Grasses as Biofactories: Scoping out the Opportunities

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Grasses as biofactories: scoping out the opportunities

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Key points

1. Plant biopharming is set to dominate commercial recombinant protein expression for specific proteins.
2. The choice of plant species depends on a multitude of factors and is determined on a case-by-case basis.
3. As a leaf based expression system grasses would have to compete predominantly with tobacco and alfalfa.
4. The grass-endophyte symbiosis offers a number of unique possibilities for biopharming.

Keywords: biopharming, endophyte, forage, transformation

Introduction

Whole genome sequencing is a remarkable scientific tool. Developing tools for recombinant protein technologies is arguably one of the strongest growth areas in applied biological research. The drive for this can be attributed to both the rising demand for highly purified proteins and secondary metabolites, and the costs associated with producing them. The list of organisms being investigated as biofactories for production of pharmaceuticals, nutraceuticals, industrial enzymes and industrial polymers is expanding; although in order to be successful in this area new organisms must first compete with the traditional systems including bacteria, yeast and Chinese Hamster Ovary cells (CHO cells). To our knowledge forage grasses have been overlooked as a potential biofactory candidate. In this paper the potential of grasses as recombinant protein expression systems for the manufacture of commercially valuable products is explored.

Advantages of plant biopharming

Costs

The main reason for investigating plants as biofactories is cost. It has been estimated that to produce a recombinant protein in a mammalian cell culture can cost up to 100 times more than a plant system. Bacterial and yeast fermentation systems fare better but even they are between 10 to 50 times more expensive than plant systems (Giddings, 2001). Furthermore, current plant-based systems are amazingly flexible and are scaleable at comparatively little cost. In comparison, up-scaling cell-based production systems requires the construction of expensive equipment, and animal-based production systems are limited by the breeding cycle of the animal.

Tissue specific gene expression

Plants can generate recombinant proteins using either prokaryotic (chloroplast) or eukaryotic (cytoplasmic and secretory) machinery; they also offer a range of expression organs, e.g. seed, leaf, root, fruit and tubers. A number of these organs can also be used as the delivery system of the final product. Examples include active and passive vaccines (antibodies). Approximately 20% of biopharmaceuticals under development are antibodies, and around 200 of these are already at the clinical trial stage. It is anticipated that the use of secretory immunoglobulin-A (sIgA) will be widely used in the future as a means of passive vaccination because of the stability of sIgA in the mucosa (Schillberg *et al*., 2003). Plants are capable of producing the most complex antibodies (sIgA) and in some cases plant produced antibodies need no further processing other than quantification.

Quality and safety considerations

Cell systems based on bacteria, yeast or animal lines have inherent risks associated with contamination of fermentation vats by a range of microorganisms. Contaminants can multiply fast, produce undesirable by-products and gases, and result in high costs for cleaning and lost production. If undetected, preparations made from infected vats impart a considerable safety risk. Plant expression systems present no such threat. Furthermore, animal cell lines may also harbour undetected viral or other infectious particles that can cause disease in humans. Again, no such concerns arise from using plant material.

Despite the advantages of cost and flexibility of using plants as mentioned above, to date there are only a relatively small number of commercial products/services manufactured through plant biopharming technologies, some of these are listed in Table 1.

Table 1 Examples of commercial biopharming in plants

Product/service	Company	Web site
Oleosin::protein fusion purification	SemBioSys Genetics Inc.	www.sembiosys.com
Human secreted alkaline phosphatase and botanical therapeutics	Phytomedics Inc.	www.phytomedics.com
Monoclonal antibodies and plasmatic proteins	MeDicaGO	www.medicago.com
CaroRx™ RhinoRx™ DoxoRx™	Planet Biotechnology Inc.	www.planetbiotechnology.com
Lipase, HSA, Lactoferrin, Collagen	Meristem ® Therapeutics Inc.	meristem-therapeutics.com
Avidin, trypsin, β-glucuronidase, aprotinin and oral vaccines	ProdiGene Inc.	www.prodigene.com

Disadvantages of plant biopharming

Expression levels of heterologous proteins

One of the most important criteria in selecting an organism as a biopharming host is adequate expression of the heterologous protein in appropriate tissues. Until recently plants (with their comparatively low expression of introduced genes), were considered to be the poor cousins of the recombinant protein expression world. The situation has changed dramatically through the use of appropriate promoters (tissue specific as well as inducible) and more astute construct design including choice of untranslated regions, insertion of introns, incorporation

of Kozac's sequence for initiation of translation, sub-cellular targeting/retention sequences, and optimal use of codons and polyadenylation signal sequences. In addition, the development of chloroplast transformation systems has enabled further increases in protein expression levels. Combined, these have facilitated accumulation of heterologous proteins to 36% of total soluble protein (TSP) in seeds (De Jaeger *et al*., 2002), 25% of TSP in chloroplast expression systems (Tregoning *et al*., 2003) and up to 21% of TSP (but more typically 0.5-2%) in vacuole, apoplast or endoplasmic reticulum (ER) -targeted systems (Conrad & Fielder, 1998).

Glycosylation

The majority of proteins that are of high value are destined for the mammalian circulatory system. One of the biggest stumbling blocks for this market is that in their natural host, many of these proteins are decorated with specific carbohydrate residues, a process known as glycosylation. Glycosylation in plants frequently includes the residues β(1,2) xylose and α(1,3) fucose that are not produced by mammals, and in some cases have been shown to be immunogenic in humans (for review see Lerouge *et al*., 2000). A number of strategies exist today where the incorporation of these motifs has been minimised and the glycosylation pattern has been partially humanised by the over expression of β(1,4) galactosyl transferase (Lerouge *et al*., 2000). Prior to secretion from the endoplasmic reticulum, the final key glycosylation reaction required for the maturing polypeptide is the incorporation of Neu5Ac N-terminal sialic acid. At present this is not performed by any of the recombinant plant expression systems; indeed it has been predicted that it will require complex engineering to generate plants with this capability (Gormond & Faye, 2004). However, even CHO cells (the most commonly used commercial mammalian expression system) do not replicate the human sialic acid glycosylation step 100% faithfully. Instead they incorporate both Neu5Ac and Neu5Gc sialic acid motifs where the latter has also been shown to be immunogenic in humans (Varki, 2001). It is predicted that our ability to manipulate this vital step in recombinant protein expression in plants will improve as our understanding of glycosylation processes advance.

Environmental and food safety

Public debate regarding plant biopharming tends to focus on potential negative issues associated with containment of genetically modified organsisms (GMOs) such as escapes, pollen spread, horizontal gene transfer, difficult site cleanup procedures and the possibility of contamination of the food chain (Conner *et al*., 2003). There is general agreement in the science community that these issues need to be addressed on a case-by-case basis when considering any GM species for release. With respect to transgenic grasses, risk of dispersal of species such as *Lolium perenne* L. (perennial ryegrass), *L. multiflorum* Lam. (Italian ryegrass), *Festuca pratensis* Huds. (meadow fescue) and *F. arundinacea* Schreb. (tall fescue) have been categorised as "substantial and widespread" (Ammann *et al*., 2001) as the species are outcrossing and the pollen dispersed by wind. Stable integration of transgenes into wild populations depends on several factors including whether the introduced gene confers any selective advantage on the progeny or if the transgenic crop is capable of being weedy in its own right. Nevertheless, there is a likelihood of gene flow from transgenic ryegrass to other grass species unless technological solutions such as chloroplast transformation (if chloroplasts are exclusively maternally inherited), terminator technology (imposes reversible sterility on reproductive plant parts), or transgenic endophytes (see later section) are developed. With regard to the issue of food contamination, the use of forage grasses as an expression system

provides an attractive alterative to other food crops used directly for human consumption, as humans are unlikely to ingest the material. Equally however, grazing animals must also be considered when risk analyses are being conducted.

Crop choice

For input traits (e.g., herbicide resistance) and output traits (e.g., modified lipid profile oil crops) the trait suits the crop, in contrast, for biopharming the crop suits the product. The crop choice depends on a large variety of factors and will be determined on a case-by-case basis for each product. In many cases the products can be targeted to a variety of subcellular compartments/tissues/organs, the requirements depend on the product itself and its designated use. Currently, biopharming utilises cell culture, root culture, root expression, leaf expression, tuber expression and seed expression with the latter three dominating current industry activity. In addition, hairy root cultures produced by *Agrobacterium rhizogenes* infection have potential as an expression system (Christey & Braun, 2004).

The species chosen must be able to quickly produce a large amount of recoverable recombinant active protein or secondary metabolite and at the same time produce low levels of toxic compounds; in essence it will provide the best compromise between production and profit. Contemporary crops are likely targets for lower-value proteins due to existing agronomic practices and processing systems, e.g. avidin is now produced in *Zea mays* (maize) at 10% of the cost compared with the extraction of native avidin from egg white (Hood *et al*., 1997).

Extraction and purification are usually the greatest cost components of biopharming. The compromise for grain products is that while it costs relatively little to store in comparison to frozen leaf or fruit material, the cost of extraction and purification from grain is higher than from leafy material. In some cases the technology developed by SymBioSys may help negate these costs. In this technology the recombinant protein is generated as a fusion with oleosin, the purification then takes advantage of the unique oleosin-oil body relationship that is formed during seed formation. A drawback is that the oleosin-fusion cannot be used directly for proteins requiring the post-translational modifications afforded in the secretory pathway.

Zea mays and other seed crops including cereals (*Oryza* spp.- rice and *Triticum* spp. -wheat) and legumes (*Pisum* spp. - pea and *Glycine* spp. - soybean) and some oil seed plants are being investigated as potential crops. While the overall protein yield/ha is lower in seeds compared with leafy systems, the proteins in seeds tend to be stable at ambient temperature and as such can make an ideal storage and delivery system for some products such as active and passive oral or topically-applied vaccines.

Current leafy plants for delivery systems include *Nicotiana tabacum* (tobacco), *Medicago sativa* (Lucerne) and *Lactuca sativa* (lettuce). The choice of *N. tabacum* is predominantly historical where the transformation systems (nuclear and chloroplast) are all well established, and there are the genetic and agronomic factors required to generate a high biomass return. Other advantages for *N. tabacum* include prolific seed yield and the fact that the risk of contamination is reduced as it is not a food crop. As a leaf crop however it must be pre-processed (frozen or dried) before transporting, or processed immediately after harvest due to the relatively unstable environment for proteins in the senescing leaf. Alternative leaf crops under investigation at this time include perennials such as *M. sativa*, which is easy to propagate; hence it is possible to generate large amounts of clonal material with relatively uniform expression. *M. sativa* also has a very consistent glycosylation pattern (Fischer *et al*.,

2004) as well as reduced fertiliser requirements due to its symbiotic relationship with nitrogen fixing bacteria. A further advantage of *M. sativa* is that it can be harvested up to nine times in a year resulting in around 12t DM/ha per year (D'Aoust *et al.*, 2004).

A modified wet fractionation of leaf material has been developed by MeDicaGo that allows the concentration (as opposed to expensive purification) of the bioactive molecule in *M. sativa* pellets to be stored for months before distribution. Combining inducible promoters with wet fractionation would make leafy crops very competitive with seed crops for certain applications such as direct feeding to animals.

Industrial enzymes, proteins and polymers: biopharming and combinatorial biopharming with output trait plants

Billion dollar pharmaceutical markets are not always needed in order to make a biopharming product commercially successful. Avidin, used in numerous laboratory-based assays and processes, until recently was purified from the egg white of chicken eggs. Prodigene Inc. has now commercialised recombinant avidin produced in *Zea mays* and is investigating the production of a number of other technical enzymes (Twyman *et al*., 2003). *M. sativa* is being investigated as a source of recombinant phytase enzyme that is normally incorporated into animal feed. Austin-Phillips & Ziegelhoffer (2001) reported they achieved economically viable expression in the field and that it could be used directly in animal feed with minimal preparation, thus replacing the microbial enzyme. Not all biopharming will be immediately profitable. An example of this has been the failure to commercialise plants producing biodegradable plastics. Monsanto purchased the rights to this technology in 1994 but has found that even with a plant generating two separate products (plastic from the leaf and canola oil from the seed) that the overall product was not commercially viable (Gross & Kalra, 2002). However, an alternative approach that may be sufficient to tip the scales in favour of commercialisation could be the combination of an inducible promoter with a dedicated perennial leafy plant thus enabling multiple high yields in a single year.

Grasses as biofactories

If grasses are to be considered as biofactories, it is necessary to examine what it would take to engineer such a plant as well as the unique advantages it might deliver in comparison to existing crops. Work performed in New Zealand is detailed below.

Genetic transformation of ryegrass

Technologies for genetic transformation of forage grasses are now well established (Wang *et al*., 2001). In general, the methodologies have been adapted from those developed for grain species such as *Oryza* spp., *Zea* spp. and *Triticum* spp. but by comparison, *Lolium perenne* is relatively recalcitrant to transformation. Efficient transformation is largely genotype-dependant due to the heterozygous nature of the plants. Consequently the generation of large plant numbers can be a laborious process. Until recently the emphasis has concentrated on development of protocols for stable integration of genetic constructs and regeneration of plants. However, with the advancement in gene isolation techniques and the rapid implementation of plant functional genomics programs, reports of successful gene expression studies in grass species have increased. Routine transformation systems now exist for *Lolium perenne*, *L. multiflorum* and *F. arundinacea* that focus on the manipulation of genes involved in the regulation of flowering and improvement of energy content (Figure 1).

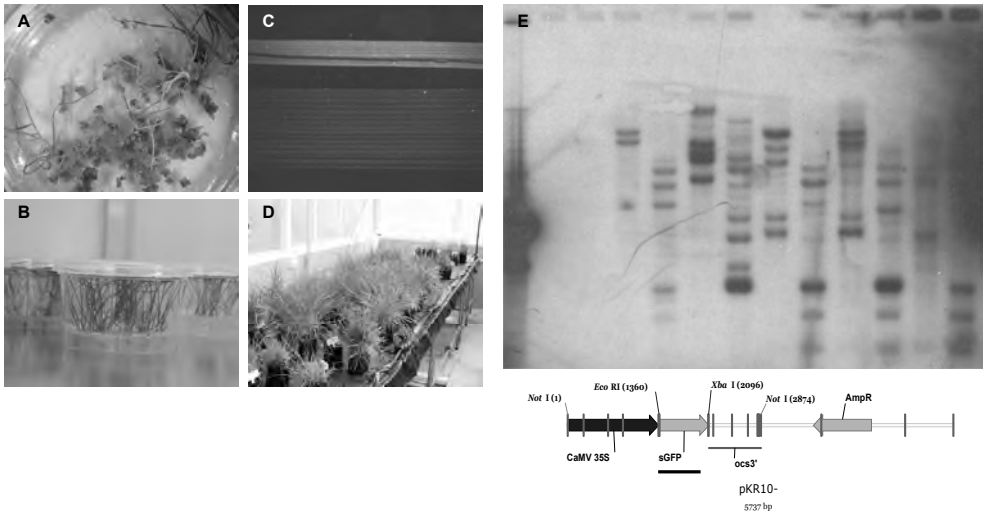

Figure 1 Production of transgenic ryegrass A) Regeneration of plants from embryogenic callus cultures. Micro-projectile bombardment was used to co-transformation calli with plasmids containing an expression cassette for the over-expression of the green fluorescent protein (GFP), and the hygromycin phosphotransferase (*hph*) gene as the selectable marker. B) Transformed *Lolium perenne* plantlets growing *in vitro* following regeneration. C) Constitutive expression of GFP in the leaf blade (top) as compared to a non-transformed plant (bottom). D) Transformed *Lolium perenne* in the greenhouse six months post transformation. E) Southern hybridisation analysis of transformants digested with *EcoRI* or *EcoRI/XbaI* and hybridised with a GFP probe.

Genotypes responsive to tissue culture and transformation remain the key component of a reproducible forage grass transformation system. The genera *Lolium* and *Festuca* are self-incompatible requiring cross-pollination; therefore a high degree of heterozygosity exists within populations that extend to responsiveness of genotypes in tissue culture. Initially this posed a limitation to transformation in *Lolium perenne* where low numbers of independently transformed lines resulted. Transformation frequencies were significantly improved by addressing the need for genotypes that perform well in tissue culture. Altpeter *et al*. (2000a) identified homozygous inbred lines of *Lolium perenne* that responded well in culture, and were subsequently used to develop an optimised transformation protocol. Manipulation of growth media components was used by Cho *et al*. (2000) to produce highly regenerative cultures for improved transformation frequency in *Fescua*. In AgResearch Ltd., the use of tissue culture responsive genotypes derived from elite *Lolium perenne* cultivars, resulted in a five-fold increase in transformation efficiency over material obtained from seedlings.

Direct DNA transfer by micro-projectile bombardment forms the primary method for transformation of many grass species. A range of transformed grasses have now been produced using this method, including *Lolium perenne* (Spangenberg *et al*., 1995b; Dalton *et al*., 1999; Altpeter *et al*., 2000b), *L. multiflorum* (Ye *et al*., 2001; Dalton *et al*., 1999), *F. arundinacea* (Cho *et al*., 2000; Spangenberg *et al*., 1995a), *F. rubra* L. (red fescue) (Cho *et a*l., 2000; Spangenberg *et al*., 1995a), *Poa pratensis* L. (Kentucky bluegrass) (Ha *et al*., 2001), *Dactylis glomerata* L. (Orchardgrass) (Cho *et al*., 2000) and *Elymus junceus* Fisch.

(Russian wild rye) (Wang *et al*., 2004). Typically, embryogenic callus cultures derived from tissue culture-responsive genotypes have been used as the target for transformation.

In the future, *Agrobacterium*-mediated transformation is likely to emerge as the favoured method for transformation of grasses. It is currently the preferred method for transforming many cereal species since the successful transformation of *Oryza sativa* using this method (Hiei *et al*., 1994). The primary advantage of *Agrobacterium* as a strategy for gene transfer is the ability to obtain plants with a simple transgene integration pattern and a higher proportion of plants containing a functional expression cassette. In general, *Agrobacterium*-mediated transformation is likely to generate plants containing 1-3 transgene loci that often contain only a single T-DNA, whereas micro-projectile bombardment frequently generates high copy number loci, complex in structure and containing up to 20 transgene copies (Kohli *et al*., 2003). *Agrobacterium*-mediated transformation has been compared to micro-projectile bombardment in *Lolium perenne* (Altpeter *et al.,* 2003). Of the 49 plants resulting from *Agrobacterium* transformation, the majority displayed two transgene inserts at independent loci whereas micro-projectile bombardment generated higher numbers of plants with multi-copy inserts.

The potential for somaclonal variation arises following the transformation process and care is required when attributing an alteration in phenotype to the inserted transgene (Conner & Christey, 1994). Analysis of transgenic ryegrass lines in the laboratory has identified chromosomal instability, including ploidy change and aneuploidy. Cytometric analysis of ploidy in a pool of 30 independently transformed plant lines revealed 6 lines to be tetraploid or aneuploid while the remaining 24 were cytologically stable with a diploid chromosome number (2n=2x=14). Further to this, molecular cytogenetics has shown rearrangement of 5S and 18S rDNA in transformed lines independent of ploidy change (Ansari & Richardson, *pers. comm*.).

Transgenic approaches are now being implemented as a tool for grass improvement. Target traits include resistance to abiotic stress, improved feed quality, control of plant development, and disease resistance. Generally the altered traits either exhibit a low heritability for the character or the trait has not been identified within the existing germplasm, limiting the ability for improvement by traditional breeding techniques.

Plants encoding genes for the accumulation of fructans have been produced. Ye *et al*. (2001) used the *sacB* gene from *Bacillus* to increase fructan levels in *L. multiflorum*. Contrary to expectations, total levels of fructose were reduced and severely stunted the growth of the resulting plants. In contrast, the *Triticum* genes encoding sucrose-fructan 6-fructosyltransferase and sucrose-sucrose 1-fructosyltransferase under the expression of the CaMV35S promoter displayed a significant elevation in fructan content and increased tolerance to freezing (Hisano *et al*., 2004).

Improvement of feed value has been approached directly via the regulation of genes associated with lignin biosynthesis to reduce lignin content of the whole plant, and also by the manipulation of flowering genes to minimise plant tissues containing a relatively higher lignin content. Down-regulation of the CAD and COMT genes from *F. arundinacea* was used to decrease lignin content (Chen *et a*l., 2003; Chen *et a*l., 2004). An increase in *in vitro* dry matter digestibility (9.8-10.8%) was associated with these plants. The vegetative phase of *F. rubra* was extended by over-expression of a *Lolium* clone of the terminal flower gene (Jensen *et al*., 2004). The expression of this gene resulted in the full inhibition of floral development over a two-year period. However, use of this strategy to improve feed value would require

the association of a switch for floral induction. In the laboratory the acceleration of flowering by up to three weeks has been demonstrated in *F. arundinacea*, by the expression of an *Arabidopsis thaliana* clone of the *FT* gene (Kardailsky, *pers. comm*.).

Introduction of a partial coat-protein gene into *Lolium perenne*, to provide resistance against ryegrass mosaic virus was achieved by Xu *et al*. (2001). Plant lines within the population of transformed lines expressing the construct displayed high, moderate, low or partial resistance.

Grass-endophyte biopharming

One of the most intriguing possibilities for biopharming temperate grasses is the presence, in many of them, of fungal endophytes, particularly those of the *Neotyphodium/Epichloë* genus. For example, *N. lolii* and *N. coenophialum* are fungal endophytes that live entirely within the intercellular spaces of *Lolium perenne* and *F. arundinacea* respectively. Infection is symptomless and the endophyte relies entirely on the host plant for dissemination via the seed or through vegetative structures (Schardl *et al*., 2004). The association is mutually beneficial since the endophyte confers a number of biotic and abiotic advantages to the host, including enhanced plant growth, protection from certain mammalian and insect herbivores, enhanced resistance to nematodes, resistance to some fungal pathogens and in some associations, enhanced drought tolerance (Scott, 2001; Schardl, 2001). Some of these benefits are due to the production of fungal secondary metabolites such as peramine (pyrrolopyrazine) and the loline (aminopyrrolizidine) alkaloids. However, endophytes also produce additional secondary metabolites such as ergovaline (ergopeptine) and lolitrem (indole diterpene) alkaloids, which cause mammalian toxicoses.

Although the endophyte comprises a very small amount of the total grass biomass (~0.5%), certain fungal secondary metabolites have been shown to accumulate to very high levels; lolines for example can accumulate to concentrations of up to 5% dry weight (Craven *et al*., 2001; Spiering *et al*., 2002). In addition, the endophytes remain metabolically active throughout the growth of the host grass (Tan *et al*., 2001); hence compounds associated with endophyte infection are continually produced which would allow for year round production. Clearly then, the potential exists to use grasses infected with endophytes to produce highly bioactive secondary metabolites.

Whilst there is considerable interest in secondary metabolites produced from endophytes, biopharming of fungal-derived secondary metabolites from infected grasses is not new. For many decades alkaloids with useful pharmacological properties were obtained from grasses infected with the ergot fungus *Claviceps purpurea* (Hoffman, 1978). Perhaps the most well-known ergot alkaloid is lysergic acid diethylamide (LSD), but other more complex ergopeptine alkaloids (similar to those produced by endophytic fungi of grasses) also accumulate to significant levels. Although nowadays these compounds can be produced directly from fermentation cultures of *C. purpurea*, the above example illustrates the potential for using infected grasses as biofactories to produce secondary metabolites not normally produced by plants themselves. Indeed, some of the enzymes associated with fungal biosynthetic pathways are novel to fungi. Crucially, although fermentation technology has been successful for producing certain secondary metabolites from some fungi, many compounds with interesting bioactive properties are only produced when the fungus is in association with its host grass. The insect feeding deterrents peramine and loline are cases in point, being barely detectable in axenic culture, but accumulating to high levels in infected

grasses. This may well be true for many other as yet to be discovered compounds where biopharming of infected grasses will be the only way of producing useful quantities.

At AgResearch Ltd., a group is interested in identifying additional endophyte-derived secondary metabolites, of which it is believed there is a vast undiscovered pool, particularly compounds which may have bioactive properties. In comparison to plants, the filamentous fungal genes involved in biosynthetic secondary metabolite pathways are usually clustered, thus by identifying particular genes frequently associated with these clusters (for example non-ribosomal peptide synthetases), it is possible to quickly isolate all the genes in a particular biosynthetic pathway. A metabolomics approach is being used to help identify the compounds produced from these 'unknown' biosynthetic pathways. Ultimately, isolated gene clusters encoding novel secondary metabolites can be manipulated at the molecular level, allowing, for example, both increased expression of the secondary metabolite, in addition to modifying the metabolite for improved properties. Modified fungal gene clusters could be transferred between different endophyte strains, considerably widening the biosynthetic potential of grasses infected with these strains. With some modification, gene clusters encoding bioactive secondary metabolites from other fungi could be transferred to the endophyte, providing an alternative to more expensive fermentation systems.

Using endophytes as surrogate transformation systems

In addition to modifying existing endophyte secondary metabolites, it is also possible to express heterologous proteins in these fungi. Transformation techniques are well established for filamentous fungi, including endophytes, and as such these can be used as surrogate hosts to introduce foreign genes into *Lolium perenne* or other temperate grasses (Murray *et al*., 1992). To demonstrate this potential, AgResearch Ltd. transformed endophyte to over-express GFP and re-introduced the endophyte into *Lolium perenne* (Figure 2). Further to this, the levels of heterologous protein produced in these associations will depend in part on the regulatory sequences used to drive the expression of the foreign genes. It is not anticipated that achieving high levels of gene expression will be a problem, since transcripts of some endophyte genes can accumulate *in planta* to levels higher than that of equivalent plant house keeping genes (Johnson *et al*., 2003).

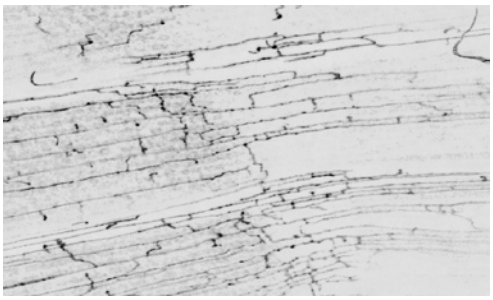

Figure 2 Endophyte (*E. fesctucae*) expressing green fluorescent protein in *Lolium perenne*

Transgenic endophytes pose reduced environmental risks

One significant factor to consider is the degree of environmental risk posed by using transgenic endophytes for biopharming. *Neotyphodium* endophytes are strictly biotrophic and live exclusively within grasses and their seed (Schardl *et al*., 2004). Since endophytes are asexual and cannot be transmitted horizontally (for example through spores) and they are not transmitted through pollen, their only mechanism for dissemination is through seed. Thus, providing seed production is controlled, the risk of large-scale spread of transgenes through other means such as wind-dispersed pollen is eliminated. The strict biotrophic relationship also assists in confining the transgenic endophyte to specific regions that are required to bulk up seed for large-scale plantings.

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