

Efficient genetic transformation of grain legumes for improved fungal resistance

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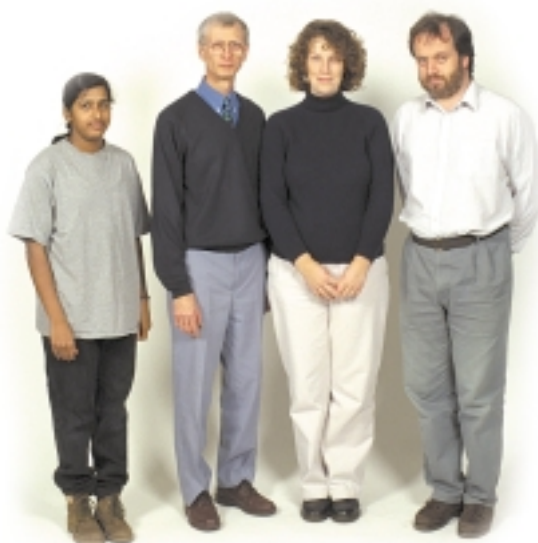
Grain legumes are one of the most important crop groups in world agriculture, improving soil fertility and yielding nutritious protein-rich seeds for human consumption or animal feed. The most widespread problems experienced by growers are diseases caused by fungal pathogens. *Botrytis* grey mould, for example, can devastate chickpea crops in the Northern part of the Indian sub-continent, risking the food security of resource-poor farmers. Even where fungicides are available and affordable, their use can render the growing of the crop less economic. In Europe, pea and beans are grown primarily on a farm scale for their protein-rich contribution to animal feed. However, home-grown grain legumes have to compete with soya meal, mostly imported from the United States. Only one third of Europe's requirement for protein-rich materials is met from within its borders, ultimately due to the cost of home-grown legumes relative to the current world trade price of soya meal. Better inherent fungal resistance in the grain legumes grown in Europe could help to improve this situation. In addition to economic factors, environmental benefits would also arise from a reduction in the use of fungicides on grain legumes.

If public concerns over the release of GM crops and the use of GM foods can be answered, new forms of fungal resistance in grain legumes provide one possible application of the technology with clear environmental benefits. Other developments of grain legumes are possible with GM technology: reduced anti-nutritional factors, improved amino acid balance for animal feed and insect resistance for example. The available genetic transformation techniques for grain legumes are, however, inefficient and unreliable. Two research projects now nearing

completion were targeted at the improvement of transformation methods for grain legumes, and their use to introduce new genes giving enhanced resistance to fungal pathogens. Here we describe the techniques developed in these projects.

Progress in the genetic transformation of grain legumes has been hampered greatly by their generally poor response in tissue culture. Even in pea, where transformation has been reported by several groups through the 1990s, methods are frequently very inefficient and not readily repeated. Although capable of being transformed, faba beans and chickpeas have been even more difficult to transform than peas. A common approach to achieving an efficient method has been taken here in all three crops. Given the poor *de novo* regeneration response seen for these crops *in vitro*, a focus was made on methods using *Agrobacterium* inoculation of meristematic tissues. *Agrobacterium* naturally performs genetic engineering to introduce its own genes into plant cells when acting as the crown gall pathogen; this process is used with disarmed forms of the bacterium to introduce new genes under the control of the investigator. Seedling tissues with pre-existing meristems are ideal starting material for meristem-based transformation, being both readily available and able to grow to form a mass of shoot buds in culture. The target meristems need to be disrupted in some way to allow the

Agrobacterium cells access to regenerable cells in the meristem. *Agrobacterium* inoculation of disrupted meristems is known to give inefficient gene transfer to plant cells with a resulting low frequency and unreliability of stable genetic transformation events. The approach taken here was to enhance the efficiency of the *Agrobacterium*-plant interaction by monitoring the expression of the GUS marker gene during the initial few days after inoculation. Various changes to the culture regime and to the inoc-



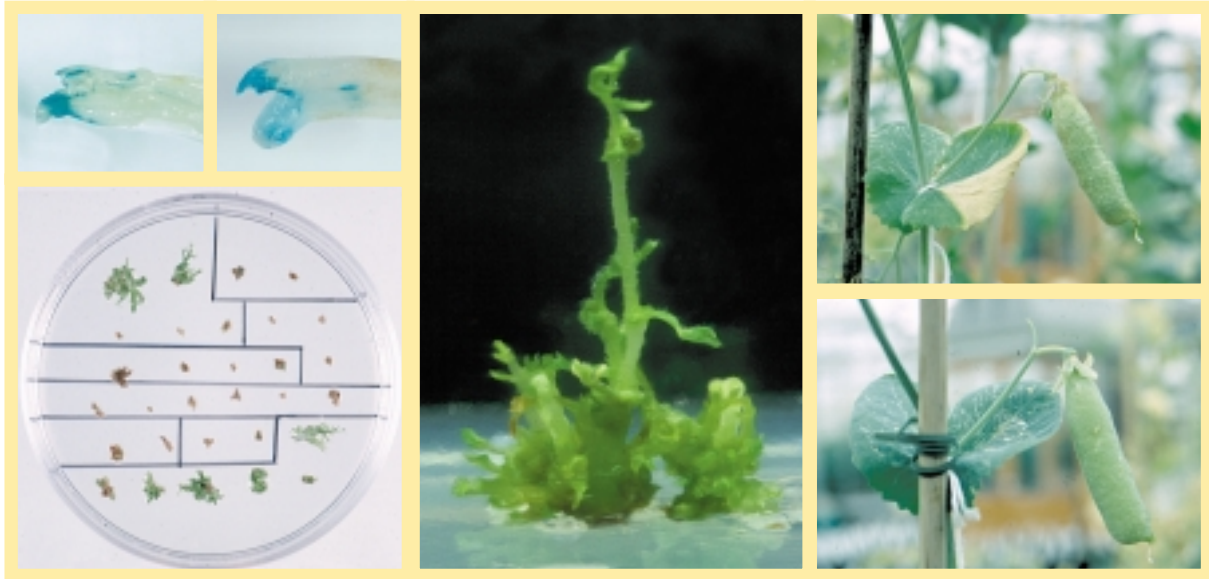


Figure 1 Transformation protocols developed for grain legumes: optimised transient expression of the GUS gene in chickpea shoot slices (upper left), selection of pea shoot clusters on medium with phosphinothricin (lower left), recovering chickpea shoot *in vitro* (centre), susceptible pea control PPT leaf paint test in pea for the *bar* gene (upper right), resistant pea leaf paint test showing expression of the *bar* gene (lower right).

ulation process have resulted in a dramatic improvement in the efficiency of this initial *Agrobacterium*-plant interaction. These, together with modifications to the subsequent tissue culture steps, combine to give efficient and repeatable transformation in both chickpeas and peas. Faba beans have also been successfully transformed by these methods.

The outline of the transformation system is given in Figure 1. Several steps in the process are important for the success of the method. The *Agrobacterium* strain/plasmid combination, the plant genotype and the kind of explant influence the outcome. The conditions of the *Agrobacterium* culture, and the plant explants exposed to it, have a large influence on the amount of GUS gene expression seen. Optimal conditions with the AGL1 strain and the pGIN1 plasmid include the use of an *Agrobacterium* culture at



the end of the logarithmic growth phase, and an induction period in a low pH medium with a modified composition. Crucial to the high levels of GUS expression seen in the explants is a careful drying of the explants after being submerged in *Agrobacterium* suspension, and subsequent culture on filter paper rather than in full contact with the

culture medium. All of these factors contribute to reliably high levels of GUS expression in the explants a few days after inoculation, as detected in histochemical assays (Fig. 1) and quantitative enzyme assays (Fig. 2). When applied to experiments with subsequent extensive shoot proliferation on thidiazuron-containing medium, followed by selection on phosphinothricin-containing selective medium, transgenic shoots were produced at a high efficiency. In one experiment, eight verified independently transformed shoot clones were produced from only 50 seeds inoculated. The long-term average is lower than this, but in 16 experiments in chickpea, 6% of seeds

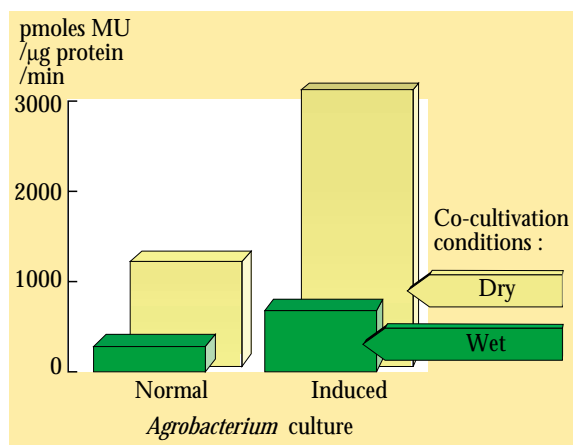


Figure 2 The effect of improvements in transformation protocols on the levels of transient expression of the GUS gene in chickpea seedling shoot slices.

overall gave rise to transformed shoot clones. Such frequencies of transformation are unprecedented, and indicate that pea and chickpea transformation is now both routine and efficient. Faba beans, previously very hard to transform, also have been transformed with this method.

The way is now open to the testing of approaches designed to enhance fungal resistance in grain legumes. Most of these approaches use genes from other food crops. Polygalacturonase-inhibiting protein (PGIP) has the ability to inhibit one of the key enzymes used by *Botrytis* and some other pathogenic fungi during invasion, and may therefore delay the pathogen long enough for other defences to take over. PGIPs from raspberry and kiwifruit are being used in this work. Phytoalexins, secondary compounds made by many plants, have a direct inhibitory effect on the growth of fungal pathogens.

Stilbene synthase uses common precursors to generate the phytoalexin resveratrol, regarded as a desirable component of red wine. This enzyme is present in groundnuts but not in the grain legumes under consideration here. The pea pathogen *Mycosphaerella pinodes* is known to have evolved specific defences against pea phytoalexins; it can be expected that such specialist pathogens may not have specific mechanisms for coping with stilbene phytoalexins. Further natural defences used by plants are targeted at lysing fungal cell walls. Glucanases and chitinases are components of normal pre-existing defence mechanisms in many plants and function in this way. The endochitinase derived from the biocontrol fungus *Trichoderma harzianum* appears to be particularly effective in inducing resistance to fungi and this version of endochitinase is being tested here. All of these genes are being used in a nine-partner European project to investigate their potential for the replacement of fungicide applications in grain legumes. The possibility of producing chickpeas resistant to *Botrytis* grey mould is also being investigated in a DfID-funded collaboration with the International Crops Research Institute for the Semi-Arid Tropics in India. Although such approaches hold much promise for more effective and environmentally-friendly means of controlling fungal pathogens, this work is still clearly at the exploratory stage, requiring further development and testing before application in the field.

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