SESSION 2B

BIOTECHNOLOGY APPROACHES TO OPTIMISE CROP DEVELOPMENT AND QUALITY

Chairman and Session Organiser:	Professor Howard Davies *Scottish Crop Research Institute, Dundee, UK*
Papers:	2B-1 to 2B-4

Genomics and molecular breeding for crop plant improvement

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ABSTRACT

Recent advances in genetic and genomic analysis of crop plants have opened up the way for deployment of marker and genomics technology in modern crop plant improvement programmes. Despite these advances we are still at an early stage in the effective use of modern technologies in plant breeding. Molecular breeding is still very much a theoretical term but examples of successful, practical outcomes using marker-assisted selection are increasing in number each year. Despite innovations such as improved marker systems, denser genetic maps and improved trait and marker mapping tools and strategies, most marker associations are not sufficiently robust for successful marker-assisted selection. This is largely due to inadequate experimental design. Molecular breeders must re-orientate their research programs so that DNA marker work leads to useful selection tools and enhanced germplasm. As more rigorous experimental guidelines and ambitious goals are adopted, the growing body of knowledge from genomics and bioinformatics can be integrated into crop improvement programmes. The deployment of transgenes, especially from crop relatives for alleviation of biotic stresses, remains an exciting possibility.

INTRODUCTION

For the past several years now the enormous promise of gene and marker technology in crop plant improvement programmes has been greatly vaunted. However, until quite recently these supposed benefits have been somewhat slow to materialise, especially in publicly-funded crop improvement programs. Several large biotechnology companies have attempted the deployment of transgene technology directly in their 'in-house' crop improvement programmes, and there have been some successes. Some private organisations have invested heavily in the development of large molecular marker sets (e.g. microsatellites) and genome or EST sequencing databases for use in modern, large-scale plant breeding programmes. These efforts and advances in the private domain have not, with a few welcome exceptions, been well disseminated to the scientific community, so it is not completely clear to what extent these efforts have been successful or cost-effective.

Publicly-funded breeding and crop improvement programmes have been slower to deploy advances in molecular genetics and genomics, despite many years of extensive trait analysis and the expenditure of vast sums of public money. The hesitation in the deployment of transgenes directly into commercial varieties is understandable and due to several causes, not all of which are scientific. For most crops we have a very limited armoury of suitable cloned genes for use directly in crop cultivars. Public programmes have focused their efforts on trait

genetics and the development and use of marker assisted breeding or selection (MAB or MAS), which is less 'risky' in that it can be used in enhanced but essentially conventional plant improvement programmes. However, despite very large investments in the generation of linkage and trait maps, there are relatively few good examples of the effective use of MAB and related technologies in crop plants, although the number of successes is increasing.

In this review I will attempt to discuss recent developments in crop plant genetics and genomics, and their use to date in crop plant improvement. I will commence by summarising the current state of crop plant genetics and genomics. In doing this I will present my own views on how some of these advances are likely to be utilised in plant improvement programmes of the future, using examples from some crops with which I am most familiar.

WHAT DO WE MEAN BY MOLECULAR BREEDING?

Plant improvement programmes have traditionally been based on phenotypic selections from large F_1 or backcross (in the case of outbreeders) or F_2 populations (inbreeders). The biology and reproductive systems of some crops greatly hinder rates of progress in terms of the time taken to breed a new variety. For example it took over 30 years to breed tetraploid potato cultivars containing a complex source of potato cyst nematode (PCN) resistance! The development of dense linkage and trait/QTL maps of crop plants in principle makes it possible to select plants possessing desirable attributes based upon genotypic selection. The advent of high-throughput marker technology has greatly enhanced our ability to generate dense linkage maps thus rendering it straightforward to find marker alleles linked in coupling to a target allele. Such maps should also be of utility for practising 'defensive' selection against undesirable chromosomal segments, for example from wild relatives used to introgress resistance to biotic or abiotic stresses into crop cultivars. It is clear that for most crops, where selections are based upon expensive, slow, and laborious phenotypic testing, the deployment of molecular markers for key traits could have a massive impact.

Molecular breeding could be defined as the use of genotypic information rather than phenotypic information to make informed choices of plants for further selection or crossing. However this is a narrow definition of the term and I would expand it to include the use of all biotechnological methodologies, including transgenesis, either for trait 'addition' or modification of biochemical pathways, for the improvement of crop plants. One could argue that for outbreeding, heterozygous crops such as potato (whose breeding is also complicated by polyploidy), all that one need is to correct obvious 'defects' in existing cultivars, such as a lack of required disease resistances to prevalent pests or pathogens, using a transgenic approach. This argument could be extended to stacking several desirable traits – incorporation of which into a single cultivar by conventional means would take an eternity! All of this is academic at the current time as we do not possess the full set of genes required to create the 'perfect' genotype for any crop.

Young (1999) has suggested that for almost every crop plant trait there has been some type of trait or QTL analysis. He discussed the particular case of soybean cyst nematode (SCN) resistance. SCN had a large effect on soybeans in the USA, causing ~8% yield loss from 1997-1997. Phenotypic assays took 5 weeks as well as a large amount of costly glasshouse space. SSR markers were developed which tagged *rhg1* a major gene responsible for SCN

resistance, and these markers are extensively used in both public and commercial breeding programmes. Young argued that this rapid uptake was due to the demonstration that *rhg1* was essential for resistance and that a single SSR marker could be used to assay its presence in breeding populations. Despite this, at the time of writing there were no commercial varieties developed using MAB for this trait.

CURRENT STATUS OF CROP PLANT GENETICS AND GENOMICS

Maps 'n' markers

For most crop plants there are now available dense molecular genetic maps, construction of which has involved the development and deployment of large numbers of markers of various types: simple sequence repeats (SSRs), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) being the most common. Some maps have targeted the mapping of 'functional' markers, usually the use of cDNAs as RFLP probes (rice, tomato, potato, pepper). The highly-multiplex AFLP marker system has allowed the construction of very high-density linkage maps of crop plants, notably in maize, tomato and potato. Such maps will, in principal, greatly accelerate map-based gene isolation and physical mapping efforts by providing a dense framework of marker points that can be anchored to BAC clones. Individual AFLP markers tightly linked to target alleles can be isolated from gels and converted to PCR-based markers, and these can be used in MAS. Recently, we have seen the initiation of single nucleotide polymorphism (SNP) marker analysis in crop plants, including potato and soybean (Zhu *et al.*, 2003) although 'global' linkage mapping using SNPs is in its infancy in most crops. SNPs are attractive in that they represent the most frequent class of DNA sequence polymorphisms and that, in all but the most monomorphic crops, can be gleaned from EST databases or by comparing PCR product sequences among parents of breeding material. Moreover, SNPs can easily be shared between labs and SNP mapping does not entail use of gel electrophoresis. However, there are still some cost issues with their deployment at high throughput but these should be overcome in the near future. As SNPs are based on nucleotide substitutions, they are eminently suitable for linkage disequilibrium (LD) mapping, whereby associations are sought between markers and trait loci in populations or collections of crop plant germplasm. Such associations are affected greatly by population structure as well as the mating behaviour of the particular crop species studied. Nonetheless LD mapping offers an exciting possibility for novel approaches to trait analysis and studies of crop evolution and may allow the partial circumvention of the time consuming practise of mapping individual pair crosses.

Map based gene isolation in plants

Many crop plant genes corresponding to Mendelian traits have been isolated by map-based cloning. For example over 30 disease resistance genes have been isolated from plants and this number is growing rapidly. Early efforts were heavily targeted on resistance gene isolation, but more recently these have been expanded to genes controlling other physiological and morphological traits. Requirements for this approach are the availability of marker-dense linkage maps around the gene of interest, as well as large-insert genomic libraries (e.g. BAC libraries) from the variety or genotype containing the targeted gene. Such libraries are now

available for almost all crop plants, although their construction is still time consuming and for some crops it remains a technical challenge.

Complex trait analysis

Quantitative Trait Loci (QTL) mapping is very well developed in crop plants as the majority of traits of interest to plant breeders, such as yield, quality, adaptation, durable disease resistance etc, are of a complex nature. A large number of biometrical and statistical approaches have been developed for the analysis of quantitative traits. Nonetheless, mapping of QTLs is still a very inexact science and is dependent on good experimental design and accurate replicated phenotyping. Most QTLs can be 'pinned down' to intervals of 3-5 cM, which correspond to very large tracts of DNA containing many hundreds of genes, making them extremely difficult to isolate. Isolation of QTLs remains a difficult endeavour although progress has been made, most notably in tomato (Frary *et al.*, 2000).

Genome sequencing and EST projects

There are now near complete genomic sequences for rice and the model crucifer *Arabidopsis thaliana*. These sequences were obtained by using physical maps of the respective genomes, based on large insert genomic clones. Perhaps of greater significance for crop plants are the large 'gene catalogues' in the form of EST databases that now exist for most of the major crops. EST databases represent a substitute for the full genome sequence for most crops, whose sequences are unlikely to be obtained in the short term, and should ideally represent a very high proportion of the 'gene-space' for any given species (Rudd, 2003). There are major issues concerning sequence quality and coverage in most EST datasets. For example potato has ~94,000 ESTs at the current time, but the number of different genes represented is probably less than 25,000. A major drawback of EST data is that virtually no information exists concerning the genetic locations or genome 'context' of ESTs. To achieve this for most crops would be a major undertaking, and for crops with low levels of genetic variability (e.g. hexaploid bread wheat) the dependence on genetic polymorphism would render it an impossible task. The genetic or physical localisation of a significant number of ESTs for each crop is an important goal, which would greatly enhance candidate gene analysis, in that gene maps and QTL maps could be directly compared. For several crops a moderate number of cDNAs have been mapped as RFLP markers and recent efforts have targeted genes involved in a particular plant process, such as cold sweetening in potato (Menendez *et al.*, 2002). Other physical mapping methods, such as Deletion Mapping, Happy Mapping or Radiation Hybrid Mapping offer a potential alternative approach to establishing the map positions of coding sequences without the need for genetic polymorphism (Waugh *et al.*, 2002).

Microarrays and other transcript profiling techniques

Expressed sequence tags (EST)-based microarrays are powerful tools for gene discovery and the analysis of signal transduction and other processes in well-characterized crop plant species. These are now becoming established for all of the major crops. Early indications are that they will help greatly in the identification of genes involved in processes important in agricultural systems. Moreover, there is evidence that such arrays developed using ESTs from one species, can be used across a wide array of related plant species. There are still statistical problems with the analysis of the large amounts of data arising from array

experiments and issues with cost and repeatability. There is insufficient space to discuss these issues here but they are reviewed by Aharoni & Vorst (2002).

The candidate gene approach

With recent advances in trait maps and increases in the size of sequence databases, the 'candidate gene' CG approach has become a feasible means for isolating major genes and QTLs (see review by Pflieger *et al.*, 2001). It comprises an alternative strategy to map-based cloning and other gene isolation approaches and is based on the hypothesis that known-function genes, the so-called candidate genes, could correspond to loci controlling traits of interest. CGs refer either to cloned genes presumed to affect a given trait ('functional CGs') or to genes suggested by their close proximity on linkage maps to loci controlling the trait ('positional CGs'). In plants, the usual means for identifying a CG is to find co-segregation between CGs and the locus affecting the trait. Statistical association analyses between molecular polymorphisms of the CG and variation in the trait of interest have also been carried out. The final validation of a CG is provided by physiological analysis, genetic transformation and genetic complementation. Good examples of this type of approach are provided by the work of McMullen *et al.* (1998). Several candidate RGA and DR gene markers were associated with QTL from the pathogens and pest. However, a note of caution is suggested by Liu *et al.* (2003). Nonetheless the development of SNP markers in candidate genes and establishing the degree of association between these SNPs and phenotypic traits seems to be a promising route for the future.

Genetic modification

Transgenic technology is now well established in the public domain for all but the most intransigent of crop plants. This has become particularly evident in cereals, such as rice, maize, wheat and barley in which facile transgenesis has been a major obstacle in recent years. The increase in the number of cloned crop plant genes has greatly increased the potential for deploying such genes transgenically to generate 'enhanced' crop cultivars. However, many of the genes isolated from crops (e.g. resistance genes) despite their biological interest, are not necessarily of any great general utility for deployment directly in crop plant cultivars. For example, the majority of plant resistance genes cloned to date are only effective against specific pest/pathogen races and would not themselves be useful in combating the pathogen effectively across a wide geographical area. The 'engineering' of race-specific or other resistance genes to be of 'broad spectrum' utility has not yet been achieved. Another problem is that many of the cross-species applications of transgenes, for example deployment of viral genes in crops to effect resistance, or deployment of genes from microbes, by their heterologous nature, are deemed as too 'risky'. There is understandable reluctance to go down this route given the experience of biotech companies! A sensible initial strategy is to deploy crop genes isolated from either the crop itself or a near relative, to overcome some of these barriers. The problem is to render such genes useful across a wide range of genotypes and environments without grossly perturbing the expressions of the transgenes themselves. To some extent this explains why the isolation of genes for durable/horizontal disease resistance genes remains the 'holy grail' for most crops as these would have widespread utility with no intrinsic requirement for up-regulation or otherwise enhanced expression.

An excellent example of a gene which may well pave the way for transgene deployment in crops is the Rb gene, isolated from potato very recently, which is active against all known races of the oomycete pathogen *Phytophthora infestans* (Song *et al.*, 2003). This gene originates from a wild diploid potato species *Solanum bulbocastanum*, and was 'introgressed' into cultivated potato using a fertile somatic hybrid between *S. tuberosum* and *S. bulbocastanum*. Transgenic plants containing the Rb gene display broad spectrum late blight resistance and this gene provides an exciting new resource for developing late blight-resistant potato varieties, which can play a major role in the fight against late blight, one of the most devastating plant pathogens. To perform the same task using conventional potato breeding using the fertile hybrid would possibly take 10-20 years as it would entail 'reshuffling' the genotypes of existing heterozygous potato cultivars during the introgression process and re-selecting for quality and other resistance traits.

Use of molecular diversity and allele 'mining'

Tanksley & McCouch (1997) advocated the great importance of wild relatives of domesticated crop plants both in terms of broadening the genetic base of such crops, but also in providing diverse alleles of important trait genes to genetically 'engineer' superior new crop plant varieties. Our ability to characterise rapidly exotic crop plant germplasm using the new genetic and genomic tools is having a massive effect on the unravelling of agronomically important traits and of the use of such germplasm in breeding and genetic studies. This is particularly true of crops with extensive collections of diverse relatives that can be crossed to the crop itself. Analysis of diverse crop plant germplasm using high-throughput molecular marker approaches has made it easier to quantify the genetic relatedness of genebank material, making it easier to identify the most diverse accessions of crop plant relatives. This allows us to ascertain which accessions should release the most genetic variation when crossed, either amongst themselves or to existing crop plant cultivars. Such studies also make it easier to identify 'working' or core collections of germplasm which can be used as primary genepools for crop improvement, either through introgression or through the use of molecular techniques to mine divergent alleles of genes coding for important traits.

In tomato, wild relatives have been used to dissect important fruit traits, using a marker assisted introgression approach to 'isolate' segments of wild species DNA in an adapted background, allowing the effects of each segment on each trait to be assessed (e.g. Bernacchi *et al.* (1998) and accompanying paper). Some surprising results have been obtained that could not have been predicted from the phenotypes of the wild relatives themselves. These introgression lines represent 'libraries' of exotic plant germplasm that can be utilised in future tomato breeding and gene isolation efforts.

PROSPECTS FOR USE OF MAB AND TRANSGENICS IN CROP PLANT IMPROVEMENT

As stated by Young (1999) for the majority of important crop traits it has been possible to develop molecular markers, which in principle can be used for MAB. These markers can be of different types and differ in their suitability for direct application by plant breeders. If one searches the literature databases there are enumerable instances of papers describing markers

suitable for use in MAB, and yet relatively few of these are concerned with the direct application of MAB in a breeding programme. However there are some good examples:

Liu *et al.* (2000) have successfully described the use of RFLP markers from different labs to pyramid three different powdery mildew resistance gene combinations in wheat. Ayoub *et al.* (2003) demonstrate the use of PCR-based markers to manipulate a malting quality component, alpha-amylase activity, in a barley breeding population. Narayanan *et al.* (2002) have used a joint MAS/transformation approach to improve the rice cultivar IR50, by adding two different resistances. Spielmeyer *et al.* (2003) show the value of using SSR markers to select for a broad-spectrum rust-resistance gene, Sr2 that is difficult to score by other means. Tar'an *et al.* (2003) have developed an index which combines QTL-linked marker scores with other data to select for complex common bean traits. Zhou *et al.* (2003a) show how Zhenshan 97, an elite parent of hybrid rice, can be improved for four quality traits using MAS. Zhou *et al.* (2003b) used flanking SSR markers and phenotypic selection to track scab resistance in the early seedling generations of a wheat breeding scheme. Lastly Datta *et al.* (2002) have crossed two homozygous transgenic IR72 rice lines to produce lines showing resistance to three different diseases and pests.

These are but a few of the recent examples of how marker and transgene technology can be used in plant breeding. Doubtless there will be many others in the next few years. Clearly complex traits and non-inbred crops present a much more serious barrier to the deployment of molecular markers. Obviously it is desirable to get markers as close to the target gene as possible. I believe that SNP markers will become of great utility for MAS in the future. For example the development of SNP markers in LD with particular R gene clusters may be of great utility for moving around resistance genes and QTL. Whether or not MAS eventually lives up to its potential will depend in part on economic considerations. In the absence of detailed empirical evidence regarding the benefits and costs of alternative breeding methods, it is hard to judge whether MAS will be attractive relative to conventional selection methods.

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Designer tubers for production of novel compounds

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ABSTRACT

Based on world-wide efforts to identify novel biopharmaceuticals, numerous low (metabolites) and high (proteins) molecular weight compounds have been described which, when used for clinical treatments, would offer substantial improvements for today's clinical therapies. As the demand for biopharmaceuticals is expected to exceed their availability, safe and cost-effective production systems are urgently needed. Plants offer several advantages over conventional production systems, but contamination of food- or feedstuff must be avoided. This can be achieved by applying inducible gene expression systems. In contrast to dry seeds, potato tubers remain metabolically active throughout the storage period, which makes them ideal as a bioreactor for biopharmaceuticals. Using a chemically inducible gene-switch post-harvest accumulation of biomolecules can be obtained, offering the possibility to grow non-pharmaceutical containing tubers in the field. To allow post-harvest induction of pharmaceutically relevant polypeptides an ethanol inducible gene switch for potato tubers has been established and optimized. As an example for the production of an edible vaccine the papillomavirus (HPV) major capsid protein L1 has been chosen. Since cervical cancer is linked to infection with HPV and is the third most common cancer among women world-wide, there is a strong demand for the development of an HPV preventive vaccine. The plant-derived L1 protein displayed conformation-specific epitopes and assembled into virus-like particles. In addition to unintended contamination of food- or feedstuff with biopharmaceuticals, unauthorized distribution of seed tubers must be avoided. Since potato plants can easily be propagated in tissue culture, the use of non-sprouting tuber bearing potato varieties offers a possible strategy to restrict seed tuber distribution. Based on current biochemical and molecular knowledge transgenic potato plants producing non-sprouting tubers for restricted production of biopharmaceuticals have been designed by interfering with long-distance sucrose transport. Combining inducible gene expression systems and non-sprouting tubers should strongly improve public acceptance of GM potatoes for the production of edible vaccines or non-plant metabolites.

INTRODUCTION

Transgenic plants represent an attractive and cost-efficient alternative to conventional systems for the production of biomolecules. Obvious advantages of transgenic plants as bioreactors can be summarized as follows: (i) due to their photo- and autotrophy plants are able to produce their biomass using inorganic substances and sunlight resulting in low energy costs for production; (ii) the production is flexible, can be done in bulk quantities and can use existing cultivation, harvesting and storage facilities; (iii) in contrast to microbial systems plants carry out post-translational protein modifications which can be essential for enzyme activity; (iv) enzymes used in food, feed, or paper industry do not necessarily need to be purified where the plant material is used directly; and (v) health risks arising from contamination with human pathogens or toxins can be minimized. However, for phytofarming to be commercially attractive a number of prerequisites need to be fulfilled. Amongst others, the polypeptides need to be produced in considerable amounts, they must be stable and remain active during harvest and subsequent enrichment/purification procedures. Within the last years a number of vaccines have been successfully expressed in plants and orally delivered to animals to determine their immunogenicity. This includes the Norwalk virus capsid protein (Mason *et al.*, 1996) the hepatitis B surface antigen (Richter *et al.*, 2000) and the heat-labile non-toxic subunit B of the *E. coli* enterotoxin (Lauterslager *et al.*, 2001). Recently, it has been shown that oral application of HPV 16 virus-like particles (VLPs) leads to the induction of capsid-specific antibodies in mice, indicating that oral vaccination against papillomaviruses is possible (Gerber *et al.*, 2001). World-wide, human papillomaviruses are responsible for approximately half a million new cervical cancer cases every year. In more than 95% of these cancers this coincides with the presence of HPV DNA of high risk genotypes. Therefore, it is an urgent need to develop a prophylactic sub-viral vaccine, preventing infection by HPV and thereby most likely the development of cervical cancer.

As outlined above, molecular farming in plants offers several advantages over conventional fermentative production systems but potential ecological and economical risks have also to be considered. Amongst those, unwanted release of biopharmaceutical containing plant material has to be prevented. With the help of advanced molecular techniques contamination of foodstuff and fodder can be minimized. Chemical regulation of transgene expression offers the opportunity to regulate gene expression. This feature is especially important if toxic substances or pharmaceutical products are to be synthesized. Various chemical-inducible systems based on activation or inactivation of target genes have been described (for recent review see Zuo & Chua, 2000). In plants several plant promoters exist which are inducible by endogenous signal metabolites. Best studied cases are abscisic acid (Marcotte *et al.*, 1989), ethylene (Ohme-Takagi & Shinishi, 1995), and salicylic acid (Uknes *et al.*, 1993) inducible promoters. The use of these promoters is limited, since the naturally occurring levels of signal metabolites and the responsiveness of the target tissue/cell may vary. Moreover, the signal metabolites can effect more than the expression of the gene of interest and the inducible genes described so far are under complex control including environmental and developmental factors. Therefore, synthetic promoters which do not respond to naturally occurring plant products, have been engineered. These include chimeric promoter systems responding to tetracycline (Gatz *et al.*, 1991), isopropyl-beta-D-thiogalactoside (IPTG) (Wilde *et al.*, 1992), ethanol (Caddick *et al.*, 1998), ecdysone (Martinez *et al.*, 1999) or dexamethasone (Aoyama & Chua, 1997). These systems offer the possibility to induce

accumulation of biopharmaceuticals after harvest, thereby minimizing the risk of food- or feedstuff contamination.

Albeit promising, molecular farming in plants is not without risk. Potential problems include the entering of pharmaceutical plants and/or products the traditional food or feed chain. This may occur by cross-contamination of commodities, inappropriate use of plant by-products, unauthorized use of seeds and cross-pollination. In addition, occupational exposure and out-crossing to wild relatives are potential risks, which need to be considered. Contamination of the traditional food or feed chains may be prevented/minimized by tissue-specific expression of the transgene (i.e. fruits versus leaves), the use of non-commercial varieties, non-food plants or plants containing visible marker genes and cultivation in containment. Furthermore, the use of male sterile plants reduces the risk of cross-pollination and out-crossing. In case of GM potatoes unwanted seed tuber distribution has to be prevented. This may be achieved by controlling tuber sprouting. After lifting, potato tubers undergo a period of dormancy. The length of the dormancy period is dependent on both the genetic background and the environmental conditions during tuber development. It has been hypothesized that dormancy is regulated by the relative concentrations of growth promoters (gibberellins and cytokinins) and inhibitors (abscisic acid and ethylene), however, results concerning the dormancy breaking or promoting activities of these phytohormons are often contradictory (discussed in Hajirezaei *et al.*, 2003). One possible explanation for the non-uniform results is that a metabolic competence of tuber cells is required before phytohormones can exert their growth control. At the onset of sprouting potato tubers turn into a source organ supporting growth of the developing sprout. During this process starch and protein degradation is initiated and soluble sugars and amino acids are formed. Initial support of sprout growth, however, most likely does not require reserve mobilisation but relies on pre-existing soluble sugars and amino acids. In any case storage parenchyma cell-derived sucrose is transported via the phloem system towards the developing sprout. Phloem transport of sucrose requires an apoplastic step and is energy dependent in potato plants (summarized in Frommer & Sonnewald, 1995). Thus, interfering with long-distance sucrose transport should allow to inhibit tuber sprouting. By removal of cytosolic pyrophosphate Lerchl *et al.* (1995) were able to inhibit long-distance sucrose transport in transgenic tobacco plants which lead to the accumulation of soluble sugars in source leaves. Sucrose export most likely was inhibited due to a reduced sucrose uptake of companion cells based on limited energy supply as a consequence of cytosolic PPi depletion. In agreement with this assumption removal of cytosolic PPi drastically delayed sprouting of transgenic potato tubers (Hajirezaei & Sonnewald, 1999).

RESULTS AND DISCUSSION

Establishment of an inducible gene-switch for post-harvest induction of target genes in potato tubers

A number of chemically inducible gene expression systems have been developed for plant use. These systems allow induction of gene expression at defined times during plant development. Although useful, most of the systems suffer from serious problems including adverse or pleiotropic effects on plant growth and limited systemic induction. The alc gene expression system, which is based on the alcohol dehydrogenase regulon from *Aspergillus nidulans*, uses the inducer ethanol or acetaldehyde, which depending on the concentration

applied have little or no pleiotropic effects on plant metabolism (Junker *et al.*, 2003). For plant expression the system is composed of two components (Figure 1). The *alcR* transcription factor is expressed under control of the constitutive 35S CaMV promoter. In the presence of ethanol ALCR induces expression of any gene fused to a modified *alcA* promoter. In the absence of ethanol no induction of the target gene occurs. The chimeric *alcA* promoter consists of a minimal 35S CaMV promoter (-31 to +1) and the upstream activator region of palcA (Caddick *et al.*, 1998). Using the β-glucoronidase reporter gene as visible marker this system has been tested in *A. thaliana* (Roslan *et al.*, 2001), *N. tabacum* (Caddick *et al.*, 1998), potato and oilseed rape (Sweetman *et al.*, 2002; Junker *et al.*, 2003). In all plant species, gene expression was strictly dependent on the external supply of ethanol or acetaldehyde, respectively. In potato, post-harvest induction of GUS activity was tested. Tubers harvested from transgenic potato lines were harvested and treated with ethanol vapor in a sealed box. During induction the ethanol concentration in the airspace was 9 μM. A time course of induction revealed detectable GUS mRNA after 6 hours of incubation, whereas GUS enzyme activity was first detectable after 24 hours starting at the outer part of the tuber (Sweetman *et al.*, 2002).

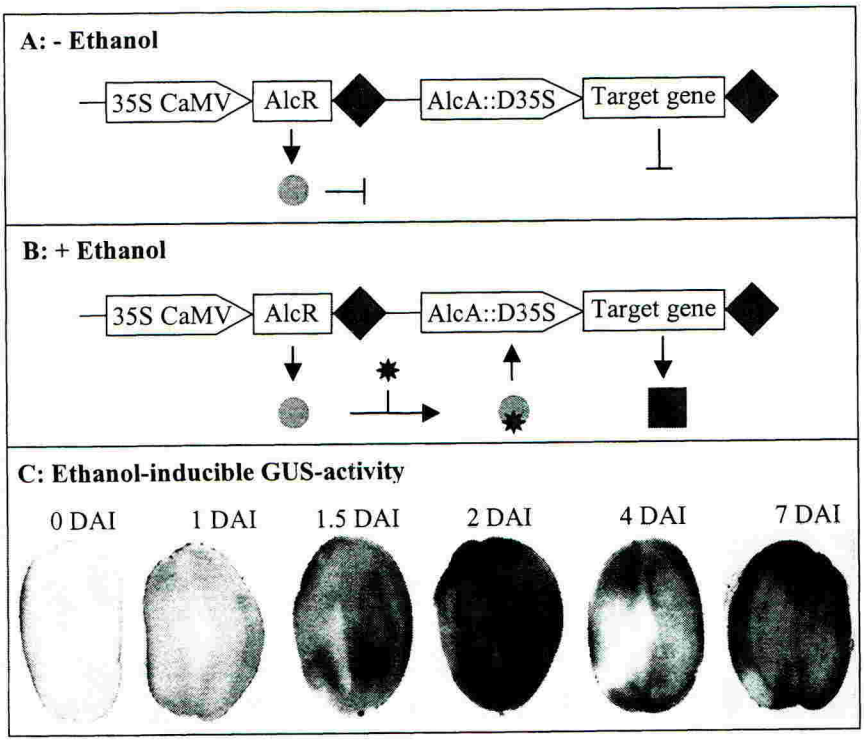

Figure 1. Schematic representation of the ethanol inducible gene switch (A, B) and visualization of reporter gene activity in tuber slices of transgenic potato plants harboring the β-glucoronidase reporter gene under control of the ethanol inducible gene switch. T, terminator of transcription; DAI, days after induction.

To verify that the chimeric *alcA* promoter would be ethanol-dependent under field conditions, field trials using three independent transgenic potato lines (50 plants each) harboring the GUS gene under alc control were conducted in the years 2001 and 2002. Basal GUS activity was histochemically tested in non-treated plants throughout the growing season. No GUS activity was found in leaves of different developmental stages (sink, source, senescence) and flowers. Furthermore, GUS activity was absent in tubers of varying size (50-400 gFW) prior induction. However, incubation of tubers with 0.02% ethanol (v/v) resulted in a progressively increasing GUS activity (Figure 1C). Based on these results we conclude that the alc system is suitable for expression of pharmaceutical proteins in transgenic potato plants.

Inhibition of potato tuber sprouting by sucrose hydrolysis in phloem cells

In contrast to cereal seeds, potato tubers are not dehydrated and metabolism remains active throughout the storage period. This allows post-harvest production of low and high molecular weight compounds, making tubers an attractive system for molecular farming. Following a period of dormancy tuber sprouting is initiated giving rise to the next generation of potato plants. Sprout development is dependent on assimilate supply which requires mobilization of carbohydrate reserves in parenchyma cells. Transport of carbohydrates from parenchyma cells to the developing sprout occurs in form of sucrose, the main transport sugar in most higher plants. Long distance sucrose transport involves apoplastic loading into companion cells and/or sieve elements (Figure 2A). This process is energy dependent and requires phloem-specific metabolism of sucrose. Recently it could be shown that removal of cytosolic pyrophosphate changed the sprouting behavior of transgenic potato tubers dramatically (Hajirezaei & Sonnewald, 1999). Since previous investigations revealed that long distance sucrose transport is pyrophosphate-dependent, it was postulated that the inhibition of sprouting was caused by a reduced sucrose export and its subsequent utilization. If true, phloem-specific removal of sucrose was expected to result in a disconnection between storage parenchyma cells and the developing bud (Figure 2B). As a consequence, sprout growth was believed to be inhibited. To achieve phloem-specific hydrolysis of sucrose, a chimeric gene consisting of the rolC promoter, the truncated suc2 gene from yeast encoding the mature invertase protein, and the octopine synthase polyadenylation signal (Lerchl *et al.*, 1995) was constructed. Following *Agrobacterium*-mediated gene transfer, transgenic potato plants expressing phloem-specific invertase were selected. Under greenhouse conditions plant growth and tuber yield was not impaired. However, as predicted, a strong delay in sprouting was observed in case of invertase expressing tubers. After 3-4 months of storage at room temperature control tubers started to sprout whereas in case of invertase expressing tubers initiation of sprouting occurred not before 8 months. Figure 2 shows representative tubers stored for five months at room temperature (Hajirezaei *et al.*, 2003).

Production of human papillomavirus (HPV) capsid protein L1 in potato tubers

Interest in transgenic plants for the production of recombinant proteins is steadily increasing. During the last years it has become evident that plants express, fold, assemble and post-translationally modify foreign proteins with high fidelity. Prototype vaccines can be produced in plants and they may trigger oral immunity if the plant tissues are consumed as food. In pre-clinical and first clinical trials it has been found that antigenic proteins may retain their antigenic properties when purified from transgenic plants and may give rise to the production of specific immune responses after immunization.

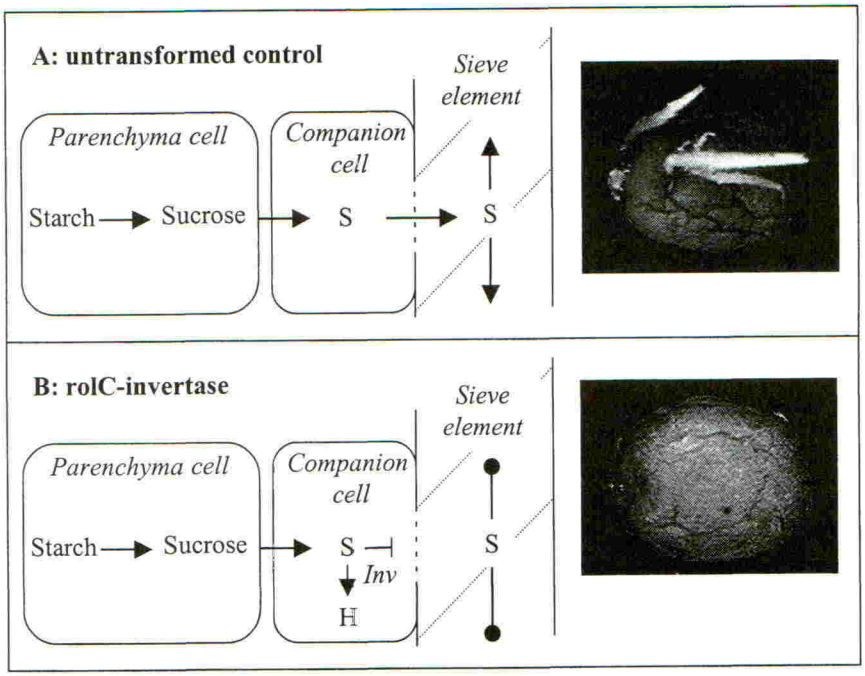

Figure 2. Phloem-specific sucrose hydrolysis via cytosolic yeast–derived invertase leads to non-sprouting tubers. S, sucrose.

To explore the feasibility of using potato tubers as bioreactors for production of edible vaccines a plant expression system to produce the HPV L1 was developed. All attempts to express either the original, unmodified L1 gene or an L1 gene with a codon usage optimized for expression in plants failed. Surprisingly, small amounts of the protein were detected using an L1 gene optimized for expression in human cells (HPV-$L1_h$). However, northern blot analysis revealed that most of the L1 transcripts were degraded. Introduction of the translational enhancer Ω (35S::HPV-$L1_h$) derived from the tobacco mosaic virus strongly increased transcript stability and resulted in accumulation of L1 protein to 0.5% and 0.2% of total soluble protein in transgenic tobacco and potato plants, respectively. The plant-derived L1 protein displayed conformation-specific epitopes and assembled into virus-like particles. Furthermore, we did not find any indications of protein modification of the L1 protein produced in plants. Most importantly, plant-derived L1 was as immunogenic as L1 expressed in baculovirus-infected insect cells (Biemelt *et al.*, 2003). Proof-of-concept was provided within this study. However, to make the system commercially applicable expression levels need to be improved. In the first approach expression of the L1 gene was driven by the constitutive CaMV 35S- promoter. Higher yield might be obtained by restricting expression to storage organs such as tubers. To achieve tuber specific expression the HPV-$L1_h$ gene was cloned behind the patatin (B33) promoter (B33::HPV-$L1_h$; Figure 3A). Accumulation of L1 protein was analysed in tubers of the transgenic lines by Western blotting (Figure 3B). The percentage of L1 protein as fraction of total soluble protein was roughly estimated to be 0.3-

0.5%. This indicates that a slight but not sufficient increase in the amount of L1 protein was achieved by tuber-specific expression of L1.

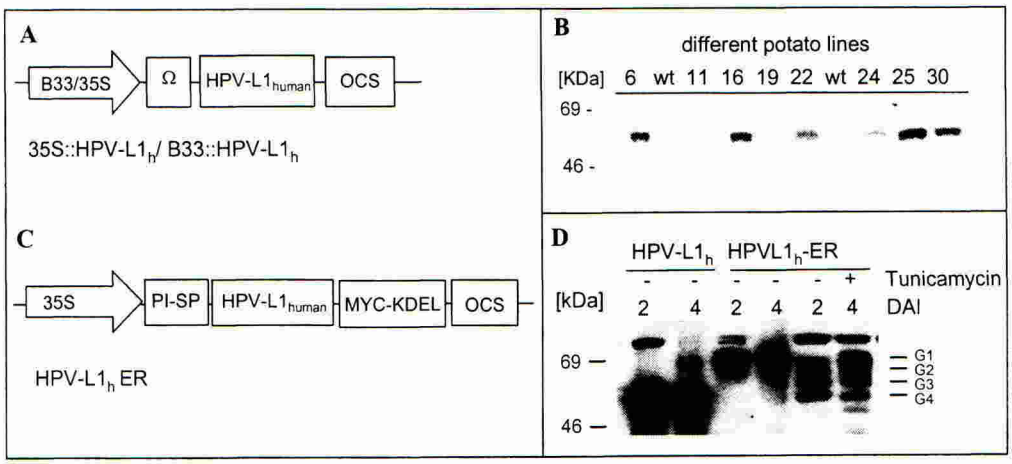

Figure 3. Schematic representation of constructs to enable HPV-L1 expression in potato tuber (A), or the ER (C) and Western blot analysis of L1 expression in plants (B, D). T, Tunicamycin; DAI, days after infiltration; G1, G2 , G3, G4, putative *N*-glycosylation products of L1.

Another approach to enhance protein yield is targeting of the protein to sub-cellular compartments such as the endoplasmatic reticulum (ER) (Wandelt *et al.*, 1992). To enable expression of L1 in the ER, the L1 gene optimised for expression in human cells was N-terminally fused to signal peptide of the potato proteinase inhibitor II to allow co-translational ER-targeting. The addition of the ER-retention signal KDEL at the C-terminus of the L1 protein aimed at accumulating L1 protein in the ER (HPV-L1$_h$ER; Figure 3C). Functionality of the chimeric gene was tested in transient *Agrobacterium* based expression system. To this end leaves of tobacco plants were infiltrated with recombinant *Agrobacterium tumefaciens* containing either the 35S::HPV-L1$_h$ or the HPV-L1$_h$ ER gene constructs. Samples were taken 2 and 4 days after infiltration and subjected to Western blot analysis using L1-specific antibodies. As shown in Figure 3D there was strong L1-specific band at *ca.* 55 kDa detectable in leaves infiltrated with 35S::HPV-L1$_h$ construct. However, transient expression of the ER-targeted L1 protein resulted in a protein migrating at *ca.* 70 kDa. This could be due to *N*-glycosylation of the L1 protein within the ER. Indeed analysis of L1 protein sequence revealed four putative *N*-glycosylation sites. To examine this hypothesis tobacco leaves were co-infiltrated with both the HPV-L1$_h$ ER construct and tunicamycin, which is a potent inhibitor of *N*-glycosylation. The addition of tunicamycin caused the appearance of four bands between 70-55 kDa most likely representing the different *N*-glycosylation products (G1-G4; Figure 3D). Since *N*-glycosylation might affect protein folding and assembling of L1 sub-units to virus-like particles, this approach is not applicable to improve production of the L1 protein.

CONCLUSION

In our study the applicability of potato tubers as bioreactors for the production of pharmaceutical proteins has been investigated. Using a tuber-specific expression system a prototype HPV vaccine could be produced and shown to trigger oral immunity when fed to mice. Based on encouraging developments in chemically-inducible gene expression it has become feasible to apply the ethanol-inducible gene switch for post-harvest induction of vaccine accumulation in transgenic potato tubers. Furthermore, the use of non-sprouting potato varieties offers the possibility to prohibit unauthorized use of the transgenic tubers and thereby the risk of contamination of food or feedstuff can be minimized.

ACKNOWLEDGEMENTS

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Transgenic approaches to study quality traits in cereals

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ABSTRACT

Since the first reports of wheat transformation in the early 1990s, protocols continue to be developed using biolistics and *Agrobacterium*-mediated DNA-delivery into regenerable explants to recover fertile adult transgenic plants. In the majority of cases, the transgene is expressed in a predictable way and segregates during meiosis giving Mendelian ratios of inheritance. Transgenic approaches involving over-expression and targeted gene silencing are increasingly used, both in model and crop species to analyse and validate gene function. We report on developments in the use of *Agrobacterium* to transform wheat and on transgenic approaches to study the role of HMW glutenin subunits in determining the mixing properties and bread making quality of flour.

INTRODUCTION

Genetic transformation is a powerful tool to study gene function and as an adjunct to conventional plant breeding. Since the first reports of successful rice and maize transformation in the late 1980s, robust and relatively efficient methods are now available for the major cereals including wheat. The early protocols used direct gene transfer methods such as biolistics to deliver DNA into protoplasts or regenerable explants. In wheat, immature embryos have proved to be good explants for transformation and regeneration. Plants generated via direct gene transfer protocols have used widely to characterise gene function. However, biolistic DNA delivery can result in high copy number insertions with rearrangements which can lead to unpredictable silencing. The advantages of transformation via *Agrobacterium* have been recognised and extensively reviewed (e.g. Hansen & Wright, 1999; Shibata & Liu, 2000; Miller *et al.,* 2002), but cereal crops, and wheat in particular, proved recalcitrant and lagged behind other plants in their ability to be transformed in this way. The first report of *Agrobacterum*-mediated transformation of wheat was by Cheng *et al.* (1997) using the model wheat variety Bobwhite. We have developed protocols for the transformation of a range of model and UK commercial wheat varieties wheat via both biolistics and *Agrobacterium*, and used this powerful technique to study genes controlling in quality traits. Our first target for improvement was bread making via manipulation of the amount and composition of the high molecular weight glutenin subunits. In this paper, we summarise our progress in developing robust *Agrobacterium*-mediated wheat transformation protocols and describe our work using biolistic transgenic approaches to study bread making quality in wheat.

MATERIALS AND METHODS

Plant material and tissue culture

Wheat (*Triticum aestivum* cv. Florida and var. Cadenza) was grown in a controlled environment room (Wu *et al.*, 2003). Immature embryos (length 0.4 mm to >2.2 mm or as otherwise indicated) were isolated from caryopses and pre-cultured, inoculated and co-cultivation on media based on Cheng *et al.* (1997) as defined fully in Wu *et al.* (2003). Silwet L-77 (Lehle Seeds USA) was added to inoculation medium at 0.01% unless specified. Freshly isolated immature embryos (IEs) were pre-cultured on co-cultivation medium for periods between less than 0.5 hours and 72 hours, then immersed in *Agrobacterium* suspension for between 0 and 5 hours in the dark. Excess bacteria were removed and explants were transferred, scutellum-side up, without blotting, to fresh co-cultivation medium. Co-cultivation was carried out in darkness at 24-25°C.

Bacterial strain and plasmids

Agrobacterium strain AGL1 containing the plasmid combination pAL154 +pAL156 was used for all experiments (Amoah *et al.*, 2001). The pGreen-based plasmid (pAL156) contained a T-DNA incorporating a modified *uid*A (GUS) gene with an intron within the ORF (to prevent expression in *Agrobacterium*) and *bar*. Both genes were driven by the maize Ubiquitin1 promoter plus ubiquitin1 intron (Christensen & Quail, 1996). The *bar* gene was adjacent to the left border and *uid*A (GUS) to the right. The growth conditions for *Agrobacterium*, inoculation and co-cultivation were as detailed in Wu *et al.* (2003).

Plant regeneration and selection

After 1-5 days co-cultivation, explants were transferred to induction medium containing the antibiotic Timentin at 160 mg/l. All subsequent media plates contained Timentin at this concentration. Induction and regeneration media, and selection regimes were detailed in Wu *et al.* (2003). Plantlets showing resistance to PPT were subjected to a PCR and/or GUS activity screen. Positive plants were transferred to soil in a containment glasshouse after 8 weeks of vernalization at 4°C.

Molecular and phenotypic analysis of marker genes

Expression of GUS was determined using immature embryos for transient expression and leaf fragments for stable expression (Jefferson, 1987). PCR was used to confirm the presence or the absence of transgenes in primary transformants and their progeny (Pastori *et al.*, 2001). Tolerance to glufosinate-ammonium herbicides was tested using leaf-painting bioassay (Barro *et al.*, 1998). Southern blot analysis was performed following Wu *et al.* (2003).

Transformation of wheat with quality trait genes

Transformation with genes conferring quality traits were done by biolistics as described by Barcelo & Lazzeri (1995) and Barro *et al.* (1997). Gel electrophoresis of seed proteins and Mixograph analysis were performed as described by Barro *et al.* (1997) and Rooke *et al.* (1999).

RESULTS AND DISCUSSION

Optimization of T-DNA delivery and subsequent tissue culture response

Transient expression of the *uidA* (GUS) marker gene was used to assess *Agrobacterium*-mediated DNA delivery. The percentage of embryos with GUS foci and the mean number of GUS foci per embryo were higher when using larger embryos (L and XL) (Table 1). However, the tissue-culture response showed the opposite trend, with the smaller embryos showing significantly higher regeneration frequency (Table 2). These data highlight the problems associated with optimisation of transformation protocols. Immature embryos of between 0.8 mm to 2.0 mm in length were chosen as a compromise between good T-DNA delivery and good recovery of regenerants.

Table 1. Effect of embryo size on survival and DNA delivery. Freshly isolated, intact embryos were grouped into four size classes: S, 0.4-0.8 mm; M, 0.8-1.5 mm; L, 1.5-2.2 mm; XL, larger than 2.2 mm. Analysis of variance was performed on log-transformed data of GUS foci per embryo. For each variety, the standard error of differences (sed) and least significant differences (5% level) (lsd) were calculated for all pairs of size classes and the maximum figure quoted. (Adapted from Wu *et al.*, 2003).

Variety	Size of embryo	No. of embryos tested	Survival rate (%)	Embryos with GUS foci (%)	Mean GUS foci/ embryo	Mean log GUS foci/ embryo
Cadenza	S	65	66.2	21.1	5.9	1.656
	M	103	92.2	88.6	16.2	2.533
	L	73	97.3	94.4	30.4	3.078
	XL	35	100	91.4	50.7	3.691
						sed=0.212
						lsd=0.438
Florida	S	65	Nd	81.5	12.0	2.252
	M	59	Nd	96.6	31.1	3.261
	L	44	Nd	100	78.6	4.343
	XL	33	Nd	100	101.4	4.491
						sed=0.124
						lsd=0.256

The effect of the surfactant, Silwet L-77, at various concentrations, was investigated during inoculation with *Agrobacterium* (Figure 1). The mortality of explants, T-DNA delivery and subsequent callus induction were measured. DNA-delivery was improved at higher Silwet L-77 concentrations, presumably due to the reduction in surface tension allowing better penetration of bacteria into the plant cells. However, at the Silwet L-77 concentrations where

this effect was most marked (0.02-0.04% v/v), phytotoxicity of the surfactant killed embryos and/or prevented callus induction. In subsequent experiments Silwet L-77 was used at a concentration of 0.01%.

Table 2. Effect of embryo size on embryogenesis, regeneration and stable transformation efficiency in wheat cv. Florida after *Agrobacterium* co-cultivation. NA – not applicable (Adapted from Wu *et al.*, 2003).

	Size of embryo	Frequency of reg. (%)	No. of transformed plants
After *Agrobacterium* co-incubation	S	73.1	0
	M	62.4	14
	L	24.0	0
	XL	1.0	0
Controls with no *Agrobacterium*	S	87.8	NA
	M	74.6	NA
	L	40.0	NA
	XL	15.5	NA

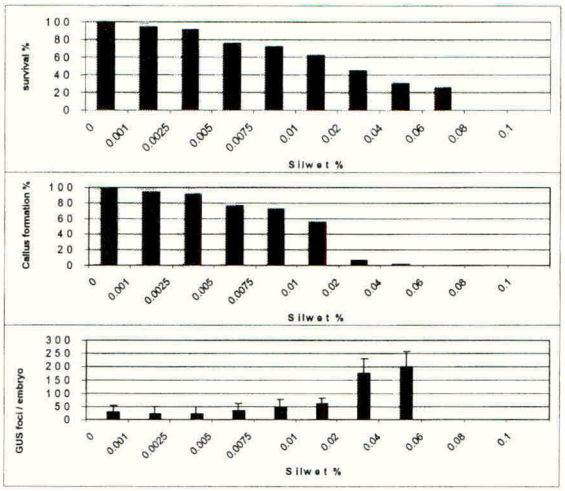

Figure 1. The survival of between 40-90 embryos per Silwet L-77 concentration was assessed 3 days after *Agrobacterium* inoculation. Callus-forming potential was assessed after 2 further weeks on induction medium. T-DNA delivery was assessed by counting embryos that had at least one GUS focus, and counting the foci per embryo (error bars represent one SD of mean). (Adapted from Wu *et al.*, 2003).

Similar conclusions were drawn by Cheng *et al.* (1997) who used 0.01-0.02% in transformation of Bobwhite, and by Clough & Bent (1998) who chose 0.05% for their floral-dip method of Arabidopsis transformation. Other variables influencing *Agrobacterium*-mediated transformation of wheat were optimised, including effect of embryo pre-culture, inoculation time, length of co-cultivation and the effect of acetosyringone as reported in Wu *et al.* (2003).

Transformation efficiencies and marker gene expression

Nine experiments, using 2,587 immature embryos, gave a total of 44 confirmed transgenic lines with efficiency for each experiment ranging from 0.3% to 3.3% (average 1.7%). This concurs with the few other reports of *Agrobacterium*-mediated wheat transformation e.g. 1.6% (Cheng *et al.*, 1997) and 1.8% (Weir *et al.*, 2001). Molecular analysis of the 44 plants revealed that 38 were PCR-positive for both *bar* and *uidA*, three were positive for *bar* only and another three were positive for *uidA* only. Plant genomic DNA was tested for contaminating DNA originating from *Agrobacterium* by PCR for the *VirC* operon and all proved negative. The 41 plants that were PCR-positive for *bar* were confirmed positive for *bar* expression via the glufosinate-ammonium herbicide leaf painting assay. Of the 41 plants PCR-positive for the *uidA* transgene, GUS expression was detectable in only 35. Of the plants tested for transgene integration and segregation, the majority (11) displayed a clear 3:1 inheritance ratio of the *uidA* trait in the T1 generation (Figure 2). The remaining lines (5) showed a skewed ratio with lower than expected number of nulls. Southern analysis of genomic DNA isolated from three primary transformants showed simple, low-copy-number integration of the *uidA* gene. *Swa*I released a fragment of the expected size (4,625) in the pAL156 control and in all of the plants tested. *Bgl*II, which cuts within the T-DNA and in the plant genomic DNA, gave bands of approximately 4.9 kb in plants C2.3 and C2.8, and approximately 5.1 kb in plant F3.1. Plants C2.3 and C2.8 originated from the same experiment and the size similarity of the bands indicate that these may be clones.

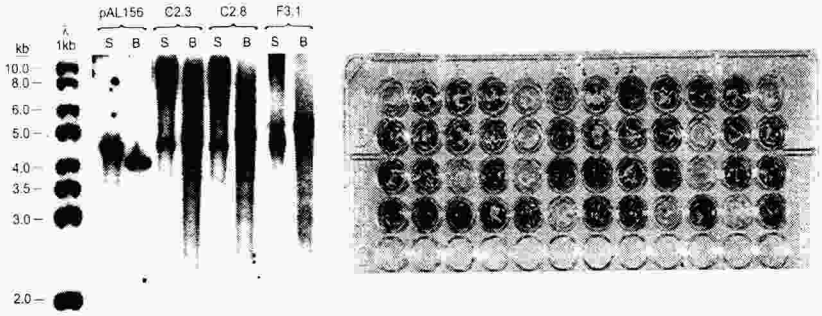

Figure 2. Molecular analysis of wheat lines made by *Agrobacterium* transformation. Southern analysis (left) of one control and three transformed plants (C2.3 and C2.8 are in wheat var. Cadenza and F3.1 is in cv. Florida). Segregation analysis of GUS expression in leaf samples of 48 T1 progeny plants from one transgenic line displays clear 3:1 transgene inheritance (right).

Transformation of the model wheat line with the high molecular weight glutenin subunit genes

Near-isogenic lines of spring wheat each possessing a specific complement of HMW glutenin subunits provide an ideal experimental model in which to test the over-expression of additional HMW glutenin subunit genes by transformation. The near-isogenic line L88-6 possesses five HMW glunenin subunits (1Ax1, 1Dx5, 1Dy10, 1Bx17 and 1By18) whereas line L88-31 possesses only two (1Bx17 and 1By18). We used these lines to test the effect of over-expressing either the 1Ax1 or the 1Dx5 subunit gene on flour mixing and bread making properties. A large number of transgenic plants have been made that appear to be morphologically normal and transmit their transgenes in Mendelian ratios. SDS-PAGE analysis of grain proteins reveal the presence of additional bands of the expected sizes (Figures 3 & 4). Transformation of L88-31 with 1Ax1 improved dough strength, mixing time and gives a small increase in loaf volume (Figure 3). However, over-expression of 1Dx5 in either the L88-6 or L88-31 background resulted in a failure to hydrate and form normal dough and a decrease in loaf volume. In particular, transgenic line B73-6-1 had an unusually high level of 1Dx5 expression (as shown by the intensely-stained band on the SDS-PAGE gel in Figure 3), and had 'over-strong' characteristics in Mixograph tests.

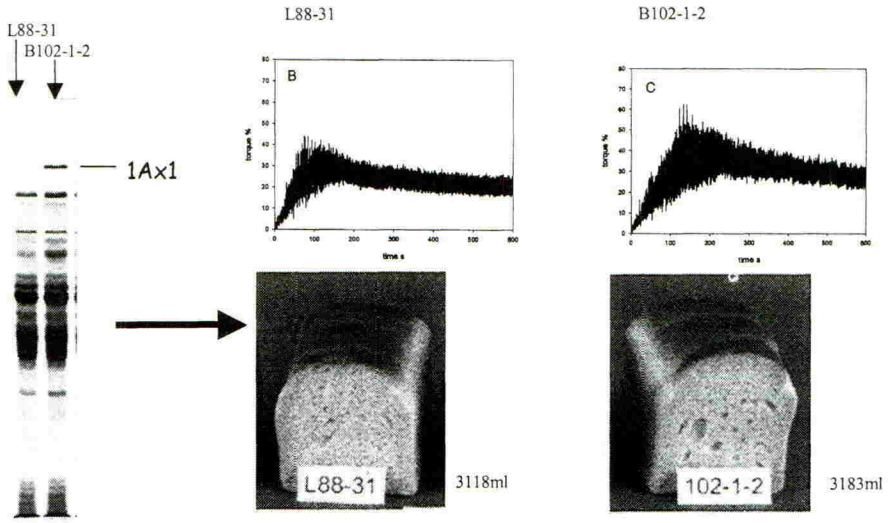

Figure 3. Transformation of the model wheat line L88-31 with the high molecular weight glutenin subunit 1Ax1 gene results in increased dough strength and loaf volume. (Adapted from Popineau *et al.* (2001) and Darlington *et al.* (2003)).

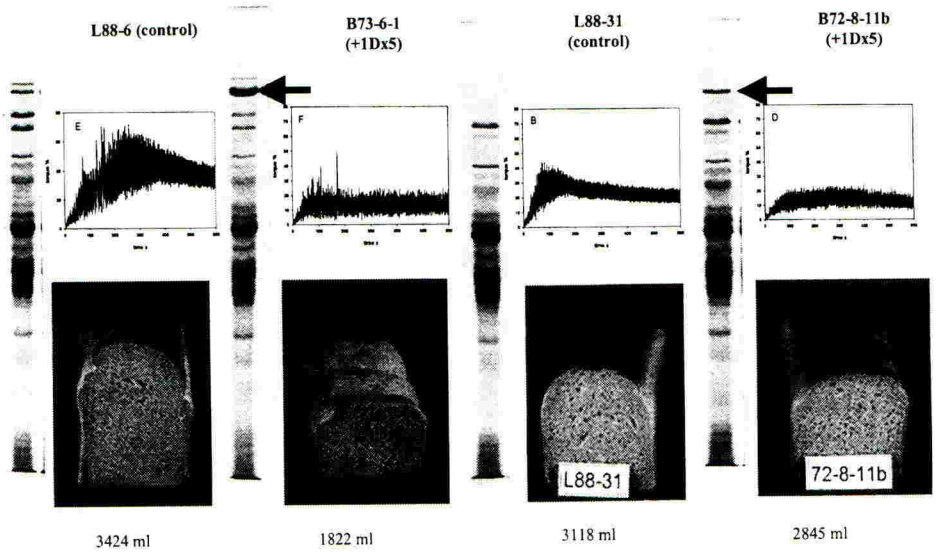

Figure 4. Transformation of model wheat lines (L88-6 and L88-31) with high molecular weight glutenin subunit genes. Over-expression of the 1Dx5 gene results in a failure to mix and lower loaf volumes in both backgrounds. (Adapted from Popineau *et al.* (2001) and Darlington *et al.* (2003).

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Cereal functional genomics and the cell cycle: improving yield

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ABSTRACT

CropDesign has developed a technology platform, TraitMill™, which closes the application gap between functional genomics and the development of improved or novel agronomic traits. The main aim of the TraitMill is to discover traits that improve the yield and yield stability of cereal crops. The TraitMill is focused on rice, which together with its relatives corn and wheat constitute the world's largest crops. As the potential agronomic value of a gene remains difficult to predict, particularly for complex traits such as growth rate, organ size, fertility, seed yield and harvest index, the TraitMill is conceived as a high throughput platform through which thousands of transgene constructs can be tested. One source of genes being tested is the cellular machinery controlling cell division, commonly denoted as the cell cycle. Two examples of cell cycle genes that modulate growth and development in rice are presented.

INTRODUCTION

Until now, plant functional genomics has largely concentrated on elucidating the role of plant genes using different approaches, such as transcriptome or proteome analysis or loss-of-function mutant collections. These approaches allowed to reach a much better understanding of gene functions, yet have failed so far to deliver products. One of the reasons for the low efficiency – from a business perspective – of functional genomics efforts, is that they are largely focused on *Arabidopsis thaliana*, which is easy to handle yet is not a good model for the most important field crops.

CropDesign has pioneered the use of rice within the agbiotech sector as a model for functional genomics of cereal crops through the development of a high-throughput technology platform (TraitMill) designed to produce and evaluate transgenic rice plants with altered growth and development traits of agronomical relevance. This technological platform comprises a high-throughput system for gene cloning, plant transformation and digital plant evaluation: (i) using a high throughput gene cloning system, expression levels of transgenes and transgene combinations can be modulated throughout entire plants or in selected tissues; (ii) an industrialized plant transformation system generates the tens of thousands of transgenic plants required annually to analyse the transgene constructs; (iii) several automated plant evaluation technologies, including digital image analysis of plants, are used to detect alterations brought about in important traits such as biomass production, and seed yield. The actual capacity of TraitMill is of 600 constructs evaluated per year.

Among CropDesign's fields of interest, is the cell cycle with its central role in plant growth, development and performance. Conceivably, manipulation of the cell cycle in plants has potential practical implications for the engineering of new agronomically important traits in crop plants.

MATERIAL AND METHODS

The TraitMill process starts out by high throughput construction of transgene vectors, derived from the Gateway® Technology (Invitrogen). Proprietary promoters are selected for constitutive, tissue-specific or inducible expression of the chosen genes. High-throughput transformation of these constructs into rice is done using Agrobacterium. For each construct, sufficient number of independent T0 plants is generated to allow for the subsequent selection of single insertion events via Q-PCR. Phenotypic evaluation is performed on T1 generation plants from five independent events, at different growth stages, from seedlings to mature plants. For each event, half of the T1 plants are null-segregants (azygous) that serve as an internal control to exclude the effects of somaclonal variation (Figure 1).

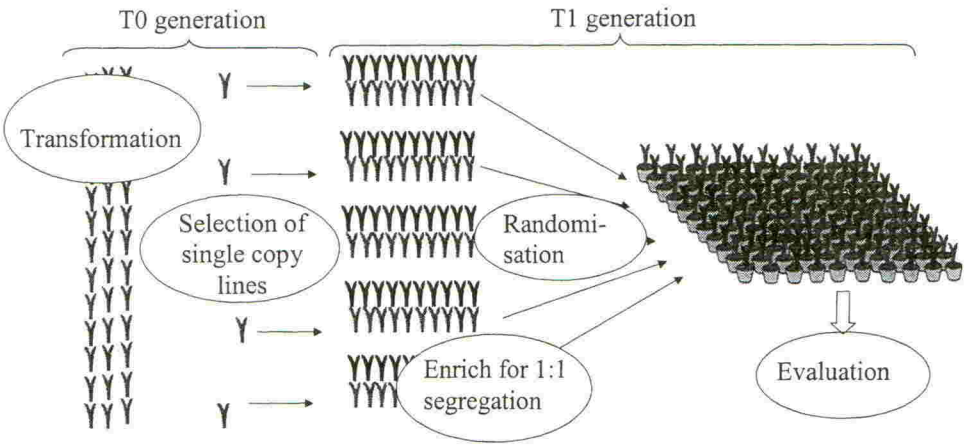
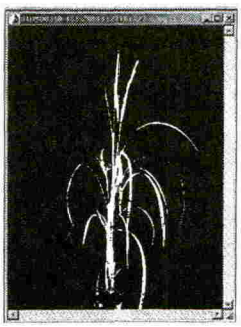


Figure 1. Scheme of the evaluation set-up. Null segregants are represented as black plantlets, transgenics are represented as grey plantlets.



Rice plants are grown on a conveyor belt system and most plant handlings are performed by robots. Plants are passed each week through a digital imaging cabinet (see right). Agronomic parameters are extracted from the digital images using various segmentation algorithms. Once plants are mature, seeds are harvested and processed through automated equipment for cleaning, counting and weighing of the seeds. All data are downloaded in a central relational database and automated statistical algorithms are deployed to visualize the performance

of transgenic plants versus in-line null segregants. For each construct, an additional analysis is performed by comparing the performance of all the null segregants versus all of the transgenics of the same evaluation (called the overall effect).

The scored parameters include plant area, plant height, number of tillers, number of panicles, growth curve (plant area/time), cycle time (time for the plant to reach 90 % of its total area), panicle length, number of spikelets per panicle, total biomass production, total number of seeds per plant, seed weight, and seed dimensions (width and length).

The evaluation of these parameters culminates in the selection of the two events in which growth parameters have been most affected, either positively (positive leads) or negatively (negative leads). The seeds of those selected plant events (leads) are then collected for the production of T2 plants. For each line, 20 to 30 T2 plants are assayed for the presence of the transgene in order to identify homozygous and heterozygous lines. T2 plants of heterozygous lines are subjected to a second evaluation in order to confirm their phenotypical alterations. In this case, 15 transgenic plants are analyzed versus 15 azygous. T2 homozygous lines (both with and without transgene) are grown for seed multiplication. The T3 seeds harvested from these lines will be used as starting material for most experiments in the project.

RESULTS

An example of a negative lead identified via the TraitMill, is the constitutive overexpression of a rice cyclin dependent kinase (CDK) inhibitor. CDK inhibitor proteins inhibit CDK activity by tight association with CDK/cyclin complexes (Figure 2).

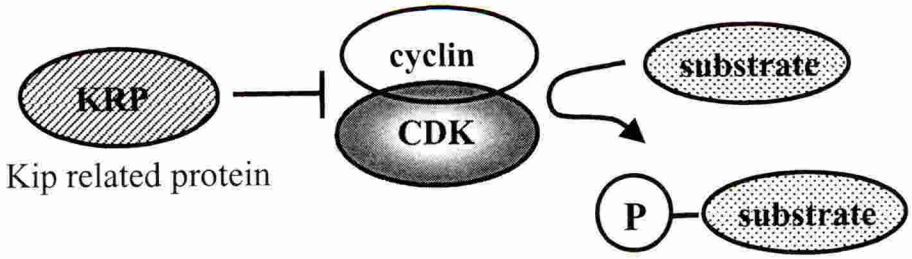


Figure 2. Kip-related proteins bind to cyclin-dependent kinase/cyclin complexes, thereby inhibiting the kinase activity of the complex, and thus affecting cell cycle progression.

Identification of Arabidopsis inhibitors (KRPs, Kip-related proteins) showed that all seven proteins display a short stretch of sequence similarity with the mammalian Kip/Cip family of CKIs (KRPs; De Veylder *et al.*, 2001). All KRP proteins bind CDKA;1, and overexpression of KRP1 and KRP2 in Arabidopsis correlates with a decrease in CDK activity, suppression of cell proliferation and growth inhibition (Wang *et al.*, 2000; De Veylder *et al.*, 2001). A rice KRP ortholog has been cloned and proceeded through the TraitMill. The negative effects are observed only with the seed-related parameters, while the plant aboveground area and height

are unchanged (data not shown). Although the total number of seeds is not significantly changed, the number of filled seeds is dramatically reduced (Figure 3A and B), thereby affecting total seed yield, and consequently harvest index (Figure 4A and B). In addition, thousand kernel weight is also very significantly reduced (data not shown), indicating in addition to poor seed number filling, the seeds eventually produced are also very reduced in size.

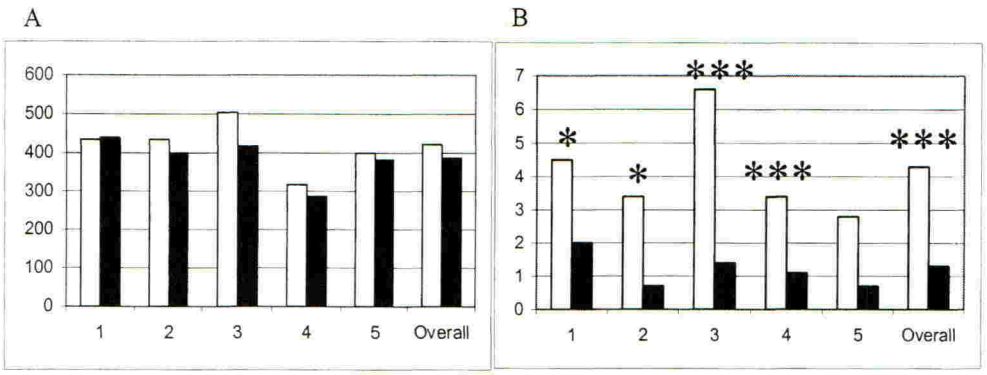


Figure 3. (A) Total number of seeds of the null segregants (open bars) compared to their corresponding transgenics (black bars) for 5 independent events (1 to 5). Overall stands for the comparison of all the nulls and the transgenics from this evaluation. One star means the P value is inferior to 0.05, two stars means the P value is inferior to 0.01, and three stars means the P value is inferior to 0.001. In this case, no significant differences have been found. (B) Total number of filled seeds of the null segregants (open bars) compared to their corresponding transgenics (black bars) for 5 independent events (1 to 5).

As an example of a positive lead discovered in the TraitMill, data are presented of transgenic rice overexpressing a cyclin A-type. Cyclins are a large family of proteins of the regulatory subunits of cyclin-dependent protein kinases (CDKs). In plants, 30 cyclin genes have been reported based on sequence homology. A comparative sequence analysis of these plant cyclin genes demonstrates four distinct cyclin groups (Type A, B, D and H cyclins) (Vandepoele et al, 2002). Cyclins from the A-group are active during S phase and G2, which would lead to suppose that elevated expression of these cyclins may stimulate DNA replication and/or accelerate G2 progression.

Tissue-specific expression of an Arabidopsis cyclin A type in rice was identified as a lead after the T1 evaluation. The positive parameters were improved seed yield due essentially to an increased number of filled seeds. As a consequence, the two most significant events were selected to proceed onwards to T2 evaluation. Again, the same two parameters that were positive during T1 evaluation, were also positive during T2 evaluation (Figure 5 A and B).

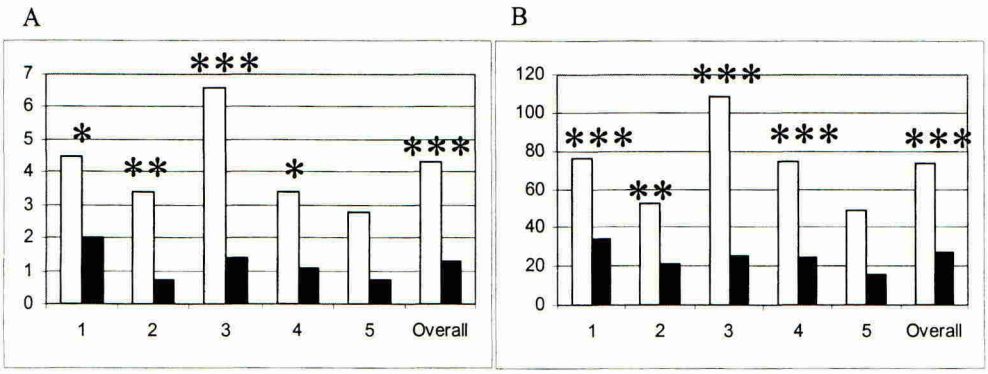


Figure 4. (A) Seed yield (g) of the null segregants (open bars) compared to their corresponding transgenics (black bars) for 5 independent events (1 to 5). Overall stands for the comparison of all the nulls and the transgenics from this evaluation. One star means the P value is inferior to 0.05, two stars means the P value is inferior to 0.01, and three stars means the P value is inferior to 0.001. (B) Harvest index of the null segregants (open bars) compared to their corresponding transgenics (black bars) for 5 independent events (1 to 5).

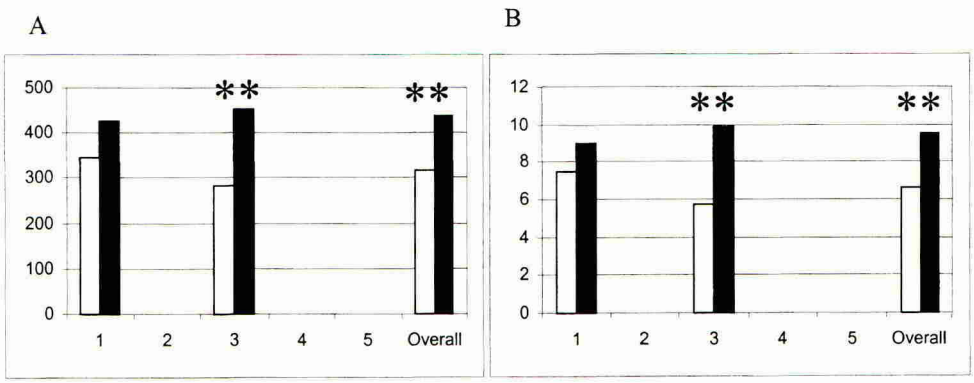


Figure 5. (A) Total number of seeds of the null segregants (open bars) compared to their corresponding transgenics (black bars) for 2 T2 independent events (1 and 3). Overall stands for the comparison of all the nulls and the transgenics from this evaluation. One star means the P value is inferior to 0.05, two stars means the P value is inferior to 0.01, and three stars means the P value is inferior to 0.001. (B) Seed yield (g) of the null segregants (open bars) compared to their corresponding transgenics (black bars) for 2 T2 independent events (1 and 3).

Transgenesis is a powerful and effective mode to study plant development. CropDesign has developed a Traitmill platform, a high-throughput technology that enables large-scale transgenesis. The TraitMill is a highly versatile tool for regulating the expression and testing the effect of genes and gene combinations on plant phenotypes. Over the last years, the CropDesign's TraitMill has generated a range of interesting rice transgenic plants with different phenotypic alterations, resulting from the altered expression of cell cycle genes. These include transgenic rice lines showing increased seed yield or increased green biomass, showing the importance of cell cycle genes for plant growth and development.

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