A Genetic Linkage Map of the Model Legume *Lotus japonicus* and Strategies for Fast Mapping of New Loci

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Manuscript received January 28, 2002
Accepted for publication May 13, 2002

ABSTRACT

A genetic map for the model legume *Lotus japonicus* has been developed. The F_2 mapping population was established from an interspecific cross between *L. japonicus* and *L. filicaulis*. A high level of DNA polymorphism between these parents was the source of markers for linkage analysis and the map is based on a framework of amplified fragment length polymorphism (AFLP) markers. Additional markers were generated by restriction fragment length polymorphism (RFLP) and sequence-specific PCR. A total of 524 AFLP markers, 3 RAPD markers, 39 gene-specific markers, 33 microsatellite markers, and six recessive symbiotic mutant loci were mapped. This genetic map consists of six linkage groups corresponding to the six chromosomes in *L. japonicus*. Fluorescent *in situ* hybridization (FISH) with selected markers aligned the linkage groups to chromosomes as described in the accompanying article by Pedrosa *et al.* (2002, this issue). The length of the linkage map is 367 cM and the average marker distance is 0.6 cM. Distorted segregation of markers was found in certain sections of the map and linkage group I could be assembled only by combining colormapping and cytogenetics (FISH). A fast method to position genetic loci employing three AFLP primer combinations yielding 89 markers was developed and evaluated by mapping three symbiotic loci, *Ljsym1*, *Ljsym5*, and *Ljhar1-3*.

THE legume family (Leguminoseae) is one of the largest families of the angiosperms. It includes several important crop plants producing protein and oil for human consumption and animal fodder. Cultivation of legumes is favored by their independence of external supply of nitrogen fertilizers and for many species by their ability to grow in poor soils. Part of this pioneer behavior stems from the genetic predisposition to develop endosymbiosis with nitrogen-fixing bacteria belonging to several genera including Rhizobium, Bradyrhizobium, and Mesorhizobium (Schultze and Kondorosi 1998; Stougaard 2001). Increasing our knowledge of the biology and genetics of the legumes may improve this important agricultural resource as well as complement the Arabidopsis model system, which is not suitable for studies of rhizobial symbiosis or fungal symbiosis leading to vesicular arbuscular mycorrhiza. A common pathway for mycorrhization and initiation of root nodule development is defined by eight symbiotic loci in pea and six loci in *Lotus japonicus* (Wegel *et al.* 1998; Borisov *et al.* 2000; Stougaard 2001). This set of "common" genes and the genes required for the specific rhizobial or mycorrhizal interactions are of particular interest and both sets are accessible in legumes. Molecular characterization of these legume symbiotic genes and comparative genome analysis toward plants forming only mycorrhiza and plants like Arabidopsis with only limited capacity for symbiosis would contribute to the description of the molecular evolution of endosymbiosis.

New possibilities for genetic studies in the *L. japonicus* model legume have recently opened as a result of the genome sequencing initiative and the espressed sequence tag (EST) sequencing programs started on *L. japonicus* (Asamizu *et al.* 2000; Cyranoski 2001; Sato *et al.* 2001). Based on the diploid genetics of *L. japonicus*, a small genome size estimated to be ~432 Mb (Pedrosa *et al.* 2002), and ample seed production from large self-fertile flowers (Handberg and Stougaard 1992), these genome initiatives will increase the efficiency of genetic linkage analysis and physical mapping. In addition, several *L. japonicus* mutant populations have been generated and many mutant classes identified (Imaizumi-Anraku *et al.* 1997; Schauser *et al.* 1998; Szczyglowski *et al.* 1998). Although both transposon (Schauser *et al.* 1999) and T-DNA tagging (Schauser *et al.* 1998; Gresshoff *et al.* 2000; Webb *et al.* 2000) have been accomplished, most of the mutant populations were

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produced by EMS mutagenesis and, to molecularly characterize the mutants from these collections, a map-based cloning procedure needs to be established. For this purpose we have developed a genetic linkage map of *L. japonicus*.

Amplified fragment length polymorphism (AFLP) marker technology (Vos *et al.* 1995) has proved to be reliable and effective for the generation of plant linkage maps (Alonso-Blanco *et al.* 1998; Haanstra *et al.* 1999; Vuylsteke *et al.* 1999; Young *et al.* 1999). The AFLP technique combines restriction fragment analysis with PCR into a multi-locus DNA fingerprinting system that is independent of prior knowledge of genome sequence. DNA fragments amplified by PCR are resolved by electrophoresis in gels or capillaries, allowing the large numbers of fragments arising from complex genomes to be detected and analyzed. We have chosen to use AFLP to provide the backbone markers of the *L. japonicus* map and to supplement this analysis with markers generated using restriction fragment length polymorphism (RFLP) and gene- or sequence-specific PCR technology.

MATERIALS AND METHODS

Plant material: The inbred *L. japonicus* B-129-S9 Gifu germplasm is described by Stougaard and Beuselinck (1996). Ecotypes Miyakojima (Kawaguchi 2000), Ashizuri, and Churui were provided by M. Kawaguchi. *L. japonicus* ecotype Funakura and *L. filicaulis* were obtained from W. F. Grant. Inter- and intraspecific crosses were made by emasculation and transfer of donor pollen to the stigma. With *L. japonicus* B-129 Gifu as the female parent, young flowers were used for the crosses to avoid selfings (Jiang and Gresshoff 1997). *L. filicaulis* is less self-fertile and older flowers can be used as pollen recipients, increasing crossing efficiency. Leaf shape and the growth habit of the F_1 plants identify successful crosses. For germination, seeds were treated for 7 min in 95–97% H_2SO_4, washed several times in water, sterilized with 0.5% NaHOCl for 20 min, and washed as before. The seeds were kept in sterile water overnight before sowing.

The *L. filicaulis* plant used in the cross had not been selfed in several generations in our laboratory. Therefore we included five individual *L. filicaulis* plants in our AFLP analysis in addition to the one used for the cross. We never observed differences in AFLP pattern between the individual *L. filicaulis* plants. So the level of heterozygosity in the *L. filicaulis* plant used for crossing is very low and markers would not be expected to segregate within *L. filicaulis*.

Progeny of the *L. japonicus* Gifu cross to *L. filicaulis* have reduced fertility. Plants in the F_1 and the F_2 generations produce 1–5 seeds per pod compared to the 15–20 seeds produced by the parents.

RAPD: The PCR program used for randomly amplified polymorphic DNA (RAPD; Williams *et al.* 1990) on a Hybaid Omnigene thermocycler was 94° for 3 min and then 94° for 20 sec, 36° for 30 sec, and 72° for 1 min for 40 cycles followed by 35° for 30 min. The products were separated on 1% agarose gels.

AFLP: AFLP was performed according to Vos *et al.* (1995) except that the restriction enzyme digest and the ligation were performed simultaneously.

Specific bases on AFLP primers were E31, AAA; E32, AAC; E33, AAG; E34, AAT; E35, ACA; E36, ACC; E37, ACG; E38, ACT; E39, AGA; E43, ATA; E44, ATC; E45, ATG; E46, ATT; M31, AAA; M34, AAT; M40, AGC; M41, AGG; M44, ATC; M45, ATG; M50, CAT; M51, CCA; M57, CGG; M58, CGT; M59, CTA; M60, CTC; M61, CTG; and M62, CTT.

For rapid mapping the following primer combinations are recommended: E32M44, E33M40, E33M58, E34M59, E34M61, E37M50, and E46M41.

RFLP: Five micrograms of genomic DNA from each of the 165 plants of the mapping population was digested with *Eco*RI, *Eco*RV, *Hin*dIII, *Xba*I, or *Bam*HI and used for genomic Southern blots. To identify RFLPs, a corresponding parental filter was tested before hybridization on the population filters. The filters were stored at −20° and stripped in boiling 0.1% SDS before hybridization with the next probe.

RFLP probes from pea were *Chs2* (Harker *et al.* 1990), *Uni* (Hofer *et al.* 1997), *Goc8* (S. Taylor and N. Ellis, unpublished results), and *SHMT* (Turner *et al.* 1993) were kindly provided by N. Ellis.

L. japonicus RFLP probes used were *Nin* (Schauser *et al.* 1999), *Enod40* (Flemetakis *et al.* 2000), nod factor binding lectin *Lnp* (Roberts *et al.* 1999), *ENBP1* (Christiansen *et al.* 1996), *Gap1* and *Gap2* (Borg *et al.* 1999), *Rac2* (Borg *et al.* 1997), *pZF* (Schauser *et al.* 1995), *Gln1* (Thykjær *et al.* 1997), Lb (leghemoglobin), and *rekin* (unpublished receptor kinase). *sym8* and *sym13* (Schauser *et al.* 1998) were mapped by closely linked markers. The 50I19 probe is from the end of a bacterial artificial chromosome (BAC) clone.

Gene-specific markers: The sequence of a number of gene fragments was determined on PCR fragments from both parents. Sequence differences [single nucleotide polymorphisms (SNPs) or size differences] were used to design specific primers and the specific PCR conditions were determined experimentally. For some of the genes it was possible to develop codominant cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel 1993) or dCAPS markers (Neff *et al.* 1998).

The following primers were used [listed by gene name, *L. japonicus* EST accession number, forward primer, reverse primer, restriction enzyme, codominant (cod.), or *L. filicaulis* (fil.) or *L. japonicus* Gifu specificity]:

2,3aldo2, gi-7745577 AV416397, GCTGCCAAGTTACAGGGG TGACAATGTG, CTGTCGTAGTCATGATCGAATGCTCA GG, *Ssp*I;

Ca-ATPase, AV420121, GATGCCCCAGCCCTGCATGAAGC AG, TGCAAGAGCAAGTGCTCCCAGGGTG, *Mse*I;

GTPase, AV426691, AV410879, AV428898, AW720321, AV409846, AV419535, AV428102, ACGATCAGATACCGT CCTAGTCTCAACC, CTGTCAGACACTCCGAGTGTACC CTCAC, cod.;

Myo1, AW720674, AW719358, AW164058, GAGATATTCATG GGTGGAAAGAACACA, CAGGAGCCAATCCAACACAAG CTCTCAG, *Hpa*II;

Myo2, AU089593, AU089207, GAGATATTCATGGGTGGAAA GAACACC, CTGGTGGAACAAGAGGAGCCTTGGTCAA, *Mse*I;

Rer, AV427249, AV423094, AV421125, CTCTTGATCGGGTT TCTTTCACCTCTC, AGCAATATTGGCCAGAAGACAGG AACA, *Apo*I;

Sdd, AV420480, CCAAGCTTCACCAACCCTTCAATTCT CAAAC, TCACATTTCCTGCTCCAATAGCAAAAACACC, *Bst*UI;

AN1, ACTTCATGCTATGTGGCGACT, CACAGCTGTCCTT GAAAATGA, cod.;

M37*Rsa*I, GAGAACTGGGGAAAAACGCAGCAGTAG, CCCTTG CTGCTGTCATCATCCTTCTTGGTCGCGAGCTCCT, *Rsa*I;

Cyc1, CAGGAGAAGCTGATGGTGAACACATTG, CGTATCT GAGATGGAGGGAACTTCAGC, cod.;

Cyc2, GACCGCAAGCTCACCAAACC, AGTATGATTCCAT CATGCTATCC, Gifu;

SO, TCGACGGATCATCATCGGTGCCGGCGTC, TCGACGA GAGCGTGAATGA, fil.;

Nlp1, GAGGAGCTTCTTGTGATTCATG, CCTGATTATGGG GGATGGTG Gifu, CCTGATTATGGGGGATGGTT fil.;

Nlp2, CCCATTTTGTAATTTGAATCTCG, GATAATGATGA GGTTGTTGAACT Gifu, GAATGATGAGGTTGTTGAATG fil.;

Imp, GGAAAGCCCCTATTTGGTACTC, GCAGTCACAGCCA TACAATGG, cod.;

Caj, TAAGGCATTTACTCTATA, CACTGGGATCCAAGTTT TTT, fil.;

50I19, CTTTCCATCATTGCCGCCAC, CATGTTGAGTTGC TATGATTTTT, fil.

Microsatellite markers: TM markers polymorphic between *L. japonicus* ecotypes Gifu and Miyakojima for each of the sequenced TAC clones were used for mapping (Sato *et al.* 2001).

Map calculation and map drawing: Joinmap version 2.0 for Apple computers (Stam and Van Ooijen 1995) was used to calculate the map at LOD score 8.25. Mapchart (Voorrips 2001) was used to draw the map. To test the quality of the data and the mapping, the colormapping procedure was used (Kiss *et al.* 1998). As most of the markers are dominant markers, separate colormaps were made for the *L. japonicus* Gifu and *L. filicaulis* markers.

BAC clones: BAC clones used for *in situ* experiments were isolated from a library of 32,000 clones made from a *Bam*HI partial digest of *L. japonicus* Gifu DNA (N. Sandal, N. Ellis and J. Stougaard, unpublished results).

RESULTS

DNA polymorphism between ecotypes and diploid Lotus species: The highly inbred *L. japonicus* B-129-S9 Gifu germplasm (Stougaard and Beuselinck 1996) was chosen for developing a linkage map. Mutant populations available in *L. japonicus* were previously generated in the Gifu background and this ecotype was also used for the Agrobacterium transformation procedure (Handberg and Stougaard 1992; Stiller *et al.* 1997). To identify a suitable partner for establishing a mapping population, the level of DNA polymorphism was estimated between Gifu and the *L. japonicus* ecotypes Funakura, Churui, Ashizuri, and Miyakojima (MG-20), named after their geographical origins in Japan. Using AFLP and seven primer combinations with 297 bands, the level of polymorphic loci in Funakura, Churui, and Ashizuri was found to be the same (3–4%). For Miyakojima (Kawaguchi 2000), tested with 39 primer combinations, we found 56 polymorphic bands out of 908 AFLP loci or a 6% difference. These initial estimates showed a relatively low level of polymorphism in the available ecotypes and the possibility of an interspecific cross to a more distantly related diploid Lotus species was investigated. Fertile crosses of *L. japonicus* and a *L. filicaulis* accession collected in Algeria were described by Grant *et al.* (1962). When tested with 31 random primer combinations, 1144 out of 2306 AFLP loci (or 49%) were polymorphic between Gifu and this *L. filicaulis* accession.

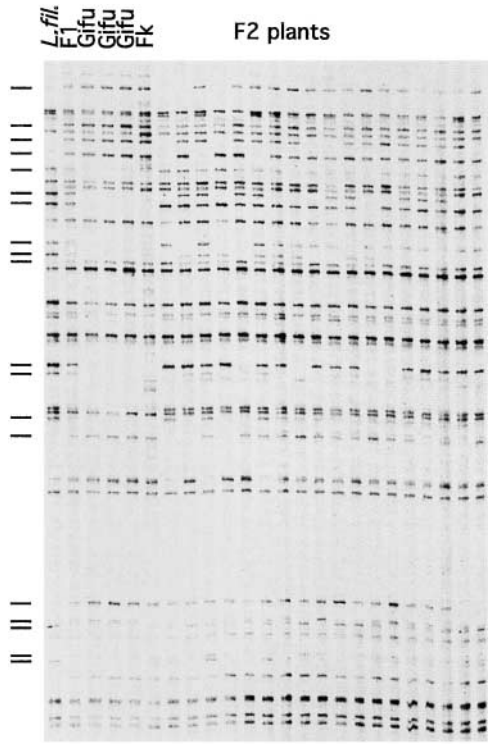

Figure 1.—Segregation of 19 markers in 18 F_2 plants. AFLP pattern on parents, F_1 and F_2 plants from *L. filicaulis* × *L. japonicus* Gifu, and *L. japonicus* Funakura (Fk) with primer set E33M40. Polymorphic AFLP bands are indicated by short thick lines at the left. Only the top part of the autoradiogram is shown.

Development of an interspecific *L. filicaulis* × *L. japonicus* linkage map: On the basis of the high level of polymorphism, a cross to *L. filicaulis* was used to develop the linkage map. A mapping population of 165 F_2 plants was raised and when necessary maintained by propagation of cuttings. DNA was extracted from the 165 F_2 plants and markers were generated primarily by AFLP but also by RFLP, RAPD, and sequence-specific PCR. In the AFLP analysis, up to 45 polymorphic loci were detected per primer combination. An example of an AFLP gel resolving 19 markers is shown in Figure 1. In total, 524 AFLP markers generated from 26 primer combinations were included in the present linkage map shown in Figure 2. The general linkage map includes 3 additional anonymous RAPD markers. Gene-specific markers corresponding to known genes were produced by RFLP and sequence-specific PCR (Figures 2 and 3). A total of 23 RFLP markers were mapped by scoring

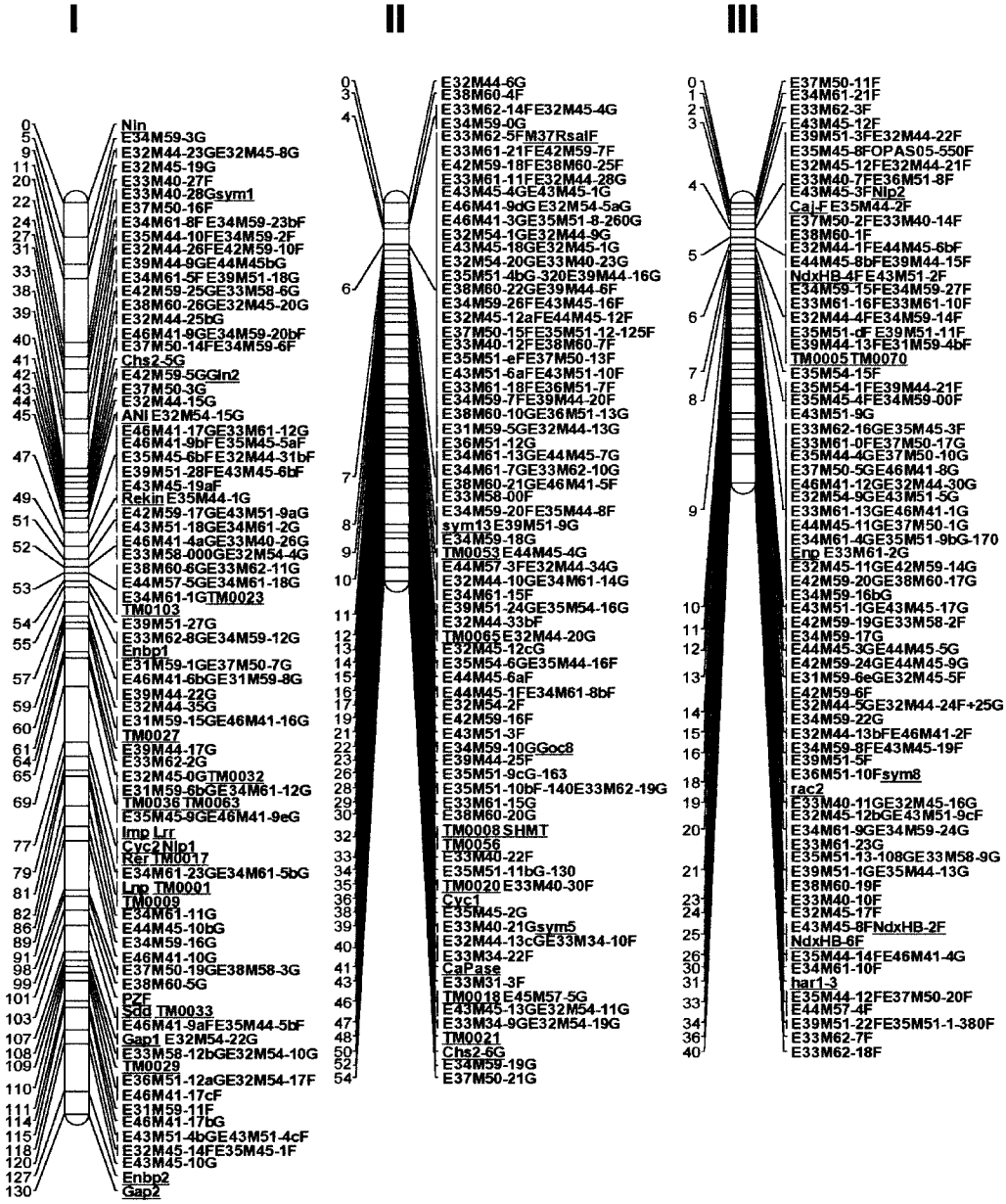

FIGURE 2.—The genetic linkage map of *L. japonicus* derived from *L. filicaulis* × *L. japonicus* Gifu. Linkage groups correspond to chromosome numbers and the short arm is shown on the top. A total of 595 markers is included. Microsatellite markers are labeled TM. The gene and TM marker names are underlined. Marker positions in centimorgans are shown on the left.

Southern blots (*Eco*RI, *Hin*dIII, *Bam*HI, *Xba*I, or *Eco*RV) hybridized with gene or cDNA probes. The 18 sequence-specific PCR markers were developed from *L. japonicus* EST and genomic sequences. The corresponding sequences from *L. filicaulis* were determined and sequence differences were used to develop dominant parent-specific SNP markers or codominant microsatellite/deletion markers. In a few cases codominant CAPS markers were generated using parent-specific restriction enzyme recognition sites. In addition, as part of the *L. japonicus* genome sequencing program, a number of microsatellite markers (TM markers) were developed for mapping in an intraspecific cross between *L. japonicus* ecotypes Gifu and Miyakojima (SATO *et al.* 2001). Many of these TM markers are codominant markers in the *L. filicaulis* cross. The codominant RFLP and PCR markers allowed the identification of heterozygotes and thus the integration of the dominant AFLP markers from the two parents into one genetic map. A summary of the different types and numbers of markers is shown in Table 1.

Initially seven linkage groups were defined by the genetic analysis, but after combining the linkage analysis with the cytogenetic chromosome characterization (PEDROSA *et al.* 2002), the genetic map was assembled into six linkage groups corresponding to the six different chromosomes in *L. japonicus* (see below). The total length of the linkage map amounts to 367 cM with markers at an average distance of 0.6 cM and a maximum distance of 11 cM (Table 1). Linkage groups were numbered according to chromosome size so that linkage group I corresponds to the largest chromosome (chromosome 1) and so

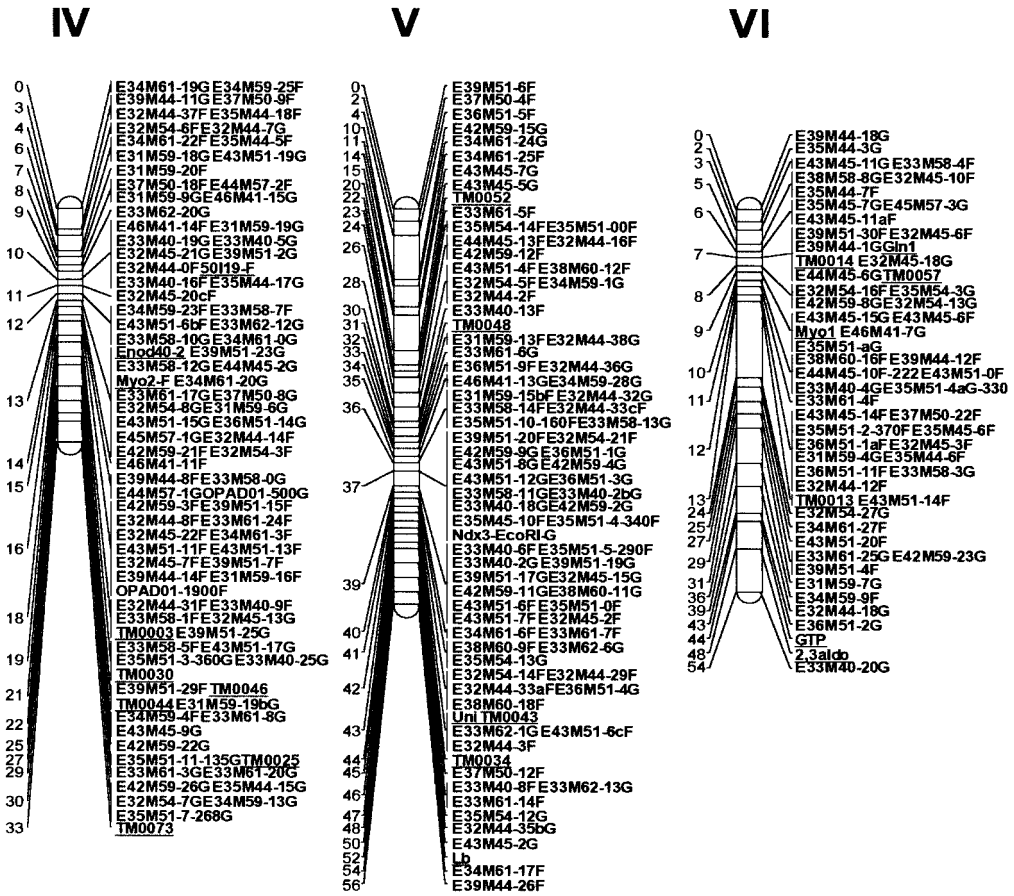

FIGURE 2.—*Continued.*

forth. Furthermore, the linkage groups were arranged with the short chromosome arm on the top of the linkage group, in agreement with the idiogram of the species (PEDROSA *et al.* 2002).

Linkage groups: The primary result of our mapping compiled by the Joinmap program was seven linkage groups, IA, IB, II, III, IV, V, and VI, one group more than expected to account for six chromosomes in *L. japonicus*. The assignment of markers to map positions and the construction of linkage groups was therefore checked manually using colormapping (KISS *et al.* 1998) combined with fluorescent *in situ* hybridization (FISH)

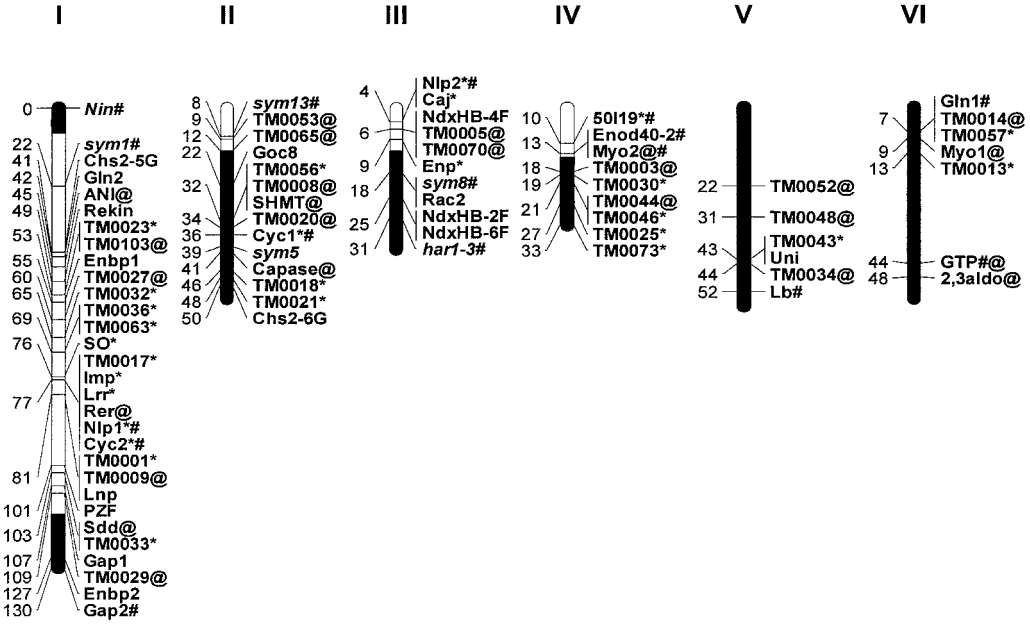

FIGURE 3.—Map positions of known *L. japonicus* genes determined by RFLP (24 genes), PCR, or TM microsatellite markers corresponding to sequenced TAC clones. (*) Dominant PCR markers; (@) codominant PCR markers; (#) markers corresponding to FISH probes used for correlating linkage groups and chromosomes (PEDROSA *et al.* 2002). The position in centimorgans is shown at the left. The chromosome regions where *L. filicaulis* is expected to be a useful mapping partner for fine mapping is indicated in black.

TABLE 1

Marker types and numbers in the six *L. filicaulis* × *L. japonicus* Gifu linkage groups

Linkage group	Chromosome	Length (cM)	AFLP markers Gifu	AFLP markers *L. filic.*	AFLP total	Gene PCR markers	RFLP markers	RAPD markers	Microsatellites (TM)	Means marker distances (cM)	Min-max (cM) distances
I	1	130	68	27	95	7	10	0	11	1.16	0–7
II	2	54	55	46	101	3	4	0	7	0.50	0–9
III	3	40	45	62	107	3	5	1	2	0.34	0–3
IV	4	33	47	40	87	2	1	2	6	0.36	0–3
V	5	56	36	45	81	0	3	0	4	0.67	0–7
VI	6	54	26	27	53	3	1	0	3	0.95	0–11
Total		367	277	247	524	18	24	3	33		

Linkage group and total map length, as well as mean, minimum, and maximum marker distances in centimorgans are also indicated.

localization of markers onto chromosome arms (Pedrosa *et al.* 2002, and below). These two approaches allowed us to merge linkage groups IA and IB into linkage group I corresponding to chromosome 1. The region joining IA and IB encompasses AFLP markers with very distorted segregation and five gene loci (*Nlp1*, *Lrr*, *Cyc2*, *Rer*, and *Imp*) where the Gifu parental alleles were present in only 1 heterozygous plant out of the 165 F_2 plants. Colormapping made it possible to order these markers and to estimate the genetic distance between the ends joining IA and IB to 10 cM. FISH localization of the two genes *Nlp1* and *Cyc2* places this region on the long arm of chromosome 1 (Pedrosa *et al.* 2002).

The genome-wide alignment of the linkage groups to chromosomes and the FISH approach used for the analysis is described in the accompanying article by Pedrosa *et al.* (2002).

Variation in marker segregation ratios along the chromosomes: A high level of DNA polymorphism is an advantage for detection of DNA-based markers but differences in DNA sequence also negatively influence the chromosome pairing and the frequency of meiotic recombination. In our mapping population the observed segregation of markers from the *L. japonicus* and *L. filicaulis* parents illustrates this paradox. Segregation of the parent-specific dominant AFLP markers close to the expected ratio (3:1) is observed only in certain sections of the genetic map, while very distorted segregation is observed in other sections. See Figure 4 for an overview of the segregation of AFLP markers along the six chromosomes. Apart from linkage group III, where Gifu alleles are overrepresented, there is generally an overrepresentation of *L. filicaulis* alleles. In linkage group IV both *L. japonicus* and *L. filicaulis* alleles are represented above 75%, indicating a selection for heterozygosity. A very distorted segregation was found for markers in linkage group I. Normal segregation was observed for markers at the chromosome ends but internally on chromosome 1 there is a very strong overrepresentation of the *L. filicaulis* alleles, culminating around 77 cM, where only 1 heterozygous plant out of 165 plants carried the *L. japonicus* allele. Judged from the almost symmetrical and gradual decrease in *L. japonicus* alleles toward the 77 cM position, a strong selection for one or more *L. filicaulis* allele(s) present here appears to occur. In an attempt to determine possible causes of this distortion we raised an F_2 population of the reciprocal cross *L. japonicus* Gifu × *L. filicaulis*. Distorted segregation in the same region of chromosome 1 indicated that the observed distortion was independent of maternal and paternal effects.

Local clustering of AFLP markers, indicative of reduced recombination frequency, was observed in all six linkage groups and judged from the position, some clusters might well be in the centromeric region of the chromosomes. One exception was a cluster of AFLP markers in linkage group III in a position corresponding

to the short arm of chromosome 3. Chromosome analysis in *L. japonicus* and *L. filicaulis* demonstrated an inversion comprising this region (Pedrosa *et al.* 2002). Clustering of markers on the linkage map positions corresponding to the top of the short arm of chromosome 2 and a part of chromosome 4 suggests suppression of recombination in these regions. On chromosome 2 lack of markers in the 45S and 5S repeats limits the genetic resolution in the region while on chromosome 4 an intercalary heterochromatic block, which is present in *L. japonicus* Gifu but absent in *L. filicaulis*, may influence recombination (Pedrosa *et al.* 2002).

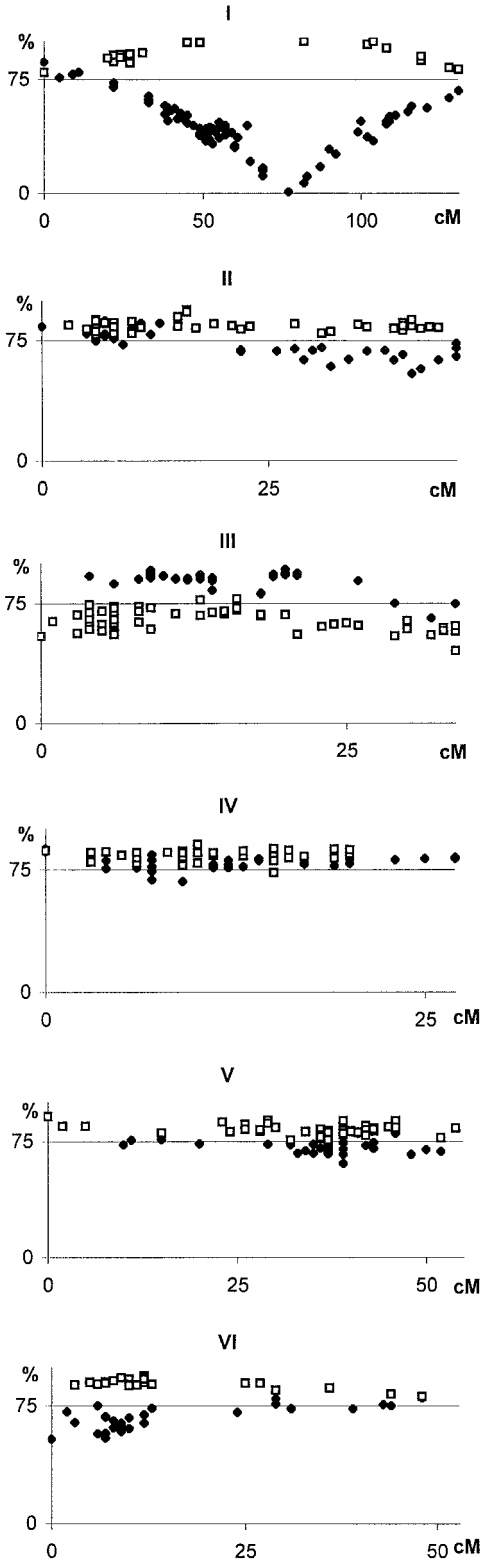

Figure 4.—Segregation of the dominant markers from *L. filicaulis* (□) and *L. japonicus* Gifu (◆) along the different linkage groups (I–VI). Position is given in centimorgans. The expected level of marker occurrence (75%) is indicated.

Judged from the degree of marker resolution, the extent of distorted marker segregation, and the relation between physical and genetic distances, we believe that map-based cloning of genes located in the following chromosomal regions can be approached in the *L. filicaulis* × *L. japonicus* Gifu cross: (a) two regions each covering ~10 cM from the ends of chromosome 1; (b) a region of ~30 cM covering the long arm of chromosome 2; (c) a region of ~25 cM covering the long arm of chromosome 3; (d) a region of ~20 cM covering the long arm of chromosome 4; (e) ~55 cM covering chromosome 5 and ~55 cM covering chromosome 6. This estimate covers a total of 205 cM or 56% of the map length (Figure 3).

Fast mapping of new loci: The advantage of *L. filicaulis* as a mapping partner is its very high level of polymorphism. In practice this makes it easy to map genes with known sequence. Furthermore, it is possible to scan the genome for markers linked to genetic loci, *e.g.*, *sym* loci, by running only a fraction of the AFLP reactions that would be needed for one of the ecotypes mentioned above. In Figure 5 we have shown the genome coverage of molecular markers obtained by only three AFLP primer combinations (E32M44, E34M61, and E37M50) in the interspecific *L. filicaulis* × *L. japonicus* Gifu F_2 population. Scanning with a few such primer combinations would quickly map a new locus to a chromosomal region and higher resolution mapping can then be accomplished with additional markers covering this section of the general linkage map. In materials and methods we have listed seven primer combinations that are useful for rapid mapping. Prescreening for cosegregation with one of the PCR markers shown in Figure 3, followed by higher resolution mapping with AFLP markers in the region, in some cases could be simpler and faster. We report here 51 sequence-specific PCR markers distributed over most of the chromosome arms.

Mapping of mutant loci: The AFLP markers available for rapid mapping as outlined in Figure 5 enable efficient mapping of mutant loci with bulked segregant analysis (Michelmore *et al.* 1997). However, distorted segregation may influence the efficiency and ease of mapping mutant loci onto the linkage map and complicate map-based cloning. To investigate the feasibility of map-based cloning using *L. filicaulis* as wild-type partner,

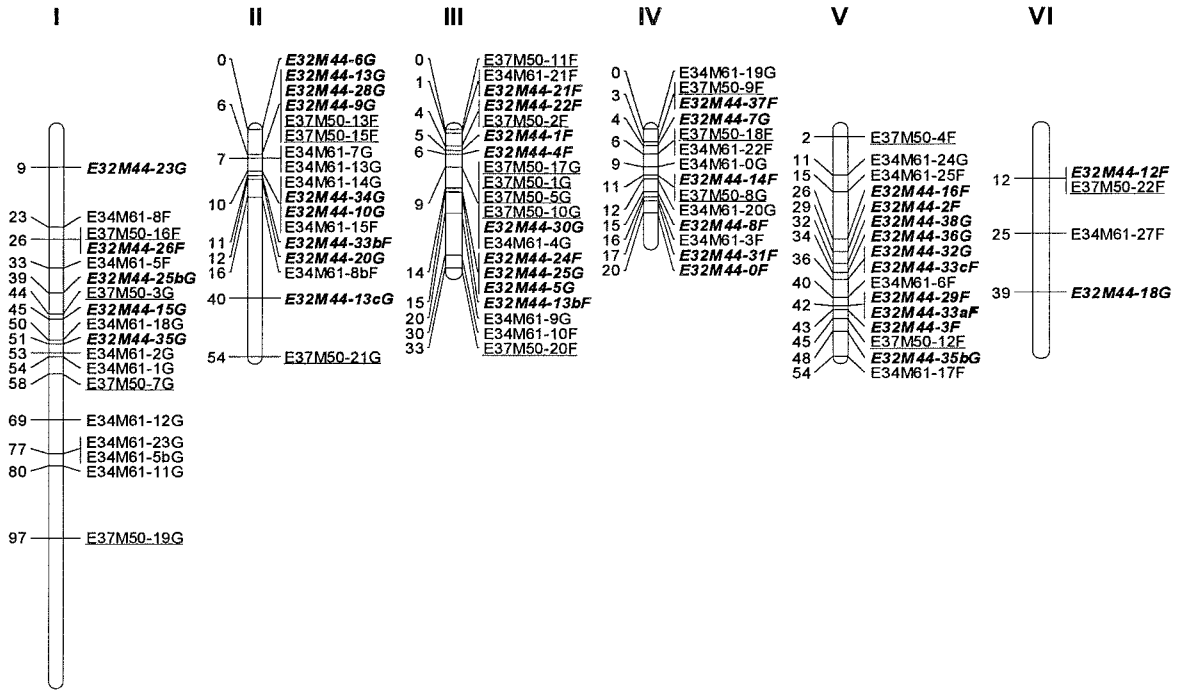

FIGURE 5.—Fast mapping: marker density with three AFLP primer combinations, E34M61, E32M44 (boldface and italic type), E37M50 (underlined).

we mapped and followed the segregation of three symbiotic mutant loci, *Ljsym1* (SCHAUSER *et al.* 1998), *Ljsym5* (SCHAUSER *et al.* 1998), and *Ljhar1-3* (SCHAUSER *et al.* 1998; SZCZYGLOWSKI *et al.* 1998; WOPEREIS *et al.* 2000). The non-nodulating *Ljsym1* locus segregated 92 wild-type to 3 mutants (31:1) and *Ljsym1* was mapped to the short arm of chromosome 1 (Figures 2 and 3). A very distorted segregation was observed and, typical for loci belonging to linkage group I, fewer plants than expected carried the *L. japonicus* Gifu mutant allele and most plants were homozygous for the *L. filicaulis* wild-type allele. Nevertheless, *Ljsym1* was rapidly mapped using only six primer combinations. The disadvantage was the low abundance of *Ljsym1* mutants that segregated, making it difficult to obtain the hundreds of mutant plants needed for fine mapping.

Segregation of the non-nodulating *Ljsym5* mapped to chromosome 2 was less distorted, namely, 60 wild-type to 7 mutants (9:1). The number of mutant plants is still low, and to investigate whether loci disturbing segregation could be segregated away, progeny from individual heterozygous plants were tested for segregation of *Ljsym5* mutants. Out of 16 heterozygous *Sym5/sym5* plants tested, 3 showed normal segregation, 21:7, 42:16, and 29:8, respectively. These heterozygous F_2 plants can now be used to develop large mapping populations for identification of closely linked markers and fine mapping.

The hypernodulating *Ljhar1-3* locus segregated 1796 wild-type to 689 mutants (2.6:1) and behaved as a recessive monogenic trait. The *Ljhar1-3* locus mapped to chromosome 3 (Figures 2 and 3) in a region with normal segregation and this has enabled fast progress in cloning the *Ljhar1-3* hypernodulating locus (L. KRUSELL, unpublished results). The analysis of these three *Sym* loci supports the estimate of chromosomal regions where map-based cloning is possible using a *L. filicaulis* cross.

Mapping of symbiosis-related genes and genes assigned by genome sequencing: One of the interests in *L. japonicus* is the study of endosymbiosis with *Mesorhizobium loti* and mycorrhizal fungi. A well-developed genetic map containing map data on both symbiotic mutant loci and genes expressed during endosymbiosis would therefore be an asset for the system. Using ESTs or genomic sequences we have mapped a number of mainly nodule-expressed genes onto the map by RFLP analysis. The position of these genes is shown in Figures 2 and 3. The *L. japonicus* leghemoglobin (Lb) genes are clustered in one area of linkage group V. This is in contrast to other legumes where the Lb genes are found on more than one chromosome. The map positions of the different nodulation-related genes do not correspond to any of the mapped *sym* mutant loci.

The *Nin* gene, which is necessary for nodule inception (SCHAUSER *et al.* 1999), mapped to the top of linkage group I. Two genes coding for Nin-like proteins (*Nlp1* and *Nlp2*) mapped to linkage groups I and III, respectively. *Nlp2* was found by FISH to be located on chromosome 3 or chromosome 5, depending on the *L. japonicus* individual (PEDROSA *et al.* 2002). We found only the linkage group III position. FISH to the *L. japonicus* × *L. filicaulis* F_1 hybrid confirmed the mapping of *Nlp2* to chromosome 3 in the *L. japonicus* individual used for

establishing the mapping population. Two putatively T-DNA tagged loci, *Ljsym8* and *Ljsym13*, were mapped to linkage group II (*Ljsym13*) and linkage group III (*Ljsym8*) using probes flanking the T-DNA insert. Mapping of *Ljsym1*, *Ljsym5*, and *Ljhar1-3* is described above.

The first release of sequences from the *L. japonicus* genome sequencing program consists of 56 randomly selected TAC clones covering 5.4 Mb of the genome. From this sequence information, 56 mainly codominant markers (TM markers) were developed for mapping the sequenced TAC clones in a Gifu × Miyakojima population (Sato *et al.* 2001). As a first approach to compare the ecotype and interspecific Lotus maps and to anchor the genome sequences obtained, we mapped 80% of the TM markers in the *L. filicaulis* × *L. japonicus* mapping population. In our population 16 of the markers are codominant and 17 are dominant markers. In addition to laying the foundation for future structural genomics on Lotus, ~330 genes can be directly assigned a map position by their presence on a mapped TAC.

DISCUSSION

We have used AFLP, RFLP, RAPD, and sequence-specific gene and microsatellite markers to develop a genetic linkage map for *L. japonicus*. The highly polymorphic species *L. filicaulis* was used as a crossing partner, allowing fast mapping of sequenced genes and rapid scanning for markers linked to genetic loci as illustrated in Figure 5. In total, 524 dominant AFLP markers, 3 RAPD markers, 23 gene-specific RFLP markers, and 51 gene-/sequence-specific PCR markers were mapped. The sequence-specific markers position ~370 known or predicted genes. The total length of the genetic map is 367 cM. We estimate that map-based cloning is possible in regions covering 56% of the linkage map length. In the accompanying article by Pedrosa *et al.* (2002) the alignment of genetically defined linkage groups and chromosomes is presented. The genome size of *L. japonicus* was estimated to be 432 Mb and on this basis the average conversion rate between physical and genetic distance can be estimated to 1.2 Mb per centimorgan. A relatively high conversion rate points to one of the disadvantages using an interspecific cross, namely, suppression of recombination in regions where differences at the DNA level influence homologous recombination negatively. The ecotype Miyakojima (Kawaguchi 2000) shows the highest level of polymorphism to *L. japonicus* Gifu (6%) among the tested ecotypes and it is so far the best alternative to *L. filicaulis*. The length of the genetic map from a *L. japonicus* Gifu × Miyakojima F_2 mapping population was estimated to be 500 cM on the basis of 575 markers (Hayashi *et al.* 2001). In an interspecific cross, a shorter length of the genetic map compared to an ecotype cross is not unusual because regional inversions and deletions lead to suppression of recombination. For the future exploitation of the different genetic maps and for map-based cloning of loci from difficult regions it is important to align the *L. filicaulis* × *L. japonicus* Gifu map to the map of the cross to *L. japonicus* Miyakojima (Hayashi *et al.* 2001). Here we have shown that 80% of the microsatellite markers generated for the cross between *L. japonicus* Gifu and Miyakojima (Sato *et al.* 2001) can also be mapped in the *L. filicaulis* × *L. japonicus* Gifu mapping population, making it possible to align the two maps. The genetic distances in linkage group I vary between them. The map distances on both ends are longer in *L. filicaulis* × *L. japonicus* than in *L.japonicus* Gifu × Miyakojima. In the central part of the linkage group I map, the opposite is the case. This is the area of the *L. filicaulis* × *L. japonicus* map where an extreme segregation distortion is found. The most extreme segregation distortion and suppression of recombination coincides with an inversion observed by Pedrosa *et al.* (2002). In the *L. japonicus* Gifu × Miyakojima map, clustering of markers is seen at the border of the translocation between chromosomes 1 and 2. For this area (position 50–60 cM in linkage group I) the resolution is better in the *L. filicaulis* × *L. japonicus* map. For linkage group III, shorter map distances can be found in the *L. filicaulis* × *L. japonicus* map caused by the inversion on the short arm of chromosome 3. In the next stage a web page containing original data and alignment of the two maps will be set up. At present details of the *L. filicaulis* × *L. japonicus* Gifu map and markers can be obtained from sandal@biobase.dk. Cuttings of our F_2 population are available to the community upon request.

For genes located in regions with very distorted segregation, map-based cloning will not be possible with the *L. filicaulis* cross and a cross to, *e.g.*, ecotype Miyakojima will be more useful. It is now possible to predict from a linked marker position which of the crossing partners will be advantageous for fine mapping and map-based cloning of that particular trait (Figure 3). Without prior knowledge of map position we recommend establishing mapping populations using both *L. filicaulis* and Miyakojima.

AFLP mapping with *Eco*RI/*Mse*I primers often gives clustering of markers. The same clustering has not been seen with *Pst*I/*Mse*I AFLP (Haanstra *et al.* 1999; Vuylsteke *et al.* 1999; Young *et al.* 1999). In Arabidopsis the map position of the *Eco*RI/*Mse*I AFLP marker clusters coincided with the centromeres (Alonso-Blanco *et al.* 1998). In *L. japonicus* only some of the clustering with *Eco*RI/*Mse*I AFLP markers could be explained by clustering around the centromeres. By comparing cytogenetic marker localization and clustering of markers on the *L. filicaulis* × *L. japonicus* map we identified additional specific chromosomal regions, most pronounced on chromosomes 1 and 3, where suppression of recombination occurs. Segregation distortions can be caused by chromosomal inversions (Lyttle 1991) and the inver-

sions found on Lotus chromosomes 1 and 3 in our cross might well be causing the segregation distortion and suppression of recombination found on these chromosomes. In general, segregation distortions are believed to be related to the evolutionary distance of the mapping parents although the level of segregation distortions can be quite high even in intraspecific crosses (Jenczewski *et al.* 1997). For a discussion of segregation distortion see Fishman *et al.* (2001).

In Figure 3 we have indicated the chromosomal regions where genetic mapping will be most informative and where map-based cloning should be possible with *L. filicaulis*-based mapping populations. The feasibility of positional cloning was confirmed for the *Ljsym5* and *Ljhar1-3* loci where map-based cloning and molecular characterization are progressing rapidly. Plants of *L. japonicus* and *L. filicaulis* differ in several morphological markers (Grant *et al.* 1962) and, considering their origin from widely separated geographic regions, they may differ in many biological characters. One example of a morphological difference is the leaf shape: *L. japonicus* has broad leaves and *L. filicaulis* has narrow leaves. The F_1 plants have broad leaves like *L. japonicus* but the F_2 plants have narrow leaves like *L. filicaulis,* although with some variation. This implies that broad leaves would be the dominant character and that the controlling gene(s) was located in the region of chromosome 1 where mainly *L. filicaulis* alleles were inherited. Most likely, this F_2 population could be useful for mapping other biological characters, for example, disease resistance.

In this perspective gene identification in crop legumes using genetic map information and genome data from more amenable model legumes is within reach. Agriculturally important traits such as seed development and pod dehiscence, for example, could be studied taking advantage of the genetic map and the straight *L. japonicus* seed pods giving easy access to the developing seeds.

We thank M. Kawaguchi and W. F. Grant for providing seeds and N. Ellis, C. Kistner, M. Koyama, and our colleagues at the Laboratory of Gene Expression, University of Aarhus, for additional probes and primer information. M. Vinther and S. Rye are thanked for assistance. A.P. was supported by a grant from the Conselho Nacional de Desenvolvimento Científico e Tecnológico/Brazil (200249/99-1). The work was supported by the Österreichische Nationalbank grant P7172 to D.S. and, in part, by the grant S8211 from the Austrian Science Foundation. L.K. was supported by Direktoratet for FoedevareErhverv (KUL97-3) and the Danish Agricultural and Veterinary Research Council (53-00-0223). M.O. was supported by the EU Socrates program. S.R. was supported by an EU Marie-Curie Fellowship (QLK5-CT-2000-51222).

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Communicating editor: V. Sundaresan