Nodules Under the Molecular Microscope

Judith Webb, Leif Skøt and Tony Gordon

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Legumes such as clover, peas and beans play a major role in sustainable agriculture throughout the world. In a symbiotic partnership with soil bacteria (Rhizobia) they are able to absorb gaseous nitrogen from the atmosphere. This process takes place in root nodules where sugars supplied by the plant fuel the fixation of nitrogen by the bacterium. Research at IGER is directed towards understanding the process of nitrogen fixation and the genetic interactions between legume and bacterium. Genetic modification of a small model legume, *Lotus japonicus*, has opened up new avenues of research. Not only can this genetic approach identify relevant new genes, it can also create mutants in specific metabolic pathways and potentially generate plants with agriculturally important traits, such as resistance to environmental stress.

Identifying new genes
A small genome, short growth cycle and self fertility make *L. japonicus* an ideal plant for a gene tagging programme. Using this plant, research at IGER forms part of an international effort which aims to identify and characterise plant genes specifically.
involved in nodulation and nitrogen fixation. However, other genes of commercial value, such as those controlling tannin biosynthesis and resistance to pests, may also be discovered.

Gene expression is controlled by portions of the genome called promoters which can be influenced by a variety of chemical and environmental signals. The approach we have used to tag and identify genes is promoter trapping. This involves inserting a reporter gene, coding for the enzyme ß-glucuronidase (GUS) into the plant genome. This reporter gene can only be expressed, and therefore detected, when it inserts close to an active plant promoter. Cells and tissues which express GUS can easily be seen using a chemical reaction which results in the production of a blue dye (Figures 4.1 & 4.2).

We have screened roots at various stages of nodule formation from about 300 transformed plants and found seven plants with stable GUS activity in root nodules. This means that some genes expressed in nodules have been tagged with the GUS gene. Some of these lines are being studied in detail. For example, in line T90 we found GUS activity in immature, mature and senescent nodules and in root hairs and root epidermal cells following inoculation with Rhizobium loti (Figure 4.1 & 4.2). This line contains one copy of the inserted gene which was transmitted to the progeny in the expected Mendelian ratio - with 72% of the progeny expressing GUS in their nodules. Observations with an electron microscope, coupled with the use of antibodies to the GUS protein, indicate that the GUS gene is expressed in the cytoplasm of various nodule cells.

Expression of the GUS reporter gene in nodules implies that it has been inserted into the plant’s genome near to a nodule-specific promoter and gene. Our next step is to use the reporter gene to isolate the
tagged promoters and, ultimately, characterise the genes from the tagged plants. Knowledge of the functions and sites of action of nodule-specific promoters and genes will help unravel the complex interactions between plant genes and \textit{Rhizobium} during the process of nodulation and nitrogen fixation.

**Creating mutants**

Traditionally, biochemical pathways are studied either by finding plants with a naturally occurring mutation, or by creating and selecting new mutants. Genetic modification can yield new mutants with absolute accuracy using antisense RNA technology (see box below).

**ANTISENSE RNA TECHNOLOGY**

RNA and DNA hybridise to one another by complementary attraction of the bases Adenosine (A) to Thymidine (T), and of Guanosine (G) to Cytidine (C). The plant is transformed by inserting an antisense gene into the genome. The base sequence is complementary to that of the natural gene so that when it is expressed, the messenger RNA produced hybridises to the normal mRNA and interferes with its translation into protein. This can result in reduced amounts of the target protein.

At IGER we have applied this technique to an enzyme which is involved in carbohydrate

![Figure 4.3](image-url)  
**Figure 4.3** Antisense sucrose synthase transformed root cultures had reduced sucrose synthase mRNA (Top panel. The bands at 2.8 and 0.9 kb are the native message and the antisense message, respectively) and reduced sucrose synthase activities (bottom) compared to controls (lines 8196.1 and 8196.8).

![Figure 4.4](image-url)  
**Figure 4.4** (A) Control plant (left) and progeny of transformant with reduced sucrose synthase activity in their nodules (centre and right), and (B) Sucrose synthase activities in nodules of progeny and control plants.
Two enzymes, alkaline invertase and sucrose synthase, are both capable of metabolizing sucrose within the nodule. We believe that sucrose synthase plays the central role in the metabolic pathway of sucrose utilization and is necessary for normal nodule function. Support for this idea comes from experiments with soybean plants. We have found that sucrose synthase (but not invertase) activity in the nodule is reduced, in parallel with reduced nitrogen fixation, when plants are subjected to a variety of environmental stresses.

We have genetically modified *L. japonicus* with the antisense sucrose synthase gene under the control of a constitutive promoter. Our first step was to test the general strategy by inserting the antisense gene using *Agrobacterium rhizogenes* which only produces root cultures. Both the normal sucrose synthase RNA message and enzyme activity in the experimental roots was greatly reduced in comparison with the control roots (Figure 4.3).

We used *Agrobacterium tumefaciens* to transform *L. japonicus* and to subsequently re-generate plants expressing the antisense gene. So far we have studied progeny from one self-fertilised transformant (T1A). Nodules from these plants showed exactly the same response as in the root cultures - reduced sucrose synthase transcript levels and reduced enzyme activity. Some of those plants with reduced nodule sucrose synthase activity were yellow and smaller than the control plants - suggesting that lower sucrose synthase activity resulted in nitrogen deficiency (Figure 4.4). This supports our hypothesis that nodule sucrose synthase is vital for nitrogen fixation. Additional evidence for this idea comes from a mutant of pea, called *rug-4*, generated by Trevor Wang and Cliff Hedley at the John Innes Centre, IPSR, Norwich. In collaboration with this group, we found that sucrose synthase activity in nodules of this pea mutant was significantly reduced and the plant’s ability to fix nitrogen was correspondingly impaired.

We are analysing other transformed plants modified with this antisense gene, under the control of either the constitutive promoter or a nodule-specific promoter. These studies will help us to confirm the importance of sucrose synthase in root nodules and also to explore its role in other aspects of plant growth and development.

Future research on nodules will also include altering the sensitivity of the sucrose synthase gene to environmental changes by substituting its own promoter with another nodule-specific promoter which is less sensitive to environmental change. It is possible that such plants could continue to fix nitrogen even when under stress conditions, such as drought or in the presence of a high concentration of fertilizer nitrate, which normally suppress nodule activity.

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