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Meeting Report

Legume Models Strut Their Stuff

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Legumes have been an integral part of sustainable agriculture for hundreds, if not thousands, of years, and they are likely to become even more important in the future. This is because legumes are able to form nitrogen-fixing symbioses with rhizobia that allow them to grow in the absence of added nitrogen fertilizers. In addition, legumes are able to form mycorrhizal symbioses with fungi that provide the plants with phosphorous and other soil nutrients, which significantly contributes to the agricultural output of legumes. The symbiotic phenotypes of legumes set them apart from other model plant species such as *Arabidopsis*.

In June 2000, a group of 120 scientists from around the world met at the John Innes Centre (JIC) in Norwich, U.K., to discuss recent developments in the molecular genetics of model legumes. The topics included comparative and functional genomics, the genetics of nitrogen-fixing and mycorrhizal symbioses, pathogen interactions, metabolism, signal transduction, development, agriculture and breeding, and the molecular systematics of legumes.

Model legumes fall into two broad classes: those that are of major importance to agriculture and those that are not. The former have been the subject of scientific investigation for many decades because of their ubiquity and economic value and include such crops as pea, soybean, and alfalfa. Unfortunately, agriculturally important legumes generally have large, complex genomes and are difficult to transform, which makes them less than ideal for molecular genetics and genomics. For this reason two species, *Lotus japonicus* and *Medicago truncatula*, have been adopted by a growing number of researchers in recent years. These models they have relatively small and simple genomes (three to four times the size of the *Arabidopsis* genome), are easy to transform, and have a number of other positive attributes for classical and molecular genetics and symbiosis research (Barker et al. 1990; Handberg and Stougaard 1992).

Leguminosae is the third-largest and possibly the most diverse family of angiosperms. Species range from small herbaceous plants to giant rainforest trees, a diversity that was beautifully illustrated in Toby Pennington's (Royal Botanic Garden, Edinburgh, U.K.) opening talk. Pennington also discussed how molecular analysis is reorganizing legume systematics, providing new insights into the evolution of important traits such as nodulation. Interestingly, it appears that the common ancestor of all legumes was not a nodulator and that this feature probably evolved independently several times in this family. The ability to nodulate also appears to have been lost from many species in multiple genera over time, possibly as a result of an increased availability of mineral or organic nitrogen in the niches occupied by legumes.

The humble pea has been a model for genetic studies since the time of Mendel and an important crop species for a much longer period. It has a relatively large and complex genome that is peppered with highly polymorphic retrotransposons (RTs). Noel Ellis (JIC) described how these RTs are being used in the genetic mapping of the pea genome. He also demonstrated substantial colinearity between the genetic linkage maps of pea and alfalfa. This theme of macro- and microsynteny between legume genomes was one that emerged throughout the meeting. For instance, there is a high degree of synteny between species in the *Medicago* genus (Thierry Huguet, CNRS-INRA, Toulouse, France) as well as between the *Medicago* species and pea (György Kiss, Institute of Genetics, Szeged, Hungary). Nevin Young (University of Minnesota, St. Paul, U.S.A.) also described microsynteny between *M. truncatula* and soybean. Synteny between the smaller genomes of model species such as *M. truncatula* and *L. japonicus* and the much larger genomes of agricultural mainstays such as soybean and pea should help to accelerate the isolation of agronomically important genes in the latter.

To facilitate the isolation of genes and quantitative trait loci involved in symbiosis and other important agricultural traits, much effort has been invested in mapping several legume genomes. The genetic map of diploid alfalfa (*Medicago sativa*, $2n = 16$) now contains 1,500 markers distributed over 754 cM (Kiss). *M. truncatula* ($2n = 16$) has at least 384 molecular markers distributed over 1,400 cM of the eight linkage groups (Huguet), whereas the *L. japonicus* ($2n = 12$) genetic map contains 500 molecular markers over 378 cM (Niels Sandal, Aarhus University, Denmark). Large bacterial artificial chromosome genomic libraries of *L. japonicus* (N. Sandal; Shinji Kawasaki, National Institute of Agrobiological Resources, Tsukuba, Japan; Peter M. Gresshoff, University of Queensland, Brisbane, Australia) and *M. truncatula* (Doug Cook, Texas A&M University, College Station, U.S.A.) will enable physical mapping of these genomes and accelerate map-based cloning of genes.

Chemical mutagenesis has generated large collections of interesting mutants in the various model legume species. Many genes involved in the development of functional sym-

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bioses between legumes and their rhizobial and mycorrhizal symbionts as well as genes involved in resistance to pathogens or tolerance to cold have been mapped and are now the focus of map-based cloning efforts. The smaller and simpler genomes of *L. japonicus* and *M. truncatula* offer obvious advantages in this regard, and it is likely that they will yield a number of interesting genes in the near future.

Efforts to isolate interesting genes via forward genetics have been accelerated by the use of transposon and T-DNA mutagenesis. In fact, the first symbiotic gene isolated from a legume was recently obtained from *L. japonicus* following transposon tagging (Schauser et al. 1999). The gene is called *Nin* to reflect its role in nodule inception. Analysis of the NIN protein sequence indicates that it may be a transcription factor. The mutation in *Nin* results in plants that cannot form nodules. The transposition of the DS transposon out of the *Nin* gene during root development, however, leads to root sectors that are able to nodulate. Approximately 1,000 transposon-tagged and a similar number of T-DNA-tagged lines of *L. japonicus* have been produced (Jens Stougaard, Aarhus University). Major concerted efforts are expected to produce tens of thousands of insertion mutants, not only in *L. japonicus* but also in *M. truncatula* (Pascal Ratet, ISV-CNRS, Gif sur Yvette, France) in the next few years.

Gene tagging with more sophisticated T-DNA constructs facilitates more than just gene inactivation. Promoter elements in the foreign, inserted DNA can activate expression of endogenous genes, leading to potentially interesting phenotypes, whereas promoterless genes like GUS and GFP can report the presence of endogenous promoters when they are inserted adjacent to such sequences. Many of these reporter lines show interesting patterns of gene expression. For example, Gresshoff and Herman Spaink (Leiden University, The Netherlands) described several different *L. japonicus* reporter lines that express GUS either exclusively in developing nodules or in other organs. Expression of GUS in nodule and root primordia but not in aerial tissues of a line called Cheetah (Gresshoff) indicates a possible overlap in the developmental programs of roots and nodules. Ratet has used a similar approach to tag potentially interesting genes in *M. truncatula*, including an MtN3-like gene that encodes a putative membrane protein that is expressed in nodules. Judith Webb (Institute of Grasslands and Environmental Research, Aberystwyth, U.K.) described a population of 200 promoter-tagged lines of *L. japonicus*. One of these has a GUS-containing T-DNA insert upstream of the coding sequence of a putative calcium-binding protein gene (*LjCbp1*). This line expressed GUS in roots and, subsequently, in nodules only after infection with rhizobia (Webb et al. 2000).

Transgenic lines in which isolated promoters of known genes are linked to reporter genes (e.g., GUS or GFP) have been produced in several species that are easily transformed, including *M. truncatula* and *L. japonicus* (Vernoud et al. 1999). Spaink described how his group is using a fusion of the ENOD40 promoter to GUS and GFP in *L. japonicus* to monitor ENOD40 gene expression during the early stages of nodule initiation and development. Such lines represent a powerful tool to explore the molecular basis of symbiotic signaling.

Nodule development is a complex process that requires multiple plant genes. Over the years, many genetic loci involved in nodule development have been identified, although all but one of the affected genes remains to be cloned. Nonetheless, substantial progress has been made in identifying the developmental stages affected by specific mutations. Igor Tikhonovich (Research Institute for Agricultural Microbiology, St. Petersburg, Russia) showed that different pea genes are critical for different stages of nodule development on the basis of the fate of bacteria entering or attempting to enter epidermal and cortical cells. Thus, *Sym* genes have been categorized as either genes essential for early infection events such as root hair curling and infection thread formation or genes involved in later events such as release of rhizobia into cortical cells or nodule persistence and/or senescence. A similar dissection of developmental steps involved in establishing mycorrhizal symbioses has been achieved with mutants of *L. japonicus* (Keishi Senoo, Mie University, Tsu, Japan; Martin Parniske, Sainsbury Laboratory, Norwich, U.K.) and *M. truncatula* (Maria Harrison, Samuel Roberts Noble Foundation, Ardmore, OK, U.S.A.).

Some of the plant genes necessary for nodule development also are required for mycorrhizal development (Duc et al. 1989). This observation has facilitated the isolation of many plant mycorrhizal mutants. Interestingly, some hypernodulating mutants of *L. japonicus*, *M. truncatula*, and pea are also hypermycorrhizal (K. Senoo; Gérard Duc, URGAP-INRA, Dijon, France). Molecular genetic studies suggest that the commonality among different symbiotic programs in plants may be much greater than predicted from classical genetics. Parniske has used a cDNA-amplified fragment length polymorphism (AFLP) approach to show that as many as 500 plant genes are upregulated during arbuscular mycorrhizal development. Some of these are also upregulated during nodulation with rhizobia. Are these genes involved in setting up house for the different microsymbionts (Parniske 2000)? Reverse genetic approaches will likely provide answers to this question in the coming years.

Legume pathogens cause great agricultural losses, and many groups are working toward reducing the extent and severity of pathogenesis. Cook is using *M. truncatula* as a model to study interactions with several different pathogens, including *Rhizoctonia solani*, *Phytophthora medicaginis*, *Colletotrichum trifolii*, and *Meloidogyne incognita*. By screening 100 ecotypes for resistance and susceptibility to these pathogens, Cook and colleagues hope to identify genes that confer resistance to the different pathogens. It is perhaps no surprise that some of the genes required for mutualistic symbioses, e.g., *dmi1-1*, are not involved in pathogen interactions. At least one gene, *skl1*, involved in ethylene signaling, however, is involved in mutualistic and pathogenic interactions. Kurt Mendgen (University of Konstanz, Germany) has used broad bean as a model to study plant–rust fungus interactions. Combining biochemistry and molecular biology, his group has demonstrated that rust haustorium is the main source and distributor of amino acids and sugars for rust fungus. Upregulation of an apoplastic invertase, an H^+-ATPase, and transporters for sugars and amino acids in haustoria appear to support these roles. β-Glucans produced by pathogenic fungi elicit a phytoalexin defense response in plants. Jürgen Ebel's group (University of Munich, Germany) is studying the role of β-glucan-binding proteins (GBPs), cytosolic calcium concentration, and MAP kinases in this defense response in soybean. They have shown that elicitor addition induces a

rapid increase in cytosolic calcium and that inhibitors of Ca^{2+} channels block this increase and the phytoalexin defense response. In a related talk, Axel Mithöfer of the same group described how cyclic β-glucans produced by the nitrogen-fixing microsymbiont of soybean, *Bradyrhizobium japonicum*, is able to antagonize the effects of linear β-glucans, presumably by competing for binding sites on GBPs. In this way, rhizobia may be able to suppress the plant defense response during nodulation and nitrogen fixation. Interestingly, mature nodules contain high concentrations of cyclic β-glucans and bacterial mutants impaired in the synthesis of these compounds form ineffective nodules that accumulate the phytoallexin glyceolin.

Symbiosis research has contributed greatly to our understanding of signaling in plants, in large part because the microbes that interact with plants produce and excrete signals that can be purified, chemically characterized, modified, and presented to the plant in a controlled manner. The response of plant cells to these signals is often easier to interpret than their response to endogenously produced signals such as the various phytohormones. The nodulation (Nod) factors produced by rhizobia, which trigger the initial events that lead to nodule development, are perhaps the best characterized of all signals produced by microsymbionts. Their chemistry, biosynthesis, and biological specificity have received a great deal of attention during the past two decades. In the past few years, attention has shifted toward the plant and, in particular, the molecular infrastructure that enables root cells to perceive and transduce Nod-factor signals. One of the earliest responses of legume root hair cells to Nod factors is the phenomenon of calcium spiking. Allan Downie (JIC) demonstrated that some non-nodulating pea mutants (*Sym 10*, *Sym 8*, and *Sym 19*) do not show calcium spiking in response to Nod factors, whereas others (*Sym 9*, *Sym 30*, *Sym 7*, and *Sym 2*) do. The genes affected in mutants that do not show calcium spiking may encode a Nod-factor receptor or some of the earliest elements of the signaling pathway. Interestingly, two of these genes (*Sym 8*, and *Sym 19)* also are essential for infection by mycorrhiza. Ethylene has been implicated as a negative regulator of nodule numbers (Penmetsa and Cook 1997). Work reported by Giles Oldroyd (Stanford University, CA, U.S.A.) indicates that ethylene suppresses Nod factor-induced calcium spiking in *M. truncatula.* Early intersection of endogenous and external signals would be an efficient way to control resource allocation to the costly enterprise of building nodules. Other groups also use the power of genetics to dissect the early events in Nod-factor perception and signaling. Jean Dénarié (CRNS-INRA) described a subset of *M. truncatula* mutants that, like the pea mutants described above, shows reduced calcium spiking in response to Nod factors. In addition, they have used the natural variation present in 120 ecotypes of this species to define gene loci involved in Nod-factor reception. With the use of these ecotypes in combination with rhizobium mutants that produce modified Nod factors, they have identified at least one locus required for O-acetyl group recognition. This gene may encode a Nod-factor receptor and is now the focus of a map-based cloning effort.

Curiosity and persistence often lead to surprising results. While characterizing genes related to nodulin 16 in *L. japonicus*, Krzysztof Szczyglowski (Michigan State University, East Lansing, U.S.A.) discovered a family of genes that contain a phosphadidylinositol transfer protein-like domain adjacent to a nodulin 16 domain. One of these, *LjPLPIV*, is present as an antisense transcript in nodules, although it is present as a normal sense transcript in flowers. Further analysis indicated that the antisense transcript resulted from the presence of a promoter embedded in an intron of the gene. Given the efficacy of engineered antisense transcripts in downregulating gene expression in transgenic plants and other organisms, one is left to wonder about the possible regulatory significance of the endogenous *LjPLPIV* "antisense" transcript in nodules. Further persistence may well lead to an answer that has important ramifications beyond legume biology.

The economic value of many crop legumes such as soybean and pea lies in their seed, which are factories for oils, protein, complex carbohydrates, and other nutritionally or industrially valuable compounds. As a result, large companies and public research organizations continue to invest in the understanding and manipulation of seed metabolism. Ted Klein (Dupont, Waltham, MA, U.S.A.) described how his company uses genomics to rapidly identify genes that are useful for modifying metabolic pathways in seeds. For instance, in order to produce vernolic acid (an epoxy fatty acid derived from Vernonia) in soybean seeds, scientists at the company identified two genes that were closely related to fatty acid desaturase (*fad*) from 2,000 expressed sequence tags (EST) from Vernonia seeds. It was hypothesized that the epoxidase from Vernonia responsible for converting oleic acid to vernolic acid would be similar to fad proteins from other species. The two genes were transformed into soybean embryos, and lipid analysis of the resulting transgenic seed showed high levels (10%) of vernolic acid in one of the lines. Steve Parry (Unilever, London, U.K.) described how a naturally occurring mutant of pea, *rug-3*, impaired in plastid phosphoglucomutase activity, altered pea seed metabolism in a way that produced sweeter peas, which of course makes them tastier, if not more nutritious, for humans.

Large-scale EST projects are not only a rich source of potentially interesting genes but are also a source of information about the relative abundance of transcripts in particular tissues or organs under defined conditions. EST collections arrayed onto nylon filters or glass slides also can be used for transcriptome analysis. During the past year, several groups have contributed to the burgeoning number of EST sequences from model legumes. A total of 97,000 soybean, 48,000 *M. truncatula*, and 27,000 *L. japonicus* ESTs were present in public databases at the start of August 2000. *M. truncatula* ESTs have come from a variety of organs, including roots, leaves, cotyledons, and symbiotic tissues such as nodules, ectomycorrhizal roots, and pathogen-infected roots or leaves (Kathryn Kate VandenBosch, Texas A&M University; Maria Federova, University of Minnesota; Etienne-Pascal Journet, CNRS-INRA, Castanet-Tolosan, France). ESTs from *L. japonicus* were derived from whole young plants or seed pods (Satoshi Tabata, Kazusa DNA Research Institute, Japan) and nodules (M. K. Udvardi). Some of these groups have begun to produce EST arrays as a prelude to transcriptome analysis.

One of the surprises of the meeting was revealed in Tabata's talk, which began with a summary of his group's work on ESTs but later included information on genome sequencing in *L. japonicus*. He revealed that his group recently shotgun sequenced the *L. japonicus* chloroplast genome (150 kbp) and

the genome of the nitrogen-fixing microsymbiont of *L. japonicus*, *Mesorhizobium loti* (7 Mbp). Sevenfold sequence coverage of both genomes was obtained in 1 week and 1 month of sequencing, respectively. Tabata also indicated that his group has begun to sequence the nuclear genome of *L. japonicus* Miyakojima MG-20-S7 and aims to produce 30 Mbp of sequence per year during the next 5 years. This represents a significant fraction of the total genome, estimated to be around 450 Mbp.

L. japonicus Miyakojima MG-20 is an accession from Miyakojima, the southern-most island of Japan. It was selected because of its ability to rapidly complete its life cycle (2 months) under weak fluorescent lights, obviating the need for special high-light growth facilities (Masayoshi Kawaguchi, University of Tokyo, Japan). This accession was put to good use to clone the *Ljsym77* gene. A mutant affected in this gene showed hypernodulation and enhanced hypocotyl elongation. The latter phenotype was reminiscent of the *hy5* mutant phenotype in *Arabidopsis*. *Arabidopsis HY5* encodes a bZIP transcription factor. A homologue of this gene was isolated from Gifu and Miyakojima MG-20. Sequence comparisons uncovered a single nucleotide polymorphism in the *HY5* homologues of these accessions, which was subsequently shown to be tightly linked to the mutant allele. The sequence of the gene from the mutant revealed a point mutation. Thus, *Ljsym77* appears to be the *HY5* ortholog in *L. japonicus*.

Proteomics is a relatively new discipline that is dedicated to the high-throughput identification and characterization of proteins from specific organs, tissues, cell types, or subcellular fractions. Recently, proteomics approaches have been used to identify differentially expressed proteins in the *Sinorhizobium meliloti–Melilotus alba* symbiosis (Natera et al. 2000) and proteins associated with the peribacteroid membrane of soybean nodules (Panter et al. 2000). Gerhard Saalbach (Risoe National Laboratory, Roskilde, Denmark) described his attempts to identify plant proteins from symbiosomes of pea. Although mass spectrometry has proved useful in identifying many proteins from pea symbiosomes, the bulk of these appear to be of bacteroid origin, which indicates that pea bacteroids may be much more fragile than those from soybean nodules.

The meeting in Norwich highlighted the broad utility of model legumes to answer not only fundamental questions about symbiosis and other aspects of plant biology, but also applied questions such as those related to pathogenesis and seed quality. The enthusiasm displayed by the participants during the scientific sessions and on the dance floor bodes well for the future of legume research.

The next meeting in this EU-sponsored series will be held 15 to 19 September 2001 at the Max Planck Institute of Molecular Plant Physiology in Golm (near Berlin), Germany.

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