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Construction of a Genetic Linkage Map of the Model Legume *Lotus japonicus* Using an Intraspecific F_2 Population

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Abstract

Among leguminous plants, the model legume *Lotus japonicus* (Regel) Larsen has many biological and genetic advantages. We have developed a genetic linkage map of *L. japonicus* based on amplified fragment length polymorphism (AFLP), simple sequence repeat polymorphism (SSRP) and derived cleaved amplified polymorphic sequence (dCAPS). The F_2 mapping population used was derived from a cross between two *L. japonicus* accessions Gifu B-129 and Miyakojima MG-20. These parental accessions showed remarkable cytological differences, particularly with respect to size and morphology of chromosomes 1 and 2. Using fluorescence *in situ* hybridization (FISH) with BAC clones from Gifu B-129 and TAC (Transformation-competent Artificial Chromosome) clones from Miyakojima MG-20, a reciprocal translocation was found to be responsible for the cytological differences between chromosomes 1 and 2. The borders of the translocations were identified by FISH and by alignment toward the *L. filicaulis* × *L. japonicus* Gifu B-129 linkage map. The markers from the main translocated region were located on linkage groups 1 and 2 of the two accessions, Gifu B-129 and Miyakojima MG-20, respectively. The framework of the linkage map was constructed based on codominant markers, and then dominant markers were integrated separately in each linkage group of the parents. The resulting linkage groups correspond to the six pairs of chromosomes of *L. japonicus* and consist of 287 markers with 487.3 cM length in Gifu B-129 and 277 markers with 481.6 cM length in Miyakojima MG-20. The map and marker information is available through the World Wide Web at http://www.kazusa.or.jp/lotus/.

Key words: *Lotus japonicus*; linkage map; translocation; AFLP; SSR; dCAPS; FISH

1. Introduction

The *Leguminosae* is the third largest family of angiosperms and comprise agronomically important crops, e.g. soybean, pea and bean. But molecular genetic analyses of agronomically important traits of crops are hampered by their large genome size or the complexity of the genome, and/or by the low transformation frequencies. The legume *Lotus japonicus* has many appropriate features as a model legume,[1] that is, diploidy (2n = 12), self fertility, small genome size (432 Mb, Pedrosa et al., manuscript submitted, 442 Mb,[2] 494 Mb[3]), short life cycle and high transformability with *Agrobacterium*.[1,4]

Genetic linkage maps are essential for genome analysis, including map-based cloning and construction of physical maps. Jiang and Gresshoff[5] tried to construct a genetic linkage map of *L. japonicus* with an F_2 population derived from a cross between two accessions, Gifu B-129-S9 and Funakura B-581 using DNA ampli-

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fication fingerprinting (DAF). As the level of DNA polymorphism between the parents was low, the number of linkage groups had not yet converged to the number of chromosome pairs. Sandal et al. developed a genetic linkage map of *L. japonicus* using an F_2 population made from an interspecific cross between *L. japonicus* Gifu B-129-S9 and *L. filicaulis* (manuscript submitted). A high level of DNA polymorphism between the parents was observed by amplified fragment length polymorphism (AFLP), and 518 AFLP markers and some additional sequence-specific PCR markers were mapped on six linkage groups. On chromosome 3, a pericentric inversion between the parental species was demonstrated by fluorescence *in situ* hybridization (FISH) and segregation distortions in the F_2 population were well described in Pedrosa et al. (manuscript submitted). In a screening of *L. japonicus* accessions for useful mapping partners, Kawaguchi et al.[6] found that Miyakojima MG-20 is the most polymorphic among the 15 accessions of *L. japonicus* when compared with Gifu B-129 by the AFLP technique. Miyakojima MG-20 was therefore selected as a suitable partner to establish a mapping population.

Based on the segregation of genotypes by AFLP, simple sequence repeat (SSR) and derived cleaved amplified polymorphic sequence (dCAPS) markers together with FISH using selected TAC clones and alignment toward the interspecific map, we have developed a genetic linkage map of *L. japonicus* using an F_2 population derived from a Gifu B-129 × Miyakojima MG-20 cross.

2. Materials and Methods

2.1. Plant materials and DNA isolation

The 127 F_2 plants of mapping population were derived from a single cross between two accessions of *Lotus japonicus*: Gifu B-129 and Miyakojima MG-20. The parents and the F_2 plants were grown in a greenhouse and leaves were collected for DNA isolation. Total DNA from leaves was extracted and purified using the DNeasy plant mini kit (QIAGEN, Germany).

2.2. AFLP analysis

The AFLP reaction was performed according to Vos et al.[7] with a slightly modified AFLP Analysis System I (Invitrogen, USA) procedure or by using *Hin*dIII/*Taq* I digestion. A total of 100–150 ng of genomic DNA of each parent and F_2 plant was completely digested with *Eco*RI and *Mse* I or by *Hin*dIII and *Taq* I. Digested DNAs were subjected to ligation reaction with adapters which correspond to the restriction sites. After ligation, reaction mixtures were diluted ten times with 1 mM Tris-HCl/0.1 mM EDTA solution. For amplification of restricted and ligated fragments, a two-step protocol was followed. The first step included the selective pre-amplification of adapter-ligated DNA with primers having one selective nucleotide. In the second step, selective amplification of pre-amplified DNA was performed with primers having two additional nucleotides (total of three nucleotides). All amplification reactions were performed with TaKaRa EX Taq (TaKaRa, Japan). Electrophoresis was conducted by high efficiency genome scanning (HEGS)[3,6] with non-denaturing 11–13% polyacrylamide running gels and 5% stacking gels. The gels were stained by Vistra Green (Amersham Pharmacia Biotech, UK) and electrophoresis patterns were detected with FluorImager 585 (Amersham Pharmacia Biotech, UK).

2.3. SSR and dCAPS markers

Strategies for the development of SSR and dCAPS markers from TAC (Transformation-competent Artificial Chromosome) clone sequences were described in the accompanying paper.[8] For the TM0088 SSR marker, we used the following set of primers that gave 151-bp and 161-bp products from Gifu B-129 and Miyakojima MG-20, respectively: Fw(5′-AGCTATAGTTCATAAAATCACCA-3′), Rv(5′-ATTACCACCATCTCTCTGGC-3′) The primer and product information about the other SSR and dCAPS markers was listed in the accompanying paper.[8]

2.4. Other PCR-based markers

Specific primer sets were developed for the Miyakojima MG-20 cDNA clones that are highly homologous to a nitrate transporter of *Arabidopsis*, a transcription factor IAA16 of *Arabidopsis* and thioredoxin of soybean. The PCR fragments showed length polymorphism between parents, and these primer sets were used as Expressed Sequence Tag (EST) markers (named LjNIT-1, LjIAA16-1, and Lj096, respectively). When primer pairs for Sctt008 and Satt567 were used for PCR, which are originally SSR markers of soybean, PCR products were observed in Gifu B-129 and absent in Miyakojima MG-20. These were also included in the map as DNA markers.

2.5. Linkage map construction

The test for segregation ratios of 3 : 1 or 1 : 2 : 1 was performed by χ^2 statistics. Codominant AFLP markers were identified, and a framework map was constructed using these markers and other codominant markers including SSR, dCAPS, and EST markers. The MAPMAKER/EXP. Ver. 3.0b[9,10] program was used for map construction. In this calculation, the 'compare' and the 'try' commands were used to determine the most likely order. Following these steps, the 'three point' command was used to confirm the marker order of the framework map. Finally, the positions for dominant markers derived from respective parents (maternal Gifu B-129 and paternal Miyakojima MG-20) were merged

into this framework map using the 'try' command. We constructed a separate linkage map for each parent. Recombination frequencies were converted into map distance in centimorgans using the Kosambi mapping function. The linkage criteria of the markers was an LOD score of > 3.0 and a maximum distance of 37.2 cM.

2.6. Cytological materials

Root tips were obtained from seeds germinating on moist filter paper or, in the case of the material from a *L. japonicus* Gifu B-129 × Miyakojima MG-20 cross, from cuttings growing in pots. Root tips were pre-treated with 2 mM 8-hydroxyquinoline for 4–5 hr at 16°C, fixed in methanol/acetic acid 3 : 1 (v/v) and stored in fixative at −20°C up to several weeks. Young flower buds from the *L. japonicus* Gifu B-129 × Miyakojima MG-20 individual were cut open, fixed and stored as described above.

2.7. DNA Probes for FISH

The probes used were: R2, a 6.5-kb fragment of a 18S-5.8S-25S rDNA repeat unit from *Arabidopsis thaliana*;[11] D2, a 5S rRNA clone, and *Ljcen1*, a centromeric repeat, from *L. japonicus* (Pedrosa et al., manuscript submitted); BAC clones from a *L. japonicus* Gifu B-129-S9 BAC library (Sandal et al., manuscript in preparation) and TAC clones from an *L. japonicus* MG-20 TAC library.[8] All probes were labeled by nick translation (Roche Diagnostics, Switzerland, Life Technologies, USA) with Cy3-dCTP or Cy3-dUTP (Amersham Pharmacia Biotech, UK). R2 and D2 were also labeled with biotin-14-dATP (Life Technologies, USA).

2.8. Fluorescence in situ *hybridization*

Mitotic preparations were obtained from root tips macerated in a 3% (w/v) cellulase 'Onozuka R-10' (Serva Electrophoresis, Germany), 10–30% (v/v) pectinase (Sigma-Aldrich, USA) enzyme solution in 0.01 M citric acid-sodium citrate buffer (pH 4.8) at 37°C for 2 hr. Meiotic preparations were obtained from flower buds macerated in a 2% (w/v) cellulase 'Onozuka R-10,' 2% (w/v) Pectolyase (Sigma-Aldrich, USA), 2% (w/v) Cytohelicase (Sigma-Aldrich, USA) enzyme solution in the same buffer, at 37°C for 1 hr. Digested material was transferred to a drop of 45% acetic acid and flamed before squashing. Slides were selected after staining with 2 μg/ml DAPI in McIlvaine's buffer (pH 7.0). Good preparations were de-stained in 3 : 1 (v/v) ethanol-acetic acid (30 min) and absolute ethanol (1 hr) and pre-treated as described in Pedrosa et al.[12]

Chromosome and probe denaturation, post-hybridization washes and detection were performed according to Heslop-Harrison et al.,[13] except for the stringent wash which was performed with 0.1 × SSC at 42°C. Hybridization mixes consisted of: 50% (v/v) formamide, 10% (w/v) dextran sulphate, 2 × SSC and 2–5 ng/μl of probe. The slides were denatured for 5 min at 75°C and hybridized up to 2 days at 37°C. Ten micrograms of salmon sperm sheared DNA and 10- to 100-fold excess *L. japonicus* $C_o t$-1 fraction[14] were added to the hybridization mix in some experiments. In those cases, slides were denatured first in 50% formamide, 2 × SSC and dehydrated in ethanol 96% for 5 min at −20°C. Denatured probe and blocking DNA were re-annealed in the hybridization mix for 3–4 hr at 37°C before being added to the slides. Biotin-labeled probes were detected using ExtrAvidin-FITC conjugate (Sigma-Aldrich, USA) in 1% (w/v) BSA. All preparations were counterstained and mounted with 2 μg/ml DAPI in Vectashield (Vector, USA). Reprobing of slides for localization of different DNA sequences on the same cell was performed according to Heslop-Harrison et al.[15] up to three times.

2.9. Image analysis

Photographs were taken on a Zeiss Axioplan (Carl Zeiss, Germany) equipped with a mono cool view CCD camera (Photometrics, USA). Images from the camera were combined and pseudo-colored using the IPLab spectrum software (IPLab, USA). For construction of the Miyakojima MG-20 basic karyotype, 10 well-spread complete metaphases were photographed and, for each chromosome, the total length and the length of one of its arms were measured in arbitrary units as described below. Relative lengths and centromeric index (short arm/total length) were calculated for each chromosome. Pairs were established according to size and morphology and mean values were calculated for each pair. At least five metaphases bearing clear signals, were photographed in order to confirm the position of a clone. Assignment of a clone to a specific chromosome arm was confirmed, when necessary, by reprobing the slides with a clone of known position. Measurements of arm and chromosome lengths, as well as the relative position of FISH signals in relation to the telomere, were performed using the 'analyze-measure length' function of the same software. Digital images were imported into Adobe Photoshop version 5.0 for final processing.

3. Results and Discussion

3.1. AFLP markers

Compared with other DNA marker systems, AFLP is an efficient technique for generating large numbers of markers for the construction of genetic maps.[16,17] While some researchers have reported relatively uniform distributions of AFLP markers,[17,18] others have observed strong clustering of AFLP markers, often in association with telomeric or centromeric regions.[19,20] To avoid clustering of markers, we used two restriction enzyme sets, *Eco*RI/*Mse* I and *Hin*dIII/*Taq* I, in our AFLP analysis.

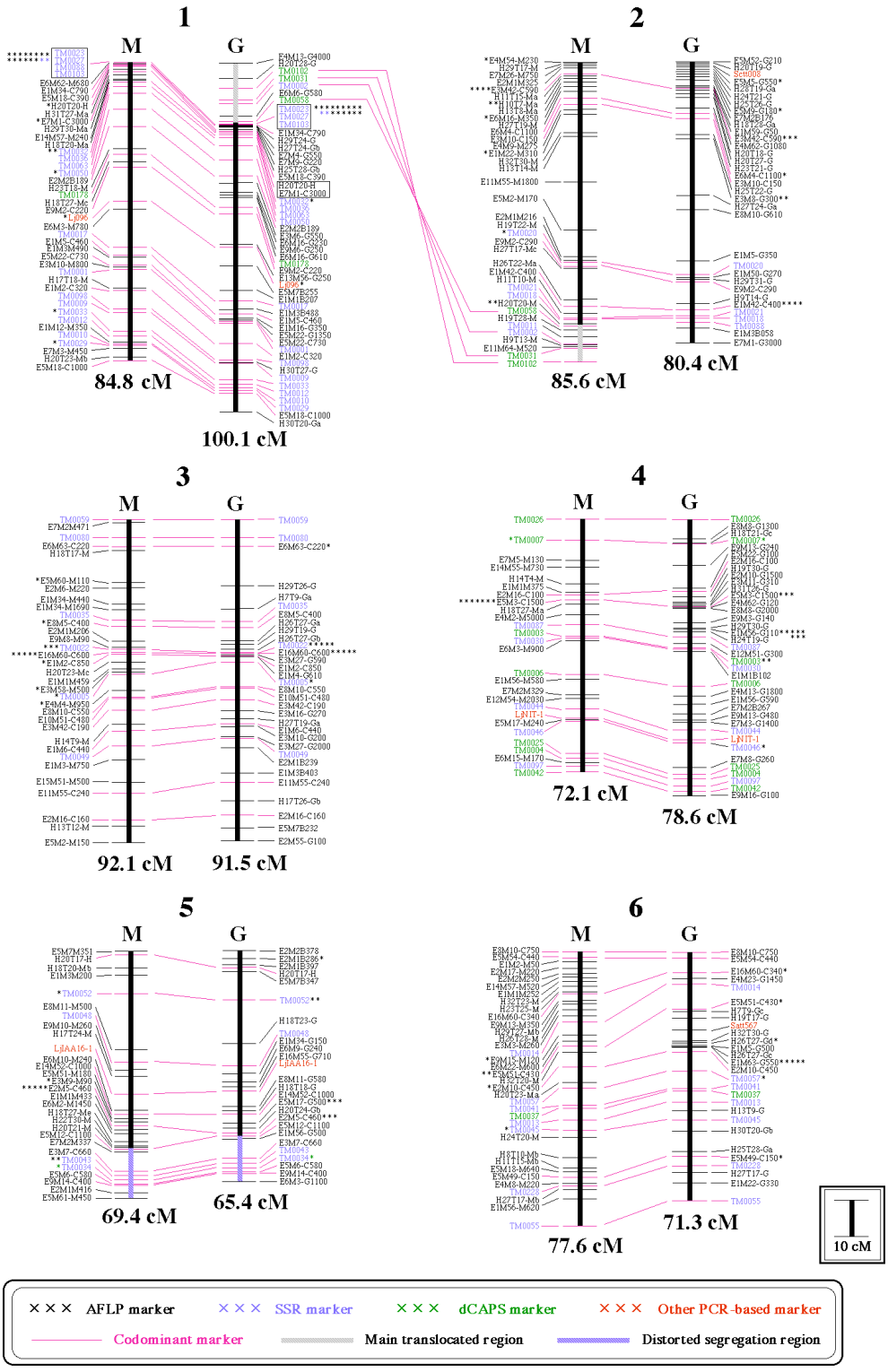

Figure 1. Genetic linkage map of *Lotus japonicus*; maternal Gifu B-129 (G) map on the right and paternal Miyakojima MG-20 (M) map on the left. Asterisks and boxed letters indicate the markers mapped on the same locus. Slanted gray color bars and slanted blue color bars show the main translocated regions and the regions of distorted segregation, respectively. AFLP markers, SSR markers, dCAPS markers and other PCR-based markers are indicated with black, blue, green and red letters, respectively. Codominant Gifu B-129 and Miyakojima MG-20 markers are connected by a pink line, except TM0088 marker mapped on Gifu B-129 linkage group 2 and on Miyakojima MG-20 linkage group 1. Bar = 10 cM.

Even with this precaution, we still observed some clusters, one of which was associated with the breakpoint of the translocation, and a few gaps (Fig. 1). Approximately 1–1.5 polymorphic bands per primer pair were observed between Gifu B-129 and Miyakojima MG-20. One hundred and four (52 sets) AFLP markers were codominantly scored. In total, 224 (Gifu B-129) and 215 (Miyakojima MG-20) AFLP markers were positioned on the linkage maps.

3.2. PCR-based markers derived from TAC clones

In addition to the AFLP markers, 45 SSR and 13 dCAPS markers (TM markers), that were developed on the basis of the sequence information from Miyakojima MG-20 TAC clones,[8] were included in the linkage analysis. These PCR-based markers can be used in an interspecific *L. filicaulis* × *L. japonicus* Gifu B-129 mapping population. Among these TM markers, 18 were codominant markers and 19 were dominant markers in the *L. filicaulis* × *L. japonicus* Gifu B-129 mapping population. They have been integrated in the linkage map of *L. filicaulis* × *L. japonicus* Gifu B-129 developed by Sandal et al. (manuscript submitted).

3.3. Identification of translocation by linkage map

The primary map that was compiled by the MAPMAKER program had five linkage groups including an unusually long linkage group and a group of excluded markers. Because there are six pairs of chromosomes in *L. japonicus*, the number of linkage groups was one less than expected. Since a translocation between *L. japonicus* Gifu B-129 and Miyakojima MG-20 was suggested from cytological information,[2] we investigated the possibility that the MAPMAKER program, through a translocation, had connected two linkage groups into the unusually long linkage group. To address this hypothesis we used two approaches: comparison with the interspecific genetic map and cytogenetic analysis using FISH.

Using the TM markers included in both Gifu B-129 × Miyakojima MG-20 and *L. filicaulis* × *L. japonicus* Gifu B-129 linkage maps, it was possible to compare these two genetic maps. Since *L. japonicus* Gifu B-129 was a parent in both mapping populations, we could determine the genome structure using Gifu B-129 as a reference. By comparing the TM marker positions in the two genetic maps, we found that most of the marker positions were well conserved. Therefore, we were able to align the two maps. Using information from this alignment, we arranged the numbering and orientation of our linkage groups according to those of the *L. filicaulis* × *L. japonicus* Gifu B-129 map. The linkage groups were numbered based on the chromosome size of Gifu B-129, and the top of each linkage group corresponded to the short arm of the chromosome (Pedrosa et al. and Sandal et al., manuscripts submitted).

As a result of this alignment, it became clear that the unusually long linkage group compiled by the MAPMAKER program from our primary data corresponded to chromosomes 1 and 2. Compared to the *L. filicaulis* × *L. japonicus* Gifu B-129 map, this long linkage group connected the top of linkage group 1 to the bottom of linkage group 2. Therefore, it was suggested that a translocation between the distal part of the short arm of chromosome 1 and the long arm of chromosome 2 distinguish Gifu B-129 and Miyakojima MG-20. Among the TM markers included in the connected linkage group, TM0020, TM0021, and TM0018 were mapped on chromosome 2 in the *L. filicaulis* × *L. japonicus* Gifu B-129 map, while TM0023, TM0103, TM0027, TM0032, TM0036, TM0063, TM0017, TM0001, TM0009, TM0033, and TM0029 were mapped on chromosome 1 (Sandal et al., manuscript submitted). Therefore, we separated the connected linkage group into two groups, putative linkage groups 1 and 2, at the position between TM0018 and TM0023. In addition, since three TM markers in the excluded marker group, TM0102, TM0031, and TM0002, were also mapped on the top region of linkage group 1 in the *L. filicaulis* × *L. japonicus* Gifu B-129 map (Sandal et al., manuscript submitted), we included the excluded markers in the putative linkage group 1. Then we carried out linkage analysis of these two groups independently. As a result, the markers that used to be in the excluded group were located on the top region of linkage group 1 (Fig. 1). And the order of TM markers in linkage groups 1 and 2 thus obtained was consistent with that in the *L. filicaulis* × *L. japonicus* Gifu B-129 map. Therefore, we adopted these linkage groups for the framework of the Gifu B-129 linkage map.

3.4. Identification of translocation by cytological analysis

In order to confirm that a chromosome rearrangement was involved in the connection of linkage groups 1 and 2 of the primary *L. japonicus* Gifu B-129 × Miyakojima MG-20 linkage map, the chromosome complements of these two accessions were compared. Gifu B-129 and Miyakojima MG-20 show noticeable cytological differences with respect to chromosome size and morphology, as reported by Ito et al.[2] Chromosome 1 of Gifu B-129 is submetacentric and is the largest chromosome of the complement. Chromosome 2 is metacentric, and is the second largest chromosome and bears the nucleolar organizing region (NOR). Both are similar in size and morphology to the homologous chromosomes 1 and 2 of *L. filicaulis* (Pedrosa et al., manuscript submitted). In the accession Miyakojima MG-20, however, the homologous chromosome 1 is acrocentric and is the second largest chromosome of the complement, whereas the NOR is located on the largest, submetacentric chromo-

some, homologous to chromosome 2 in Gifu B-129. Additionally, the heterochromatin content was increased in Miyakojima MG-20 in comparison to Gifu B-129, as already suggested by differences in their prophase condensation patterns.[2] In Miyakojima MG-20, besides the centromeric heterochromatic blocks also present in Gifu B-129, additional blocks were observed at the ends of both arms of chromosomes 3 and 4 and of the long arm of chromosome 5, as well as interstitially at the long arm of chromosome 2 (Figs. 2, 3). On the other hand, Gifu B-129 has one interstitial band on the long arm of chromosome 4, which is not present in Miyakojima MG-20 (Pedrosa et al., manuscript submitted).

Using both 45S and 5S rRNA genes and BAC clones that were assigned to different *L. japonicus* Gifu B-129 chromosomes (Pedrosa et al., manuscript submitted), it was possible to confirm by FISH the homologies among chromosomes of both accessions. 45S rDNA was localized at the short arm of chromosomes 2, 5, and 6 and the 5S rRNA cluster was located at the short arm of chromosome 2 (Fig. 2a). BACs from the long arm of chromosome 1 (BAC 72G19 (*Lnp*), Fig. 2b), long arm of 2 (BAC 58K7) and long arm of 3 (BAC 85D15, Fig. 2b) were located on the corresponding regions of Miyakojima MG-20 chromosomes. However, when BAC S1.1 was used, a clone from the short arm of chromosome 1 of Gifu B-129, signals were detected in the long arm of Miyakojima MG-20 chromosome 2 (Fig. 2c), showing that a translocation was responsible for the main cytological difference between both accessions. This result was also confirmed when probing cells from one individual originating from the cross between Gifu B-129 and Miyakojima MG-20 with this probe (Fig. 2d). Furthermore, BAC 11O21, which contains the *Nin* gene,[21] showed that the distal region of the short arm of chromosome 1 in Gifu B-129 remained distal after its translocation to chromosome 2 in Miyakojima MG-20.

TACs localized along the short arm of chromosome 1 and the long arm of chromosome 2 in Gifu B-129 (Sandal et al. manuscript submitted), were used to determine the borders of the translocation and, therefore, to establish the genetic map of *L. japonicus* Miyakojima MG-20. TM0023 (Fig. 2e) and TM0088 were shown to be located on the short arm of Miyakojima MG-20 chromosome 1. TM0020 and TM0021 (Fig. 2f), on the other hand, were detected on the long arm of chromosome 2, more proximally and more distally than the intercalary heterochromatic block, respectively. Since both clones are also located on the long arm of chromosome 2 of Gifu B-129, this result shows that the interstitial heterochromatic block of Miyakojima MG-20 chromosome 2 does not coincide with the position of the translocation breakpoint. TAC clones TM0058 (Fig. 2g), TM0002 (Fig. 2h) and TM0102, however, which were assigned to linkage group 1 of Gifu B-129, were localized at the distal region of the long arm of chromosome 2 of Miyakojima MG-20 and, thus, are affected by the translocation event (Fig. 3).

Mapping of the same TAC clones in Gifu B-129 confirmed that TM0058, TM0002, and TM0102 are contained in the translocated segment, since they were located on chromosome 1 of Gifu B-129. Furthermore, their positions confirm the inverted orientation of the translocated segment in both accessions. TM0023 and TM0021 were localized on the same chromosomes in Gifu B-129 and in Miyakojima MG-20 and, therefore, were not involved in the rearrangement. However, TM0088, which was mapped to Miyakojima MG-20 chromosome 1, was localized on Gifu B-129 chromosome 2, showing that a small segment was translocated reciprocally. This segment is probably also in an inverted orientation in both accessions, since it is likely that rejoining occurred at existing breakpoints and that telomeres were maintained at chromosome ends. These results together demonstrate that a reciprocal translocation occurred between both accessions that it defines the borders of chromosomes 1 and 2 in Miyakojima MG-20.

To understand the effect of this translocation to the genetic map, meiotic analysis was performed in an F_1 plant from the Gifu B-129 × Miyakojima MG-20 cross, using BAC S1.1 as a marker for the translocation, and the R2, 45S rDNA probe, as a marker for chromosome 2. From 100 diakineses analyzed, 94 showed the formation of normal six bivalents, indicating that in the majority of cells, no crossing-over occurs along the translocated region. The heteromorphism of bivalent 2, and sometimes of bivalent 1, was evident on those cells (Fig. 2i). However, in six diakineses, association of bivalents 1 and 2, involving the larger translocated segment, was observed. Five cells showed four bivalents and one chain quadrivalent (Fig. 2j), and one cell showed four bivalents, one trivalent and one univalent (on Gifu B-129 chromosome 2). No ring quadrivalent was observed, probably due to the small size of the minor translocated segment. The association of bivalents 1 and 2 through chiasmata demonstrates the presence of recombination along the translocated region and explains the joining of linkage groups 1 and 2 in the genetic map of Gifu B-129 × Miyakojima MG-20.

3.5. Development of a linkage map

Based on the results of comparison with the *L. filicaulis* × *L. japonicus* Gifu B-129 linkage map and the FISH analyses, we assigned the region and relative orientation of the translocated region between Gifu B-129 and Miyakojima MG-20. By including the information about translocation, two separate linkage maps corresponding to maternal Gifu B-129 and paternal Miyakojima MG-20 were obtained (Fig. 1). Each linkage map consisted of 6 linkage groups and contained 113 codominant markers that were used for construction

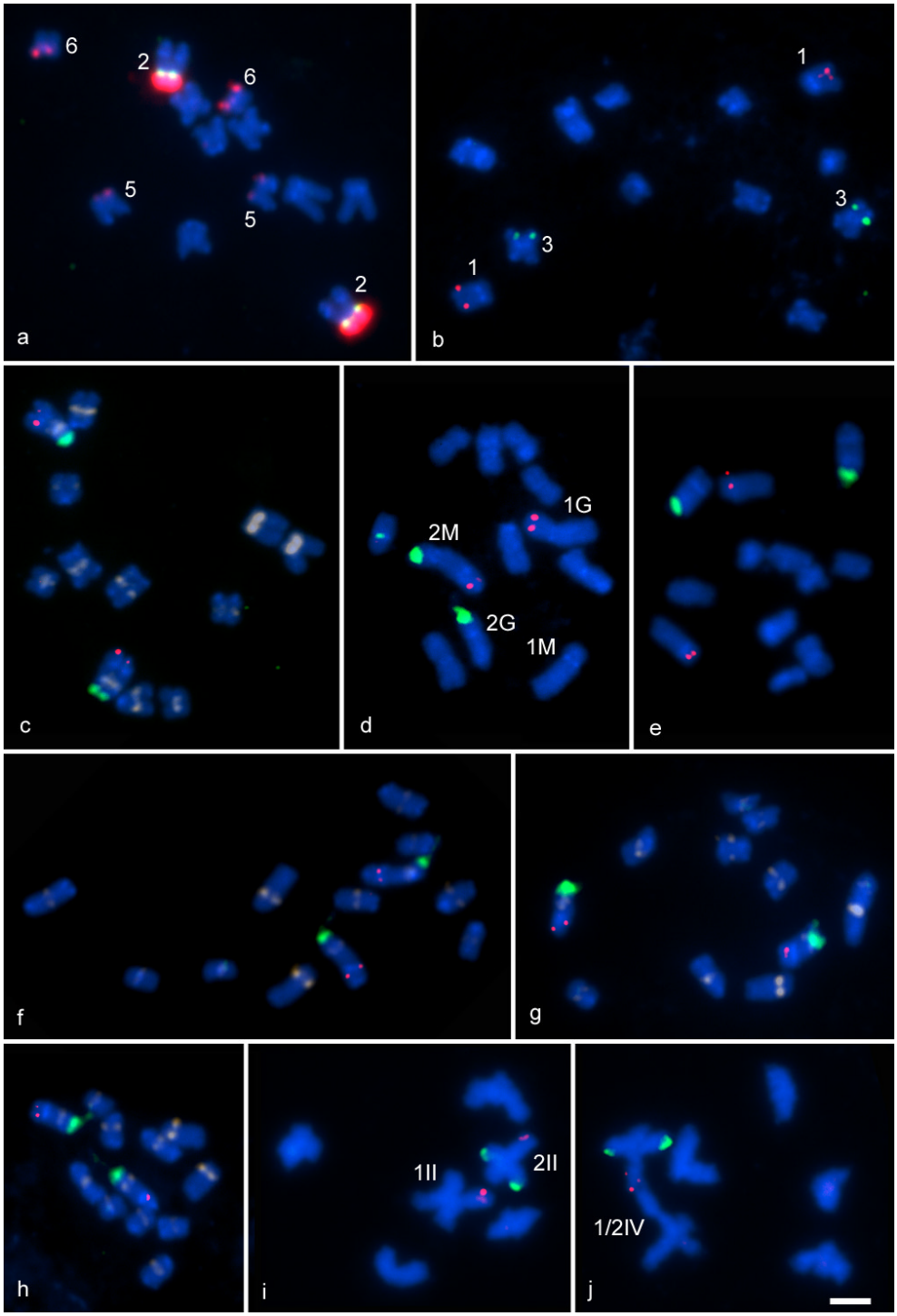

Figure 2. *In situ* hybridization to mitotic (a–h) and meiotic (i, j) chromosomes of *Lotus japonicus* Miyakojima MG-20 (a–c, e–h) and of the *L. japonicus* Gifu B-129 × Miyakojima MG-20 F_1 cross (d, i, j). a. rDNA (red) and 5S rRNA gene (green) on chromosomes 2, 5, and 6; b. *Lnp* (BAC 72G19, red) and BAC 85D15 (green) on chromosomes 1 and 3, respectively; c. BAC S1.1 (red) and rDNA (green) on chromosome 2; d. BAC S1.1 (red) and rDNA (green), indicating translocated chromosome region between Gifu B-129 chromosome 1 and Miyakojima MG-20 chromosome 2; e. TM0023 (red) on chromosome 1 and rDNA (green) on chromosome 2; f. TM0021 (red) and rDNA (green) on chromosome 2; g. TM0058 (red) and rDNA (green) on chromosome 2; h. TM0002 (red) and rDNA (green) on chromosome 2; i, j. Diakineses with BAC S1.1 (red) and rDNA (green) as markers for chromosomes 1 and 2, respectively, showing 6 bivalents (II, i) and 4 bivalents and 1 quadrivalent (IV, j). Centromeres in pictures c, f, g, and h were labeled using BAC 10F21 (c) and the *Ljcen1* repeat (f–h) as probes (light gray). Bar = 2.5 μm.

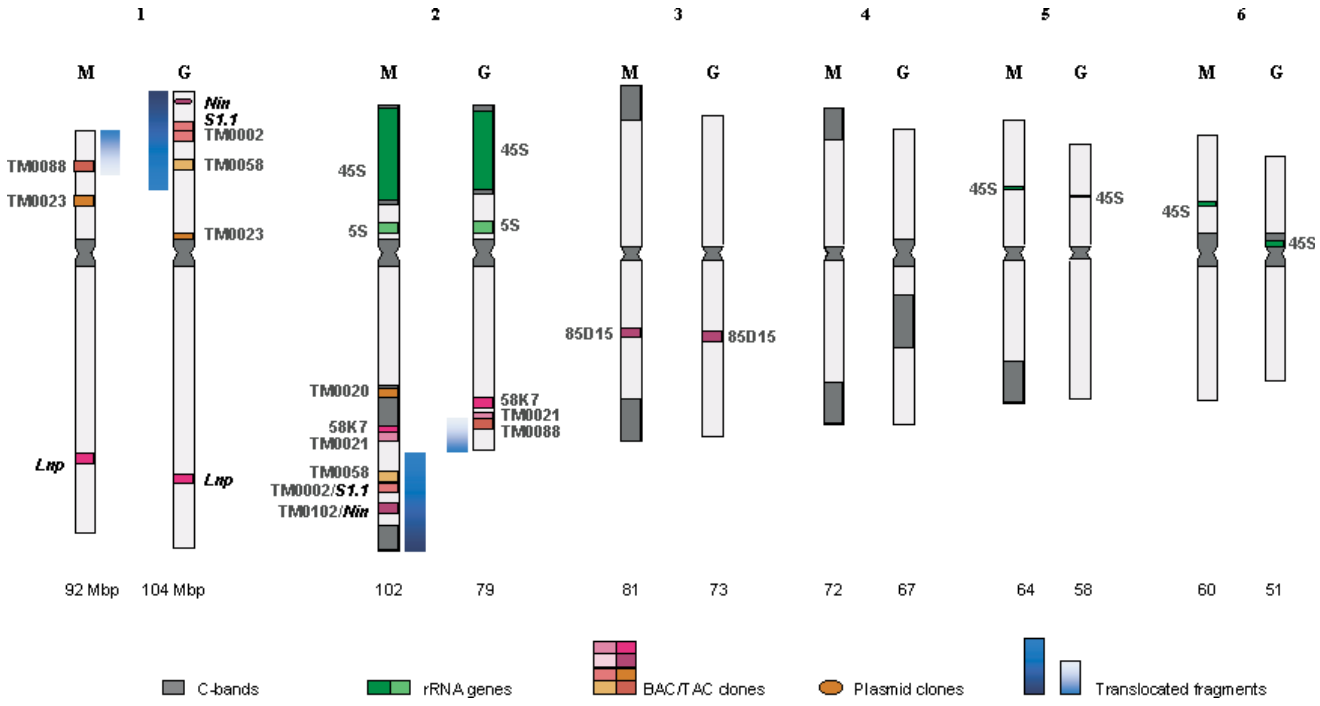

Figure 3. Idiogram of *Lotus japonicus* Miyakojima MG-20 (M) in comparison to the idiogram of the accession Gifu B-129 (G), as proposed in Pedrosa et al. (manuscript submitted). Idiogram shows relative chromosome length, position of centromeres, distribution of heterochromatin and mapping of plasmid, BAC and TAC clones. Chromosome complement of Miyakojima MG-20 was normalized in relation to the complement of Gifu B-129 according to the published difference in genome size.[2] Segments aligned with blue bars in chromosomes 1 and 2 indicate the translocated segments and their inverted orientation in both genomes. Chromosome numbers are listed above and the estimated chromosome sizes in Mbp are listed below each chromosome.

Table 1. Descriptive data of Gifu B-129 and Miyakojima MG-20 map.

	Gifu B-129	Miyakojima MG-20
Number of markers	287	277
Number of genetically separated loci	206	201
Total length of linkage groups	487.3 cM	481.6 cM
Average distance between markers	1.70 cM	1.74 cM
Average distance between genetically separated loci	2.38 cM	2.41 cM

of framework map, and the total number of markers were 287 and 277 in the Gifu B-129 and Miyakojima MG-20 linkage maps, respectively (Fig. 1 and Table 1). The total lengths of the linkage maps are 487.3 cM for Gifu B-129 and 481.6 cM for Miyakojima MG-20, and the average distance between markers (genetically separated loci) was 1.70 cM (2.38 cM) for Gifu B-129 and 1.74 cM (2.41 cM) for Miyakojima MG-20 (Table 1). Markers used for map construction are shown in Table 2. The map and marker information is available through the World Wide Web at http://www.kazusa.or.jp/lotus/.

In linkage group 1, characteristic clustering of markers was observed in the region corresponding to the short arm of chromosome 1, where 20 markers each of Gifu B-129 and Miyakojima MG-20 were mapped at 0 cM distance (Fig. 1). This feature suggests a suppressed rate of meiotic recombination and can at least in part be explained by the presence of the small-translocated segment in this region. At present, however, we cannot exclude the possibility that an additional rearrangement, such as an inversion, contributes to this suppression. Remarkable clustering was also observed on the top region of linkage group 2 and relatively long gaps were detected in linkage groups 2 and 5 (Fig. 1). A region of distorted segregation was identified on the bottom part in linkage group 5 (Fig. 1). The segregation ratios of all the markers mapped on this region were significantly different from the theoretical one. These markers showed high genotypic frequencies for maternal Gifu B-129. Because we can exclude the possibility of mistakes in genotyping, loci involved in gametogenesis and sterility might be located in this region.

We succeeded in constructing a linkage map of *L. japonicus* based on an intraspecific cross. This map will provide the basis for molecular genetic analysis of

Table 2. Number of markers along linkage group.

Linkage group		AFLP		SSR	dCAPS	Other PCR-based marker	Total
		*Eco*R I /*Mse* I	*Hind* III /*Taq* I				
1	Gifu	38 (13)	9 (1)	18 (18)	4 (4)	1 (1)	70 (37)
	Miyakojima	35 (13)	11 (1)	18 (18)	1 (1)	1 (1)	66 (34)
2	Gifu	29 (8)	12	4 (4)	0	1	46 (12)
	Miyakojima	23 (8)	18	5 (4)	3 (3)	0	49 (15)
3	Gifu	28 (10)	10	6 (6)	0	0	44 (16)
	Miyakojima	31 (10)	10	6 (6)	0	0	47 (16)
4	Gifu	31 (3)	8	5 (5)	8 (8)	1 (1)	53 (17)
	Miyakojima	18 (3)	3	5 (5)	8 (8)	1 (1)	35 (17)
5	Gifu	24 (9)	6 (1)	4 (4)	1 (1)	1 (1)	36 (16)
	Miyakojima	25 (9)	8 (1)	4 (4)	1 (1)	1 (1)	39 (16)
6	Gifu	16 (7)	13	7 (7)	1 (1)	1	38 (15)
	Miyakojima	21 (7)	12	7 (7)	1 (1)	0	41 (15)

Values in parentheses indicate number of codominant markers.

the traits in *L. japonicus*, especially nitrogen-fixing symbiosis, mycorrhization, host-pathogen interaction, secondary metabolism, seed components and morphogenesis. If a trait is identified to be controlled by a single gene, the gene could be mapped on the linkage map using the codominant markers first, and bulked segregant analysis with HEGS[3,6] using AFLP markers around the mapped locus could efficiently locate the responsible gene. By analysis of genome synteny with other leguminous crops, this map will provide clues for the identification and isolation of agronomically important genes.

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