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New techniques for studying competition by Rhizobia and for assessing nitrogen fixation in the field

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Abstract

One of the key factors limiting the proper assessment and use of rhizobial strains in the field is the lack of suitable methodology to screen the success of individual isolates in competing for nodule occupancy with different cultivars of legumes and in different soil and agronomic conditions. The use of marker genes enables individual rhizobial strains to be identified by a simple colour assay, thus enabling a dramatic increase in throughput of strain screening. One such marker system for rhizobial ecology, the GUS system, is already in use to facilitate rapid screening of rhizobial isolates. Other markers, which will allow the competitive behaviour of several strains to be studied at once, are under development.

Likewise, breeding of the host legume for a high efficiency of nitrogen fixation is hampered by the difficulty in assessing this property. The method which currently gives the highest throughput of analysis, and has been successfully used in soybean breeding programs, is the ureide technique. However, it remains somewhat laborious for use in routine breeding programs. In this paper we discuss the potential use of reporter genes to provide information on the relative levels of ureides and other nitrogenous compounds in plants growing in the field. This would greatly increase the rate at which this trait could be scored, and would thus enable routine assays for increased symbiotic nitrogen fixation for breeding or management purposes in legume crops such as soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*).

Introduction

Everyone who has worked in the field of biological N_2 fixation (BNF) knows what a dramatic effect it can have on plant growth. We have all carried out controlled experiments in the glasshouse and demonstrated the "big plant, little plant" phenomenon — luxuriant healthy green plants that have been inoculated with a compatible *Rhizobium* strain contrasting with slight, pale green plants that are starved of nitrogen (N).

The problem is transferring such spectacular results to the field. There are at least two limitations. Firstly conditions in the field are never entirely devoid of N, and secondly there are usually also some compatible rhizobia present; hence, the big plant-little plant difference is rarely apparent. Nevertheless, even where no major yield increases occur as a result of BNF, some plants may be obtaining more of their N from symbiotic N_2 fixation than their neighbours. In fact, an equally important challenge may be not to increase overall N in the crop dramatically, but to increase the proportion of N that comes from BNF, either through breeding (Herridge and Danso, 1994) or through effective management strategies (Peoples et al., 1995b).

This is difficult to achieve however, because of limitations in methodology. It is easy to measure N_2 fixation in the glasshouse because it is reflected very directly in differences in shoot weight — the N-difference method. Likewise, because we can use single strain inocula and there are no competing rhizobia in vermiculite, sand or perlite, we can determine the fixation efficiency of individual rhizobial strains with ease. The problem comes in transferring these measurements to the field. How do we assess the effect of an inoculant strain in a background of competing rhizobia? How do we measure the proportion of N derived from fixation in a plant that may have no more total N content than its poorly fixing neighbour?

In this paper we discuss novel methodologies based on the use of reporter genes that will greatly expand our ease of measuring these factors in the field and will help us to make better use of this free and renewable source of N in agriculture.

Reporter genes: The fundamental tool

A reporter gene is exactly that — a gene which reports on some biological phenomenon that the researcher wants to gain information about. The original concept was developed in the early 1960s to facilitate the study of gene regulation, and is a very powerful and pervasive one.

Most gene products — proteins — are very difficult to detect or assay. Hence, study of the regulation of a gene — determining when, and in response to which developmental and/or environmental signals it makes its product — is very tedious. The trick underlying the use of reporter genes is to substitute the part of the DNA that codes for the difficult-to-assay protein, with a piece of DNA that specifies a protein which is easy to detect (Fig. 1). This gene is the reporter gene. It almost invariably codes for an enzyme which can be assayed in a simple, standardized, spectrophotometric, fluorometric or colour assay.

This use of reporter genes is possible because the region of DNA coding for the protein is (by and large) physically distinct from that which regulates activity of the gene. Thus, substituting one "coding region" with another (the reporter gene) has little impact on the regulation of the gene. It is then possible to monitor expression of the reporter gene using well defined procedures, and be confident that it will only be produced in response to the same developmental and environmental triggers as the original gene.

Reporter genes were developed by molecular biologists and have primarily been used by them to minutely examine the DNA sequences that determine the when, where, how and why of gene expression. However, because the synthesis of many specific gene products

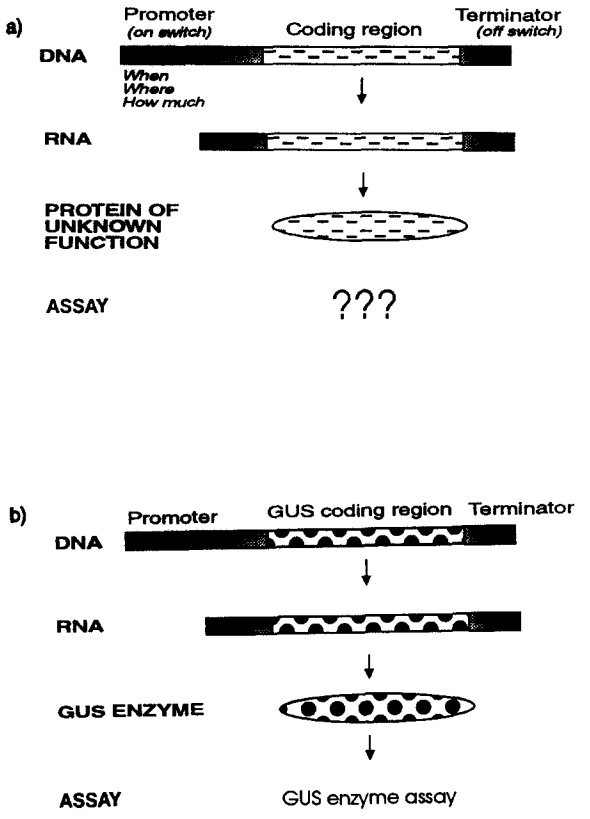

Fig. 1. Structure and functioning of a reporter gene. **a)** represents the synthesis of RNA and protein from a gene of unknown function. It is therefore difficult to assay the resulting protein. In **b)**, the coding region of the unknown gene has been substituted with the GUS coding region — the *gusA* reporter gene. This is still regulated in the same way as the original gene, but the assay showing expression of the gene becomes a routine GUS assay.

is known to be responsive to specific environmental signals, we can turn this concept on its head, and use production of the reporter gene as a beacon indicating when a bacterium or a plant has received a particular environmental signal. The reporter genes are then being used as bioindicators.

Bioindicators are already used routinely in diagnostic tests. For example, in mammalian physiology, pregnancy tests rely on detection of the hormone chorionic gonadotropin through an enzyme-linked immunoassay. In effect the hormone is the bioindicator of pregnancy and its presence is being detected through a combination of antigenic detection with an enzyme assay. A reporter gene can be used in a way that is highly analogous — the enzyme is reporting on the production of a protein which is diagnostic of a particular physiological state. The difference is that, rather than having to be coupled to a immunological assay, the reporter enzyme encoded by a reporter gene is produced directly by the organism at the same time and place as other molecules that reflect a specific physiological state.

There are already some examples where a reporter gene has been used to measure an environmental parameter. For example, Heitzer et al. (1992) engineered bacteria which luminesce in response to naphthalene and salicylate bioavailability, and Reches et al. (1994) have developed reporter bacteria to monitor levels of the trace element selenium. Later in this paper we will discuss how reporter genes could be used to report on the efficiency of N_2 fixation.

Choice of reporter genes

The key requirement for an effective reporter gene is that it is easy to assay. Thus most reporter genes encode enzymes with straightforward, quantitative, assays. In the examples being discussed in this paper, the constraints imposed on detection of the reporter gene are quite severe, as we wish to be able to detect their activity at the very least in a simple laboratory which might be available on an agricultural research station, and ideally directly in plants growing in the field. Additionally, the assay must he extremely high throughput and must be inexpensive.

The primary reporter gene used in plant molecular biology at present is *gusA* encoding the enzyme β-glucuronidase or GUS (Jefferson et al., 1987). GUS is a hydrolase which will cleave a very wide range of substrates — almost any aglycone conjugated to D-glucuronic acid in the β configuration. Thus a variety of histochemical and spectrophotometric assays are available. The greatest advantage of GUS is the nearly complete lack of endogenous activity in plants and most agriculturally important organisms, coupled with the simple assays for its activity with spatially restricted chromogenic substrates — leaving insoluble coloured compounds at the site of enzyme activity (Jefferson and Wilson, 1991).

At present, GUS assays must be carried out in a destructive manner, as cells must be permeabilized with destructive detergents to allow entry of hydrophilic GUS substrates. However, modifications are underway to convert GUS to a secreted enzyme such that exogenously applied substrates can gain free access to the enzyme trapped between the cell membrane and the cell wall. This leads in principle to the possibility that enzyme activity could be detected by the simple expedient of painting substrate onto

the leaf blade and looking for an easily visible colour change.

A second reporter gene which is under development is another hydrolytic enzyme, an arylsulfatase (ARS), which cleaves a sulfate residue from almost any sulfate-substituted organic ring compound (an arylsulfate). ARS is produced by a wide variety of animals and microbes, but has not been detected in plants, and thus will serve as a second reporter gene with no background activity in plants. Most importantly, the enzyme appears to be naturally secreted and localized to the cell wall in yeast strains which we have isolated. Addition of substrate to cells that express ARS does not kill or inhibit growth of the cells, presumably because the substrates do not have to penetrate the living cell and its membrane system to come in contact with the enzyme. Thus, once the gene is isolated and characterized, this will provide a second reporter system that will allow in vivo detection of the enzyme.

As well as using secreted reporter enzymes to detect reporter gene activity in living cells, another approach is to use reporter enzymes which act on endogenous substrates within the cell to give rise to a coloured — and hence easily visible — product. A potential source of such genes is from bacteria which are naturally pigmented. For example, a single gene has been isolated from *Rhodococcus* which confers production of the pigment indigo on the laboratory bacterium *Escherichia coli* using the endogenous substrate indole (created from tryptophan by *E. coli* tryptophanase; Hart et al., 1992). Another bacterial pigment system with potential as a reporter system is the violacein operon from *Chromobacterium violaceum* (Pemberton et al., 1991). A set of six genes has been identified which confers production of violacein on *E. coli* from the substrates tryptophan and oxygen. The role of each gene is not yet known, but subsets of the genes will give rise to different coloured compounds, and a single gene from this pathway can lead to production of an unknown blue pigment in *E. coli* (D Quiggin, P Keese and L Graf, pers. commun.).

The relative advantages of both approaches have yet to be tested. The advantage of using a secreted reporter enzyme which requires exogenous substrate is that the plants are largely unaffected, assuming that there are no natural substrates for the enzymes (which is the case for GUS and arylsulfatase). On the other hand, the assay still requires some kind of action to be performed on each plant. The use of reporter genes which act on endogenous compounds means that a plant — or plant parts — will naturally change colour in response to the chosen physiological or environmental parameter. This allows for extremely inexpensive and rapid screening, but has the disadvantage that the assay could be cumulative — depending on how stable the coloured product is. It is also possible that metabolism of endogenous substrates by the reporter gene will have an adverse effect on the plant. Nevertheless, this type of reporter system could be invaluable in designing sacrificial "sentinel" plants which are scattered throughout the field and report visually on particular physiological stresses.

Having introduced the concept of reporter genes and their use to report on physiological and environmental assays, we will now discuss one example — rhizobial ecology — where they are already in use — and subsequently the possibility of using reporter genes to enhance the ureide assay for efficiency of N_2 fixation.

Microbial ecology: detection of inoculant strains using simple colour coding

One of the key limiting factors in ecological analysis of field performance of inoculated rhizobial strains is the difficulty in distinguishing the introduced strain from indigenous rhizobia. Current methodologies, including various antigenic techniques and the use of antibiotic resistance markers, all require individual analysis of single nodules and hence are labour intensive and limit the amount of data that can be gathered. This is true also of other molecular biological approaches that determine nodule occupancy by analysis of the DNA present in a nodule. This labour intensity means that sample sizes analysed are generally very small — typically 20-30 nodules per plot (e.g. Somasegaran and Hoben, 1985) in a plot that might contain 10^3–10^4 nodules in total, and, coupled with the fact that there is known horizontal (e.g. Wollum and Cassel, 1984) and vertical (e.g. McDermott and Graham, 1989) variation in the profile of strains occupying nodules, this means that obtaining highly accurate values for nodule occupancy is extremely difficult (see Wilson, 1995) Perhaps as importantly, the laboriousness of current assays means that there is no routine screening procedure to assess how successful a given highly effective strain would be in diverse soil types or with novel cultivars of a host plant.

We have developed a system which uses the GUS reporter system to report on the presence or absence of a specific strain in a nodule (Wilson et al., 1991;

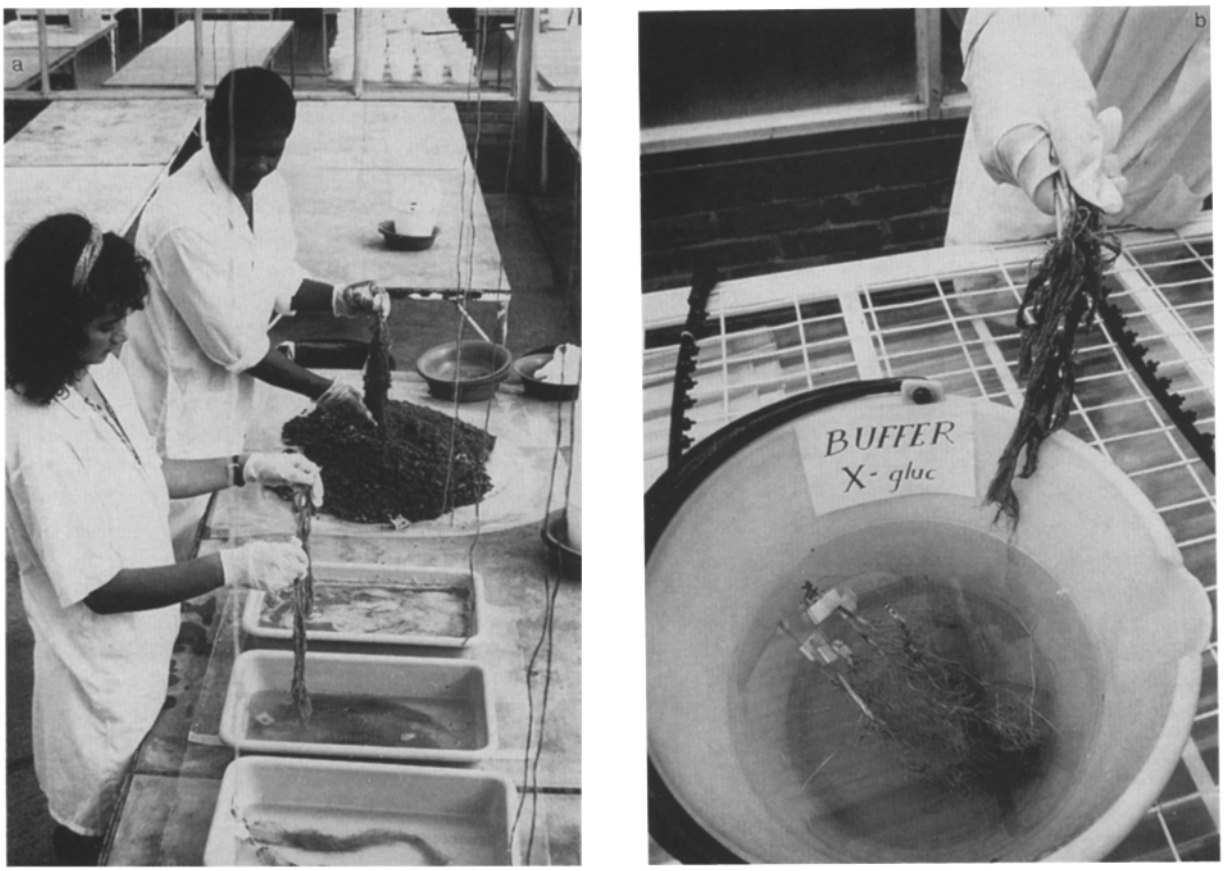

Fig. 2. Reporter gene assays in a bucket. Roots from soil-grown *Phaseolus vulgaris* plants inoculated with GUS-marked strains of *Rhizobium etli* are first washed (**a**) to remove excess soil, then assayed directly in X-Gluc buffer in a bucket (**b**) to determine nodule occupany by the inoculant strain at CIAT in Colombia (photograph courtesy of Doug Beck).

Wilson, 1995) Here again, the reporter gene is being asked to report visually on something that is otherwise difficult to detect and assay.

First a rhizobial strain of interest is marked with GUS in a simple bacterial mating procedure which requires only routine microbiological equipment. The assay for nodule occupancy then becomes trivial: the whole root system of an inoculated plant is incubated in buffer containing an indigogenic GUS substrate and nodules occupied by the marked rhizobia are detected by virtue of a simple colour change There are now a number of indigogenic substrates that give rise to differently coloured products These include: 5-bromo-4-chloro-3-indolyl glucuronide (X-GlcA or X-Gluc) which gives rise to a blue coloured precipitate on cleavage by GUS; 5-bromo-6-chloro-3-indolyl glucuronide (Magenta-GlcA), giving rise to a magenta colour; and 6-chloro-3-indolyl glucuronide (Salmon-GlcA) giving rise to a salmon colour. Thus nodules occupied by a GUS-marked strain can be detected as blue, magenta or pink-coloured (see Figure 1 in Wilson, 1995; and front cover of this volume).

As there is no endogenous GUS activity in either the plant or in any rhizobial strain tested so far, development of the characteristic colour is completely diagnostic of nodule occupancy by the inoculant strain. Moreover the assay is so straightforward that at CIAT (Centro Internacional de Agricultura Tropical) in Colombia, this assay is carried out by immersing whole root systems in buckets containing the GUS substrate X-GlcA (Fig. 2). The use of buckets as a reaction vessel for a "molecular" reaction is clearly novel, and illustrates the extent to which tools based on molecular biology can really be taken to field-level, large-scale analysis. This assay greatly increases the number of nodules that can be analysed for nodule occupancy — it now

becomes logistically feasible to analyse all the nodules on all the plants sampled from a plot — and also retains information regarding the positional location of nodules induced by the inoculant strain along the root.

The assay has so far been used with *Bradyrhizobium* strains that nodulate cowpea (*Vigna unguiculata* (Wilson et al., 1991)) and soybean (*Glycine max*) (G Hardarson, pers. commun.), and with *Rhizobium* sp. inoculated onto siratro (*Macroptilium atropurpureum*) and pigeonpea (*Cajanus cajan*) (Wilson, 1995; Wilson et al., 1995) and various *R. tropici* (Streit et al., 1992; Sessitsch et al., 1995) and *R. etli* strains (Streit et al., 1995) on *Phaseolus vulgaris*. Examination of the competitive ability of marked strains indicates that derivatives can rapidly be identified with competitive ability that differs little from the wild-type (Sessitsch et al., 1995; Streit et al., 1995), and that these marked strains then allow rapid, and highly reproducible competition assays. Enhancements underway for the near future include the development of additional marker genes that will give rise to differently coloured products; these will allow multi-strain competition trials by such straightforward assays, and will also allow ready assessment of dual strain occupancy of nodules.

As well as being used for rhizobial nodule occupancy studies, marker genes can be used for studies of rhizosphere colonization. For example, the GUS system allows detection of some of the earliest stages of root colonization and infection (de Boer and Djordjevic, 1995). It has recently been used in this manner to monitor infection of roots by the fungus *Fusarium oxysporum* (Couteaudier et al., 1993) and of tomatoes (*Lycopersicon esculentum*) by the fungus *Cladosporium fulvum* (Oliver et al., 1993) and to study colonization of maize (*Zea mays*) para-nodules by an *Azospirillum* species (Christiansen-Weniger and Vanderleyden, 1993). In an analogous manner, the *lacZ* gene encoding β-galactosidase was recently used to monitor colonization of wheat (*Triticum aestivum*) roots by *A. lipoferum* — although here it is necessary to first treat the roots so that the endogenous β-galactosidase activity in the plant is eliminated (Katupitiya et al., 1995).

Perhaps the most powerful demonstration of the practical utility of the marker gene approach to date is the use of GUS-marked *Cladosporium fulvum* as a bioassay to rapidly screen a mutagenized population derived from a fungus-resistant tomato line, and to readily identify susceptible derivatives (Hammond-Kosack et al., 1994). These examples all demonstrate that there is also great potential for using GUS and other marker genes in studies of associative N_2 fixation between diazotrophs and non-legumes such as cereals or C4 grasses (Boddey et al., 1995).

Bioindicators: reporter genes to measure N_2 fixation efficiency in the field

The use of legume plants in cropping systems can serve to greatly reduce the need for application of chemical nitrogenous fertilizers. The potential agronomic, economic and environmental benefits are great, particularly in less developed countries where economic constraints are often the overwhelming factor — if a farmer simply cannot afford to buy chemical fertilizers, inclusion of legumes in the cropping system may be the only possibility for maintaining, let alone increasing, soil N fertility. However, no legume plant derives 100% of its N from atmospheric N_2 unless it is grown in a totally N-free environment in the greenhouse; rather a proportion varying from 0 to 90% is usually derived from fixation and the remainder is taken up from the pool of available N in the soil, so exploiting soil N reserves just like any other plant. Clearly the extent to which N benefits can be derived from use of legumes depends on the proportion of N which is actually derived from BNF. However, in the field it may not be possible to easily identify poorly-fixing plants from actively fixing neighbours on the basis of crop growth or N content alone unless the soil is depleted of plant-available N (Table 1).

There are many factors governing the extent of N_2 fixation in a legume crop. One critical factor is the availability of compatible, effective and competitive rhizobia in the soil, whether indigenous or inoculated. The second key component is the genotype of the plant. The proportion of nitrogen (P_{fix}) derived from fixation for a field-grown crop can vary immensely even within a single species (see Giller and Wilson, 1991; Peoples and Herridge, 1990; Peoples et al., 1995a) indicating that there is strong genetic potential for improving the contribution of BNF. However, emphasis on N_2 fixation as one of the important traits in breeding programs for legumes has been relatively rare despite its link with yield potential in infertile soils. This could be for a number of reasons, but one of the overriding limitations is the difficulty in actually measuring the amount of N_2 fixation (Herridge and Danso, 1995).

The one technique which can be readily and relatively inexpensively used to evaluate N_2 fixation in most tropical legumes involves the measurement of

Table 1. Effect of prior management and inoculation with *Bradyrhizobium japonicum* on the accumulation of crop nitrogen, the proportion of plant nitrogen derived from N_2 fixation (P_{fix}) and the amount of N_2 fixed by soybean (*Glycine max*)[a]

Pre-treatment	Inoculation rate (bacteria seed^{-1})	Total crop N (kg N ha^{-1})	N_2 fixation	
			P_{fix} (%)	Amount[c] (kg N ha^{-1})
Cropped to cereal (18)[b]	0	108	0	0
	5×10^7	267	56	149
Fallow (38)[b]	0	246	0	0
	5×10^7	347	9	32

[a] Data of Bergersen et al. (1989).

[b] Levels of soil mineral N (mg N g dry soil^{-1}) in the top 10 cm of soil at the time of sowing soybean.

[c] Amount of N_2 fixed = (Crop N)) $\times \frac{(P_{fix})}{100}$.

ureides. This technique relies on the observation that in certain legumes pathways of assimilation of N derived from fixation and from soil are different: ammonia derived from symbiotic fixation is converted into the ureides, allantoin and allantoic acid, in the nodule and then transported to the shoot in this chemical form in the transpiration stream; in contrast, N taken up from the soil, which is primarily nitrate, is transported either directly as nitrate or is assimilated into the amino acids asparagine or glutamine in the root prior to transport (Herridge and Peoples, 1990). Therefore, xylem sap composition changes from one dominated by ureides in fully symbiotic plants to one dominated by nitrate and amino acids in poorly nodulated plants utilizing soil N for growth. The proportion of total sap N formed by ureides has been shown to be a reliable indicator of the P_{fix} for a number of legume species (Table 2), and comparable field estimates of N_2-fixation can be obtained using either ureide or ^{15}N-based methodologies (Herridge et al., 1990).

The ureide technique: present use and future potential

The application of the ureide technique to measurement of N_2 fixation is discussed extensively in Peoples et al. (1989a). In essence, a sample of xylem sap containing N being transported from root to shoot is collected, and the amounts of the different N-containing compounds within that sample are measured. The sample can be obtained in one of three ways: root-bleeding sap, shoot tissue extract, or vacuum extracted sap (Herridge and Peoples, 1990). Approximately 150 samples can be collected per day with a team of six people working in the field. The samples are then taken to the laboratory and the determinations of the different N-solutes are done using spectrophotometric methods. A different set of colorimetric reactions have to be carried out on each sample to determine separately ureides and nitrate, and if necessary amino acids.

The ureide technique has been successfully applied to breeding programs involving soybean (Herridge and Danso, 1995). The procedure was used to identify individual lines and crosses with enhanced capacity to fix atmospheric N_2 in the presence of soil nitrate. Using the currently existing technique, a complete set of analyses can be undertaken for about 800 plants in four weeks (Herridge and Danso, 1995). To be truly useful to a legume breeder wishing to select for improvements in symbiotic performance, it would be necessary to be able to screen 1,000–2,000 plants per day (10–20,000 per fortnight) (D Herridge, pers. commun.). One way to achieve this would be to build on the great power of the ureide technique for measuring N_2-fixation by simplifying the assay procedure using reporter genes so that such rapid screening is possible. The ureide assay would then become an even more powerful tool for breeding of high N_2-fixing legumes.

Another powerful application of the use of bioindicators would be to use transgenic "sentinel plants" scattered among the main crop in the field to report

Table 2. Legume species where the relative abundance of ureides in xylem sap have been correlated to the proportion of plant nitrogen derived from N_2 fixation (P_{fix}) estimated using ^{15}N techinques

Species		Reference
Crop legumes		
Soybean	*Glycine max*	McClure et al. (1980)
		Herridge and Peoples (1990)
Common bean/dry bean	*Phaseolus vulgaris*	Peoples and Herridge (1990)
		Hansen et al. (1993)
Pigeonpea	*Cajanus cajan*	Peoples et al. (1989b)
Cowpea	*Vigna unguiculata*	Pate et al. (1980)
		Peoples and Herridge (1990)
Rice bean	*V. umbellata*	Rerkasem et al. (1988)
Green gram	*V. radiata*	Peoples and Herridge (1990)
Black gram	*V. n.ungo*	Peoples and Herridge (1990)
Forages and legume cover crops		
Calopogonium caeruleum		Faizah and Peoples, unpubl.
Desmodium ovalifolium		
Macroptilium atropurpureum		
Shrub legumes		
Desmodium rensonii		Herridge et al. (1995)
Codariocalyx gyroides		

on levels of BNF. In this case, the main crop would not be engineered with the reporter genes, and the sentinel plants would be used for rapid evaluation of the impact of management on N_2 fixation. It is clear that it may be possible to impose a range of management strategies to manipulate and enhance the proportion of N derived from BNF (Peoples et al., 1995b), and evaluation of the impacts of different strategies could be streamlined using such sentinel plants.

Use of bioindicators to measure N_2 fixation efficiency

In this example the strategy would be to develop reporter genes to report on the levels of different nitrogenous compounds in the sap of an N_2-fixing plant. As discussed earlier, bioindicators can be either endogenous molecules or enzymes that result from the expression of an introduced gene. Thus, in the conventional ureide assay, allantoin and allantoic acid are bioindicators indicating the reliance of a legume on N_2-fixation for growth. An alternative route, which could both speed the assays and make them more reliable and also would avoid the dangerous or toxic chemicals normally required for colorimetric assays in the laboratory, would be the use of reporter genes whose expression would reflect the relative levels of the different nitrogenous compounds.

Engineering response of reporter genes to reflect levels of nitrogenous compounds

Two different reporter gene strategies would be possible. One would be to engineer reporter bacteria either bacteria which report on the levels of nitrogenous compounds in a tissue extract, or endosymbiotic bacteria which could report on the levels of these compounds directly in the plant. The second approach, and the long-term goal, would be to engineer the plants themselves to report directly on their N status while growing in the field.

The concentration of amino-N in sap does not vary much with varying dependence on N_2 fixation (Herridge and Peoples, 1990), so the relative ureide index, the indicator of P_{fix} (see Peoples et al., 1989a), can be based on the two N-solutes which are most sensitive to changes in the source of N for growth:

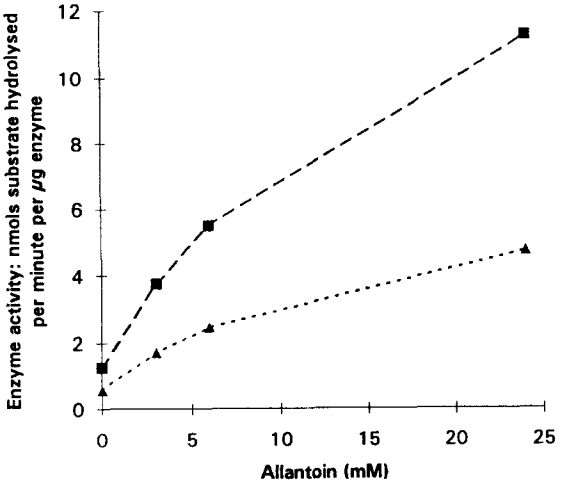

Fig. 3. Induction of allantoinase and hypothetical induction of a GUS reporter gene in response to different levels of allantoin (figure based on Venkateswara Rao et al., 1990). –▲– allantoinase activity; –■– GUS activity.

$$\text{Relative ureide index}(\%) = \frac{4(\text{ureide}) \times 100}{4(\text{ureide}) + \text{nitrate}} \quad (1)$$

Hence the most important compounds to measure are the ureides and nitrate. The approach therefore would be to engineer reporter systems to respond quantitatively to the levels of these two compounds.

a) Use of bacterial reporters

The bacterial approach is most likely to succeed in the short term, because N metabolism and its regulation are better understood in bacteria than in eukaryotes, and because it would not involve production and use of transgenic plants.

Little work has been carried out on ureide metabolism in bacteria. There is one report on allantoin metabolism in *Bradyrhizobium* strain NC92, a symbiont of peanut and pigeonpea as well as other legume crops (Venkateswara Rao et al., 1990). Allantoinase activity was identified in this species and activity of the enzyme was reported to be inducible by allantoin. Importantly for the reporter gene strategy, the extent of induction was proportional to the concentration of allantoin in the medium over a range from 0.3 to 24 m*M* allantoin (Fig. 3). Since levels of ureides reported in xylem sap of nodulated soybean, cv Bragg, ranged from 0.3 to 2.5 m*M* (Herridge and Peoples, 1990), the implication is that the bacterial gene regulatory sequences would be sensitive enough to respond to physiological levels of ureides.

By contrast, a great deal of work has been carried out on general N metabolism in bacteria, particularly in enteric bacteria and in rhizobial species (reviewed in Merrick, 1992). The system is highly complex and is affected (in different species) by factors such as whether bacteria are growing aerobically or anaerobically, and what N sources are available. In *E. coli*, induction of nitrate reductase is primarily regulated by the ambient oxygen tension, being stimulated in microaerobic conditions (2% oxygen). However, it is also responsive to levels of nitrate (Dong et al., 1992), and the genetics of *E. coli* is sufficiently well understood that it would be a relatively simple matter to break these two control circuits apart and engineer an *E. coli* strain in which nitrate reductase was induced directly in response to levels of nitrate. If a reporter gene was engineered to respond to nitrate in the same way, such a strain could then act as the sensor of the soil N component in the xylem sap of a plant.

As well as using existing knowledge to identify genes which are induced in response to the presence of specific nitrogenous compounds, it is possible to use a "promoter-probe" approach to screen for genes which respond in the desired way. A reporter gene without DNA regulatory elements is introduced into a bacterium — *E. coli*, or perhaps a *Rhizobium* strain, and the bacteria are screened for ones in which the desired response is obtained. For example, a *Rhizobium* strain with an introduced promoterless GUS gene could be screened in the presence and absence of allantoin and allantoic acid for strains which made blue colonies, indicating induction of the reporter gene, in the presence of these ureides, but not in their absence.

Whichever approach is followed, construction of bacterial strains carrying easily detectable and distinguishable reporter genes which respond to ureides, and to nitrate, is certainly feasible. It would then be necessary to fine tune the system so that activation of these genes parallels levels of the inducer compounds over a certain range, and to ensure that the induction is not affected by other molecules which might be present in the plant sap. It would also be important to combine the two reporter genes in a single indicator bacterial strain so that the two reporter gene activities directly reflected relative levels of ureides and nitrate and complications were not introduced by varying bacterial population sizes. The end result should be a bacterial indicator strain which allows rapid, reliable and safe analysis of relative ureide index.

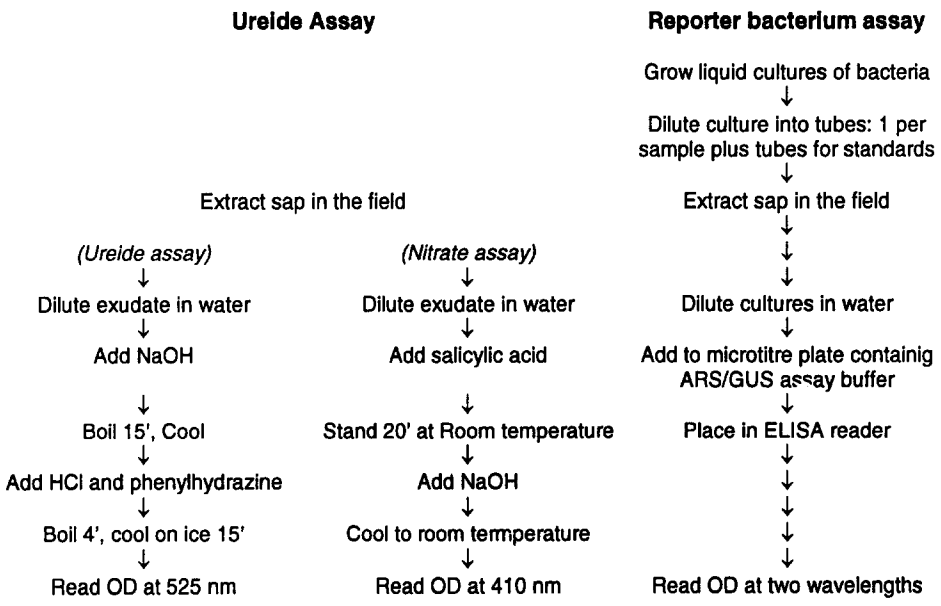

Fig. 4. A comparison of the conventional ureide assay and the steps involved in a reporter bacterium assay.

The procedure for using a reporter bacterial strain to analyse relative ureide index could be envisaged as follows. Prior to the assay, a culture of the reporter bacteria would be grown up, diluted in fresh medium to a concentration low enough to enable fresh growth, and distributed among enough tubes to allow one tube per sample of sap. The sap would be collected in the field as normal for the ureide assay, and a given volume would be placed into a tube containing the reporter bacterial strain. The assay would effectively begin at this point, as the bacteria would then be being incubated in a given concentration of allantoin, allantoic acid and nitrate, and would grow and induce the reporter enzymes during transport from the field to the laboratory. In contrast to the conventional ureide assay, where the speed of transport of the sap sample to the laboratory or storage on ice is important so that the key compounds do not break down prior to analysis, the warm field conditions that are often prevalent will actually be an advantage as they will facilitate growth of the bacteria and expression of the reporter enzymes.

Once in the laboratory, aliquots of the bacterial/xylem exudate mix would be added to a non-toxic buffer containing a colorigenic substrate for each reporter enzyme. Ideally, if substrates with different absorption maxima are available, both enzymes could be assayed in a single reaction vessel. Alternatively, two parallel reactions could be set up, one for each reporter enzyme. The reaction(s) would then be allowed to proceed until colour that is clearly visible to the eye develops. At that point, the reaction would be stopped and the absorbance read on a spectrophotometer. Calculation of the rate of hydrolysis of substrate for each of the two reporter enzymes will reflect the level of induction of each reporter gene, and hence the approximate concentration of the inducer compounds. Since both reporter genes would be in a single bacterial strain which was responding to a single sap sample, the ratio of these two reporter gene activities would directly reflect the relative levels of ureides and nitrate. For example, if GUS induction reflects ureides, and ARS induction reflects nitrate, the relative ureide index can be calculated as: $(4 \times \text{GUS}/(4 \times \text{GUS} + \text{ARS})) \times 100$.

The steps involved in the current procedure and a reporter gene one are compared in Figure 4. Advantages are: the reduced number of steps; the fact that the assay now depends on measurement of the reporter enzymes which are robust rather than that of the signature compounds, which are fragile; the complete avoidance of toxic or dangerous compounds. The laboratory infrastructure required is very similar to the conventional ureide and nitrate assays. However, because this procedure has fewer steps, it lends itself more readily to automation using microtitre plates and automatic reading and calculation of the data. Given that it is pos-

sibly to carry out 96 assays in a conventional microtitre dish, if these are processed at the rate of 50 per day using an ELISA reader, this gives a rate of assay of 4,800 samples per day, approximately 60 times higher than the maximal rate of conventional ureide analysis. In this case, it is the rate of sampling of xylem sap which becomes the limiting factor, rather than the rate of analysis of the sap contents.

b) Use of eukaryotic systems

A longer-term goal would be to engineer plants to report directly on their N status in the field. In this case, prokaryotic regulatory circuits might be adapted for use in eukaryotes — there are precedents. However, a far more powerful approach would be to identify genetic regulatory circuits in the plants themselves that respond to the different N sources, and to link these to reporter genes that would function to give distinct colour changes in living plants.

There is a lot of background data indicating that this approach is highly feasible. As far as measurement of nitrate goes, both fungal and higher plant genes encoding the enzyme nitrate reductase are known to be regulated in response to the available levels of nitrate, and hence these regulatory circuits could be adapted to construct gene fusions that would detect different levels of nitrate (e.g. Banks et al., 1993; Cheng et al., 1991).

Genes which respond to ureide levels have not been identified in plants (Winkler et al., 1988), but precedents exist in yeast. *Saccharomyces cerevisiae* has the ability to use allantoin (or allantoic acid) as a sole N source, degrading it in five enzymatic steps to ammonia, glyoxylate and carbon dioxide (the first intermediate is allantoic acid), and the regulation of this pathway has been extensively studied. The enzymes of allantoin degradation and utilization were shown to be induced in response to the presence of allantoin (Cooper and Lawther, 1973), and, while strictly-speaking the inducer is allophanate, the last intermediate in the degradative pathway, the net effect is induction of these enzyme activities in response to levels of allantoin and allantoic acid. There is also an allantoin permease, induction of which is directly caused by the presence of allantoin (Sumrada et al., 1978). This regulation has subsequently been shown to be transcriptionally mediated i.e. the mRNA and hence protein are not made in the absence of inducer, and the DNA sequences required for this response have been identified (Cooper et al., 1987; Yoo and Cooper, 1989). These data indicate that the strategy of looking for analogous regulatory sequences that respond to ureides in plants is highly plausible.

There are numerous methods for isolation of genes whose expression is modulated relative to environmental or physiological parameters. All of them rely on obtaining plant material from each of the two opposite conditions which are under consideration. For example, to identify genes that respond to ureide levels, ureide-producing plants such as soybean would be grown that were either 100% dependent on BNF (maximum ureide production) or which had no N_2 fixation activity (minimum ureide condition). RNAs can then be prepared from both sets of plants and used in a variety of molecular protocols e.g. subtractive cDNA libraries (Duguid and Dinauer, 1990) or arbitrarily primed PCR fingerprinting of RNA (Welsh et al., 1992), to identify mRNAs which are produced specifically under the target conditions (high ureides, in this example). This in turn will lead to identification of the genes and the regulatory sequences, and would enable construction of the appropriate reporter gene fusions.

Thus it would be possible to develop transgenic plants containing two different reporter constructs. One, fused to the GUS gene, produces GUS under high nitrate conditions. The second, fused to the ARS gene, produces ARS under high ureide conditions. Since substrates are available for each which give rise to different colours, it is possible to envisage painting a mixture of the two substrates onto a stem and petiole and looking to see whether it turns predominantly blue (high GUS, hence high nitrate and low fixation) or red (high ARS, hence high ureide and high fixation). Such plants might be used directly in a breeding program, or as sentinel plants to indicate the effect of different farm management practices on the extent of N_2 fixation.

Clearly a vast amount of work would have to be undertaken to fine-tune such bioindicator reporter genes to accurately reflect relative ureide levels, but the examples given here clearly illustrate that at least the basis for this approach exists in the amount of information already available about genetic circuits that respond to N compounds in eukaryotes.

Conclusions

Molecular biology is now reaching a stage of maturity where it can provide tools to facilitate analysis of population dynamics and physiological responses of

microbes and plants in the natural environment, and hence can facilitate decision-making regarding inoculation, management or breeding strategies. This is practically demonstrated by the use of marker gene technology by rhizobial ecologists to rapidly screen the competitive ability of a number of potential inoculum strains for *Phaseolus vulgaris*. In addition, the concept is extended to the future scenario of using reporter genes to report on the extent of N_2 fixation in a crop, either by way of bioindicator bacteria, or directly in transgenic sentinel plants. This concept moves away from the idea of using genetic manipulation only to engineer solutions, but rather uses it as a tool to aid our understanding of the natural environment.

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