Lotus burttii Takes a Position of the Third Corner in the *Lotus* Molecular Genetics Triangle

Masayoshi Kawaguchi,[1,8,*,§] Andrea Pedrosa-Harand,[2,§] Koji Yano,[3,§] Makoto Hayashi,[3,8] Yoshikatsu Murooka,[3] Katsuharu Saito,[1] Toshiyuki Nagata,[1] Kiyoshi Namai,[4,†] Hiroshi Nishida,[4] Daisuke Shibata,[4] Shusei Sato,[4] Satoshi Tabata,[4] Masaki Hayashi,[7] Kyuya Harada,[7] Niels Sandal,[5] Jens Stougaard,[5] Andreas Bachmair,[2,‡] and William F. Grant[6]

Department of Biological Sciences, Graduate School of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-0033, Japan,[1] *Department of Cell Biology and Genetics, Institute of Botany, University of Vienna, Rennweg 14, A-1030 Vienna, Austria,*[2] *Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamdaoka, Suita, Osaka 565-0871, Japan,*[3] *Kazusa DNA Research Institute, 2-6-7 Kazusa-kamatari, Kisarazu, Chiba 292-0818, Japan,*[4] *Laboratory of Gene Expression, Department of Molecular Biology, University of Aarhus, Gustav Wieds Vej 10, DK-8000 Aarhus C, Denmark,*[5] *Department of Plant Science, Macdonald Campus of McGill University, Ste. Anne de Bellevue, Quebec H9X 3V9, Canada,*[6] *Faculty of Horticulture, Chiba University, 648 Matsudo, Matsudo, Chiba, 271-8510, Japan,*[7] *and Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0112, Japan*[8]

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Abstract

In order to consolidate molecular genetic system in *Lotus japonicus* and to further access the biological diversity in Lotea, we introduce here *Lotus burttii* B-303 derived from West Pakistan as the third crossing partner of the Gifu ecotype (B-129-S9) for a genetic analysis. *L. burttii* is a relatively small and early flowering plant with non-shattering behavior. The general chromosome morphology is very similar to Gifu, and fluorescence *in situ* hybridization (FISH) analysis revealed that the short arm of chromosome 1 in *L. burttii* is comparable to that of Gifu, indicating that the translocation event involving chromosomes 1 and 2, which was observed in *L. japonicus* Miyakojima MG-20, is not present in *L. burttii*. In addition *L. burttii* has a higher level of DNA polymorphism compared to Gifu and MG-20 enabling design of codominant markers such as SSR, CAPS and dCAPS. Using an F_2 population from a cross between Gifu and *L. burttii*, codominant makers that co-segregated at the translocation site could be expanded. In order to normalize the genetic background, *L. burttii* was inbred for nine generations and the germplasm *L. burttii* B-303-S9 was established.

Key words: *Lotus burttii*; *Lotus japonicus*; molecular genetics; fluorescence *in situ* hybridization (FISH); crossing partner

The Leguminosae comprise the third largest family with more than 18,000 species and a remarkable biodiversity. Features of this biodiversity range over seed proteins; carbohydrates; oils; secondary metabolites; morphological traits and biological interactions with insects, animals and microorganisms such as symbiotic nitrogen fixing bacteria and mycorrhizal fungi. The family also comprises crops including many important resources sustaining human life. However, molecular characterization of genes responsible for these properties is still only in its infancy. In order to identify these genes, the infrastructure of molecular genetics and genomics in several legumes has been studied extensively since the early 1990s. Among them, *Lotus japonicus* is today well known as a typical model legume,[1,2] together with *Medicago truncatula*.[3,4] In *L. japonicus*, a substantial effort has been devoted to establishing infrastructures and technology platforms for molecular genetics and genomics. Chromosomes were analyzed,[5–7] molecular linkage maps assembled,[8–14] large-scale ex-

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* To whom correspondence should be addressed. Tel. & Fax. +81-3-5841-4458, E-mail: masayosi@biol.s.u-tokyo.ac.jp

† Present address: Tochigi Prefecture Agricultural Experiment St., 1,080 Kawaraya-cho, Utsunomiya 320-0002, Japan

‡ Present address: Department of Plant Developmental Biology, Max Planck Institute for Plant Breeding Research. Carl-von-Linné-Weg 10, D-50829 Cologne, Germany

§ These authors contributed equally to this work.

pressed sequence tags (EST)[15–19] and cDNA array analyses performed,[20–23] gene tagging initiated,[24–26] BAC clones constructed,[27,28] and a whole genome sequencing project[9,11–14] focused on gene-rich regions is progressing rapidly. Based on the genomic data, RNA interference[29] and TILLING (Target-induced local region)[30] systems were also developed recently for approaching reverse genetics.

L. japonicus is distributed from subtropical to subarctic regions of Asia.[31] One ecotype Gifu derived from a riverbank of Gifu prefecture in Japan has been used worldwide, owing to its model characteristics and its stable transformation system.[1,32–34] A number of mutants affecting root symbiosis with microorganisms,[35–38] photorespiration[39] and nyctinastic movement[40] were isolated in this genetic background.

In order to map these mutant alleles and identify the corresponding genes, *L. japonicus* Miyakojima MG-20 derived from the southern most island of Japan[41,42] and a related species *L. filicaulis*[10] originating from Algeria were used as crossing partners for Gifu. Two high-density linkage maps were developed from genotyping of these F_2 mapping populations[8–14] and symbiotic genes such as *SYMRK*,[43] *HAR1*,[44,45] *ASTRAY*,[46] *NFR1*,[47] *NFR5*,[48] *CAS* and *POL*[49] have been cloned. Both genetic maps have advantages and disadvantages. Among the Japanese accessions, MG-20 was chosen as a partner because of its relatively high level of polymorphism (4.5% to 6% for AFLPs [amplified fragment length polymorphisms]),[8,42] allowing molecular markers to be generated, but due to a translocation of the short arm of Gifu chromosome 1 to the bottom of chromosome 2 in MG-20, recombination is significantly suppressed in a chromosomal segment close to the translocation site.[8] On the other hand, *L. filicaulis* has a very high level of DNA polymorphism by AFLP analysis (up to 49%),[10] but two paracentric and pricentric inversions on chromosomes 1 and 3, respectively,[7] lead to distorted segregations. In order to overcome these structural problems limiting recombination frequencies and consolidate the infrastructure of molecular genetics in *L. japonicus*, we introduce here *L. burttii* B-303 as a third crossing partner in a *Lotus* genetic triangle. *L. burttii* is a relatively small and early-flowering plant with non-shattering behavior of pods that was originally collected from banks of the Kabul River, Peshawar, West Pakistan.[50]

1. *L. burttii* as a Possible Crossing Partner for *L. japonicus* Gifu

It was reported in the early 1970s that diploid and self-fertile species such as *L. burttii*, *L. alpinus*, *L. krylovii*, *L. filicaulis* and *L. shoelleri* could cross with *L. japonicus* Gifu and produced F_1 hybrids.[51] Among them, we obtained seeds of two *Lotus* species, *L. burttii* B-303 and *L. krylovii* B-568, and grew them in a growth cabinet (Biotron LH-300, Nippon Medical & Chemical Instruments, Japan). Under these conditions, *L. burttii* flowered 5 weeks after sowing and produced a number of seeds under 18 hr/6 hr day/night cycle, at a light intensity of approximately 150 $\mu Esec^{-1}m^{-2}$ at 25°C, 70%–90% humidity, whereas *L. krylovii* hardly flowered under these conditions. Based on the advantageous short flowering time and the ease of seed production, we continued experiments with *L. burttii*. Interspecific crosses were done by emasculation and transfer of donor pollen to stigma. *L. burttii* was successfully crossed with Gifu and MG-20. The resulting F_1 hybrids were easily discriminated from their parents because they were morphologically intermediate between the parents. For example, leaflet width of F_1 plants is wider than *L. burttii* and narrower than *L. japonicus*. No significant reciprocal differences were observed. These observations were consistent with the previous observations by O'Donoughue et al. (1989).[52] The F_1 plants have normal fertility and produced a number of F_2 seeds.

2. FISH Analysis of *L. burttii* Chromosomes

The chromosome complement of *L. burttii* was analysed by FISH using some of the probes that were previously mapped onto *L. japonicus* Gifu chromosomes.[7] Using this approach, it was possible not only to establish an idiogram for *L. burttii*, but also to directly compare its chromosomes to the homologous chromosomes of the model legume. The probes used were: R2, a 6.5-kb fragment of an 18S-5.8S-25S rDNA repeat unit from *Arabidopsis thaliana*,[53] and D2, a 5S rRNA clone, *Ljcen1*, a centromeric repeat, and seven plasmid and bacterial artificial chromosome (BAC) clones from *L. japonicus* Gifu B-129-S9.[7] All probes were labelled by nick translation (Roche Diagnostics, Vienna, Austria; Life Technologies, Vienna, Austria) with Cy3-dUTP (Amersham Pharmacia Biotech, Vienna, Austria). R2 was also labelled with biotin-14-dATP (Life Technologies, Vienna, Austria).

Mitotic preparations, slides selection and pretreatment, and image analysis were performed according to Pedrosa et al. (2002).[7] Chromosome and probe denaturation, post-hybridization washes and detection were performed according to Heslop-Harrison et al. (1991),[54] with modifications described in Pedrosa et al. (2002).[7] Reprobing of slides for localisation of different DNA sequences on the same cell was performed according to Heslop-Harrison et al. (1992).[55]

The general chromosome morphology was very similar between *L. japonicus* Gifu and *L. burttii*. Both complements can be divided into one large, three medium and two small chromosomes. The 45S rDNA probe detected two rDNA loci on *L. burttii* chromosomes: a major locus at the short arm of the second largest chromosome (chr. 2) and a minor locus close to the centromere of a small chromosome, which was designated as chromo-

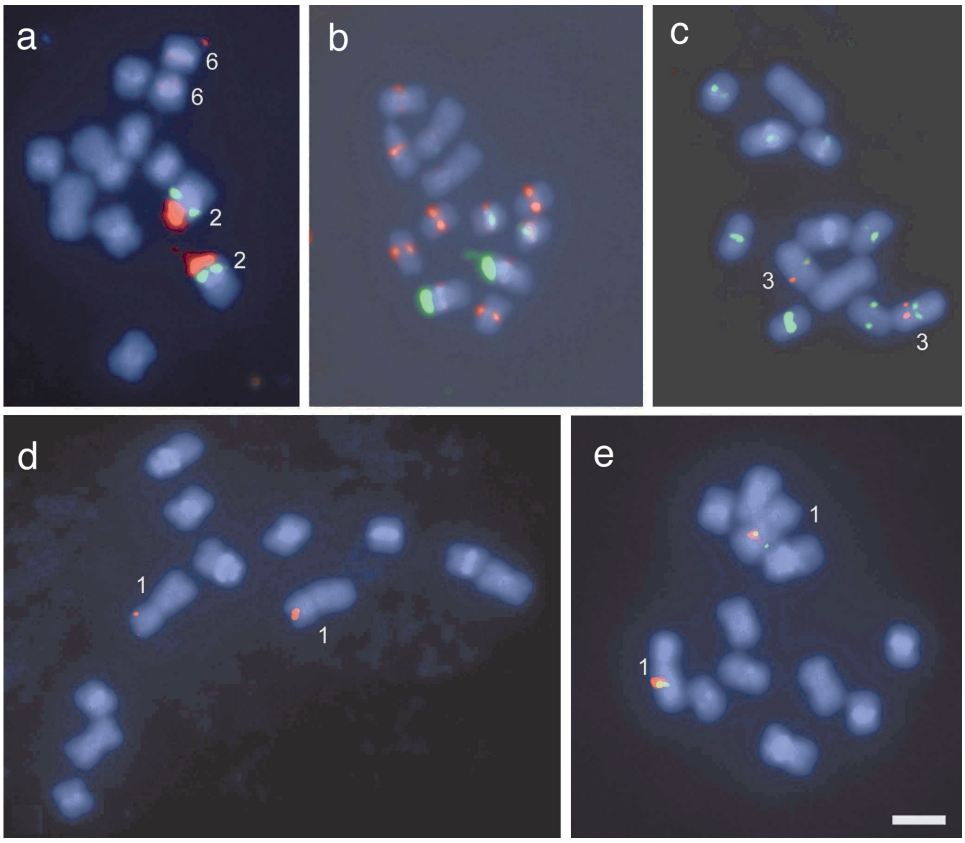

Figure 1. *In situ* hybridization to mitotic chromosomes of *Lotus burttii*. **a**. 45S (red) and 5S rRNA genes (green) on chromosomes 2 and 6; **b**. *Ljcen1* repeat distribution (red). Chromosomes were identified with the help of 45S rDNA (green); **c**. BAC 85D15 (red) on metacentric (*Ljcen1* repeat, green) chromosome 3; **d**. BAC S1.1 (red) on chromosome 1; **e**. Co-localisation of BACs 81J4 (red) and 53C15 (green) on chromosome 1. Bar=2.5 μm.

some 6. One 5S rRNA site was located proximally to the major rDNA site on chromosome 2 (Fig. 1a). 45S and 5S rRNA gene clusters were located in similar positions with Gifu, but *L. burttii* did not have a third, very small 45S rDNA site on chromosome 5 that could be detected in *L. japonicus*. This third rDNA site was also not detected in *L. filicaulis*, suggesting that this additional site may be exclusively present in *L. japonicus*. Hybridization of the *Ljcen1* repeat revealed the expected centromeric distribution pattern, confirming that all chromosomes were metacentric, except for chromosome 1, which was submetacentric. The number of *Ljcen1* repeats in chromosome 1 in *L. burttii* appears to be relatively low, comparing the strength of the signals on different centromeres (Fig. 1b).

Seven single-copy clones were used to confirm the identification of chromosomes and to evaluate the conservation in position of those clones in comparison to Gifu. BAC clone 85D15 was located interstitially in the long arm of the metacentric chromosome 3 (Fig. 1c), as observed in Gifu. BAC clone S1.1 was located terminally on the short arm of the largest chromosome 1 (Fig. 1d), indicating that the translocation event involving chromosomes 1 and 2, which was observed in MG-20,[8] is not present in *L. burttii*. However the long arm of chromosome 1 of *L. burttii* was altered in comparison to that of Gifu. Two BAC clones 53C15 (*cyclin2*) and 81J4 (*Nin-like protein 1* [*Nlp1*]) were located interstitially on the long arm of chromosome 1 in Gifu[7] and in *L. burttii*, but these clones were localised very close to each other only in the latter species (Fig. 1e). Figure 2 summarizes the localisation of all clones that were mapped on *L. burttii* chromosomes. The close proximity between BAC clones 53C15 and 81J4 on the long arm of *L. burttii* chromosome 1 resembles the position of these clones in *L. filicaulis*.[7] This observation suggests that both clones had been in close proximity in the ancestor of the two species and a paracentric inversion in the long arm of chromosome 1 might have occurred during the speciation of *L. japonicus*.

3. Genetic Resolution of a Recombination-Suppressed Region at a Chromosome Translocation Site

Although a relatively small paracentric inversion in the long arm of chromosome 1 has altered the colinearity of *L. burttii* and Gifu chromosomes 1, *L. burttii*

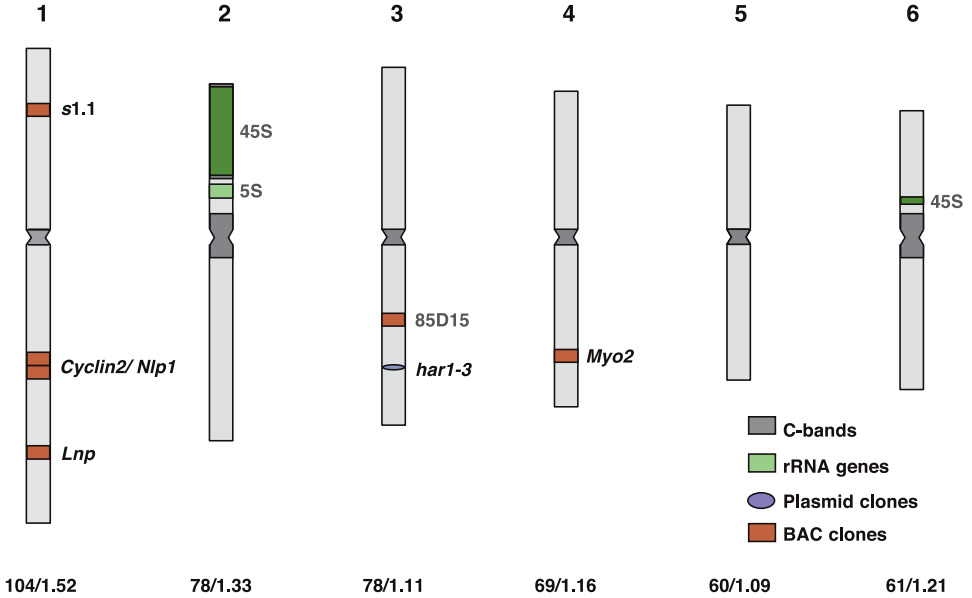

Figure 2. Idiogram of *Lotus burttii* showing relative chromosome length, position of centromeres, distribution of heterochromatin, and mapping of plasmid and BAC clones. Numbers above chromosomes indicate chromosome numbers. Numbers below chromosomes indicate chromosome sizes in megabase pairs (based on Sz.-Borsos 1973 and Pedrosa et al. 2002) and arm ratios, respectively.

may be well suited as a parent in a mapping population resolving regions with suppression of recombination in the MG-20×Gifu population. To analyse this possibility, we focused on a segment surrounding the chromosome 1/2 translocation site. In the Gifu×MG-20 mapping population, there is zero recombination between markers in this segment and, due to the translocation, markers in the segment can not be assigned to either linkage group I (chromosome 1) or linkage group II (chromosome 2). Previously generated markers belonging to this zero-recombination segment and some newly developed markers (see below) were used to determine the genetic resolution of linkage groups I and II in the Gifu×*L. burttii* population. A *L. burttii*×Gifu mapping population of 154 plants were genotyped and 18 markers out of 26 SSR (simple sequence repeats) and 3 dCAPS (derived cleaved amplified polymorphic sequence) markers that mapped at the translocation site in the MG-20×Gifu map exhibited DNA polymorphism between Gifu and *L. burttii*.

Prior to precise linkage analysis we surveyed insertion/deletion (in/del) or single nucleotide polymorphisms (SNPs) in the translocation region as follows. First, primers were designed by Primer3 program[56] based on the genomic clones from MG-20 in order to amplify corresponding genomic regions of Gifu and *L. burttii*, and the resultant PCR fragments were sequenced. For in/del polymorphisms between Gifu and *L. burttii*, flanking regions of primers were designed by Primer3. When only SNPs were found, primers were designed by dCAPS Finder program[57] to generate appropriate CAPS of PCR products. The amplification condition for PCR was 94°C for 5 min, followed by 45 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, and finally 72°C for 10 min. For CAPS or dCAPS markers, an equal amount of the appropriate enzyme mixture including 3 U of an enzyme was added to 10 μl of PCR products, and then incubated for 3 hr. DNA fragments were electrophoresed in 13% native polyacrylamide, 3% NuSieve 3 : 1 agarose, or 3% MetaPhor agarose gel. We finally identified 16 SSR, 5 in/del, 4 CAPS, and 2 dCAPS markers (Tables 1 to 4). Map construction was carried out by JoinMap 3.0. Linkage analysis of these markers, together with newly developed markers, allowed us to assign previously inseparable markers to either linkage group I or II. Four markers (TM1473, TM0591, TM0771, TM0358B) expanded the bottom of linkage group II by 2.0 cM, while the other markers expanded linkage group I (short arm of chromosome 1) by 8.9 cM (Fig. 3). Also approximately 8.0 cM from TM0002 to TM0036 markers calculated using the Gifu×MG-20 F2 population could be expanded by 27.1 cM using the Gifu×*L. burttii* population (Fig. 3).

4. DNA Polymorphism

In order to assess the degree of DNA polymorphism between Gifu and *L. burttii*, AFLP analysis was conducted. Sixty-four primer combinations for selective amplification derived from *Eco*RI-CAA, -CAC, -CAG, -CAT, -CTA, -CTC, -CTG, -CTT and *Mse* I-GAA, -GAC, -GAG, -GAT, -GCA, -GCC, -GCG, -GCT were employed. The AFLP analysis and electrophoresis were performed as described in Hayashi et al. (2001). As a result, 360 out of 2752 bands derived from 64 primer combinations showed polymorphisms. Thus, the polymorphism rate between

Table 1. List of SSR markers.

Marker name	Forward primers	Reverse primers
TM0002	AGCGATCTACATTCAAGAG	AGCGTTCTCTCAGTGTTG
TM0021	GGTCATCTTTGTGATAGTAAGTAA	CTGTTGTATCAAGCCACAAG
TM0023	CATAAGCACAACAATTCATAG	GTTGTGGTTCAAAGTTAGGG
TM0027	AGGATAATTACATTCCACCTC	TCTTGCAATATCTATATGACTGG
TM0036	GATGTGACGGTGAGTTATTG	AGAGAGAAGTGGAGCTTACG
TM0078B	ATCCGTCATCTATAGGGTGG	TCCCTATGAGCAGGGTTATG
TM0103	TGACAAGAGCTTCATAAGAG	GATGAAGTACAGACACCGAC
TM0123	AATGAAAAGATGTATAACAGTCAC	TTTTCCAATCGAACTTGCCG
TM0145	ACTTCTCCACCACACCAGCC	ATGCAACCAGAAAACTCAGC
TM0166	TCCTTACAAACTGATCTCCG	GAATTCTCTCTTGAGAAAATAGTC
TM0181	CTGCTGATCGATTCCTGGAC	AATCATTCATTAATGGCATC
TM0289B	CTTTGTAAGATGGCTTAATTG	ATGAAAGAACATTGAGCGGG
TM0358B	TCATTCCAAGGTTGACCGAG	CCTCAAGCTTAATTTCTCCC
TM0591	GGCTTTACACTGAAGCTATG	TTTCCTTAGAATCAATTCTGAC
TM0771	CACTTCCTTTGAGAGCAGTC	GTTCCCTTGAAAATTGAATG
TM1473	TCTTTACCGAGTCCATACGC	CTCCATCAGATTGTTGTCAG

Table 2. List of insertion/deletion markers.

Marker name	Forward primers	Reverse primers
TM0016B	GGGGTTGTTTCCATTTTCTGT	GCTCCAATTAACCCAATGGA
TM0174B	AAAAGGGGAAAAGGACCAGA	CCTGCCATAATTCGAGGTGT
TM0252B	CAAATTGAGGGACTTGATTGC	TGCATTCTGTCTGGCCTATG
TM1265B	TTTTCAGCCACACCTTTTACA	TCTTGCTTCCATTCCTTTCAA
TM1436B	TGACAGCTTGGTTATCCACTTC	CGTTGAATGGTTCGCTCTTT

Table 3. List of CAPS markers.

Marker name	Enzyme	Forward primers	Reverse primers
TM0189B	*Hinf* I	TTGACGTGTCTTGGAAGCTG	CAATCACCACACAAGGCATC
TM1049B	*Hae* III	GTGACATCGCTGGAAAAGGT	ATGATGTTGACGACGGTGAA
TM1475B	*Hae* III	ATTTTCAGGGGTGCATTGTC	CCGCCAATCTCGTATTTGAT
TM1542B	*Bgl* II	TGGAAGAGGTCTGGGAAATG	CCACGGTCTCTTGTCCCTTA

Table 4. List of dCAPS markers.

Marker name	Enzyme	Forward primers	Reverse primers
TM0171B	*Hinf* I	ACCATAAGCCTCGGTCATGAG	CCGCTGCATTTCCTATCATT
TM0454B	*Hinf* I	GCAGTGTTTTCTCTTCACTCTTGAG	TCGTCTTCATCACCGGATAC

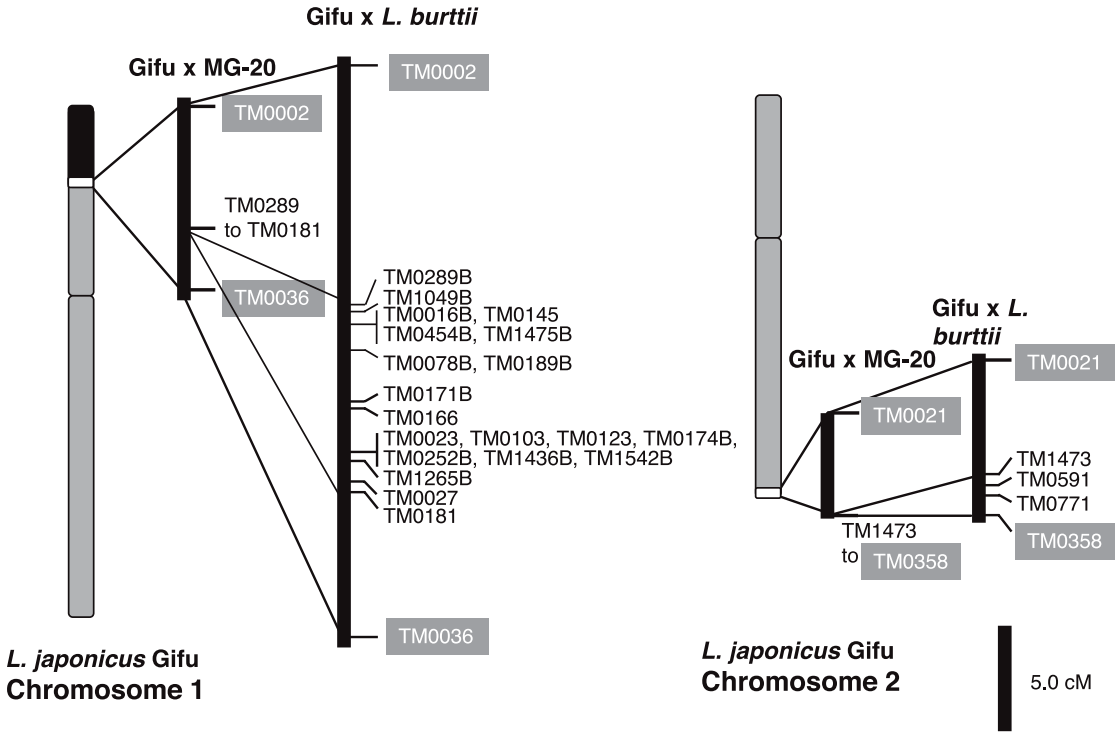

Figure 3. Genetic resolution of the *L. japonicus* Gifu/MG-20 chromosome 1/2 translocation site (dotted line). The Gifu × MG-20 linkage maps with markers flanking the zero-recombination segment are shown closest to the chromosomes. The same flanking markers, the expanded linkage maps from the Gifu × *L. burttii* population and assignment of markers between chromosomes 1 and 2 are shown next to the Gifu × MG-20 map.

the two species was 13.1%, and an average of 5.6 bands per primer pair showed polymorphisms. The polymorphism rate between Gifu and *L. burttii* is approximately three times higher than that between Gifu and MG-20,[42] indicating that *L. burttii* has a suitable DNA polymorphism to generate the codominant markers.

5. *L. burttii* Germplasm B-303-S9

In order to normalize the genetic background, an inbred line of *L. burttii* B-303 was established, referring to the method by Stougaard and Beuselink (1997).[58] From the seeds obtained from a single plant, approximately 10 seeds were germinated. Among them, one well-grown plant was selected and cultivated to collect the next-generation seeds. Single-seed-descent was practiced from the S_1 to S_9 generations. The self-pollination was performed in a greenhouse or a growth cabinet without pollinators. Finally, *L. burtii* B-303-S9 was established.

L. burttii B-303-S9 is relatively small in size compared to Gifu and MG-20. It develops a solitary flower at the top of each peduncle. The flowers of *L. burttii* are very pale yellow but turn red during maturation (Fig. 4a). The timing was accelerated by touch stimuli. Pods hardly shatter even under dry conditions, whereas those of *L. japonicus* shatter very easily after pod maturation (Fig. 4b). Seeds are freckled and light brown in color (Fig. 4c). The length of leaflets and bracts of *L. burttii* is almost the same as that of *L. japonicus* while the width is approximately one-half compared to *L. japonicus* (Fig. 4d). These phenotypic characteristics of *L. burttii* B-303-S9 are identical to previous phenotypic characterization,[50,52] indicating that there is no phenotypic modification through the establishment of the germplasm. Furthermore, *L. burttii* flowered early as observed in *L. japonicus* MG-20 naturally growing in southern parts of Japan.

In this report, we evaluated *L. burttii* as the third crossing partner of Gifu. *L. burttii* easily crossed with *L. japonicus* and the F_1 progeny produced ample F_2 seeds. Except for an inversion on the long arm of chromosome 1, chromosomes and representative marker positions of *L. burttii* were very similar to those of Gifu. In addition, *L. burttii* has a level of DNA polymorphism in between MG-20 and *L. filicaulis*, making it easy to generate co-dominant makers such as SSR, in/del, CAPS and dCAPS. Using such codominant markers that in the MG-20×Gifu population cosegregated at the translocation site between *L. japonicus* B-129 and MG-20 in the short arm of chromosome 1, we could expand this site to approximately 10 cM after assigning markers to both linkage groups I and II. Several mutants that define legume characteristics such as *Ljsym24*[37] (nod$^-$ myc$^-$; Martin Parniske, personal information), *Ljsym73*[38] (low nod; Makoto Hayashi, personal information), *Ljsym74* (*alb1*)[35,38]

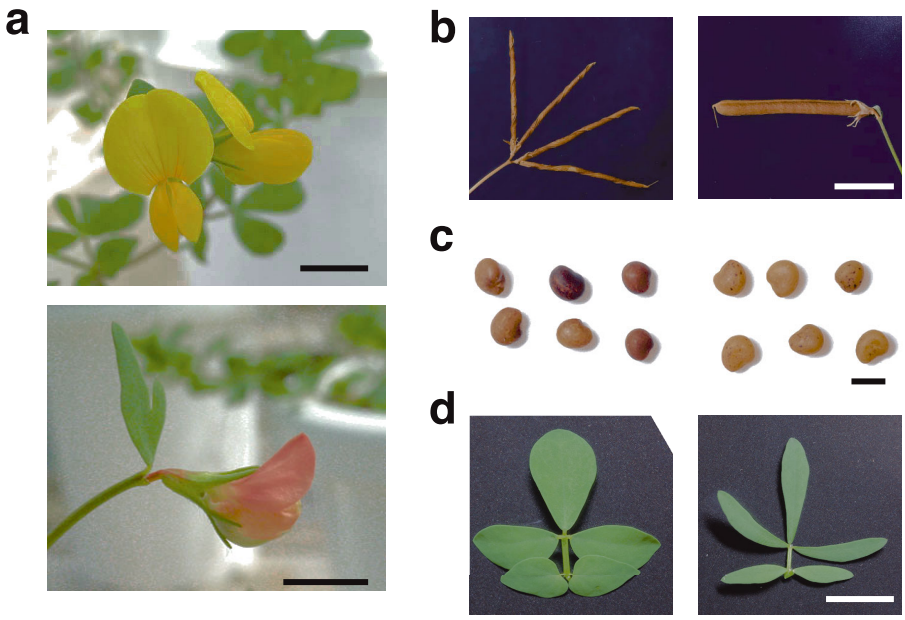

Figure 4. *Lotus burttii* germplasm B-303-S9. **a**. flowers of *L. japonicus* Gifu B-129 (upper) and *L. burttii* B-303-S9 (lower), *Bar* = 5 mm; **b**. pods of *L. japonicus* MG-20 (left) and *L. burttii* B-303-S9 (right), *Bar* = 10 mm; **c**. seeds of *L. japonicus* Miyakojima MG-20 (left) and *L. burttii* B-303-S9 (right), *Bar* = 1 mm; **d**. leaves of *L. japonicus* MG-20 (left) and *L. burttii* B-303-S9 (right) *Bar* = 10 mm.

(hist$^-$; Makoto Hayashi, personal information), *lot1* (low nod and distorted trichomes),[60] *sleepless*[40] (nyctinastic movement$^-$), *Ljsym85* and *Ljsym87* (unpublished nod$^-$ myc$^-$ mutants) derived from Gifu were mapped in or near this translocation site. Therefore, it is expected that *L. burttii* will facilitate molecular identification of these causal genes by positional cloning. Recombinant inbred lines of Gifu×*L. burttii* are currently propagated to improve and ease genetic linkage analysis.

During the evaluation of *L. burttii*, we recognized that *L. burttii* itself has several traits suitable for molecular genetics, because it is relatively small in size and flowers easily under fluorescent light. Furthermore, it shows strong non-shattering behavior of pods that could reduce the seed contamination and make it easier to harvest them prior to shattering. The genome size of *L. burttii* is almost the same as that of Gifu.[59] Hence molecular genetics using *L. burttii* will make a contribution to isolation and functional characterization of leguminous genes in the future.

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