Chapter 2

User Guide for the *LORE1* Insertion Mutant Resource

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Abstract

Lotus japonicus is a model legume used in the study of plant-microbe interactions, especially in the field of biological nitrogen fixation due to its ability to enter into a symbiotic relationship with a soil bacterium, *Mesorhizobium loti*. The *LORE1* mutant population is a valuable resource for reverse genetics in *L. japonicus* due to its non-transgenic nature, high tagging efficiency, and low copy count. Here, we outline a workflow for identifying, ordering, and establishing homozygous *LORE1* mutant lines for a gene of interest, *LjFls2*, including protocols for growth and genotyping of a segregating *LORE1* population.

Key words *Lotus japonicus*, *LORE1*, Reverse genetics, Mutagenesis, Genotyping

1 Introduction

Lotus japonicus is a well-characterized model legume [1] that is widely used in the study of biological nitrogen fixation when entering a symbiotic relationship with its compatible symbiont *Mesorhizobium loti* [2]. The published genome sequence of *L. japonicus* [3], combined with the public release of *Lotus* Base, a central information portal for the model legume [4], enables researchers to tap into the wealth of genomics and expression data from *Lotus*. Additional proteomic data from *Lotus* are available separately [5].

Since the discovery of mobile genetic elements in maize [6], their mutagenic nature has been widely utilized for large-scale mutagenesis in various model plants, such as *Tnt1* in *Medicago truncatula* [7] and *Tos17* in rice [8]. The endogenous *Lotus* retrotransposon element 1 (*LORE1*) was first discovered in a nodulation mutant, *Nin* [9]. Its subsequent successful derepression in tissue culture [10] culminated in the establishment of large mutant populations, comprising more than 134,000 mutant lines and 640,000 annotated insertions [11–13]. The non-transgenic nature of the *LORE1* element, its low copy number, and its high tagging efficiency posit *LORE1* as a valuable resource in forward and reverse

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genetic studies in *L. japonicus* [12]. The *LORE1* resource has been used in various forward [11, 12] and reverse genetics studies [14–18], but applications in the former are considered beyond the scope of this chapter and will not be discussed further.

Here, we describe the complete workflow of a typical researcher aiming to generate homozygous *LORE1* mutants for the purpose of downstream characterization and genetic studies of a gene of interest. We have selected a gene encoding the putative *Lotus* ortholog of the flagellin receptor, *FLS2* from *Arabidopsis thaliana* (AT5G46330), as a candidate. In this workflow, a researcher will be guided through the procedure for identification of *Lotus* orthologs/homologs for genes of interest by (1) searching for exonic *LORE1* insertions in the candidate gene; (2) ordering the *LORE1* mutants of interest; (3) germinating, growing, and genotyping a segregating *LORE1* F0 population; and finally (4) selecting and setting up homozygous *LORE1* mutants for seed production.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature and away from direct sunlight unless otherwise stated.

2.1 Seed Cleaning, Scarification, and Germination

1. Sandpaper.
2. Ceramic mortar.
3. 1% (v/v) hypochlorite solution.
4. Conserve (Dow Agroscience, Denmark).
5. Sterile forceps.
6. Greiner square petri dishes (120 × 120 × 10 mm; Sigma-Aldrich, Denmark).
7. Sterile aluminum foil (cut to 30 × 120 mm).
8. Parafilm M (Bemis Company Inc., USA).
9. Growth chamber or room, with the ability to regulate day/night cycle, light intensity, temperature, and humidity.
10. UV light.
11. Simple household blender.
12. 1 mm and 2 mm Metal gauzes.
13. Wax paper bag.

2.2 Plant Growth

1. Solution A: 291.4 g/L $CaCl_2.2H_2O$.
2. Solution B: 68.5 g/L KH_2PO_4, 113.4 g/L K_2HPO_4.
3. Solution C: 4.9 g/L ferric citrate.

4. Solution D: 123.3 g/L $MgSO_4 \cdot 7H_2O$, 87.0 g/L K_2SO_4, 0.338 g/L $MnSO_4 \cdot 7H_2O$, 0.247 g/L H_3BO_4, 0.288 g/L $ZnSO_4 \cdot 7H_2O$, 0.100 g/L $CuSO_4 \cdot 5H_2O$, 0.056 g/L $CoSO_4 \cdot 7H_2O$, 0.048 g/L $Na_2MoO_4 \cdot 2H_2O$.
5. 1/4 B&D medium: Per 1 L 1.4% (w/v) of Agar Noble, add 125 µL of solutions A, B, C, and D (in that order) [19]. Optional nitrate supplemented is achieved with 1 mM KNO_3 (*see* **Note 1**).

2.3 DNA Extraction Components

1. Tungsten carbide beads.
2. Tissuelyzer (QIAGEN, Denmark).
3. Isopropanol.
4. 70% ethanol.
5. Nanodrop (Thermo Fisher Scientific, USA).
6. Chloroform:isoamyl alcohol 24:1.
7. Rapid DNA extraction buffer: 200 mM Tris–HCl adjusted to pH 7.5, 250 mM NaCl, 25 mM 0.5 M EDTA adjusted to pH 8.0, and 0.5% (w/v) sodium dodecyl sulfate.
8. CTAB DNA extraction buffer: 2% (w/v) CTAB, 0.1 mM Tris–HCl adjusted to pH 8.0, 1.4 M NaCl, and 20 mM EDTA adjusted to pH 8.0. Add 0.5% (v/v) of β-mercaptoethanol immediately before use.
9. TE buffer: 10 mM Tris–HCl adjusted to pH 7.5 and 1 mM EDTA adjusted to pH 8.0.

2.4 Genotyping PCR

1. λ DNA (Fermentas, Germany).
2. Pst1 restriction enzyme (Fermentas, Germany).
3. Gel visualization equipment.
4. Genotyping PCR master mix (per reaction):
   2 µL of each forward and reverse primers (2.5 µM).
   0.1 µL of 20 µM dNTP.
   2.0 µL of manufacturer-supplied 10× reaction buffer.
   0.1 µL of Taq polymerase.

The master mix should be topped up to a total of 15 µL with ultrapure water. Each PCR reaction comprises 15 µL of master mix and 5 µL of extracted DNA.

2.5 Gel Electrophoresis Components

1. 5× loading buffer: 25% (v/v) glycerol, 0.8% (w/v) bromophenol blue, and 0.8% (w/v) xylene cyanol.
2. TAE buffer: 4.84 g/L Tris, 10% (v/v) 0.5 M EDTA adjusted to pH 8.0, and 5.71% (v/v) glacial acetic acid.
3. DNA ladder: Add 333 µL of 0.3 mg/mL λ DNA to 40 µL of 10× Pstl enzyme buffer. Add 5 µL of 40 unit μL^{-1} of Pstl. Top up the final mixture to a total volume of 400 µL, and incubate mixture overnight at 37 °C. Add 100 µL of 5× loading buffer before storing in −20 °C.

3 Methods

All wet lab procedures are performed at room temperature unless otherwise stated. In this section, we outline the workflow of a researcher interested in generating homozygous *LORE1* mutants of a gene of interest, in this case a putative *Lotus* ortholog of the *AtFLS2* gene.

3.1 Identification and BLAST Search for the Lotus Ortholog(s) of AtFLS2

1. Retrieve the amino acid sequence of *AtFLS2* (AT5g46330) from Araport [20]. The sequence is available from https://apps.araport.org/thalemine/report.do?id=1097852.
2. Search the retrieved sequence against the *L. japonicus* MG20 v3.0 protein database on *Lotus* BLAST (https://lotus.au.dk/blast/).
3. Retrieve the amino sequence of the top candidate (Lj4g3v0281040.1, *LjFls2*) from the SeqRet tool on *Lotus* Base (https://lotus.au.dk/tools/seqret), and BLAST against *Arabidopsis* TAIR protein database to validate the orthologous relationship.

3.2 Search for LORE1 Lines with Exonic LORE1 Insertions in LjFls2

1. Search for all *LORE1* mutant lines that contain genic insertions in *LjFls2* either by (1) using the TREX tool on *Lotus* Base (https://lotus.au.dk/tools/trex), selecting version 3.0 as the genome to be searched against, and then selecting "*LORE1* lines" in the drop-down contextual menu when hovering over the gene name on the results page, or (2) using the *LORE1* search page (https://lotus.au.dk/lore1/search), selecting version 3.0 as the reference genome and using "Lj4g3v0281040" as the gene ID in the filtering option. You should be presented with 45 mutant lines.
2. Select the *LORE1* lines of interest containing exonic insertions in *LjFls2* for further study (*see* **Note 2**).
3. Download the results by exporting a CSV file from the "download options" at the top of the page. You may download the entire search or check specific rows on the results page. The CSV file will contain other useful metadata for each insertion, such as the forward and reverse primer sequences used for genotyping (*see* Subheading 3.7).

3.3 Order LORE1 Lines of Interest

1. If required, apply for the necessary phytosanitary certificate(s) for the destination country by contacting the *Lotus* Base team (https://lotus.au.dk/meta/contact). The person placing the order shall bear the cost of said certificate(s). Should a phytosanitary certificate be required, the *LORE1* seeds shipment will only be dispatched when the relevant authorities have issued the certificate.

2. Place an order for *LORE1* lines of interest at the *LORE1* order page (https://lotus.au.dk/lore1/order). Currently, *LORE1* seeds are being shipped without the need to sign any material transfer agreements. The shipping time will usually be between 2 and 6 weeks depending on the geographical location of the recipient.

3.4 Seed Scarification and Germination

1. Scarify 16–20 *LORE1* seeds by abrasion of seed coats with sandpaper in a mortar until superficial layers of the seed coat have been removed and the seeds turn a lighter shade of brown.
2. Shake scarified seeds for 10 min at room temperature on a rotary shaker at 200 rpm in the presence of 1% (v/v) hypochlorite solution. Wash seeds four times with sterile water.
3. Stratify seeds by overnight incubation at 4 °C in 0.075% (v/v) Conserve (Dow Agroscience, Denmark, *see* **Note 3**) in the dark (*see* **Note 4**).
4. Germinate seeds on sterile filter paper wetted with 0.075% (v/v) Conserve under a 16 h/8 h day/night regime at 21 °C, with a light intensity of 200 μmol m^{-2} s^{-1} at the plant level, for at least three days [1].

3.5 Lotus Growth

1. Remove seed coat with a pair of sterile forceps (*see* **Note 5**).
2. Transfer seedlings onto square petri dishes (measuring 120 × 120 × 10 mm) poured with 50 mL of plant growth medium covered by a layer of wet sterile filter paper. The medium should be poured slanted and allowed to set as such, by propping up one end of the plate by around 8 mm. Shield roots from light by (1) placing a sterile wedge, made from a 30 × 120 mm aluminum foil folded into half lengthwise, onto the roots, and (2) wrapping upright petri dishes with opaque materials—black cupboard paper or aluminum foil—up till the height of the sterile aluminum wedge. Close the square petri dishes with parafilm tape (*see* **Note 6**). Incubate upright petri dishes at 21 °C under a 16 h/8 h day/night regime with a light intensity of 200 μmol m^{-2} s^{-1} in a growth chamber for the entire growth period. The simplified stepwise setup of plates for plant growth is summarized in Fig. 1.

3.6 DNA Extraction from Lotus Plants

Depending on the required quality of the DNA preparations, either Subheading 3.6.1 or 3.6.2 is performed.

3.6.1 Rapid DNA Extraction from Lotus Plants

Perform all centrifugation steps at 4 °C. This section describes a quick and dirty protocol used for DNA extraction. If a purer sample is desired, i.e., for sequencing, the CTAB protocol is recommended (*see* Subheading 3.6.2).

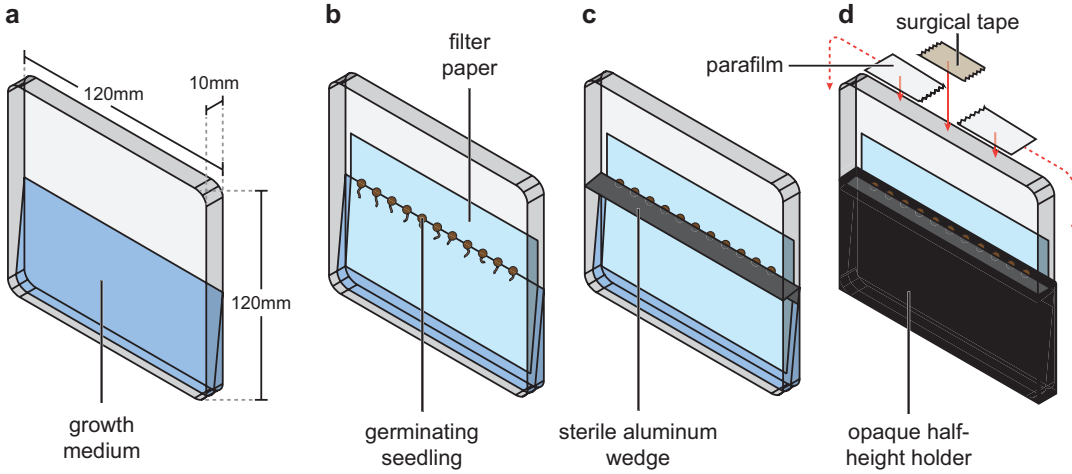

Fig. 1 The stepwise setup of the slanted plate system for growing Lotus plants: (**a**) 50 mL plant growth media is poured slated onto a square petri dish and allowed to set; (**b**) a maximum of ten germinating seedlings are transferred onto the medium and are equally spaced apart; (**c**) a sterile aluminum wedge is placed on top of the seedlings to shield roots from light above; and (**d**) the plates are wrapped in opaque paper or aluminum foil to cover roots from lateral light sources. Objects in the diagram are not drawn to scale. Broken red lines indicate stretching of parafilm around perimeter of plate to achieve a seal. Solid red lines indicate adhesion to plate perimeter

1. Remove a single trefoil from a plant, and place it in an Eppendorf tube (EPT) or collection microtube (CMT), depending on the number of samples that have to be processed (*see* **Note 7**). Harvesting of plant material from *Lotus* plants can be performed as soon as the first or second trefoil appears.
2. Add 400 μL DNA extraction buffer and a single tungsten carbide bead to each sample, and grind samples for 2 × 3 min at 30 Hz in a Tissuelyzer.
3. Centrifuge lysed samples for 10 min at 13,200 × *g* (EPT) or 40 min at 2700 × *g* (CMT).
4. Transfer 300 μL of the supernatant into new tubes, and add an equivalent volume of ice-cold isopropanol.
5. Incubate mixture at room temperature for at least 2 min with occasional mixing by inverting the tubes.
6. Precipitate DNA by centrifuging samples for 10 min at 13,200 × g (EPT) or 40 min at 2700 × *g* (CMT).
7. Wash pellet using 300 μL of 70% ethanol, and then centrifuge samples again for 5 min at 13,200 × *g* (EPT) or 40 min at 2700 × *g* (CMT).
8. Discard supernatant and allow pellet to dry at 65 °C for 15–30 min.
9. Resuspend pellets in 75 μL of TE buffer by heating at 65 °C for 15 min.

10. Check the quality and quantity of extracted DNA using a Nanodrop (Thermo Fisher Scientific, USA), following manufacturer's protocol.
11. Use 5 μL of DNA for the genotyping PCR amplification.

3.6.2 High-Quality DNA Extraction from Lotus Plants

If a purer DNA sample is required, we recommend using the CTAB method of DNA extraction, previously described in Urbanski et al. [13] and adapted from Rogers and Bendich [21].

1. Add a single tungsten carbide bead per sample, and homogenize plant tissues using a Tissuelyzer for 2 × 45 s.
2. Add 600 μl of CTAB extraction buffer. Incubate samples for 20 min in a 65 °C hot water bath.
3. Add 600 μL of chloroform:isoamyl alcohol 24:1. Shake mixture vigorously on a rotary shaker for 15 min at room temperature.
4. Centrifuge mixture for 10 min at 12,000 × *g*.
5. Transfer supernatant to new sterile tubes, and add 600 μL of isopropanol. Invert mixture three times.
6. Centrifuge mixture for 1 min at 12,000 × *g*. Discard supernatant, and wash DNA pellet with 500 μL of 70% ethanol.
7. Centrifuge samples for 10 min at 12,000 × *g*.
8. Dry pellet for at least 15 min in a fume hood, and resuspend in 30 μL of ultrapure water.

3.7 Genotyping PCR and Gel Electrophoresis to Identify Homozygous LORE1 Mutants from a Segregating F0 Population

LORE1 mutants can be genotyped using a modified touchdown PCR protocol [22], outlined in Table 1 (*see* **Note 8**). Each *LORE1* mutant should be genotyped with two pairs of primers: one comprising the forward and reverse primers and the other comprising the forward primer and the *LORE1* P2 primer, 5′-CCATGGCGGTTCCGTGAATCTTAGG-3′ (*see* **Note 9**).

1. Add 5 μL of loading buffer to 20 μL of PCR sample, and load samples onto a 1.5% agarose gel with 0.5 mg/L ethidium bromide. Load 8 μL of PstI-digested λ DNA marker per row.
2. Resolve DNA bands for 30–90 min at 150 V in TAE buffer.
3. Visualize DNA bands under UV light. *LORE1* homozygous mutants are identified by successful amplification with the forward and P2 primer pair, but not by the forward and reverse primer pair (Fig. 2; also *see* **Note 10**).

3.8 Establishing Homozygous fls2 Mutants from the LORE1 Collection

1. Scarify, germinate, and grow homozygous *LORE1* mutants as described in Subheadings 3.4 and 3.5. Perform PCR genotyping as per Subheading 3.7 to select mutants homozygous for the *LORE1* insertion.

Table 1
The modified touchdown PCR program for *LORE1* genotyping

Step	Temperature/°C	Duration	Cycle(s)
Activation	95	3 min	1
Denaturation Elongation	95 72	30 s 1 m 15 s	5
Denaturation Annealing Elongation	95 72–62 (−1 °C per cycle) 72	30 s 30 s 45 s	10
Denaturation Annealing Elongation	95 62 72	30 s 30 s 45 s	20–30
Termination	72	10 m	1
Storage	10	∞	1

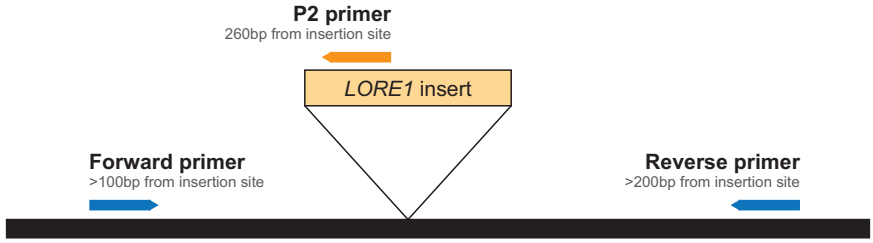

Fig. 2 The design of LORE1 genotyping primers. Note that the figure is not to scale. Forward and reverse primers are designed using Primer 3 [23] and are located at least 100 and 200 bp away from the LORE1 insertion site, respectively. The P2 primer binds to a region 264 bp downstream of the LORE1 5′ LTR

2. Sow homozygous mutants out for seed production in the greenhouse (14/10 h light/darkness and >70% relative humidity). If relative humidity drops below 65%, mature seed pods will burst, dispersing the seeds before they can be collected.
3. Transfer plants into larger pots when the root systems are well developed and extend throughout the pot volume. Two liter pots are well suited for growing plants for seed production. As *Lotus* have rather weak stems to support the relatively heavy and abundant foliage, tying the stems against vertical supports will help to control horizontal spread of plants.
4. Seed production in F0 homozygous plants will start around 1.5–2 months after sowing out in the greenhouse. Harvest seed pods, and allow them to dry for a week at room temperature and away from direct sunlight prior to processing.
5. Extract seeds by processing seed pods in a blender for 5–10 s.

6. Pass contents of the blender through a 2 mm metal gauze, which allows for *Lotus* seeds to pass through but not larger plant or pod debris.
7. Pass seeds through a 1 mm metal gauze, and shake to remove smaller particles. Seeds are typically larger than 1 mm, and do not pass through the second filter.
8. Store seeds in folded wax paper, at room temperature and away from direct sunlight and high levels of moisture. These seeds can be scarified, germinated, and grown (as described in Subheadings 3.4 and 3.5) for future studies.

3.9 Deposit Validated Homozygous LORE1 Lines at Legume Base

To support research activities in the community and to ensure the continued availability of characterized *LORE1* mutants, we strongly encourage users of the LORE1 resource to deposit seeds of validated mutant lines at Legume Base, following the procedure outlined below.

1. Prepare a table in Microsoft Excel format with the following information for each homozygous *LORE1* line: name of mutant allele, ±1000 bp flanking sequence, original *LORE1* line ID, brief phenotype description, optional comments, and optional publication reference.
2. Send the table to the Legume Base curators at legume@brc.miyazaki-u.ac.jp.
3. Please enclose a phytosanitary certificate when shipping the seeds. If no phytosanitary certificate is available, please print "*Lotus japonicus* seeds enclosed" on the envelope.
4. Send the seeds to The National BioResource Project (*L. japonicus* and *Glycine max*) Office, Faculty of Agriculture, University of Miyazaki, Miyazaki 889–2192, Japan. Please mark the envelope "*LORE1* mutants."

4 Notes

1. Nitrate supplementation is only recommended for long-term growth of plants, and should not be used *in lieu* of inoculation with nodulating symbionts due to nitrate-based inhibition of nodulation [24, 25].
2. When establishing new *LORE1* homozygous mutant lines, we recommend selecting three or more alleles in order to confidently eliminate the effect of background mutants in future phenotyping or characterization experiments. For the same reasons, *LORE1* lines with a low number of exonic and total insertions are typically preferred.
3. Use of Conserve is recommended to eliminate any possible seed-borne thrips.

4. Stratification of scarified Lotus seeds is optional but strongly recommended to ensure uniform germination.
5. Removing the seed coat helps to prevent contamination of the sterile growth medium. Dried-out seed coats provide optimal growth conditions for fungal spores that might be harbored within.
6. Avoid sealing square petri dishes too tightly. To allow proper ventilation, either (1) cut a slit on the curved sides on the top of the petri dish or (2) leave a 3 cm area on the top edge free from parafilm, and cover instead with surgical tape, to allow venting of accumulated ethylene gas. The presence of ethylene, a pleiotropic and potent plant hormone, is known to inhibit root and shoot elongation, affect gravitropism, and inhibit nodulation in *Lotus*. We find that with this modification, it is still possible to retain sufficient moisture within the square petri dishes for up to six weeks, before the growth medium and filter paper start to dry out.
7. The choice between individual Eppendorf tubes (EPT) and arrays of collection microtubes (CMT) depends on the scale of the experiment. For a large number of samples to be collected and genotyped, the use of CMT is strongly recommended due to the ease of handling and processing. Individual EPTs can be overwhelmingly cumbersome and time-consuming for sample sizes exceeding 24.
8. The touchdown PCR protocol is used to ensure specificity of the PCR amplification process. However, if no temperature steps or ramping is available on the machine, a simple three-step PCR protocol may be used, with annealing temperature set to 62 °C.
9. The two genotyping PCR reactions performed per *LORE1* line should not be carried out together, i.e., mixing all three primers—forward, reverse, and P2—in the same reaction. Due to how the genotyping primers are designed, the PCR product of the forward + reverse and the forward + P2 primers may have similar sizes that cannot be resolved on the agarose gel.
10. Due to the reliance on the absence of DNA bands in the forward and reverse primer mix, *LORE1* homozygous mutants, when identified as such in the first PCR run, should be re-genotyped at least once more to confirm their genotype prior to sowing out for seed production.

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