LOTUS JAPONICUS HANDBOOK

Lotus japonicus Handbook

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Springer

A C.I.P. Catalogue record for this book is available from the Library of Congress.

ISBN-10 1-4020-3734-1 (HB)
ISBN-13 978-1-4020-3734-4 (HB)
ISBN-10 1-4020-3735-X (e-book)
ISBN-13 978-1-4020-3735-1 (e-book)

Published by Springer,
P.O. Box 17, 3300 AA Dordrecht, The Netherlands.

www.springeronline.com

Printed on acid-free paper

Printed in the Netherlands.

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Lotus japonicus literature

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Preface

Legumes are very important plants (VIPs). They account for about one-third of the world's primary crop production, human dietary protein, and processed vegetable oil, and are a major source of feed for livestock and raw materials for industry. Legumes are a key component of sustainable agricultural systems because of symbiotic nitrogen fixation, which provides these plants and associated or subsequent crops with a free and renewable source of nitrogen. Legumes are also able to establish beneficial symbioses with soil fungi that enable them to mine phosphorous and other essential nutrients from the soil more effectively. However, despite their importance in agriculture, increases in legume yield through breeding over the past few decades have lagged behind those of cereals. While classical plant breeding can and will lead to further improvements in legume genotypes in the future, the genomics revolution offers alternative and complementary approaches that can aid and accelerate plant breeding. Genomics and functional genomics, together with the more classical scientific disciplines of genetics, biochemistry, physiology, and molecular and cell biology, have already accelerated discoveries in legume molecular and systems biology.

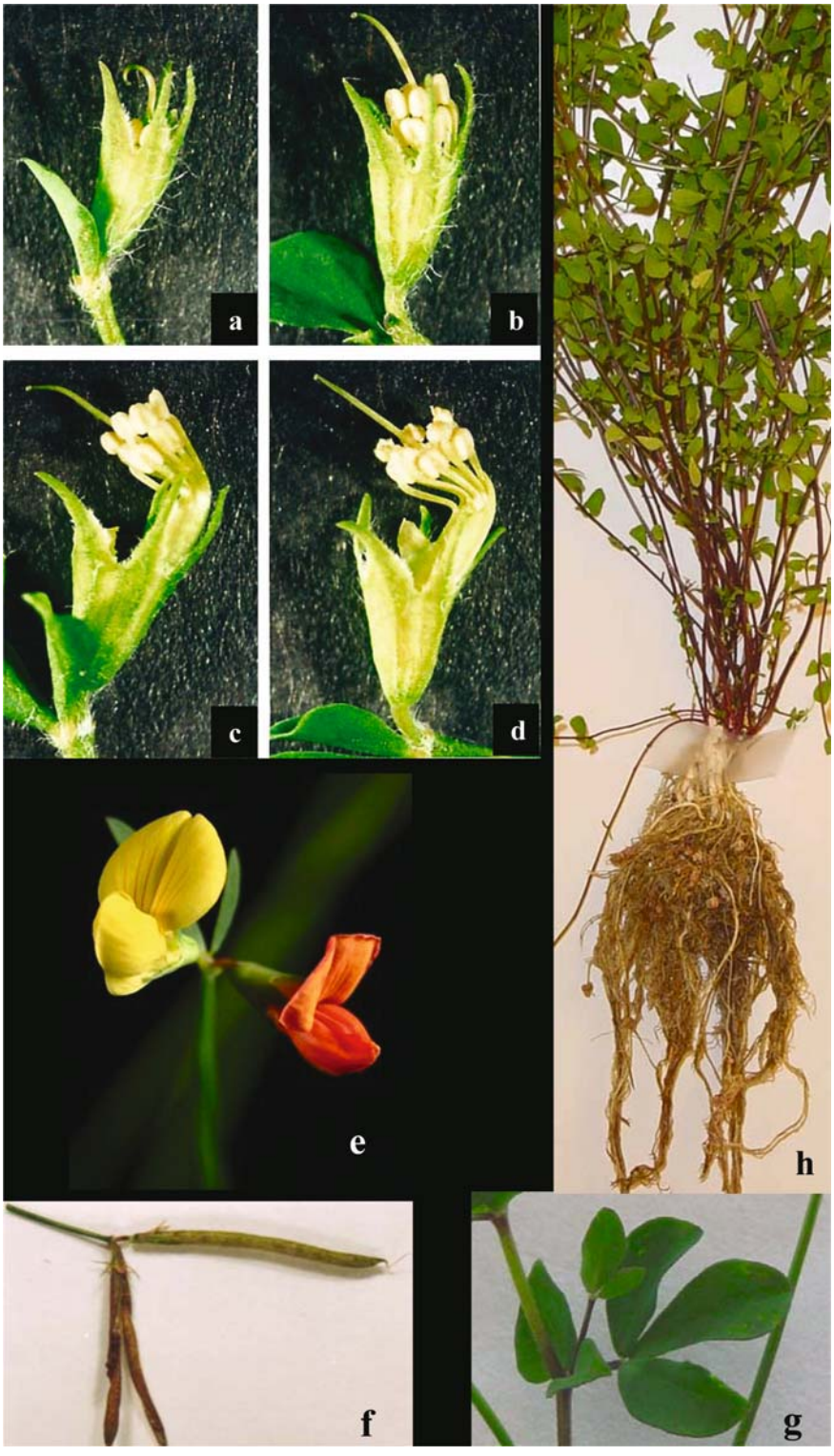
Legumes have played a key role in biological research, a good example of which is Mendel's work on the common garden pea that provided the groundwork for modern genetics. Mendel's work also highlighted the importance of choosing a tractable model species for scientific research. While diploid pea was a fine model for classical genetics, the large size of the pea genome together with other less than optimal features have hindered the isolation and characterisation of genes with important roles in legume biology and agriculture. For this reason *Lotus japonicus* was chosen as a model species for legume research some ten years ago. Since then, many groups within Europe and around the world have adopted *L japonicus* as a model and have developed numerous resources and protocols to facilitate basic and applied research on this species. Over 200 research papers focusing on *L japonicus* have now been published, many of them groundbreaking. Amongst the most important discoveries that have come from Lotus research have been the isolation of genes with crucial roles in symbiosis development, which have provided amazing new insight into the nature and evolution of signalling in plant-microbe symbioses.

This handbook represents the first effort to compile basic descriptions and methods for research in *L japonicus*. We wish to thank all the authors for their efforts in making the book as complete and up-to-date as possible. We are also grateful to the EU, which has provided funding to promote Lotus research, including the research and training networks HPRN-CT2000-00086 (coordinated by Michael Udvardi) and MRTN-CT-2003-505227 (coordinated by Martin Parniske). In fact, this book was conceived within the framework of the first of these networks. Finally, we wish to thank Megan McKenzie, our Technical Editor for her extraordinary professional work, as well Junta de Andalucía (Spain) for support in preparing the camera-ready version of this handbook for publication.

THE EDITORS

COLOUR PLATES

Colour plate 1. Different plant organs of Lotus japonicus *plants. a-d) Developmental stages of* Lotus japonicus *flowers relevant to hand pollination (reproduced with permission from Jiang and Gresshoff, 1997). Flowers in a and b are too young and inappropriate for fertilisation as female flower. The correct age of the flower is shown in c: optimal flowers are 6-7 mm in length and the standard petal is still fused, they show no curvature of the style as well as complete extension of the anther filament and ripening of the pollen. The flower in d is too old, pollen has been released from the anther and self-pollination has already taken place. e) Flowers from Gifu (yellow) and Filicaulis (red) ecotypes. (a courtesy of K. Szczyglowski) f) Seedpod containing up to 20 seeds. g) Trifoliate leaf. h) 2 months old* Lotus japonicus *plant. See chapter 1.1.*



Colour plate 1.

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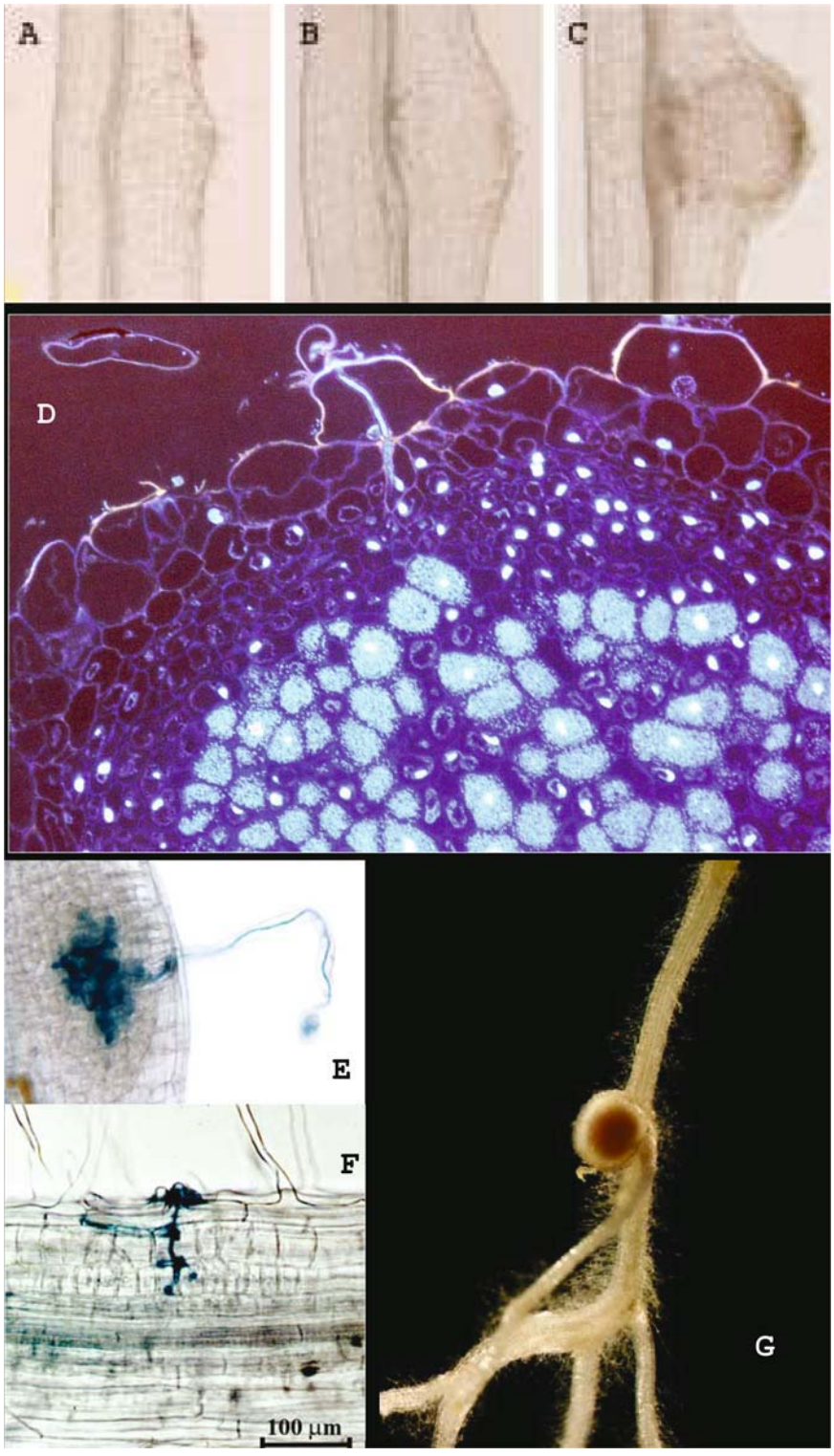

Colour plate 2.

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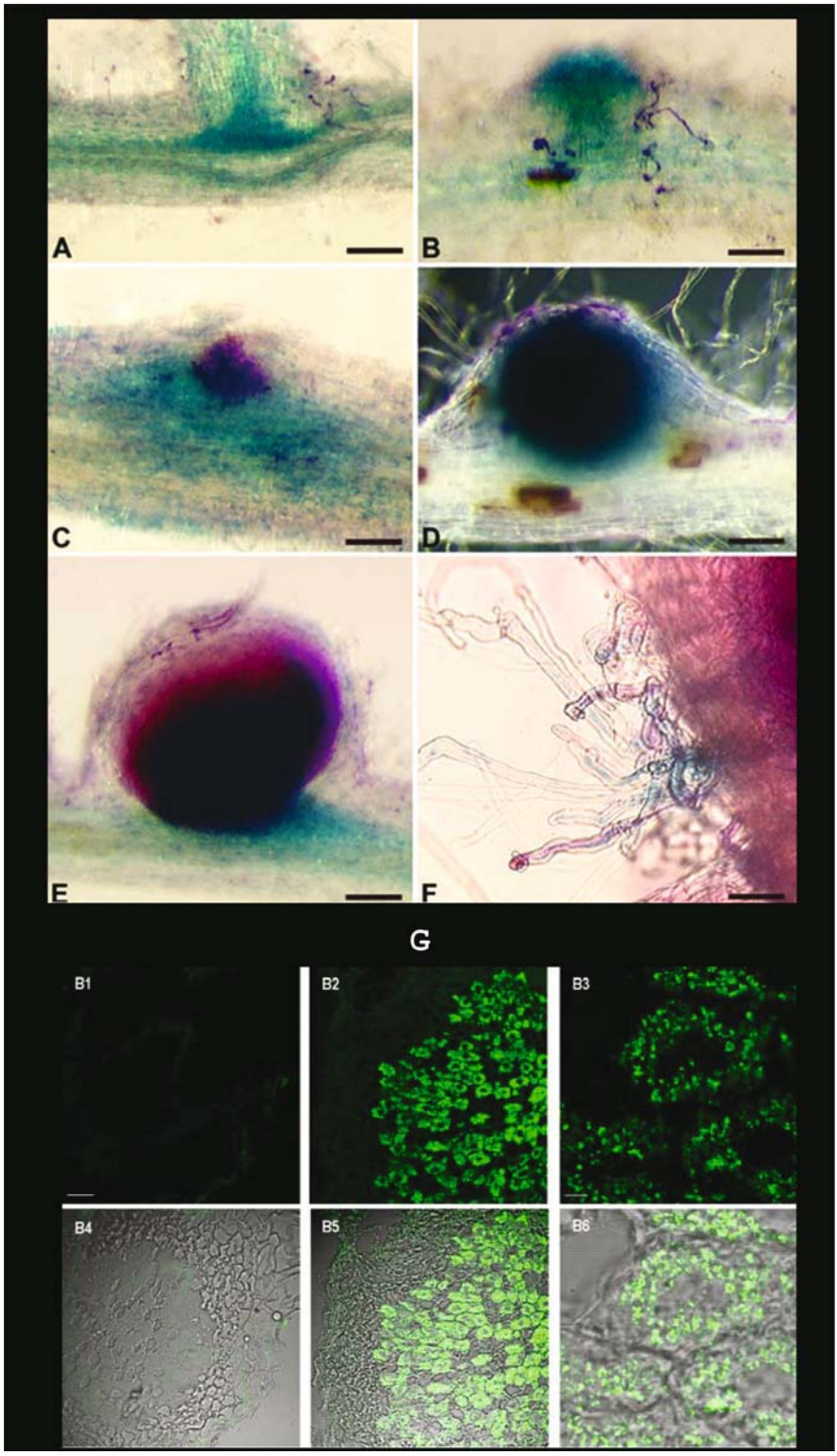

Colour plate 3.

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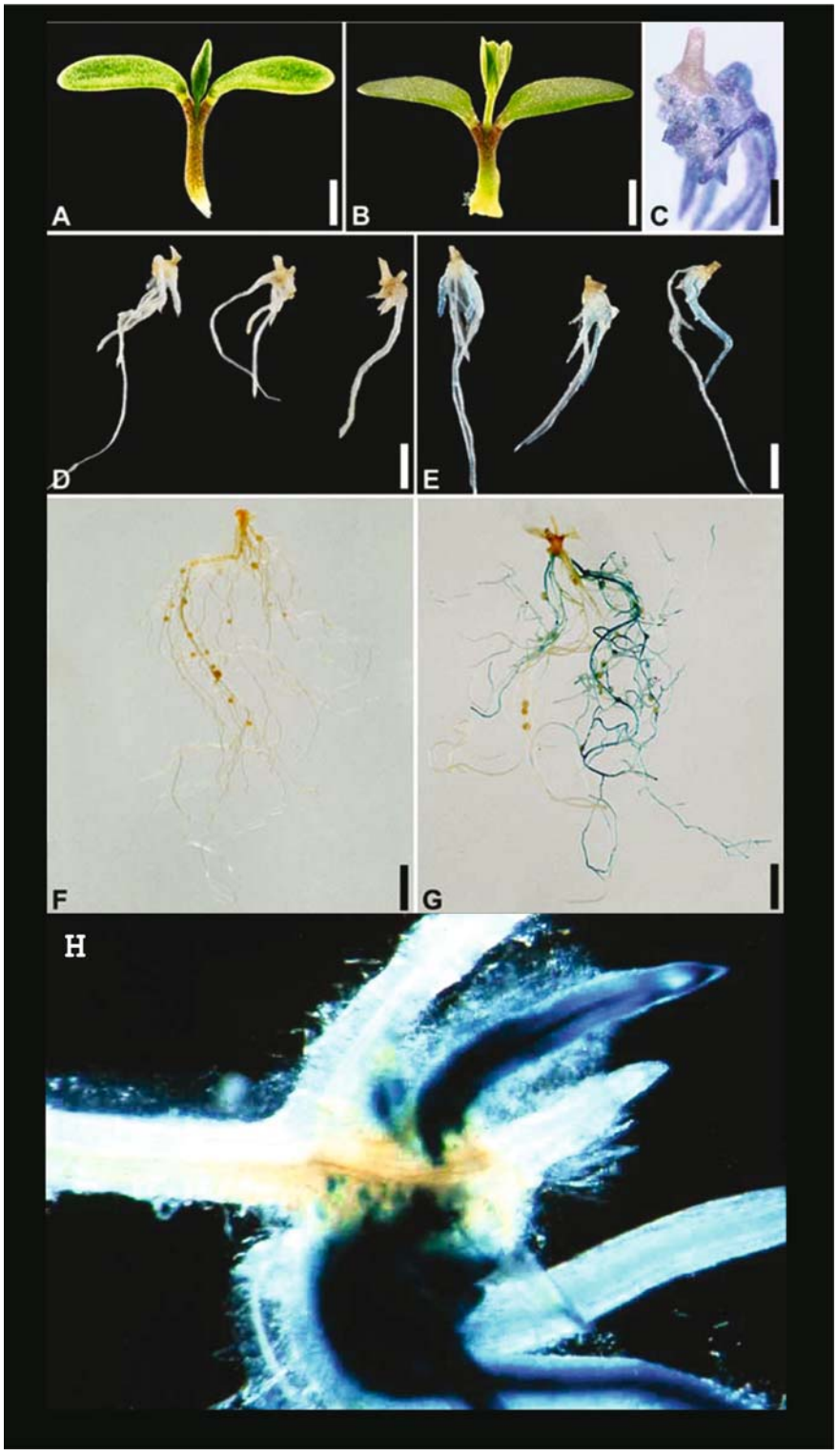

Colour plate 4.

SECTION 1

LOTUS JAPONICUS, A GENERAL INTRODUCTION

Chapter 1.1

LOTUS JAPONICUS AS A MODEL SYSTEM

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Keywords: Model legume, Lotus, symbiosis, ecotype, microsynteny

Lotus japonicus *was proposed as a model legume twelve years ago, because of a number of characteristics making this system very amenable for legume research. These characteristics include small plant, large and abundant flowers, easy hand pollination, high seed production, short generation time, easy cultivation, amenable to plant transformation and regeneration from tissue culture. At present, a set of genetic resources and tools has rapidly become available, including ecotypes, mutant lines, genetic maps, RIL lines, transformation procedures, EST sequences, and a whole genome-sequencing project. In these twelve years, research on* L. japonicus *has greatly contributed to the understanding of both symbiotic processes, i.e. with Rhizobium and mycorrhiza, making possible the cloning of several key genes involved in both symbioses. Now Lotus is regarded as one of the most useful plants for legume study and researchers who have interests in nodulation and other aspects of legume biology use it worldwide. In this introductory chapter we deal with the most general aspects and possibilities of the biology of* L. japonicus*, plant growth conditions, culture media, nitrogen supply and symbiotic partners; plant tissue culture; genetic transformation and regeneration of transgenic plants, contribution to the understanding of symbiotic processes and the role of this model plant for other research topics and exploiting microsynteny.*

INTRODUCTION

The family Leguminosae (Fabaceae), which is the third largest Angiosperm family, contains a number of important crop plants and woody trees, producing protein, carbohydrates, and oil for human consumption and animal fodder. Legumes are 'pioneer' plants that are able to grow in nutrient-poor soils by virtue of their ability to establish symbioses with nitrogen-fixing rhizobia bacteria, and with nutrient scavenging soil fungi, which provide the plants with phosphorous and other essential

A.J. Márquez (ed.), Lotus japonicus Handbook, 3-24.

nutrients. These natural features favoured the adoption of legumes in ancient agriculture, and make them an important part of sustainable agricultural systems to this day.

Among the plant families, symbiotic interaction with rhizobia is unique to legumes. The only known exception is *Parasponia andersonii* belonging to the elm family. Another symbiosis providing nitrogen to host plants is between gram-positive bacteria *Frankia* and several woody plant species forming actinorhizal symbiosis. Phylogenetic analysis based on DNA sequencing of the *rbcL* genes places all plants involved in rhizobial or actinorhizal symbiosis in the same clade (Rosid I), thus indicating that predisposition for nodulation evolved only once (Soltis et al, 1995, Doyle, 1998). However, different morphology and biochemistry of several types of nodules, indicate multiple origins of nodulation, perhaps even within the legume family (Doyle, 1998; Doyle and Luckow, 2003). The symbiotic interaction of legume plants with mycorrhizal fungi, though it is not exclusive to this family, is also an important interaction, which provides phosphorus and other nutrients to the plant. Obtaining sufficient nutrients from the soil is a common problem facing land plants, and 80% to 90% of all land plants are assisted in nutrient mining through association with symbiotic arbuscular mycorrhizal fungi. Both rhizobial and mycorrhizal symbiosis have been investigated previously, but modern molecular tools have only recently become available to study the genetics and genomics of root symbiosis. Increasing our knowledge of the biology and genetics of the legumes may improve this important agricultural resource as well as complement Arabidopsis, which does not develop rhizobial symbiosis or fungal symbiosis leading to vesicular arbuscular mycorrhiza.

BIOLOGY

With close to 18,000 different species belonging to the subfamilies Mimosoideae, Caesalpinioideae and Papilionoideae the legume family is a large and diverse plant family that includes several species of agricultural interest. The importance of the family from applied, botanical, and basic scientific perspectives merits a focused effort on a model legume. The poor ability of *Arabidopsis* to enter into symbiotic interaction with mycorrhiza and the inability to form nitrogen fixing root nodules in symbiosis with *Rhizobium* or it allies has been a further incentive to develop a model legume. A clear illustration of the need for "family specific" model plants is the relatively low levels of microsynteny between *Arabidopsis* and *L. japonicus* suggesting that the structure of legume genomes might be quite different from *Arabidopsis*. Recent bioinformatic based studies of genome evolution supports this view and indicates that legumes may not have undergone one of the genome duplication events inferred for *Arabidopsis*. Genomic research on models such as *Lotus japonicus*, should allow a fundamental understanding of the nodulation process and identification of genes involved in nodule formation and mycorrhizal symbiosis. Together with soybean, bean, cowpea, and *Sesbania*, *L. japonicus* belongs to legumes forming determinate nodules. Cortical cell divisions leading to the formation of the determinate nodule primordium initiate in the outer cortex. A meristem starts dividing to form the nodule primordium, and afterwards the meristematic activity ceases. Nodule size then increases via cell expansion.

Molecular characterisation of 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.

Many cultivated legumes are either tetraploid, self-incompatible, or have a pod morphology making access to the seeds difficult, such as spiral pods in *Medicago*. *L. japonicus* is diploid, self-fertile, develops straight seedpods with two valves and seeds are arranged along a simple linear axis. Mature seedpods break along the rim, making seed harvest very easy. Seeds have no dormancy period and in the Lotus model, pod morphology is similar to the morphology of the most important grain legumes like soybean, pea, and cowpea making it a prime model for comparative studies of seed development.

L. japonicus (Regel) Larsen was first recognized at the ancient capital of Japan, Kyoto, centuries ago. The natural habitat for *L. japonicus* is in East- and Central-Asia including the region around Japan, Korea, China, and stretching into Afghanistan. In the 1950s, Professor Isao Hirayoshi (Kyoto University) collected *L. japonicus* plants growing on a riverbank in Gifu. Professor William F. Grant (McGill University, Montreal) collected its progeny as the accession B-129. In 1992, Kurt Handberg and Jens Stougaard of University of Aarhus (Denmark) obtained B-129 and established Lotus as a valuable tool for modern legume research. Many ecotypes of Lotus can be found growing in its natural habitat. Researchers in Japan have taken advantage of this and dozens of accessions have recently been collected (www.beansbase.agr.miyazaki-u.ac.jp/). Resources for investigating genotypic variability contributing to actual and potential traits of agronomic importance, such as seed yield, plant height, cold tolerance, and disease resistance is thus available for identification by mapping of quantitative trait loci (QTLs). In this context the possibility of obtaining fertile F1 plants and segregating F2 populations after crossing *L. japonicus* to diploid relatives like *L. filicaulis*, *L. frondosus*, *L. schoelleri*, *L. burttii* and others (Grant et al, 1962, Sz-Borsas et al, 1972, Sandal et al. 2002) is an additional source of biodiversity.

Ecotypes

A number of *L. japonicus* ecotypes have been collected. This material is a rich source of biological (phenotypical) and genetic variability. At present, 75 *L. japonicus* ecotype lines from different areas of Japan can be requested on the *L. japonicus* seed center at Miyazaki University Japan web site, http://www.beansbase.agr.miyazaki-u.ac.jp. The ecotype Miyakojima (MG-20) found on the island of the same name, has been used as a crossing partner with Gifu in order to establish populations for map-based cloning, since it has the highest degree of polymorphisms, about 6%, among other 15 ecotypes tested (Kawaguchi, 2000). Miyakojima displays several traits that distinguish it from other Japanese accessions: low concentrations of anthocyanin in the stem and petals, few trichomes, a more upright habit, broad leaflets and petals, and large black seeds. The first two traits, which are controlled by single recessive genes, serve as useful markers for following mutant crosses. The Kazusa DNA Research Institute is currently sequencing the MG-20 genome.

Morphological description of plant developmental stages

L. japonicus is a small prostrate perennial that flowers profusely. Flowers are yellow and relatively big, generally two per peduncle. It is self-fertile with a straight, cylindrical, and dehiscent pod containing up to 20 seeds.

L. japonicus grows relatively slowly during the first weeks after germination. The primary plant is small, allowing high-density germination, mutant plant screening on agar plates, nodulation tests, selection of transformants on selective conditions, etc. However, the mature plant has a bushy growth with many branches up to 30 cm long and many leaves, which makes it very useful for biochemical, chemical and physiological studies, since abundant material can be harvested. The mature plant exhibits abundant indeterminate flowering, with large yellow flowers, which are usually arranged in pairs and less frequently one or three flowers per peduncle. Mature flowers on the ecotype Gifu are about 8-12 mm in length. Under greenhouse conditions, *L. japonicus* has a generation time of approximately 3 months. Fertilisation and seed set occur without manipulation of the flowers. Pod formation in fertilised flowers occurs within 3-4 days. Mature seedpods are about 3 cm in length and contain up to 20 seeds. Mature seed weight is around 1.0-1.2 mg. Because *L. japonicus* is a perennial plant, new branches and flowers continue to develop after removing the old branches.

Colour plate 1 illustrates the main morphological characteristics of *L. japonicus* plants.

Cross-pollination

In order to use *L. japonicus* for genetic studies and map based cloning a cross-pollination method is required. The method described by Jiang and Gresshoff (1997) is generally used. Forceps are used to cut the tip of the petals and emasculate the flowers, gently removing the anthers. Pollen from the donor flower is deposited onto the stigma of the emasculated young flower, whose stigma should be slightly bent.

In Figure 1, from Jiang and Gresshoff (1997), the correct age of the flower is shown in C whereas the flowers in A and B are too young and inappropriate for fertilisation as female flower. The flower in D is too old (pollen has been released from the anther and self-pollination has already taken place). Optimal flowers for cross-pollination are 6-7 mm in length and the standard petal is still fused, they show no curvature of the style as well as complete extension of the anther filament and ripening of the pollen. The flowers used for crossing can be protected against drying out with small bags. It is important that the humidity is high during the development of the pod from the cross. *L. japonicus* ecotypes are generally easy to cross. *L. japonicus* can also be crossed to related diploid Lotus species like *L. filicaulis*.

Figure 1. Developmental stages of Lotus japonicus *flowers relevant to hand pollination (reproduced with permission from Jiang and Gresshoff, 1997). See colour plate 1 for this figure in colour.*

As a legume model plant, *L. japonicus* offers a suit of advantages; high transformability, self pollinating proliferation, small genome size (ca. 450 MB), small plant size, large seed set and short life cycle (ca. three months) (Table 1). In addition to its model status, *L. japonicus* belongs to the Loteae and represents the genus *Lotus* consisting of approximately 180 distributed worldwide (Kirkbride Jr. J.H. 1999). Among the genus *Lotus,* one of the species of greater interest from an agricultural point of view is the tetraploid *Lotus corniculatus* L. (birdsfoot trefoil) 2n=24. *L. uliginosus/L. pedunculatus* Schkuhr (Big trefoil) (2n=12), *L. tenius/L. glaber* (Waldts&Kit) (Narrow-leaf birdsfoot trefoil) (2n=12 or 24) and *L. subbiflorus* (2n=12 or 24) are also used in agriculture (Swanson et al., 1990). All of these cultivated species are either self-incompatible or have marginal to low self-fertility. *L. corniculatus* (birdsfoot trefoil) is an herbaceous perennial dehiscent species native to Europe, where it is widely distributed, and has extensively colonized in North America and South America. *L. corniculatus* has become a successful forage crop during the past century both in Europe and America. While *L. corniculatus* has been reported as having diploid ecotypes (Grant 1995), it is generally known as a tetraploid species (2n = 24). The anti-bloating properties due to tannin content, ability to grow in unfertile, acid or alkaline soils, and drought/flooding tolerance are the main agricultural advantages. In addition, the species has become widely used to prevent roadside erosion.

Growth	Growth in a variety of conditions	
	Short generation time	3-4 months
	Primary small plant, but bushy plant after secondary shoot formation, branching	
	Perennial	
	Straight seed pods, indeterminate flowering	
	Small seeds (1.2 g per 1,000 seeds)	
Propagation	Self-fertile	
	Large and abundant flowers	
	High seed production	Up to 20 seeds per pod, 6000 seeds per plant
	Vegetative propagation from nodal sections	
	Easy hand pollination	
Genome characteristics	Small genome	450 Mbp
	Diploid	2n=12
Plant tissue culture	Regeneration from callus	
	Amenable to plant transformation with *A. tumefaciens* and *A. rhizogenes* and regeneration of transgenic plants.	
	Hygromycine, kanamycine and phosphinothricin selection possible	
	Transgene stable in subsequent generations	
Symbiosis	Rhizobial and mycorrhizal symbiosis	*Glomus intraradices, Gigaspora margarita*?
	Determinate nodules	
	Fast growing *Mesorhizobium loti*	R7A, NZP2235, MAFF303099 TONO, NGR234
Tannin content	Low levels of tannin accumulation	No tannins in leaves
Galactomannan content in seed endosperm	Endospermic seeds	Galactomannan with a high degree of Gal substitution (Man/Gal=1.23)

Insect interaction	Cyanogenic and nitrile glucosides content: Lotaustralin and linamarin Rhodiocyanosides A and D	Lotaustralin:linamarin ratio approx. = 10
Spider mite interaction	Emission of volatile compounds (terpenoids) to attract mites	(*E*)-*β* -ocimene
Nematode interaction	Root-knot and cyst nematodes	Sensitive to root-knot nematodes: *M. incognita*, *Meloidogynes sp*. Resistant to cyst-nematode: *H. glycines* (hypersensitive response)

Table 1. L. japonicus *model plant features (taken from Handberg and Stougaard, 1992).*

PLANT GROWTH CONDITIONS, CULTURE MEDIA, NITROGEN SUPPLY, AND SYMBIOTIC PARTNERS

L. japonicus is a species able to grow under a variety of conditions. Many growth systems have been developed depending on the final use of the plant, for instance, microscopical observation, biochemical analysis, genetic screening, etc. Usually, the best conditions for plant cultivation are temperatures between 18-22ºC and to avoid seedpod dehiscence, humidity around 70%. Photoperiods of 16 h light and 8 h dark are common plant growth chamber conditions. Among the inert materials used as support for plant growth are clay granules (leca), a 50% mix of perlite:vermiculite, or even vermiculite alone. *L. japonicus* is somewhat sensitive to over-watering. Watering from below is generally better than flooding from above and a "semidry" air-filled root support is better than a dense water soaked support. The pillow system is another system for plant growth and nodulation experiments and consists of bags filled with sand: vermiculite and submerged in plant growth solution (Szczyglowski et al., 1998). Seeds are planted in between two bags, and roots develop in between the bags. This system is also good for root and nodulation studies, since it is easy to pull out the plant without distorting roots and nodules. When grown on peat, plants can develop a bigger size, with stems up to 30 cm long and many branches and side shoots, giving a bushy appearance. This is the best condition for seed production. After a round of flowering and seed set, old branches can be removed and plants can regenerate new branches and start flowering again.

Plants are sufficiently small during the first 4-6 weeks to be grown on plates under sterile conditions on plant growth medium-agar. In this case, covering the root of the plants with dark paper or keeping the lower part of the plant under the dark is recommended. These conditions are especially good for nodulation studies, since it is easy to see nodules without taken the plant out of the plate. Magenta boxes filled with vermiculite or perlite:vermiculite are also suitable. In all these closed systems, the addition of ethylene inhibitors as L-α-(2-aminoetoxivinil)glycine (AVG) has been recommended in nodulation experiments (Pacios-Bras et al., 2000).

An alternative growth system is hydroponic culture. In this case, seeds are placed on a mesh and submerged in plant growth solution, under suitable temperature and photoperiod conditions. A similar system is aeroponic cultures, where plants are also germinated on a mesh, and plant growth solution is misted around the roots. Plants develop a big root system under these conditions, which is useful for experiments requiring a large amount of root tissue.

Plant growth solutions

Broughton & Dilworth solution (1971) often diluted one fourth and also Jensen solution have been used extensively for *L. japonicus* nodulation experiments, since they are N-free. Plants inoculated with compatible *Rhizobium* can grow well without additional nitrogen. A detailed study on nitrogen metabolism in this plant is included elsewhere in this book. For non-inoculated plants, Hornum solution has also been used (Handberg and Stougaard, 1992). Plants grow better in Hornum solution containing both ammonium and nitrate as nitrogen sources. Nitrate alone can also be used as nitrogen source, when the plant is not inoculated. Ammonium alone is not an optimal nitrogen source for *Lotus*, and growth in the presence of ammonium is slower. Nevertheless, it has also been used as nitrogen source in the form of ammonium succinate, which supports plant growth reasonably well. Seedlings are sensitive and may turn yellow on high nutrient concentrations. The best growth is often obtained by germination and seedling growth on water or low nutrients followed by application of nutrient solution after plants start to develop trifoliate leaves.

Frequently used culture solutions

- Hornum solution (100x stock): 40 g/l NH_4NO_3, 30 g/l KNO_3, 30 g/l $MgSO_4.7H_2O$, 10 g/l $NaH_2PO_4.H_2O$, 2 g/l Fe-EDTA (9% Fe), 120 mg/l $MnSO_4.H2O$, 120 mg/l H_3BO_3, 40 mg/l $CuSO_4.5H_2O$, 40 mg/l $ZnSO_4.7H2O$ and 8 mg/l $Na_2MoO_4.2H_2O$. Diluted in tap water and pH adjusted to 6.8.
- Broughton & Dilworth solution: $CaCl_2$ 1 mM; KH_2PO_4 0.5 mM; Fe-citrate 10 µM; $MgSO_4$ 0.25 mM; K_2SO_4 0.25 mM; $MnSO_4$ 1 µM; H_3BO_3 2 µM; $ZnSO_4$ 0.5 µM; $CuSO_4$ 0.4 µM; $CoSO_4$ 0.2 µM; Na_2MoO_4 0.2 µM.
- Jensen medium: 0.1 g/l $CaHPO_4$; 20 mg/l K_2PO_4; 20 mg/l $MgSO_4.7H_2O$; 20 mg/l NaCl; 10 mg/l Fe-citrate; 100 µg/l $Na_2MoO4.4H_2O$; 200 µg/l $MnSO_4.4H_2O$; 10 µg/l $CuSO_4.5H_2O$; 25 µg/l $ZnSO_4.7H_2O$; 300 µg/l H_3BO_3.

Nodal propagation

The perennial character of this plant enables easy vegetative propagation: individual plants can be multiplied from nodal cuttings with a rooting frequency of 90%, even in the absence of hormones. For clonal propagation of individual plants, nodal sections 2 cm long with one trifoliate leaf are firmly pressed into vermiculite watered with Hornum solution and covered with a transparent plastic bag for a few

days. Growth stops for a few days until a small root develops and then resumes, so it can be possible to get flowers in 1.5 months. Alternatively, we have also obtained plants from nodal sections in hydroponic cultures. A 5-6 cm long branched cutting is placed on a piece of polyurethane provided with a hole, which is floating on the Hornum nutrient solution, so the section can be submerged in the solution inside a plastic tray filled with solution and covered with a transparent tap. The cuttings quickly develop roots under these conditions, and can be transferred to peat or vermiculite afterwards.

Symbiotic partners

Both "fast-growing" *Mesorhizobium loti* and "slow-growing" *Bradyrhizobium* sp. (*Lotus*) strains can nodulate *L. japonicus*. Fast-growing strains induce nodules effective in nitrogen fixation while Bradyrhizobium sp. induce only ineffective nodules. Among the fast growing *M. loti* strains, the following are commonly used: R7A, NZP2235, JRL501 (Niwa et al., 2001; Kawaguchi et al., 2002), MAFF 303099 (complete genome sequence NC002678) (Kaneko et al., 2000; Stracke et al, 2002), and TONO (Kawaguchi et al., 2000). The fast-growing broad host range NZP2037 induces effective nodules but the frequency of nodulation is relatively low. Many of these strains are available with reporter genes such as *lacz*, *GFP* or *GUS*, in order to follow the different steps of the nodulation process, as visualisation of infection and nodule colonisation. Nodules are visible 7-10 days post-inoculation, clustered along the taproot. Three weeks after inoculation, nodules are about 1 mm in diameter. If inoculation is done with a GUS-carrying strain, extensive blue coloration in the nodule interior caused by GUS expression and infection thread structures can be visualised after staining. New rounds of nodulation can occur in the younger part of the root, so nodules at different developmental stages can be found in the same plant. The broad host range *Rhizobium* sp. NGR 234 has also been reported to nodulate *L. japonicus*, (Hussain et al., 1999). *Rhizobium etli* (Banba et al., 2001) nodulates but nitrogen fixation efficiency is poor and nodules senesce rapidly.

The major Nod-factor secreted by *M. loti* consists of a pentameric N-acetylglucosamine, which carries a cis-vaccenic acid and a carbamoyl group at the non-reducing end of the molecule and a 4-O-acetyl fucose at the reducing terminal residue (López-Lara et al., 1995, Niwa et al., 2001).

PLANT TISSUE CULTURE

Protocols for plant tissue culture and regeneration of plants were given in Handberg and Stougaard (1992). Calli can be obtained from different plant tissues, including hypocotyls, leaves, roots, and petals cultivated on B5 medium containing 2,4-D and kinetin. However, regeneration of the plant is better when done from hypocotyl explants. Shoot induction is achieved in B5 supplemented with 0.2 mg/ l BAP and 10 mM ammonium sulphate, and then 3-4 cm long shoots are transferred to soil mixture in the greenhouse for rooting using the conditions for cuttings (see chapter from Tirichine et al Ibid).

GENETIC TRANSFORMATION AND REGENERATION

Plant transformation and regeneration is a prerequisite for the molecular analysis of genes involved in nodulation and symbiotic nitrogen fixation. The molecular analysis of plant genes involved in nodulation has been hindered by the inability to produce high numbers of transgenic legume lines, since some legume plants are very recalcitrant to plant transformation and regeneration of stable transformants. Furthermore, efficient regeneration of transgenic plants is required for studies such as promoter/enhancer trapping and insertional mutagenesis (Thykjaer et al., 1995; Schauser et al., 1998; Martirani et al., 1999; Webb et al., 2000).

Species, Genotype	DNA Delivery	Explant	Selection		Citation
			Marker	Agent	
Bird's foot trefoil (*L. corniculatus* Leo)	At (LBA4404)	Leaves	*aphIV*	Hyg	Webb et al. (1996)
L. japonicus Gifu	At (LBA4404, C58C1, GV2260)	Hypocotyls	*nptII, hpt*	Kan, Hyg	Handberg and Stougaard (1992)
	Ar (9402, AR10)	Seedlings	*nptII*	Kan	Stiller et al. (1997)
	At (AGL1)	Hypocotyls	*bar*	Phosphinothricin (PPT)	Lohar et al. (2001)
	At	Root	*hpt*	Hyg	Lombari et al (2003)

Table 2. Summary of legume transformation systems yielding transformed plants. At = A. tumefaciens*; Ar* = A. rhizogenes*. (Modified from Somers et al., 2003).*

Table 2 summarises *Lotus* transformation systems. The first *L. japonicus* transformation-regeneration protocol was reported in 1992 by Handberg and Stougaard, along with the proposal to use this species as a model legume. Transformation of *L. japonicus* has been achieved by *Agrobacterium*-mediated hypocotyl transformation (Handberg and Stougaard, 1992; Thykjaer et al., 1995; Stiller et al., 1997). In these methods, selection for transgenics is accomplished using antibiotic-resistant selectable markers such as *nptII* and *hptII* genes. This method was further optimised and the time to produce whole plants reduced to about 4 months by Stiller et al. (1997). A collection of wild-type *A. rhizogenes* strains was tested for infectiveness and the most virulent strains were selected for further use. To open the possibility of searching for mutant phenotypes, a regeneration system from hairy roots cultures was developed enabling the regeneration of large numbers of transgenic plants in about 5-6 months. However, the whole regeneration process although effective is relatively expensive and time-consuming. Additionally, about 10-20% of the transgenic lines regenerated through these schemes are sterile,

probably due to somaclonally-induced abnormalities. Somaclonal variation and sterility were significantly reduced by use of the bar gene as selectable marker and selection with phosphinothricin (PPT) (Lohar et al., 2001). The bar gene encodes Phosphinothricin Acetyl Transferase that detoxifies phosphinothricin (PPT). Transgenic *L. japonicus* plants resistant to PPT were positive upon PCR by bar gene-specific primers and in most cases PPT resistance segregated as a single dominant allele indicating a single T-DNA insertion into the plant genome. All regenerated plants were fertile and void of visible somaclonal abnormalities. More recently a new transformation-regeneration procedure has been reported (Lombari et al., 2003) in which dedifferentiated root explants are a source of large numbers of cells susceptible to *Agrobacterium*-mediated transformation resulting in a tenfold increase in the number of transformants within 4 months. *L. japonicus* is easily transformed by *A. rhizogenes* and hairy roots can form nodules 3-4 weeks after emergence. Composite plants with a transgenic root system can thus be used for complementation of plant mutants and other transgene studies.

L. JAPONICUS: UNDERSTANDING SYMBIOTIC PROCESSES

Since *L. japonicus* was proposed as a model legume a set of genetic resources and tools have rapidly become available, including ecotypes, mutant lines, genetic maps, RIL lines, transformation procedures, EST sequences and sequence of the gene rich regions of the genome (VandenBosch and Stacey, 2003). Techniques such as plant tissue culture, nodulation assay, crossing and histology have already been established for *L. japonicus* research (Jiang and Gresshoff, 1993; Szczyglowski et al., 1998; Hussain et al., 1999). Now, Lotus is regarded as one of the most useful plants for legume study and researchers who have interests in nodulation and other aspects of legume biology use it worldwide. Other resources developed for use in *L. japonicus* like forward and reverse genetics, gene tagging, a high-density genetic linkage map, establishment of inbred populations for map-based cloning of genes, functional genomics projects for the completion of the genome sequence of *L. japonicus*, microarrays, proteomics, metabolomics, and exploiting microsynteny are described in individual chapters of this book.

Forward genetics in *Lotus* has been approached in several laboratories (Schauser *et al.,* 1998; Martirani et al., 1999; Webb *et al.* 2000, T. Aoki, unpublished data). T-DNA tagged lines have been obtained and the maize *Activator* transposon was reported to be mobile in *L. japonicus* and could be used as a mutagen (Thykjaer et al., 1995; Schauser *et al.* 1999). This has allowed the cloning of the *Nin* (nodule inception) nodulation gene. The molecular characterization of the *nin* mutation is considered a scientific milestone in the knowledge of nodulation, since it was the first plant gene for nodule formation to be cloned using molecular genetic methods. The *Nin* gene encodes a regulator protein that triggers the initiation of infection threads and the onset of cortical cell division (Schauser *et al.* 1999). The putative protein has a DNA binding/dimerization domain showing strong homology to Mid (Minus dominance) proteins from *Chlamydomonas reinhardtii*, which is involved in regulation of sexual reproduction in response to nitrogen starvation. *Ljcbp1* has been isolated by promoter tagging as a transgenic line showing GUS reporter activity in roots only after infection with *M. loti* or after colonisation by *G.*