Mutagenesis and Beyond! Tools for Understanding Legume Biology

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The family Leguminosae is one of the largest families of flowering plants and includes important crop legumes such as soybean (Glycine max) and lentil (Lens culinaris) and forage legumes like alfalfa (Medicago sativa). Legumes vary in habit from annual to perennial and in their genomes from simple diploids to large and complex polyploids. Two legume species, Medicago truncatula and Lotus japonicus, are being used as models to study legume genetics and genomics. Both of these species belong to the Galegoid clade that includes pea (Pisum sativum) and alfalfa but is also closely related to the other agriculturally important major clade, the Phaseoloid, that includes bean (Phaseolus vulgaris). M. truncatula and L. japonicus thus represent very useful model systems to study pertinent issues that are relevant to economically vital legumes. These two species were chosen as models because of their diploid, relatively small genomes (450–550 Mb), self-fertility, relative ease of genetic transformation, and short generation time. The success of any species as a molecular genetic model depends on the availability of versatile genetic and genomic resources. The genomes of both of these species are being sequenced by international consortia and are approaching completion (Sato et al., 2008; Young and Udvardi, 2009). In addition to the excellent transcriptomics, proteomics, metabolomics, and bioinformatics tools being developed for both species by different groups around the world, a sizeable number of mutants have been collected using various mutagenesis approaches. In this update, we will briefly describe the current mutant populations and draw particular attention to two of the most frequently used mutant resources for reverse genetics: the Tnt1-tagged population of M. truncatula and the targeting-induced local lesions in genomes (TILLING) population of L. japonicus.

LEGUME MUTAGENESIS RESOURCES

Mutagenesis is a fundamental approach in biology to identify gene function, and in plants it may involve using chemicals, ionizing radiation, or specific DNA insertion sequences. All of these have been attempted in legumes including the models M. truncatula and L. japonicus, and comprehensive tools for M. truncatula have been described in a recent review (Ane et al., 2008).

Chemical Mutagenesis

Alkylated agents such as ethylmethane sulfonate (EMS) have been widely used in the past for producing mutagenized populations, which can then be used for forward genetic screens. It is applicable readily to most plant species, inducing single base pair G/C-to-A/T substitutions in nucleotides. TILLING (McCallum et al., 2000; Colbert et al., 2001) is a reverse genetic tool that is used to identify these single base pair changes in target DNA sequences and is likewise readily applicable to most plants (Henikoff and Comai, 2003). It is frequently carried out in parallel with forward screens, permitting preselection of deleterious mutations (Perry et al., 2003). TILLING involves the identification of mismatches in heteroduplexes formed by single-stranded DNAs from wild-type and mutant sequences, and key to the technology are an appropriately mutagenized population of M2 plants and a method for identifying the mismatches. The sequences are generated by PCR amplification from a population of DNAs isolated from single M2 plants using labeled primers appropriate for the type of detection employed. The DNAs can be pooled to increase the efficiency of the process. They are then heated, causing strand separation, renatured to form the heteroduplexes, and cleaved at the mismatch, and the products are separated by electrophoresis. Cleavage is carried out using endonucleases, the most commonly employed, Cell, being readily obtained from celery (Apium graveolens; Till et al., 2003), although several others have also been examined (Till et al., 2004;
Deletion Mutagenesis

Fast-neutron bombardment (FNB) and γ-rays result in deletion of DNA fragments of variable length from the genome (Li and Zhang, 2002). FNB populations of *M. truncatula* var Jemalong A17 are being developed by the groups of G. Oldroyd at the John Innes Centre (Rogers and Oldroyd, 2008) and R. Chen at the Samuel Roberts Noble Foundation (Wang et al., 2006). Approximately 156,000 M2 families at the John Innes Centre and 460,000 M2 families at the Noble Foundation have been generated so far, and both populations have been publicly available for forward and reverse genetic screening. A reverse screening strategy called deletion TILLING has been developed. It uses a very sensitive PCR-based protocol to screen deletion mutants and detects mutants at a frequency of 20% to 50% using a portion of the mutant population. Forward genetic screens have also been used to identify mutants with phenotypic alterations from the wild type. Developments are under way to expedite gene cloning during seed-to-seed generation in greenhouses; transposition can be activated by tissue culture; new transposable elements can target any part of the genome, but it does so more frequently in the gene-dense regions than the centro-meric and other repetitive sequence regions. The preference for exons is a valuable attribute for efficient saturation mutagenesis (as the *M. truncatula* genes are not uniformly distributed in the genome) and may simply be due to a bias for GC-rich against AT-rich sequences (Tadege et al., 2008).

The advantages of *Tnt1* as an insertional mutagen in *M. truncatula* are numerous: insertions are stable during seed-to-seed generation in greenhouses; transposition can be activated by tissue culture; new transpositions show preference toward transcribed regions without a strong preference for a unique target site sequence (d’Erfurth et al., 2003a; Le et al., 2007; Tadege et al., 2008); it inserts always as a single copy at one locus, generating only a 5-bp duplication (target-site duplication), with none of the deletion or local genomic rearrangements observed with the T-DNA. These latter characteristics are important features for the identification of the tagged loci. *Tnt1*, therefore, can target any part of the genome, but it does so more frequently in the gene-dense regions than the centro-meric and other repetitive sequence regions. The preference for exons is a valuable attribute for efficient saturation mutagenesis (as the *M. truncatula* genes are not uniformly distributed in the genome) and may simply be due to a bias for GC-rich against AT-rich sequences (Tadege et al., 2008).

**M. TRUNCATULA TINT1-TAGGED RESOURCES**

The *Tnt1* retroelement was initially introduced into *M. truncatula* and shown to transpose in tissue culture by the group of P. Ratet (d’Erfurth et al., 2003a). The group of K. Mysore at the Noble Foundation then...
collaborated with Dr. Ratet’s group and launched the first large-scale Tnt1 tagging in the R108 background at the end of 2003. Since then, a multinational European group consisting of 10 laboratories, organized in the framework of the European Union Grain Legumes Integrated Project (GLIP), has successfully generated 6,000 Tnt1 lines in the M. truncatula Jemalong 2HA background (www.eugrainlegume.org). An Italian group has also recently reported the generation of 1,000 Tnt1 lines in the R108 background (Porceddu et al., 2008). To date, approximately 15,000 independent Tnt1 lines have been generated in the United States, and the average copy number per genome estimated by Southern-blot analysis and sequencing of flanking sequence tags (FSTs) from a limited number of lines is approximately 25 (Tadege et al., 2008), suggesting that the existing population contains approximately 375,000 Tnt1 inserts. Assuming the M. truncatula genome size to be 500 Mb and the average gene length to be 1.7 kb, it was previously estimated that 14,000 Tnt1 lines would provide approximately 90% probability of tagging any average-sized single-copy gene. To date, 90% of the genes that have been targeted were found to be tagged in a subpopulation of 10,000 lines, confirming this estimate. This suggests that the Tnt1 population should provide a very high probability of tagging any genes in M. truncatula except very small genes, which unfortunately are not uncommon in the M. truncatula genome. This is a very efficient resource that the legume community is encouraged to make the most use of it. The vast array of Tnt1-tagged genes already identified by FST sequencing that have been described in other species can be searched at http://bioinfo4.noble.org/mutant/database.php. Other Tnt1-tagged genes that are identified by forward and reverse screening include symbiotic genes (such as NIN, NSP1/2, ERN1, DMII/3, NFP, and SUNN), flowering time and floral development genes, leaf and inflorescence development genes, and several transcription factors that belong to homeobox, MADS box, MYB, and zinc finger classes as well as several metabolic enzymes and various transporters, which are the subject of active investigation by a large number of groups.

The Tnt1 population at the Noble Foundation is generated from a single parental line, Tnk88-7-7, that contains five copies of the Tnt1 element. Tnk88-7-7 was obtained by introducing the Tnt1-containing construct into the R108 genotype and subsequent selfing. This parental line contains only transposed Tnt1 copies, not the original construct. Extensive PCR analysis using sequence-specific primers and sequence analysis of thermal asymmetric interlaced (TAIL)-PCR fragments found no antibiotic resistance markers, left and right T-DNA borders, or any other sequences of the transformation vector. The vector was detected in the progenitor of Tnk88-7-7 but not in Tnk88-7-7 itself, suggesting that the Tnt1 element had transposed during the transformation process. The vector and the transposed Tnt1 copies ended up in different plants by segregation during the selfing process in soil. The pipeline for generation of the population is schematically depicted in Figure 1 and has three components for community utilization: forward genetic screening of mutant phenotypes; reverse genetic screening of DNA pools; and a FST database. In brief, mutagenesis is accomplished by cultivating leaf explants from the parental line on auxin- and cytokinin-containing culture media and regenerating new plants via somatic embryogenesis (Tadege et al., 2005, 2008). Each new plant is derived from a single leaf explant and represents a truly independent line.

At the Noble Foundation, an annual open and free Tnt1 screening workshop have been organized to catalogue mutant phenotypes and make them available to the scientific community. The first such screening was conducted in the summer of 2005 involving various groups from France, the United Kingdom, and the United States primarily interested in nodulation biology. To date, approximately 7,000 lines have been screened for phenotypes ranging from symbiotic and root development to leaf architecture and shoot meristem functions. Some representative root and nodule phenotypes are shown in Figure 2. Tnt1 tagging pro-
vides more allelic series of mutants compared with other insertional mutagenesis due to the higher density of Tnt1 insertions. The highest frequencies of confirmed alleles found so far are for the nodulation gene NIN (nine), the lamina gene STENOFOLIA (eight), and the unifoliate gene SINGLE LEAFLET1 (six). Interestingly, no two alleles of the nine inserts in the NIN gene (C. Pislaru and M. Udvardi, personal communication) or the eight inserts in the STENOFOLIA gene (M. Tadege and K. Mysore, unpublished data) have been found to have insertions in the same physical location. The insertion site sequences within the gene or between these two genes also do not show any particular homology or specific pattern consistent with the absence of a strong sequence-specific target site for Tnt1 insertion.

To customize reverse screening, genomic DNA has been extracted from 13,500 lines and is being pooled in one dimension with superpools of 500 lines, smaller pools, and mini pools that are convenient for PCR-based screening. Two pairs of one Tnt1-specific and one gene-specific primer combination are used in a standard PCR screening to look for Tnt1 insertion in a gene of interest. The screening starts by running the PCR with the superpools. Once a positive signal is identified in the superpool(s), the screening continues down to the smallest pool until the individual line(s) containing the insert is identified. So far, 20 superpools from 10,000 lines have been optimized for the community service. FST sequencing is in progress to recover most of the plant sequences flanking the Tnt1 insert. Tnt1-flanking plant sequences are obtained by TAIL-PCR using a combination of two or three nested Tnt1-specific primers and one arbitrary primer. PCR products are then cloned and sequenced. Sequences are deposited in a Tnt1 FST database (currently 15,476 FST sequences) that is equipped with GBrowser and BLAST search functions and is publicly available (http://bioinfo4.noble.org/mutant/database.php). Protocols for an efficient 454 sequencing platform are being optimized to significantly increase the number of FSTs available to the community. Seeds corresponding to the FSTs of interest can be ordered online for a small handling charge per line. Nondestructive phenotype screening of the Tnt1 lines and reverse genetic screening of DNA pools can be arranged by contacting K. Mysore (ksmysore@noble.org) until an online service is established.

**TILLING RESOURCES**

The first plant TILLING service was developed for Arabidopsis using LICOR sequencers (Till et al., 2006a) at the Fred Hutchinson Cancer Research Center in Seattle following the pioneering work of J.G. Henikoff and colleagues and is still in operation today (http://tilling.fhcrc.org/). The first outside the United States was for a legume model, L. japonicus. Lotus TILLING was a collaborative venture between the

Figure 2. Some examples of root and nodule mutant phenotypes identified by forward genetic screening of a Tnt1-tagged M. truncatula population. Plants were 6 weeks old at the time of screening and grown in Perlite/sand mix supplemented with low-phosphorus and low-nitrogen nutrient solution. A, A portion of a wild-type (WT) R108 root with two nitrogen-fixing nodules. The rest are mutant phenotypes. B, Very dwarf whole root showing deformed main and lateral root formation. C, Another type of severely dwarf root showing almost the whole root system in which main and lateral root development is arrested. D, Super-nodulator showing an unusually high number of nodules on a portion of normal root system. E, Nonnodulator with a short root in which main and lateral roots start expanding but further growth is arrested. F, A nodule mutant on a portion of a normal root in which a new root emerges from the nodule apex.
Sainsbury Laboratory and the John Innes Centre in Norwich (http://www.lotusjaponicus.org/tillingpages/homepage.htm). Initially, an ABI377 gel-based platform was employed (Perry et al., 2003), but this was superseded by LICOR 4300 instruments, which are now the most commonly used. Similar platforms were also set up by D. Cook (University of California, Davis) and under the European Union Grain Legumes Integrated Project program for the partner model, M. truncatula (http://www.glttp.com/products_services/technical_services/genomic_resources_from_glip/functional_genomics/medicago_tilling_platform), and for pea (http://www.glttp.com/products_services/technical_services/genomic_resources_from_glip/functional_genomics/pea_tilling_platform). Since then, there have been several platforms established for a range of legume species, including phenotype classification for some (Table I). The L. japonicus service currently operates as RevGenUK (http://revgenuk.jic.ac.uk), which also carries out TILLING in M. truncatula and Brassica rapa, all using a capillary electrophoresis ABI3730 sequencing platform. For this platform, specialized protocols had to be developed to obtain sufficient lengths of sequence reads (Le Signor et al., 2009). Improvements to all platforms are ongoing. For example, costs can be reduced on the LICOR instrument using the dye Cy5 and internal labeling of primers (K. Meksem and F. Gresshoff, personal communication). Lotus TILLING established tools for both forward and reverse genetic analyses. Arguably, it is the most extensively used platform and has generated much data. A structured population of M2 progeny of 4,904 EMS-mutagenized M1 embryos was generated in the ecotype Gifu, but thematic populations were also assembled (Perry et al., 2009) for development, starch accumulation, and the nitrogen-fixing root nodule symbiosis. The phenotypes (Perry et al., 2003) can be interrogated via a Web-based database (http://data.jic.bbsrc.ac.uk/cgi-bin/lotusjaponicus/). Similar databases are available for other legumes (Table I). A new population of approximately 3,500 lines in L. japonicus ecotype MG20 is currently being assembled. The mutation load of the original population has been calculated to be one per 502 kb, equivalent to approximately 940 mutations per genome based on subsets of genes. Lotus TILLING carried out mutation detection for 23 research groups across 11 countries and analyzed 158 targets. Interestingly, in examining these mutations in detail, a 1:10 ratio between homozygous and heterozygous mutations in the M2 progeny was observed. This result is in stark contrast to the 1:2 ratio observed for Arabidopsis (Greene et al., 2003) and indicates an unusual number of genetically effective cells in L. japonicus producing up to six gametes. Furthermore, by analyzing the distribution of amino acids that were replaced by EMS mutagenesis in the missense and nonsense alleles in functionally impaired mutant lines, they revealed a significant bias for replacements of Gly residues in the defective alleles. This may be explained by the exceptional structural features of Gly, since Gly allows peptide chains to adopt certain conformations that are not possible once it has been replaced. TILLING in legumes has been used largely for functional genomics research to date. This has been either to confirm, by generating additional alleles, a lesion in forward screened mutants, especially those associated with the Rhizobium-legume symbioses, or to generate unique mutants. As examples of the former, Perry et al.

Table 1. Examples of current EMS mutagenesis resources for forward and TILLING reverse genetics in legumes

<table>
<thead>
<tr>
<th>Species</th>
<th>Mode</th>
<th>Site</th>
<th>URL</th>
<th>Contact</th>
<th>Associated Refs.</th>
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<tbody>
<tr>
<td>Arachis hypogaea</td>
<td>F/R</td>
<td>UGA, Tifton</td>
<td><a href="http://www.soybeantilling.org/index.jsp">http://www.soybeantilling.org/index.jsp</a></td>
<td><a href="mailto:pozias@uga.edu">pozias@uga.edu</a></td>
<td>Ramos et al. (2009)</td>
</tr>
<tr>
<td>Cicer arietinum</td>
<td>F</td>
<td>WSU, Pullman</td>
<td></td>
<td><a href="mailto:w-chen@wsu.edu">w-chen@wsu.edu</a></td>
<td>Muehlbauer and Rajesh (2008)</td>
</tr>
<tr>
<td>Glycine max</td>
<td>F/R</td>
<td>SIU, Carbondale</td>
<td><a href="http://www.tilling.jic.ac.uk/#">http://www.tilling.jic.ac.uk/#</a></td>
<td><a href="mailto:meksemk@siu.edu">meksemk@siu.edu</a></td>
<td>Cooper et al. (2008)</td>
</tr>
<tr>
<td>Lotus japonicus</td>
<td>F/R</td>
<td>JIC, Norwich</td>
<td><a href="http://data.jic.bbsrc.ac.uk/cgi-bin/">http://data.jic.bbsrc.ac.uk/cgi-bin/</a></td>
<td><a href="mailto:mutant.finder@bbsrc.ac.uk">mutant.finder@bbsrc.ac.uk</a></td>
<td>Perry et al. (2003)</td>
</tr>
<tr>
<td>Medicago truncatula</td>
<td>S/R</td>
<td>INRA, Evry</td>
<td><a href="http://www.versailles.inra.fr/urvg/tilling.htm">http://www.versailles.inra.fr/urvg/tilling.htm</a></td>
<td><a href="mailto:bendahm@evry.inra.fr">bendahm@evry.inra.fr</a></td>
<td>Le Signor et al. (2009)</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>F/R</td>
<td>USDA-ARS, Puerto Rico</td>
<td><a href="http://www.igv.cnrs.fr/welcome/">http://www.igv.cnrs.fr/welcome/</a></td>
<td><a href="mailto:sergio.arcioni@igv.cnrs.fr">sergio.arcioni@igv.cnrs.fr</a></td>
<td>Porceddu et al. (2008)</td>
</tr>
<tr>
<td>Pisum sativum</td>
<td>F</td>
<td>USDA-ARS, Idaho</td>
<td></td>
<td><a href="mailto:victor.raboy@ars.usda.gov">victor.raboy@ars.usda.gov</a></td>
<td>Porch et al. (2009)</td>
</tr>
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Currently EcoTILLING (TILLING on ecotypes; Till et al., 2006b), with a full TILLING population under development.
(2009) recently reported allelic series for 12 L. japonicus genes involved in the symbiosis for use in structure-function studies, and Hofer et al. (2009) used pea TILLING to support the identification of the Tendril-less gene, whereas for the latter, Welham et al. (2009) used TILLING alone to show that a cytosolic invertase was crucial for normal plant growth and cellular development but did not affect nodule function.

Since TILLING is readily applicable to most plant species, it is especially suited to species where there are few genomic resources and where insertion mutagenesis to create knockout mutants is difficult either through a lack of appropriate elements or an inefficient transformation system. Moreover, since it is a non-genetically modified (GM) technology, it is also especially desirable in those crops/countries where application of GM technology is restricted. These advantages have facilitated its swift move from models to legume crops and have meant that some crops have been used in depth already. Pea was used as a pan-European platform under the European Union Grain Legumes Integrative Program (http://www.euorganiclegumes.org/), and this has a direct link to the breeders and other stakeholders under its Technology Transfer Platform (http://www.gl-ttp.com/products_services/technical_services/genomic_resources_from_glip/functional_genomics). Soybean TILLING, established with support from the U.S. Department of Agriculture (USDA), has now targeted almost 130 amplicons across 4,600 lines (divided between ‘Forrest’ and ‘Williams82’ plants), one mutation on average occurring every 130 or 190 kb, respectively. The soybean platform has extensive agronomic targets (K. Meksem, personal communication), including low phytate, higher seed yield and improved seed oil quality, improved nodulation (based on the NARK mutants, in collaboration with P. Gresshoff and colleagues at the University of Queensland), and improved resistance to cyst nematode.

Phaseolus, chickpea (Cicer arietinum), and lupin (Lupinus albus) TILLING are in different degrees of development. A population of Phaseolus ‘BAT93’ has been established jointly by two groups (Porch et al., 2009), and populations of U.S. market-class varieties are being developed by V. Raboy (USDA-Agricultural Research Service [ARS], Aberdeen, MD) and K. Cichy (USDA-ARS, East Lansing, MI). These will be ready for forward genetics screening in 2010. F.J. Muehlbauer and P.N. Rajesh obtained 21,000 M1 plants of chickpea (‘ICC12004’, a brown desi type) and have 2,855 lines (albeit at a lower frequency than insertions) give it advantages over other technologies. Its non-GM technology provides a further asset that has facilitated its rapid move from model to crop legumes. Similarly, the beauty of Tnt1 insertion mutagenesis in M. truncatula in generating multiple knockouts with a high density of allelic series will make it a very useful and handy tool critical for gene function analysis. Since insertion sites can be readily mapped using tags, libraries of mutations can be created for browsing by the community. For TILLING this is not so easy, but one can envisage that the use of next-generation sequencing will come into play as costs diminish, so that it will be more effective to use this technology to resequence whole EMS-mutagenized populations and database their full mutation landscapes. There are research efforts in this area currently for plants (e.g. http://tilling.ucdavis.edu/index.php/TILLING-by-Sequencing).

**BEYOND MUTAGENESIS**

The prediction of Henikoff and Comai (2003) that TILLING had the potential to become a standard reverse genetic strategy for plant functional genomics is well on its way to being realized. Its ease of use, widespread applicability, relatively low cost (when plant population costs are taken into account), and ability to generate allelic series including knockouts (albeit at a lower frequency than insertions) give it advantages over other technologies. Its non-GM technology provides a further asset that has facilitated its rapid move from model to crop legumes. Similarly, the beauty of Tnt1 insertion mutagenesis in M. truncatula in generating multiple knockouts with a high density of allelic series will make it a very useful and handy tool critical for gene function analysis. Since insertion sites can be readily mapped using tags, libraries of mutations can be created for browsing by the community. For TILLING this is not so easy, but one can envisage that the use of next-generation sequencing will come into play as costs diminish, so that it will be more effective to use this technology to resequence whole EMS-mutagenized populations and database their full mutation landscapes. There are research efforts in this area currently for plants (e.g. http://tilling.ucdavis.edu/index.php/TILLING-by-Sequencing).

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