate nodules (Hirsch, 1992).

The *LATD/NIP* gene provides a strong piece of evidence for the hypothesis that nodules evolved from lateral roots, as it is required for the development of both roots and nodules in *M. truncatula* (Bright, Liang, Mitchell, & Harris, 2005). In *MtLATD/NIP* is involved in root meristem organization, abscisic acid (ABA) signaling, and symbiosis with rhizobia (Bright et al., 2005; Liang, Mitchell, & Harris, 2007). *Mtlatd* mutants initiate the formation of nodules, but these nodules never reach maturity. These mutants also initiate the formation of lateral roots, which arrest shortly after initiation. The primary root tip eventually becomes disorganized and abnormal (Bright et al., 2005). Because there are meristems in *M. truncatula* nodules, lateral root tips, and primary root tip, these three mutant phenotypes all demonstrate that *LATD/NIP* plays a key role in meristem organization in *M. truncatula*.

The *LATD/NIP* gene is part of the Nitrate and Peptide Transporter Family (NPF) (Léran et al., 2014). The NPF family is incredibly widespread, as its genes are present in bacteria, fungi, animals, and plants (Léran et al., 2014). In plants, it consists of eight subfamilies and encodes transporters and transceptors for a diversity of substrates, including nitrates, peptides, amino acids, hormones, and more. Subfamily 1 (*NPF1*) is comprised of five clades (A, B, C, D1, and D2) based on their evolutionary relationships. *MtLATD/NIP* falls in *NPF1C* (Figure 1) (G. Sassi, unpublished data).

The *NPF1* subfamily originated at the base of the flowering plants (G. Sassi, unpublished data). The duplication event that gave rise to *NPF1B* most likely occurred very early in the flowering plants, before the divergence of the magnolids, and the *NPF1B* gene was lost in monocots. *NPF1A* is present only in monocots, and was most likely lost at the base of the
eudicots. Genes of clades C, D1 and D2 appear at the base of the eudicots, suggesting that these duplication events occurred in rapid succession. Subsequently, the clades D1 and D2 have diversified considerably. The species that contain genes from clades C, D1, and D2 have lost clade A genes, but most contain a clade B gene. This maintenance of NPF1B genes throughout angiosperm radiation, even in species with genes from other NPF1 clades, suggests that NPF1B’s function may be unique and important. In addition, plants often contain multiple copies of clade C, D1, and D2 genes, but the majority have only one copy of NPF1B, suggesting a dosage limit. NPF1D genes from Arabidopsis thaliana have been shown to transport abscisic acid, gibberellin1, and jasmonoyl-isoleucine into yeast cells (Chiba et al., 2015). However, MtLATD/NIP is the only phenotypically characterized gene in planta in the NPF1 subfamily.

![Figure 1. Nitrate and Peptide Transporter Family Subfamily 1 (NPF1). The five clades of the NPF1 subfamily of genes. NPF1B and NPF1C are marked with stars. There are 4 NPF1D1 genes and 3 NPF1D2 genes in L. japonicus (G. Sassi, unpublished data).](image)

The function of NPF1B is unknown, but our laboratory has shown that its retention is strongly correlated with the ability to form both rhizobial and mycorrhizal symbioses (G. Sassi, unpublished data). MtLATD/NIP is the only characterized gene in the NPF family that is required for meristem function. It is unknown whether its function is conserved in other legumes, in particular those that do not maintain meristems in their nodules. It is also unknown whether other NPF1 genes play a role in root and nodule development. Are the root and nodule functions of LATD/NIP in M. truncatula conserved in L. japonicus? Given the LATD/NIP and NPF1B genes are so closely related, do they share some functions?

Here I show that in L. japonicus, LATD/NIP is indeed involved in lateral root and nodule formation. I also show that LjNPF1B is involved in the development of both lateral and primary roots, but does not seem to be involved in nodule formation.

Methods

Plant Material and Growth Conditions

The Lotus japonicus ecotype B-129-S9 Gifu (Handberg & Stougaard, 1992) was used as the wild-type in all experiments and the LORE1 mutants (Urbański, Malolepszy, Stougaard, &
Andersen, 2011) were all in this background. LORE1 lines 30034842, 30010404, 30133827, 30008750, 30003350, and 30052308 were obtained from the LORE1 collection (Figure 3) (Mun, Bachmann, Gupta, Stougaard, & Andersen, 2016; Urbański et al., 2011). Plants in the segregating population were genotyped and homozygous mutants were grown up for a seed increase. Seeds were scarified 20 minutes with sulfuric acid, rinsed twice with sterile water, sterilized with 5 mL of a 70% ethanol, 3% hydrogen peroxide, and 27% water solution, rinsed five times with sterile water, and imibed in sterile water for four hours while shaking (Harris, Wais, & Long, 2002). After imbibition, seeds were transferred to an inverted petri dish sealed with Parafilm. If grown for a seed increase, the petri dish contained 1% PhytoAgar in water, covered with 2 layers of sterile filter paper. Seeds were vernalized at 4°C for 10 days and then transferred to pots. If grown for an experiment, the petri dish was kept in a dark drawer for 3 days, and then plated on nitrogen-free 1/4x Broughton and Dilworth (B&D) 1% agar plates on filter paper (Broughton & Dilworth, 1971) (Márquez et al., 2005) in 12.5 cm by 12.5 cm square plates (Phenix Research Products). All plants were grown in a growth chamber with a 16-hour day, 50% humidity, and 20°C.

Ten plants were grown on each plate. After plants were plated, the plates were sealed with 3M Micropore tape, to allow gas exchange, completely covered with tin foil, and placed vertically, at a slight angle in a growth chamber with a 16-hour day, 50% humidity, and 20°C. After two days, the tin foil was removed from the upper portion of the plates leaving only the roots covered. The following day, plants were inoculated with Mesorhizobium loti strain NZP2235 (Jarvis, Pankurst, & Patel, 1982).

M. loti was grown between 18 and 22 hours in 2 mL of TY (Shirling & Gottlieb, 1866), shaking, at 28°C until it reached an optical density of between 0.021 nm and 0.081 nm at A600nm. This overnight culture was centrifuged in 15 mL of 10 mM MgSO4 for 4 minutes at 4°C and 8000 rpm. The pellet was resuspended in 25 mL of 10 mM MgSO4. Subsequently, 2.8 mL of this solution was pipetted over the roots for flood inoculation, and the excess inoculum removed.

Genotyping Mutants

To determine whether the LORE1 transposon was inserted in both copies of the gene of interest, tissue was collected from each LORE1 line plant, genomic DNA was extracted, and the plants were genotyped via PCR using gene-specific primers flanking the site of the LORE1 insertion. If the transposon had inserted between the gene-specific primers, it would be too big to amplify across. However, if the transposon was present, the gene-specific primer and a LORE1 transposon-specific primer, P2, would amplify this region. When genotyping for insertions in LjNPF1B, a pair of primers that amplified LjNPF1C was done as a positive control. When genotyping for insertions in LjNPF1C, a pair of primers that amplified LjNPF1B was done as a positive control. Primers are listed in Table 3. Primers were designed using Primer3 (Koressaar & Remm; Untergasser et al., 2012). Twenty-five mL PCR reactions began with an initial activation step of 3 minutes at 94°C, cycled 35 times through 30 seconds at 94°C, 30 seconds at various annealing temperatures (Table 2), and 1 minute at 72°C. The reactions finished at 72°C for 5 minutes. The amplified products were run on 1.25% agarose gels and visualized using ethidium bromide (Figure 3).
Figure 2. Organization of the LjNPF1B and LjNPF1C genes. (A) and (B) Scale bar represent 100 base pairs. Exons are numbered. LORE1 retrotransposon insertion sites are indicated with red triangles. Primer locations are shown with arrows and labeled in tables below diagrams. (A) LjNPF1B gene diagram. Insertion sites of the LORE1 retrotransposons in the 30034842, 30010404, and 30133827 lines are indicated. (B) LjNPF1C gene diagram. Insertion sites of the LORE1 retrotransposons in the 30008750, 30003350, and 30052308 lines are indicated.
Plates were photographed at days 0, 5, 10, 17, and 24. Primary root length, primary lateral root length and count, adventitious root length and count, and root tip count were measured using ImageJ software (Abramoff, Magalhaes, & Ram, 2004). At days 17 and 24, number, color, and location of nodules were measured and recorded.

Expression Analysis

To test for expression of LjNPF1C, nodulated roots from 14 days post inoculation (dpi) plants were cut just below the hypocotyl, frozen and ground in liquid nitrogen, and mRNA extracted using the RNeasy® Plant Mini Kit (Qiagen Cat. No. 74904). RNA was then treated with DNase to remove any genomic DNA contamination using the TURBO DNA-free™ Kit (ThermoFisher Scientific Cat. No. AM1907), and cleaned up using the RNeasy® MinElute® Cleanup Kit (Qiagen Cat. No. 74204). cDNA was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad Cat. No. 1708890). Expression was checked using primers LjNPFI C_exon4_F and LjNPFI C_exon4_R to amplify a region on exon 4. ATP synthase-F and ATP synthase-R were used as a positive control and the –RT reaction as a negative control. Primers can be seen in Table 3. PCR conditions were the same as described above, except 32 cycles were performed.

Statistical Analysis

Each experiment tested Gifu and the three mutant lines. For each experiment, three biological replicates were performed. Each of these biological replicates included two technical replicates for each genotype, in which up to ten plants of that genotype were grown on each plate. For the LjNPFI B experiment, 58 Gifu plants, 43 LjLORE 842-7 plants, 50 LjLORE 404-3
plants, and 44 LjLORE 827-7 plants were phenotypically analyzed. For the LjNPF1C experiment, 49 Gifu plants, 57 LjLORE 75-3 plants, 37 LjLORE 350-1-3 plants, and 56 LjLORE 308-2-3 plants were phenotypically analyzed. Statistical analyses, an ANOVA followed by a post-hoc Tukey’s HSD test, were done using JMP Pro 13. The same replicates are compared at multiple time points, and therefore significant differences that are observed at different days are not independent.

Results

Isolation of LORE1 mutants

To determine the functions of LjNPF1B and LjNPF1C, three independent LORE1 insertion mutants were isolated from the LORE1 line collection (Urbański et al., 2011). Lines were genotyped using PCR. If the transposon had inserted between the gene-specific primers, it would be too large to amplify. However, if the transposon was present, another reaction with the gene-specific primer and a LORE1 transposon-specific primer, P2, would amplify. Plants homozygous for the LORE1 insertion event were grown up for a seed increase. At least one homozygous mutant was found for each line (Tables 1 and 2).

Characterization of root phenotypes for LjNPF1B and LjNPF1C mutants

Since the NPF1C gene is critical for lateral root development in M. truncatula, I tested whether LjNPF1B and LjNPF1C had similar roles in plant development. In order to determine the functions of these genes in root development, I examined root growth from days 5 to 24. Plants were grown on petri plates containing 1/4x B&D agar lacking Nitrogen (see Methods for details). All plants were inoculated on day 3 by flood inoculation. Plates were photographed and I subsequently analyzed images to explore aspects of root architecture and growth.

Both the LjNPF1B and LjNPF1C mutants had reduced root branching compared to Gifu, measured by root tip count (which includes both the primary root as well as primary, secondary, and tertiary lateral roots). The mutant phenotype of reduced root tip count was more consistent for the LjNPF1C mutants, as all three had significantly reduced root branching compared to Gifu at days 17 and 24, while only two of the LjNPF1B mutants (LjLORE 842-7 and LjLORE 827-7) had significantly reduced root branching at days 17 and 24 (Figures 4C and 5C).

Two of the LjNPF1B mutants had reduced total length of lateral roots off of the primary root at days 17 and 24. Again, it was LjLORE 842-7 and LjLORE 827-7 which had this phenotype, while LjLORE 404-3 had an intermediate phenotype between these two mutants and Gifu (Figure 4D). At days 10 and 17, both LjLORE 842-7 and LjLORE 827-7 had fewer lateral roots off of the primary root, and LjLORE 404-3 again had an intermediate phenotype. By day 24, all three LjNPF1B mutants had fewer lateral roots off of the primary root than Gifu (Figure 4E). All three LjNPF1C mutants had significantly reduced total length of lateral roots off of the primary root at days 10, 17, and 24 (Figure 5B).

From day 0 to day 5, all three LjNPF1B mutants had slower primary root growth than Gifu. After day 5, the LjNPF1B mutants trended towards having faster primary root growth than
Gifu, but for each time point, only one mutant grew significantly faster than Gifu (LjLORE 827-7 for days 10 and 17, and LjLORE 404-3 for day 24) (Figure 4B). The LjLORE 350-1-3 and LjLORE 308-2-3 LjNPF1C mutants had significantly reduced primary root growth at day 5, but had a wild-type growth at the other time points (Figure 5H). All three mutants had significantly shorter primary root lengths at days 10 and 17, and both LjLORE 350-1-3 and LjLORE 308-2-3 had significantly shorter primary root lengths at all of the time points (Figure 5I).

The LjNPF1C mutants trended towards having reduced lateral root density (number of lateral roots off of the primary root per cm of primary root); at day 17, both LjLORE 750-3 and 350-1-3 had reduced lateral root density than Gifu, while LjLORE 308-2-3 had an intermediate phenotype. At day 24, LjLORE 750-3 had reduced lateral root density as compared to Gifu, while LjLORE 350-1-3 had an intermediate phenotype (Figure 5D). For lateral organ density, which is the number of lateral roots and nodules off of the primary root per cm of primary root, all three LjNPF1C mutants had significantly reduced lateral organ density as compared to Gifu at days 17 and 24. LjLORE 350-1-3 had the most severe phenotype at day 17 (Figure 5E). The LjNPF1B mutants had wild-type levels of lateral root density and lateral organ density.

Characterization of symbiotic nodule phenotype for LjNPF1B and LjNPF1C mutants

Because the NPF1C gene is required for nodulation in *M. truncatula*, I tested whether LjNPF1B and LjNPF1C play a role in nodulation. In order to determine the functions of these genes in nodulation, I examined the nodules in the same plants as described in the previous section. On days 17 (14 dpi) and 24 (21 dpi), I counted the number, location, and color (pink as a indicator of maturity and white as an indicator of immaturity) of nodules.

The LjNPF1C mutants had decreased nodulation compared to Gifu, while LjNPF1B mutants formed the same number of nodules as Gifu. At day 17, all LjNPF1C mutants had fewer nodules than Gifu, and LjLORE 350-1-3 had the fewest. At day 24, both LjLORE 350-1-3 and LjLORE 308-2-3 had fewer nodules than Gifu (and again LjLORE 350-1-3 had the most severe phenotype), while LjLORE 750-3 had an intermediate phenotype (Figure 4F). The LjNPF1C mutants made pink nodules in the same pattern as the total number of nodules, indicating that the nodules that they made were likely mature (Figure 4G). In contrast, the LjNPF1B mutants formed the same number of total and pink nodules as Gifu at days 17 and 24 (Figures 3F and 3G).

Expression of NPF1B and NPF1C in Gifu

I noticed that there were differences in the severity of the phenotype between the different LORE1 lines, and wondered if this could be due to altered expression. In order to determine a good time point for checking expression, I tested when and where NPF1C and NPF1B are expressed in Gifu. I found that 14 dpi nodulated roots of Gifu express NPF1C (Figure 6). NPF1B is not expressed in the nodulated roots of Gifu at 2 dpi, 5 dpi, 10 dpi, or 14 dpi. It is known that LjNPF1B is highly expressed in nodules of rhizobia-inoculated nodule development mutants (Ljsen1 and Ljsst1) (Høgslund et al., 2009), as well as in roots of wild-type *L. japonicus* that have been inoculated with mycorrhizal fungi (Guether et al., 2009; Mun et al., 2016).
Figure 4. Root and nodule phenotypes of *LjNPF1B* mutants. (A) Inoculated wild-type (Gifu) and mutant plants at Day 24. Bars = 1 cm. (B) to (G) Quantitative analysis of root and nodule phenotypes of *LjNPF1B* mutants. Different letters denote a statistically significant difference (P < 0.05) among averages at each time point according to a one-way ANOVA followed by Tukey’s HSD test. Graphs show the average of three biological replicates. Error bars represent the average ± standard error. (B) Rate of primary root growth (cm/day) (C) Total lateral root length off of primary root. (D) Number of lateral roots off of primary root. (E) Total number of nodules. (G) Total number of pink nodules.
Figure 5. Root and nodule phenotypes of LjNPF1C mutants.
(A) Inoculated wild-type (Gifu) and mutant plants at day 24. Bars = 1 cm. (B) to (I) Quantitative analysis of root and nodule phenotypes of LjNPF1C mutants. Different letters denote a statistically significant difference (P < 0.05) among averages at each time point according to a one-way ANOVA followed by a Tukey's HSD test. Graphs show the average of three biological replicates. Error bars represent the average +/- standard error. (B) Total lateral root length off of primary root (C) Count of root tips, a measure of root branching (D) Lateral root density off of primary root (E) Lateral organ density off of primary root (lateral organs include nodules and roots) (F) Total number of nodules (G) Total number of pink nodules (H) Rate of primary root growth (cm/day) (I) Primary root length (cm)
LORE1 insertions reduce expression of LjNPF1C

I noticed that the LjLORE 350-1-3 mutant had a more severe phenotype than the other two LjNPF1C mutants, and we wondered if this was due to the LORE1 insertion reducing expression of NPF1C more in the LjLORE 350-1-3 line than the other lines. To determine whether the LORE1 insertions altered expression of the NPF1C gene in the mutants, part of the fourth exon of the NPF1C gene was amplified out of the cDNA of 14 dpi nodulated roots. All three of the LjNPF1C mutants express NPF1C in the nodulated roots at 14 dpi, but at very different levels. LjLORE 750-3 appeared to express NPF1C at similar levels to Gifu, LjLORE 350-1-3 expressed NPF1C at much lower levels than Gifu, and LjLORE 308-2-3 expressed NPF1C at lower levels than Gifu (Figure 6).

Discussion

Overview

The relationship between the genetic control of root architecture and symbiotic nodule development is not fully understood, but the LATD/NIP gene suggests that the two processes are related. I report here the characterization of the NPF1B and LATD/NIP genes in L. japonicus. I show that the L. japonicus LATD/NIP gene affects both root architecture and noduleation, which is consistent with its role in M. truncatula. I also show that the L. japonicus NPF1B gene affects both primary and lateral root growth, but does not affect nodule number. This observation indicates that the LjNPF1B gene and the LjLATD/NIP gene have similar effects on root growth but different effects on nodulation. I also demonstrate that in 14 dpi nodulated roots, the 3000350 LORE1 insertion in the LjLATD/NIP gene strongly reduces expression of the LATD/NIP

Figure 6. Expression of NPF1C in LjNPF1C mutants.

cDNA from 14 dpi nodulated roots of Gifu, LjLORE 750-3, LjLORE 350-1-3, and LjLORE 308-2-3 was tested for expression of NPF1C using primers LjNPF1C_exon4_F and LjNPF1C_exon4_R. ATP Synthase expression was tested as a positive control using primers ATP Synthase-F and ATP Synthase-R. The –RT negative control came from RNA in which Reverse Transcriptase was not added to the cDNA synthesis reaction.
gene and the 30052308 insertion somewhat reduces expression. These reductions in expression were consistent with the severity of the phenotypes that I measured.

**LATD/NIP**

When starting this project, I was curious if the functions of LATD/NIP in *M. truncatula* would be conserved in *L. japonicus*. Specifically, I wondered if LATD/NIP would be involved in nodulation in *L. japonicus*, because although *L. japonicus* requires a meristem to initiate nodule formation, it does not maintain a meristem in its nodule. Therefore, the result that *LjNPF1C* mutants form fewer nodules than Gifu was surprising. It is difficult to know whether the nodules that the *LjNPF1C* mutants did form were functional; I observed whether the nodules were pink, which is a marker for maturity, but this assay is not completely reliable. Therefore, I will test whether the bacteria successfully infect the nodules using a hemA: lacZ reporter strain of *M. loti*, and whether the bacteria differentiate into bacteroids inside the nodule using a different strain, containing a nifH: lacZ reporter. This test will help clarify whether or not the nodules that *LjNPF1C* mutants do form are functional nitrogen-fixers.

*LATD/NIP* is also required for lateral root formation in *M. truncatula*; without it, lateral roots arrest soon after emergence (Bright et al., 2005). In *L. japonicus*, *LATD/NIP* is clearly involved in lateral root formation, but the mutant phenotype is not as severe as it is in *M. truncatula*. This decrease in lateral root count and number was not accompanied by hypernodulation, as in the *Ljhar1* mutant (Wopereis et al., 2001). It seems as if the lateral root function of *MtLATD/NIP* is somewhat conserved in *L. japonicus*; while the mutant phenotype is not as severe as in *M. truncatula*, it is consistent.

**LjNPF1B**

Another goal of this project was to determine if *LjNPF1B* and *LjLATD/NIP* have similar functions. As *MtLATD/NIP* was the only phenotypically characterized gene in the NPF1 subfamily, learning about the *NPF1B* gene provides significant insight into this subfamily of genes. I found that the *NPF1B* gene does not play a role in nodule number in *L. japonicus*, unlike the *LjLATD/NIP* gene. However, it is involved in lateral and primary root growth. Its role in lateral root growth is consistent with the function of *LjLATD/NIP*, suggesting that there is some overlap between the functions of these two genes.

**Future Directions**

The consistent phenotypes across various *LORE1* lines and the expression levels of *LjLATD/NIP* correlating with the severity of the mutant phenotype suggest that insertions in *NPF1B* and *NPF1C* are responsible for the mutant phenotypes I observed. However, to confirm these results, I plan to rescue each gene with the *L. japonicus* genes and perform a phenotypic analysis.

The *LjNPF1B* and *LjLATD/NIP* genes have some overlapping functions, but also have some unique functions. To test the extent to which the *LjNPF1B* and *LjLATD/NIP* genes are
redundant, I plan to do a cross between a Lj\textit{NPF1B} mutant and a Lj\textit{NPF1C} (Lj\textit{LATD/NIP}) mutant to generate a double mutant. This will help us to determine to what degree the functions of these two genes overlap.

Conclusion

Using the functional analysis of the mutants, I have demonstrated a shared function between Lj\textit{NPF1B} and Lj\textit{LATD/NIP} in controlling lateral root number and elongation. I have also shown that Lj\textit{NPF1B} and Lj\textit{LATD/NIP} have different functions in primary root growth and nodulation and that LATD/NIP functions to control both root architecture and nodule development in both \textit{M. truncatula} and \textit{L. japonicus}.

\textbf{Table 1.} Summary of genotyping for Lj\textit{NPF1B} mutant lines.

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\textbf{Table 2.} Summary of genotyping for Lj\textit{NPF1C} mutant lines.

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### Table 3: Primers used for this study

#### Primers for Lj\textit{NPF1B}

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#### Primers for Lj\textit{NPF1C}

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<td>TGCAAGCAAAATACGTACAAGGAGGC</td>
<td>(Mun et al., 2016)</td>
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<tr>
<td>Lj_LORE-308F</td>
<td>TCTGCATCATTTACTGTGCAAGATCACA</td>
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<tr>
<td>Lj_LORE-308R</td>
<td>ATAGCTGAGGCCTCAGGCGGT</td>
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<td>Lj_LORE-350F</td>
<td>TGATAGCCAGCTCCCCGTTCCTCC</td>
<td>(Mun et al., 2016)</td>
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<td>CAAACCATGGAGCCTCTGCACTG</td>
<td>(Mun et al., 2016)</td>
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<tr>
<td>Lj_NPF1C_exon4_F</td>
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#### LORE1 specific primer

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<tr>
<th>Primer Name</th>
<th>5’ to 3’ Sequence</th>
<th>Reference</th>
<th>Annealing Temperature (°C)</th>
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<td>Lotus-LORE-P2</td>
<td>CCATGGCGGTTCCTCAGATCTTAGG</td>
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#### Other

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<td>ATP synthase-F</td>
<td>GGTGATAAGCAGAGTGAAGCA</td>
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<td>ATP synthase-R</td>
<td>AAGACCAGTGAGACCAACACG</td>
<td>(Yano et al., 2016)</td>
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</tbody>
</table>
References


Koressaar, T., & Remm, M. Enhancements and modifications of primer design program Primer3. *Bioinformatics, 23*(10), 1281-1291.


