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Genetics of Symbiosis in *Lotus japonicus*: Recombinant Inbred Lines, Comparative Genetic Maps, and Map Position of 35 Symbiotic Loci

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Development of molecular tools for the analysis of the plant genetic contribution to rhizobial and mycorrhizal symbiosis has provided major advances in our understanding of plant–microbe interactions, and several key symbiotic genes have been identified and characterized. In order to increase the efficiency of genetic analysis in the model legume *Lotus japonicus,* we present here a selection of improved genetic tools. The two genetic linkage maps previously developed from an interspecific cross between *L. japonicus* Gifu and *L. filicaulis,* and an intraspecific cross between the two ecotypes *L. japonicus* Gifu and *L. japonicus* MG-20, were aligned through a set of anchor markers. Regions of linkage groups, where genetic resolution is obtained preferentially using one or the other parental combination, are highlighted. Additional genetic resolution and stabilized mapping populations were obtained in recombinant inbred lines derived by a single seed descent from the two populations. For faster mapping of new loci, a selection of reliable markers spread over the chromosome arms provides a common framework for more efficient identification of new alleles and new symbiotic loci among uncharacterized mutant lines. Combining resources from the *Lotus* community, map positions of a large collection of symbiotic loci are provided together with alleles and closely linked molecular markers. Altogether, this establishes a common genetic resource for *Lotus* spp. A web-based version will enable this resource to be curated and updated regularly.

Additional keywords: symbiotic mutants.

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Seeds of *L. filicaulis* × Gifu recombinant inbred lines may be obtained by E-mailing the corresponding author.

Genetic analysis and application of genetic approaches in the model legume *Lotus japonicus* (Handberg and Stougaard 1992) has progressed rapidly. Several key genes important for symbiosis with mycorrhizal fungi, root nodule development, and other developmental processes have been identified using molecular

genetic techniques. The developmental regulators *Nin* (Schauser et al. 1999) and *Pfo* (Zhang et al. 2003) were isolated by transposon tagging, whereas map-based cloning led to the molecular characterization of *Har1*, *SymRK*, *Nfr1, Nfr5*, *Castor,* and *Pollux* involved in autoregulation, Nod-factor signal perception or signal transduction (Imaizumi-Anraku et al. 2005; Krusell et al. 2002; Madsen et al. 2003; Nishimura et al. 2002a; Radutoiu et al. 2003; Schauser et al. 1999; Stracke et al. 2002). Genetic loci required for the early stages of endosymbiosis have attracted particular interest. Diallelic crosses together with phenotypical studies defined seven loci (*SymRK, Nup133, Castor, Pollux, Sym6, Sym15,* and *Sym24*) in the common pathway required for both rhizobial and mycorrhizal symbioses (Kistner et al. 2005). Map-based cloning of these loci either has been accomplished or is well advanced. A similar interest is now advancing the genetic dissection of nodule organogenesis and function by using the Fix^- mutants arrested at later stages of nodule development or impaired in nodule function. Cloning of the *L. japonicus Sst1* sulfate transporter, which is required in nitrogen-fixing root nodules, represents the first successful example of such an approach (Krusell et al. 2005).

Continuous isolation of new plant mutant lines is important for comprehensive genetic dissection of symbiosis. Thus far, seven independent mutant populations have been generated by chemical (ethyl methane sulphonate) mutagenesis (Kawaguchi et al. 2002; Márquez et al. 2005; Perry et al. 2003; Szczyglowski et al. 1998; Webb et al. 2005; P. M. Gresshoff, *unpublished data*), four populations were obtained after T-DNA or transposon insertion mutagenesis (Buzas et al. 2005; Schauser et al. 1998; Thykjær et al. 1995; Webb et al. 2000), and two populations were made by either fast neutrons (P. M. Gresshoff, *unpublished data*) or tissue culture (Y. Umehara and H. Kouchi, *unpublished data*). More than 400 symbiotic *Lotus* mutant lines were identified and more are likely to follow. Assignment of these mutant lines to complementation groups is the next logical step in order to determine the overall number of loci involved, to identify all alleles that contribute to phenotypic variation of the mutants, and for genotyping of loci. However, diallelic crossing is a relatively slow process where progress is determined by the length of a generation time and slowed by a continuously increasing number of individual crosses necessary to keep up with mutant isolation programs. Given the number of symbiotic mutant lines already available and considering the time used to define seven complementation groups with a total of 26 alleles constituting the common symbiotic pathway (Kistner et al. 2005), this approach is unlikely to encompass all alleles in the near future. Detection of alleles in already cloned genes using the TILLING facility (Perry et al. 2003) is one way forward to significantly decrease the number of diallelic crosses needed, and this technique will become increasingly effective as mutational saturation is approached. Further improvement of genetic maps and mapping approaches also can diminish the effort needed. So far, two genetic maps have been established, one based on an interspecific cross between *L. filicaulis* and *L. japonicus* ecotype Gifu (Sandal et al. 2002) and one from an *L. japonicus* ecotype cross between Gifu and Miyakojima MG-20 (Asamizu et al. 2003; Hayashi et al. 2001; Kaneko et al. 2003; Nakamura et al. 2002; Sato et al. 2001). Both maps have advantages, and alignment of the two maps enables direct transfer of information between them. This will be especially useful in regions where suppression of recombination or segregation distortion limits genetic resolution in one of the crosses.

In this article, we present improved genetic tools, such as recombinant inbred lines (RILs), alignment of genetic maps through anchor markers, a selection of reliable markers for faster mapping of new loci, and map positions of a collection of symbiotic loci, providing a common framework for more efficient identification of new alleles and new symbiotic loci among uncharacterized mutant lines.

RESULTS

Map alignment.

Three ecotypes of *L. japonicus* (Gifu, MG-20, and Funakura) and two related species (*L. filicaulis* and *L. burttii*) have been used or suggested as parents for various aspects of genetics (Handberg and Stougaard 1992; Jiang and Gresshoff 1997; Kawaguchi 2000; Kawaguchi et al. 2005; Sandal et al. 2002). All have advantages and disadvantages to be considered before choosing crossing partners to establish an appropriate segregating population for a particular genetic analysis. Positional cloning relies on genetic linkage analysis and, at present, two maps provide marker information. In order to optimize positional cloning efforts, it is crucial to choose a crossing partner and a linkage map with genetic resolution in the chromosomal region of interest. Regions of distorted segregation and suppression of recombination were found in both of the genetic linkage maps available in *Lotus* spp. (Hayashi et al. 2001; Sandal et al. 2002). In the interspecific *L. filicaulis* × *L. japonicus* Gifu map, distorted segregation was observed in the middle part of linkage group I and suppression of recombination on the upper part of linkage groups II and III and in the lower middle part of linkage group I (Pedrosa et al. 2002; Sandal et al. 2002). In the Gifu × MG-20 ecotype map, suppression of recombination was found in a region of Gifu linkage group I at 10.8 centimorgans (cM) corresponding to the MG-20 linkage group I 0-cM region. This region is close to the translocation break point between chromosome 1 of Gifu and chromosome 2 of MG-20 (Hayashi et al. 2001). To provide detailed information on genetic resolution, alignment of genetic maps through anchor markers is required. A source of such anchor markers was established by the *Lotus* genome-sequencing program. More than 1,000 microsatellite markers were developed to anchor sequenced TAC clones onto the MG-20 × Gifu genetic map (Asamizu et al. 2003; Hayashi et al. 2001; Kaneko et al. 2003; Nakamura et al. 2002; Sato et al. 2001). We took advantage of these markers and tested them in the *L. filicaulis* × Gifu F2 mapping population. Among microsatellite markers tested, 39% were codominant markers, 44% were dominant markers, and 17% showed no size differences in the gel system used to separate polymerase chain reaction (PCR) fragments. The set of 160 anchor markers identified made it possible to align the two genetic F2 maps. This alignment reveals colinearity along all *Lotus* linkage groups. More noticeable are differences in genetic distances between the *L. filicaulis* × Gifu and MG-20 × Gifu F2 maps, especially in linkage group I (Fig. 1). Inversions on the long arm of chromosome 1 and the short arm of chromosome 3 between *L. filicaulis* and Gifu and the translocation between chromosome 1 of Gifu and chromosome 2 of MG-20 (Hayashi et al. 2001; Pedrosa et al. 2002) might explain some of these differences. The aligned genetic maps are shown in Figure 1 and the regions where one of the linkage maps provides better genetic resolution are indicated.

The total length of the genetic map for *L. japonicus* Gifu × *L. japonicus* MG-20 is 439 cM and for *L. filicaulis* × *L. japonicus* Gifu it is 490 cM. The major difference between the previous version of the *L. filicaulis* × *L. japonicus* Gifu map (Sandal et al. 2002) and the new version is the addition of 62 cM on the top of linkage group IV from TM0525 to TM0075.

Recombinant inbred lines.

Stabilized mapping populations, so-called RILs, are important resources for future mapping of single genes as well as quantitative trait loci (QTLs). The advantage of RILs is the genetically stabilized recombination events fixed in their ge-

nomes. Almost all loci are homozygous and, furthermore, the number of recombination events represented is expected to be doubled compared with the F2, giving a better resolution of the map (Burr and Burr 1991). Progeny of RILs will have an identical genotype and can be shared between laboratories, making direct comparison of mapping data and phenotypic characterization from different laboratories possible. Furthermore, the increase in the number of homozygous loci will be expected to increase the phenotypic differences between lines and make mapping of dominant markers more precise. The RILs have been developed from the two original F2 mapping populations (discussed above) to the S8 generation (theoretically, 99.22% homozygotic loci). Until now, 79 RILs have been developed for the *L. filicaulis* × Gifu population. The final number of RILs is expected to be approximately 100. In addition, 149 RILs are available from Gifu × MG-20 and the final number of these RILs is expected to be 205. Primary mapping data for these two RIL populations are available online.

Alignment of the *L. filicaulis* × Gifu RIL-based genetic linkage map and the two F2 maps was performed by mapping 108 microsatellite markers on the 79 *L. filicaulis* × Gifu RILs. To ascertain the quality of mapping data and maps, both Mapmaker and Joinmap programs were used together with manual color maps. Generally, the order of markers from the F2 maps was confirmed by the *L. filicaulis* × Gifu RIL map. Some chromosomal regions were better resolved in the RIL. One example is in a part of linkage group VI (chromosome 6) that was not resolved in the MG-20-based F2 map, in which 11 markers mapped to the same position at 48.6 cM. Using RILs, it was possible to resolve some of these markers in the order listed from the top down: (TM0066, TM0437, TM0944), TM0367, TM0630, TM1525, (TM1116, TM0139), TM1035 (Fig. 1, TM0367 and TM0139). The markers that still cannot be resolved are shown in brackets.

Direct comparison of allele distributions of 48 loci of the MG-20 × Gifu and 60 loci in *L. filicaulis* × Gifu RIL populations allowed assessment of allele representations along the six linkage groups. In RILs, 50% representation of each of the homozygous alleles is expected for individual loci. Gifu × MG-20 RILs showed only minor distortions, whereas *L. filicaulis* × Gifu RILs had some segregation distortion on parts of linkage groups I, II, and VI (Fig. 2). In the F2, *L. filicaulis* × Gifu

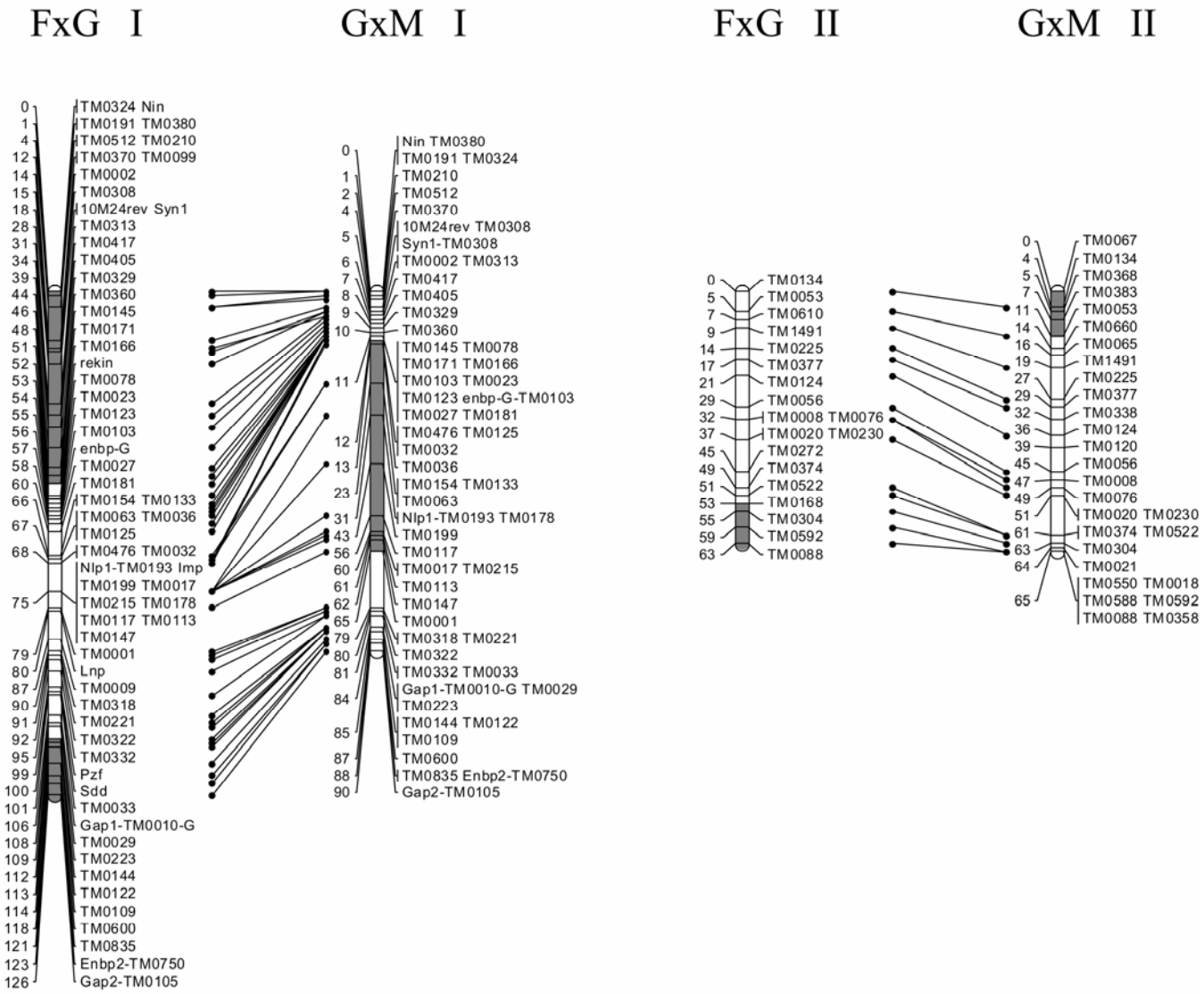

Fig. 1. Alignment of *Lotus filicaulis* × *L. japonicus* Gifu (F×G) and *L. japonicus* Gifu × *L. japonicus* MG-20 (G×M) map distances on the six linkage groups. The map distances were determined by Mapmaker based on the F2 populations. The map positions are from *L. japonicus* Gifu where the region from *Nin* (0 centimorgans [cM]) to TM0360 (10 cM) is located on the top of linkage group I. TM numbers refer to codominant microsatellite markers. Regions where one of the linkage maps provides better genetic resolution are indicated in gray.

showed a strong preference for the Gifu allele on linkage group III (Sandal et al. 2002). Unexpectedly, this has stabilized to a certain degree in the RILs, giving rise to an identical number of Gifu and *L. filicaulis* alleles from 1 to 40 cM and a preference for either *L. filicaulis* or Gifu alleles from 40 to 70 cM and 70 to 85 cM regions, respectively.

In theory, the number of heterozygous loci in the RILs should be very low (0.78%). Surprisingly, RILs based on the *L. filicaulis* cross still have several heterozygous loci present in S8, especially for the upper part of linkage group III. In this region, 24 lines are homozygous *L. filicaulis*, 27 lines are homozygous Gifu, and 29 lines are heterozygous. Although the frequency of heterozygous plants is lower than in the F2, there apparently is a selection for heterozygous plants throughout the generations. The only other region showing more heterozygous plants than expected is the upper part of linkage group II in the *L. filicaulis* cross (Fig. 2). These two regions, linkage group II from TM0134 (2.4 cM) to TM0053 (9.2 cM) and linkage group III from the top to TM0005 at 35.6 cM in the Gifu × MG-20 map, correspond to two regions with suppression of recombination in the F2 for the *L. filicaulis*-based map. Recombination in this region of chromosome 2 remains rare in RILs, whereas the region from chromosome 3 remains without recombinations in RILs. Excess heterozygozity could be caused by an advantage in viability for heterozygotes in a particular locus. An overlapping suppression of recombination caused by the large inversion found on chromosome 3 (Pedrosa et al. 2002) would expand such a selection to encompass a larger chromosomal segment. This phenomenon was found in *Drosophila* spp., where some populations had a high proportion of heterozygotes in inverted regions (Dobzhansky and Pavlovsky 1958). The excess of heterozygotes probably is related to heterosis and functional overdominance or to pseudo-overdominance, where the viability advantage of opposite parental genotypes at two linked loci leads to a selection for heterozygotes (Mitchell-Olds 1995).

Map positions of *Sym* genes.

L. japonicus has been chosen as a model plant for symbiotic interactions with rhizobia and mycorrhizal fungi. This has led to the isolation and characterization of important symbiotic genes such as *Nin*, *Har1*, *Astray*, *SymRK*, *Castor*, *Pollux*, *Nfr1,*

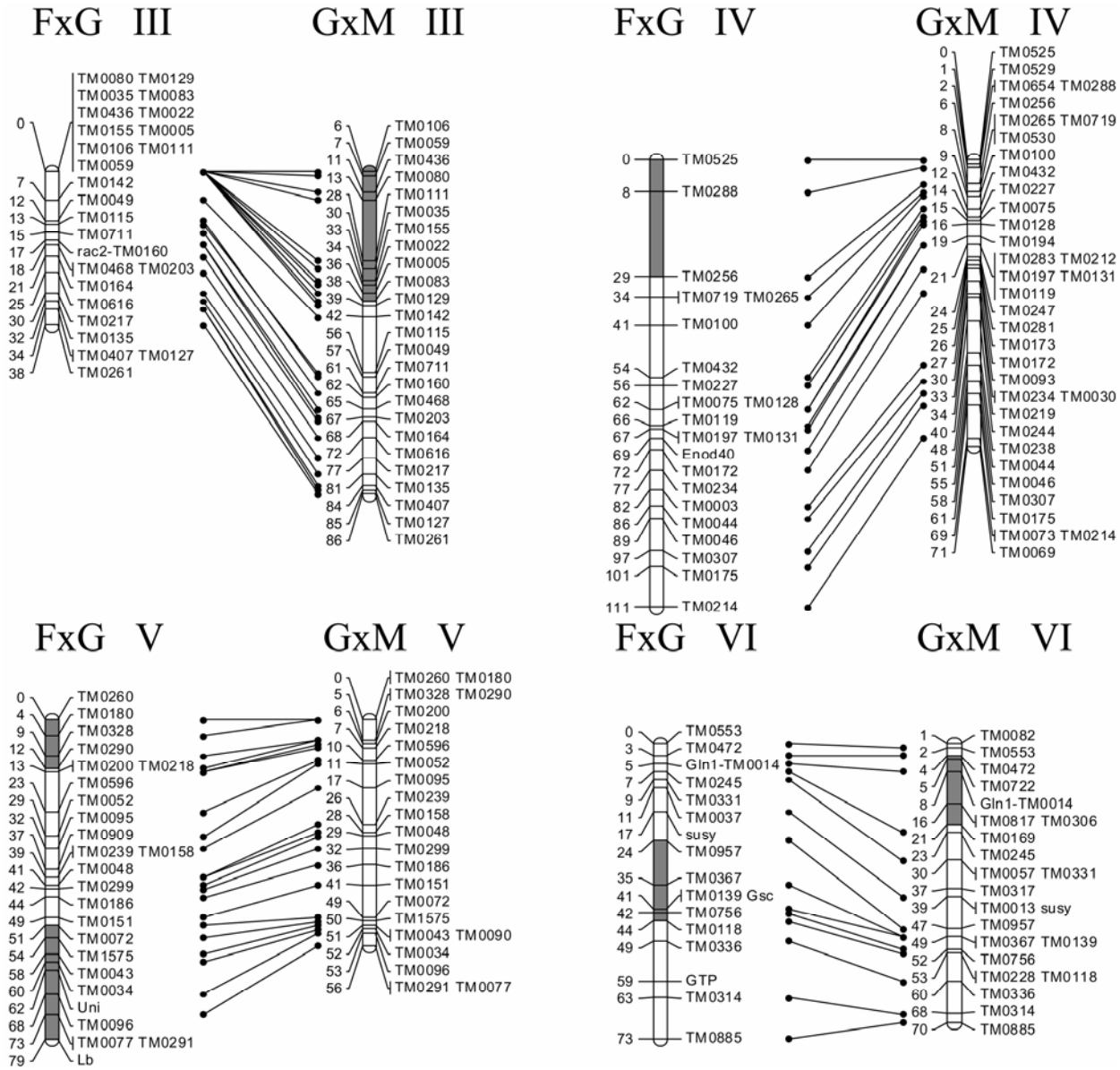

Fig. 1. *Continued from preceding page.*

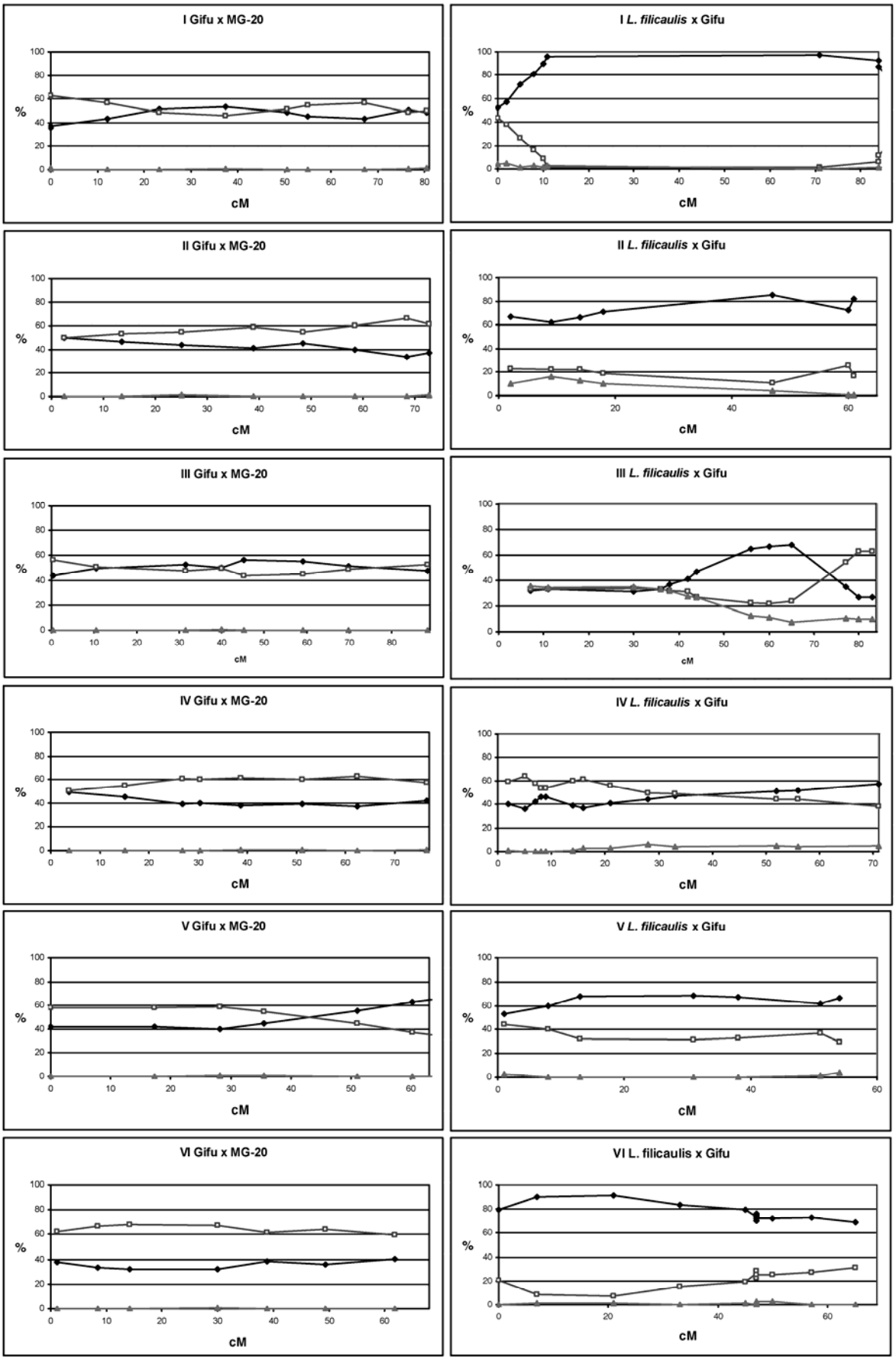

Fig. 2. Segregation of codominant markers in recombinant inbred lines based on *Lotus japonicus* Gifu (◆)× *L. japonicus* MG-20 (□), on the left, and *L. filicaulis* (□) × *L. japonicus* Gifu (◆), on the right. Allele distributions are shown along linkage groups I to VI. Heterozygous (h) plants are indicated with △.

Nfr5, and *Sst1* (Imaizumi-Anraku et al. 2005; Krusell et al. 2002, 2005; Madsen et al. 2003; Nishimura et al. 2002a and b; Radutoiu et al. 2003; Schauser et al. 1999; Stracke et al. 2002) and additional genes are being cloned. Several groups are pursuing positional cloning and characterization of different genes that are important for endosymbiosis. To avoid unproductive duplication of work, it is important to establish a common reference of *Sym* loci and to compare map positions for new mutations isolated by different groups. A number of *Sym* gene map positions already have been published for *Lotus* spp. (Imaizumi-Anraku et al. 2005; Kawaguchi et al. 2005; Nishimura et al. 2002a; Sandal et al. 2002; Stracke et al. 2002; Suganuma et al. 2003; Tansengco et al. 2004). We have expanded the number of mapped loci by mapping 16 new loci and indicating the tentative alleles mapping in the corresponding genetic region as defined by flanking microsatellite markers (Tables 1 and 2). The combined map of 35 *Sym* genes is shown in Figure 3. This combined effort shows that at least 35 *Sym* loci, including 11 that are characterized by a Nod$^-$ phenotype, 9 with a Hist$^-$ phenotype, 8 Fix$^-$ lines, and 3 hypernodulating mutants, have been found in *L. japonicus*. Based on the proposed genetic nomenclature (Stougaard et al. 1999), Table 2 summarizes information on loci and alleles.

Fast mapping of new loci.

Using one or two microsatellite markers from each linkage group for cosegregation analysis, it is possible to determine the chromosomal position of a locus very quickly. We have compiled a list of recommended microsatellite markers for linkage group assignment (Table 3). In addition, a red (Gifu)/green (recessive MG-20) stem color (*Vic6*) locus mapping between TM0377 (29.0 cM) and TM1403 (34.7 cM) provides a visible marker on linkage group II. A low-resolution mapping procedure using, for example, 16 to 30 mutant plants can rapidly indicate an approximate map position. Note that the top of Gifu chromosome 1 and the bottom of MG-20 chromosome 2 will appear linked during a segregation analysis due to crossover between translocated regions. Map-based selection of candidates for complementation analysis has become easier and faster than performing more classical complementation tests to all *sym* mutants with a similar phenotype (Sandal et al. 2005). This approach has been tested with three new symbiotic mutant lines, 282-641, 282-643, and LKL486, that showed a Nod$^-$ phenotype. This low-resolution mapping resulted in map positions close to *SymRK*, *Nup133*/*Nin,* and *Castor*, respectively (Table 2). These new, likely alleles, as indicated from

Table 1. Phenotype and flanking microsatellite markers for *Lotus japonicus Sym* genes[a]

sym locus	Phenotype	Chr.	Marker at the gene	cM	Upper flanking marker	cM	Lower flanking marker	cM	Reference
nin #	Nod–, Ami+	1,2	TM0102	0	…	…	TM0324	0.4	Schauser et al. 1999
nup133 #	Nod–, Ami–	1,2	…	0.8	TM0324	0.4	TM0370	3.6	Kanamori et al.*
nfr1 #	Nod–, Ami+	1,2	TM0545, TM0546	4.8	TM0370	3.6	TM0002	5.6	Radutoiu et al. 2003
lot1	Nod+–, Ami+	1	…	…	TM0166	10.8	TM0027	10.8	Ooki et al. 2005
sym24	Nod–, Ami–	1		…	TM0016	10.8	TM0027	10.8	Szczyglowski et al. 1998; Kawaguchi et al. 2005
alb1	Hist–, Ami+	1	…	…	TM0016	10.8	TM0027	10.8	Kawaguchi et al. 2005
sym70	Nod–, Ami+	1	…	…	TM0016	10.8	TM0125	12.4	Murakami et al.*
sym102	Fix–	1	…	…	TM0671	19.6	TM0498	22.9	Umehara and Kouchi*
klavier	Nod++	1	…	…	TM1325	43.0	TM0215	49.0	Oka-Kira et al. in press
fen1	Fix–, Ami+	1	…	…	TM0001	65.3	TM0064	67.3	Suganuma*
sym80	Hist–, Ami+	1	…	…	TM0122	83.8	TM0105	89.0	Yano, Kawaguchi, and Hayashi*
sym40	Hist–	1	…	…	TM0109	84.6	TM0105	89.0	Yokota, Sandal, and Stougaard*
castor #	Nod–, Ami–	1	TM1494	89.4	TM0105	89.0			Senoo et al. 2000; Imaizumi-Anraku et al. 2005
sst1 #	Fix–, Ami+	2	TM0610	13.2	TM0153	12.4	TM0660	14.4	Krusell et al. 2005
symRK #	Nod–, Ami–	2	TM0541	51.0	TM0076	45.3	TM0230	51.4	Stracke et al. 2002
sym6	Hist–, Ami–	2			TM0076	45.3	TM0021	64.2	Schauser et al. 1998; Yano, Kawaguchi, and Hayashi; Vickers, Pike, and Parniske*
nfr5 #	Nod–, Ami+	2	TM0522	61.4	TM0257	58.6	TM0250	62.6	Madsen et al. 2003
sym8	Hist–, Ami+	3	…	…					Schauser et al. 1998
sym104	Fix–, Ami+	3	…	…	TM0996	21.9	TM0111	27.6	Hossain, Umehara, and Kouchi*
sym15	Nod–, Ami–	3			TM0022	33.6	TM0005	35.6	Senoo et al. 2000; Yoshida and Parniske; Imaizumi-Anraku and Kawasaki*
har1 #	Nod++, Ami++	3	TM0258	78.0	TM0217	76.4	TM0135	80.8	Krusell et al. 2002; Nishimura et al. 2002a; Solaiman et al. 2000
srh1	…	3	…	…	TM1419	84.0	TM0127	84.8	Karas et al. 2005
vrh1	…	3	…	…	TM1419	84.0	TM0127	84.8	Karas et al. 2005
sen1	Fix–, Ami+	4	…	…	TM0530	8.4	TM0182	8.8	Suganuma et al. 2003
sym10	Hist–, Ami+	4	…	…	TM0182	8.8	TM0283	20.5	Petersen, Sandal, and Stougaard*
sym67	Hist–	4	…	…	TM0227	14.4	TM0347	15.6	Yokota, Sandal, and Stougaard*
sym105	Fix–, Ami+	4	…	…	TM0126	24.6	TM0173	25.8	Hossain, Umehara, and Kouchi*
ign1	Fix–, Ami+	5	…	…	TM0260	0	TM0180	0.4	Kumagai et al.*
sym7	Hist–, Ami+	5	…	…	TM0095	16.9	TM0913	22.3	Schauser et al. 1998; Shibata et al.*
crinkle	Hist–, Ami+	5	…	…	TM0151	43.1	TM0024	43.4	Tansengco et al. 2004
prh1		5	…	…	TM0494	41.8	TM0696	47.0	Karas et al. 2005
sym43	Fix–, Ami+	5	…	…	TM0072	49.0	TM0043	51.0	Petersen, Sandal, and Stougaard; Umehara and Kouchi*
astray #	Nod++, Ami+	6	TM0085	29.6	TM0821	27.8	TM1374	29.8	Nishimura et al. 2002b
rhl1		6	…	…	TM0037	34.9	TM0140	38.1	Karas et al. 2005
pollux #	Nod–, Ami–	6	TM0885	70.2	TM0314	68.1	…	…	Imaizumi-Anraku et al. 2005

[a] Map positions shown are for *L. japonicus* Gifu from the cross to MG-20. Chromosome (Chr.) 1,2 means that the gene is located in the region that is translocated between Gifu chromosome 1 and MG-20 chromosome 2; cM = centimorgans; * = this publication; and # = cloned genes.

low-resolution mapping, are shown in Table 2. The table also lists confirmed alleles together with a summary of relevant information. New alleles identified through sequencing of previously isolated *Sym* genes are shown in Table 4 together with the nature of the identified mutation.

DISCUSSION

In this article, we present the map positions of 35 *Sym* genes from *L. japonicus* (Fig. 3) and compile a list of possible alleles awaiting confirmation by complementation or sequencing (Table

Table 2. Summary list of *Lotus* mutants and alleles[a]

Sym locus	Phenotype	Alleles	Likely mutant alleles	Original line names of confirmed mutant lines	Previous name	Reference
Nin	Nod–, Ami+	*nin-1* and *nin-7*	…	96.1M2, KL577	*sym20*	Schauser et al. 1999
Nfr1	Nod–, Ami+	*nfr1-1* to *4*	…	282-118, 282-665, N38, G71-23	*sym1, sym106*	Radutoiu et al. 2003; Chen, Umehara, and Kouchi; Kawaguchi*
Nfr5	Nod–, Ami+	*nfr5-1* to *-5*	…	282-894, KL865, EMS223, S4-1, S58-1, B31-1	*sym5*	Madsen et al. 2003; Murray, Karas, and Szczyglowski*
Sym70	Nod–, Ami+	*sym70-1 and 2*	…	cAc18.4	*sym35*	Kawaguchi et al. 2002; Murakami et al.*
SymRK	Nod–, Ami–	*symRK-1* to *-11*	*sym84,* 282-641	282-287, 282-288, cAc41, KL481, cAc67.6, EMS34, EMS61, SL160-2,3,4,5,6, SL605-2,3, SL1951-2,3,4,5,6,7, SL3472-2, 8-4H	*sym2*	Stracke et al. 2002; Yoshida and Parniske 2005
Castor	Nod–, Ami–	*castor-1* to *-20*	S41-1, S46-1, LKL486, B68-B, B22-A, B32-BA	282-227, EMS1749, EMS46, 25-5A, 24-8B, N5, N10, G00472, G00716, G00862, M89-27, SL3251-2, SL820-3, SL1715-2, SL1966-3, SL3160-3, SL6812-2, KL549, G00532-21, LKL186-4	*sym4, sym22, sym71, sym64*	Senoo et al. 2000; Imaizumi-Anraku et al. 2005
Pollux	Nod–, Ami–	*pollux-1* to *-10*	S49-DA, LKL302, S30, B50-E, SL729-5	EMS70, EMS167, 29-2A, SL571-2, SL3130-2, SL5691-3, SL1899-2, SL159-3, SL405-6, SL1070-2	*sym23*	Imaizumi-Anraku et al. 2005
Nup133	Nod–, Ami–	*nup133-1* to *-4*	un3, Sup12, B9-A, B80-A, B88-B2, B83-B, B62-D	5371-22, 2557-1, cac33.1, EMS247	*sym3, sym26*	Schauser et al. 1998; Kanamori et al.*
Sym15	Nod–, Ami–	*sym15-1* to *2* and *sym72*	S95-B	cAc57, 282-1078, 8-1G, N20	…	Schauser et al. 1998; Senoo et al. 2000; Kawaguchi et al. 2002; Imaizumi-Anraku and Kawasaki*
Sym24	Nod–, Ami–	*sym24*, *sym73*, and *sym85*	…	EMS76, 1-1E, 1-6F	*sym73, sym85*	Szczyglowski et al. 1998; Kawaguchi et al. 2002; Kawaguchi et al. 2005; Pike and Parniske*
Sym6	Hist–, Ami–	*sym6-1, sym6-2, sym30, sym82*	pASacI124	10512.9, 1962-124, EMS126, N4	*sym30, sym82*	Schauser et al. 1998; Szczyglowski et al. 1998; Yano, Kawaguchi, and Hayashi; Vickers, Pike, and Parniske*
Sym7	Hist–, Ami+	*sym7* and *sym101*	*sym7-1* to *11*	312-133, G85-21, G90-23, G101-22, G106-21, cAc61, KL224, KL360, KL379, KL611, KL519	*sym101, 41, 55, 56, 57, 60, 64*	Schauser et al. 1998; Shibata et al.*
Sym8	Hist–, Ami+	…	…	5361-33	…	Schauser et al. 1998
Sym10	Hist–, Ami+	…	…	2572-77.1	…	Schauser et al. 1998
Alb1	Hist–, Ami+	*alb1-1* to *-3*		16-3F, fix1, S64	*sym74*	Imaizumi-Anraku et al. 1997; Kawaguchi et al. 2002; Yano, Kawaguchi, and Hayashi*
Crinkle	Hist–, Ami+	*crinkle-1* to *-3*	*LKL515(sym58)*	1-1H, S66, 1-5A	*sym79*	Kawaguchi et al. 2002; Tansengco et al. 2003; Yano, Kawaguchi, and Hayashi*
Sym80	Hist–, Ami+	…	…	N2	…	Yano, Kawaguchi, and Hayashi*
Sen1	Fix–, Ami+	*sym11, sym61, sym75(sen1)*	…	KU3-13, KL1174, fix6, fix9, s88	*sym11, sym61, sym75*	Schauser et al. 1998; Kawaguchi et al. 2002; Suganuma et al. 2003
Sst1	Fix–, Ami+	*sst1-1* to *5*	…	282-936, fix7, fix13, s89	*sym13, sym81*	Krusell et al. 2005; Schauser et al. 1998; Kawaguchi et al. 2002
Sym43	Fix–, Ami+	…	*sym103*	cAc17.2.1, G629-21		Umehara, Kumagai, and Kouchi*
Fen1	Fix–, Ami+	…	…	9-5B	*sym76*	Imaizumi-Anraku et al. 1997; Kawaguchi et al. 2002
Sym104	Fix–, Ami+	…	…	M89-21	…	Hossain, Umehara, and Kouchi*
Sym105	Fix–, Ami+	…	…	M202-24	…	Hossain, Umehara, and Kouchi*
Ign1	Fix–, Ami+	…	…	56M	…	Kumagai et al.*
Sym40	Hist–	*sym40*and *sym44*	…	cAc38.1, cAc39.1	…	Yokota, Sandal, and Stougaard*
Sym67	Hist–	…	…	KL770	…	Yokota, Sandal, and Stougaard*
Sym102	Fix–	…	…	…	…	Umehara and Kouchi*
Prh1	Fix–	…	…	…	…	Karas et al. 2005

[a] Likely mutant alleles have been found by low-resolution mapping or with very few complementation crosses of mutants with similar phenotypes; * = this publication.

2). Flanking markers for each locus are indicated (Table 1). This collection of data will provide a framework for the future assignment of new loci and the characterization of alleles of already assigned loci. Altogether, this platform will speed up the early categorization of mutant lines and enable a faster and more efficient characterization of new mutants. In addition, the resource described in this work provides an overview of available alleles (Table 2) and various ongoing activities in different laboratories. The mutant resource described here is comparable to the pea collection (Borisov et al. 2004) and, with the genetic tools available in *Lotus* spp., it is predicted to have a significant impact throughout the legume family. A high level of synteny between different legumes has already been shown in the *Har1* region between the genus *Lotus* and soybean, extending even to *Arabidopsis* (Krusell et al. 2002; Searle et al. 2003). In the *SymRK* and *Nfr5* regions on chromosome 2, synteny also was found in the genus *Lotus* and pea (Madsen et al. 2003; Stracke et al. 2003). Furthermore, genome sequencing data and mapping data have been used to make syntenic comparisons among *Lotus* and *Medicago* spp. and other legumes (Choi et al. 2004; Udvardi et al. 2005; Young et al. 2005). Therefore, map positions provided here can be expected to predict which *Sym* genes or loci in *Lotus* spp., pea, and *Medicago* spp. are likely to be orthologous. This, in turn, should enable characterization of genes from more recalcitrant legumes, once a gene has been isolated from a model legume, forming a basis for comparative genetics.

New tools for *Lotus* genetics and molecular characterization of isolated mutants have been developed. We have shown that 83% of the tested microsatellite markers from the MG-20 × Gifu linkage map were directly transferable to the *L. filicaulis* × Gifu map as either dominant or codominant markers. Because microsatellite markers were developed from MG-20 sequence information, it was not surprising that dominant markers, in most cases, detected the Gifu allele. As expected, transfer of markers from an intraspecific map to a more polymorphic interspecific map is relatively easy. Transfer of markers and linkage mapping allowed a direct comparison of the Gifu × MG-20 and *L. filicaulis* × Gifu F2 maps as well as the *L. filicaulis* × Gifu RIL map with the Gifu × MG-20 F2 map. Co-linearity was very pronounced, suggesting, for example, that positional cloning projects can be running in parallel between mapping populations with different parents. This was used in the map-based cloning of *Har1* and *Nfr5* (Krusell et al. 2002; Madsen et al. 2003). The alignment of F2 and RIL maps also identified regions where genetic resolution is possible or advantageous in only one parental combination. By combining the information from the two mapping populations, it is possible to map and use positional cloning in most of the *L. japonicus* genome. One region that remains problematic in the MG-20-based maps localizes to the top of MG-20 chromosome 1, where a large region with suppressed recombination is located. Some "stacked" markers of this region can be ordered in the *L. filicaulis*-based maps; however, in *L. filicaulis* × Gifu crosses, there is significantly distorted segregation on most of linkage group I (chromosome 1), with a preponderance of *L. filicaulis* alleles. Fortunately, *L. burttii* recently was identified as a third crossing partner (Kawaguchi et al. 2005) and the *L. burttii* × Gifu map has only minor suppression of recombination on the top of chromosome 1. *L. burttii* × Gifu populations provide the

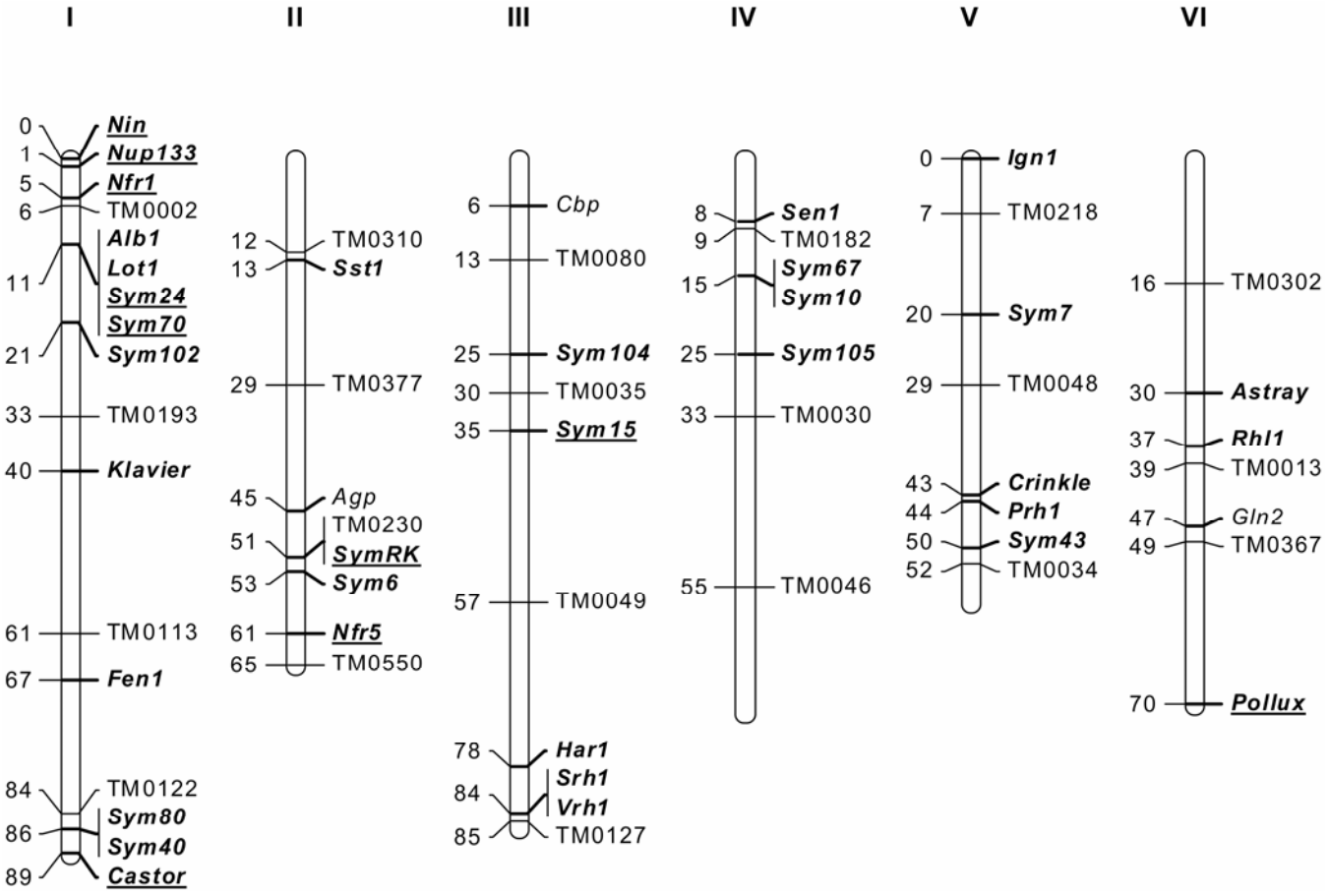

Fig. 3. Position of symbiotic (*Sym*) genes and selected microsatellite markers on the genetic map of *Lotus japonicus* Gifu. Loci with a Nod⁻ mutant phenotype are underlined. *Ljcbp* and *Ljagp* were isolated from two promoter-trap lines tagging putative symbiotic genes. *Ljgln2* is mutated in a photorespiratory mutant.

necessary resolution and can be used for map-based cloning of genes in this region. The level of polymorphism between Gifu and *L. burttii* is intermediate between that found in Gifu and *L. filicaulis* and Gifu and MG-20 (Kawaguchi et al. 2005).

In this article, we describe the development of, and mapping with, two RIL collections developed with an ecotype MG-20 and a related diploid *Lotus* sp. (*L. filicaulis*). These RILs will be a valuable resource to determine the segregation of various traits, such as disease resistance, symbiotic properties, and many other biological characters, and for subsequent mapping and map-based cloning efforts. For the *L. filicaulis*-derived RILs, phenotypic differences are expected to be more pronounced than for RILs based on MG-20. *L. filicaulis* originates from the Mediterranean area (Algeria), which has a very different environment than Japan (where Gifu and MG-20 were collected). Therefore, these parent plants are expected to have adapted to quite different environmental conditions. The variations among RILs might be even bigger than between the parents due to new combinations of alleles. *L. burttii* was crossed to *L. japonicus* Gifu and the development of RILs from the F2 population is ongoing. *L. burttii* was collected in Pakistan and represents another source of genetic and biological variation. Recombinant inbred lines were also developed from a Gifu × *L. japonicus* Funakura F2 population. Although Gifu and Funakura are relatively closely related (2 to 3% polymorphism in amplified fragment length polymorphism), these RILs turned out to be valuable for examining the role of glutamine synthetase in plant biomass accumulation (Harrison et al. 2004; Limami et al. 1999).

With the development of several hundred microsatellite markers corresponding to sequenced TAC clones with approximately 10 genes on each clone, the genetic maps available for the genus *Lotus* are directly related to the genomic sequence. Combining information from various linkage maps and the genome sequence, map-based cloning in *L. japonicus* is now quite straightforward. We have shown that a number of important genes for mycorrhizal and rhizobial symbiosis can be isolated using this system. Exploiting biological diversity reflected, for example, in RILs can be used to address other biological questions relevant to both model and crop legumes which should benefit the whole legume community.

Table 3. Microsatellite markers for low-resolution mapping[a]

Marker	Chr.	Map position	Bottom of chr.
TM0002	1	5.6	…
TM0193	**1**	**32.5**	…
TM0113	**1**	**60.9**	…
TM0122	1	83.8	89.4
TM0310	2	12.4	…
TM0377	**2**	**29.0**	…
Vic6	2	29–35	…
TM0230	2	51.4	…
TM0550	2	64.6	65.4
TM0080	3	13.3	…
TM0035	**3**	**30.0**	…
TM0049	**3**	**56.7**	…
TM0127	3	84.8	86.4
TM0182	4	8.8	…
TM0030	**4**	**33.0**	…
TM0046	4	55.4	70.5
TM0218	5	7.2	…
TM0048	**5**	**29.0**	…
TM0034	5	51.8	56.6
TM0302	6	16.0	…
TM0013	**6**	**38.5**	…
TM0367	6	48.6	70.2

[a] Indicated in bold is a minimal set of primers to cover the six chromosomes (Chr.) of *Lotus* spp. The map positions shown are for *Lotus japonicus* Gifu from the cross to MG-20. The red stem or green stem locus *Vic6* is a phenotypic marker.

MATERIALS AND METHODS

Plant material.

The inbred *L. japonicus* B-129 S9 Gifu germplasm is described by Stougaard and Beuselinck (1996). Ecotype Miyakojima MG-20 is described by Kawaguchi (2000). In the crosses for low-resolution mapping, MG-20 normally is used as the female parent because this gives a higher number of successful crosses. *L. filicaulis* was obtained from W. F. Grant. The *L. filicaulis* × *L. japonicus* Gifu F2 mapping population was described by Sandal and associates (2002). By selfing of this mapping population until the S8 generation, recombinant inbred lines were obtained. The mutants have been named according to Stougaard and associates (1999). For germination, seeds were treated for 7 min in 95 to 97% H_2SO_4, washed several times in water, sterilized with 0.5% NaHOCl for 20 min, and washed as before. Seeds were kept in sterile water overnight before sowing. The screening for symbiotic mutants with a Nod^- phenotype (no nodule development) was done after 6 to 7 weeks and, for the $Hist^-$ (partially developed nodules) and Fix^- (nodules that cannot fix nitrogen) mutants, after 4 to 6 weeks. The arbuscular mycorrhizal infection (Ami) phenotype for some of the mutants was determined in previous studies (Kistner et al. 2005; Senoo et al. 2000; Solaiman et al. 2000; Wegel et al. 1998).

DNA preparations.

DNA from the general F2 and RIL mapping populations was prepared with a modified cetyltrimethylammonium bromide (CTAB) method. Leaf tissue (1 g) was crushed in liquid nitrogen with two porcelain beads in a 10-ml plastic centrifuge tube by vortexing. The beads were removed and 5 ml of CTAB buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, pH 8.0, and 100 mM Tris-HCl, pH 8.0) preheated to 60ºC was added. The tubes were incubated at 60ºC for 30 to 60 min and mixed a few times during the incubation. Chloroform/isoamylalcohol (5 ml at 24:1) was added. The tubes were mixed gently and centri-

Table 4. New alleles of previously published *sym* mutants confirmed by sequencing are listed together with the identified mutation[a]

sym locus	Phenotype	New allele	DNA mutation	Amino acid change
nfr1-3	Nod–, Ami+	N38	G 1003 to A	G 335 to R
nfr1-4	Nod–, Ami+	G71-23	G 1145 to A	C 382 to stop
nfr5-4	Nod–, Ami+	S4-1	G 1415 to A	G 472 to E
nfr5-5	Nod–, Ami+	S58-1	G 1212 to A	W 404 to stop
nfr5-6	Nod–, Ami+	B31-E	C 237 deleted	Frameshift
har1-6	Nod++	SL419-4	T 737 to A	L 246 to H
nup133-4	Nod–, Ami–	EMS247(*sym26*)	G 4496 to A, 3′ splice site	R993 to V, E994 to stop
symRK-12	Nod–, Ami–	8-4H	Large insertion at 5′ untranslated region	…
castor-19	Nod–, Ami–	G00532-21	Deleted	…
castor-20	Nod–	LKL186-4	C 2234 to G	S745 to stop

[a] Position of the mutation is indicated in the coding region (for *nup133-4* in the gene); A of the initiator methionine codon is position 1.

fuged at 8,000 rpm for 10 min. The water phase was transferred to a new tube and 5 µl of RNAse (10 mg/ml of stock) was added and incubated at 37ºC for 30 min. The tubes were placed on ice for 5 min and 0.6 volumes of ice-cold isopropanol was added. The tubes were mixed gently by turning, incubated at –20ºC overnight, centrifuged for 6 min at 6,000 rpm at 4ºC, washed with 70% ethanol, and centrifuged again. The ethanol was removed and the pellets were air dried. The DNA was redissolved in 100 to 300 µl of 10 mM TrisHCl, pH 8.0.

DNA for mutant populations was prepared by placing a leaflet (or trifoliate for small leaves) in an Eppendorf tube containing 35 µl of 0.5 N NaOH. The tissue was ground using a plastic pistil and a drilling machine until no large tissue parts were present. The tube was centrifuged for 1 min at 13,000 rpm and 10 to 20 µl of the supernatant was transferred to a new Eppendorf tube containing 490 µl of 100 mM TrisHCl, pH 8.0. From this DNA preparation, 5 µl was used in a standard PCR reaction. This type of preparation works only for small PCR fragments like microsatellite markers.

Markers.

Microsatellite markers polymorphic between *L. japonicus* ecotypes Gifu and Miyakojima for each of the sequenced TAC clones were used for mapping (Asamizu et al. 2003; Kaneko et al. 2003; Kato et al. 2003; Nakamura et al. 2002; Sato et al. 2001). In PCR reactions for the populations from *L. filicaulis* crosses, the annealing temperature was lowered from 55 to 54ºC, the number of cycles was 37, and the products were separated on 2 or 2.5% agarose gels. Initially, PCR on DNA from 44 plants was used to determine the linkage group and the approximate map position. For the informative markers, the remaining plants were also tested by PCR.

The plastid glutamine synthetase gene (*Gln2*) (Márquez et al. 2005; Orea et al. 2002) was mapped in the *L. filicaulis* × *L. japonicus* Gifu mapping population as a cleaved amplified polymorphic sequence (CAPS) marker amplified with the primers 5′-CCTTCGCCACCAGGACC-3′ and 5′-CATCAATGTTT GTCCAGATGC-3′ with an annealing temperature of 55ºC and subsequently digested with *Rsa*I. Mutation of *Gln2* created a photorespiratory mutant (Márquez et al. 2005; Orea et al. 2002).

Ljcbp (Webb et al. 2000) (E. Tuck, *unpublished data*) and *Ljagp* (N. Rispail, *unpublished data*) were isolated from two promoter-trap lines tagging putative symbiotic genes. The *Ljcbp* sequence is present on a contig containing TM0106 and *Ljagp* was mapped as a CAPS marker (*Taq*I) in the *L. filicaulis* × *L. japonicus* Gifu mapping population with the primer set: 5′-CTAGACACACCCAGGCCATAGC-3′ and 5′-GTCCAAGAC CACTGTACTTATGCTC-3′.

Map calculation and map drawing.

The linkage groups have been numbered according to Gifu chromosome size. F. ex. linkage group I corresponds to the largest Gifu chromosome (chromosome 1) (Sandal et al. 2002). MAPMAKER/EXP. version 3.0b (Lander et al. 1987; Lincoln et al. 1992) and Joinmap version 2.0 for Apple computers (Stam and Van Ooijen 1995) were used to calculate the maps. Mapchart 2.1 (Voorrips 2002) was used to draw the map. In the alignment, the *L. japonicus* Gifu map was used. To test the quality of the data and the mapping, the color mapping procedure was used (Kiss et al. 1998).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Kazusa DNA Research Institute's primary mapping data for *L. japonicus* Gifu × *L. japonicus* MG-20 RILs: www.kazusa.or.jp/lotus/RIline/RI_map.html
National BioResource Project Legume base *Lotus japonicus* website: www.shigen.nig.ac.jp/lotusjaponicus/index_e.html
University of Aarhus Bioinformatics Research Center's primary mapping data for *L. filicaulis* × *L. japonicus* Gifu RILs: www.lotusjaponicus.dk