Classical and Molecular Genetics of the Model Legume *Lotus japonicus*

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The model legume *Lotus japonicus* was demonstrated to be amenable to classical and molecular genetic analysis, providing the basis for the genetic dissection of the plant processes underlying nodulation and nitrogen fixation. We have developed an efficient method for the sexual hybridization of *L. japonicus* and obtained F₁ progeny derived from a cross of *L. japonicus* B-129-S9 Gifu × B-581 Funakura. Over half of the cross-pollinations resulted in fertile hybrid seed, which were confirmed morphologically and by single arbitrary primer DNA amplification polymorphisms using the DAF technique. Molecular and morphological markers segregated in true Mendelian fashion in a F₂ population of 100 plants. Several DAF loci were linked using the MAPMAKER software to create the first molecular linkage groups of this model legume. The mapping population was advanced to generate a set of immortal recombinant inbred lines (F₆; RILs), useful for sharing plant material fixed genetically at most genomic regions. Morphological loci for waved stem shape (*Ssh*), dark leaf color (*Lco*), and short flowering period (*Fpe*) were inherited as single dominant Mendelian loci. DAF markers were dominant and were detected between Gifu and Funakura at about one per primer, suggesting that the parents are closely related. One polymorphism (270G generated by single octomer primer 8.6m) was linked to a morphological locus controlling leaf coloration. The results demonstrate that (i) *Lotus japonicus* is amenable to diploid genetic analysis, (ii) morphological and molecular markers segregate in true diploid fashion, (iii) molecular polymorphisms can be obtained at a reasonable frequency between the related Gifu and Funakura lines, and iv) the possibility exists for map-based cloning, marker assisted selection and mapping of symbiotic mutations through a genetic and molecular map.

Additional keywords: morphological markers, genome analysis, symbiosis, nitrogen fixation, linkage map, recombinant inbred lines.

Like the model plant *Arabidopsis thaliana*, the legume *Lotus japonicus* possesses many biological and genetic advantages (Handberg and Stougaard 1992; Jiang and Gresshoff 1993). For example, it has a small genome size (about 400 Mb per haploid genome), is a true diploid, has a short generation

time, large self-fertile flowers (Fig. 1A), large number of small seeds per pod (Figs. 1B,D), and, because of large flower size and apparent absence of incompatibility, is easy to cross sexually. Some lines, like "Gifu," have the ability of high frequency regeneration from tissue cultures of root, hypocotyl, and leaf sections; furthermore the line is easily transformed by *Agrobacterium tumefaciens* and *A. rhizogenes* facilitating insertional mutagenesis and gene tagging (Thykjaer et al. 1995; Stiller and Gresshoff 1996). *L. japonicus* has a low chromosome number ($n = 6$), suggesting the presence of just few genetic and molecular linkage groups. In contrast to *Arabidopsis*, *L. japonicus* is able to provide information related to plant processes governing the symbiotic interactions during nodulation and nitrogen fixation (Kolchinsky et al. 1994) and mycorrhizal symbiosis (Parniske et al. 1996). Nodules develop quickly (within 7 to 10 days) after inoculation with *Rhizobium loti* (Fig. 1C,E). The plant develops to a good size (Fig. 1D) allowing biochemical and chemical analyses, necessary to transit from molecular biological studies to functional investigations.

Genetic and biological features make this plant an attractive experimental organism. Initial studies by several laboratories studying nitrogen fixation and nodulation have led to the proposal (Handberg and Stougaard 1992) to use *L. japonicus* as a model legume for the study of determinate nodulation, through molecular genetic means such as the generation of symbiotic mutants using EMS mutagenesis and the utilization of nodule-specific expressed sequenced tags (ESTs; Szczygłowski et al. 1995). Already over 20 plant mutations conferring non-nodulation, supernodulation and non-fixation phenotypes have been isolated (F. de Bruijn, personal communication; J. Webb, personal communication; J. Stougaard, personal communication). Transformation and plant regeneration protocols for both hypocotyl co-culture with *Agrobacterium tumefaciens* and from *A. rhizogenes* transformed roots provide a means of high efficiency gene transfer and a useful system for plant gene analysis (e.g., gene trapping, silencing, or activation; Walden and Schell 1994). It was possible to demonstrate insertional mutagenesis using the maize transposable element *Ac* (Thykjaer et al. 1995). Insertion mutagenesis by transposable elements or by T-DNA may allow the tagging of genes involved in symbiosis. Alternative model legumes, such as *Medicago truncatula* (Barker et al. 1990; Gallusci et al. 1991; Sagan et al. 1995), exist for indeterminate nodulation.

While many plant genes involved in nodulation and nitrogen fixation may be detected through insertional mutagenesis, differential display (Goormachtig et al. 1995), or subtractive

hybridization, many new functional elements remain to be detected through map-based cloning (e.g., Martin et al. 1993; Leyser et al. 1993). Thus it is of value to build a genetic map of *L. japonicus*, demonstrate the application of marker technology capable of detecting molecular polymorphisms efficiently, and to develop means of positional cloning through the construction of libraries containing high molecular weight DNA (Gresshoff 1995). The latter was recently achieved by Pillai et al. (1996) who constructed the first YAC and BAC clones carrying "Gifu" DNA in vector pYAC4 and pBeloBAC11.

DNA polymorphisms are of great value as molecular markers, finding applications in gene mapping, map-based cloning, marker-assisted breeding, and varietal diagnosis. Classically, polymorphisms were detected by RFLP probes. PCR-based methods provided a tool to find amplification polymorphisms. DNA amplification fingerprinting (DAF; Caetano-Anollés et al. 1991), random amplified polymorphic DNA (RAPD; Williams et al. 1990), and arbitrarily primed polymerase chain reaction (AP-PCR; Welsh and McClelland 1990) use PCR-

based technology to produce DNA fingerprints. More recently, the AFLP method (Vos et al. 1995) combining RFLP and PCR amplification was applied to plant genome mapping. In principle, the arbitrary techniques involve the enzymatic amplification of template DNA directed by one or more arbitrary oligonucleotide primers to produce a characteristic spectrum of products, a portion of which can be polymorphic. Each technique has differences in DNA amplification conditions, the length of primers used, and the resolution of products obtained.

DAF uses short arbitrary oligonucleotide primers to generate amplification products which are routinely separated on thin polyacrylamide gels, which are stained with silver (Bassam et al. 1991; Caetano-Anollés and Gresshoff 1994a). DAF markers in soybean have been mapped (Prabhu and Gresshoff 1994), isolated, and cloned from dried silver-stained gels (Weaver et al. 1994), and sequenced to generated SCARs (sequence characterized amplified regions; Kolchinsky et al. 1997) linked to the supernodulation gene *nts-1* of soybean (Landau-Ellis et al. 1991).

Fig. 1. Developmental stages of *Lotus japonicus*. **A**, A mature flower on Gifu (about 8 to 12 mm in length); **B**, mature seed pod containing about 10 seeds (about 3 cm in length); **C**, stained, 3-week-old nodule and root segment after inoculation with *Rhizobium loti* strain QJ101 containing a *gus* gene construct for visualization of colonization and infection. The *gus*-plasmid was kindly supplied by Kate Wilson (Canberra). The nodule is about 1 mm in diameter and shows extensive blue coloration in the nodule interior caused by GUS expression. Note regions of root surface colonization and infection thread structures (lower root surface). **D**, Mature plant exhibiting abundant flowering. Note the abundance of flowers and pods as well as abundant leaf material, useful for biochemical studies; **E**, Nodulated seedlings 3 weeks after inoculation. Note clustered nodulation pattern along the tap root.

Here we describe the construction of the first genetic map of *Lotus japonicus* using morphological and molecular markers based on an F₂ population of 50 to 100 plants. We describe efficient methods for *Lotus japonicus* hybridization and the detection of amplification polymorphisms in two ecotypes, B-129 Gifu and B-581 Funakura (called Gifu and Funakura hereafter). We intentionally chose two parents that were closely related to decrease interference during recombination generated in wide crosses because of non-pairing chromosomal regions. Such wide crosses yield genetic maps with many markers but regional contraction of map distances and clustering of markers may distract from their application in

marker assisted selection or map-based cloning. We accepted a lesser degree of genetic polymorphism, counting on multiplex methods capable of detecting variation at high efficiency (such as DAF and AFLP) to provide the framework for the map. Further work will be required to place ESTs, microsatellites (Akkaya et al. 1992), and RFLP (Handberg and Stougaard 1992) markers on the map.

RESULTS

Crossing and reproductive biology.

Gifu and Funakura were chosen for further investigation

Fig. 2. Stages of flower development relevant to emasculation and cross-fertilization. **A** and **B**, Young flowers inappropriate for fertilization. **C**, Proper flower for cross-pollination; **D**, flower too mature for cross-fertilization. Note the difference to flower shown in **C** is the complete extension of the anther filament and the ripening of the pollen, as well as the curvature of the stigma.

because of their good genetic compatibility, the regeneration and transformation potential of Gifu, while the relative vigor of Funakura made it a good male parent. All parental plants are presumed to be homogeneous based on their breeding history and uniformity of seeds and plants. Both ecotypes have profuse flowering (Fig. 1D), although for differing duration.

L. japonicus grows relatively slowly during the first 4 weeks after germination. However, Funakura had faster seedling growth rate than Gifu. Under Knoxville, TN, greenhouse conditions, both ecotypes have a generation time of approximately 3 mo. Fertilization and seed set occur without manipulation of flowers. The primary plant is small (Fig. 1E) al-

lowing high density germination, determinations of toxic levels for selective compounds, nodulation tests and mutant plant screens on agar plates of varying sizes (Rolfe et al. 1980). Mature plants are "bushy" and consist of several branches up to 30 cm long (Fig. 1D); abundant material can be harvested for biochemical, chemical, and molecular analysis. Large yellow flowers are usually arranged in pairs (Fig. 1A), and pod formation in cross-fertilized flowers requires as little as 2 to 3 days. Each nonshattering pod contains up to small 20 easily released seeds (Fig. 1B). In the typical growth cycle, seeds collection is 10 to 12 weeks postgermination. Because *Lotus japonicus* is a perennial plant, new shoots and flowers continue to develop after cutting the old branches. Plants suffer from some insects pests in greenhouses and require good hygiene and occasional chemical treatment.

L. japonicus ecotypes were crossed efficiently and throughout the entire flowering period in the greenhouse using a manual emasculation method (Fig. 2). Only if the flower and anthers were at the stage as shown in Figure 2C, were high frequency pollinations possible. Special attention needed to be given to the stickiness of the stigma (see Materials and Methods). The crossing history for the mapping population in the F_2 and the further development of the recombinant inbred lines (RILs) is outlined in Figure 3. RILs are at F_6 and are available for sharing along with the data spread sheet for segregating molecular markers. At the F_6 stage, theory suggests that, in the absence for heterosis, about 97% of the genome of any one plant should be homozygous for either one or the other parental marker. Thus the RIL families reflect recomb-

Table 1. Crossing ability of *Lotus japonicus*^a

Parents	Crosses (no.)	Pods (no.)	Seeds (no.)	Success (%)
B-129 "Gifu" × B-581 "Funakura"	29	15	194	52
B-129 "Gifu" × B-177 "Korea"	18	8	109	39
B-177 "Korea" × B-129 "Gifu"	11	1	15	9
B-129 "Gifu"(control)	20	0	0	0
B-177 "Korea" (control)	25	0	0	0

^a Data from 1992 season, greenhouse grown. Forceps method. Control were emasculated but not pollinated.

Fig. 3. Crossing history of *Lotus japonicus* F_2 population. Recombinant inbred lines (at F_6 level) are available now for international sharing. Parental seeds, growth and culture conditions, and inoculum are available from the authors' laboratory upon written request.

Fig. 4. DAF marker analysis for *Lotus japonicus*. **A (above)**, DNA amplification profiles for F_1 progeny of several independent crosses between *L. japonicus* B-129-S9 Gifu × B-581 Funakura generated with the primer 8.6e (5'GACGTAGG3'). Male parent Funakura had polymorphic markers 210F and 250F. Female parent Gifu showed a polymorphic band 260G. The polymorphic markers from both parents were found in all F_1 hybrids shown in the 3 lanes to the right of the parents (G and F). Note the reproducibility of banding patterns between different F_1 isolates and the parents. In total about 30 markers are scored per average DAF run resolved on polyacrylamide and silver stained. The F_1 hybrid nature was confirmed by DAF polymorphic products and morphological characteristics. **B (right)**, DAF polymorphic markers of cross F_1 -6 of *L. japonicus* generated with different primers 10.6d 10.6e 10.6g HpA17, HpA38, HpA41, HpA49, HpA54, HpA57 HpA59, HpB38, HpB41, HpB55, HpB56, HpB61. These primers revealed polymorphic markers respectively at different size. In all cases inheritance confirmed the dominant nature of the DAF marker. G = B-129-S9 Gifu, F = B-581 Funakura.

B

nation events which gave rise to variation in the original F₂. Homozygous regions remain “fixed.” This permits the repeated analysis of the population in different laboratories and the progressive increase of marker density on a “master map.” New loci, such as those controlling supernodulation or non-nodulation, need to be associated with a mapped marker in separate F₂ families; by intrapolation new loci can be introduced into the “master map.”

We have made crosses between Gifu, Funakura, and B-177 “Korea” and obtained F₁ seeds at different frequencies (Table 1). Line Gifu served as the most effective female parent giving crossing frequencies around 40 to 50%. In contrast, the reciprocal cross with Gifu as the male parent gave only 9% seed set. Control emasculations gave no seed set, showing that the method described here is very efficient and effective in preventing selfing.

Nomenclature for genetic loci.

DAF nomenclature used here indicates the size of the polymorphic band (in base pairs) as determined by separation on 5% PAGE gels and silver staining, and the genotype in which it is present (abbreviated as the initial; e.g., 270G-1).

We here suggest a three-letter code (in italics) for the genetic nomenclature of *L. japonicus* genes. Thus, stem shape is controlled by *Ssh1*, in which the upper case initial signifies the dominant allele. *Lco1* controls leaf color, and *Fpe1* flowering period. An attempt is made to signify the organ affected by the mutation in the first letter of the code. Accordingly, mutations

altering symbioses would be given a symbol ‘sym’, followed by a number. To avoid confusion with other ‘sym’ mutations in other legume, the prefix Lj should be used. Numbering of ‘sym’ mutant alleles should be by chronology of discovery (e.g., *Lj-sym-5*). Once the molecular function of a locus is defined, alternative nomenclature appears to be warranted.

Detection of morphological and molecular polymorphisms.

Gifu and Funakura differ morphologically in stem shape, leaf color, and flowering period. Gifu develops a straight, erect stem, whereas Funakura has a waved, horizontal stem for the first 30 days postgermination. This juvenile characteristic disappears once long branches formed. Gifu has dark green leaves of small size, while Funakura has light green leaves with bigger size. The flowering period of Gifu starts 7 days earlier than that of Funakura and lasts for 35 days. Gifu still blooms as flowers of Funakura senesce. This property may explain why Gifu is an excellent female parent as the stigma is receptive for a long time. In contrast, Funakura is receptive for a specific time only, making the timing of the emasculation very critical. These morphological markers, namely stem shape, flower period, and leaf color, were used for investigation of Mendelian segregation of F₂ progeny. Variation for the three morphological traits was discontinuous and clearly distinguished into two phenotypic classes in the F₂.

Since the natural lines of *Lotus* have few naked eye polymorphisms (NEPs), and since there is a need to establish markers to verify crosses and to generate diagnostic markers

Table 2. DAF polymorphisms detected between *Lotus japonicus* line B-129 “Gifu” and B-581 “Funakura”

Primer	Sequence (5'-3')	DAF marker ^b	Primer	Sequence (5'-3')	DAF marker ^b
8.6m	GTAACGGC	270G	HpB45	GCGACAGC-GAC	180F
10.6d	GAAACGGGTC	250F	HpB46	GCGACAGC-GAT	290G
10.6e	GTGACGTAGG	220F	HpB55	GCGACAGC-ATG	330G
10.6g	CAATCGCCGT	170G	HpB61	GCGACAGC-AAC	220F
10.6m	AGGTGACCGT	280G	HpC1	GCGAGAGC-CCC	310G
HpA17	GCGAAAGC-TCC	300F	HpC7	GCGAGAGC-CTG	230G
HpA38	GCGAAAGC-GTT	240F	HpC8	GCGAGAGC-CTA	470G
HpA40	GCGAAAGC-CGA	280G	HpC10	GCGAGAGC-CGT	300G
HpA41	GCGAAAGC-CCA	400F	HpC25	GCGAGAGC-TGC	600F
HpA49	GCGAAAGC-ACC	150G	HpC28	GCGAGAGC-TGA	320G
HpA54	GCGAAAGC-ATT	250F	HpC38	GCGAGAGC-GTT	270G
HpA57	GCGAAAGC-AGC	220G	HpC40	GCGAGAGC-GTA	170G
HpA59	GCGAAAGC-AGG	270F	HpD12	GCGATAGC-CGA	150G
HpB38	GCGACAGC-GTT	380F	HpD13	GCGATAGC-CAC	240F
		360G	HpD37	GCGATAGC-GTC	340G
		210F	HpD38	GCGATAGC-GTT	500G
HpB41	GCGACAGC-GGC	370F	HpD42	GCGATAGC-GGT	460G
HpB43	GCGACAGC-GGG	330G	HpD59	GCGATAGC-AGG	370G

^a Above DAF markers obtained by screening 276 primers. All polymorphisms were dominant in the F₁ (see Fig. 4b).

^b Indicates molecular size (in bp in 5% PAGE) and genotype (G = B-129-S9 “Gifu”, F = B-581 “Funakura”) in which the band was detected.

Table 3. Mendelian segregation in 100 F₂ plants of *Lotus japonicus*

Morphological marker		Mendelian segregation		Calculated chi-square	Table chi square α = 0.05
		Expected	Observed		
1.	Stem Shape (Ssh) (waved : straight)	75:25	82:18	1.28	3.84
2.	Leaf Color (Lco) (dark green : light green)	75:25	76:24	0.05	3.84
3.	Flowering Period (Fpe) (short period : long period)	75:25	72:28	0.48	3.84

^a Five F₁ plants showed waved stem, short flowering period and dark green leaf. Female parent B-129 “Gifu” showed straight stem, long flowering period and dark green leaf. Male parent B-581 “Funakura” showed waved stem, short flowering period and light green leaf.

^b Stem shape: Observed 30 days postgermination. This characteristic disappeared with branches formed. Leaf color: B-129 “Gifu” showed dark green and B-581 “Funakura” showed light green. Flowering period: The flowering period of B-581 “Funakura” and F₁ progeny lasted 25 days and all flowers disappeared, the flowers of B-129 “Gifu” still bloomed.

for newly discovered phenotypes, we chose to look for molecular polymorphisms. Handberg and Stougaard (1992) already detected RFLP differences between *L. japonicus* ecotypes; however, none of these were studied for their heritability or linkage. Preliminary results from RFLP analysis of Gifu and Funakura shows close relatedness as RFLPs are rare, requiring a broader restriction nuclease spectrum.

DAF, as a multiplex method, provided many amplification products per single primer amplification. Our preliminary screen used 276 arbitrary oligonucleotide primers. Many belonged to the mini-hairpin series (Caetano-Anollés and Gresshoff 1994b), in which a self-closing mini-hairpin forms the 5' end of the primer and a three nucleotide 3' extension provides selectivity (similar to 3' extensions used in AFLP and DD-RT-PCR). Because of the small region of direct template matching, a higher degree of genetic polymorphism is detected (Caetano-Anollés and Gresshoff 1996), valuable for the analysis of crosses between cultivars or other closely related parents. Scoring only major DAF polymorphisms, as seen as heavy band differences on silver-stained polyacrylamide gels, 34 primers gave 36 polymorphisms (Table 2).

A more detailed look at some of the polymorphisms listed in Table 2 reveals further facts. Primers HpA59 and HpD59 share the same 3' extension, but differ by a single A:T to T:A change in the central part of the primer loop; yet they produce different polymorphisms at 270 bp (in Funakura) and 370 bp (in Gifu), respectively. The primer series HpA/B/C/D38 contains the same terminal GTT 3' extension. These primers only differ by subtle changes in the loop, yet they produced six polymorphisms (240F, 210F, 350G, 380G, 270G, and 500G). In other words, the mini-hairpin, although unable to direct amplification by itself, influences the annealing characteristics. It is possible that the 350G-380G pair produced by HpB38 is co-dominant. Polymorphisms for HpA38 and HpA59 are linked at 5 cM distance (Fig. 6, below).

We have not determined whether some of these polymorphisms define the same locus. Since the number of short primers and primer mixing combinations has not been exhausted, we expect an abundance of further markers. Additionally, existing gels may be scored for weaker molecular DAF markers.

Confirmation of F₁ hybrids.

We used six replicate F₁ seeds from crosses between Gifu × Funakura for DAF analysis (three F₁ plants shown Fig. 4A).

None of the amplification product patterns from F₁ plants matched the one obtained from the female parent; accordingly, selfing was eliminated. Furthermore, the DAF patterns revealed the inheritance of polymorphic bands from both parents. F₁ plants were morphologically identical exhibiting characters from both parents.

Figure 4A shows the amplification profile using primer 8.6e (5'GACGTAGG3') resolved by silver staining. In the F₁ progeny, common and monomorphic bands of apparent molecular size of 140, 150, 155, 185, 200, 205, 230, 255, 270, 310, 350, 360, 390, 400, 420, 460, 480, 550, and 600 bp were resolved. No nonparental bands observed. Gifu showed an additional band at 260 bp, but lacked markers at 210 and 250 bp, which were present in Funakura. The polymorphic markers from both male and female parents were found in all F₁ hybrids, confirming nuclear fusion.

We randomly selected cross F₁-6 for DNA amplification using a further 10 nucleotide long primers and arbitrary mini-hairpin primers (Caetano-Anollés and Gresshoff 1994b). We have obtained 36 DNA polymorphic markers by screening 276

Fig. 5. Inheritance of DAF polymorphic marker in an F₂ population of *L. japonicus*. Fifty F₂ plants (selected from the 100 used for other steps) were analyzed for segregation of the 250F DAF marker with primer HpA54 giving a ratio of 38:12 (presence vs. absence of band). Thirteen randomly chosen F₂ plants and the two parents and F₁-6 are shown. G = B-129-S9 Gifu, F = B-581 Funakura.

Table 4. The inheritance of DAF markers in F₂ plants of *Lotus japonicus*

DAF Marker	Primer (5' to 3')	Mendelian segregation		Calculated chi square	Table chi square $\alpha = 0.05$
		Expected	Observed		
270G	8.6m (GTAACGGC)	75:25*	75:25	0	3.84
220F	10.6e (GTGACGTAGG)	75:25	77:23	0.12	3.84
250F	10.6d (GAAACGGGTC)	75:25	78:22	0.33	3.84
170G	10.6g (CAATCGCCGT)	37.5:12.5	40:10	0.43	3.84
300F	HpA17 (GCGAAAGC-TCC)	37.5:12.5	41:9	0.67	3.84
240F	HpA38 (GCGAAAGC-CCT)	37.5:12.5	43:7	2.13	3.84
280G	HpA40 (GCGAAAGC-CGA)	37.5:12.5	40:10	0.43	3.84
400F	HpA41 (GCGAAAGC-CCA)	37.5:12.5	38:12	0	3.84
150G	HpA49 (GCGAAAGC-ACC)	37.5:12.5	40:10	0.43	3.84
250F	HpA54 (GCGAAAGC-ATT)	37.5:12.5	38:12	0	3.84
270F	HpA59 (GCGAAAGC-AGG)	37.5:12.5	41:9	0.67	3.84
225G	HpB55 (GCGACAGC-ATG)	37.5:12.5	34:16	0.96	3.84

* Presence of band vs. absence of band.

primers (Table 2). Fig. 4B showed that 15 primers generated unique distinguishing DAF bands of great clarity. Polymorphisms with weaker intensity differential were discovered as well but are not highlighted here. For this initial study we focused on DAFs of the strongest category, i.e., a major band differed between the parents.

F₁-6 DNA, amplified with primer 10.6g (5'CAATCGCCGT3'), showed a strong band at 270 bp derived from Gifu. With primer 10.6e (5'GTGACGTAGG3') Funakura had an unique marker 220F. F₁-6 maintained this band at the same position. Likewise, primer 10.6d (5'GAAACGGGTC3') exhibited a polymorphism at 250F which was inherited in a dominant fashion in F₁-6 (Fig. 4B). There are 22 DAF markers derived from Gifu and 14 DAF markers derived from Funakura. These polymorphic markers were distributed from 150 to 500 bp (average 297 bp). This range is controlled by amplification, gel separation, and silver staining parameters.

The inheritance of DAF polymorphic products from both parents confirmed the F₁ hybrid nature. No maternal inheritance was observed, although it may eventually be found as Prabhu and Gresshoff (1994) showed about 25% cytoplasmic inheritance of DAF markers in soybean. However, two DAF markers were inherited in a 49:1 and 48:2 ratio, suggesting repeated DNA variation or abnormal cytoplasmic inheritance (data not shown).

The reliability of inheritance and the clarity of scoring indicated that DAF products 270G, 220F, and 250F could be used directly as dominant markers in DAF analysis of the F₂ generation.

Mendelian segregation of F₂ progeny.

One hundred lines of F₂ population were grown in greenhouse for morphological observation and DAF analysis. All morphological and DAF polymorphisms investigated here exhibited Mendelian inheritance in the F₁ and F₂ progeny. Table 3 shows phenotypic Mendelian segregation in 100 plants of F₂ population. The same F₂ was maintained through single seed descent to produce recombinant inbred lines (RILs) for further mapping and sharing of the mapped population among interested laboratories (see Lark et al. (1993) for soybean). This population is at the F₆ stage, providing an expected 97% homozygosity for fixed genomic regions.

Morphological characteristics were inherited in a dominant fashion. Dark green leaf was dominant over light green leaf, waved stem was dominant over erect stem, and short flowering period was dominant over long flowering period. These traits segregated 76:24, 82:18, and 72:28, respectively. Chi-square analysis of the segregation ratio confirmed true Mendelian inheritance, indicating that single nuclear loci are affected.

Mendelian inheritance in an F₂ subpopulation was also observed for DAF polymorphisms (Table 4). For example, marker 250F, revealed through primer HpA54, segregated 38:12 in the F₂ (13 F₂ plants shown, Fig. 5). Markers 270G with primer 8.6m derived from Gifu and 250F with primer 10.6d derived from Funakura, segregated 75 : 25 and 78 : 22. In general, chi-square analysis of the segregation ratio of 12 amplification markers (Table 4) in the F₂ confirmed true dominant nuclear inheritance.

Twelve DAF and three morphological markers, which segregated from 50 F₂ plants from the Gifu × Funakura cross were used to identify genetic linkages. The linkage map was created by using maximum likelihood analysis and "MAPMAKER." A total of 11 linkage groups was obtained; Figure 6 showed three linkage groups of these. Cosegregation and linkage analysis revealed that DAF markers 270G (8.6m) and 225G(HpB55) were linked to the morphological marker *Lco* (Fig. 4). *Lco* is positioned about 16 cM from 225G(HpB55) and about 26 cM from 270G (8.6m). Expansion of the marker set will combine many of the linkage groups.

DISCUSSION

This study demonstrated that the model legume *Lotus japonicus*, characterized by a small genome size, high transformation efficiency, and self-fertility as well as high seed set and short generation time, is amenable to both classical and molecular genetic analysis. The ease with which molecular polymorphisms were found, mapped, and associated with morphological characters is reminiscent of *Arabidopsis* research and further demonstrates the utility of this organism for fundamental studies pertaining to nodulation, nitrogen fixation, and mycorrhizal associations. We propose that ecotype B129-S9 Gifu is accepted as the reference type for further investigations.

Hybrid seeds were produced easily at high efficiencies. Hybrid plants were confirmed by the inheritance of morphological and molecular markers from both parents. Molecular markers generated by single primer amplification were inherited in dominant manner and segregated as single loci in a population of 100 F₂ plants. Visual observation revealed morphological differences between the parents, which were also inherited as single dominant loci. A system for designating loci, and molecular markers generated by DAF, in *L. japonicus* was proposed. For the first time, linkage between a DAF marker and a morphological character is reported.

For sexual crossing of *L. japonicus*, the choice of flowers with pollen and stigma in the proper stage of development was crucial for obtaining high percentages of seed set. We suspect a genetic component to the success rate as reciprocal crosses favored the long flowering genotype as a female parent.

Fig. 6. Example of three linkage groups of *Lotus japonicus*. Several other markers are unlinked. This linkage map was created by using MAPMAKER and 50 to 100 plants.

Younger flowers (Fig. 2A,B) have green petals, unripe anthers, and curled styles. Their size was similar to those from soybean (about 1 mm in length). Using this kind of flower for emasculation, almost 100% of the flowers dropped 3 to 4 days after pollination. Because *L. japonicus* Gifu is a very self-fertile, mature flowers (Fig. 2D) were also inappropriate for emasculation as self-pollination would have been achieved. For crossing we selected flowers with ripe anthers and unreleased pollen (preventing self-pollination), a straight style and sticky stigma (easier to touch pollen from the male parent) for emasculation (Fig. 2C). At this stage, the standard petal was still fused. This kind of flower was best for pollination if used as the female parent.

Breeders differ in their timing for performing emasculations and subsequent pollination. We prefer to emasculate in the late afternoon and pollinate early next morning. Some people may prefer air suction emasculation technique to the forceps method described here. Our results indicated that the method described here is simpler and effective; it even does not need spraying with 2-4-5-trichlorophenoxypropionic acid, which prevents flower dropping (Grant et al. 1962).

We have made crosses between Gifu, Funakura, and B-177 "Korea" and got F_1 seeds. One cross between Gifu and Funakura was advanced to an F_2 population which is being mapped. The same population is being advanced through single seed descent to produce recombinant inbred lines which will facilitate further studies and seed sharing. It is anticipated that a skeletal map of *L. japonicus* containing morphological, RFLP, microsatellite, telomere, and amplification markers will provide the molecular and phenotypic landmarks to provide the framework for further investigation of the plant's role in symbiotic processes (Gresshoff 1993).

MATERIALS AND METHODS

Plant growth and DNA extraction.

Lotus japonicus seeds were obtained from Jens Stougaard, University of Aarhus, Denmark. *L. japonicus* B-129 Gifu was originally collected by I. Hirayoshi, Kyoto University, on a riverbank near Gifu, Japan. *Lotus japonicus* B-177 "Korea" originated from Korea and is also kept at the Plant Introduction Station, Geneva, NY (number G-7359). The B-581 Funakura was collected on Jima Island, Japan by N. Satomi.

Seeds of *L. japonicus* were scarified by a gentle treatment with sand paper, then germinated on the top of several layers of water-soaked filter paper in a petri dish, kept moistened by addition of some water at the side. Incubation at 25°C (in dim light) allowed seeds to swell overnight. Germinated seedlings were transferred to pots when the rootlet was prominent. The soil was Fafard Mix No. 4 (Conrad Fafard, Inc., Agawam, MA). All plants were grown in the greenhouse with an 18/6 h day/night cycle and a 24°C/18°C day/night temperature regime.

Young tissues were harvested from plant material 35 to 40 days postgermination. Genomic DNA was extracted and purified according to Dellaporta et al. (1983).

Crossing method.

A forceps method (Grant et al. 1962) was used to emasculate *L. japonicus* flowers. In the late afternoon, "proper" flowers were selected (Fig. 2C). "Proper" flowers are about 6 to 7 mm in length, have ripe anthers with unreleased pollen, a

straight style and sticky stigma, and the standard petal is still fused. Small pointed forceps were used to cut standard, wing and keel petals (sometimes only cut at the tip). Then all anthers were removed carefully. At this step, two points were important. First, pollen must not have released. Second, the excised anthers were not left around the pistil. Pots were moved to the growth chamber after emasculation to avoid insect-mediated pollination.

During the early morning of the next day, pollen was harvested from the donor plant by releasing it onto the thumb nail. Then the stigma of the emasculated flower was gently appressed to the fresh pollen. Unpollinated flowers (only emasculated) were used as controls. Small pods were visible 3 to 4 days after pollination, signifying that the cross was successful. Flowers from the entire flowering period were receptive for emasculation and pollination.

DNA amplification fingerprinting.

DNA amplification fingerprinting (DAF) was carried out according to Caetano-Anollés et al. (1991). In short, the reaction mixture in a total volume of 25 μ L containing 1 to 2 ng of template DNA (i.e., 40 μ g μ L⁻¹; note the low DNA concentration used for DAF in conjunction with high primer levels), 1.5 μ M oligonucleotide primer (National Biosciences, Plymouth, MN), three units of Stoffel fragment polymerase (Perkin-Elmer Corp., Emeryville, CA), 200 mM of each dNTP, 10 mM Tris-HCl (pH 8.3), 10 mM KCl and 3.75 mM MgCl₂. The total reaction mixture was overlaid with a drop of heavy mineral oil and amplified in an Ericomp thermocycler (Ericomp Inc., San Diego, CA) for 35 cycles (using two-step cycles of 1 s at 96°C and 1 s at 30°C, then one final cycle at 72°C for 5 min; heating and cooling rates were 23°C/min and 14°C/min, respectively). Amplification products were separated by 5% polyacrylamide gel (7 M urea), backed by a plastic Gel-Bond film (for stability and later handling) and detected by silver staining (Bassam et al. 1991; Caetano-Anollés and Gresshoff 1994a). Amplification profiles may vary in thermocyclers with different ramping speeds, but have not been matched in a MJ Research Pt-200 unit, set to the Ericomp ramping rates.

Linkage analysis.

All DAF markers from segregating F_2 population were analyzed. The computer program "MAPMAKER" (Lander et al. 1987) was used to establish these linkage group.

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LITERATURE CITED

- Akkaya, M. S., Bhagwat, A. A., and Cregan, P. B. 1992. Length polymorphism of simple sequence repeat DNA in soybean. *Genetics* 132:1131-1139.
- Barker, D. G., Bianchi, S., Blondon, F., Dattée, Y., Duc, G., Essad, S., Flament, P., Gallusci, P., Génier, G., Guy, P., Muel, X., Tourneur, J., Dénarié, J., and Hugué, T. 1990. *Medicago truncatula*, a model plant for studying the molecular genetics of the *Rhizobium*-legume symbiosis. *Plant Mol. Biol. Rep.* 8:40-49.
- Bassam, B. J., Caetano-Anollés, G., and Gresshoff, P. M. 1991. A fast and sensitive silver-staining for DNA in polyacrylamide gels. *Analytical Biochem.* 196:80-83.

- Caetano-Anollés, G., and Gresshoff, P. M. 1994a. Staining nucleic acids with silver: An alternative to radioisotopic and fluorescent labeling. *Promega Notes* 45:13-18.
- Caetano-Anollés, G., and Gresshoff, P. M. 1994b. DNA amplification fingerprinting using arbitrary oligonucleotide mini-hairpin primers. *Bio/Technology* 12:619-623.
- Caetano-Anollés, G., and Gresshoff, P. M. 1996. Generation of sequence signatures from DNA amplification fingerprints with mini-hairpin and microsatellite primers. *Bio/Techniques* 20:1044-1056.
- Caetano-Anollés, G., Bassam, B. J., and Gresshoff, P. M. 1991. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Bio/Technology* 9:553-557.
- Dellaporta, S. L., Wood, J., and Hicks, J. B. 1983. A plant DNA mini-preparation: Version II. *Plant Mol. Biol. Rep.* 1:19-21.
- Gallusci, P., Dedieu, A., Journet, E. P., Huguet, T., and Barker, D. G. 1991. Synchronous expression of leghaemoglobin genes in *Medicago truncatula* during nitrogen-fixing root nodule development and response to exogenously supplied nitrate. *Plant Mol. Biol.* 17:335-349.
- Goormachtig, S., Valerio-Lepiniec, M., Szczyglowski, K., van Montagu, M., Holsters, M., and de Bruijn, F. J. 1995. Use of differential display to identify novel *Sesbania rostrata* genes enhanced by *Azorhizobium caulinodans* infection. *Mol. Plant-Microbe Interact.* 8:816-824.
- Grant, W. F., Bullen, M. R., and De Nettancourt, D. 1962. The cytogenetics of *Lotus*. 1. Embryo-cultured interspecific diploid hybrids closely related to *L. corniculatus* L. *Can. J. Genet. Cytol.* 4:105-128.
- Gresshoff, P. M. 1993. Molecular genetic analysis of nodulation genes in soybean. *Plant Breeding Rev.* 11:275-318.
- Gresshoff, P. M. 1995. Moving closer to the positional cloning of legume nodulation genes. Pages 431-436 in: *Nitrogen Fixation: Horizons and Application*. I. Tikhonovich, N. Provorov, V. Romanov, and W. E. Newton, eds. Kluwer Academic Publishers, Dordrecht, Netherlands.
- Handberg, K., and Stougaard, J. 1992. *Lotus japonicus*, an autogamous, diploid legume species for classical and molecular genetics. *Plant J.* 2:487-496.
- Jiang, Q., and Gresshoff, P. M. 1993. *Lotus japonicus*: A model plant for structure-function analysis in nodulation and nitrogen fixation. Pages 97-110 in: *Current Topics of Plant Molecular Biology*, II. P. M. Gresshoff, ed. CRC Press, Boca Raton, FL.
- Kolchinsky, A., Funke, R. P., and Gresshoff, P. M. 1994. Dissecting molecular mechanisms of nodulation: taking a leaf from *Arabidopsis*. *Plant Mol. Biol.* 26:549-552.
- Kolchinsky, A., Landau-Ellis, D., and Gresshoff, P. M. Map order and linkage distances of molecular markers close to the supernodulation (*nts-1*) locus of soybean. *Mol. Gen. Genet.* In press.
- Lander, E. S., Green, P., Abrahamson, J., Barlow, A., Daly, M., Lincoln, S. E., and Newburg, L. 1987. MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* 1:174-181.
- Martin, G. B., Brommonschenkel, S., Chunwogse, J., Frary, A., Ganai, M. W., Spivey, R., Wu, T., Earle, E. D., and Tanksley, S. D. 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* 262:1432-1436.
- Landau-Ellis, D., Angermüller, S. A., Shoemaker, R., and Gresshoff, P. M. 1991. The genetic locus controlling supernodulation co-segregates tightly with a cloned molecular marker. *Mol. Gen. Genet.* 228:221-226.
- Lark, K. G., Weisemann, J. M., Mathews, B. F., Palmer, R., Chase, K., and Macalma, T. 1993. A genetic map of soybean (*Glycine max* L.) using an interspecific cross of two cultivars 'Minsoy' and 'Noir 1'. *Theor. Appl. Genet.* 86:901-906.
- Leyser, H. M. O., Lincoln, C. A., Timpte, C., Lammer, T., and Estelle, M. 1993. Arabidopsis auxin-resistance gene AXR1 encodes a protein related to ubiquitin-activating enzyme E1. *Nature* 364:161-163.
- Pillai, S., Funke, R. P., and Gresshoff, P. M. 1996. Constructions of YAC and BAC clones from the model legume *Lotus japonicus*. *Symbiosis* (in press).
- Prabhu, R. R., and Gresshoff, P. M. 1994. Inheritance of polymorphic markers generated by DNA amplification fingerprinting and their use as genetic markers in soybean. *Plant Mol. Biol.* 26:105-116.
- Parniske, M., Wulff, B., Hughes, D., Webb, J., Szczyglowski, K., de Bruijn, F. J., Stougaard, J., and Wegel, E. 1996. Mycorrhizal mutants of *Lotus japonicus*. Satellite Meeting on 8th International Congress of Molecular Plant-Microbe Interaction: Emerging Model Legume System: Tools and Recent Advances.
- Rolfe, B. G., Gresshoff, P. M., and Shine, J. 1980. Rapid screening for symbiotic mutants of *Rhizobium* and white clover. *Plant Sci. Lett.* 19:277-284.
- Sagan, M., Morandi, D., Tarengui, E., and Duc, G. 1995. Selection of nodulation and mycorrhizal mutants of the model legume *Medicago truncatula* (Gaertn.) after γ -ray mutagenesis. *Plant Sci.* 111:63-71.
- Stiller, J., and Gresshoff, P. M. 1996. Trapping of symbiotic genes in the model legume *Lotus japonicus*: the establishment of the system. 1996. Abstract Book on 8th International Congress of Molecular Plant-Microbe Interaction, H-121.
- Szczyglowski, K., Hamburger, D., Kapranov, P., Shaw, R. S., and de Bruijn, F. J. 1995. Symbiotic mutants and nodule-specific ESTs of the model legume *Lotus japonicus*. Page 484 in: *Nitrogen Fixation: Fundamentals and Achievements*. I. Tikhonovich, N. Provorov, V. Romanov and W. E. Newton, eds. Kluwer Academic Publishers, Dordrecht.
- Thyckjaer, T., Stiller, J., Handberg, K., Jones, J. D. G., and Stougaard, J. 1995. The maize transposable element *Ac* is mobile in the legume *Lotus japonicus*. *Plant Mol. Biol.* 27:981-993.
- Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: A new technique for DNA fingerprinting. *Nucleic Acid Res.* 23:4407-4414.
- Walden, R., and Schell, J. 1994. Activation T-DNA tagging—a silver bullet approach to isolating plant genes. *Agro-Food Ind. Hi-Tech* 5:9-12.
- Weaver, K., Caetano-Anollés, G., Gresshoff, P. M., and Callahan, L. M. 1994. Isolation and cloning of DNA amplification products from silver stained polyacrylamide gels. *BioTechniques* 16:226-227.
- Welsh, J., and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* 18:7213-7218.
- Wicking, C., and Williamson, B. 1991. From linked marker to gene. *Trends Genet.* 7:288-290.
- Williams, J. G. K., Kubelik, A. E., Livak, K. J., Rafalski, J. A., and Tingey, S. C. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18:6531-6535.