大阪大学蛋白質研究所セミナー

マメ科のモデル植物ミヤコグサを用いた分子細胞生物学

平成12年11月16日(木)～17日(金)

場所：大阪大学蛋白質研究所１階講堂

吹田市山田丘３－２

【演者】

Peter Gresshoff（Queensland 大・オーストラリア）
Jens Stougaard（Aarhus 大・デンマーク）

林　誠（阪大工）／原田久也（千葉大園芸）／河内　宏（農業生物資源研）／
川口正代司（東大総合文化）／菅沼教生（愛知教育大）／
今泉（安楽）温子（農業生物資源研）／妹尾啓史（三重大生物資源）／
青木俊夫（日大生物資源）／高林純示（京大農／畑　信吾（京大生命科学）／
金子貴一（かずさDNA研）／佐伯和彦（阪大理）／南澤　究（東北大遺伝生態研）／
佐藤修正（かずさDNA研）／渡辺正夫（岩手大農）／福井希一（阪大工）／
川崎信二（農業生物資源研）／田畑哲之（かずさDNA研）

【オーガナイザー】

佐伯和彦（阪大理）／林　誠（阪大工）／中井正人（阪大蛋白研）

１１月１６日（木）

12:00-13:00 参加登録受付

13:00-13:05 永井克也（大阪大学蛋白質研究所所長）・・・・・・・・・・・・・・・・・開会の挨拶

13:05-13:25 林 誠（大阪大学工学研究科）・・・・・・・・・・はじめに・ミヤコグサ研究の概観・①

13:25-13:45 原田 久也（千葉大学園芸学部）・・マメ科作物のゲノム研究から見たミヤコグサの意義・②

13:45-14:15 河内 宏（農業生物資源研究所）・・根粒形成初期応答：*Mesorhizobium loti* JRL501株の Nod factorの構造とミヤコグサの応答について・・③

14:15-14:45 川口 正代司（東京大学総合文化研究科）・・根粒共生変異体スペクトラムと根粒形成を負に制御する原因遺伝子のクローニング・・④

14:45-14:55 コーヒーブレイク

14:55-15:15 菅沼 教生（愛知教育大学生命科学部）・ミヤコグサの窒素固定発現に関する変異体 *fix6*(*Ljsym75*)と*fix7*(*Ljsym81*)の表現型解析・・⑤

15:15-15:35 今泉（安楽）温子（農業生物資源研究所）・・*alb1*/*sym74-1*、*sym72*の表現型解析及びpositional cloningへのアプローチ・・⑥

15:35-16:00 妹尾 啓史（三重大学生物資源学部）アーバスキュラー菌根菌の共生に異常を示す変異体・⑦

16:00-16:10 コーヒーブレイク

16:10-16:55 【特別講演】Peter M. Gresshoff（Queensland 大学・オーストラリア）
Isolation of nodule and root genes using promoter-trapping in *Lotus japonicus*・・⑧

16:55-17:40 【特別講演】Jens Stougaard（Aarhus 大学・デンマーク）
Structural and functional genomics in *Lotus japonicus*・・・・・・・・・・・・・・⑨

18:00- 懇親会

１１月１７日（金）

9:30- 9:50 青木 俊夫（日本大学生物資源科学部）・ミヤコグサを用いたマメ科フラボノイドの研究・⑩

9:50-10:10 高林 純示（京都大学農学研究科）・・・・・・・・・・ハダニに対するミヤコグサおよびマメ科植物の誘導間接防御応答・・⑪

10:10-10:30 畑 信吾（京都大学生命科学研究科）・・・・・インゲン根粒菌によって形成されたミヤコグサの早期老化型根粒について・・⑫

10:30-10:40 コーヒーブレイク

10:40-11:00 金子 貴一（かずさDNA研究所）・・・・・・・・ミヤコグサ根粒菌のゲノム構造解析・⑬

11:00-11:20 佐伯 和彦（大阪大学理学研究科）・ミヤコグサ根粒菌遺伝子の系統的不活性化に向けて・⑭

11:20-11:40 南澤 究（東北大学遺伝生態研究センター）・・・・・・ミヤコグサ根粒菌の網羅的な遺伝子発現解析を目指して・・⑮

11:40-12:00 佐藤 修正（かずさDNA研究所）・・ミヤコグサゲノム研究のためのリソースの整備・・⑯

12:00-13:15 ランチ

13:15-13:35 渡辺 正夫（岩手大学農学部）・・・・・・DNAマイクロアレイを用いたミヤコグサ花器官成熟関連遺伝子の探索・・⑰

13:35-13:55 福井 希一（大阪大学工学研究科）・・・・ミヤコグサの染色体およびゲノムサイズ・・⑱

13:55-14:15 川崎 信二（農業生物資源研究所）・ミヤコグサ全ゲノムライブラリーの整列化に向けて・⑲

14:15-14:35 田畑 哲之（かずさDNA研究所）・・・・・・・・・・・・ミヤコグサゲノム解析の展望・⑳

14:35-14:50 佐伯 和彦（大阪大学理学研究科）・・・・・・・・・・・・・・・・・・・・まとめと挨拶

日本人演者発表の梗概

１１月１６日（木）

13:05-13:25 林 誠 (大阪大学工学研究科) はじめに・ミヤコグサ研究の概観:ミヤコグサに期待すること①

ミヤコグサ（*Lotus japonicus*）は近年、マメ科植物のモデルとして広くもちいられるようになってきた。ライフサイクルが２～３ヶ月と短く、ゲノムサイズが約500Mbpと比較的小さく、２倍体、自殖性、形質転換が可能なことなどがその理由としてあげられる。最近ゲノム関連の基盤が整いつつある中で、ミヤコグサの様々な変異体の単離・クローニング・機能解析ができる可能性が見えてきた。マメ科の特徴である根粒菌との共生は言うに及ばず、菌根菌との共生、病原生物との相互作用、2次代謝産物、葉や花の形態形成などの解析が期待されている。また、マメ科は双子葉植物の中で2番目に大きな科をなしており、その多様性に興味がもたれ、あるいは作物としてマメ科植物は広く利用されており、ミヤコグサを材料に研究することでマメの育種に応用できると考えられる。

13:25-13:45 原田 久也（千葉大学園芸学部）・・マメ科作物のゲノム研究から見たミヤコグサの意義・②

主要なマメ科作物はマメ科のマメ亜科に属している。マメ科作物の多くはゲノムサイズが大きかったり、形質転換が困難であるため、分子遺伝学的な研究が遅れている。ミヤコグサはマメ亜科に属し、モデル植物としての優れた性質をもっている。ミヤコグサとのゲノムシンテニーを利用してダイズのゲノム研究を進展させる計画について述べる。

13:45-14:15 河内 宏（農業生物資源研究所）・・根粒形成初期応答：*Mesorhizobium loti* JRL501株の Nod factorの構造とミヤコグサの応答について・・③

ミヤコグサ根粒形成ミュータントの表現型を解析していく上で、根粒菌Nodファクターに対する応答の解析は不可欠である。ミヤコグサ根粒菌M. loti JRL501 (MAFF303099)から単離されたNod factorの構造と、それに対するミヤコグサの応答について報告する。

14:15-14:45 川口 正代司（東京大学総合文化研究科）・・根粒共生変異体スペクトラムと根粒形成を負に制御する原因遺伝子のクローニング・・④

ミヤコグサにEMS処理を行い３２系統の根粒形成変異体を単離した。遺伝解析を通して今までに１３の遺伝子座を同定している。今回は，その中から，新規な根粒を多く着生する変異体*Ljsym77*とその原因遺伝子（マメ科植物に特徴的な転写因子）のクローニングについて紹介する。

コーヒーブレイク

14:55-15:15 菅沼 教生（愛知教育大学生命科学部）・ミヤコグサの窒素固定発現に関する変異体*fix6*(*Ljsym75*)と*fix7*(*Ljsym81*)の表現型解析・・⑤

根粒は形成されるが形成された根粒が窒素固定活性を示さない２種類のミヤコグサFix-突然変異体fix6(Ljsym75)とfix7(Ljsym81)の生育、根粒着生、窒素固定活性、形態といった共生に関わる基本的な特性を紹介する。

15:15-15:35 今泉（安楽）温子（農業生物資源研究所）・・*alb1*/*sym74-1*、*sym72*の表現型解析及びpositional cloningへのアプローチ・・⑥

マメ科植物の根粒形成過程に光を当てることを目的として単離した、３系統の共生変異体*sym70, sym72, sym74-1*/*alb1*について、ミヤコグサAFLPマーカー地図へのマッピングを行っている。*sym72*については、ポジショナルクローニングへ向けての解析経過についても報告する。

15:35-16:00 妹尾 啓史（三重大学生物資源学部）アーバスキュラー菌根菌の共生に異常を示す変異体・⑦

根粒形成・窒素固定に異常を示すミヤコグサ変異体について、アーバスキュラー菌根菌（植物の根に共生してリン酸吸収を促進する土壌糸状菌の一種）の感染状況を調べたところ、変異体のいくつかは菌根菌の感染にも異常が示され、両者の共生成立の初期に必要な遺伝子群に共有部分のある事が示唆された。

１１月１７日（金）

9:30- 9:50　青木 俊夫（日本大学生物資源科学部）・ミヤコグサを用いたマメ科フラボノイドの研究・⑩

フラボノイドは，維管束植物に分布する主要な代謝成分で，様々な機能を担っていると考えられる．縮合型タンニン／アントシアニン生合成の遺伝学的解析とマメ科固有のイソフラボノイド生合成の生理学的解析について，ミヤコグサを用いて得られた最近の成果を紹介する．

9:50-10:10　高林 純示（京都大学農学研究科）・・・・・・・・・ハダニに対するミヤコグサおよびマメ科植物の誘導間接防御応答・・⑪

害虫に食害された植物が積極的に天敵を誘引している場合がマメ科の植物などで明らかになりつつある．我々はミヤコグサに注目し，それがハダニの被害を受けたときに天敵を誘引する成分を生産するかどうかについて野生型及びミュータントを用い検討を行った．その結果について報告する．

10:10-10:30 畑 信吾（京都大学生命科学研究科）・・・・・インゲン根粒菌によって形成されたミヤコグサの早期老化型根粒について・・⑫

インゲン根粒菌（Rhizobium etli CE3）はミヤコグサに根粒を形成した。菌接種後3週目の若い根粒は、レグヘモグロビンによる薄いピンク色を呈し、アセチレン還元活性も有意に発揮した。しかし接種後4週目以降には根粒は緑に変色し、活性の低下と内部構造の著しい崩壊が観察された。すなわち、共生関係が一旦成立したあとに、防御反応が引き起こされたと思われた。

コーヒーブレイク

10:40-11:00 金子 貴一（かずさDNA研究所）　・・・・・・・・ミヤコグサ根粒菌のゲノム構造解析・⑬

ミヤコグサ根粒菌（*Mesorhizobium loti* MAFF303099）のゲノム構造解析の状況について報告する。ミヤコグサ根粒菌は３つの複製単位をもち、合計長は7.76 Mbである。このゲノムの全塩基配列解析をホールゲノムショットガン法により進めており、これまでに約78%を確定した。

11:00-11:20 佐伯 和彦（大阪大学理学研究科）・ミヤコグサ根粒菌遺伝子の系統的不活性化に向けて・⑭

Mesorhizobium loti MAFF303099のゲノム機能解析を行う一つのアプローチとして、塩基配列決定の際に作製された整列コスミド・ライブラリーを使用する、オペロン単位欠失株の作製プロジェクトについて紹介する。相同組換えに用いるコスミド派生物による相補実験とミニトランスポゾンを用いる個別遺伝子の破壊についても触れる。

11:20-11:40 南澤 究（東北大学遺伝生態研究センター）・・・・・・ミヤコグサ根粒菌の網羅的な遺伝子発現解析を目指して・・⑮

根粒菌は、(1)低栄養環境である土壌における単生生活、(2)土壌中の飢餓状態の生活から宿主植物への感染へ向かう増殖ステージ、(3) 宿主マメ科植物根粒内における共生生活（バクテロイド）の多様な生活環を持つグラム陰性の細菌である。しかし、それらの単生および共生生活とその移行の遺伝的、生理的な背景は断片的にしか分かっていない。そこで、このような観点からα-*Proteobacteria*にする*Mesorhizobium loti*の全レプリコンを、カズサDNA研究所のゲノム解析の際に作られたM13クローンからカバーするマクロアレーの作製を行い、上記の各種条件下におけるミヤコグサ根粒菌の網羅的な遺伝子発現解析を関連研究者のコンソーシアム（東北大学遺伝生態研究センター、 鹿児島大学理学部、東京農工大学、 帯広畜産大学）で行っている。

11:40-12:00 佐藤 修正（かずさDNA研究所）　・・ミヤコグサゲノム研究のためのリソースの整備・・⑯

ミヤコグサのゲノム研究を進める上で基盤となるリソースの整備を進めている。そのうち包括的な遺伝子情報を提供し、またアレイによる発現解析の材料にも用いられるcDNAクローンの大量解析と、クローニング断片を植物に導入することが可能なTACベクターを用いたゲノムライブラリーの作製について紹介する。

ランチ

13:15-13:35 渡辺　正夫（岩手大学農学部）・・・・・・DNAマイクロアレイを用いた
ミヤコグサ花器官成熟関連遺伝子の探索・・⑰

ミヤコグサの花器官、特に生殖器官特異的遺伝子の網羅的解析を目的として、EST解析、DNAマイクロアレイによる発現解析を行った。他植物の花粉特異的遺伝子と相同性を示したクローンは、DNAマイクロアレイによる発現解析でも葯で特異的にシグナルが検出された。一方、EST解析による相同性からは機能の推定できない新規な葯特異的遺伝子も数多く単離された。

13:35-13:55 福井 希一（大阪大学工学研究科）　・・・・ミヤコグサの染色体およびゲノムサイズ・・⑱

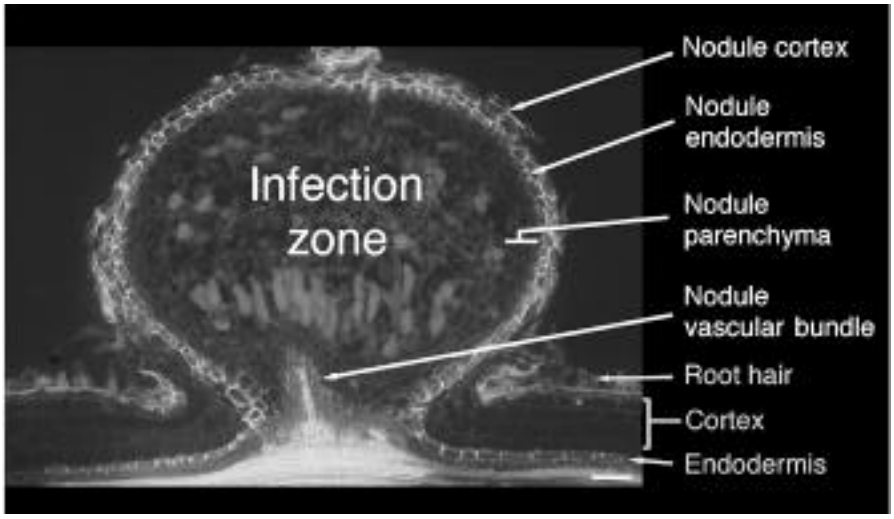
ミヤコグサのゲノム解析の基盤となるゲノムサイズの決定と定量的染色体地図の作成を行った。その結果、ミヤコグサの２つのアクセッション、ミヤコジマとギフ間でゲノムサイズに差があり、染色体の核型においても違いが認められることが明らかになった。その原因として染色体上の反復配列塊が考えられた。

13:55-14:15 川崎 信二（農業生物資源研究所）・ミヤコグサ全ゲノムライブラリーの整列化に向けて・⑲

モデル植物としてのミヤコグサを活用して根粒形成過程を分子遺伝学的に分析するに当たり、必要な道具としての高能率ゲノム走査法と大容量BACライブラリー（平均インサート140kb）の構築を行った。今後、ゲノムライブラリーをマップと統合しつつ全ゲノムcontigの構築を目指す。

14:15-14:35 田畑 哲之（かずさDNA研究所）　・・・・・・・・・・・ミヤコグサゲノム解析の展望・⑳

マメ科植物のもつ遺伝情報を明らかにするため、ミヤコグサゲノムの大規模塩基配列決定プロジェクトを開始した。得られたゲノムの塩基配列は、その位置情報やcDNA情報と共に、ミヤコグサの遺伝解析を加速することが期待される。



What you can do with *Lotus japonicus*

Makoto Hayashi
Department of Biotechnology, Graduate School of Engineering, Osaka University

Lotus japonicus is one of the model plants. It has a short life cycle (2 to 3 months), relatively small genome (about 500Mbp), and features as diploidy, autogamy and easy transformation. These are the fundamental aspects when dealing with molecular genetics. Because of recent advance on genome projects of *L. japonicus* and its symbiont *Mesorhizobium loti*, we are now able to isolate and characterize various kinds of mutants such as those of nodulation, mycorrhiza interaction, pathogen interaction, isoflavonoid production, leaf and flower development, etc. Diversity of legumes (the family is the second biggest in dicots) and its use as crops indicate the application of *L. japonicus* studies to all legume plants.

Significant impact of *Lotus japonicus* on the genome analyses of leguminous crops.

Kyuya Harada (Faculty Horticulture, Chiba University)

The Leguminosae is the third largest family in angiosperms comprising three subfamilies, Caesalpinioideae, Mimosoideae and Papilionoideae. Most of leguminous crops are included in subfamily Papilionoideae. Leguminous crops have special features including nitrogen fixing symbiosis and accumulation of a large quantity of protein and/or lipid in their seeds. Leguminous seeds supply lysine which is deficient in gramineous seeds and combination of pulse and cereal is very important for food and feed. To understand the mechanisms of special physiological functions and increase the productivity of leguminous crops, it is necessary to identify and isolate the responsible genes for many traits, such as, flowering and seed development, nitrogen fixing symbiosis and nitrogen metabolism, defense against pests and pathogens and tolerance to cold and water stresses. But molecular genetic analyses of leguminous crops are hampered by larger genome size, low transformation frequencies or both.

Lotus japonicus is also included in Papilionoideae and has many appropriate features as a model legume, that is, diploidy, self fertility, small genome size short life cycle, high transfomability. High degree of macro and microsynteny could be expected between *L. japonicus* and many leguminous crops.

We are developing, as a first step, molecular genetic map of *L. japonicus* based on 127 F2 plants derived from a single cross between Gifu B-129 and Miyakojima MG-20-S7 using high efficiency genome scanning(Kawasaki *et al*. PAG8 P482). This map at present comprises 202 markers including 197 AFLP markers and consists of 6 major linkage groups that may correspond to 6 pairs of *L. japonicus* chromosomes covering 791.0cM of the genome in the Kosambi function. We will analyze genome synteny between *L. japonicus* and *Glycine max*, the most important leguminous crop in Japan, using cDNA markers.

Structures of Nod factors of *Mesorhizobium loti* JRL501(MAFF303099) and their biological activities

Kouchi, H.[1)], Niwa, S.[2)], Imaizumi-Anraku, H.[1)], Ikuta, A.[2)], Ishizaka, M.[3)] and Kawaguchi, M.[4)]

1) National Institute of Agrobiological Resources, Tsukuba; 2) Science University of Tokyo, Noda; 3) National Institute of Agroenvironmental Sciences, Tsukuba; 4) The University of Tokyo, Tokyo 163-8902

Analysis of the responses of *Lotus japonicus* to purified Nod factors (lipo chitin oligosaccharides, LCOs) is indispensable to characterize the phenotypes of the nodulation mutants isolated so far. We analyzed LCOs produced by *M. loti* JRL501 (a derivative of MAFF303099, a true symbiont of *L. japonicus*) by transferring pMp2112 containing *nodD* from *R. leguminosarum* bv. *trifolii* followed by induction of *nod* genes by naringenin. The HPLC profile indicated two major naringenin-inducible peaks. Collision induced dissociation tandem mass spectrometry and HPLC-mass spectrometry revealed that NodMlo-V(C18:1, Me, Cb, AcFuc) as previously identified as a major LCO molecule in *M. loti* E1R (Lopez-Lara et al. 1995) was present as a major component of the second peak. Additional novel structures were identified in the first peak bearing fucose instead of acetylfucose at the reducing end.

The responses of *L. japonicus* B-129 “Gifu” upon application of LCOs purified from *M. loti* JRL501 are analyzed. The mixture of LCOs from the two HPLC peaks induced abundant distortion, tip swelling and branching of root hairs within 6-12 hours at 10^{-7} to 10^{-9}M. Spot inoculation of 10-30 ng LCOs induced the formation of nodule primordia within 3 to 6 days, in which early nodulin genes, *ENOD40* and *ENOD2* are induced with the same spatial expression patterns as in nodules formed by the infection with *M.loti*. These responses of *L. japonicus* are essentially comparable to but occur at much higher frequencies than the case of *Glycine soja* described previously (Minami et al. 1996). In addition, a pre-infection thread (cytoplasmic bridge) is formed in the outermost cortical cell of *L. japonicus* in response to purified LCOs alone. This may be a unique feature of *L. japonicus* among legume plants because the peculiar swelling of the outermost cortical cells at the inoculation site and induction of preinfection thread formation with LCOs alone has been observed in legumes bearing indeterminate nodules but not in those bearing determinate nodules to date.

References

Imaizumi-Anraku, H. et al. (1997). *Plant Cell Physiol*. **38**: 871-881.

Lopez Lara, I.M. et al. (1995) *Mol. Microbiol.* **15**: 627-638.

Minami, E. et al. (1996) *Plant J.* **10**: 23-32.

Spectrum of mutations affecting nodule number and organogenesis in *Lotus japonicus* and molecular cloning of *LjSym77* gene acting as the negative regulator.

Masayoshi Kawaguchi[1,7], Reiko Nishimura[1], Masayuki Ohmori[1], Haruko Imaizumi-Anraku[1,6], Hiroyuki Koiwa[2], Sinobu Niwa[3], Akira Ikuta[4], Kunihiko Syono[5] and Shoichiro Akao[6].

[1]Dept. of Life Sci., Univ. of Tokyo; [2]Iwate Biotechnology Research Center; [3]Dept. Biol. Sci. Tech., [4]Res. Inst. Sci. Tech., Sci. Univ. of Tokyo; [5]Fac. Sci., Nihon Women's Univ; [6]National Inst. of Agrobiol. Res.; [7]PRESTO, JST.

In order to get an overview of plant factors controlling nodule number and organogenesis, an extensive screening was performed using a model legume, *Lotus japonicus*. Totally 40,000 M2 seeds of *L. japonicus* 'Gifu' B-129 by treatment of ethylmethan sulfonate (EMS) were sown under nitrogen-deficient condition either in vermiculite or on agar plates. These screening succeeded in the isolation of 32 mutant lines. These mutants were largely divided into two categories, symbiotic mutants affecting nodule organogenesis (27 mutant lines) and pleiotropic mutants affecting the number of an effective nodule (5 mutant lines). Using the half mutant lines 13 loci affecting the number and organogenesis were identified. Among them a novel mutant, *Ljsym77*, will be reported. The *sym77* mutant is rather intriguing in that it not only fails to control nodulation, but also is deficient in stimulus responses. Nodules of *sym77* mutant were two to three times higher than those of wild type. When grown on an agar plate in the absence of bacteria, the hypocotyls and shoots of *sym77* mutant were longer than those of wild type. In addition the direction of lateral root growth was nearly horizontal. Since these traits except nodulation are very similar to those in *hy5* mutant in *Arabidopsis*, we hypothesized that the *sym77* phenotype is due to a mutation in *HY5* ortholog of *L. japonicus*.

A full length of cDNA showing high similarity with *HY5* gene was isolated from *L. japonicus* and named *LjBZF*. The majority of the identical residues in bZIP motif and the casein kinase II phosphorylation site, which are characteristic to *HY5*, were well conserved in *LjBZF*. Interestingly, *LjBZF* included zinc finger motif and acidic region, which are absent from *HY5*. In addition *LjBZF* showed high similarity with *STF* in *Glycine max* and CGATG-motif binding protein in *Vicia faba* over their entire length.

In order to examine whether nucleotide polymorphism of *LjBZF* cosegregates with mutant phenotype, genomic regions of *LjBZF* were sequenced in Gifu and Miyakojima accessions. A single nucleotide polymorphism (SNP) was found in the forth intron. The linkage was examined by dCAPS method using this SNP in 40 plants of the F2 generation [*sym77* (Gifu) x Miyakojima MG-20]. All mutants cosegregated with a nucleotide sequence of Gifu.

Sequencing of genomic region of *LjBZF* in *sym77* revealed the single substitution in the splicing acceptor site of the second intron. This result suggested that the substitution caused the aberrant RNA processing in *sym77* mutant. We evaluated *LjBZF* transcript by RT-PCR. The greater length of the transcript, 0.91kb, was observed in the *sym77* mutant in the contrast with the 0.84 kb transcript in wild type. Based on these mutant analyses, we will focus on the significance of *L. japonicus* as a model plant in elucidating the gene function conspicuous with legumes.

Phenotypic characterization of *Lotus japonicus* mutants, fix6 (*Ljsym75*) and fix7 (*Ljsym81*), that form ineffective nodules

Norio Suganuma[1], Futoshi Iwamatsu[1], Mamiko Yamamoto[1], Shoichiro Akao[2] and Masayoshi Kawaguchi[3]

[1] Department of Life Science, Aichi University of Education, Kariya, Aichi 448-8542
[2] National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305-8602
[3] Department of Life Sciences, Graduate School of Arts and Science, University of Tokyo, Komada, Tokyo 153-8902

Legume-*Rhizobium* symbiosis is controlled by genes of both partners. Plant mutants that form ineffective nodules (Fix$^-$ phenotype) are useful to study roles of host plants for establishing symbiotic nitrogen fixation. Dozens of Fix$^-$ mutants have been isolated from several legume plants. However, none of the gene defective in those mutants have been elucidated. *Lotus japonicus* has a small genome size, a short generation time, large self-fertile flowers and a ability to be transformed by *Agrobacterium* infection, proposing a model legume. Therefore, *L. japonicus* Fix$^-$ mutants have advantages compared with those of other legume species for characterization of genes controlling symbiotic nitrogen fixation. In this study, basic symbiotic characteristics of two Fix$^-$ mutants of *L. japonicus*, fix6 (*Ljsym75*) and fix7 (*Ljsym81*), were investigated.

Both mutants formed nodules by inoculation with *Mesorhizobium loti* under nitrogen-free conditions, but growth of plants were retarded and showed symptoms of nitrogen deficiency. Application with combined nitrogen recovered the growth of both plants, indicating that genes defective in both mutants concern symbiotic nitrogen fixation. The three strain of *M. loti*, JRL507, TONO and NZP2235, all induced formation of nodules and retardation of plant growth. Both nodules formed on fix6 and fix7 were smaller than those on the parent Gifu and colored white or pale pink. Acetylene reduction activity of nodules induced on fix6 mutant was not detectable, but that on fix7 was detected as much as about 15% of that on Gifu. The number of nodules formed on fix7 was almost similar to that on Gifu. However, fix6 formed about two times higher numbers of nodules. When those phenotypes were examined during plant development, acetylene reduction activities of nodules on fix6 were not detectable throughout plant development and higher numbers of nodules were formed from the early stages of plant development. Acetylene reduction activities of nodules on fix7 were always lower than those on Gifu, but fluctuated in a similar way to those on Gifu during plant development. Light microscopy showed that infected cells were observed in nodules induced on both mutants and their structures were similar to that on Gifu. This indicates that rhizobia invades normally into nodules cells of both mutants.

From these results, it is suggested that *LjSym75* and *LjSym81* are involved in the later stages of nodule development. The nodules on fix7 were structurally normal and exhibited lower nitrogenase activity, indicating that *LjSym81* has an effect on activity of nitrogenase like *LjSym76*, *Pisum sativum Nof1* and *Medicago sativa* By contrast, *LjSym75* may be a novel type of symbiotic gene controlling both nitrogenase activity and number of nodules. Biochemical analyses, such as protein composition, enzymatic activity and nodulin gene expression are intended for more detailed characterization of *LjSym75* and *LjSym81*.

Symbiotic mutants *sym70*, *sym72* and *sym74-1/alb1*; phenotype characterization and progress toward positional cloning

Haruko IMAIZUMI-ANRAKU [1,2], Masayoshi KAWAGUCHI[1], Keishi SENOO[3], Kyuya HARADA [4], Syoichiro AKAO[2] and Shinji KAWASAKI[2]

1. Dept. of Life Science, Univ. of Tokyo, Komaba, 2. Dept. of Physiology, NIAR Tsukuba, 3. Faculty of Bioresources, Mie Univ., Tsu, 4. Dept. of Horticulture, Chiba Univ., Matsudo.

For the molecular genetic analysis of nodulation process, our group have developed a mutant library by EMS-mutagenetis of *Lotus japonicus*. Among them, 3 mutant lines were selected and their phenotypes and map positions of the causative genes were analyzed. Genetic analysis indicated that these mutants were all due to single, recessive mutations at three different loci, respectively. *sym70* shows no response to *Rhizobium* infection and Nod factor treatment, indicating that the mutated gene is responsible for Nod factor signal transduction (Kawaguchi et al. in preparation). *sym72* was isolated as a non- nodulating mutant, but this line rejects also mycorrhizal colonization (Senno et al, 2000). *sym74-1/alb1* forms mainly empty nodules, in which the bacteria remain in abnormally enlarged infection threads, and are not released to the host plant cells. In addition to the developmental arrest of infection thread, *alb1* was also found to be defective in differentiation of ramified nodule vascular bundles (Imaizumi-Anraku et al. 1997 and 2000).

F_2 analysis was done to map these genes with populations derived from crosses between these mutant lines (Gifu; B-129) and the wild type of Miyakojima (MG-20), using the HEGS (High efficiency genome scanning) system. As a reference, the HEGS map of *Lotus japonicus* (Harada et al. in preparation) was used. The *Ljsym70* and *Ljsym74-1/alb1* were mapped on the linkage group 2. The fine mapping of *Ljsym72*, as a step for positional cloning, is in progress. Results of these ongoing experiments will be presented and discussed.

(References)

Senoo *et al*. *Plant Cell Physiol*. 41: 726-732 (2000)

Imaizumi-Anraku *et al*. *Plant Cell Physiol.* 38: 871-881 (1997)

Imaizumi-Anraku *et al*. *Mol. Gen. Genet*. (*in press*)

Characterization of mycorrhizal mutants in *Lotus japonicus*

Keishi Senoo
Faculty of Bioresources, Mie University, Tsu, Mie 514-8507, Japan
senoo@bio.mie-u.ac.jp

Arbuscular mycorrhizal (AM) fungi belong to the Zygomycetes and are capable of making a non-specific relationship with roots of more than 80% of terrestrial plant species. Although extensive research efforts has been made, current knowledge on the molecular and genetic aspects of the development of the AM symbiosis is very limited.

We use a model legume *Lotus japonicus* to screen mycorrhizal mutants and ultimately isolate plant genes of functional relevance in AM development. Mycorrhizal mutants of *Lotus japonicus* were screened from a collection of 45 mutant lines showing non-nodulating (Nod^-), ineffectively nodulating (Fix^-) and hypernodulating (Nod^{++}) phenotypes induced by EMS-mutagenesis. To infect *L. japonicus* roots, six seedlings were planted per nursery tray containing equal volume of autoclaved sand and Andosol subsoil. Spores of a commercial inoculum *Glomus* sp. R-10 were used. Plants were cultivated for two months in a growth chamber.

We have screened 13 mycorrhizal mutants. Among them, 11 mutants are defective in colonization and all of them were derived from Nod^- phenotypes. These mutants have significantly reduced mycorrhizal root colonization compared with wild-type parent. We determined percentage of root colonized by arbuscules, vesicles, internal hyphae, external hyphae and appressoria of these defective mutants, and grouped them into 4 categories based on microscopic observations: (i) mycorrhizal colonization blocked in epidermis (*mcbep*); (ii) mycorrhizal colonization blocked between epidermis and exodermis (*mcbee*); (iii) mycorrhizal colonization blocked in exodermis (*mcbex*); and (iv) mycorrhizal colonization blocked at cortex (*mcbco*).

In *mcbep* mutant lines, root colonization by *Glomus* sp. is characterized by poorly developed external hyphae on root surface and completely no fungal structures found inside the root. Appressoria formed on root surface were extremely abnormal in shape, showing extraordinary swelling and branching. The mutants in *mcbee* showed similar phenotype to those in *mcbep*, except that hyphae penetrated to the intercellular space between epidermis and exodermis and arrested there. Unique characteristics in mutant lines *mcbex* were overproduction of deformed appressoria with arrested hyphal penetration within exodermis. Small amounts of internal colonization including degenerated arbuscule formation were occurred infrequently in these types of mutant. Not only fungal development on the root surface but also that in root exodermis and cortex was affected by the mutation. The final category of mutants in *mcbco* have reduced colonization blocked at cortex and are characterized by early senescent of arbuscules, with or without vesicle formation but have considerable growth of internal and external hyphae.

Two hypermycorrhizal mutants were obtained from Nod^{++} mutants. These mutants produced large number of arbuscules compared with wild-type (Arb^{++}). The majority of individual arbuscule was active in terms of succinate dehydrogenase activity, and had well-developed and seemingly tough in morphology.

Five of Nod^- mutants used in this study were found to have the phenotype that the early steps of nodule formation prior to nodule primordia formation were blocked. Among them, three showed defective mycorrhizal phenotype but the other two showed normal mycorrhization. Nod^{++} mutants showed Arb^{++} phenotype. These results suggest the existence of both shared and unshared genetic systems between early steps of nodule formation and those of mycorrhizal colonization.

Isolation of nodule and root genes using promoter- trapping in *Lotus japonicus*

Peter M. Gresshoff[a], Artem E. Men[a], Tina Maguire[a], Sean Grimmond[b], Dasharath Lohar[d], Sandra Ayanru[a], Khalid Meksem[c], David Lightfoot[c] and Jiri Stiller[a]

[a]Department of Botany; [b]Institute of Molecular Biosciences, The University of Queensland, Brisbane St. Lucia, QLD 4072, Australia; [c]Department of Plant, Soil, and Agronomy, Southern Illinois University, Carbondale, IL, USA; [d]Center for Legume Research, University of Tennessee, Knoxville, TN, USA

Abstract

Recent advances of high throughput DNA sequencing, bioinformatics, robotics, BAC libraries, microarrays, insertional mutagenesis as well as promoter trapping open the opportunity for an integrated function and structure analysis of the genomes of soybean and the model legume ***Lotus japonicus***. We are specifically interested in the plant's role during the establishment of nodule morphogenesis, and the genes shared during seemingly related developmental programs leading either to nodule or lateral root formation. Additionally our research seeks to elucidate plant genetic controls over plant–microbe interactions and symbiotic signaling of both ***Rhizobium*** and mycorrhizal symbioses. The challenge is to understand the plant's genetic contribution to this symbiosis with the aim to improve natural associations of benefit for agriculture and the environment. Plant mutations were induced using EMS, fast neutron deletion as well as insertional mutagenesis. Single recessive loci were mapped using molecular markers, which were used to isolate soybean BAC clones to generate contigs spanning mutant deletions. Special emphasis was given to the ***Nts-1*** locus of soybean that governs autoregulation of nodulation. If mutated, this locus leads to abundant nodulation (supernodulation) as well as nitrate tolerance in nodulation. Expression analysis of nodulation events using 4,200 micro-arrayed root ESTs was initiated to detect gene products temporarily expressed during early nodulation. ***Lotus japonicus*** as compared to soybean facilitates high throughput insertional mutagenesis and promoter trapping. Insertion of a promoter-less ***gus***-reporter gene allowed the isolation of activated plant lines that showed development specific ***gus***-gene expression. Isolation of flanking DNA sequences provided information of potential promoters and gene function as well as providing a link between structural and functional elements of nodulation-related genes. Evidence suggests that many nodule initiation functions evolved or are shared with lateral root related processes. The possibility exists that several non-legumes share such genes.

Jiang Q, Gresshoff PM (1997) Classical and molecular genetics of the model legume *Lotus japonicus*. Mol. Plant-Microbe Interact**. 10**: 59-68.

Lohar D, Schuller K, Gresshoff PM, Stiller J (2001) Transformation of *Lotus japonicus* by the *bar* gene as a selectable marker. J. Exp. Bot. (submitted).

Martirani L, Stiller J, Mirabella R, Alfano, Lamberti A, Radutoiu S, Iaccarino M, Gresshoff PM,, Chiurazzi M (1999) T-DNA tagging of nodulation- and root–related genes *in Lotus japonicus* expression patterns and potential for promoter trapping and insertional mutagenesis. Mol. Plant-Microbe Interact. **12**: 275-284.

Men A, Meksem K, Kassem MA, Lohar D, Stiller J, Lightfoot D, Gresshoff PM (2000) A bacterial artificial chromosome (BAC) library of *Lotus japonicus* constructed in an *A. tumefaciens* – transformable vector. Mol. Plant-Microbe Interact. (in press).

Stiller J, Martirani M, Tuppale S, Chian R, Chiurazzi M, Gresshoff PM (1997) High frequency transformation and regeneration of transgenic plants in the model legume *Lotus japonicus*. J Exp Botany **48**: 1357-1365.

Wopereis J, Pajuelo E, Dazzo FB, Jiang Q, Gresshoff PM, de Bruijn FJ, Stougaard J, Szczyglowski K (2000) Short root mutant of *Lotus japonicus* with a dramatically altered symbiotic phenotype. Plant Journal **23**: 97-114.

STRUCTURAL AND FUNCTIONAL GENOMICS IN *LOTUS JAPONICUS*

Jens Stougaard, José Antonio Herrera-Cervera, Maria Josefa Lorite-Ortega,
Lene Krusell, Niels Sandal, Lene Heegaard Madsen.

Laboratory of Gene Expression, Department of Molecular and Structural Biology,
University of Aarhus, Gustav Wieds Vej 10, 8000 Aarhus C, Denmark

Following the emergence of model legumes the molecular genetic analysis of symbiosis is now rapidly developing. In *Lotus japonicus* a genetic map is constructed to allow map based cloning of mutations arising from chemical mutagenesis procedures. AFLP and RFLP markers are mapped in a F2 mapping population from a cross between *Lotus japonicus* and *Lotus filicaulis*. The resulting linkage groups are correlated to chromosomes by *in situ* hybridization on metaphase chromosomes. Different methods for positional cloning of symbiotic loci is currently applied in order to clone genes involved in nodule initiation, nodule function, autoregulation as well as mycorrhization. Attempts towards map based cloning of the *Ljsym16* gene will be presented to exemplify these approaches.
Several novel methods for genetic analysis of gene function have been established in eukaryotes and a subset of these can be applied to investigate the function of plant genes. Insertion mutagenesis with T-DNA and transposable elements have for example been used in *Lotus japonicus*. A summary of the transposon insertion approaches and the future perspectives will be given. The cloning and characterization of the first transposon tagged symbiotic locus (*Nin*) will be presented together with the first tagged flower mutant. The *n*odule *in*ception (*nin)* mutants did not develop nodules after inoculation with *Mesorhizobium loti*. Genetically, the *nin* allele segregates monogenic recessive and due to the continued activity of the autonomous *Ac* tag, both stable mutants and stable revertants were identified. The excision footprints detected in this allelic series confirmed that the mutation was caused by *Ac* insertion. A gene inventory for *L. japonicus* is accumulated from EST sequencing programs and functional analysis of these genes is now on the priority list. Approaches for converting this structural information into functional genomics will be discussed.

Perspective of molecular genetic studies of leguminous flavonoids with *Lotus japonicus*

Toshio Aoki, Department of Applied Biological Sciences, Nihon University, Fujisawa, 252-8510 Japan, taoki@brs.nihon-u.ac.jp

Flavonoids are common to most vascular plants and are involved in a number of plant functions. Leguminous flavonoids are intriguing subjects for several reasons: 1) They have specific chemical structures; 2) They are postulated to play important roles both as defensive compounds against pathogenic microorganisms and as chemical signals in symbiotic nitrogen fixation; 3) They have striking biological activity in humans and animals as constituents of food and forage. This presentation will introduce our recent achievements in genetic analysis of anthocyanin/condensed tannin biosynthesis and the characterization of cytochrome P450 monooxygenases (P450s) in legume-specific flavonoid biosynthesis.

Artificial and spontaneous mutants with lowered anthocyanin accumulation in the stem were obtained by ethylmethane sulfonate mutagenesis and the collection of wild grown variants, respectively. Genetic and chemical analysis identified several loci involved in anthocyanin accumulation, including two loci which are also essential for condensed tannin production. These mutants will provide good tools for genetic and molecular studies of the biological roles and the biosynthetic regulation of leguminous flavonoids.

A PCR-based strategy using degenerate primers yielded cDNAs encoding two cytochrome P450s (CYP81E6 and CYP93C17) from two-day old *L. japonicus* seedlings treated with reduced glutathione as an elicitor. Microsome fractions of yeast cells transformed with these cDNAs showed isoflavonoid 2'-hydroxylase (I2'H) and 2-hydroxyisoflavanone synthase (IFS) activity, respectively. Both P450s are involved in the biosynthesis of an isoflavan phytoalexin, vestitol. Gel blot analysis of genomic DNA revealed that IFS is encoded by a small number of genes, while a single copy of the I2'H gene was present in *L. japonicus*.

Induced indirect defense responses of bean plants against herbivorous mites

Junji Takabayashi (1), Rika Ozawa (1,2), Takeshi Shimoda (1,2), Gen-ichro Arimura (1,2), Jun-ichro Horiuchi (3) and Takaaki Nishioka (3)

(1) Laboratory of Ecological Information, Graduate School of Agriculture, Kyoto University, (2) Bio-oriented Technology Research Advancement Institution, (3) Laboratory ofInsect Physiology, Graduate School of Agriculture, Kyoto University

Abstract

It is known that plants can defend themselves against herbivores indirectly by emitting volatiles that attract carnivorous natural enemies of herbivores. One of the well-documented systems on the above interaction consists of Lima bean plants, two-spotted spider mites Tetranychus urticae and predatory mites Phytoseiulus persimilis. The predatory mites are attracted to volatile infochemicals emitted by plants infested by spider mites. In these studies, a Y-tube olfactometer was used to detect the olfactory responses of predatory mites to HIPV. To clarify whether or not Lotus japonicus show such indirect defense against herbivorous mites T. urticae, we investigated the responses of predatory mites P. persimilis to volatiles from L. japonicus infested by T. urticae in Y-tube olfactometer. The plants infested by the spider mites attracted more P. persimilis than uninfested plants. When L. japonicus infested by the spider mites, the plants started emitting (Z)-3-hexenyl acetate, (E)-4,8-dimethyl-1,3,7-nonatriene, germacrene d and methyl salicylate (MeSA). These compounds were considered to be T. urticae-induced plant volatiles. Among these compounds, (E)-dimethyl-1,3,7-nonatriene and MeSA but (Z)-3-hexenyl acetate are known to attract P. persimilis. When three L. japonicus mutants on nodule organogenesis were infested by the spider mites, they all attracted P. persimilis. However, two of the infested mutants emitted qualitatively different blend of induced volatiles when compared with the blends of the induced volatiles emitted from the wild type. Our finding demonstrated the feasibility of molecular genetic approaches to the mechanisms that underlie the production of specific volatile compounds in response to herbivores.

Lotus japonicus forms early senescent root nodules with *Rhizobium etli*

Shingo Hata

Laboratory of Plant Physiology, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

(ABSTRACT)
Mesorhizobium loti and *Rhizobium etli* are microsymbionts of *Lotus* and *Phaseolus* species, respectively, that secrete essentially the same Nod factors. *Lotus japonicus* formed root nodules efficiently with *R. etli* CE3 irrespective of the presence or absence of an flavonoid-independent transcription activator *nodD* gene. On a nitrogen-free medium, however, the host plant inoculated with *R. etli* showed a severe nitrogen deficiency symptom. The nodules formed with *R. etli* were initially pale-pink, leghemoglobin mRNA being detectable at significant levels. Nevertheless, they became greenish with time. Acetylene reduction activity of nodules formed with *R. etli* was comparable to that formed with *M. loti* three weeks after inoculation, but thereafter it decreased rapidly. The nodules formed with *R. etli* contained much more starch granules than those formed with M. loti. *R. etli* developed into bacteroids in the *Lotus* nodules, although the density of bacteroids in the infected cells was lower than that in the nodules formed with *M. loti*. The nodules formed with *R. etli* were of the early senescence type; membrane structures were drastically disintegrated in the infected cells of the greenish nodules. Thus, *L. japonicus* started and then ceased a symbiotic relationship with *R. etli* at the final stage.

Genome sequence analysis of *Mesorhizobium loti.*

Takakazu Kaneko, Yasukazu Nakamura, Shusei Sato and Satoshi Tabata
(Kazusa DNA Research Institute)

In order to understand the genetic control mechanisms involved in the symbiotic nitrogen fixation, we have initiated genome sequencing of *Mesorhizobium loti* MAFF303099, a symbiont of *Lotus japonicus*.

The genome of *M. loti* MAFF303099 consists of three circular replicons: a chromosome of 7.2 Mb and two plasmids of 360 Kb and 200 Kb long. An average GC content of the genome was estimated to be 61.7%. The nucleotide sequence of the genome was determined by the whole genome shotgun method. The random sequences were accumulated until seven folds of the genome size were covered, which were assembled into 370 sequence contigs after assembly. These contigs were then assigned to the genome in order, and the genome size was estimated to be 7,589 Kb. As of Oct. 31 2000, most of the sequence gaps were filled, and the sequence editing is under way (78% completed).

To assign the potential protein coding regions on a part of the completed sequence (1,970 Kb), similarity search against the “nr” database and gene prediction by the glimmer-program were performed preliminarily. As the result, a total of 2,314 potential protein-coding genes were assigned on the sequence. The average size of the potential genes was 871 bp long. The gene density was 1 gene per 1.2 Kb, which was similar to many of the other full-sequenced bacterial genomes.

In this presentation, the latest status of the sequencing and gene prediction will be discussed.

Towards systematic inactivation of total operons in *Mesorhizobium loti*

Kazuhiko Saeki
Department of Biology, Graduate School of Science, Osaka University, Toyonaka 560-0043

During the past decade, great progress has been made in understanding the structural organization, function and regulation of *nod* and other symbiotic genes mainly using three representative rhizobium bacteria, *Sinorhizobium meliloti*, *Rhizobium leguminosarum* bv *viceae*, and *Bradyrhizobium japonicum*. Compared to these rhizobia, however, the *Lotus* symbiont *Mesorhizobium loti* is far less well studied despite the recent attention given to its host *Lotus japonicus*. The information lack is more severe on the true symbiont of *L. japonicus*, since most studies have been performed with *M. loti* strains isolated from *Lotus corniculatus.* These rhizobial-side disadvantages of the *Lotus-Mesorhizobium* model should be overcome by systematic molecular genetic study of the of *M. loti,* as supported with structural information on the whole genome of strain MAFF303099 which is the only established true symbiont of the host plant. We are working on two approaches to make systematic operon-level knock out/deletion of the *M. loti* MAFF303099 genome.

One approach is based on conventional homologous recombination between disrupted copy on plasmid and wild-type copy on the chromosome. It is at the validation stage and designed to utilize the ordered cosmid library constructed with pKS800 (a derivative of the pLAFR1 vector) by the genome sequencing project at Kazusa DNA Institute. Briefly, a gentamycin (or chloramphenicol) resistance gene cassette is inserted to a particular gene (or replace an entire operon) in a selected cosmid clone, the resulting cosmid derivative is then conjugatively transferred into MAFF303099 and then recombined mutant is screened with a helper plasmid incompatible to pKS800. Obtained mutant can be subjected to systematic complementation analysis on symbiotic and other phenotype with ordered deletion or mini transposon insertion derivatives of the cosmid used.

The other approach is currently under development to inactivate individual genes or to delete relatively large genomic region by a combination of ‘plasposon’ technology (Dennis & Zylstra 1998 *Appl. Env. Microbiol.* 64:2710-2715) and yeast, *Zygosaccharomyces rouxii*, recombinase specific for a 58-bp AT-rich sequence (Araki *et al.* 1992 *J. Mol. Biol.* 225:25-37). Technical outlines and overall rationale will be presented to be criticized for improvement.

Towards global analysis of gene expression in *Mesorhizobium loti*

Kiwamu MINAMISAWA[1], Toshiki UCHIUMI[2], Tadashi YOKOYAMA[3], and Takuji OOWADA[4]

1 Institute of Genetic Ecology, Tohoku University, 2 Kagoshima University, 3 Tokyo University of Agriculture and Technology, 4 Obihiro University

Recent progress of microbial genomics enables us to understand globally environmental adaptations and plant-microbe interactions of symbiotic bacteria. The establishment of nodules during the *Rhizobium*-legume symbiosis requires the induction of new developmental programs within each partner. A number of rhizobial genes that has been found for the establishment of functional nodules have been identified as *nod*, *nif* and *fix* genes by traditional analyses. Although other housekeeping genes in rhizobia appear to take part in infection in the rhizosphere and bacteroid development in the plant roots, their knowledge is fragmentary. Bacterial populations including rhizobia in soil are frequently exposed to stress due to limitations and change in nutrient availability. The existence and nature of a viable but nonculturable (VBNC) state of bacteria under starvation conditions has been the topic of intense debate for over 20 years in microbial ecology. Indeed, *Mesorhizobium loti* fell into a VBNC state under nutrition starvation. When the stressed rhizobia in soil gain access to the rhizosphere of host plants, it is possible that their proliferation and signal perception changes drastically.

To address the above questions, we aimed to analyze global gene expression of *Mesorhizobium loti* MAFF303099 under nutrient-starvation, rhizosphere and bacteroid conditions. We have selected 3832 clones of M13 phage that covers three replicons (chromosome, 7.2 Mb; plasmids, 0.36 and 0.20 Mb) of *Mesorhizobium loti* MAFF303099, and are preparing a macroarray system.

Development of the resources for genome analysis of *Lotus japonicus*

Shusei Sato, Erika Asamizu, Yasukazu Nakamura and Satoshi Tabata
(Kazusa DNA Research Institute)

Lotus japonicus has been shown to be a suitable model legume for molecular and genetic analysis, because of its small genome size, self-fertile nature, short generation time and high transformability. In order to investigate the legume-specific biological processes such as symbiotic nitrogen fixation, we have launched a large-scale genome analysis of *Lotus japonicus* by sequencing both cDNA and the genome.

We have constructed normalized and size-selected (>3kb) cDNA libraries from various sources. As of October 2000, a total of 22,983 5' end and 22,560 3' end expressed sequence tags (ESTs) from libraries of 2 week old plants, pods and nodulated-roots have been obtained. The 3' ESTs were grouped into 12,675 non-redundant groups. Similarity search against public non-redundant protein database indicated that 27% of the groups showed similarity to genes of known function, 11% to hypothetical genes, and 62% were novel sequences. Homologus of nodule-specific genes which have been reported in other legume species were contained in the collected ESTs, suggesting that the EST source generated in this project will become a useful tool for identification of genes related to legume-specific biological processes. The sequence data of individual ESTs are available at the web site: [http://www.kazusa.or.jp/en/plant/lotus/EST/].

As well as cDNA analysis, we have constructed a genomic library of *L. japonicus* in a transformation competent artificial chromosome (TAC) vector as a source of genome sequencing clones. The average insert sizes were 87kb, 96kb, 105 kb and 106kb for four independent preparations, a total of which is 7.7 haploid genome equivalent. The TAC libraries thus generated were arrayed in ninety-three 384 well microtiter plates, and 48 DNA pools each containing 384 clones were subjected to PCR screening. Seed clones for sequencing were selected by high throughput PCR screening using primer sets designed on the basis of ESTs and cDNA markers of *L. japonicus* and other legumes. Along with sequence and map position data produced through the project, the TAC clones will facilitate map-based cloning in *L. japonicus.*

Molecular characterization of genes expressed in floral organs of *Lotus japonicus* --- Analysis of expression pattern by using DNA microarray ---

Masao Watanabe[1], Makoto Endo[1], Hitoshi Matsubara[1], Yoshihito Takahata[1], Atsushi Higashitani[2] and Tohru Tsuchiya[3]

[1]Faculty of Agriculture, Iwate University, Ueda, Morioka 020-8550, Japan.
[2]Institute of Genetic Ecology, Tohoku University, Katahira, Sendai 980-8577, Japan.
[3]Faculty of Bioresources, Mie University, Kamihama, Tsu 514-8507, Japan

Introduction

Lotus japonicus has emerged as a model legume, because it has good characteristics for genome analysis. The flower size of *L. japonicus* is larger than that of other model plant species, such as *Arabidopsis* and rice, thus this feature may be useful in dissecting the gene expression of floral reproductive organs. Anther and pistil are highly differentiated sexual reproductive organs, which contain male and female gametophytes. To date, several male and female organ-specific genes have been identified and characterized in several plant species. In recent year, DNA microarray adds new dimension to gene expression studies. This type of analysis provides a powerful tools for studying the expression profiles of large subsets of genes in a given tissues under specific physiological and environmental conditions.

In this report, we determined partial sequences of 842 randomly selected cDNA clones from mature flower buds of *L. japonicus* B-129, Gifu, and analyzed the gene expression patterns in the vegetative organ (leaf) and reproductive organs (anthers and pistils) using DNA microarray, which was constructed from the EST clones.

Results and Discussion

A cDNA library was constructed from mRNA derived from flower buds removed sepals. The 5' ends of 842 clones, which contained inserts of more than 500 bp, were sequenced, and subjected to database analysis. When the 5' end sequences from 842 clones were compared with a dataset of itself, 718 non-redundant groups were generated.

In order to identify the putative function of 718 non-redundant EST groups, they were subjected to similarity search against the non-redundant protein database. In the analysis, 58.5% of the EST groups showed significant sequence similarity to known genes, 30.1% of EST groups showed similarity to protein and/or DNA sequences of unknown functions. The remaining 11.4% of EST groups showed no significant similarity to the sequences of registered genes, and were classified as novel sequences.

Comparison of these EST sequences with those derived from the whole plant of *L. japonicus*, revealed that 64.8% of EST sequences from the flower bud were not found in EST sequences of the whole plant. The result indicates that the cDNA library generated from mature flower bud is a good source to discover reproductive-related genes.

In order to determine the expression profile in reproductive organs, we constructed DNA microarray with 960 cDNA clones (842 EST clones and 118 non-EST clones). The DNA microarray was independently hybridized with three kinds of Cy3-labeled cDNAs, which were reverse transcribed from mRNA derived from leaves, anthers, and pistils. The intensities of fluorescence at the immobilized probes on DNA microarray were determined from images. In order to compare with the expression profiles among three organs (leaves, anthers, and pistils), scatter plots were made based on the data of fluorescence intensities. The scatter plots showed that 76 and 6 EST clones were characterized as specifically and/or dominantly expressed genes in anthers and pistils, respectively. In the case of anthers, some clones, which have been characterized as pollen-specific genes based on the similarity to pollen-specific genes in other plant species, were specifically (dominantly) expressed in anther tissues in this DNA microarray analysis.

The DNA microarray is a powerful tool for identification of reproductive organ-specific genes. Now we are starting to dissect the function of the specific gene with loss-of-function strategy.

Acknowledgement

This work was supported in part by a grant from Rice Genome Projects (Gene Discovery and Elucidation of Functions of Useful Genes in Rice Genome by Gene-Expression Monitoring System) from the Ministry of Agriculture, Forestry and Fisheries, Japan.

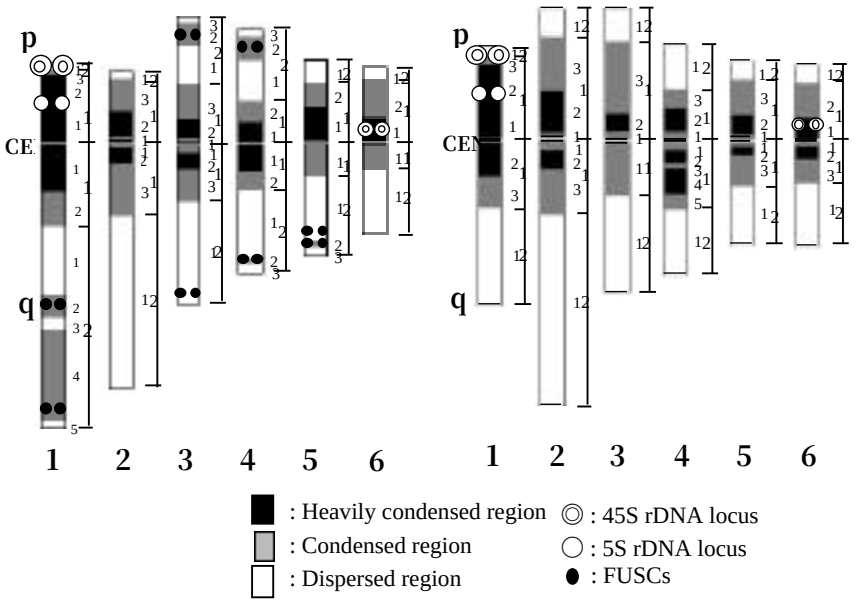
Genome and Chromosome Dimensions of ***Lotus japonicus.***

Kiichi Fukui[1], Mikako Ito[1], Junko Miyamoto[2] and Toshiki Uchiumi[2]

1. Dept. Biotechnology, Grad. Sch. Engineering, Osaka Univ.,

2. 2. Fac. Science, Kagoshima Univ.

Genome and chromosome dimensions were determined using two accessions of ***Lotus japonicus***, Miyakojima MG-20 and Gifu B-129. The genome sizes of Miyakojima and Gifu were determined as 463 and 415 Mbp, respectively. Both the accessions were diploid (2n=12) and six chromosomes were identified and characterized based on the condensation patterns and the locations of rDNA loci. The obvious polymorphism observed in the genome size and the chromosome morphology between the two accessions, revealed specific accumulation of heterochromatin in the Miyakojima, or elimination in the Gifu. The chromosome number of ***L. japonicus*** was first designated according to their length order of the Miyakojima. A quantitative chromosome map was also developed by the imaging methods using the condensed pattern. 45S rDNA loci were located on chromosomes 1 and 6, and 5S rDNA locus on chromosome 1 by fluorescence ***in situ*** hybridization (FISH). FISH using the mixture of approximately 500 cDNA clones as the probes revealed that the signals were located more on the terminal regions than the centromeric or proximal regions of the chromosomes. Identification of the chromosome and genome sizes and development of the quantitative chromosome map contribute to the genome project of ***L. japonicus*** as the basic information.



A: Miyakojima B: Gifu

Towards construction of the full genome contig of *L. japonicus*

-Providing infrastructures for the functional genomics -

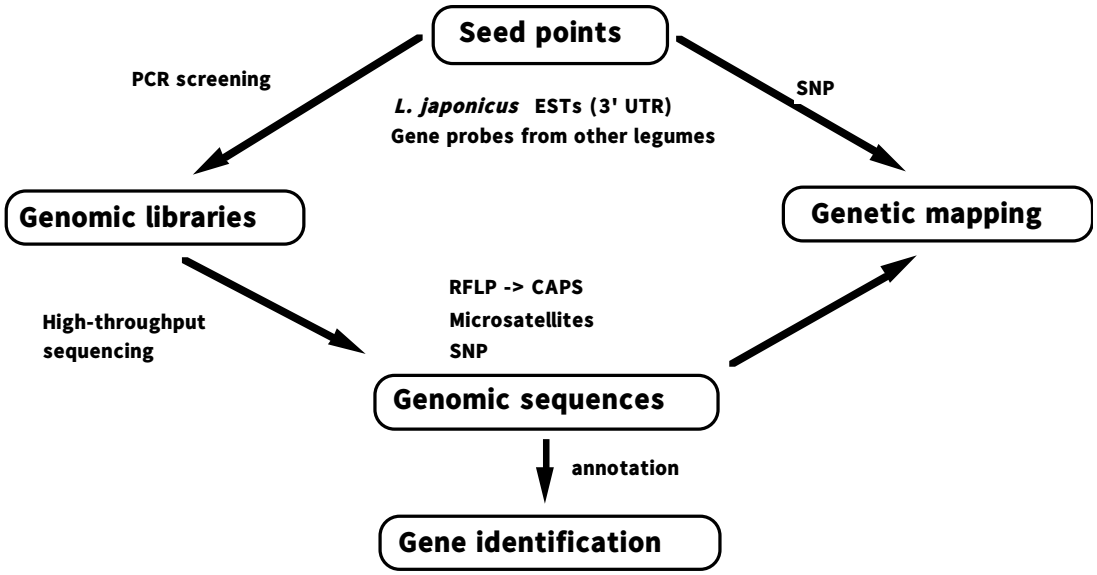
Shinji Kawasaki National Institute of Agrobiological Resources (NIAR)
Kannon-dai, Tsukuba, Ibaraki 305-8602

Lotus japonicus is attracting interest as a model plant of legume, with special interest on elucidation of the molecular process of nodulation. Functional genomics will be the most prospective approach, at present, for this purpose, and is consisting of systematic mutagenesis and genomic analysis of the causative genes. As a technical procedure for this, we preferred systematic positional cloning of various mutation genes due to chemical or physical mutagenesis, and culture mutations, than insertion or activation tagging, because the former process can be easily systematized further. For this approach, presence of an efficient marker analysis system and a high quality of genome library are indispensable. We have provided the HEGS (High Efficiency Genome Scanning) system for various genetic analyses with genome markers. Assessment of the polymorphism between accessions Gifu and Miyakojima with HEGS revealed a modest but probably sufficient polymorphic rate (4%) between them. Using this cross and HEGS, a genome map of ***L. japonicus*** is being constructed in collaboration with the group of Harada and others. The fine mapping of the major mutated genes are also in progress (see Imaizumi-Anraku of this symposium). On the other hand, large insert genome library of ***L. japonicus*** was constructed at the average insert size of 140 kb, and 8.1 genome equivalent scale. The library's genome coverage was calculated from the precise genome size of ***L. japonicus***, determined flowcytometrically by us; 494 MB for Gifu and 512 MB for Miyakojima using ***Arabidopsis*** (130 MB) as the reference. These infrastructures will facilitate systematic positional cloning of ***Lotus*** genes. For further convenience of functional genomics, we are planning to construct a full genome contig of ***L. japonicus***. This will put a great impetus to advance the functional genomics of nodulation and symbiotic nitrogen fixation. Methodologies for this end will be discussed in the symposium.

Genome analysis of *Lotus japonicus*

Satoshi Tabata, Takakazu Kaneko, Yasukazu Nakamura, Erika Asamizu, Tomohiko Kato, and Shusei Sato (Kazusa DNA Research Institute)

To obtain information on the structure of the genome as well as those of entire gene constituents of legume species, we have initiated large-scale sequencing of the genome of *Lotus japonicus*. The TAC (Transformation-competent Artificial Chromosome) genomic libraries of *L. japonicus* MG-20 were arrayed in ninety-three 384 well microtiter plates, and 48 DNA pools each containing 384 clones were subjected to PCR screening. Seed clones for sequencing were selected by high throughput PCR screening using primer sets designed on the basis of ESTs and cDNA markers of *L. japonicus* and other legumes. As of October 2000, a total of 158 seed clones have been selected using 203 primer sets. Fifty-six of them are ready for sequencing, 5 are being sequenced, and 15 are in the finishing phase. The expected rate of the sequence production is approximately 30 Mb per year. The sequenced clones were then subjected to genetic mapping using DNA markers such as microsatellite and SNPs (Single Nucleotide Polymorphisms) generated utilizing the sequence information obtained. A large quantity of information on ESTs as well as genomic sequences of known map locations will provide a basis of genetic analysis in *L. japonicus*, which will accelerate gene mapping and isolation.



Institute for Protein Research WorkShop

Molecular and Cell Biology of the Model Legume *Lotus japonicus*

November 16-17, 2000

at Lecture Hall, Institute for Protein Research, Osaka University

Organizer, Kazuhiko Saeki (Osaka Univ.) , Makoto Hayashi (Osaka Univ.),
Masato Nakai (Osaka Univ.)

November 16, Thursday

12:00-13:00	Registration	
13:00-13:05	Katsuya Nagai (Director, Institute for Protein Research)	・・・・・Opening Remark
13:05-13:25	Makoto Hayashi (Osaka Univ.)	・・*Lotus japonicus* as a model legume: an overview ・①
13:25-13:45	Kyuya Harada (Chiba Univ.)	・・・・・Significant impact of *Lotus japonicus* on the genome analyses of leguminous plants. ・②
13:45-14:15	Hiroshi Kouchi (Nat. Inst. Agro. Res.)	・・Responses of *Lotus japonicus* to Nod factors purified from *Mesorhizobium loti* JRL501 ・③
14:15-14:45	Masayoshi Kawaguchi (Univ. of Tokyo)	・・Spectrum of symbiotic mutants and molecular cloning of the gene responsible for negative regulation of nodules. ・④
14:45-14:55	**Coffee Break**	
14:55-15:15	Norio Suganuma (Aichi Univ. of Edu.)	・Phenotypic characterization of *Lotus japonicus* mutants, *fix6*(*Ljsym75*) and *fix7*(*Ljsym81*), that form ineffective nodules ・⑤
15:15-15:35	Haruko-Imaizumi Anraku (Nat. Inst. Agro. Res.)	・Symbiotic mutants of *alb1*/*sym74-1* and *sym72*; phenotypes characterization and progress toward positional cloning ・⑥
15:35-16:00	Keishi Senoo (Mie Univ.)	Characterization of mycorrhizal mutants in *Lotus japonicus* ⑦
16:00-16:10	**Coffee Break**	
16:10-16:55	**[Special Lecture]** Peter M. Gresshoff (Queensland Univ., Australia)	Isolation of nodule and root genes using promoter-trapping in *Lotus japonicus* ・・⑧
16:55-17:40	**[Special Lecture]** Jens Stougaard (Aarhus Univ., Denmark)	Structural and functional genomics in *Lotus japonicus* ・・・・・・・⑨
18:00-	**Light Repast and Drink**	

November 17, Friday

9:30- 9:50	Toshio Aoki (Nihon Univ.)	Studies on leguminous flavonoids using *Lotus japonicus* ・⑩
9:50-10:10	Junji Takabayashi (Kyoto Univ.)	・・・Induced indirect defense responses of bean plants against herbivorous mites ・・・⑪
10:10-10:30	Shingo Hata (Kyoto Univ.)	・・・*Lotus japonicus* forms early senescent root nodules with *Rhizobium etli* ・・・・⑫
10:30-10:40	**Coffee Break**	
10:40-11:00	Takakazu Kaneko (Kazusa DNA Res. Inst.)	Genome sequencing of *Mesorhizobium loti* ⑬
11:00-11:20	Kazuhiko Saeki (Osaka Univ.)	・・・・・・Towards systematic inactivation of total operons in *Mesorhizobium loti* ・⑭
11:20-11:40	Kiwamu Minamisawa (Tohoku Univ.)	・・・・Towards global analysis of gene expression in *Mesorhizobium loti* ・⑮
11:40-12:00	Shusei Sato (Kazusa DNA Res. Inst.)	・・・・Development of the resources for genome analysis of *Lotus japonicus* ・⑯
12:00-13:15	**Lunch**	
13:15-13:35	Masao Watanabe (Iwate Univ.)	Molecular characterization of genes expressed in floral organs of *Lotus japonicus*: Analysis of expression pattern by using DNA microarray ・⑰
13:35-13:55	Kiichi Fukui (Osaka Univ.)	Genome and Chromosome Dimensions of *Lotus japonicus* ⑱
13:55-14:15	Shinji Kawasaki (Nat. Inst. Agro. Res.)	・・Towards construction of the full genome contig of *Lotus japonicus* ・⑲
14:15-14:35	Satoshi Tabata (Kazusa DNA Res. Inst.)	・・ Genome analysis of *Lotus japonicus*: Future perspectives ・・・・・・⑳
14:35-14:50	Kazuhiko Saeki (Osaka Univ.)	Closing Remark